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Global Infectious Diseases and Response Systems

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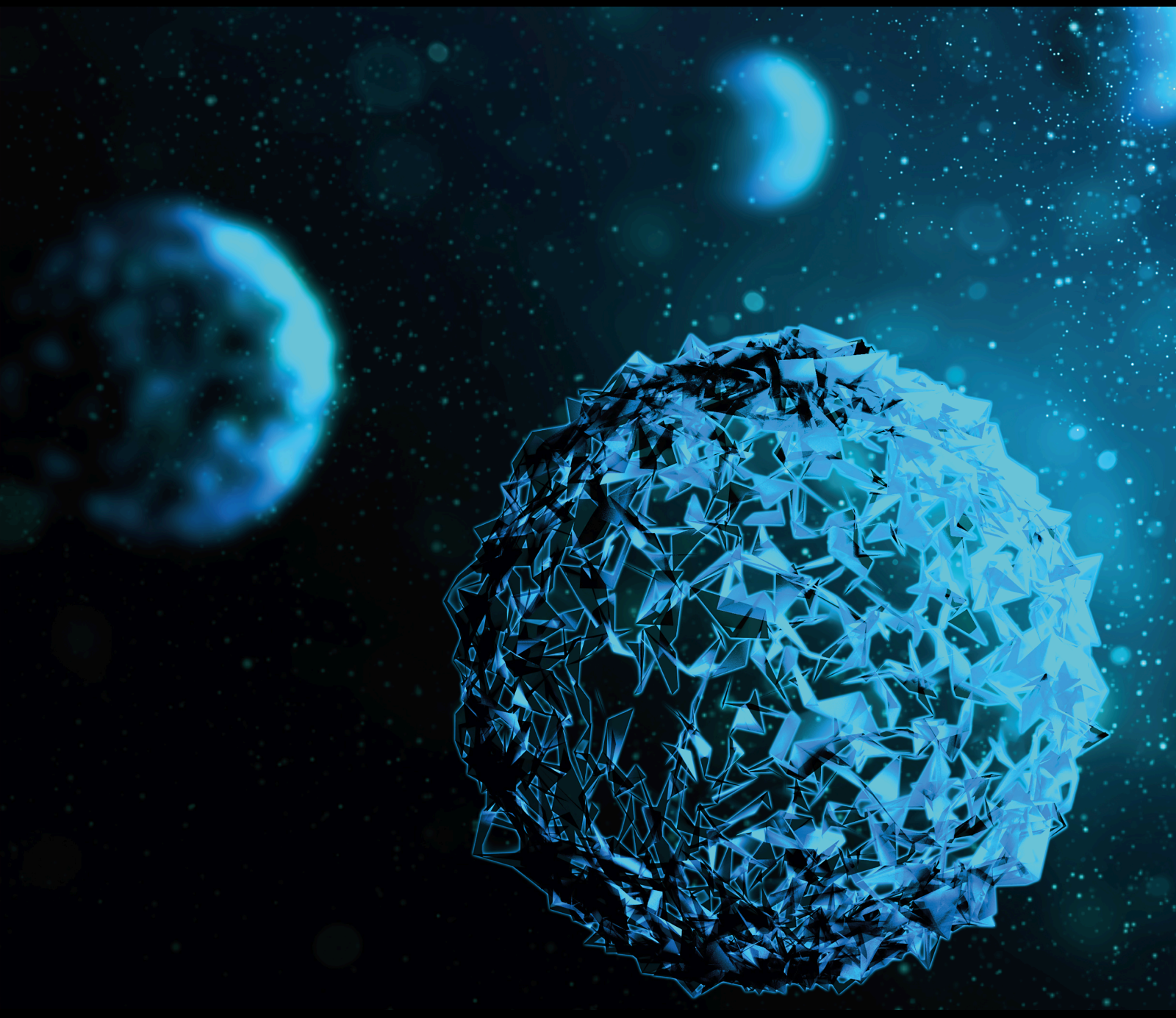

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Research Article

Costs and Cost Drivers of Providing Option B+ Services to Mother-Baby Pairs for PMTCT of HIV in Health Centre IV Facilities in Jinja District, Uganda

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Guest Editor: Hugo Turner

Background. In 2013, the World Health Organization (WHO) revised the 2012 guidelines on use of antiretroviral drugs (ARVs) for the prevention of mother-to-child transmission (PMTCT) of human immunodeficiency virus (HIV). The new guidelines recommended lifelong antiretroviral therapy (ART) for all HIV-positive pregnant and breastfeeding women irrespective of CD4 count or clinical stage (also referred to as Option B+). Uganda started implementing Option B+ in 2012 basing on the 2012 WHO guidelines. Despite the impressive benefits of the Option B+ strategy, implementation challenges, including cost burden and mother-baby pairs lost to follow-up, threatened its overall effectiveness. The researchers were unable to identify any studies conducted to assess costs and cost drivers associated with provision of Option B+ services to mother-baby pairs in HIV care in Uganda. Therefore, this study determined costs and cost drivers of providing Option B+ services to mother-baby pairs over a two-year period (2014–2015) in selected health facilities in Jinja district, Uganda. *Methods*. The estimated costs of providing Option B+ to mother-baby pairs derived from the provider perspective were evaluated at four health centres (HC) in Jinja district. A retrospective, ingredient-based costing approach was used to collect data for 2014 as base year using a standardized cost data capture tool. All costs were valued in United States dollars (USD) using the 2014 midyear exchange rate. Costs incurred in the second year (2015) were obtained by inflating the 2014 costs by the ratio of 2015 and 2014 USA Gross Domestic Product (GDP) implicit price deflator. *Results*. The average total cost of Option B+ services per HC was 66,512.7 (range: 32,168.2–102,831.1) USD over the 2-year period. The average unit cost of Option B+ services per mother-baby pair was USD 441.9 (range: 422.5–502.6). ART for mothers was the biggest driver of total mean costs (percent contribution: 62.6%; range: 56.0%–65.5%) followed by facility personnel (percent contribution: 8.2%; range: 7.7%–11.6%), and facility-level monitoring and quality improvement (percent contribution: 6.0%; range: 3.2%–12.3%). *Conclusions and Recommendations*. ART for mothers was the major cost driver. Efforts to lower the cost of ART for PMTCT would make delivery of Option B+ affordable and sustainable.

1. Background

Globally, HIV infection continues to pose a serious health risk for pregnant women and their children, especially in high HIV burden countries [1]. Mother-to-child transmission (MTCT) of HIV accounts for over 90% of new HIV infections among children. One in five new HIV infections is through preventable vertical transmission from the mother to the baby during pregnancy, labour, delivery, or breastfeeding [2]. In 2015, an estimated 150,000 children globally acquired HIV through MTCT. Of all these new infections, 88% occurred in sub-Saharan Africa [3]. In Uganda, MTCT of HIV accounted for 18% of new HIV infections [4]. In 2015, slightly over 95,000 children were estimated to be living with HIV in Uganda. Of these, 4% were new infections. In the same year, acquired immunodeficiency syndrome (AIDS) was responsible for 17% of deaths among children [5].

In 2013, the WHO revised the 2012 guidelines and made new recommendations on the use of ARVs for PMTCT of HIV [6, 7]. The revised guidelines included a new, third option (Option B+) which recommended lifelong ART for all HIV-positive pregnant and breastfeeding women irrespective of CD4 cell count or clinical stage. This recommendation was identified as high priority for countries with high HIV prevalence and fertility rates like Uganda [8, 9]. In 2012, Uganda had a total fertility rate of 6.2 (6.2 children per woman aged 15-49 years) and HIV prevalence of 7.3% [10]. Between 2009 and 2015, Uganda documented up to an 86% reduction in the number of new HIV infections in children indicating the great reduction of the MTCT as a public health threat [11]. Much of this progress is attributed to the adoption of Option B+ in 2012, which was initiated in the central districts of Uganda and was extended to the entire country by 2013. This led to a dramatic increase in the number of HIV-positive pregnant and breastfeeding women able to access ART. For example, the number of HIV-positive mothers who received ART for PMTCT increased from 112,909 in 2014 to 117,854 in June 2016 [5]. This increased access to ART led to both an improvement in the health of mothers and in a reduction of MTCT of HIV.

Sustaining the delivery of Option B+ requires an insight into and an appreciation of factors that support treatment, care, and retention of mothers and their babies in care which includes a strong understanding of costs and cost drivers of providing such services [12]. Health economic evaluations are important for understanding the cost implications for the service provider and clients, and can assist in determining effective approaches and informing healthcare resource allocation choices.

As advances in HIV treatment are embraced by national healthcare systems globally, it is essential for program managers and policy makers to know the long-term trade-offs between their costs and benefits. To this end, cost analysis of retention in Option B+ programs has been conducted for generalized epidemics in low- and middle-income countries. In South Africa, cost studies highlighted the importance of HIV prevalence and existing resources as an important determinant in resource use in relation to PMTCT [13, 14]. Overall studies have demonstrated the cost-effectiveness of the transition to and implementation of the Option B+ guidelines. They concluded that Option B+ interventions are inexpensive and compared favourably to other interventions with respect to efficacy [15–19]. Bratt et al. in 2011 assessed annual costs of antenatal care (ANC) including PMTCT, but this was before the Option B+ era [20]. To the researchers' knowledge, as implementation and scale up of Option B+ continues in Uganda, no studies have been conducted to assess costs and cost drivers associated with the provision of Option B+ to mother-baby pairs in Uganda. Therefore, this study set out to determine costs and cost drivers of providing Option B+ services to mother-baby pairs over a two-year period (2014-2015) in selected health facilities in Jinja district, Uganda.

2. Methods

2.1. Study Setting. The study was conducted in Jinja district, East-central Uganda, which has a high prevalence of HIV among ANC attendees [21]. The study was conducted in four HC IVs: Bugembe, Mpumudde, Budondo, and Walukuba. These sites were chosen as they had the required data, were among the first facilities to implement Option B+ in the district, and were using the innovative approach detailed below. The HCs were also chosen due to their geographic diversity—they are located in urban (Walukuba and Mpumudde), periurban (Bugembe), and rural (Budondo) areas of the district. All these sites provide PMTCT services. In Jinja district, implementation of Option B+ services started in April 2013. The district uses an innovative interdisciplinary approach engaging healthcare workers (HCWs), village health team members, mentor mothers, and linkage facilitators in the provision of Option B+ services. Mentor mothers are volunteers living with HIV who are trained to provide psychosocial support and health education and to empower pregnant and breastfeeding mothers to improve the health of their families and themselves. Linkage facilitators are campaigners living with HIV who are trained to create awareness and increase uptake of HIV/AIDS services. Figure 1 shows the structure of the public healthcare system in Uganda [22].

2.2. Data Collection. Cost data for providing Option B+ services to mother-baby pairs in 2014 were collected between November 2015 and May 2016 (data were collected retrospectively). Data collection involved conducting face-to-face structured interviews with purposively selected Option B+ service providers, health facility accountants, district officials, and program managers using a structured questionnaire adapted from STAR EC/John Snow Inc. The initial purpose of the survey was the evaluation of outcomes and the impact of the mentor mother model as well as determining the cost-benefit of scaling up the model to the national level [23]. It was administered in English by one of the authors (EB) and trained research assistants (RAs).

Option B+ service providers who participated in the study were drawn from the selected HC IVs while district officials were selected from the district health offices. Program managers who participated in the study were involved in supervising and supporting the HCs in the provision of

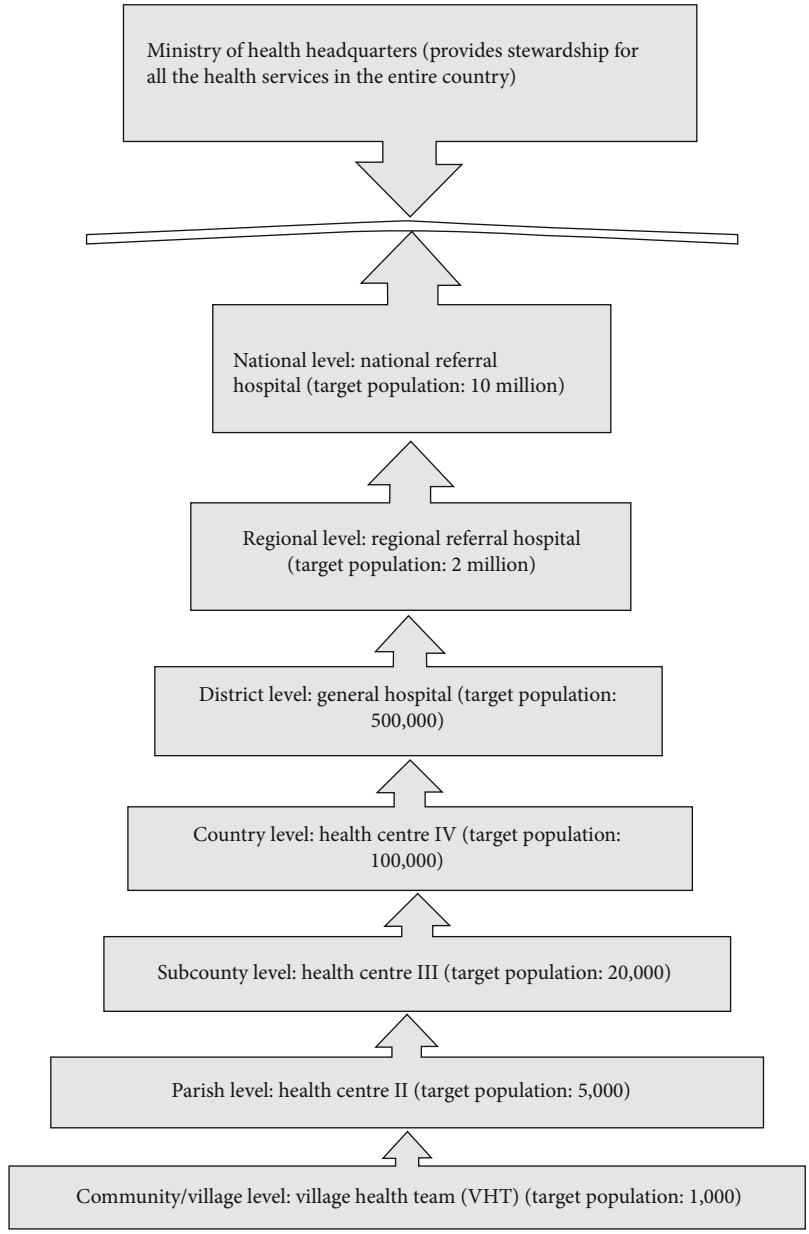

Figure 1: Structure of the health care system in Uganda.

Option B+ services. They were from the Ministry of Health (MoH) and the two implementing partners, namely, The AIDS Support Organization (TASO) and the Strengthening Uganda's Systems for Treating AIDS Nationally (SUSTAIN) project, who supported implementation of Option B+ in Jinja district. TASO is a nongovernmental organization that offers a comprehensive package of HIV prevention and AIDS care and support services [24]. The SUSTAIN project supports the MoH to strengthen sustainable and innovative approaches for HIV service delivery (including implementation of Option B+ services) at select public healthcare facilities in Uganda. The Option B+ services include high-quality HIV counselling and testing; enrolling HIV-positive women and their infants in care; family support groups for HIV-positive pregnant and postpartum women and their families; integration of family planning into HIV care; HIV-exposed infant monitoring and early infant diagnosis (EID) services; infant and young child feeding in the context of HIV counselling and support; and retention monitoring of HIV-positive women and their infants [25]. Additionally, secondary data were abstracted from health facility accounting records, district payroll records, MoH salary scales, District Health Information Software 2 (DHIS2), and PMTCT registers by one of the authors (JK) and RAs.

Cost categories, parameters, and data sources are summarized in Table 1.

2.3. Cost Analysis. All costs were determined from the provider's perspective. All direct healthcare costs and costs of nongovernmental organizations were included, but those incurred by the clients (such as client travel and time costs) were excluded [26]. This enabled the researchers to establish

Table 1: Cost categories, cost items, and source.

Cost category	Cost item	Source
Personnel	Health facility staff	District Health Officer (DHO), accountants, personnel in charge of health facility, facility accounting records, and district payroll records
Medications	ART: tenofovir disoproxil fumarate (TDF)+lamivudine (3TC)+efavirenz (EFV)	ART registers, DHIS2, and National Medical Stores (NMS)*
	Nevirapine syrup	ART registers, dispensing logs, and NMS
	Cotrimoxazole	Dispensing logs and NMS
Laboratory tests	(a) HIV tests (b) HIV deoxyribonucleic acid polymerase chain reaction (DNA PCR) tests (c) Rapid HIV tests (d) CD4 counts	Laboratory registers, DHIS2, NMS, and Gaston Co.
	Dried blood spot (DBS) sample transportation for HIV DNA PCR testing	Laboratory registers, SUSTAIN records, and NMS
Above-site coordination and supervision	MoH coordination	MoH manager, salary scales
	TASO coordination	TASO manager, salary scales
	Option B+ training	DHO, district PMTCT focal person, MoH manager
	Facility-level monitoring and quality improvement	DHO, district PMTCT focal person, and MoH and TASO managers
	Overhead costs (maintenance, utilities, stationery, and fuel)	Accountants, district accountant, personnel in charge of health facility and facility accounting records

*District Health Information System 2 (DHIS2): a free and open source health management data software platform.

costs borne by the providers and implementers of Option B+ services to mother-baby pairs in Jinja. All costs were converted to USD using the 2014 midyear exchange rate of 2544.6 Ugandan shillings. A time horizon of 2 years was used to cover an antenatal period of six months and a postnatal period of 18 months, after which the mother-baby pairs would be discharged from the mother-baby care point.

Costing was done using the ingredient-based approach, i.e., costing each component of an activity, including capital and recurrent costs [27, 28]. This approach identifies each resource input required and values its market or economic costs [26, 29]. Main ingredients and costs in providing Option B+ services to mother-baby pairs were identified, and a unit cost was calculated. We obtained the mean facility total cost of Option B+ and mean facility cost per cost category by averaging the respective cost over the four health facilities. Overall and cost category mean unit costs per mother-baby pair were calculated as a ratio of respective mean facility costs and average mother-baby pairs per facility. The study estimated program financial costs, reflecting actual costs derived from market prices. All analyses were done using Microsoft Excel.

2.3.1. Allocation of Shared Costs. Costs were allocated as part of cost data analyses following data collection. Direct costs to the PMTCT program were allocated fully to PMTCT. Shared costs were allocated to the MoH coordination office, TASO above-facility coordination and supervision, SUSTAIN transportation of DBS samples from facilities to the regional laboratory/hub, district and facility personnel, facility quality improvement, and overheads from the primary healthcare fund. Coordination and supervision costs were allocated using the proportion of each facility Option B+ clientele to the total district Option B+ clientele for district-level costs and proportion of facility Option B+ clientele to Option B+ national clientele for national-level costs. Facility HIV quality improvement costs were allocated using facility Option B+ clientele as a proportion of total clientele for the HIV program at the facility. Personnel costs for midwives were allocated using Option B+ clientele as a proportion of total ANC attendance, while other facility personnel costs and overheads were allocated using Option B+ clientele as a proportion of total out-client attendance.

2.3.2. Costing of Assets. The cost of the motorcycle used by SUSTAIN to transport DBS specimens was annuitized using an annuity factor of 6.23.

2.3.3. Future Costs. All data on costs incurred by the PMTCT program was determined using 2014 as base year. Costs incurred in the second year (2015) were determined by inflating the 2014 costs by the ratio of 2015 and 2014 USA GDP implicit price deflator [30], as described by Turner et al. [31].

2.4. Ethical Considerations. This study was approved by Makerere University School of Public Health Higher Degrees Research and Ethics Committee (protocol number: 308) and Uganda National Council for Science and Technology. Permission to conduct the study was obtained from district and health facilities. Participants were assured of anonymity and confidentiality. All information obtained was kept confidential and used only for study purposes.

Table 2: Health facility client parameters in 2014.

Client parameters	Name of HC IV				Total
	Mpumudde	Walukuba	Budondo	Bugembe	
Number of mothers started on Option B+	179	122	64	237	602
Number of ANC attendees	1967	1391	1480	2570	7408
Number of out-patient attendees	22395	36404	18828	28982	106,609
Number of HIV DNA PCR tests done	52	102	42	159	355

Table 3: Cost parameters and unit costs at a national level, and data source.

Cost parameter	Unit cost in 2014 USD	Data source
Monthly ART for the mother (TDF+3TC+EFV)	12.0	NMS*
Nevirapine syrup	1.7	NMS*
CD4 test	8.0	NMS*
HIV DNA PCR test	30.0	NMS*
HIV testing (Determine+STAT-PACK test kits)	2.5	Gaston Co.
Testing using Uni-Gold test kit	2.6	Gaston Co.

*National Medical Stores; parameters at the national level including cost of medications, unit costs of laboratory tests (CD4, DNA PCR, and HIV testing), gestation at start of Option B+, useful life of motorcycle (7 years), and the discount rate (3%) were applied to all facility-level cost estimations.

3. Results

Table 2 shows health facility client parameters. According to data from DHIS2, a total of 50,049 and 1,617 pregnant and lactating mothers in Uganda and Jinja district, respectively, were initiated on Option B+ in 2014. In the same year, 602 pregnant or lactating mothers were initiated on Option B+ in the four HC IVs where the study was conducted. Of these, majority (39.4%, 237/602) came from Bugembe HC IV and Budondo HC IV contributed the least (10.6%, 64/602). Mpumudde and Walukuba HC IVs had 29.7% (179/602) and 20.3% (122/602) mothers, respectively. Infants of these mothers had a final rapid HIV test done from the four health facilities.

Table 3 shows cost parameters and unit costs at the national level and data source.

HIV DNA PCR had the highest unit cost followed by ART for the mother and CD4 cell count test.

Table 4 shows cost drivers, costs, and percentage contribution of cost drivers to the total cost per health facility. The average cost of Option B+ services per facility over 2 years was USD 66,512.7 (range: 32,168.2–102,831.1). The total average 2-year unit cost of Option B+ services per mother-baby pair was USD 441.9 (range: 422.5–502.6).

ART for mothers was the biggest driver of costs (mean contribution: 62.6%; range: 56.0–65.5%). At all sites, the cost of facility personnel was the next highest cost driver (mean contribution: 8.2%; range: 7.7–11.6%), followed by facility-level monitoring and quality improvement (mean percent contribution: 6.0%; range: 3.2–12.3%).

4. Discussion

This study determined costs and cost drivers of providing Option B+ services to mother-baby pairs over a two-year period in four HC IVs in Jinja district. Information on costs and cost drivers of providing Option B+ services is vital for policy makers, managers, funders, and implementers to ensure appropriate state and donor fund allocation with clear knowledge of priority areas. This could contribute to the elimination of MTCT of HIV [32].

The study found that the average cost of providing Option B+ services in the second year was higher compared to the first year. The finding could be attributed to the solitary additional cost incurred in the second year to perform the follow-up HIV DNA PCR. The follow-up HIV DNA PCR is done six weeks after an HIV-exposed infant ceases to breast feed [4], which commonly occurs in the second year of provision of Option B+ services to mother-baby pairs.

In our study, the total mean cost per mother-baby pair was USD 441.9 (range: 422.5–502.6) which is congruent with findings from a study conducted in Ethiopia in urban high HIV prevalence health facilities [33]. The Ethiopian study reported that the cost of providing a PMTCT service per woman-infant pair ranged from USD 319 to USD 1099 (2014 cost prices). Correspondingly, another study conducted in Namibia and Rwanda during the pre-Option B+ era found the cost per mother-infant pair in Namibia in the range of USD 203–1030, (2009 cost prices) which is comparable to the current study [34]. The wide range in the cost per mother-infant pair could be due the differences in the PMTCT packages in the Ethiopian and Namibian studies.

Medications, laboratory tests and health facility personnel were found to be the main cost categories in this study.

4.1. Medications. ART for mothers was the biggest cost driver, consistent with findings in Côte d'Ivoire where ART cost for Option B+ contributed 68% of the annual treatment cost [35]. Furthermore, our findings are similar to a study done in Ethiopia [33] and a systematic review conducted by Galarraga [36]. The percent contribution in these studies are comparable to those of the current study although there

Table 4: Cost drivers, mean cost, and percent contribution to total costs of Option B+ services per health facility.

Cost drivers	Mean year 1 costs*	Mean year 2 costs*	Total 2-year cost	Mean cost per mother-baby pair	Mean percent contribution (range) %
Personnel					
Facility personnel	2,711.8	2,774.1	5,485.9	36.5	8.2 (7.7–11.6)
Medications					
ART (TDF+3TC+EFV)	20,589.4	21,062.6	41,652.0	276.8	62.6 (56.0–65.5)
Nevirapine syrup	195.4	199.9	395.3	2.6	0.6 (0.5–0.6)
Cotrimoxazole	1,820.6	1,862.5	3,683.1	24.5	5.5 (49–5.8)
Laboratory testing					
HIV enzyme immunoassay (EIA) tests	402.2	411.5	813.7	5.4	1.2 (1.1–1.3)
CD4 counts	1,204.0	1,231.7	2,435.7	16.2	3.7 (3.3–3.8)
1st HIV DNA PCR	1,710.0	1,749.3	3,459.3	23.0	5.2 (2.2–6.7)
2nd HIV DNA PCR	—	337.5	337.5	2.2	0.5 (NA)
DBS sample transportation for HIV DNA PCR testing**	593.8	607.4	1,201.2	8.0	1.8 (1.6–1.9)
Above-site coordination, supervision, and training					
MOH	104.4	106.8	211.2	1.4	0.3 (0.2–0.3)
TASO	1,155.4	1,181.9	2,337.3	15.5	3.5 (3.2–3.9)
Option B+ training	135.3	138.4	273.8	1.8	0.4 (0.2–0.4)
Facility-level monitoring and quality improvement	1,973.6	2,019.0	3,992.5	26.5	6.0 (3.2–12.3)
Overheads	115.7	118.4	234.2	1.6	0.4 (0.2–0.7)
Grand total	32,711.7	33,801.0	66,512.7	441.9	100.0%

All costs are in 2014 USD. *Cost incurred in the second year (2015) were inflated by the ratio of 2015 and 2014 USA GDP implicit price deflator. **SUSTAIN supported transportation of DBS samples.

are slight differences in the cost ranges due to possible variations in ART producers and suppliers. The cost of ART is met by the provider and given free to the mothers and their infants. The cost of ART has been significantly reduced over the past decades, but there is still a need for further reduction to prevent ARV shortages [37] which could interrupt provision of Option B+ services. Interruption in the provision of services discourages mothers, which would subsequently result in mother-baby pairs lost to follow-up [38]. ART remains a cornerstone in PMTCT of HIV, and the government should therefore ensure availability of funds to procure sufficient ARVs.

Cotrimoxazole, which plays a key role in preventing opportunistic infections and malaria [39], had a percent contribution of 5.5% to the total mean cost. It is prescribed to HIV-positive mothers and their infants as a prophylaxis. Cotrimoxazole is cost effective in averting opportunistic infections [40–43]. Consequently, higher costs of treating opportunistic infections are averted. The cost found by this study is affordable though slightly higher compared to other literature. A study conducted in Ethiopia with findings closely comparable to the current results combined the cost of all drugs used in Option B+ [33]. Other literature only considered costs of Cotrimoxazole use by HIV-exposed infants, whereas the current study evaluated costs incurred on both mothers and their infants [16, 44].

4.2. Laboratory Tests. Significant costs (12.4%) were incurred on laboratory tests. Infant HIV testing (HIV DNA PCR) was the biggest cost driver among the laboratory tests. The cost incurred on the initial HIV DNA PCR was higher than that of the follow-up PCR. This might be attributed to the fact that more mothers bring their infants for the initial PCR as opposed to the follow-up one [45]. Consequently, more initial HIV DNA PCR tests are done compared to follow-up testing. This could be because the initial HIV molecular test is performed when the exposed infant is six weeks old or the earliest opportunity thereafter and coincides with the second immunization visit for the baby and the mother's postnatal care visit [46]. Furthermore, many mothers want to know the HIV status of their exposed infant as soon as possible. The mothers are therefore motivated to do the initial HIV DNA PCR. Studies have reported that some mothers do not return or do not bring their babies with them for subsequent HIV tests if the initial HIV DNA PCR is negative [47].

Monitoring the mothers' CD4 cell counts was a big cost driver. It is important to note that it is not a requirement for a mother to start on Option B+; however, CD4 cell count was the cornerstone in assessing HIV disease progression, making clinical decisions, and monitoring the response to ART [48]. However, since WHO recommended the use of viral load testing as the preferred monitoring tool for people on ART in 2013 [6], many countries have adopted it [49]. Uganda adopted routine viral load testing in 2014 and gradually scaled it up country wide [50]. Viral load testing is more costly than CD4 cell count, and this is anticipated to increase the cost of monitoring mothers on Option B+. Similar to this study, laboratory testing was the second largest cost

component of direct costs for providing key services at the facility level to prevent MTCT of HIV in Ghana [51] and in a systematic review by Galarraga et al. [36]. Accordingly, funds should be allocated to sustain laboratory testing.

4.3. Health Facility Personnel. Facility personnel costs accounted for 8.2% of the total mean cost per mother-baby pair. Personnel included those at the frontline of delivering care to pregnant and lactating mothers, for example midwives, village health team members, linkage facilitators, and mentor mothers whose role is to ensure smooth implementation of the PMTCT program leading to uptake of Option B+ services and retention in HIV care [52, 53]. In a study conducted in 212 PMTCT facilities in Kenya, Rwanda, South Africa, and Zambia, health personnel costs were reported to be a major cost driver along the PMTCT service cascade [54]. Health personnel play a key role in the provision of Option B+ services. A shortage of healthcare workers is likely to dent the quality of service provision and consequently have a negative effect on patient's adherence to ART and retention in HIV care [53]. This calls for more and sustained funding to cater for the health workforce amidst an increasing number of patients in the era of test and treat.

4.4. Monitoring and Quality Improvement. Facility-level monitoring and quality improvement activities accounted for a mean cost contribution of 6.0%. Quality improvement activities are critical in ensuring the provision of standard Option B+ services along the entire PMTCT cascade. This ensures client satisfaction, which potentially leads to uptake of services and retention in care [55]. Indeed, WHO recommends quality HIV care services to achieve desired health outcomes such as the uptake of HIV services, retention in care, and reduction in MTCT and related morbidity and mortality [56].

4.5. Coordination and Supervision. A mean percent contribution of 3.8% was incurred on coordination and supervision of Option B+ services. In the current study, above-site coordination and supervision were undertaken by the MOH and the implementing partner (TASO). Proper coordination in the provision of Option B+ services ensures that implementation of the strategy involves all the stakeholders, and is effective and efficient. The Interagency Task Team (IATT) on the prevention of HIV infection in pregnant women, mothers, and their children recommend a well-functioning national coordination mechanism to ensure a successful PMTC program implementation [57]. A well-functioning coordination mechanism is crucial to guide PMTCT program design, implementation, reporting, and monitoring. Studies have highlighted the role of coordination and supervision to address health system challenges in PMTCT programs [58–60]. Ensuring availability of resources to strengthen coordination and supervision is recommended.

5. Strengths and Limitations

The study team used program data which gave the real context of providing Option B+ to mother-baby pairs by program implementers. Jinja district has five HC IVs, but the study team failed to access cost data for one of these HC IVs. Nevertheless, the four HCs that were studied gave a representative evaluation. Therefore, this information may be applicable to other HC IVs in the region. However, findings from this study may not be applicable to health facilities that are at a lower or higher level than HC IVs.

6. Conclusions and Recommendations

The mean cost for providing Option B+ services per mother-baby pair per health facility was USD 441.9. The three major cost drivers of providing Option B+ services to mother-baby pairs were ART for the mothers, facility personnel, and facility-level monitoring and quality improvement. We recommend that sufficient funds should always be in place to ensure that ARVs are continually in stock, laboratory tests are performed, health facility personnel are remunerated, and quality improvement activities are conducted. In addition, efforts to lower cost of ART for PMTCT would make delivery of Option B+ affordable and sustainable.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Disclosure

The contents of this article are solely the responsibility of the authors and do not necessarily represent the official views of the funders, the Ministry of Health, or the Makerere University School of Public Health.

Conflicts of Interest

The authors declare that there is no conflict of interest.

Authors' Contributions

AM, CM, FM, HK, DB, AK, PS, SF, and SK were involved in development of the study concept. AM, CM, FM, HK, EB, DB, JM, AK, PS, SF, SK, and JK were involved in development of the proposal. AM, CM, EB, and DB carried out field work. AM, CM, EB, and JK undertook data analysis. AM conceptualized the research question and wrote the first draft of the paper. AM, CM, FM, HK, EB, DB, JM, AK, PS, SF, SK, and JK revised the draft manuscript to strengthen its intellectual content. AM, CM, FM, HK, EB, DB, JM, AK, PS, SF, SK, and JK approved the final draft.

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Research Article

Enhancing Preparedness for Arbovirus Infections with a One Health Approach: The Development and Implementation of Multisectoral Risk Assessment Exercises

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Background. One Health is receiving attention for arbovirus infection prevention and control and for defining national "intersectoral" priorities. Increasing awareness of intersectoral priorities through multisectorial risk assessments (MRA) is promising, where data are not systematically shared between sectors. Towards this aim, the MediLabSecure project organized three MRA exercises (hereby called exercises): one on West Nile virus, one on Crimean–Congo haemorrhagic fever, and one on Rift Valley fever, assessing the added value of this approach. *Methods*. The exercises relied on RA methodologies of international organisations. Country representatives of the human and animal virology, medical entomology, and public health sectors (hereby called "sectors") involved in the surveillance of vector-borne diseases participated in the exercises. Background documentation was provided before each exercise, and a guide was developed for the facilitators. All three exercises included technical and methodological presentations and a guided RA directed at bringing into play the different sectors involved. To assess the added value of the approach, each participant was asked to rank the level of perceived benefit of the multisectoral collaboration for each "risk question" included in the exercises. *Results*. In total, 195 participants from 19 non-EU countries in the Mediterranean and Black Sea regions took part in the exercises. The participants assessed the multisectoral approach as valuable in analysing comprehensively the situation by having access to information and knowledge provided by each of the sectors involved. Sharing of information and discussion facilitated reaching a consensus on the level of risk in each country. *Conclusions*. Increasing awareness of intersectoral priorities, including cross-border ones, through MRA is relevant to reduce gaps due to unavailability of shared data and information. Given that six out of the ten threats to global health listed by WHO are occurring at the human-animal-environmental interfaces, comprehensive regional RA with a One Health approach made by national authorities can be a relevant added value for the global health security.

1. Introduction

Integrated surveillance is considered a promising working strategy [1–4] to enhance early warning of emerging infections such as arboviral diseases. In addition to providing early signals, integrated surveillance by systematically integrating multiple sources of surveillance data in a timely manner (indicator- and event-based surveillance, case-based surveillance, vector surveillance, and virus and environmental data and information) could contribute more effectively to accurate risk assessments (RA) [5]. Unfortunately, very few countries worldwide [6–8], and in the Mediterranean Region [910], have managed to collect and analyse surveillance data across sectors related to arbovirus transmission, and even fewer have interoperable databases. Ultimately, this limits early warning and risk assessment capacity with impact on the prevention and control of arbovirus infections. This is in line with the recognised challenges of sharing data and information, although the evidence for the public health benefits of sharing is growing with well-documented instances of an improved outcome as a result of sharing surveillance data [11–16]. Efficient data sharing also prompted an early response to the emergence of the H7N9 influenza virus in humans in China [14]; conversely, reluctance to share can hinder or slow down the response and global outbreaks have shown that inadequate surveillance and response capacity in a single country can endanger national populations and the public health security of the entire world [13]. One relevant issue is how to enhance trust within and between countries, considering that trust facilitates the sharing of data and information. Trust-building measures can take the form of face-to-face meetings, regular regional workshops, desktop exercises, joint outbreak investigations, and networking activities. These promote the sense of working towards a common goal [11].

To this aim, working with a multisectoral and transdisciplinary approach, often mentioned as a One Health approach, can help to mediate different assumptions and views and to fill knowledge gaps [17–20].

Focusing specifically on those threats which occur at the animal-human-ecosystem interfaces, several international organisations, including the World Health Organization (WHO), Food and Agriculture Organization of the United Nations (FAO), World Organisation for Animal Health (OIE), and World Bank, have recognised the critical role of multisectoral risk assessment (MRA) (multisectoral risk assessment (MRA): assessment with the concomitant participation of all the relevant sectors involved in the surveillance of a given arbovirus infection) to enhancing cross-sectoral collaboration and improving data collection and data-sharing from different sectors [21–24].

In fact, for health threats that are either emerging or existing at the interface, including food safety issues, neither the technical data nor other information important to conduct a comprehensive assessment nor the appropriate breadth of technical expertise and experience are routinely available within a single agency or sector [22].

In the dimension of capacity building and training, risk assessment exercises implemented with a multisectorial approach can foster data and information sharing across sectors reducing information gaps, highlight experiences and contributions across countries, develop the concept of a national/regional "cross-sectoral" risk assessment outcome, and guide prioritisation of actions and allocation of funds also taking into account the cross-border dimension.

In fact, regional public health threats are often presenting common characteristics such as the need of joint prevention and response activities, common coordination, and comprehensive lessons learned analysis across the actors involved, especially at borders (see the cases of Crimean–Congo haemorrhagic fever cluster in 2008 at the borders between Greece and Bulgaria [25] and in 2009 between Georgia and Turkey [26]).

These characteristics can be easily integrated in the framework of MRA.

Towards this aim, we organized three MRA exercises: one on West Nile virus (WNV) infection, one on Crimean–Congo haemorrhagic fever (CCHF), and one on Rift Valley fever (RVF) in the framework of the MediLabSecure (MLS) project [27].

The aim of these exercises was not only to formulate more reliable risk assessments but also to promote a process leading to a homogenous understanding of risk across different sectors in a given country, and across neighbouring countries, using a structured strategy of assessment. This article describes their implementation and discusses the added value of the adopted multisectoral approach.

2. Materials and Methods

The MLS project started in 2014 and aims at consolidating a regional network of public health institutions and laboratories, belonging to 19 non-European Union (EU) countries (Albania, Algeria, Armenia, Bosnia and Herzegovina, Egypt, Georgia, Jordan, Kosovo, Lebanon, Libya, Moldova, Montenegro, Morocco, Palestine, Former Yugoslav Republic of Macedonia, Serbia, Tunisia, Turkey, and Ukraine), for the control of zoonotic emerging viruses. It represents a cluster for awareness, risk assessment, surveillance, monitoring, and control of relevant emerging diseases, with special focus on arbovirus infections.

In this context, we designed three MRA exercises in coordination with the MLS working group and the subject-matter experts of the European Centre for Disease Prevention and Control (ECDC) and of the Italian Animal Health Institute "Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise (IZSAM)."

For the development of the three MRA exercises, we relied on the following existing RA methodology and guidance documents: the ECDC "WNV risk assessment tool" [28], the ECDC "operational guidance on rapid risk assessment (RRA) methodology" [29], and the Food and Agriculture Organization of the United Nations (FAO) methodology of "The RVF in Niger: Risk Assessment"

[30]. All mentioned tools and guidance documents were developed by subject-matter experts, had been piloted in other contexts, and were in line with the pathogens and methodological priorities identified by the MLS countries.

We invited country representatives of the human virology, animal virology, medical entomology, and public health sectors (hereby called "*sectors*") involved in the surveillance of vector-borne diseases to participate in the three MRA exercises. Background documentation (including selected references) was sent by e-mail to participants one week before each exercise. An exercise implementation guide was also developed and sent to the facilitators together with the background documentation. The participants were asked to send national epidemiological data on the concerned pathogens that were then shared with all participants. At the start of each exercise session, participants were provided with a participant's guide.

All three exercises developed in three phases, the first always consisted in technical and methodological presentations by subject-matter experts. The second and third phases differed as shown in Table 1.

Additional details on the developed exercises, background documents, and guidance for facilitators and participants are available in the MRA exercise reports [32–34].

The added value of the multisectoral approach during the CCHF and the RVF assessments was collected by asking each participant to rank (high, medium, or low) the level of perceived benefit of the multisectoral collaboration when answering each "risk question" included in the exercises.

Pre- and posttest questionnaires, designed to assess if the MRA had increased the participant's knowledge, were prepared and submitted for the CCHF (Annex 5 of [33]) and RVF (Annex 6 of [34]) exercises. We deemed that, considering the aim of the exercises, it would have been particularly important to assess knowledge of participants on key parameters on which to rely on for the assessment, notably, surveillance data, source, and type of information and disease/infection risk factors.

Participants were also asked to compile an exercise evaluation form (Annex 6 of [34]) at the end of each exercise to provide MediLabSecure project with feedback on the quality and the pertinence of the training sessions.

2.1. The WNV Exercise. All the 19 countries involved in the MLS network took part in the exercise (Table 1). The participants were divided in smaller groups by country according to regional proximity.

Each participant was asked to identify the risk area typology that was mostly representative of his/her country on the basis of the six risk area types defined by ECDC for WNV transmission (Figure 1).

Subsequently, the participants discussed the reasons for their identified risk area in groups, considering both national and cross-border factors. They were allowed to modify their risk area after the discussion. Then, the participants discussed in country groups the level of risk with regard to national surveillance system characteristics using the SWOT [35] analysis framework (strengths, weaknesses, opportunities, and threats analysis) guided by the ECDC tool [28]. The final risk area typology and the main aspects that had emerged from all the national SWOT analyses were presented and discussed in plenary with all other groups [31].

2.2. The CCHF Exercise. CCHF MRA was implemented with the countries of the Balkans and Black Sea Region of MLS (Table 1) that considered this disease as a priority for the area. The exercise was developed by adapting the information table for rapid risk assessment and the risk-ranking algorithm of the ECDC operational guidance on rapid risk assessment methodology (Annex 2 and 3 in [33]) to rate the potential of CCHF virus transmission in each participating country integrating the views of the different sectors. The assessment was done in two steps: first, the participants assessed the risk in small groups of neighbouring countries on the basis of the information delivered with the technical presentations, available national data, and the background document sent in advance; second, an assessment was made by each country over the different sectors. Each country provided the multisectoral added value to the rapporteur for plenary audience restitution.

2.3. The RVF Exercise. The RVF exercise was implemented with the countries of North Africa and the Middle East Region of MLS (Table 1) which considered RVF a priority for the area. The RVF exercise was developed by adapting the risk questions of the FAO RVF in Niger Risk assessment (Annex 3 in [34]) to identify the risk of RVF virus infection introduction, spread and/or persistence in each participating country. As for the CCHF exercise, the participants were divided in small groups of neighbouring countries to discuss the regional situation with the colleagues of the other sectors in the group.

For the last phase, the group was divided by country with all sectors represented because the expected outcome was the level of risk by country. Each country provided the multisectoral added value to the rapporteur for plenary audience restitution.

3. Results

A total of 159 participants from the 19 non-EU countries of the MLS network took part in the three exercises: 73 participants in the WNV, 42 in the CCHF, and 44 in the RVF exercise.

3.1. The WNV Exercise. The WNV exercise highlighted a high heterogeneity in assessing the level of risk across the involved sectors. The sharing of information and discussion between sectors and neighbouring countries reduced intersectoral variability towards a single level of risk in each country.

Each participant was provided with dots coloured as per his/her sector (i.e., yellow for human virology, blue for animal virology, green for medical entomology, and red for

Table 1: Overview of the three multisectoral risk assessment exercises conducted, Source: [31].

Exercise (place and date)	Participant countries from MediLabSecure network	Objectives	Methodology	Guidance documents
West Nile virus exercise (Paris, December 2015)	Albania, Algeria, Armenia, Bosnia and Herzegovina, Egypt, Georgia, Jordan, Kosovo, Lebanon, Libya, Moldova, Montenegro, Morocco, Palestine, former Yugoslav Republic of Macedonia (FYROM), Serbia, Tunisia, Turkey, and Ukraine	(i) Describe risk level assessment between sectors and countries (ii) Assess the cross-sectoral collaboration during the initial phase of the MediLabSecure project (iii) Make participants aware of the ECDC tool (iv) Provide indications for the next MRA exercises	(1) Map the assessment of WNV risk across four sectors (human and animal virology, medical entomology, and public health) by country and by regions (2) Conduct a SWOT analysis to assess strengths, weaknesses, opportunities, and threats in relation to the surveillance systems in place at national level, to support the risk assessment (3) Compile an evaluation questionnaire on exercise satisfaction	ECDC "West Nile virus risk assessment tool" [28]
Crimean–Congo haemorrhagic fever exercise (Belgrade, November 2016)	Albania, Armenia, Bosnia and Herzegovina, Former Yugoslav Republic of Macedonia (FYROM), Georgia, Kosovo, Moldova Montenegro, Serbia, Turkey, and Ukraine	(i) Enhance knowledge and capacity on MRA (ii) Encourage multisectoral collaboration and exchange, also among neighbouring countries and assess the related added value (iii)Provide consensus on a single national level of risk across all the sectors (iv) Make participants aware of ECDC RRA guidance and FAO RA methodology (v) Make participants aware of ECDC RRA guidance and FAO RA methodology	(1) Table0top exercise on multisector risk assessment with four sectors (human and animal virology, medical entomology, and public health) by country and by regions (2) Questionnaire on the value of multisector approach (3) Evaluation questionnaire on exercise satisfaction	ECDC "operational guidance on rapid risk assessment methodology" [29] FAO "RVF in Niger risk assessment" [30]

public health), and these dots were used to mark the identified risk area on a wall poster.

As an example, we report here the outcomes of two groups. In Figure 2, country 1 assessed risk level 5 (affected risk area), country 2, risk level 2 (imperilled risk area), and country 3, risk level 1 (predisposed risk area) with final good agreement between different sectors within country. In Figure 3, countries 1 and 2 assessed risk level as 1 and 2, respectively, without final agreement between different sectors in one country.

The SWOT analysis underlined the critical role of integrated surveillance systems, laboratory capacity, and intersectoral collaboration for reliable risk assessments of arbovirus infections. The implementation of the first MRA exercise on WNV highlighted the need for enhancing the collaboration between sectors to reduce heterogeneity in risk assessment and for analysing the added value of a multisectoral approach.

3.2. The CCHF Exercise

3.2.1. Knowledge and Capacity. The results of the pre- and posttests completed by thirty-five (83%) participants of CCHF exercise showed that the exercise led to improvements in the capacity to determine *risk factors* and to identify *sources of reliable information* to assess the risk. For example, with reference to the question of the test "*Would CCHF be an unusual or unexpected threat in your country?*"10 out of 35 (29%) of the respondents replied "yes" in the pretest, while in the posttest, all the respondents (35) replied "no" to this question. This suggests that the discussion between countries and the assessment exercise helped to identify possible risk factors also at cross-border or regional level (i.e., knowledge that neighbouring countries host the pathogen).

Regarding documentation for risk assessment, we reported in Table 2 the documents mentioned by the participants to assess the level of risk for CCHF in their country.

3.2.2. The Added Value of the Multisectoral Approach. The added value of the concomitant participation of several sectors to the RA for each risk question of the exercise is reported in Figure 4. These specific aspects related to the added value of the exercise were considered particularly relevant by the project's stakeholders and therefore reported

Corresponding risk area	Risk level	Description
Free area	0	No historical circulation of WNV
Predisposed area	1	Ecological conditions suitable for WNV circulation but no historical circulation of WNV
Imperilled	2	Past evidence of WNV circulation
	3a	Evidence of WNV circulation in mosquitoes or birds in the second part of the current season (August-September-October)
	3b	Evidence of WNV circulation in mosquitoes or birds in the first part of the current season (May-June-July)
	4	WNV-specific IgM detected in local nonvaccinated horse(s) or WNV isolated from a local horse
Affected	5	Detection of at least one human case according to the EU case definition

Figure 1: Seasonal risk levels of WNV transmission to humans with the corresponding risk area and the indicators used to define the level (source ECDC), Source: [28].

in the MediLabSecure Strategic Document [31] for further developments.

The multisectoral approach was assessed as particularly valuable in "setting the scene" and in analysing comprehensively the situation having access to information and knowledge provided by each of the sectors involved in the exercise (see the added value for risk questions 1 and 5 in Figure 4 and data analysis in additional file 1).

3.3. The RVF Exercise

3.3.1. Knowledge and Capacity. The results of the pre- and posttests, completed by twenty-one (48%) participants of the RVF exercise, showed that the exercises led to improvements in the capacity to determine risk factors. Although the participants were all able to identify several relevant risk factors, some specific risks were only identified in the posttest. Among them "*animal movements*" included by 11 (52%) and 10 (48%), as relevant risk of spread of the virus in endemic and new areas, respectively, "*social and economic instability*" included by six (29%) both as relevant risk of endemic and new areas, and "*climate changes*" included by eight (38%) and seven (33%) as relevant risk of endemic and new areas, respectively. In relation to "list kind of documents to rely on to assess the level of risk for RVF in your country," in total, 18 (88%) and 19 (90%) of participants were able to mention kind of documents useful for RA of RVF in their countries in pre- and posttest, respectively.

3.4. The Added Value of the Multisectoral Approach. The country perception of the added value of the multisectoral approach is reported in Figure 5. Also, for this exercise, the multisectoral approach was particularly valuable in "setting the scene" and in analysing comprehensively the situation having access to wide range of information and knowledge provided by each of the sectors involved in the exercise (see the added value for risk questions 3, 4, and 6 in Figure 5 and data analysis in additional file 1). As for CCHF and also for RVF, the aspects related to the added value of the exercise were considered particularly relevant by the project's stakeholders and therefore reported in the MediLabSecure Strategic Document [31].

3.5. Results of the Evaluation of Three Exercises. Response rates to the evaluation questionnaire were 90% (66/73), 88% (37/42), and 68% (30/44) for WNV, CCHF, and RVF exercises, respectively.

Overall, 92% (WNV), 94% (CCHF), and 83% (RVF) of respondents found the exercise satisfactory. Ninety percent or more of respondents for each exercise found the discussion between sectors useful to identify the level of risk.

Almost all respondents reported that the objectives of the exercises were clearly communicated (99% for the WNV MRA exercise, 89% for the CCHF MRA, and 83% for the RFV MRS), while agreement on the appropriateness of the time allotted for the exercises was 92% for both WNV and CCHF and just 54% for the RVF exercise (see data analysis in additional file 1).

4. Discussion

As reported, the main aims of these exercises were to increase knowledge on MRA and raise awareness of multisectoral collaboration for conducting risk assessment of arbovirus infection with a One Health approach in the Mediterranean region. Using available tools and guidance documents allowed to avoid duplications and to refer to existing recognized published guidance.

Also, using different guidance documents helped to identify methods needed to facilitate risk assessments. For

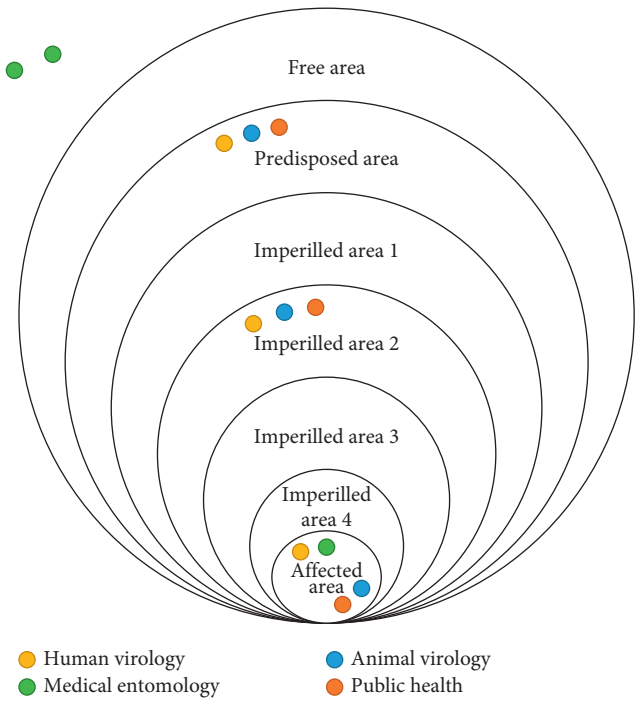

Figure 2: Perceived risk of West Nile virus using the ECDC risk assessment tool. Risk areas identified by three countries with consensus between sectors.

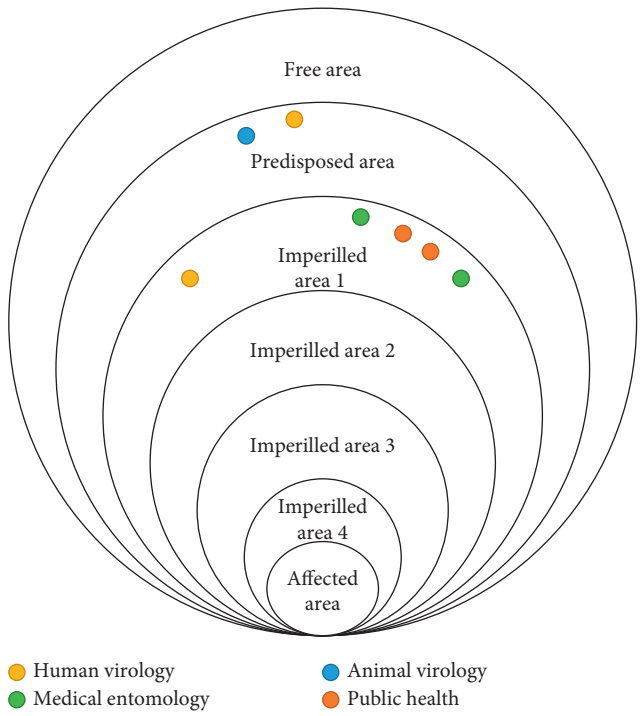

Figure 3: Perceived risk of West Nile virus using the ECDC risk assessment tool. Risk areas identified by two countries with less consensus between sectors.

example, the WNV and CCHF exercises seem to have been facilitated by the concomitant presence of "risk questions" and algorithms in the method that guide in a stepwise manner the participants towards the final assessment. The RVF exercise instead relied only on "risk questions" to guide the participants. Replying to those questions might be difficult for people not familiar with RA methodologies and/or without access to relevant information and data. This has probably generated the perception of lack of adequate time allotted for the RVF exercise, and it is also in line with the best practice identified for a joint risk assessment by WHO-OIE-FAO [21]: "at least one member of the Joint Risk Assessment (JRA) Technical Team should have experience in risk assessment to guide the process and advise on the JRA methodology."

Considering that different sectors may rightfully assess the risk differently, this approach has the advantage of enabling actors in each sector to recognize this variability and the reasons behind it. This awareness is a first step towards the identification of national intersectoral priorities in terms of surveillance and response that, in turn, can guide a OneHealth approach to resource allocation. In fact, MRA can facilitate prioritization of zoonosis in line with other proposed integrated approaches [19, 21, 36] and, in addition, allow joint evaluation of the risk of a specific zoonosis and prepare for a coordinated integrated response.

The pre- and posttests implemented during the exercises have highlighted that many participants did not perceive the relevance and need of recent published and unpublished documents (including those from neighbourhood countries) to support risk assessments. The exercises helped in understanding the relevance of different sources of information and data for RAs. However, it has to be noted that, in order to save time during the implementation, the ISS team searched and analysed in advance the available relevant documentation and synthetized the outcomes of the research in background documents distributed to participants. Data review was therefore not fully simulated. The identification of relevant sources of information by each of the sectors involved in the assessment and their sharing is the first step of the RA, and it should be considered among the relevant outcomes of intersectoral collaboration.

As highlighted by the WNV exercise, the multisectoral collaboration helped in the identification of the level of risk, and with the CCHF and RVF exercises, we explored at what stage of the RA this collaboration was more beneficial. Our findings suggest that the strategic added value of the multisectoral approach lies in its ability to create a common base of comprehensive and critical information, filing knowledge gaps, and to reduce uncertainty in risk assessment. This, in turn, facilitates the achievement of consensus on the comprehensive level of risk for the country taking into account the perspective of all sectors involved. The concomitant participation to the assessment of other countries of the region has also contributed to the identification of possible cross-border risk factors and to the assessment of a "regional" risk level. Similar outcomes were reported following the 2003 International Workshop [37] on the possibility, benefits, and obstacles of integration of ecological and health risk assessments based on the WHO "framework for integrated assessment of human health and ecological risks"

Table 2: Number of participants of the CCHF exercise who identified useful documents for RA, by type of document.

Type of document	Pretest		Posttest	
	N participants	Percentage	*N* participants	Percentage
No documents mentioned	9	25	2	6
Guidance, law decrees, plans	22	63	17	49
Guidance, law decrees, plans, scientific articles, unpublished documents, studies	2	6	14	39
Scientific articles	2	6	2	6
Total responders	35	100	35	100

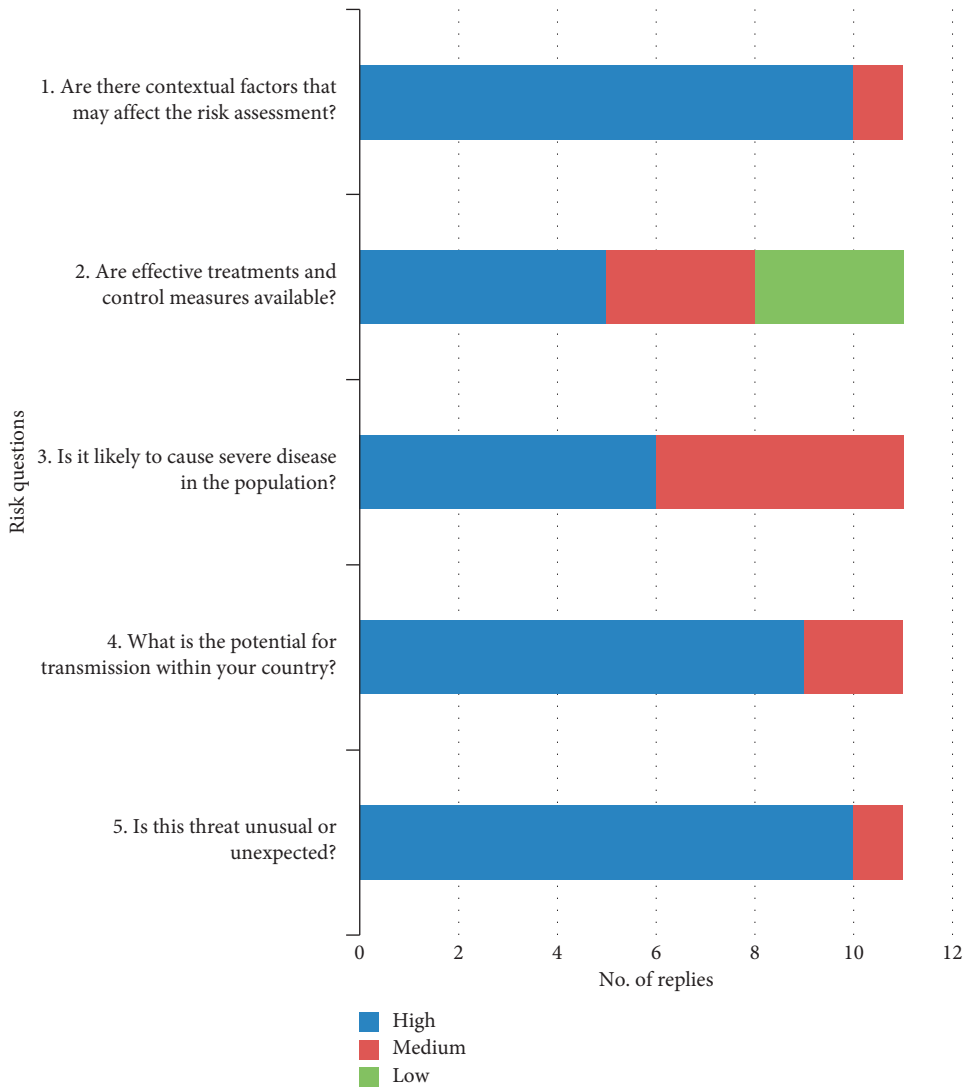

Figure 4: Added value of the multisectoral approach as assessed by participants to the CCHF exercise (11 countries).

[38]. Improved assessment quality, efficiency, and predictive capability were considered to be principal benefits of integration of risk assessments. Unfortunately, some of the obstacles to the acceptance and implementation of this approach, identified at the time, such as disciplinary and organizational barriers between disciplines, are still present. The workshop's recommendations, such as harmonization of exposure characterization, surveillance methods and models, and development of methods to facilitate comparison of risks, are still being addressed [21, 31, 39] underlying both the relevance and the complexity of the issue.

5. Conclusions

Increasing awareness of intersectoral priorities, including cross-border ones, through MRA is a new frontier which

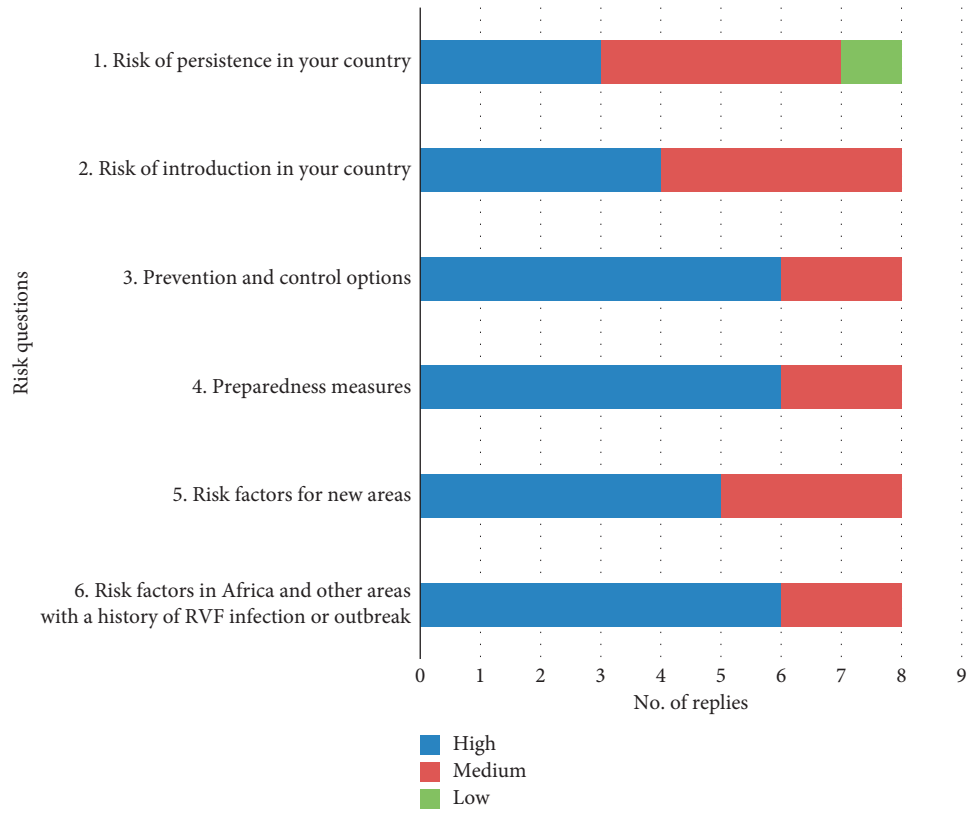

Figure 5: Added value of the multisectoral approach as assessed by participants to the RVF exercise (8 countries).

can support early warning capacities. This approach is relevant to reduce gaps due to unavailability of shared data and information, and it can also promote the use of multiple sources of information across sectors and facilitate consensus on operational arrangements for the RA, e.g., as recommended by the World Health Organisation (WHO) in the Western Pacific Regional Action Plan for Dengue Prevention and Control [5]. Given that six out of the ten threats to global health listed by WHO [40] are issues occurring at the human, animal, and environmental interface, the implementation of comprehensive regional assessments with a One Health approach made by national authorities using similar frameworks is promising in terms of the potential added value for the global health security agenda. This justifies further efforts in fine-tuning methodological approaches and addressing implementation challenges.

Abbreviations

CCHF: Crimean–Congo haemorrhagic fever
ECDC: European Centre for Disease Prevention and Control
EU: European Union
FAO: Food and Agriculture Organization of the United Nations
IZSAM: Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise
MLS: MediLabSecure project
MRA: Multisectoral risk assessment
RA: Risk assessment
RRA: Rapid risk assessment
RVF: Rift Valley fever
SWOT analysis: Strengths, weaknesses, opportunities, and threats analysis
WHO: World Health Organisation
WNV: West Nile virus.

Data Availability

The data generated or analysed during this study, including documentation and tools prepared for the exercises, are available in the references reported in this published article (refer to [32–34]) and its supplementary information files. Pre-test and post-test forms filled in by participants are in hard copies available from the corresponding author and can be provided on reasonable request making the copies anonymous.

Ethical Approval

The implementation of the exercises reported in this manuscript did not need formal ethical approval and informed consent and complies with national guidelines as per the code of ethics of Istituto Superiore di Sanità https://www.iss.it/wp-content/uploads/2017/12/CE_Codice_di_etica_2015_01_13.pdf

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

MGD developed and implemented the exercises and wrote the manuscript; FR supported the development of the exercises, the implementation of WNV exercise, and the drafting of the manuscript; WVB and LM supported the development and the implementation of the WNV exercises and critically revised the manuscript; TM and TD supported the development and the implementation of the CCHF exercise and critically revised the manuscript; BS and PC supported the development and the implementation of the RVF exercise and critically revised the manuscript; SD supported the development and the implementation of all the three exercises and critically revised the manuscript; the persons of the MediLabSecure Working Group collaborated in the exercise development and implementation. All authors reviewed and approved the final manuscript.

Acknowledgments

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Supplementary Materials

Additional file 1: file format: excel; title of data: data analysis of the evaluation of the exercises and the added value of multisectoral approach. Description of data: replies of the participants to the questions related to the evaluation of the exercises and perceived participants' added value of the multisectoral approach. (*Supplementary Materials*)

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Research Article

Qualitative Research: Institutional Preparedness During Threats of Infectious Disease Outbreaks

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Background. As demonstrated during the global Ebola crisis of 2014–2016, healthcare institutions in high resource settings need support concerning preparedness during threats of infectious disease outbreaks. This study aimed to exploratively develop a standardized preparedness system to use during unfolding threats of severe infectious diseases. *Methods.* A qualitative three-step study among infectious disease prevention and control experts was performed. First, interviews ($n = 5$) were conducted to identify which factors trigger preparedness activities during an unfolding threat. Second, these triggers informed the design of a phased preparedness system which was tested in a focus group discussion ($n = 11$). Here preparedness activities per phase and per healthcare institution were identified. Third, the preparedness system was completed and verified in individual interviews ($n = 3$). Interviews and the focus group were recorded, transcribed, and coded for emerging themes by two researchers independently. Data were analyzed using content analysis. *Results.* Four preparedness phases were identified: preparedness phase green is a situation without the presence of the infectious disease threat that requires centralized care, anywhere in the world. Phase yellow is an outbreak in the world with some likelihood of imported cases. Phase orange is a realistic chance of an unexpected case within the country, or unrest developing among population or staff; phase red is cases admitted to hospitals in the country, potentially causing a shortage of resources. Specific preparedness activities included infection prevention, diagnostics, patient care, staff, and communication. Consensus was reached on the need for the development of a preparedness system and national coordination during threats. *Conclusions.* In this study, we developed a standardized system to support institutional preparedness during an increasing threat. Use of this system by both curative healthcare institutions and the (municipal) public health service, could help to effectively communicate and align preparedness activities during future threats of severe infectious diseases.

1. Background

The four pandemics (SARS, Influenza A/H1N1, MERS, Ebola) that have emerged since the beginning of this century [1] underpin the necessity of global awareness and optimal control strategies. These outbreaks showed the potential for the worldwide spread of such severe diseases [2] and led to social unrest and large economical consequences for the affected countries [3]. During the spread of the Ebola viral disease (EVD) outbreak in West Africa, the likelihood of imported cases in Europe increased, and European countries advised their healthcare institutions to prepare for patients with suspicion of EVD. Admission of a patient suspected for EVD, or another transmittable viral hemorrhagic fever (VHF), requires a large pool of trained healthcare workers and of specialized medical facilities [4, 5]. Therefore, in the Netherlands, the care for these patients was designated to a few highly specialized hospitals.

An evaluation by the Harvard-LSHTM Independent panel on the global response to ebola concluded that international response to EVD was inadequate [6]. A multidisciplinary national EVD outbreak evaluation in the Netherlands concluded that better guidance on preparedness during threats of outbreaks was needed for diseases, such as EVD, where patients only can be admitted to highly specialized hospitals [6]. In the Netherlands, as in other countries [7, 8], it had been unclear among curative and public health institutions which preparations during a developing threat of EVD were

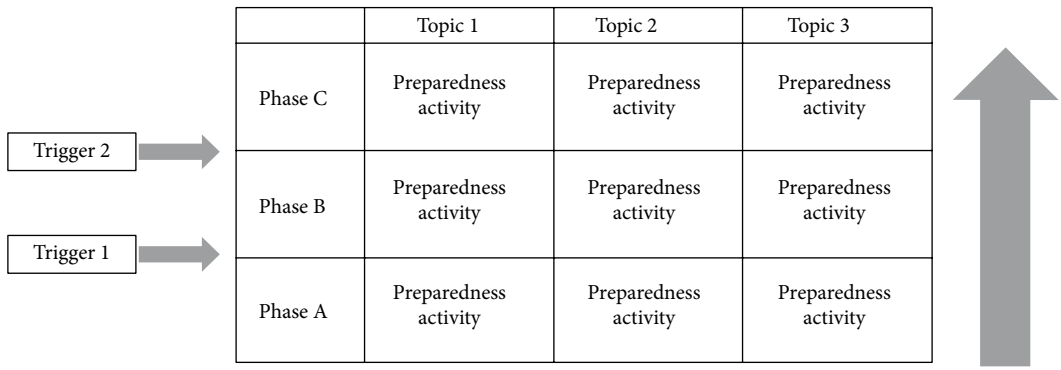

Figure 1: Concept preparedness system. The system consists of preparedness phases, defined by certain triggers; and by corresponding preparedness activities per phase, which are grouped per topic.

necessary, and for which preparations, the institutional or national level should have guidance [9].

While many studies describe preparedness for outbreaks, preparedness during a remote threat is not discussed separately in literature. The European Center for Disease Control defines preparedness for infectious diseases as "the knowledge and capacities […] to effectively anticipate, respond to, and recover from, the impacts of a likely, imminent or current crisis" [10]. Preparedness includes the development of institutional, national or international plans, communication and collaboration among different (types of) healthcare institutions, training and simulation, and surge capacity. The EVD outbreak, however, showed that successful preparedness during a threat requires flexibility and adaptations to be able to respond to differences in the probability of occurance of the disease. What these adaptations should be, has been unclear.

There is a need to clarify what preparedness entails in healthcare institutions during threats of severe infectious diseases whose patients can, due to the severity of the disease, only be admitted to designated, highly specialized hospitals (from now on described as diseases "which require centralized care"). Therefore, the aim of this study was to define preparedness during an unfolding threat of an infectious disease that requires centralized care. Second, we aimed to exploratively develop a standardized preparedness system describing preparedness activities for healthcare institutions in different preparedness phases.

2. Methods

We conducted a qualitative three-step study with an iterative design of in-depth interviews (steps 1 and 3) and a focus group (step 2), in order to identify the key elements of a preparedness system. The system includes (a) the triggers for healthcare institutions to initiate extra preparedness activities during different levels of a threat, which define preparedness phases, and (b) preparedness activities for each preparedness phase. We aimed at finding generic triggers for preparedness, applicable to different types of healthcare institutions, such as hospitals, ambulance services, general practitioners and the municipal health services. The outline of the preparedness system is shown in Figure 1. In the first round of in-depth interviews, we explored the phases and triggers of the preparedness system, which we validated in the consensus meeting. In the second round of interviews, we aligned the preparedness activities per phase and grouped them per topic. We obtained ethical approval from the medical ethical committee of the UMC Utrecht (WAG/mb/17/028319). All participants provided informed consent and were informed that their responses would be used for research purposes.

2.1. Study Population and Recruitment. For all three steps, we invited professionals working at various levels and in various healthcare institutions and public health organizations. Included healthcare institutions were academic and peripheral hospitals, ambulance services, general practitioners, and municipal health services. Figure 2 shows how healthcare institutions are involved in the case of a potential patient requiring centralized care [11]. The municipal health service (MHS) was additionally involved because of their coordinating role between all partners at the regional level. Professionals with the following backgrounds were invited:

(i) Academic hospitals: microbiologists and infectious disease specialists;
(ii) Peripheral hospitals: infection preventionists;
(iii) Ambulance services: medical managers at the regional and national level;
(iv) General practitioners: respresentattives of the National Association for General Practitioners (LHV) and Dutch College of General Practitioners (NHG);
(v) Municipal health services: regional communicable disease control consultants and infectious disease control specialists.

We used purposeful sampling by approaching the key players in the Netherlands, from the above-mentioned healthcare and public health organizations, who were involved in preparedness and/or response during the EVD outbreak. When the invited key player was not able to participate, we asked him or her to nominate a colleague in the same type of healthcare institution with comparable expertise. All professionals had, in this way, expertise in EVD preparedness or response. For step 1 and 3 we aimed for one participant per

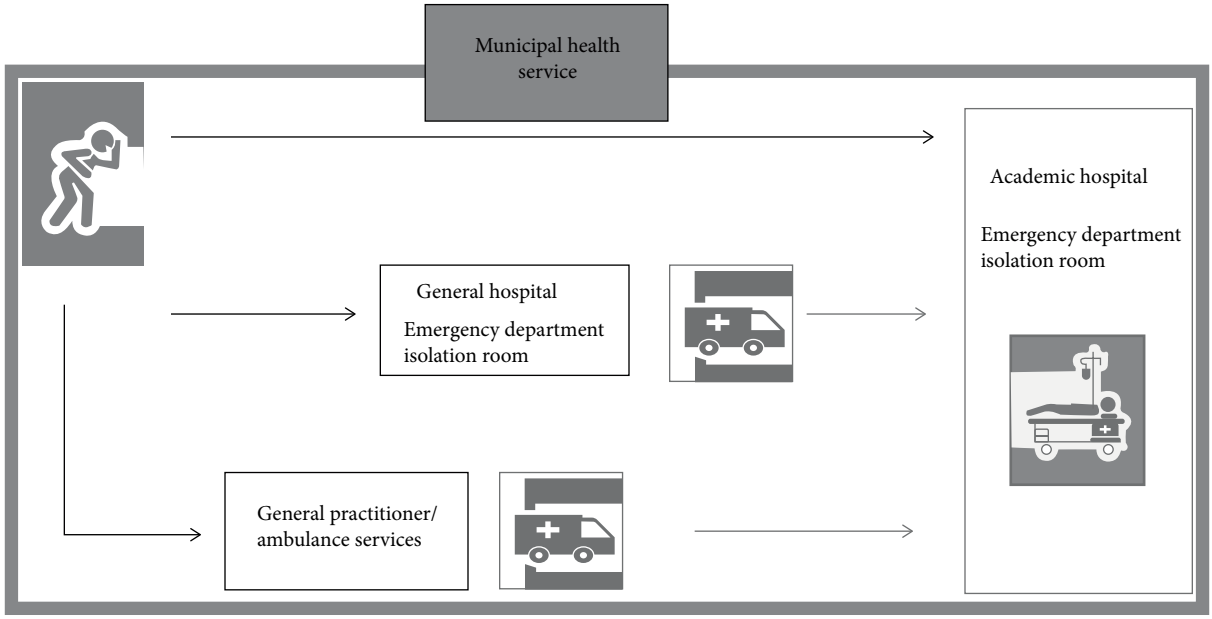

Figure 2: Healthcare institutions involved with a patient suspected for an infectious disease requiring centralized care. (Adapted from Swaan et al. [9].)

healthcare institution. For step 2, we aimed for 1–3 participants per healthcare institution. Participants were invited by e-mail and a consecutive telephone call during September–November 2017.

2.2. Data Collection. Data collection comprised three steps: individual interviews, a focus group and another individual interview round. A hypothetical scenario of a VHF outbreak was used for data collection, which was developed by experts on outbreak control of the National Institute for Public Health and the Environment (RIVM). The scenario described a fictitious Marburg virus outbreak in Uganda that spread towards neighboring countries and continuously led to exported cases throughout the world. The outbreak scenario consisted of three stages, each hypothetically representing a preparedness phase of the preparedness system, as shown in Figure 1. Based on the identified triggers, preparedness phases for the preparedness system were developed. For each of these preparedness phases, again a corresponding scenario of the Marburg virus outbreak was developed to discuss in the focus group of step 2. The preparedness system was adjusted based on the results from step 2 and was sent by e-mail to the participant to discuss in step 3. The results of step 3 were used to finalize the preparedness system by grouping preparedness activities into overarching topics, and in institutional and collaborative activities.

2.2.1. Step 1. Step 1 consisted of individual, in-depth, semi-structured interviews. To ensure that collected data was as reliable and consistent as possible, an interview guide was developed beforehand (additional file 1). The interview guide for step 1 was piloted with a microbiologist at a Dutch academic hospital. All interviews were conducted by one researcher (DdR), to safeguard inter-observer reliability. Interviews took between 35 and 50 minutes. During the interviews, the interviewer presented the outbreak scenario to the participant. Per preparedness phase, the participant was asked if and why preparedness activities would be necessary for their healthcare institution. In this way, the triggers for preparedness activities were explored. Alongside, questions covered terminology for preparedness during a threat, responsibility for preparedness during a threat, and collaborative preparedness activities carried out together with other healthcare institutions.

2.2.2. Step 2. In step 2, a mixed focus group discussion with 1–3 representatives per type of healthcare institution was organized to validate the concept preparedness system. A focus group guide was developed beforehand (additional file 1). The focus group was guided by two researchers (DdR and CS), and supported by an expert in guiding focus groups (RE). The focus group took place at the RIVM and lasted 2 hours and 15 mintues. Per preparedness phase, the corresponding scenario was presented. Participants were asked if and why preparedness would be necessary within their institutions, what they would expect the other healthcare institutions to do, and where cooperation and support between healthcare institutions was needed. In the second phase of the focus group, preparedness activities identified in step 1 were presented to representatives of each type of healthcare institution separately. Representatives of one type of healthcare institution debated if and in which preparedness phase these preparedness activities were needed. Subsequently, terminology for preparedness during a threat was discussed among all participants, since the interpretation of terminology had shown to be different.

2.2.3. Step 3. Step 3 consisted of individual, in-depth, semi-structured interviews. We included one participant per type of healthcare institution out of the participants in step 2. An

interview guide was developed beforehand (additional file 1). Interviews were conducted by telephone and were all conducted by the same researcher (DdR). Interviews took between 15 and 20 minutes. The preparedness system was reviewed during the interview by discussing preparedness activities per phase. The participants discussed specific needs and adaptations per preparedness phase of the preparedness system. Further analyses included comparing preparedness activities between healthcare institutions, to see whether their expectations matched.

2.3. Data Analysis. Each interview and focus group was voice-recorded, with permission from the participants, and transcribed. Transcription started directly after the first interview and continued parallel to further data collection. Data were processed anonymously using a coding system. A summary of every interview and focus group was sent to the participants to verify their input. All interviews and focus group sessions were coded using content analysis. A coding guide (additional file 2) was developed beforehand, based on the structure of the interview guide. For each step, the guide was expanded and adapted. Coding was done by two researchers independently (DdR, and DR), using ATLAS.ti [12], and differences were discussed until consensus was reached. Data collected from each step of the study were analyzed and interpreted before the beginning of the subsequent step.

3. Results

3.1. Study Population. In step 1, we invited 8 participants, of whom 3 could not be included. Five experts participated in the interview round: a microbiologist of an academic hospital, an infection preventionist in a general hospital, a medical manager of the national ambulance service with extensive experience as an ambulance nurse, a practicing GP and representative of the LHV, and a regional communicable disease control consultant of a municipal health service. In step 2, we invited 24 participants of whom 13 could not be included. Eleven experts participated in the focus group: 1 microbiologist and 1 infectious disease specialist of two different academic hospitals, 3 infection preventionists of different general hospitals, 2 medical managers of different regional ambulance services, 1 GP who also was representative of the NHG, and 3 regional communicable disease control consultants of different municipal health services. In step 3, we re-invited 7 participants from step 2, of whom only 3 accepted participation: one of the infection preventionists, the GP and representative of the NHG, and one of the regional communicable disease consultants. All representatives of academic hospitals and medical managers of the national ambulance services were either not responding or not able to participate due to time constraints. For all steps, reasons why professionals could not be included were the absence of reaction to the invitation ($n = 7$), unavailability during the data collection period ($n = 11$), completeness of inclusions ($n = 2$). Figure 3 provides an overview of the different steps, the number of included participants and their backgrounds.

3.2. Terminology. During the first interview round, "scaling up" and "enhanced preparation" were used as synonyms by the interviewer for different preparedness phases. In the focus group, we observed differences in interpretation between curative and municipal health services. According to the curative partners, the Dutch term for upscaling that was used, applied to the response phase "with the presence of an actual potential patient". In contrast, for the MHS, the term could also be used for preparedness during an increasing threat. The need for congruent language was highly stressed by the participants, and consensus was reached on the definition of *"enhanced preparedness"* to describe preparedness activities during a threat.

3.3. Triggers. During the first interview round several factors that trigger preparedness activities were identified for different healthcare institutions. The microbiologist at an academic hospital reported that they were at all times ready for such cases. However, they would enhance preparations if the likelihood of admitting a VHF patient increases, such as or with repatriated staff from the outbreak area. Municipal health services started with preparedness activities as soon as the outbreak somewhere in the world occurred and would be further enhanced when health institutions in their region were likely to become involved. For academic hospitals, general hospitals and ambulance services, (1) the likelihood that an unexpected potential patient was presented to their healthcare institution, and/or (2) unrest among the general population and staff, triggered preparedness activities. For general practitioners, only an outbreak in their would lead to preparedness activities.

In the focus group, trigger 1 and 2 were accepted as main triggers distinguishing between preparedness phases. Besides, a third trigger was the situation of several (potential) patients hospitalized within the country, conceivably leading to different referral pathways between healthcare institutions. Not all triggers would lead to the same intensity of extra preparations in all healthcare institutions, but all healthcare institutions would be involved in these phases. And most importantly, they all agreed upon the need for univocal communication.

The final preparedness system based on these three triggers consists of four preparedness phases and is shown in Figure 4. Preparedness phase green is a situation without the presence of the infectious disease threat that requires centralized care, anywhere in the world. Preparedness phase yellow is the occurrence of the disease somewhere in the world but without triggers one and two. In preparedness phase orange, trigger one or two applies, and in preparedness phase red trigger number three applies.

3.4. Institutional Preparedness Activities. The preparedness activities as derived from step 1 and 2 and tested in step 2 and 3, were divided by topic as shown in the institutional preparedness column in Figure 4. All participants needed preparedness activities on infection prevention, such as the right type and stock of personal protective equipment, donning and doffing procedures, and waste management. Regarding diagnostics, academic hospitals described preparedness activities. These consisted mostly of extra checks whether

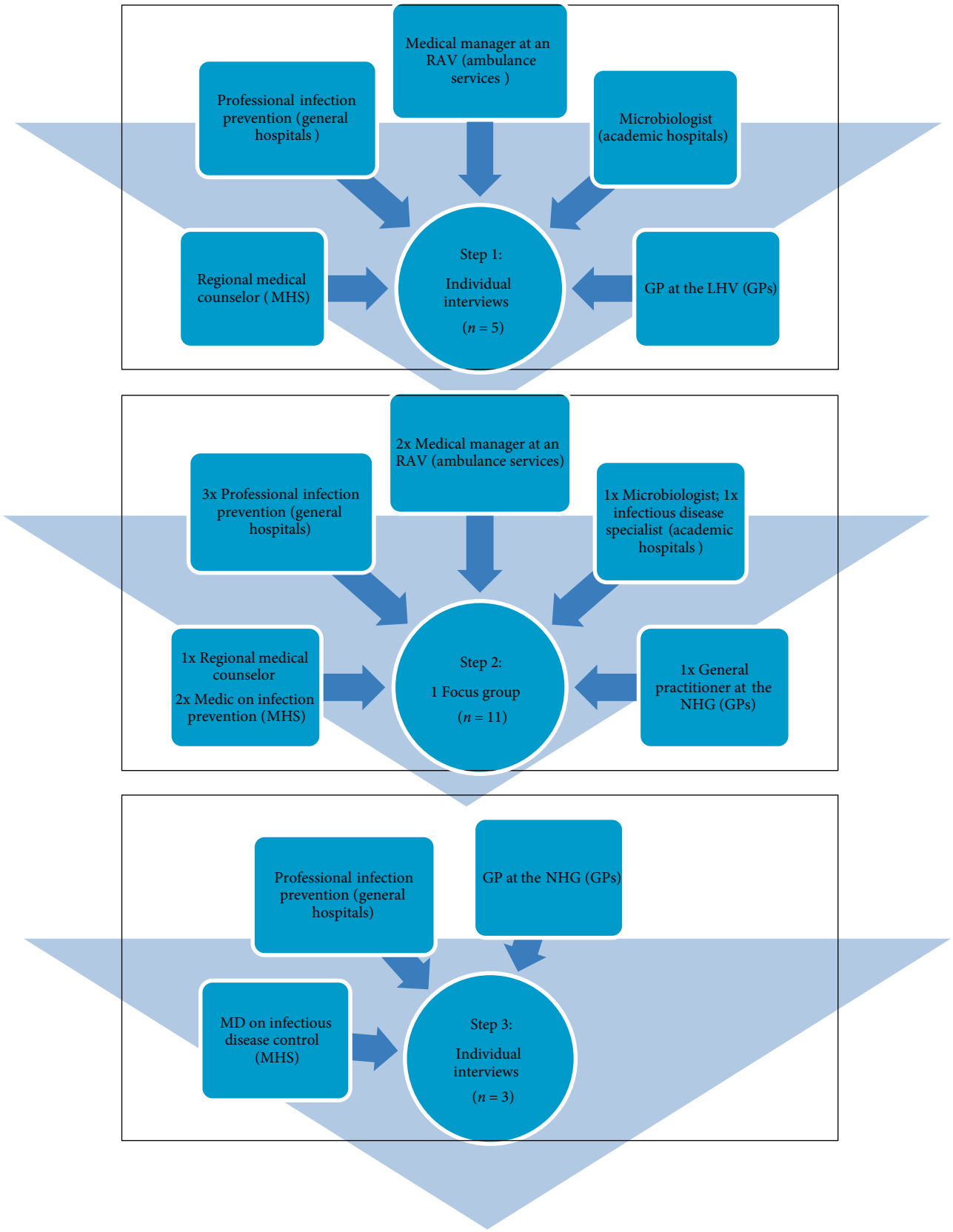

Figure 3: Included participants per step. N = number of participants, MD = medical doctor, RAV = region of ambulance services, MHS = municipal health service, GP = general practitioner, LHV = national association for general practitioners, NHG = dutch college of general practitioners.

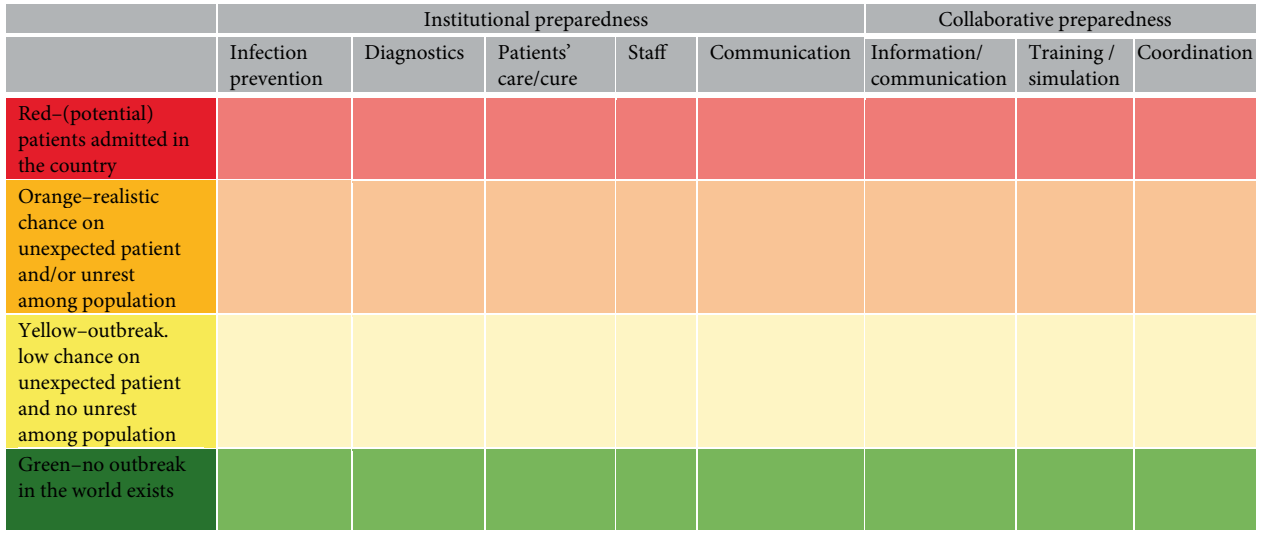

Figure 4: Preparedness system for threats of infectious diseases requiring centralized care. The system provides in rows the four ascending preparedness phase, and in columns the preparedness activities divided by content and grouped in institutional preparedness within or collaborative preparedness among healthcare institutions.

differential diagnoses for these patients could run. Academic hospitals, peripheral hospitals and ambulance services named preparedness activities for patient care. Academic hospitals and peripheral hospitals discussed the need to prepare their personnel for extra working hours, or the need for extra supplies. General practitioners and municipal health services needed preparedness for controlling unrest among their staff and the population, e.g., by informing staff and the availability of a telephone line for questions. In additional file 3a, an overview of identified preparedness activities that resulted from step 2 and 3 are shown per type of healthcare institution. We identified the following trends:

(i) Academic hospitals start with all preparedness activities from preparedness phase yellow on. In preparedness phase orange, a sub-commission on preparedness for the admittance of patients needs to be installed, and in preparedness phase red, there will be a need to consider more ethical challenges related to the threat and challenges related to a shortage of staff.

(ii) Peripheral hospitals inform triage staff and professionals at the gate during preparedness phase yellow. In preparedness phase orange, preparedness activities should start, except for diagnostics and patients' care/cure. In preparedness phase red, mainly a more intense communication among hospital departments and healthcare institution in the region is needed.

(iii) For ambulance services, preparedness activities start in preparedness phase orange and no clear difference in preparedness activities were identified between preparedness phase orange and preparedness phase red.

(iv) For general practitioners, phase orange is most important. The preparedness activities of general practitioners are limited to triage and primary infection prevention in all preparedness phases. Ethical considerations start in preparedness phase orange as well, but policy on this should ideally be made in the green phase.

(v) For MHS, diagnostics and training are required from preparedness phase yellow on, depending on the type of pathogen. In preparedness phase orange and red, they start to prepare their internal communication and personnel capacity.

3.5. Ethical Considerations. During step 1 and 2, ethical considerations were mentioned by representatives of all institutions except the municipal health services. The considerations of GPs, academic hospitals and ambulance services described how to deal with suspected cases in life-threatening situations. They need guidance on when concerns for their own safety would overrule their duty as a care provider, and based on which criteria. Another aspect named several times by representatives of ambulance services, general hospital and academic hospitals was the priority of care: to respectively transport, temporarily accommodate, or care for one EVD suspected patient, meant that many other patients could not receive care because an ambulance, an emergency department or large parts of intensive care had to close due to cleaning procedures, panic reduction or lack of staff or resources. Participants explicitly stated that these were dilemmas they faced during the latest EVD outbreak, and they would still face them should a case be admitted today.

3.6. Collaborative Preparedness. In addition to institutional preparedness activities, collaborative preparedness activities were discussed in all interview rounds. These are activities that overarch individual healthcare institutions or should be performed by multiple healthcare organizations together. We identified collaborative preparedness activities in information, training and simulation, and coordination, as shown in the

columns headed "collaborative preparedness" in Figure 4. The expectations of the different types of healthcare institutions regarding information and coordination match well between healthcare institutions. This implies that information exchange between organisations is reported to be adequate. Furthermore, information on case definitions and information on the current preparedness phase is expected from the national centre for disease control. What did not match were the expectations of the different types of healthcare institutions regarding training and simulation exercises. The need to perform training or exercises together was mentioned by ambulance services and academic hospitals towards each other. But for peripheral hospitals, the need to practice together with ambulance services varied among participants. An overview of collaborative preparedness activities per preparedness phase is shown in additional file 3b. Participants of all types of healthcare institutions stressed that aligned preparedness activities are preferred over institutional autonomy. However, healthcare institutions with a specific function should be able to deviate from the preparedness system activities. Examples are healthcare institutions serving points of entry or those with national tasks such as the academic hospital with the reference laboratory.

4. Discussion

The aim of this study was to define preparedness during an unfolding threat of an infectious disease that requires centralized care. Second, we aimed to develop a standardized system describing preparedness activities per preparedness phase for healthcare institutions. We developed this standardized system by defining phases of preparedness during a threat and their corresponding preparedness activities, within both the perspective of individual healthcare institutions and of the collaborative network in which these institutions need to function. The four identified preparedness phases were based on (a) the likelihood of presentation of an infected patient to one of the healthcare institutions and (b) the unrest among the general population and staff. Phases ranged from no outbreak to the situation in which several potential or confirmed patients were hospitalized, conceivably leading to other referral pathways in the country. This system could be used for any future threat from an infectious disease requiring centralized care.

Using phases in preparedness to threats is not new. For terrorist attacks, for example, a level system using numbers 1–5 is common in several European countries, with 1 being considered a low threat, and 5 being a critical one [13]. In the Netherlands, a code system using colors is used in the weather forecasting, ranging from green ("business as usual"), through yellow and orange, to code red ("high impact on society") [14]. And the WHO announced a pandemic phase system during the influenza outbreak (H1N1) in 2009, reaching from 0 to 6 [15]. However, by our knowledge, explicit preparedness phases following an unfolding threat caused by an infectious disease that offers concrete measures for frontline institutions have not been identified in literature. Certainly, we acknowledge the existence of the pandemic phases of the WHO [15]. These phases reflect the preparedness activities at a global, international and national level, rather than the institutional level within a country. The need for such specific phases for frontline institutions emerged during the evaluation of the Ebola threat [16], since all types of healthcare institutions experienced the need to perform extra activities to stepwise increase their level of operational response as the threat evolved. Healthcare institutions need thus to adapt their preparedness activities to the level of a threat.

The identified triggers for enhanced preparedness match with other studies and theory. Schol et al. identified higher fear among Dutch healthcare workers during the threat of Ebola and identifies a relation between fear and the need for information. This study provides support for our finding that unrest is a trigger for enhanced preparedness (in this case by providing additional information) [17]. The founding risk classification theory of Kinney and Wiruth (1976) [18]states that risk is the chance that something happens times the impact of that event. Within this formula, the presentation of an unexpected patient is the event that could happen, and the unrest among healthcare workers and the population represents impact. Together they define the risk, which is then translated in the urge to prepare. In this way, the phase system in this study builds upon the literature on risk classification.

Studies showed that extra preparedness was needed for countries during threats with increasing severity of outbreaks elsewhere in the world [7, 8, 19, 20]. However, these studies report on disease-specific preparedness activities and, therefore were, not necessarily applicable to other threats. This study used Marburg virus disease in the scenario and included experts with EVD outbreak experience. We worked in the aftermath of the EVD outbreak, but used a case of another disease. Hereby, we strongly aimed to work towards a generic preparedness system.

While specific preparedness activities differ between types of healthcare institutions and threat phases, in this study, a uniform enhanced preparedness system has been developed. During interviews, the focus group, healthcare institutions expressed the need to communicate explicitly and uniformly about preparedness activities. It became clear that there is no uniform terminology among experts from different healthcare institutions. For example, the term "scale-up" applies in curative care to the act of responding to an actual patient, while in public healthcare, the term could also be used during the preparedness. Absence of uniform terminology impedes communication between public and curative health care, while smooth communication between the two is a must, especially during threats or outbreaks. With clear definitions of phases, our system offers this uniformity both within institutions, as well as among institutions. It could therefore be used to effectively arrange communication about the required specific enhanced preparedness.

Although this specific study was conducted in the Netherlands, the results are also applicable in other countries with a comparable organization of healthcare. Centralized care in dedicated health centers for patients suspected for an infectious disease requiring centralized care, is described in Israel [7], the United States (New York State) [19], and in Canada [20]. Besides, they can be of value in other countries, because

past experience with outbreaks has shown that presentation or even the likelihood of imported patients, indeed led to unrest among the general population and hospital staff [21, 22].

Our study has several limitations. There was a high attrition rate between the focus group and last interview round, leading to a lack of representation of academic hospitals and ambulance services. This has led to gaps in the completeness of the review of preparedness activities per stakeholer and per phase. However, to increase validity the outcomes of this study were presented and discussed in a 1,5-hour slot during the regular national meeting on EVD preparedness. During this meeting with national representatives of academic and peripheral hospitals, ambulance services and MHS who had been involved with preparedness and response during the EVD outbreak of 2013–15, the findings of this study were endorsed. This strongly supports further generalizability for both institutional as well as collaborative preparedness.

Another limitation is that data collection was only through interviewing, whereby direct observation of preparedness activities might yield additional findings. Also conducting a simulation exercise might lead to other insights. Moreover, we need to acknowledge the chance of recall bias. Although we used a new scenario, participants referred to activities they had performed two years before, during the EVD outbreak. This could have resulted in the identification of preparedness activities in phase yellow, orange and red that should not be performed in that phase. Participants might have reported from previous EVD experience where sometimes activities were performed in phase yellow, organge or red, whereas ideally these should be tackled in the green phase. An example are the ethical considerations, which indeed turn up during higher phases, but which should be covered in standard guidelines or procedures. An additional limitation is that most participants worked in the most urbanized parts of the Netherlands. Although regional organization might be different in the more rural regions, we have chosen to approach healthcare institutions with most experience with infectious diseases requiring centralized care. The expertise of the participants can be mentioned as a strength.

Since there was a strong need for a system that identifies different phases of a threat and the corresponding activities, this preparedness system could be used as a communication tool on a national or regional level. Future research should focus on identifying all activities for each phase. The completed preparedness system can be used by healthcare instiutions as a checklist of all preparedness activities they should perform during unfolding threats. In addition, it can be used as an agenda-setting for regional meetings to discuss the collaboration between healthcare institutions for unfolding threats. Finally, we recommend investigating the applicability of this system to other severe infectious diseases, not requiring centralized care. Examples could be, the recent outbreak of plague in Madagascar [23] or the ongoing threat of the Middle East Respiratory Syndrome-coronavirus [24]. Roles and responsibilities among types of healthcare institutions, in case of outbreaks of these diseases, vary and it is possible that other triggers and preparedness activities are required. Our phased preparedness system may also be applicable in these situations.

5. Conclusion

This study investigated preparedness during threats of infectious diseases requiring centralized care. This is the first study that explicitly defines preparedness activities during a threat for different frontline healthcare institutions. We reached consensus that a standardized preparedness system is required. A phased preparedness system has been developed, which can be used for improving institutional preparedness in curative healthcare institutions, and collaborative preparedness among curative healthcare institutions and the public health services.

Abbreviations

EVD: Ebola virus disease
GP: General practitioner
LHV: National association for general practitioners
MD: Medical doctor
MHS: Municipal health service
N: Number
NHG: Dutch college of general practitioners
PPE: Personal protective equipment
RAV: Region of ambulance services
RIVM: National institute for public health and the environment
VHF: Viral hemorrhagic fever
WHO: World Health Organization.

Data Availability

The datasets generated and/or analyzed during the current study are not publicly available due to the privacy protection of the participants, but are available from the corresponding author on reasonable request.

Ethical Approval

We obtained ethical approval from the medical ethical committee of the UMC Utrecht (WAG/mb/17/028319).

Consent

All participants provided informed consent and were informed that their responses would be used for research purposes.

Disclosure

Earlier versions of this manuscript have been presented in oral presentations two congresses: the 3rd Northern European Conference on Emergency and Disaster Studies, The Netherlands, Amsterdam 21–23 March 2018; and 11th European Public Health Conference Winds of change: towards new ways of improving public health in Europe Ljubljana, Slovenia 28 November–1 December 2018. The study was funded by the National Institute for Public Health and the Environment (RIVM).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

DdR collected, analyzed and interpreted the data and has drafted the manuscript. EB has been involved in designing the data collection and in revising the manuscript critically for important intellectual content. RE has been involved in the conception of the data collection and guided the focus group. DR has performed coding of the data. CS has made substantial contributions to the design of the study and interpretation of data and had supervision over the research project. AT has critically revised the manuscript and has given final approval of the version to be published. All authors read and approved the final manuscript.

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Supplementary Materials

Additional File 1a: Guide for individual, semi-structured interviews, belonging to step 1 (written in Dutch). Additional File 1b: Guide for the mixed focus group, belonging to step 2 (written in Dutch). Additional File 1c: Guide for individual, semi-structured interviews, belonging to step 3 (written in Dutch). Additional File 2: Coding guide used for qualitative content analysis of data in step 1–3. (written in Dutch). Additional File 3a: Institutional preparedness activities per type of healthcare institution and per preparedness phase (written in Dutch). Additional File 3b: Collaborative Preparedness activities per preparedness phase. (*Supplementary Materials*)

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Research Article

Preventive CTLA-4-Ig Treatment Reduces Hepatic Egg Load and Hepatic Fibrosis in *Schistosoma mansoni*-Infected Mice

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Background. Hepatic fibrosis and granuloma formation as a consequence of tissue entrapped eggs produced by female schistosomes characterize the pathology of *Schistosoma mansoni* infection. We have previously shown that single-sex infection with female schistosomes mitigates hepatic fibrosis after secondary infection. This was associated with an increased expression of cytotoxic T-lymphocyte-associated protein-4 (CTLA-4), known as a negative regulator of T cell activation. Based on these findings, we hypothesized that administration of agonistic CTLA-4-Ig (Belatacept) is capable to prevent and/or treat hepatic fibrosis during schistosomiasis. *Methods*. Mice were infected with 50 *S. mansoni* cercariae and CTLA-4-Ig, or appropriated control-Ig was administered for 4 weeks. Preventive treatment started 4 weeks after infection, before onset of egg production, and therapeutic treatment started 8 weeks after infection when hepatic fibrosis was already established. *Results*. When given early after infection, livers of CTLA-4-Ig-treated mice showed significantly reduced collagen deposition and decreased expression of profibrotic genes in comparison to controls. In addition, administration of CTLA-4-Ig suppressed the inflammatory T cell response in infected mice. If therapy was started at a later time point when fibrogenesis was initiated, CTLA-4-Ig had no impact on hepatic fibrosis. *Conclusion*. We could demonstrate that an early preventive administration of CTLA-4-Ig suppresses effector T cell function and therefore ameliorates liver fibrosis. CTLA-4-Ig administration after onset of egg production fails to treat hepatic fibrosis.

1. Introduction

Schistosomiasis is a debilitating tropical disease caused by infection with trematode worms of the genus *Schistosoma* spp.. Currently, more than 200 million people, mostly in the tropic and subtropics, are affected; more than 700 million people in 78 countries are at risk of the infection [1]. The larvae of *Schistosoma (S.) mansoni*, one of the most common *Schistosoma* species besides *S. haematobium*, penetrate the skin of their hosts and migrate via the blood circulation, transiting the lungs to reside as adults in the mesenteric veins, where they mate and lay eggs around 5 weeks after infection. Parts of these eggs enter the portal circulation and are entrapped within the small liver sinusoids. Here, they induce a perioval granulomatous reaction resulting in severe hepatic fibrosis characterized by the excessive deposition of extracellular matrix proteins [2].

S. mansoni-induced hepatic fibrosis is of high relevance among chronic liver diseases worldwide. The pathology is mainly induced by cellular immune responses to tissue-entrapped eggs and orchestrated by $CD4^+$ T cells. The early immune reaction to adult worm antigens is dominated by proinflammatory Th1 cytokines, but shifts towards a Th2-biased immune response following egg deposition. The granulomatous reaction is characterized by the infiltration of $CD4^+$ Th2 cells, eosinophils, and alternatively activated macrophages, as well as the production of Th2 cytokines (IL-4, IL-5, and IL-13) promoting tissue fibrosis [3, 4]. During subsequent chronic infection, granuloma formation is downregulated by an upcoming regulatory T cell

response, while sustained Th2-driven fibrotic response to egg antigens leads to clinical anomalies such as portal hypertension and subsequent ascites and life-threatening esophagus varices ruptions [5, 6].

The cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) is known as a crucial negative regulator of T-cell activation and proliferation. It acts as an antagonist of CD28-ligand interactions by competitive binding to CD80 and CD86 on antigen-presenting cells [7]. The ability of CTLA-4 to selectively suppress T-cell-mediated immune responses has made it a promising target for therapeutic interventions. Antagonistic CTLA-4 antibodies, such as ipilimumab, increase immune activation and are successfully used in tumor therapy [8, 9], whereas agonistic CTLA-4 fusion proteins, like commercially available belatacept and abatacept, act immunosuppressive. Treatment with belatacept has been shown to prevent rejection of allografts, particularly renal transplants [10, 11], and abatacept is used to treat rheumatoid arthritis [12, 13].

With respect to murine *S. mansoni* infections, blocking of CTLA-4 during acute infection was associated with significant weight loss and altered type 2 cytokine responses indicating the crucial importance of this regulator during *S. mansoni* infection [14]. Moreover, we recently reported that single-sex infection with female *S. mansoni* cercariae mitigates hepatic fibrosis after secondary infection, which was associated with an increased expression of CTLA-4 in these mice [15, 16].

We therefore hypothesized that a primary infection with female *S. mansoni* and the related antifibrotic effect can be mimicked by a CTLA-4-Ig administration. We performed two experimental approaches: (i) preventive CTLA-4-Ig treatment, starting at week 4 after infection and (ii) therapeutic CTLA-4-Ig treatment starting at week 8 after infection to investigate the therapeutic potency of CTLA-4-Ig in counteracting the profibrotic immune reactions. We herein demonstrated that preventive, but not therapeutic, CTLA-4-Ig treatment ameliorated hepatic fibrosis.

2. Methods

2.1. Ethics Statement. All animal experiments were performed in strict accordance with the German regulations of the Society for Laboratory Animal Science and the European Health Law of the Federation of Laboratory Animal Science Associations. The protocol was approved by the local committee on animal care and use (7221.3-1-034/18-1). All efforts were made to minimize the suffering of animals.

2.2. Mice Infection and Study Design. Eight-week-old female C57BL/6 mice were percutaneously infected with 50 cercariae of *S. mansoni* (Belo Horizonte strain) obtained from our in-house cycle of infected *Biomphalaria glabrata* snails (Brazilian strain) as previously described [15]. For treatment, belatacept (Nulojix, Bristol-Myers Squibb, Germany) and appropriate control antibodies (MP Biomedicals/Fisher scientific, Germany) were diluted in PBS (100 μg/ml). Mice were administered 10 mg/kg belatacept or control-Ig three times a week by intraperitoneal injection for four weeks [16]. Preventive treatment started 4 weeks after infection (p.i.), and mice were sacrificed 8 weeks p.i.. Therapeutic treatment was started after egg deposition 8 weeks p.i., and mice were sacrificed 12 weeks p.i. (Figure 1(a)). We determined the following group sizes: uninfected naïve mice, $n = 6$; *S. mansoni*-infected mice, $n = 12$. Since not all animals in the therapeutically treated groups (scarification 12 weeks p.i.) were successfully infected (no granuloma formation and no increase of spleen and liver), we excluded these mice from the analysis. Due to the step-by-step experimental procedure, the results of this study were reproduced several times. On each processing day, we analyzed at least 2 to 3 animals of each group to ensure comparability.

2.3. Assessment of Pathology. The total amount of collagen in weighted liver fractions was quantified based on the colorimetric detection of hydroxyproline using a Quickzyme Total Collagen assay kit (Quickzyme Biosciences) according to the manufacturer's instructions. For histological evaluation, a standardized part of the right liver lobe was fixed in 10% neutral buffered formalin solution (Sigma-Aldrich) and embedded in paraffin. Thin sections of 5 μm were stained for collagen with Sirius red (SR) or with haematoxylin/eosin (H&E). Granuloma size was determined using ImageJ software (v1.47v; National Institutes of Health, USA).

The relative weight of spleens and livers was expressed as the ratio of organ to body weight. Serum biochemistry for alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (AP) was performed using a UniCel® DxC 800 Synchron® Clinical System (Beckman Coulter GmbH). Total egg numbers were assessed by microscopic evaluation (100-fold magnification) of weighted liver fractions.

2.4. Quantitative RT-PCR. Total RNA was isolated from liver tissue (RNeasy Plus Mini Kit, Qiagen) and reversely transcribed into cDNA using a High-capacity cDNA Reverse Transcriptase Kit (Thermo Fisher) according to the manufacturer's instructions. Real-time PCR (RT-PCR) was performed using the following TaqMan Gene Expression Assays: *Col1a2* Mm00483888; *Acta2* Mm00725412; *Mmp2* Mm00439498; *Timp1* Mm01341361; *Il13* Mm00434204; *Il4* Mm00445259; *Infg* Mm01168134; and *Il10* Mm01288386 (Thermo Fisher). Gene expression values were normalized to the endogenous reference gene *Gapdh* (Rodent GAPDH control reagent, ThermoFisher) and presented as normalized, relative expression values to naive controls.

2.5. Cell Preparation. Single-cell suspensions were prepared by passing the spleen through a cell strainer (100 μm) followed by PBS washing and erythrocytes lysis with RBC lysis buffer (BioLegend). Cells were washed twice with PBS and cell numbers were quantified using a CASY TT cell counter (OLS-Omni Life Science).

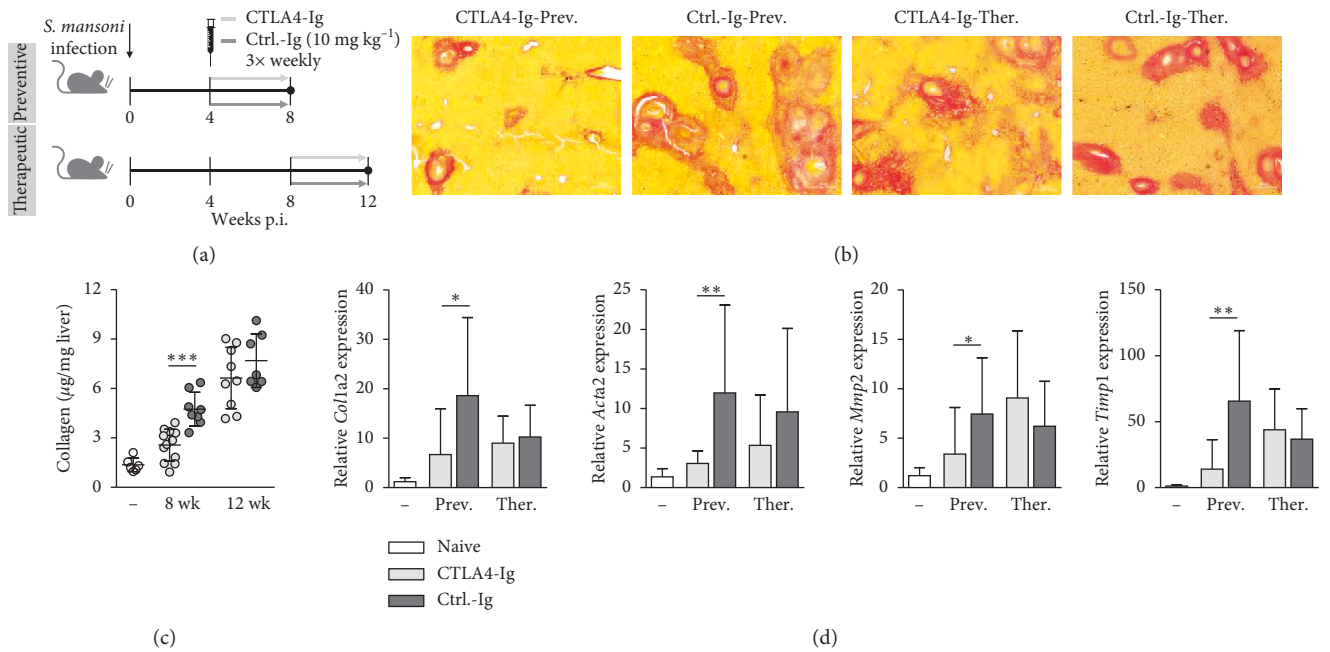


FIGURE 1: Preventive CTLA-4-Ig treatment reduces hepatic fibrosis, but has no therapeutic effect. (a) Schematic of *S. mansoni*-infected mice receiving either CTLA-4-Ig or ctrl.-Ig. (10 mg/kg) for 4 weeks 3 times weekly (preventive and therapeutic approach). (b) Representative images of liver sections stained with Sirus-Red are shown; 100x magnification. (c) Collagen deposition in livers of mice was quantified by measurement of hydroxyproline ($n = 6$–12 out of 4 independent experiments). (d) Relative gene expression of *Col1a2, Acta2, Mmp2,* and *Timp1* in livers of mice was determined by real-time PCR ($n = 6$–9, performed in triplicates). Data are represented as mean ± SD from triplicate data.

2.6. Flow Cytometry. Cells were stained with a Zombie Red™ Fixable Viability Kit (BioLegend) for 15 min at RT in PBS followed by incubation with appropriate fluorochrome-conjugated antibodies for 20 min at 4°C in FACS buffer (PBS + 3% FCS). The following antibodies were used: lymphoid panel anti-CD3-APC (clone 145-2C11), anti-CD4-PerCP-Cy5.5 (clone RM4-4), anti-CD8-PE-Cy7 (clone 53-6.7), anti-CTLA-4-PE (clone UC10-4B9), IgG Isotope Ctrl.-PE (clone HTK888); myeloid panel anti-CD11b-APC (clone M1/70), anti-CD11c-Alexa488 (clone N418), anti-F4/80-PE-Cy7 (clone BM8), and anti-CD86-BV605 (clone GL-1). All antibodies were purchased from BioLegend. After washing, flow cytometric analysis was performed using FACS Aria™ IIIu (BD Bioscience), and data were analyzed by FlowJo software (v10.0.7, Tree Star Inc., CA, USA). Live cells were differentiated by gating on the following cell populations: T cells ($CD3^+/CD4^+$ or $CD3^+/CD8^+$), macrophages ($CD11b^+F4/80+$), and dendritic cells ($CD11c^+$). A representative gating strategy for CTLA-4 is given in supplementary Figure 1.

2.7. Cytokine ELISAs. For assessment of cytokine production, isolated splenocytes were cultured in RPMI 1640 medium supplemented with 10% FCS, 25 mM HEPES and antibiotics. Cells were stimulated by an in-house generated 10 μg/ml *S. mansoni* soluble worm antigen prepartion (SWAP) for 72 h at 37°C [16]. Cytokines in cell-free supernatants were quantified using DuoSet ELISAs Kits (R&D Systems) detecting IL-13, IL-4, INF-γ, or IL-10 according to the manufacturer's protocol.

2.8. Statistics. Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA). Values are expressed as mean + SD. Differences between groups were analyzed by Mann–Whitney *U* test, and the *p* values < 0.05 were considered statistically significant. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

3. Results

3.1. Preventive CTLA-4-Ig Treatment Reduces Hepatic Fibrosis but Has No Therapeutic Effect. To investigate whether CTLA-4 impacts the development of hepatic fibrosis during Schistosomiasis, we treated *S. mansoni*-infected mice with CTLA-4-Ig or appropriate control antibodies (ctrl.-Ig) for 4 weeks starting at 4 weeks p.i. (preventive approach) or 8 weeks p.i. (therapeutic approach) (Figure 1(a)). Histological evaluation of Sirus-Red (SR) stained liver slices revealed that preventive administration of CTLA-4-Ig resulted in a reduction of SR-positive areas and portoportal bridging compared to the control group. However, there were no pronounced differences between the CTLA-4-Ig and ctrl.-Ig treated groups when therapeutic treatment was performed (Figure 1(b)). To underpin these observations, hepatic fibrosis was quantified by the measurement of hydroxyproline as a marker for collagen deposition. Hydroxyproline levels were significantly diminished in mice preventively, but not therapeutically, treated with CTLA-4-Ig compared to controls. As expected, collagen deposition increased over time and was the highest in livers of mice at 12 weeks p.i.

(therapeutic approach) (Figure 1(c)). In line with these findings, expression levels of fibrosis-associated genes *collagen type I alpha 2 (Col1a2,) alpha-actin-2 (Acta2), matrix metalloproteinase-2 (Mmp2),* and *tissue inhibitor of metalloproteinases (Timp1)* were significantly decreased in livers of mice after preventive, but not therapeutic, CTLA-4-Ig treatment in comparison to controls (Figure 1(d)). Overall, these data demonstrate that preventive CTLA-4-Ig administration efficiently ameliorates hepatic fibrosis of *S. mansoni*-infected mice.

3.2. Hepatosplenomegaly and Egg Load Are Decreased in Mice Preventively Treated with CTLA-4-Ig. Since preventive CTLA-4-Ig treatment was capable to reduce hepatic fibrosis during schistosomiasis, we further analyzed the impact of the treatment on disease progression. Hepatosplenomegaly, characterized by a simultaneous enlargement of the liver and the spleen, was significantly less pronounced in mice preventively, but not therapeutically, treated with CTLA-4-Ig compared to controls (Figure 2(a)). In addition, hepatic egg load was significantly reduced in these mice (Figure 2(b)). Infection with *S. mansoni* resulted in a uniform appearance of egg granulomas in the livers of infected mice (Figure 2(c)). Perioval granulomas displayed comparable sizes in all experimental groups (Figure 2(d)). Liver transaminases (AST and ALT), as a marker for hepatocellular damage, were slightly elevated in the serum of infected mice compared to naïve mice. However, all values were below clinical relevance and not significantly different. Serum levels of AP were not affected by the infection (Figure 2(d)). These results indicate that preventive CTLA-4-Ig treatment improves the clinical picture of schistosomiasis.

3.3. CTLA-4-Ig Treatment Leads to a Reduction in Total Cell Counts and $CD4^+$ T Cells in the Spleens of Mice. To characterize the role of immunosuppressive CTLA-4 on inflammatory immune cell recruitment, we analyzed the spleens of mice by flow cytometry. Preventive administration of CTLA-4-Ig led to a significant reduction of total cell numbers in the spleens compared to controls. However, administration of CTLA-4-Ig had no impact on cell numbers when the treatment was started after egg deposition (therapeutic approach) (Figure 3(a)). Treatment with CTLA-4-Ig selectively decreased the percentage of $CD4^+$ T cells, but not $CD8^+$ T cells, in *S. mansoni*-infected mice (Figures 3(b) and 3(c)). Moreover, we observed that mice receiving ctrl.-Ig upregulate the expression of CTLA-4 on the surface of $CD4^+$ T cells during infection in contrast to CTLA-4-Ig-treated mice (Figure 3(d)). With regard to myeloid cells, CTLA-4-Ig administration neither affected the percentage of antigen-presenting $F4/80^+$ macrophages and $CD11^+$ dendritic cells within spleens nor the expression of the costimulatory molecule and CTLA-4-ligand CD86 on these cells, which indicates that the functionality of macrophages and dendritic cells is not affected by the treatment (Figure 3(e)–3(h)). Taken together, these observations prove that the administration of CTLA-4-Ig efficiently suppresses the $CD4^+$ T cell response *in vivo*.

3.4. Preventive CTLA-4-Ig Treatment Impairs Cytokine Production by Splenocytes and Gene Expression of Cytokines in the Livers. Considering the fact that CTLA-4-Ig suppresses the cellular immune response in *S. mansoni*-infected mice, we next examined whether the treatment influences the cytokine production of isolated splenocytes and the expression of cytokines in the liver. The secretion of Th2 key cytokine IL-13, known to promote fibrogenesis, as well as regulatory IL-10, was decreased in the supernatants of SWAP-stimulated splenocytes of preventively CTLA-4-Ig-treated mice in comparison to ctrl.-Ig-treated control group, whereas INF-γ secretion was not affected by the treatment (Figure 4(a)). In addition, expression levels of *Il13, Il4, Infg,* and *Il10* in the livers of these mice were significantly downregulated (Figure 4(b)). The cytokine response of mice in the therapeutically treated groups was diminished in comparison to the preventive ctrl.-Ig group. However, cytokine levels were not affected by the therapeutical CTLA-4-Ig treatment (Figure 4(a) and 4(b)). These data show that the production of profibrotic cytokines is impaired in mice preventively treated with CTLA-4-Ig.

4. Discussion

The current study was performed as a proof-of-principle study. We previously demonstrated that the antifibrotic effect of single-sex infection with female schistosomes is associated with increased CTLA-4 expression in livers of these mice [15]. Based on these data, it was obvious for us to investigate the potential direct antifibrotic effect of CTLA-4 on *S. mansoni*-induced hepatic fibrosis by mimicking the effect of female worms. Herein, we demonstrated that an early preventive treatment with CTLA-4-Ig (belatacept) in mice leads to (i) reduction of hepatic fibrosis, (ii) diminished disease progression, (iii) reduced hepatic egg load, and (iv) an impaired immune response characterized by decreased immune cell recruitment and cytokine production. However, CTLA-4-Ig administration was not capable to treat ongoing hepatic fibrosis.

In mansonian schistosomiasis, hepatic fibrosis is initiated by vigorous granulomatous responses to tissue-entrapped parasite eggs that is mainly orchestrated by cross-regulatory $CD4^+$ T cell. Although an appropriate Th1 response to *S. mansoni* larvae is associated with high protection levels [17] and Th2 response to soluble egg antigens is known to promote excessive fibrotic organ damage [18], there are actually no good or bad T cell responses to *Schistosoma* larvae or eggs. Earlier studies in experimental schistosomiasis have shown the essential role for $CD4^+$ T cells in granuloma formation and disease [19]. Excessive polarization to either Th1 or Th2 by knocking down certain type 2 cytokines was shown to impede granulomatous response leading to 100% mortality [20–22]. The picture of formally announced Th1/Th2 paradigm is obsolete due to the discovery of a more complex pattern of regulation and interplay of different $CD4^+$ T cell subsets [23]. It has been shown that immunotherapy by administration of specific antibodies or agonists have remarkable effects on the establishment of hepatic fibrosis following *S. mansoni* infection.

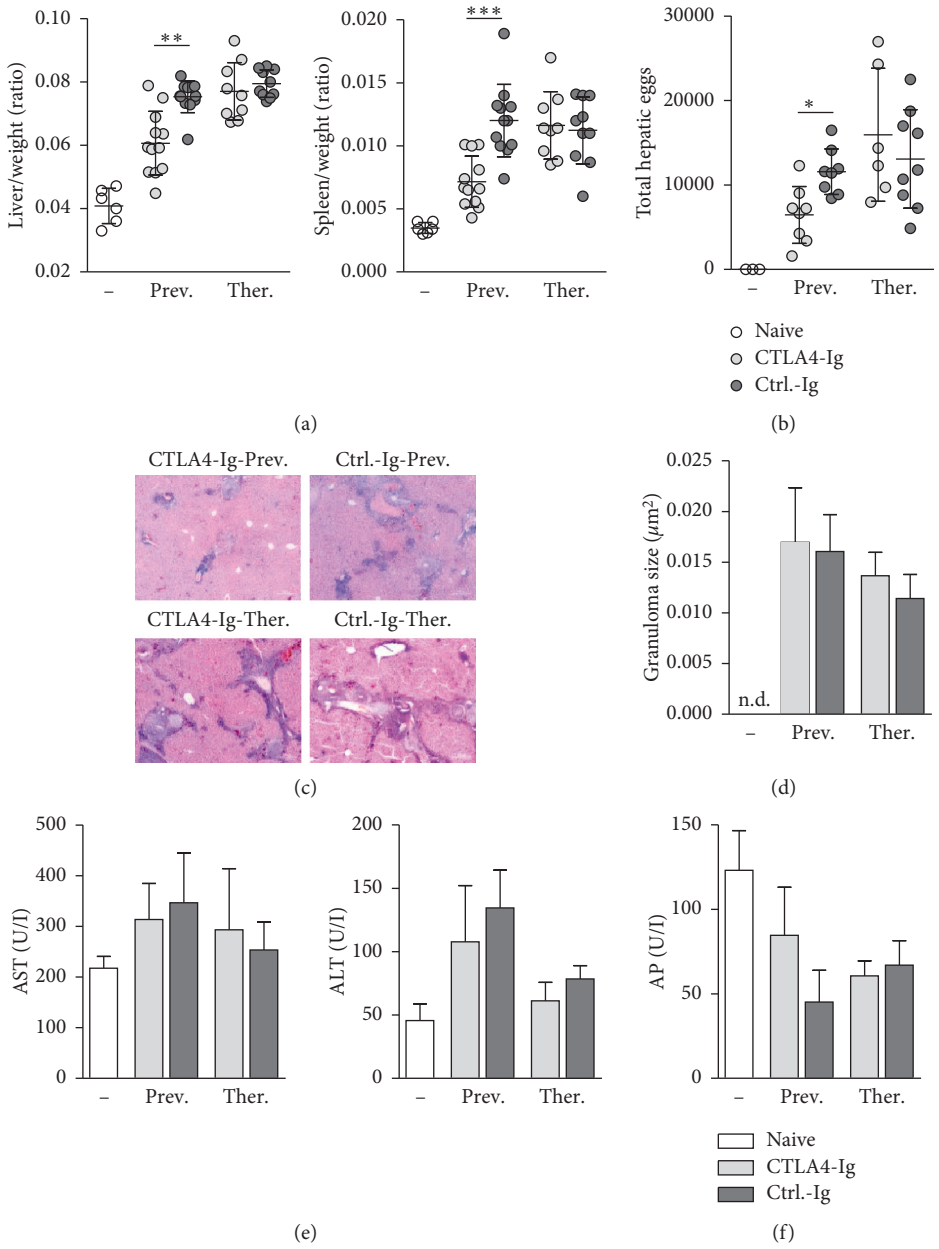
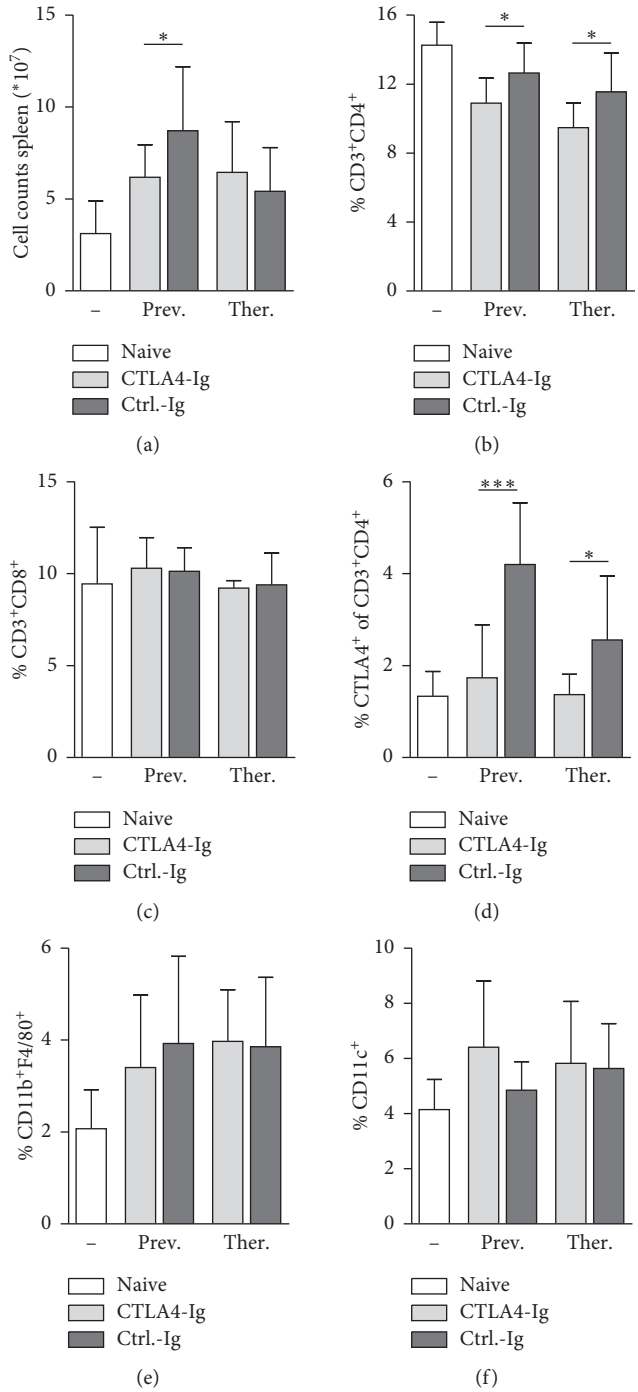


Figure 2: Hepatosplenomegaly and egg load are decreased in mice preventively treated with CTLA-4-Ig. (a) Relative organ size of livers and spleens was expressed as a ratio to body weight ($n = 6$–12 out of 4 independent experiments). (b) Numbers of total hepatic eggs were counted ($n = 6$–9 out of 4 independent experiments). (c) Representative images of liver sections stained with haematoxylin/eosin are shown; 100x magnification. (d) Size of hepatic granulomas were quantified by using Image*J* software ($n = 5$–7 out of 4 independent experiments). (e) Serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and (f) alkaline phosphatase (AP) were determined ($n = 4$–6 out of 4 independent experiments). Data are represented as mean ± SD from duplicate data.

Substitution of Th2 response by Th1, induced by IL-12 administration, is able to prevent excessive tissue fibrosis [24]. In addition, direct interference with the type 2 cytokine IL-13 by IL-13 inhibitor sIL-13Rα2-Fc leads to significant reduction of hepatic fibrosis in a mouse model of *S. mansoni* infection [25].

One important regulator of T cell function is the CTLA-4 contributing to the suppressor function of regulatory and conventional $CD4^+$ T cells [26]. CTLA-4 is expressed on the T cell surface and binds to the B7 molecule on antigen-presenting cells leading to an inhibitory signal to the activated T cell by limiting the production of the T cell growth factor IL-2 [27]. $CTLA\text{-}4^{-/-}$ mice developed a fatal disease characterized by massive proliferation of lymphocytes [28]. Vice versa, the administration of CTLA-4-Ig (belatacept or abatacept) exerts beneficial effects on a range of autoimmune disorders such as airway inflammation [29, 30], rheumatoid arthritis [31], and dermal fibrosis [32] or to prevent rejection of allografts, particularly renal transplants [33].



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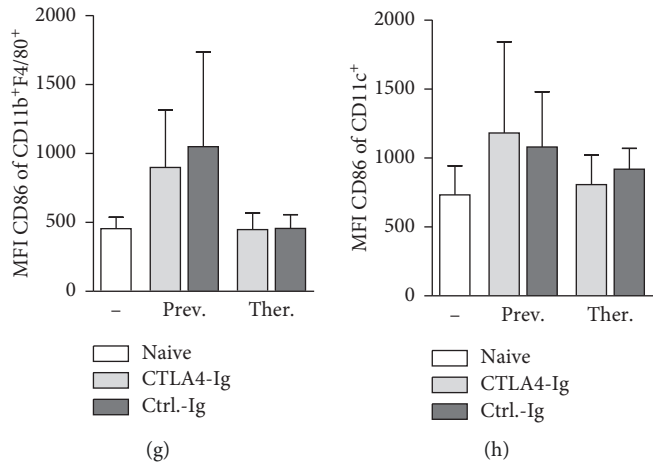
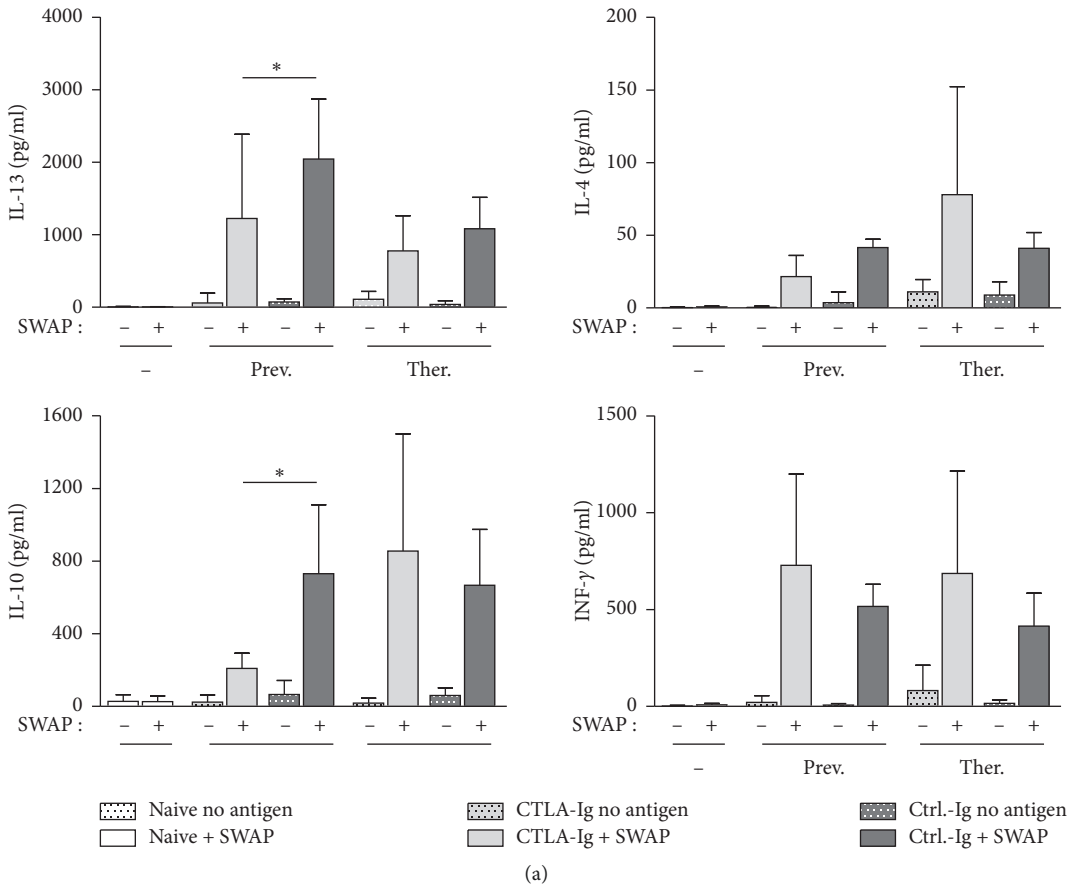


Figure 3: CTLA-4-Ig treatment leads to a reduction in total cell counts and $CD4^+$ T cells in the spleens of mice. (a) Numbers of spleen cells were quantified using a CASY TT cell counter and ((b)-(h)) analyzed by flow cytometry ($n = 6$–12 out of 4 independent experiments). The percentage of (b) $CD4^+$ T cells, (c) $CD8^+$ T cells, (d) CTLA-4^+ $CD4^+$ T cells, (e) macrophages (Mϕs), and (f) dendritic cells (DCs) of viable cells was depicted. The mean fluorescence intensity (MFI) of CD86 expression on Mϕs and DCs was quantified. Data are represented as mean + SD from duplicate data.



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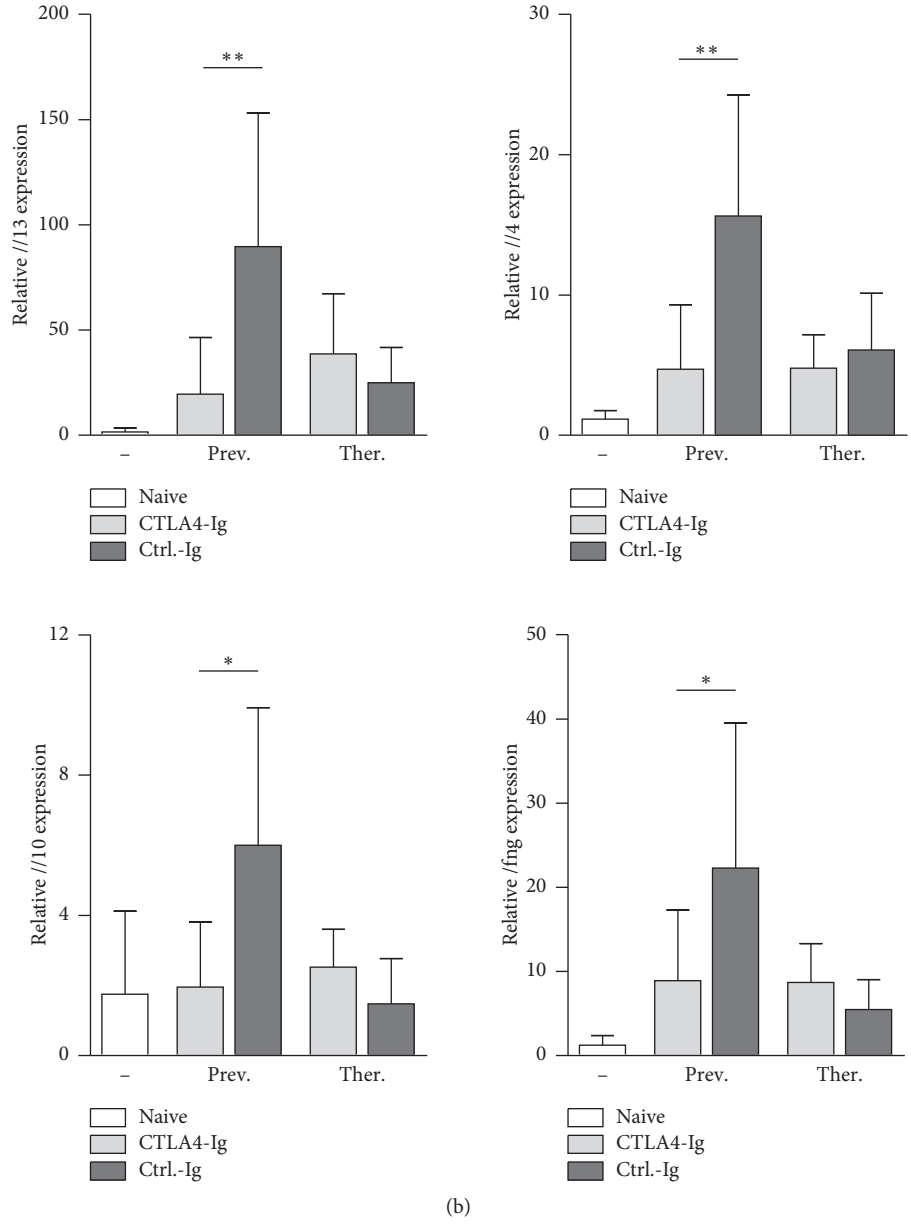

(b)

Figure 4: Cytokine production by splenocytes and expression of cytokines in the livers of mice preventively treated with CTLA-4-Ig are impaired. (a) Splenocytes were isolated and stimulated with 10 μg/ml SWAP (soluble worm antigen preparation). Supernatants were collected after 72 h and amounts of IL-13, IL-4, INF-γ, and IL-10 ($n = 4$–9 out of 3 independent experiments) were quantified by ELISA ($n = 4$–9). (b) Relative gene expression of *Il13*, *Il4*, *Ifng,* and *Il10* in livers of mice was determined by real-time PCR ($n = 6$–9). Data are represented as mean + SD from duplicate data.

In its proper use (prevention of kidney transplant rejection), immunosuppressant Nulojix® (belatacept, CTLA-4) is a very expensive therapy. In consideration to *S. mansoni* infection in mice, it has been shown that blocking of CTLA-4 by anti-CTLA-4 antibodies leads to significant weight loss and altered Th2 response in these mice when administered during the acute stage of infection [14]. Using a single-sex infection model of *S. mansoni*, we have recently shown that an initial infection with female *S. mansoni* reduces hepatic fibrosis of a bisexual challenge infection [15]. We concluded that this inhibited Th2 response and the antifibrotic effect was due to a significant increase of CTLA-4 expression in the livers of these mice. To our knowledge, there is only one study in mice showing similar results in regard to impairment of Th2 responses due to CTLA-4-Ig injection in experimental *Nippostrongylus brasiliensis* infection [34]. In humans, it has been shown that CTLA-4 is upregulated following *Schistosoma* spp. infection, which was associated with a reduced incidence of Th2-driven allergic diseases [35, 36].

The reduction of hepatic fibrosis, observed in this study, must be viewed from different angles. On one hand side, we have shown a significant reduction of the hepatic egg load in the prevention group. This alone can be the cause of the

reduction of hepatic fibrosis. The reason for the reduced egg load might be related to a disturbed maturation of the worms due to the immunosuppressive effect of CTLA-4. Harrison and Doenhoff have shown that a disturbed CD4 T cell response by the administration of immunosuppressive substances negatively affects the fecundity of female worms, which in turn leads to reduced egg production [37]. On the other hand side, a selective costimulatory blockage of CD28-mediated activation of T cells might exert a direct antifibrotic effect. The spreading of fibrosis mainly depends on Th2 expansion. Therefore, fibrogenesis, as a fibroproliferating process, might be directly affected. The granulomas consist of different cell types that are not influenced by the specific effect of costimulatory blockage of CD28-mediated T cell activation. This could explain the unchanged granuloma sizes in our experimental groups. However, if the egg numbers are correlated to the amount of hydroxyproline, the differences in hydroxyproline levels were fading. This fact points rather to the reduced egg load as the main cause for the reduced fibrosis. Another point that could play a role in the development of hepatic fibrosis is the fact that in this study the right hepatic lobe was used to quantify hydroxyproline levels, although in human infections the left hepatic lobe is more conspicuous. However, this study focused on the analysis of relative changes in the degree of fibrosis and less with absolute changes. Several studies indicate on the interaction of regulatory T cells and matrix metalloproteinases (MMP) with emphasis on tumorigenesis. Anti-CTLA-4 treatment, not at least due to the removal/reduction of immune tolerance, is a very promising anticancer therapy [38]. Increased collagen synthesis along with a downregulation of proteolytic enzymes, especially matrix metalloproteases (MMP), and upregulation of tissue inhibitors of MMP (TIMP) may contribute to hepatic fibrosis [39–41]. During *S. mansoni* infection, MMP-2 and TIMP-1 expression levels are generally upregulated, where the expressions levels for TIMP-1 are usually higher than those for MMP-2, as evidence of an ongoing fibrotic process. Interestingly, we could observe inverted proportions in both, the preventive and the therapeutic group, indicating fibrolytic processes. Since $CD4^+$ T cells itself produce MMPs [38], a reduction of $CD4^+$ cells in our setting might explain the observed reduction in MMP-2. However, the reduction of hepatic fibrosis was seen in the preventive group, exclusively. In the "therapeutic" group, we could only see a trend reduction of fibrosis in all measured parameters.

In the current study, we could show that there is an overall reduction of $CD4^+$ T cells within spleens of infected mice that received CTLA-4-Ig during acute infection. These $CD4^+$ T cells express less CTLA-4 compared to ctrl.-Ig groups, indicating that the treatment was effectively working *in vivo*. In addition, *ex vivo* analysis of SWAP-stimulated splenocytes displayed a reduced Th2 cytokine production, as an important trigger of fibrosis. The use of SWAP instead of SEA in this study is due to experiments prior to the presented study which showed that SWAP elicits the most reproducible results compared to SEA or T-cell-receptor-specific (CD3/CD28) or nonspecific (PMA/ionomycin) stimulation methods. This may be explained by the fact that the protocol used for SWAP production [42] involves the processing of worm pairs, and therefore parasite egg antigens are most likely present.

The reduced inflammation observed in livers and spleens due to immunosuppressive CTLA-4 administration led to reduction of regulatory IL-10, which is crucial for emergence of Treg responses during *Schistosoma* spp. infection [43, 44]. It has been show that reduced IL-10 levels are associated with increased resistance to reinfection [45, 46]. However, the observed low IL-10 values, artificially induced by belatacept in this study, might have the opposite effect as well and induce a higher vulnerability to the infection in human infections. IL-10 is undoubtedly a major molecule involved in immunosuppression and development of a modified Th2 response [43]. In this study, the development of an inflammatory response against the parasites and eggs has been severely disarranged in the prevention group. We suggest that a certain level of inflammation or a sequence of inflammatory processes during the infection is needed to initiate the regulatory potential of IL-10. In a system of artificially induced immunosuppression, regulation by IL-10 might not be initiated or failed. We therefore assume that an artificial immunosuppression by belatacept will disturb the balance between Th1/Th2/Treg considerably seen by an antifibrotic effect in the prevention group but no effect in the therapeutic group.

The question remains whether the effect of belatacept on $CD4^+$ T cell response in regard to the reduction of hepatic fibrosis is restorable in case of a CTLA-4-Ig treatment stop. We designed a follow-up study to analyze a potential long-term effect of belatacept (follow-up: CTLA-4-Ig, $n = 6$ and ctrl.-Ig, $n = 4$). We had to abandon that study 12 weeks p.i. since the animals reached critical termination criteria in regard to weight loss and mobility. It has been shown that in the absence of $CD4^+$ T cells the granulomatous response is limited leading to increased hepatocyte damage [19]. However, the surrogate marker for liver injury (ALT and AST) did not indicate severe organ damage. We assume that these mice, due to the early administration of CTLA-4, generate a severe systemic inflammation due to the inability to develop an appropriate $CD4^+$ T cell response.

In conclusion, this study points clearly to the important role of CTLA-4 as an important regulator of *S. mansoni*-induced fibrosis by modulating T-cell responses. However, our data show that early preventive CTLA-4-Ig treatment ameliorates liver fibrosis, but was not sufficient to treat ongoing fibrotic processes. In endemic areas, *S. mansoni* infection is not a once-only event but is very likely to occur regularly. A therapeutic effect of belatacept would therefore be, although rather unlikely due to its expense, a promising approach, especially in severe disease progressions. The more effective preventive treatment will be difficult to implement, considering that young children in particular are affected by the infection. Therefore, this study does not provide a probable tool, which could be considered for therapeutic treatment in human natural infection, but

contributes a puzzle piece to the effect of CTLA-4 in *S. mansoni*-associated fibrogenesis and its role in disease progression.

Abbreviations

ALT: Alanine aminotransferase
AP: Alkaline phosphatase
AST: Aspartate aminotransferase
CTLA-4: Cytotoxic T-lymphocyte-associated protein-4
S. mansoni: *Schistosoma mansoni*
Th1: T helper cell response type 1
Th2: T helper cell response type 2

Data Availability

All data (original data) used to support the findings of this study are an integral part of this manuscript.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

MS, AR, and ECR conceived and designed the experiments. MS, AR, MB, FW, and CS performed the experiments. MS, AR, MB, and FW analyzed the data. MS, AR, and ECR contributed reagents/materials/analysis tools. MS, AR, and ECR wrote the paper.

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Supplementary Materials

Supplementary Figure 1: representative FACS gating strategy for $CD4^+/CTLA\text{-}4^+$ cells in spleen homogenates. FACS plots: (A) gating of $CD4^+$ T cells and (B) extracellular staining of CTLA-4 and appropriate control-IG on $CD3^+CD4^+$ T cells. (*Supplementary Materials*)

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Research Article

Effect of *Lonicera caerulea* var. *emphyllocalyx* Fruit on Biofilm Formed by *Porphyromonas gingivalis*

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Porphyromonas gingivalis is an important pathogenic anaerobic bacterium that causes aspiration pneumonia. This bacterium frequently forms biofilms in the oral cavity and in respiratory tract-associated medical devices. Bacterial colonization that occurs in association with this biofilm formation is the main reason for incurable aspiration pneumonia. The *Lonicera caerulea* var. *emphyllocalyx* (LCE) fruit has been used in folk medicine in Hokkaido, the northern part of Japan. The aim of this study was to elucidate one of the antimicrobial mechanisms of LCE methanol extract (LCEE)—the inhibitory effect of LCEE on biofilm formation by *P. gingivalis*. Our results show that LCEE significantly reduced biofilm formation by three different *P. gingivalis* isolates in a concentration- and time-dependent manner that were quantified by the adsorption of safranin red. When LCEE was added to biofilms already formed by *P. gingivalis*, LCEE did not degrade the biofilm. However, treatment with LCEE significantly promoted the removal of existing biofilm by vibration compared to that of control. We also confirmed biofilm formation in LCEE-treated *P. gingivalis* in tracheal tubes using scanning electron microscopic (SEM) analysis. Cyanidin 3-O-glucoside (C3G), one of the components of LCE, also inhibited the formation of biofilm by *P. gingivalis* in a concentration-dependent manner. Our results reveal that LCEE may be an effective antibacterial substance for *P. gingivalis*-induced aspiration pneumonia because of its role in the suppression of bacterial biofilm formation in the oral cavity.

1. Introduction

Healthcare-acquired infections are a major cause of mortality and morbidity. According to the existing data, about 10% bedridden patients in developed countries contract hospital-acquired infections [1]. Healthcare-acquired pneumonia, especially ventilator-associated pneumonia, is the leading cause of death in intensive care units (ICUs), with a mortality rate from one-fourth and three-fourth [2].

Aspiration pneumonia is a severe lethal condition caused by aspiration of oral bacteria during medical procedures or by misswallowing of food. It can lead to the development of necrotizing pneumonia or lung abscesses, which may require a prolonged course of antibiotics and surgery [3]. Mortality was about 90% if two or more lobes of the lung were involved and 41% if only one lobe was affected in the previous study [4]. The most common microorganisms isolated from aspiration pneumonia are anaerobes found in the oropharynx [5]. As bacteria are also observed in edentulous elderly patients, the coating of the tongue by bacteria is associated with the risk of aspiration pneumonia [6]. The teeth may also be related as a reservoir for bacterial colonization and nosocomial pneumonia. These anaerobes were found by the colonization of dental plaque in hospitalized intensive care and nursing home [7].

Colonization of the oropharynx and stomach by Gram-negative pathogens increases in critically ill patients immediately after hospitalization [8]. Since bacterial colonization in the mouth and pharynx is a threat for bedridden patients in ICU, several strategies have been used to prevent colonization, such as nonabsorbable antibiotics. Nevertheless, prolonged use of prophylactic antibiotics can lead to increases in resistant organisms and thus is not recommended [9]. Longer ICU admission and longer duration of connection to ventilators are prominent causes of accumulation of infectious agents. Increased duration of patient connection to the ventilator, which is an infection source, causes transmission of infectious agents from the ventilator to the lung [10].

Porphyromonas gingivalis is an anaerobic bacterium that is significantly related to periodontitis and several systemic diseases such as aspiration pneumonia. This pneumoniae is responsible for crucial morbidity and mortality in the elderly [11–13]. Numerous clinical case reports and animal models show that this Gram-negative bacterium plays an important role in the development of aspiration pneumonia [14]. Dental plaque biofilm may serve as a persistent reservoir for respiratory diseases. Oral bacteria can be aspirated into the lung to cause aspiration pneumonia. *P. gingivalis* expresses several virulence factors such as lipopolysaccharide (LPS), fimbriae, and cysteine proteinases. In particular, *P. gingivalis* is a popular bacterium isolated from aspiration pneumonia, lung abscesses, and periodontitis in the elderly [15].

Lonicera caerulea var. *emphyllocalyx* (LCE), also known as blue honeysuckle or haskap, is a plant belonging to Caprifoliaceae family that grows naturally in cool temperate regions such as high mountains or wet areas in the Northern Hemisphere, such as Hokkaido in Japan, and has been cultivated [16, 17]. Its fruits are purple-colored, hard berries, which are about 1–2 cm long and 1 cm wide and can resist temperatures below −40°C [18]. These fruits of honeysuckle plants have been used in folk medicine in the countries of their origin [19]. Recently, LCE has been widely harvested in many countries including Japan and consumed as a part of the human diet [18]. Previously, we partly described the effect of LCE extract (LCEE) on *Streptococcus pyogenes* infection not only *in vitro* but also *in vivo* [19, 20]. However, the precise mode of antibacterial activity of LCEE against other bacteria has not been unclear.

In this study, we tried to clarify whether LCEE is useful for antiaspiration pneumonia-associated anaerobic bacteria therapy.

2. Materials and Methods

2.1. Preparation of LCEE. *Lonicera caerulea* var. *emphyllocalyx* (LCE) was harvested in Atsuma, Hokkaido, in the northern part of Japan in 2017. The methanol extract of LCE fruit (LCEE) used in this study was the same one as that in our previous study [19, 20]. C3G was purchased from Tokiwa Phytochemical (Sakura, Japan). *Lonicera caerulea* var. *emphyllocalyx* fruit extract (5 μg), cyanidin 3-O-glucoside (C3G, 28.8, 57.5, and 115 ng), was injected to HPLC with the following conditions: system, Shimadzu LC–10A_{VP} (Kyoto, Japan); column, TSK-GEL ODS-80$_{TS}$ (4.6 × 250 mm, Tosoh, Tokyo); mobile phase, 0.5% AcOH/0.5% AcOH in CH_3OH 85 : 15; flow rate, 1.0 mL/min; column temperature, 40°C; and detection, 520 nm. Retention time of C3G was 9.0 min. The range of C3G was calibrated by the peak area using the least-squares method ($r^2 = 0.997$) (Figure 1). The concentration of C3G in LCEE was 1.12 (w/w)%.

2.2. Bacteria and Chemicals. *Porphyromonas gingivalis* JCM12257 (ATCC33277), JCM8525, and JCM19600 were purchased from RIKEN BioResource Research Center (Ibaraki, Japan). Anaerobes were grown at 37°C under anaerobic conditions (AnaeroPack System, Mitsubishi Gas Chemical, Tokyo, Japan) using Gifu anaerobic medium bouillon (Nissui, Tokyo, Japan), supplemented with 5 μg/mL hemin (Sigma-Aldrich, St. Louis, MO, USA) and 1 μg/mL menadione (Fujifilm Wako Pure Chemical Industries, Osaka, Japan) (GAM) [21]. Ampicillin sodium (Wako Pure Chemical Industries, Osaka, Japan) was used in a final concentration of 50 μg/mL as a positive control.

2.3. Bacterial Growth Analysis. Before broth culture analysis, bacteria were incubated in CDC Anaerobe Blood Agar (Nihon BD, Tokyo, Japan) under anaerobic conditions for 48 h. For the determination of the growth inhibitory activity, about 1×10^6 bacteria were incubated in 2 ml of GAM with LCEE or C3G for 24 h. For culturing, 5 mL polypropylene tubes (#34180005D, As-One Corporation., Osaka, Japan) were used. As the determination of bacterial growth, we measured the turbidity of cultured medium (optical density (OD) 600 nm) [21].

2.4. Biofilm Assay by Safranin Red Analysis. Biofilm formation was quantified using a polypropylene tube assay specifically for *P. gingivalis* adhesion [22, 23]. Briefly, overnight cultures of *P. gingivalis* were adjusted to 1×10^6 CFU in GAM with or without LCEE and C3G. Aliquots of 200 μL were anaerobically incubated for 72 h at 37°C in a polystyrene tube. To remove planktonic cells, wells were gently washed 3 times with phosphate-buffered saline (PBS, pH 7.2, 0.15 M) and air-dried. After that, remaining bacteria were stained for 15 min with 5 mL of 0.2% (w/v) safranin red. Excess dye was removed by washing 2 times with PBS and then with water. Dye taken up by cells was eluted using 5 mL 95% ethanol, and OD (490 nm) was measured to assess the mass volume of the biofilm. Tubes incubated without bacteria were used as blanks. The absorbance for the blank wells was subtracted from the test values. In dose-dependent analysis, the half inhibitory concentration (IC_{50}) was calculated from the least-squares regression line made from 3 points that crossed 50% of the control logarithmic concentration values.

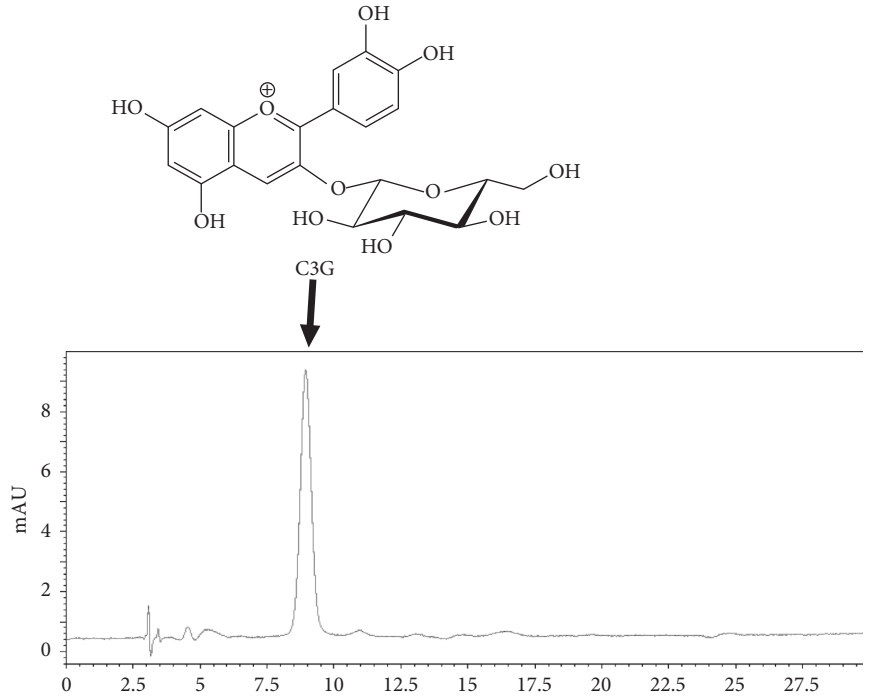


Figure 1: Chromatogram of LCEE. LCEE (5 μg) was injected onto HPLC with the following conditions: system, Shimadzu LC–10A$_{VP}$ (Kyoto, Japan); column, TSK-GEL ODS-80$_{TS}$ (4.6 × 250 mm, Tosoh, Tokyo); mobile phase, 0.5% AcOH/0.5% AcOH in CH_3OH 85 : 15; flow rate, 1.0 mL/min; column temperature, 40°C; and detection, 520 nm. Peak at 9.0 min was identified as cyanidin 3-O-glucoside (C3G). LCEE: *Lonicera caerulea* var. *emphyllocalyx* extract; HPLC: high-performance liquid chromatography.

2.5. Biofilm Assay by Scanning Electron Microscopic (SEM) Analysis. Scanning electron microscopic preparation was performed as described elsewhere [24]. A tracheal tube (DYND48050J, Medline Japan Inc. Tokyo, Japan) was uniaxially cut at a length of 1 cm, and *P. gingivalis* treated with or without LCEE and C3G were anaerobically incubated in it for 72 h at 37°C. After that, those tubes were immediately placed in 2.5% glutaraldehyde (Nisshin EM, Tokyo, Japan) prepared in 0.1 M phosphate buffer (pH 7.4) for 24 h at 4°C as a prefixation step. They were rinsed 2 times with 0.1 M phosphate buffer (pH 7.4), postfixed using 2% osmium tetroxide (Nisshin EM, Tokyo, Japan) for 2 h at 25°C, and finally rinsed with distilled water. Next, the specimens were dehydrated using graduated concentrations of ethyl alcohol (30%, 50%, 70%, 90%, 95%, and 100%) for 30 min, each followed by absolute alcohol for 30 min. The specimen was dried using the critical point dryer CPD300 (Leica, Wetzlar, Germany). For mounting, carbon conductive paint was used; for specimens, osmium coating was completed using an Osmium Coater (NL-OPC-AJ, Filgen, Nagoya, Japan). Finally, each sample was examined using a microscope (SEM: S-4800) (Hitachi High-Technologies Corporation, Tokyo, Japan).

2.6. Statistical Analysis. Experimental data were expressed as mean values with standard deviation (SD). Statistical analysis of the differences between the mean values obtained was performed using unpaired Student's *t*-test for the comparison between two groups or Tukey/Bonferroni's multiple comparison test for differences among multiple groups (EZR version 1.36). The statistical difference was considered significant with $p < 0.01$.

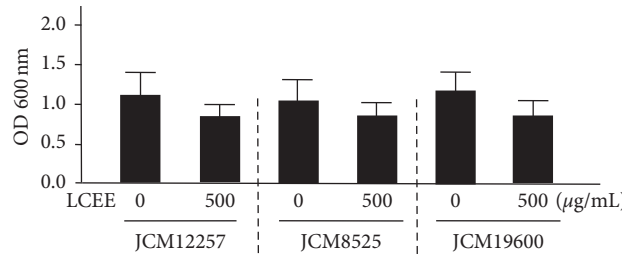


Figure 2: Effect of LCEE on the growth of three *P. gingivalis* isolates. Three *P. gingivalis* isolates (JCM12257, JCM8525, and JCM19600) were treated with or without LCEE (500 μg/mL) for 24 h and their growth was quantified by measuring absorbance at 600 nm. Data represent the mean ± SD ($n = 3$). LCEE: *Lonicera caerulea* var. *emphyllocalyx* extract.

3. Results

3.1. Bacteria Growth Analysis for LCEE. It was evaluated whether LCEE could inhibit the growth of anaerobic bacteria grown in GAM with LCEE. The result showed that LCEE could not inhibit the growth of *P. gingivalis* significantly (Figure 2).

3.2. Biofilm Assay with LCEE. Although LCEE did not suppress the growth of *P. gingivalis*, we considered other antibacterial effects, including the inhibitory effect on

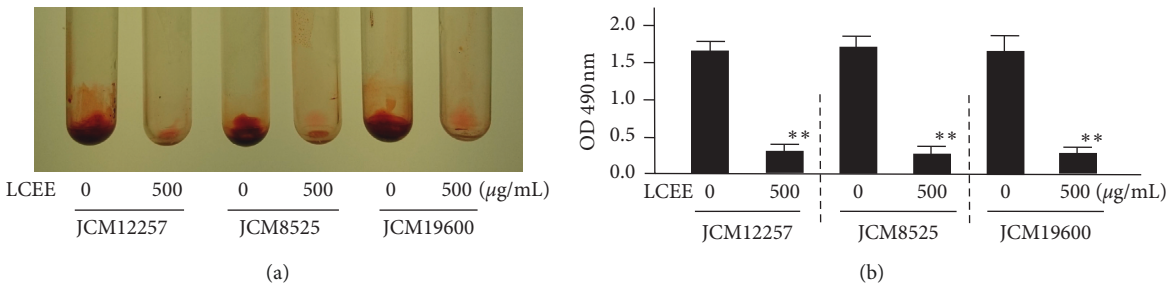


Figure 3: Inhibitory effect of LCEE on biofilm formation by three *P. gingivalis* isolates. Three *P. gingivalis* isolates (JCM12257, JCM8525, and JCM19600) were treated with or without LCEE (500 μg/mL) for 72 h. (a) Image of polypropylene tube. (b) Biofilm formation was quantified by safranin red adsorption at 490 nm. Data represent the mean ± SD ($n = 3$). $^{**}p < 0.01$ compared to each untreated group evaluated by Student's *t*-test. LCEE: *Lonicera caerulea* var. *emphyllocalyx* extract.

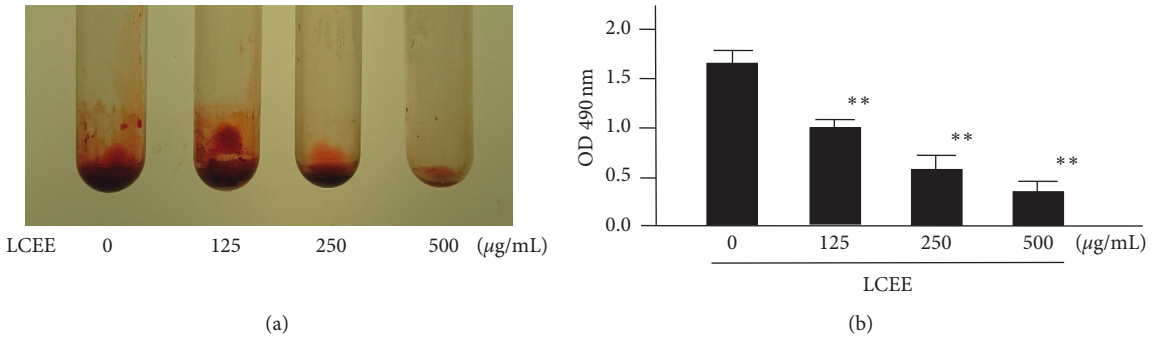


Figure 4: Dose-dependent inhibitory effect of LCEE on biofilm formation by *P. gingivalis*. *P gingivalis* JCM12257 was treated with or without LCEE (125, 250, and 500 μg/mL) for 72 h. (a) Image of polypropylene tube. (b) Biofilm formation was quantified by safranin red adsorption at 490 nm. Data represent the mean ± SD ($n = 3$). $^{**}p < 0.01$ compared to untreated group evaluated by Turkey/Bonnferoni's multiple comparison test. LCEE: *Lonicera caerulea* var. *emphyllocalyx* extract.

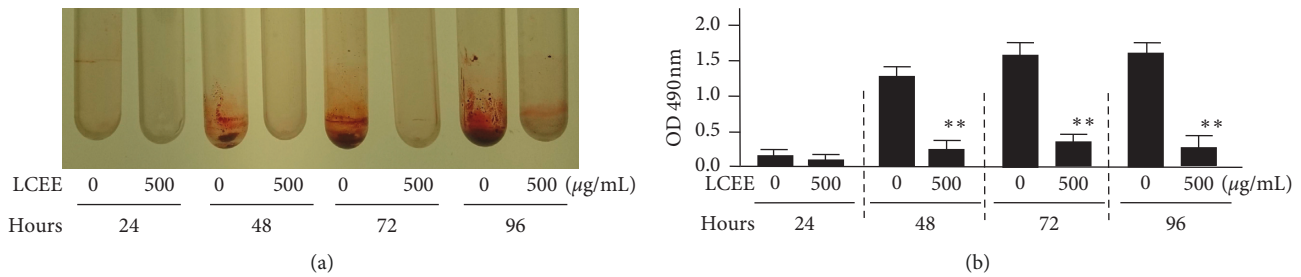


Figure 5: Time-dependent inhibitory effect of LCEE on biofilm formation by *P. gingivalis*. *P gingivalis* JCM12257 was treated with or without LCEE (500 μg/mL) for 24, 48, 72, or 96 h. (a) Image of polypropylene tube. (b) Biofilm formation was quantified by safranin red adsorption at 490 nm. Data represent the mean ± SD ($n = 3$). $^{**}p < 0.01$ compared to each untreated group evaluated by Student's *t*-test. LCEE: *Lonicera caerulea* var. *emphyllocalyx* extract.

biofilm formation. To evaluate whether LCEE could inhibit biofilm formation or not, pathogenic anaerobes were grown in GAM with LCEE, and the ability to form biofilm in a polypropylene tube was assessed by safranin staining. Safranin red assays showed that untreated anaerobic bacteria formed biofilms intensely. As expected, LCEE significantly inhibited the formation of biofilm by anaerobic bacteria. We confirmed the significant difference of this inhibitory ability of LCEE among 3 bacteria (JCM12257, JCM8525, and JCM19600) (Figure 3). From these universal results, future experiments were focused on *P. gingivalis* JCM12257. More than 125 μg/mL of LCEE inhibited biofilm formation by anaerobic bacteria significantly ($p < 0.01$). The IC_{50} value of LCEE was calculated as 178 μg/mL (Figure 4). We could not find any differences in biofilm formation by *P. gingivalis* by either LCEE-untreated or treated assays at 24 h. However, LCEE inhibited biofilm formation by anaerobic bacteria after 48 h (Figure 5). Thus, it was confirmed that antibiofilm activity of LCEE was present in a concentration- and time-dependent manner. Next, it was examined whether LCEE could affect the biofilm that had already been formed by *P. gingivalis* or not. When *P. gingivalis* was cultured in a

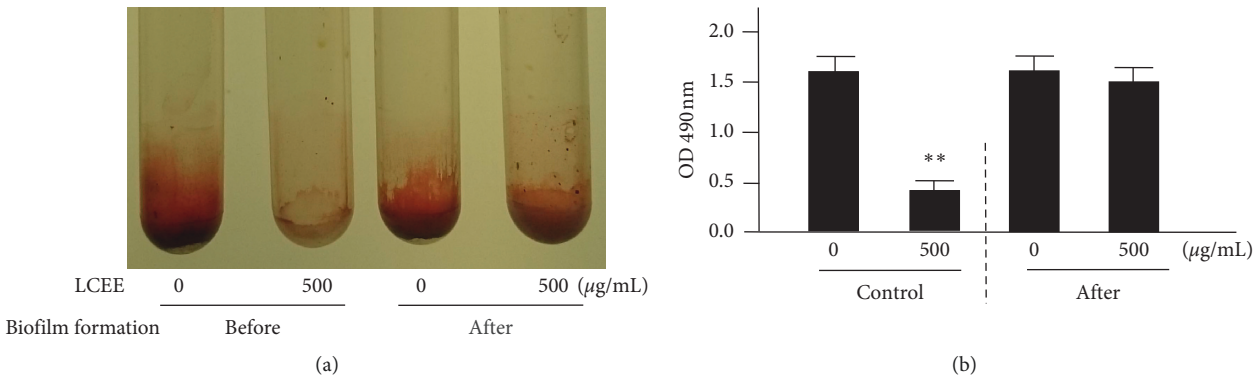


Figure 6: LCEE did not degrade the biofilm already formed by *P. gingivalis*. *P. gingivalis* JCM12257 was treated with or without LCEE (500 μg/mL) for 72 h (control). In order to evaluate the effect of LCEE on the biofilm already formed by bacteria, control bacteria were further treated with or without LCEE for other 72 h (after). (a) Image of polypropylene tube. (b) Biofilm formation was quantified by safranin red adsorption at 490 nm. Data represent the mean ± SD ($n = 3$). $^{**}p < 0.01$ compared to each untreated group evaluated by Student's *t*-test. LCEE: *Lonicera caerulea* var. *emphyllocalyx* extract.

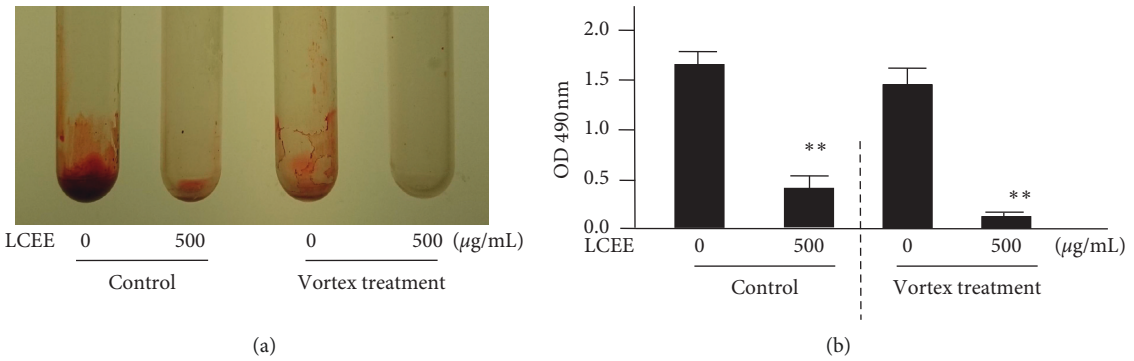


Figure 7: LCEE promoted the degradation of biofilm already formed by *P. gingivalis* subjected to vibrational stimulation. *P. gingivalis* JCM12257 was treated with or without LCEE (500 μg/mL) for 72 h (control). In order to evaluate the effect of LCEE on the biofilm already formed by bacteria, control bacteria were further treated with or without LCEE for other 72 h. Then, a polyethylene tube was vibrated using a vortex mixer for 10 seconds. (a) Image of polypropylene tube. (b) Biofilm activity was quantified by safranin red adsorption at 490 nm. Data represent the mean ± SD ($n = 3$). $^{**}p < 0.01$ compared to each untreated group evaluated by Student's *t*-test. LCEE: *Lonicera caerulea* var. *emphyllocalyx* extract.

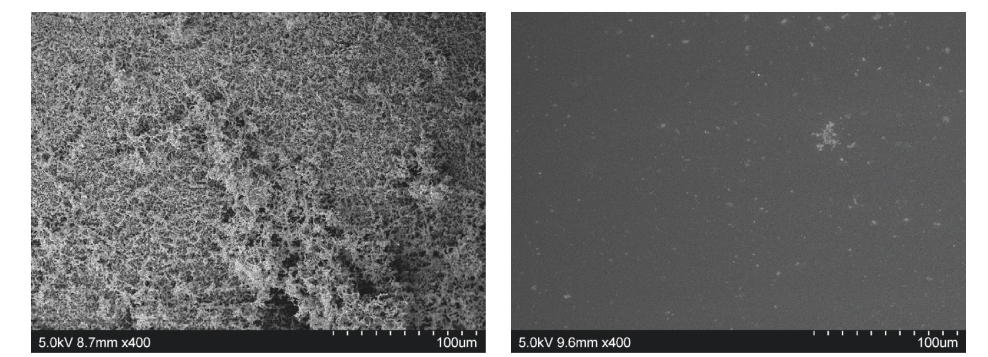
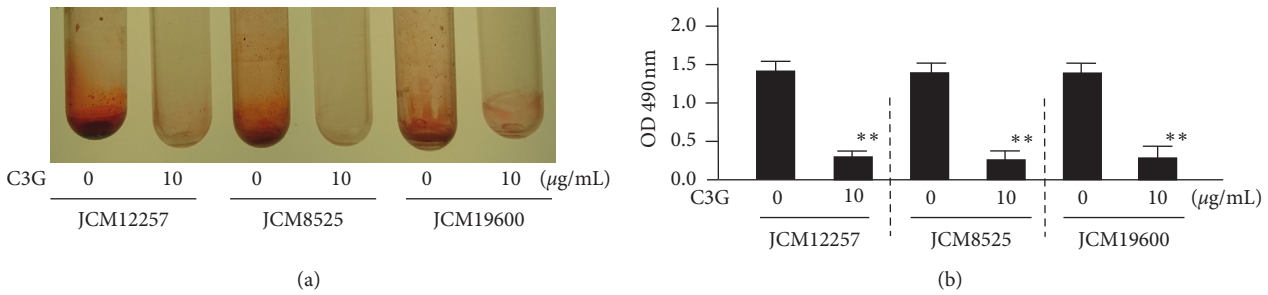
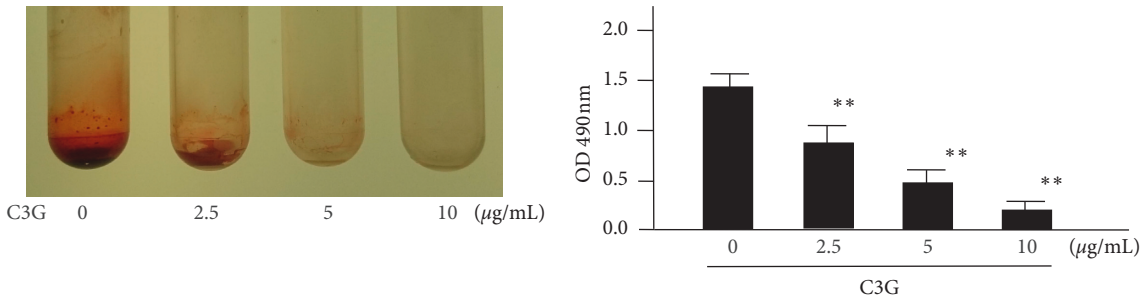


Figure 8: Inhibitory effect of LCEE on biofilm formation by *P. gingivalis* evaluated by SEM assay. *P. gingivalis* JCM12257 was treated with or without LCEE (500 μg/mL) for 72 h. Tracheal tube was involved in culture medium. Biofilm was analyzed by scanning electron microscopy (SEM). (a) Untreated bacteria. (b) LCEE-treated bacteria. LCEE: *Lonicera caerulea* var. *emphyllocalyx* extract.



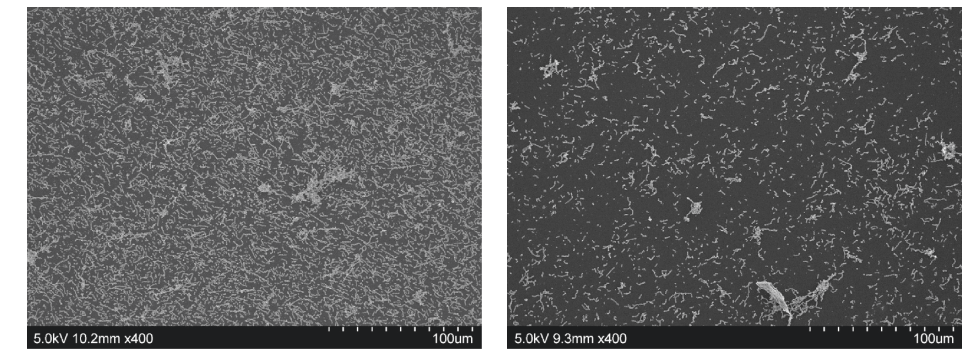
(a) (b)

Figure 9: Inhibitory effect of C3G on biofilm formation by three *P. gingivalis* isolates. Three *P. gingivalis* isolates (JCM12257, JCM8525, and JCM19600) were treated with or without C3G (10 μg/mL) for 72 h. (a) Image of polypropylene tube. (b) Biofilm formation was quantified by safranin red adsorption at 490 nm. Data represent the mean ± SD ($n = 3$). $^{**}p < 0.01$ compared to each untreated group evaluated by Student's *t*-test. C3G: cyanidin-3-O-glucoside.



(a) (b)

Figure 10: Dose-dependent inhibitory effect of C3G on biofilm formation by *P. gingivalis*. *P gingivalis* JCM12257 were treated with or without C3G (2.5, 5, and 10 μg/mL) for 72 h. (a) Image of polypropylene tube. (b) Biofilm formation was quantified by safranin red adsorption at 490 nm. Data represent the mean ± SD ($n = 3$). $^{**}p < 0.01$ compared to untreated group evaluated by Turkey/Bonnferoni's multiple comparison test. C3G: cyanidin-3-O-glucoside.



(a) (b)

Figure 11: Inhibitory effect of C3G on biofilm formation by *P. gingivalis* evaluated by SEM assay. *P. gingivalis* JCM12257 was treated with or without LCEE (500 μg/mL) for 72 h. Tracheal tube was involved in culture medium. Biofilm was analyzed by scanning electron microscopy (SEM). (a) Untreated bacteria. (b) C3G-treated bacteria. C3G: cyanidin-3-O-glucoside.

medium containing LCEE before biofilm formation, the biofilm of *P. gingivalis* was significantly suppressed. However, the addition of LCEE did not suppress the biofilm already formed (Figure 6). When LCEE was added to the biofilm that had already been formed, LCEE significantly promoted the removal of biofilm by vibration using a vortex mixer for 10 sec compared to control (Figure 7). It was also confirmed the effect of LCEE on biofilm formation in tracheal tubes using SEM scanning. Although *P. gingivalis* formed a monospecies biofilm in control, those cultured with LCEE (500 μg/mL) did not form biofilm significantly (Figure 8).

3.3. Bacterial Biofilm Analysis with C3G. Next, it was evaluated whether C3G, one of the components of LCEE, could inhibit biofilm formation by *P. gingivalis* [20, 25]. Three *P.*

gingivalis isolates (JCM12257, JCM8525, and JCM19600) were grown in GAM with or without C3G. As expected, C3G exhibited significant inhibitory effects on biofilm formation by three anaerobic bacteria (Figure 9). More than 2.5 μg/mL of C3G significantly ($p < 0.01$) inhibited biofilm formation by anaerobic bacteria in a concentration-dependent manner. The IC_{50} value of C3G was calculated as 3.3 μg/mL (Figure 10). Finally, it was examined the presence or absence of the biofilm inhibitory effect of C3G on bronchial tubes used in clinical practice. SEM scanning showed that although control *P. gingivalis* formed a monospecies biofilm, *P. gingivalis* cultured with C3G (10 μg/mL) did not form biofilm; the difference was statistically significant (Figure 11).

4. Discussion

Within the scope of our investigation, this study is the first scientific research on the antibiofilm formation effect of LCEE against *P. gingivalis*. We demonstrated the antibiofilm formation effect of LCEE on polypropylene tubes by safranin red staining and on tracheal tubes by scanning electron microscopy. First, LCEE significantly suppressed the biofilm formation of three independent *P. gingivalis* isolates. Next, our study demonstrated that LCEE significantly inhibited the formation of biofilm by *P. gingivalis* in a concentration- and time-dependent manner. In addition, the pretreatment effect of LCEE was superior to the posttreatment effect. However, we confirmed that applying vibrational stimulation is enough to remove the biofilm even when treated with LCEE after biofilm formation. Furthermore, LCEE reduced the bacterial biofilm in tracheal tubes. The use of C3G, one of the constituents of LCEE, also showed antibiofilm formation effects against three anaerobic bacteria. The effect of C3G was expressed in a dose-dependent manner, and the biofilm inhibitory effect in the bronchial tube was also recognized by SEM analysis. As there are several reports of potential antibiofilm candidate drugs against *P. gingivalis* such as the fruit and seed of *Elettaria cardamomum*, resveratrol, azithromycin, carvacrol and terpinen-4-ol, and curcumin [26–30], there are no established antibiofilm drugs for inhibiting biofilm formation of *P. gingivalis* in western medicine. These results suggest that LCEE treatment would be more beneficial for antibiofilm formation therapy of *P. gingivalis* as well as plaque control.

There are several scientific reports of the antibacterial effect of plant fruits on *P. gingivalis* worldwide. *Phyllanthus emblica* (PE) fruit extract exerts antibacterial effects, and the assessment of *P. gingivalis* revealed significant differences between the PE and control groups [31]. Kapadia et al. showed the antimicrobial activity of banana peel extract on *P. gingivalis*. Using a well diffusion method, *P. gingivalis* showed a 15 mm inhibition zone against an alcoholic extract of banana peel [32]. Seneviratne et al. focused on the mode of antibacterial actions of *Prunus mume* fruit extract against periodontal pathogens. A total of 15 oral pathogens including *P. gingivalis* were investigated to screen the antibacterial activities of *Prunus mume* fruit extract by an agar diffusion assay. *P. gingivalis* was the most susceptible species for *Prunus mume* fruit [33]. Several compounds isolated from the fruits of *Melia toosendan* exhibited significant antibacterial activity against *P. gingivalis* ATCC 33277 [34]. However, we could not confirm the scientific reports of the antibiofilm effect of fruit on *P. gingivalis*. Additionally, our results regarding the antibiofilm effect of LCEE on *P. gingivalis* seem to be beneficial to both basic science and clinical medicine.

We confirmed C3G, one of the compounds found in LCEE, is effective against *P. gingivalis*. Previous reports showed that the content of C3G in LCE fruit was significantly higher than that in other common berries [25]. In the present study, IC_{50} of LCEE was 177 μg/mL. Since LCEE contains 1.12% of C3G, the concentration of C3G in 177 μg/mL of LCEE was calculated to be 1.98 μg/mL. From the approximation formula of the relationship between the antibiofilm activity and the concentration of C3G shown in Figure 10(b), the inhibition percentage of C3G at 1.98 μg/mL was calculated to be 36.3%. Since this concentration was the IC_{50} of LCEE, the contribution of C3G to the activity of LCEE was calculated to be 72.6%. Therefore, C3G plays an important role as the active ingredient in LCEE. To date, there have been few reports of antibacterial effects of C3G against bacteria. C3G suppressed the secretion of CagA and VacA because of intracellular accumulation of CagA and VacA in *H. pylori*. C3G did not inhibit CagA and VacA expression except SecA transcription in *H. pylori*. Although SecA is associated with translocation of bacterial proteins because of the downregulation of SecA expression by C3G, *H. pylori* may reduce the toxin secretion [35]. Yao et al. demonstrated that bayberry fruit extract possessed antibacterial activity against *Salmonella*, *Listeria*, and *Shigella* significantly. The fraction of bayberry with the most activity comprised of flavonoids, which included C3G [36]. Lacombe et al. evaluated the antimicrobial effect of the contents of the American cranberry (the fruit of *Vaccinium macrocarpon*) against *Escherichia coli* O157 : H7; it was demonstrated that anthocyanins produced significant bacterial reductions with minimum inhibitory concentrations of anthocyanins of 14.8 μg/mL (C3G equivalent) [37]. However, it is also clear from our results that C3G has an antibiofilm formation effect, as C3G suppressed bacterial biofilm formation as much as LCE upon SEM analysis. It may be speculated that natural compounds which have antibiofilm formation effects other than C3G are present in LCE. Research on the unknown natural compounds in LCE would be beneficial.

LCEE inhibits the biofilm formation of *P. gingivalis*. Among the components of LCEE, C3G plays an important part in suppressing biofilm formation by this bacterium. Our results reveal that LCEE may be an effective antibacterial substance for *P. gingivalis*-induced aspiration pneumonia because of its ability to suppress bacterial biofilm formation in the oral cavity.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Research Article

Neopterin and CXCL-13 in Diagnosis and Follow-Up of *Trypanosoma brucei gambiense* Sleeping Sickness: Lessons from the Field in Angola

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Human African Trypanosomiasis may become manageable in the next decade with fexinidazole. However, currently stage diagnosis remains difficult to implement in the field and requires a lumbar puncture. Our study of an Angolan cohort of *T. b. gambiense*-infected patients used other staging criteria than those recommended by the WHO. We compared WHO criteria (cell count and parasite identification in the CSF) with two biomarkers (neopterin and CXCL-13) which have proven potential to diagnose disease stage or relapse. Biological, clinical, and neurological data were analysed from a cohort of 83 patients. A neopterin concentration below 15.5 nmol/L in the CSF denoted patients with stage 1 disease, and a concentration above 60.31 nmol/L characterized patients with advanced stage 2 (trypanosomes in CSF and/or cytorachia higher than 20 cells) disease. CXCL-13 levels below 91.208 pg/mL denoted patients with stage 1 disease, and levels of CXCL-13 above 395.45 pg/mL denoted patients with advanced stage 2 disease. Values between these cut-offs may represent patients with intermediate stage disease. Our work supports the existence of an intermediate stage in HAT, and CXCL-13 and neopterin levels may help to characterize it.

1. Introduction

It is hoped that sleeping sickness will become manageable within the next decade, as suggested by the WHO [1, 2]. In 2016, the number of patients with sleeping sickness has been reported as fewer than 4,000 but there are still unreported cases, and the estimate of actual cases is around 20,000 infected people in the remaining endemic countries in Africa. The availability of easy-to-use molecules such as fexinidazole (oral intake) will help to reduce the remaining burden of the disease [3, 4].

Pentamidine (for *gambiense*-HAT) remains the molecule indicated for the treatment of stage 1 of the disease. Thus, stage diagnosis is still necessary. Furthermore, fexinidazole has so far only been used in the context of field trials. What will happen when it is used routinely? Moreover, the infection mechanism remains incompletely understood; the disease may reappear at cyclic intervals as chronically infected individuals without clinical signs of disease or animal reservoirs can lead to reemergence [5, 6]. Recently, the skin has been suggested as possible reservoir of latent infection, leading to relapse [7], and several authors have

suggested that relapses can occur from parasites living in the meningeal spaces and recirculating through the cervical lymph node system [8, 9]. As the knowledge of pathogen-invasion mechanisms in the central nervous system (CNS) improves, new concepts help us to integrate data from the field and enhance our knowledge of sleeping sickness infection mechanisms. Establishing disease stages is necessary for effective treatment, and the discovery of new markers in order to do this relies on the understanding of mechanisms of CNS invasion [10].

During stage 1 of the disease, the invasion of the host by the trypanosome is accompanied by inflammation caused by recognition of two pathogen-associated molecular patterns (PAMPs), the parasite VSG coat and DNA, by the immune system [11]. This triggers a cascade of activation of various immune cells, which is regulated by cytokines and chemokines [11–15]. During the course of sleeping sickness, trypanosomes cross the blood–brain barrier and invade the CNS, leading to the second stage of the disease. This tropism of the parasite for CNS induces neuroinflammation and lymphocyte infiltration in the cerebrospinal fluid (CSF). At present, only a lumbar puncture showing cytorachia of greater than 5 cells/μL or evidence of trypanosomes in the CSF allows the diagnosis of stage 2 of the disease [3, 16]. Alternatively, expression levels of various cytokines and chemokines may track inflammatory changes earlier and may be more relevant for the diagnosis as already demonstrated in various inflammatory conditions, including sleeping sickness [17]. Neopterin is a pyrazino-pyrimidine compound that is synthesized by monocytes and macrophages in response to the production of interferon-γ by activated T cells. The increase in the level of expression of neopterin therefore follows the increase in macrophage and T cell activation [18]. In fact, increased neopterin levels are seen early in various pathologies, including malignant tumours such as CNS lymphomas [19] and viral infections [20, 21] including HIV [22, 23], justifying its use as a screen to exclude infected blood from blood donors [24, 25]. CXC-chemokine ligand 13 (CXCL-13) is expressed by B cells and dendritic cells and is involved in B cell migration [26]. Perturbation of plasma levels of CXCL-13 is associated with activation of the host's immune response [27], and CXCL-13 is used as an indicator of breast cancer, cutaneous vasculitis, and various lupus-like condition and bacterial infections [28–31]. CSF CXCL-13 levels are clearly associated with neuroborreliosis [32], neurosyphilis concomitant with HIV [33], and multiple sclerosis [34]. Previous studies have shown that increased levels of CXCL-13 and neopterin could be a good diagnostic marker for sleeping sickness and an indicator of the disease stage [35–37]; rapid detection tests (RDTs) based on the neopterin concentration in the CSF are under investigation.

In this study, classical staging criteria, clinical signs, and new potential biomarkers (namely, neopterin and CXCL-13) were reevaluated in patients from a cohort study in Angola [36, 37]. These results are discussed on the base of the immune response and the physiopathological context of the diseases, arguing in favor of an intermediate stage [38].

2. Patients and Methods

2.1. Ethics Statement. Ethical clearance was obtained from the Angolan Direccao Nacional de Saude Publica, Ministerio da Saude. Written informed consent was received from these individuals prior to enrolment and/or from their parents or guardians for participants below 18 years of age. Any individual who declined to participate was followed up according to the standard procedures of the national control programme of Angola.

The clinical data used to support the findings of this study are restricted by the Angolan Direccao Nacional de Saude Publica, Ministerio da Saude in order to protect patient privacy. Data are available from the authors (bertrand.courtioux@unilim.fr) for researchers who meet the criteria for access to confidential data.

2.2. Case Definition, Inclusion, and Exclusion Criteria. Samples were obtained retrospectively from a cohort study conducted in Angola between 2008 and 2011 (Figure 1). The study aimed to collect appropriate clinical, neurological, psychiatric, and biological data from a cohort of 247 *Trypanosoma brucei gambiense*-infected patients ($n = 228$) from diagnosis to the end of follow-up (6, 12, 18, and 24 months) and noninfected controls ($n = 19$).

It is not acceptable to perform a lumbar puncture on a patient for whom no neurological pathology is suspected. Seronegative Angolan controls for HAT could therefore not be included in this study. Thus, the 19 controls are derived from the 601 patients with a positive CATT test. Initially, these individuals were suspected of HAT and finally considered healthy following further negative tests.

Subjects were enrolled during both active and passive screening activities by teams of the national sleeping sickness control programme. The CATT (Card Agglutination Trypanosomiasis Test) screen was used [39] and followed by confirmation using microscopy of concentrated blood and CSF. Patients with sleeping sickness were defined as individuals in whom trypanosomes were demonstrated in either blood, lymph node aspirate, or CSF by microscopy. Disease cases were classified as stage 1 when no trypanosomes were observed in CSF and when the CSF white blood cell (WBC) count was lower than or equal to 5 cells/μL, whereas those with trypanosomes in the CSF and/or a CSF WBC count above 20 cells/μL were classified as advanced stage 2. Patients who were deemed to be in the intermediate stage were those between stage 1 and advanced stage 2 with a CSF WBC count between 5 and 20 cells/μL and/or with trypanosomes in CSF without an increase in CSF WBC numbers. The Angolan national control programme differentiates the intermediate stage, as they treat those patients with first-stage drugs unless some clinical signs are suggestive of CNS involvement, in which case second-stage drugs are used. All participants were examined clinically, and a questionnaire was used to note all clinical and neurological characteristics (Romberg, Babinski's reflex, dysmetry, meningeal syndrome, etc.), including sleep and psychiatric disturbances using the Mini-International

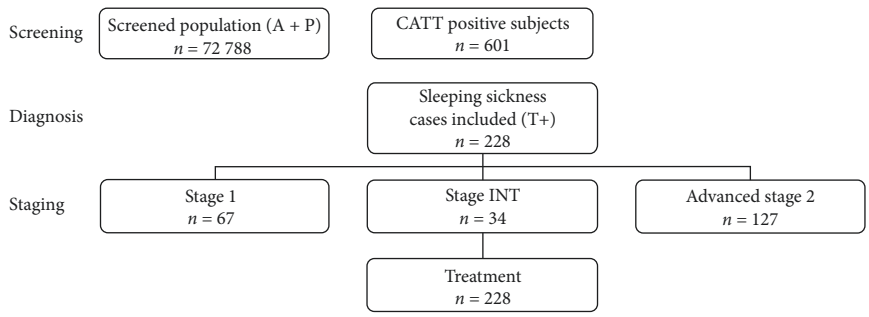

Figure 1: Cohort study conducted in Angola between 2008 and 2011. The enrolment process of 247 *Trypanosoma brucei gambiense*-infected patients and controls is shown. This cohort is composed of 19 controls, 67 patients with stage 1, 34 patients with intermediate stage, and 127 patients with advanced stage 2. All HAT patients were treated according to their stage.

neuropsychiatric interview [40] and the Hamilton rating scale for depression. These two scales include sleep examination criteria. All participants were checked clinically and microscopically for the presence of the main parasitic coinfections (malaria by blood smear, filariasis during blood examination by capillary tube centrifugation, and schistosomiasis when blood was detected in urine samples). The HIV and syphilis status was determined retrospectively on stored samples using the VIKIA HIV 1/2 test (Biomérieux, France) and the RPR-Nosticon II (Biomérieux, France) and TPHA (Biorad, France) tests, respectively. These analyses aimed to exclude individuals with interfering parameters for CSF analysis. Children under 12 years were also excluded to avoid complications in obtaining lumbar puncture samples and also possible difficulties in interpretation [41, 42].

For our analysis of neopterin and CXCL-13, we selected 83 patients for whom we had maximum of neopterin and CXCL-13 CSF concentrations data. When the concentration data were missing and we had CSF samples available in the biobank, we determined the neopterin and CXCL-13 concentration in the CSF via ELISA. The ELISA kits for the dosage of neopterin and CXCL-13 used are the same throughout the study [42].

2.3. Sample Collection and Selection. A lymph node aspirate was taken from any subject who presented with swollen lymph nodes and examined for trypanosomes by microscopy. 10 mL venous blood with heparin as anticoagulant was collected from CATT-positive individuals as well as from patients with swollen lymph nodes. 600 μL blood was used to perform capillary tube centrifugation test (4 capillary tubes of 75 μL), and 300 μL blood was used for the miniature anion exchange centrifugation technique [43]. For individuals who were positive by CATT on whole blood, 1 mL plasma was used to perform CATT dilutions. Confirmed cases of parasite infection and/or individuals found positive by CATT at a dilution of 1/16 who had negative results for all other performed tests for parasite infection underwent a lumbar puncture, in accordance with national guidelines for stage determination and/or parasitological confirmation in CSF, when there were suggestive neurological signs. Parasitological examination of CSF was done using the modified single centrifugation technique. This optimized parasitological confirmation method permits sensitivity of parasite detection similar to that of molecular testing [42, 43].

All plasma, buffy coat, and CSF samples that remained after the diagnostic procedures were aliquoted and stored in liquid nitrogen before being transported to Limoges on dry ice and then stored at −80°C. Patient medical data were anonymized. Samples were further selected for neopterin and CXCL-13 determination at diagnosis [42].

2.4. Test Procedures. Commercially available ELISA assays were used to measure CSF levels of neopterin (Brahms, Thermo Fisher Scientific, Germany) and CXCL-13 (R&D Systems, UK, and RayBiotech, GA, USA) as performed previously by Tiberti et al. [36, 37]. All assays were performed according to the manufacturer's instructions and the interassay variability was evaluated using quality controls (coefficient of variation (CV) < 20%). A limit of detection corresponding to the mean concentration measured for the lowest standard less than 2 standard deviations was calculated for each assay. All data were obtained using the same ELISA assays than previous authors and data were compared to those obtained by Tiberti et al. [36, 37].

2.5. Statistical Analysis. Descriptive analysis was carried out on Microsoft Excel. Receiver operating characteristic (ROC) curves were drawn to determine cut-off points as the best sensitivity/specificity ratio for each marker (CXCL-13, neopterin, leucocyte count, sleep disturbances, neurological signs, and presence of trypanosomes). ROC curves were further used to determine the disease stage (that is, stage 1, intermediate, or advanced stage 2) on diagnostic samples. The significance was assessed using the Kruskal–Wallis test and Fisher's exact test for testing the null of independence ($p < 0.05$). Tests were performed using R Core Team version 3.3.2 [44] and the following packages were used: ROCR for drawing curves and determining the values of specificity and sensitivity that will make it possible to find the cut-off [45] and PMCMR for the pairwise comparisons post hoc test of the Kruskal–Wallis test [46].

When the values of the CXCL-13 and neopterin biomarkers from the stage 1 patients are represented on the same curve (Figure 2(a)), there is no definite cut-off value with an acceptable sensitivity and specificity. For the

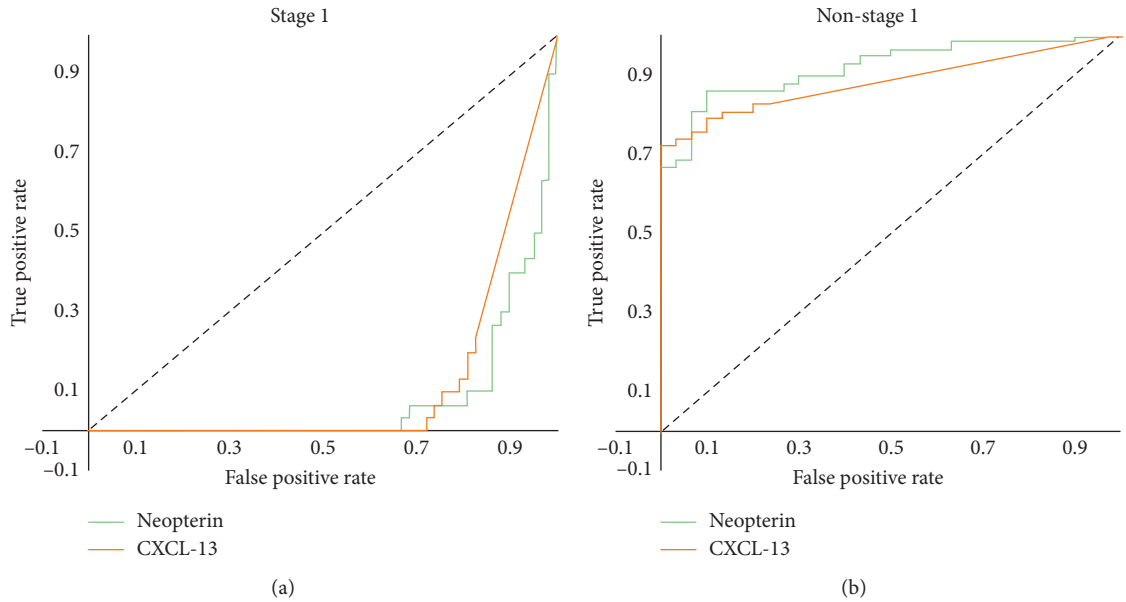

Figure 2: ROC curves for all threshold values for neopterin and CXCL-13 in patients with stage 1 disease and in patients with non-stage 1 disease (intermediate stage and advanced stage 2). ROC curves represent sensitivity as a function of the complement of specificity (1 – specificity) for all threshold values for neopterin and CXCL-13 in patients with stage 1 disease (a) and in patients with non-stage 1 disease (b).

feasibility of our analysis, we have thus grouped all patients not in stage 1 (that is patients with intermediate stage and advanced stage 2 disease) and defined a cut-off discriminating non-stage 1 patients (Figure 2(b)). Patients with values below these cut-off points are considered to be in stage 1.

Cut-off points used for comparison have been used in accordance with previous data for neopterin and CXCL-13 [36, 37].

3. Results

3.1. Population Characteristics at Inclusion. Demographic, clinica, l and biological data are summarized in Figure 3. Sex ratio was 0.44 (37/83), and ages ranged from 13 to 83, with median ages of 42.7, 35.37, and 34.38 years for stage 1, intermediate, and advanced stage 2 disease groups, respectively. CATT serology was positive in all individuals, as it was the screening method used, and titres ranged from 1/8 to 1/32. Trypanosomes were found in 48 lymph node aspirates, 50 blood samples, and 34 CSF samples. Patients with stage 1 disease had a mean CSF WBC count of 1.83 cells/μL, and no trypanosomes were detected in the CSF. Patients with intermediate stage disease had a mean WBC count of 10.05, and three patients had trypanosomes in their CSF, although the WBC counts of these patients were less than 5 cells/μL. Patients with advanced stage 2 disease had a mean cell count of 226 cells/μL, and trypanosomes were detected in the CSF of 31 out of 34 patients. Sleep disorders were present in 27% of patients with stage 1 disease, 16% of patients with intermediate stage disease, and 88% of patients with advanced stage 2 disease, with a clear increase in their severity with disease stage. Eleven (37%) patients in stage 1 and four (21%) patients in the intermediate stage showed neurological signs, and there was an increase in neurological signs in advanced stage 2 patients (32 out of the 34 (94%)) compared with previous stages (Figure 3). All patients with stage 1 disease were treated with pentamidine; 33 out of 34 patients with advanced stage 2 disease were treated with eflornithine (DFMO), and one was treated with pentamidine; 16 out of 19 patients with intermediate stage disease were treated with pentamidine, and three of them were treated with DFMO. The choice of treatment for intermediate stage patients was made by the medical team based on the severity of the clinical symptoms of these patients.

3.2. Neopterin and CXCL-13 Levels during Diagnosis. Neopterin and CXCL-13 concentrations at inclusion are presented in Table 1. At diagnosis, the mean concentrations of neopterin and CXCL-13 in the CSF were higher in patients with advanced stage 2 disease (280.62 nmol/L and 3919.32 pg/mL, respectively) than patients with intermediate disease (18.72 nmol/L and 111.85 pg/mL, respectively) and patients with stage 1 disease (12.15 nmol/L and 26.42 pg/mL, respectively).

3.3. New Neopterin and CXCL-13 Cutoff Points for Defining Disease Stages. Our study suggests that a CSF neopterin concentration less than or equal to 15.56 nmol/L defines stage 1 disease with 84% sensitivity and 90% specificity (AUC = 0.91), whereas a concentration above 60.31 nmol/L characterizes advanced stage 2 disease (AUC = 0.99; 97% sensitivity and 100% specificity). Values between these two cut-off points could be used to characterize an intermediate stage (Table 2).

	Stage 1	Int stage	Ad stage 2
Number of patients	30	19	34
Sex (male/female)	11/19	7/12	19/15
Age (median, range)	42.7 (13–83)	35.37 (13–71)	34.38 (13–62)
CATT positive (positive/number tested)	30/30	19/19	34/34
CATT titre range	1–32	1–32	1–32
Presence of trypanosomes (blood; lymph; CSF)	(30; 26; 0)	(9; 7; 3)	(11; 15; 31)
CSF WBC per μL (median; range)	1.83 (1; 0–4)	10.05 (9; 1–20)	266.24 (220; 30–999)
Sleep disturbances	8/30	3/19	30/34
Neurological signs	11/30	4/19	32/34

Figure 3: Samples and patients' follow-up with population characteristics at inclusion. Table represents the epidemiological and biological data of the 30 patients with stage 1 disease, the 19 patients with intermediate stage disease, and the 34 patients with advanced (Ad) stage 2 disease who constitute the inclusion cohort.

Table 1: CXCL-13 and neopterin levels in the three stages of disease.

		Inclusion Mean (SD)
Stage 1	CXCL-13[1]	26.42 (36.38)
	Neopterin[2]	12.15 (4.95)
Int stage	CXCL-13[1]	111.85 (119.30)
	Neopterin[2]	18.72 (8.5)
Ad stage 2	CXCL-13[1]	3919.32 (1856.06)
	Neopterin[2]	280.62 (220.69)

The mean and standard deviation (SD) of CSF concentration of neopterin and CXCL-13 are shown for patients with stage 1, intermediate (Int), and advanced (Ad) stage 2 disease at inclusion. [1]pg/mL. [2]nmol/L.

Table 2: Sensitivity and specificity of new tests for staging.

	Neopterin (nmol/L)	CXCL-13 (pg/mL)
Non-stage 1	15.57	91.21
Sensitivity [IC]	0.90 [0.73; 0.98]	0.90 [0.73; 0.98]
Specificity [IC]	0.86 [0.74; 0.94]	0.79 [0.66; 0.89]
Int stage		
Sensitivity [IC]	0.65 [0.43; 0.84]	0.52 [0.31; 0.73]
Specificity [IC]	0.94 [0.85; 0.98]	0.95 [0.87; 0.99]
Ad stage 2	60.31	395.45
Sensitivity [IC]	0.97 [0.85; 1.00]	0.90 [0.85; 1.00]
Specificity [IC]	1.00 [0.93; 1.00]	0.86 [0.93; 1.00]

The cut-off values established for neopterin and CXCL-13 are expressed in nmol/L and pg/mL, respectively. The sensitivity and specificity values for each cut-off points determined by ROC analysis are shown in the table. The authors defined cut-off values of neopterin and CXCL-13 for non-stage 1 patients, under which patients are considered to be in stage 1 of sleeping sickness.

Our findings also suggest that CSF CXCL-13 levels below 91.21 pg/mL define stage 1 disease (AUC = 0.89; 81% sensitivity and 90% specificity), and CXCL-13 levels above 395.45 pg/mL define advanced stage 2 disease (AUC = 0.93; 97% sensitivity and 100% specificity). Values between these two cut-off points could be used to characterize an intermediate stage (Table 2).

3.4. Comparison of Neopterin and CXCL-13 Concentrations to Clinical Manifestations (Neurological Signs, Sleep Disorders), Leucocyte Count, and Presence of Trypanosomes in CSF. ROC curves show that neurological signs and sleep disorders appeared when CSF neopterin concentrations were above 21.20 nmol/L (AUC = 0.79 and 0.81, respectively) and/or CXCL-13 concentrations were above 330.26 pg/mL (AUC = 0.78 and 0.80, respectively). Trypanosomes were found in the CSF when neopterin concentrations were higher than 31.40 nmol/L (AUC = 0.94) and/or CXCL-13 concentrations were higher than 688.85 pg/mL (AUC = 0.94). Sleep and neurological disorders were associated with the presence of a minimum of 16 cells/μL of CSF (AUC = 0.80), and the invasion of the CNS by trypanosomes was associated with the presence of more than 50 cells/μL of CSF (AUC = 0.93). The sensitivity and specificity of each of these cut-off points are shown in Table 3.

4. Discussion

Our study addressed and characterized a specific group of patients with intermediate stage sleeping sickness, or early stage of CNS involvement. There have been reports of patients cured with first-line drugs at this disease stage, which suggests

Table 3: Comparison of neopterin and CXCL-13 results to classical markers (quantitative and qualitative data).

Cut-off	Neurological signs	Sleep disorders	Trypanosomes in CSF
CXCL-13[1]	310.52	330.26	310.52
Sensitivity [IC]	0.63 [0.49; 0.76]	0.63 [0.48; 0.77]	0.79 [0.63; 0.90]
Specificity [IC]	0.92 [0.77; 0.99]	0.92 [0.78; 0.98]	0.90 [0.78; 0.97]
Neopterin[2]	21.20	21.20	31.41
Sensitivity [IC]	0.69 [0.54; 0.81]	0.69 [0.55; 0.82]	0.84 [0.69; 0.94]
Specificity [IC]	0.83 [0.67; 0.94]	0.82 [0.68; 0.92]	0.90 [0.78; 0.97]
WBC count[3]	12	16	50
Sensitivity [IC]	0.71 [0.56; 0.83]	0.71 [0.57; 0.83]	0.82 [0.66; 0.92]
Specificity [IC]	0.92 [0.78; 0.98]	0.89 [0.76; 0.97]	0.84 [0.70; 0.93]

The determined cut-off values of neopterin, CXCL-13, and leucocyte numbers at the appearance of clinical signs of the disease (neurological signs and sleep disorders) and CNS invasion by the parasite. [1]pg/mL. [2]nmol/L. [3]cells/μL CSF.

that either pentamidine [38, 47, 48] or host immunity can contribute to eradication of the parasite [49, 50]. Our results showed that 75% (12/16) of patients with intermediate stage disease were cured following pentamidine treatment, confirming earlier data and supporting evidence of the existence of that stage [38, 51]. The physiopathology of this intermediate stage remains unknown. Question remains about the presence and the numbers of parasites in the CNS, the host immune response. Some hypothesis can be done, especially about the capacity of the parasite to resist in the CNS but its localisations into cells were demonstrated *in vitro* [52, 53]. In the present state of knowledge, this intermediate stage (between the hemolymphatic stage and the meningoencephalitis stage) can be defined only on the basis of our biomarker levels CXCL-13 and neopterin. But we can hypothesized that it corresponds to the presence of the parasite into cells as it was shown previously *in vitro* and/or the beginning of the BBB crossing with the presence of some parasites in the CSF which have not been detected yet on CSF after lumbar puncture. However, the relevance and the importance of the intermediate stage are still not completely understood [10]. In three patients with intermediate stage disease, trypanosomes were present in the CSF or at the interface between the blood system and the CSF which are endothelial cells, without an increase in WBC count. Current WHO recommendations for clinical trials do not usually take the intermediate stage of disease into account in order to simplify treatment protocols; therefore, stage 2 disease is characterized as having a CSF WBC of above 5 cells/μL [52]. In the next years, those criteria will not be necessary for treatment, as fexinidazole will be proposed for all patients. But the accuracy of using neopterin and CXCL-13 as biomarkers for sleeping sickness has been demonstrated previously in larger cohorts, and their links with early inflammatory processes of sleeping sickness have been shown [35–37]. In our study, we assessed the ability of those markers to potentially characterize disease stages; it was shown that levels of CSF neopterin and CXCL-13 can define stage 1 and advanced stage 2 diseases and that the range of biomarker concentrations between those assigned to stage 1 and advanced stage 2 could indicate an intermediate disease stage.

One of the difficulties encountered was to define cut-off points for markers of stage 1 disease in the absence of seronegative Angolan controls for HAT in our cohort. This is why we have grouped all patients in the intermediate stage and in stage 2 of the disease and therefore defined cut-offs to discriminate patients who are not in stage 1 (non-stage 1). Values below these thresholds are therefore characteristic of stage 1 patients.

Previously, CSF neopterin levels below 14.3 nmol/L were indicative of stage 1 disease, and neopterin levels greater than this were indicative of stage 2 disease (determined from 412 samples) [37]. Similarly, CSF concentrations of CXCL-13 lower than 125.5 pg/mL were indicative of stage 1 disease, whereas levels greater than this were indicative of stage 2 disease (determined from 97 samples) [36]. In our study, it was shown that the cut-off point of CSF neopterin to define stage 1 disease is below 15.56 nmol/L, which is close to that established by Tiberti et al. in 2013 [37]. However, we have established a cut-off point marking the entry of patients in advanced stage 2 at 60.31 nmol/L, introducing a gap between two cut-off values that represents the levels of neopterin found in the intermediate stage of disease.

In our study, the cut-off point of CSF CXCL-13 to define stage 1 disease was found to be a concentration of less than 91.21 pg/mL; a CSF CXCL-13 concentration greater than 395.45 pg/mL was indicative of advanced stage 2 disease. Concentrations of CXCL-13 between these two values were indicative of the intermediate stage. The concentrations of CXCL-13 that define stage 1 and stage 2 diseases were originally found in the study by Tiberti et al. in 2012 [36]; their data suggested that a CSF CXCL-13 concentration below 125.5 pg/mL was indicative of stage 1 disease, whereas values above this threshold were indicative of stage 2 disease. This cut-off value falls between the two cut-off points found in our study (below 91.21 pg/mL for stage 1 disease and above 395.45 pg/mL for advanced stage 2).

This observed difference between the CXCL-13 and neopterin cut-off points is due to the fact that in the studies by Tiberti et al. [36, 37], patients in intermediate stages of disease are included in stage 2. Here, we have demonstrated that a change in the CSF neopterin and CXCL-13 concentration does not correspond simply to stage 1 or 2 (a WBC count of greater than 5 cells/μL), but rather to an intermediate stage between stage 1 (a WBC count below 5 cells/μL) and an advanced stage 2 (a WBC count greater than 20 cells/μL).

The differences in levels of neopterin and CXCL-13 in patients with sleeping sickness may be explained by their production mechanisms. Neopterin is produced by

microglial cells in response to the parasite [54, 55], whereas CXCL-13 occurs when the parasite is already in the CNS and induces, in conjunction with CXCL-12, leucocyte extravasation into the CSF [56]. This is correlated with a late response of the B cell populations, as described in the literature [57, 58]. This mechanism can explain why, in serum, no correlation can be made with stage diseases.

The main criticism of this work is the absence of a HAT-seronegative Angolan controls population, which is justified because it is not ethical to perform a lumbar puncture on a healthy person. It is for this reason that we had to define cut-off points for neopterin and CXCL-13 above which patients are considered non-stage 1; all patients with values below these thresholds are thus stage 1. According to the literature, the normal concentration of neopterin is less than 5.1 nmol/L of CSF [19], and the normal concentration of CXCL-13 is less than 11.5 pg/mL of CSF [59], but we do not know the true concentration values for these two biomarkers in healthy Angolan individuals. In our study, cut-off values (as well as their sensitivities and specificities) were defined on a limited sample size. These values would be improved using a larger sample of patients.

The difficulty in all studies of neurological stage discrimination during sleeping sickness is the definition of the gold standard as recently reviewed [60]. The current gold standard is defined by the criteria of the WHO [54] based on the cytorachia and/or the presence of the parasite in the CSF. The only specific test confirming stage 2 is the presence of trypanosomes in the CSF (neurological invasion) [61]. In our study, the presence of trypanosomes is a good marker when CSF WBC count is over 50 cells/μL of CSF (91% sensitivity and 96% specificity) and confirms the lack of sensitivity of parasite detection. The most sensitive and current test to define CNS involvement is cytorachia but this test lacks specificity [54, 62]. The onset of neurological signs and sleep disorders may be associated with cytorachia of less than 5 cells/μL; this has been reported previously [63, 64], confirmed in our study with 37% of stage one patients with neurological signs, and shown also more recently in T. b. *rhodesiense* infection where patients are cured with first-stage drugs [16]. Thus, the appearance of WBC in the CSF does not always correlate with the appearance of neurological signs and sleep disorders; this would also suggest that cytorachia is not a reliable biomarker for staging. In our study, neurological signs and sleep disorders appeared when mean cytorachia was greater than 16 cells/μL. We established cut-off points for neopterin and CXCL-13 related to the onset of clinical signs of the disease instead of using cytorachia but the neopterin and CXCL-13 cutsoff points were not discriminative enough to be used as diagnostic markers.

5. Conclusion

Our work suggests that the levels of the two biomarkers, neopterin and CXCL-13, are good markers of disease staging, in the patient. Using the two biomarkers in conjunction, rather than individually, could be a stronger predictor of disease stage after its diagnosis.

Biomarker identification should not be limited to the CSF and should be continued on other biological fluids to avoid lumbar puncture. Even if no correlations were observed in serum, neopterin and CXCL-13 must be in urine and lacrimal fluid. With the development of new methods to identify specific markers, as proteomics, diagnosis algorithms should be simplified in the near future.

Data Availability

The clinical data used to support the findings of this study are restricted by the Angolan Direccao Nacional de Saude Publica, Ministerio da Saude, in order to protect patient privacy. Data are available from the authors (bertrand.courtioux@unilim.fr) for researchers who meet the criteria for access to confidential data.

Conflicts of Interest

BC and SB were employed for FIND at the moment of the samples collection. JMN is an employee of FIND and accepts the publication of this study.

Acknowledgments

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Research Article

Epidemiological and Clinical Features of Dengue Infection in Adults in the 2017 Outbreak in Vietnam

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Purpose. The clinical features and laboratory results of dengue-infected adult patients admitted to the hospital during the 2017 outbreak were analyzed in this study. *Method*. This is a cross-sectional study. 2922 patients aged 18 years or more with dengue fever in National Hospital for Tropical Diseases (NHTD) in the North and Hospital for Tropical Disease (HTD) in the South of Vietnam were recruited in this study. *Result*. Patients were admitted in the hospital around the year and concentrated from August to December, in 53/63 (84.0%) provinces in Vietnam, and patients in all ages were affected. The number of patients with dengue fever was 1675 (57.3%), dengue with warning signs 914 (31.3%), and severe dengue 333 (11.4%), respectively. Among patients with severe dengue, severe plasma leakage and dengue shock account for 238 (8.1%), severe organ impairment 73 (2.5%), and severe bleeding 22 (0.75%). The rate of mortality was 0.8%, and the outcome of dengue patients is worse in the elderly and people with underlying diseases. *Conclusion*. The 2017 dengue outbreak occurred in a larger scale than in the previous years in terms of time, location, and number of patients. More elderly patients were infected by dengue in this outbreak, and this may contribute to the mortality rate. Clinical manifestations of dengue patients in Southern Vietnam are more typical than the northern, but the rate of severe dengue is not different. The mortality risk and underlying conditions associated with dengue-infected elderly patients are worthy of further investigations in the future.

1. Background

According to the WHO, dengue is one of the mosquito-borne viral diseases that poses high medical burden in many regions worldwide recently. Before 1970, limited number of countries reported severe dengue epidemics [1]. However, the disease is now endemic in more than 100 countries in the regions of Africa, America, Eastern Mediterranean, South East Asia, and Western Pacific [2]. America, South East Asia, and Western Pacific regions are the most seriously affected [1, 2]. In recent years, there is an increasing number of dengue infection cases detected predominantly in urban and semiurban areas and therefore has become a major international public health concern. Severe dengue has become a leading cause of hospitalization and death among children and adults in many regions, especially Asian and Latin American countries [3, 4].

In Vietnam, dengue was first recognized since the 1960s, thanks to the dengue epidemics in the Hanoi (North of Vietnam) and Cai Be (South of Vietnam). Recently, dengue has been reported to affect most provinces of the country [5], and the peak of infection is in June to October every year. Due to the wide geographic distribution of the mosquito vector and circulation of all four types of Dengue virus, dengue could rapidly spread across the country [6–9]. Therefore, the Viet Nam's National Dengue Control

Program was established in 1999, and Vietnam has also been successfully recorded in controlling mortality from dengue fever [9].

Although the disease is now endemic in Vietnam, the knowledge of adult dengue virus infection is still limited and therefore requires a nationwide extensive analysis of clinical and epidemiological results. Such data will also provide useful information for establishing the dengue fever prevention program in Vietnam. In the early year of 2017, an outbreak of dengue fever transmitted throughout the country with much higher number of cases than in previous years. This study was undertaken to examine the clinical and laboratory profile of dengue infection in adult patients and to determine any new insights into the 2017 outbreak.

2. Materials and Methods

2.1. Population Study. The study included patients from 18 years old, diagnosed with dengue during outbreak of the disease from 1 January to 31 December 2017. Patients were recruited from the two largest centers for infectious diseases in Vietnam: National Hospital for Tropical Diseases (NHTD) in the North and Hospital for Tropical Disease (HTD), Ho Chi Minh City, in the South of Vietnam.

2.2. Study Design. This is a cross-sectional study. The case definition was based on compatible clinical history and examination based on WHO criteria, confirmed by positive for the NS1 antigen (rapid test, SD Bioline) or dengue IgM antibodies (rapid test, SD Bioline) or PCR method to detect dengue virus serotype. The PCR can detect dengue virus serotyping using specific primers and probe for each dengue serotypes from 1 to 4. All subjects were classified according to WHO guidelines 2009 [1]. We excluded the patients with confirm other acute infectious diseases such as measles, influenza, or chikungunya. Demographic data and details of clinical history and careful clinical examination were performed. Besides the routine test such as hematocrit, total leucocyte count, platelet count, liver enzyme (ALT and AST), blood urea, and serum creatinine, other investigations were performed according to the clinical conditions of the patients. Patients having nonspecific manifestations were grouped in expanded dengue syndrome category [2].

Sample size: we used a sample calculation formula for a descriptive study:

$$n = \frac{z^2_{1-(\alpha/2)}\,(1-p)}{p\varepsilon^2}, \quad (1)$$

where n is the minimum sample size; p is the rate of severe dengue patients, estimated $p = 10\%$; ε is the relative error, we choose $\varepsilon = 18\%$ (15–20%); and $z_1-\alpha/2$ is the reliability factor, with 95% confidence, $z_1-\alpha/2 = 1.96$.

The calculated sample size in this study was 1100 patients, at one study site. At each study site, based on a sample size of 1100 patients, the numbers of patients per month were randomly selected proportional to the number of hospitalized patients in the year. The total 2922 patients were selected for this study, including 1738 (59.5%) patients in NHTD site and 1184 (40.5%) patients in HTD site.

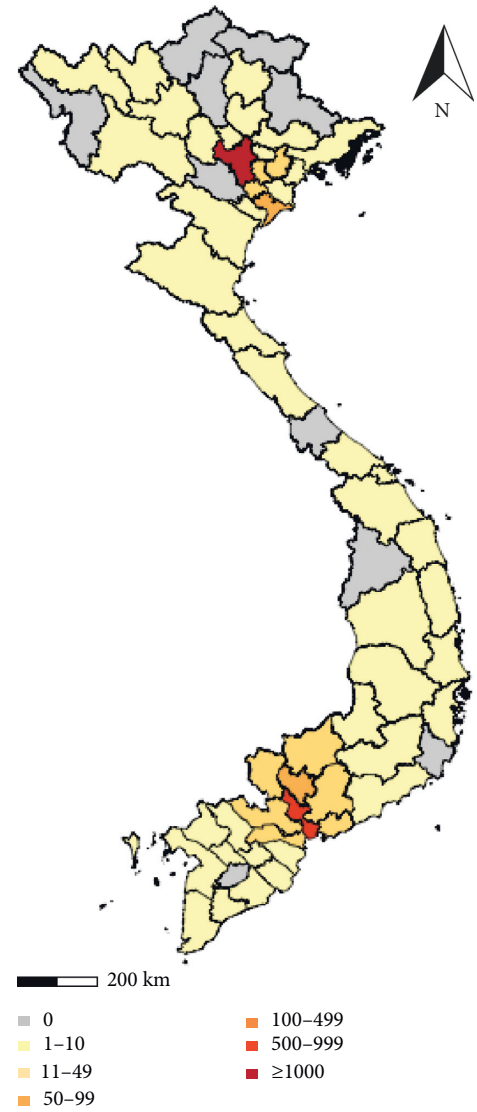

Figure 1: Map of dengue outbreak 2017 in Vietnam. Hanoi (in the North) and Ho Chi Minh city (in the South) are highlighted in red with higher number of dengue cases than other provinces.

All statistical analyses were performed using the SPSS statistical version 17.0. Descriptive statistics like numbers and percentages were enumerated for all categorical variables. The chi-square (χ^2) test was used to evaluate statistical differences in categorical variables between the groups. A p value <0.05 was considered significant.

The study protocol was approved by the ethical committee at NHTD, and all patients were provided informed consents.

3. Results

3.1. Demographic Characteristics. Of the 2922 patients 1392 (47.6%) were male and 1530 (52.4%) were female. The rate male: female = 1 : 1.1. In the dengue outbreak in 2017, dengue patients were found in all ages, from 18 to over 80 years old,

Table 1: Demographic of patients enrolled in the study.

	NHTD *n* (%)	HTD *n* (%)	Total *n* (%)
Total	1738(59.5)	1184(40.5)	2922 (100)
Gender			
Male	842 (48.4)	550 (46.6)	1392 (47.6)
Female	896 (51.6)	634 (53.4)	1530 (52.4)
Age			
≤20 years	184 (10.6)	139 (11.7)	323 (11.1)
21–30 years	652 (37.5)	570 (48.1)	1222 (41.8)
31–40 years	382 (22.0)	304 (25.7)	686 (23.5)
41–50 years	228 (13.1)	118 (10.0)	346 (11.8)
51–60 years	183 (10.5)	42 (3.5)	225 (7.7)
61–70 years	75 (4.3)	10 (0.8)	85 (2.9)
71–80 years	25 (1.4)	1 (0.1)	26 (0.9)
>80 years	9 (0.5)	0 (0.0)	9 (0.3)
Underlying diseases	166 (9.6)	79 (6.7)	245 (8.4)
Pregnant women	73 (4.2)	111 (9.4)	184 (6.3)
Months			
January	1 (0.05)	85 (7.2)	86 (2.9)
February	2 (0.11)	77 (6.5)	79 (2.7)
March	1 (0.05)	71 (6.0)	72 (2.5)
April	3 (0.17)	59 (5.0)	62 (2.1)
May	4 (0.2)	59 (5.0)	63 (2.2)
June	11 (0.6)	102 (8.6)	113 (3.9)
July	79 (4.5)	113 (9.5)	192 (6.6)
August	492 (28.3)	121 (10.2)	613 (20.9)
September	598 (34.4)	108 (9.1)	706 (24.1)
October	357 (20.5)	145 (12.2)	502 (17.2)
November	144 (8.3)	128 (10.8)	272 (9.3)
December	46 (2.6)	116 (9.8)	162 (5.5)
Day admission			
1–3 days of illness	443 (25.5)	328 (27.7)	771 (26.4)
Day 4–6 of illness	1136(65.4)	771 (65.1)	1907 (65.4)
>6 days	159 (9.1)	85 (7.2)	244 (8.4)
Distribute by regions			
North	19/25 (76.0%) provinces		53/63 (84.0%) provinces
Centre	12/14 (86.0%) provinces		
High land	4/5 (80.0%) provinces		
South	18/19 (95.0%) provinces		

although most patients were less than 40 years old. Patients admitted hospital around the year, concentrated from August to December of that year, and inhabited in 53/63 (84.0%) provinces in Vietnam. Hanoi and Ho Chi Minh cities were the area with highest number of dengue patients. The map color in each province was in accordance with the number of dengue cases found (Figure 1). In this study, there were 184 (6.3%) pregnancy and 245/2922 (8.4%) patients with underlying diseases such as liver disease (chronic hepatitis and cirrhosis), kidney disease (chronic nephritis and renal failure), diabetes, hyperthyroidism, cardiovascular disease, and hypertension (Table 1).

3.2. Clinical Presentations. In this epidemic, the common manifestations were fever (96.9%), skin erythema, (69.7%), myalgia (48.7%), and hemorrhagic manifestation (48.4%). In addition, other signs of nonspecific infections were encountered with low frequency (Table 2).

3.3. Laboratory Findings in Patients with Dengue Fever. From day 4 of fever, hematocrit began to rise and platelets began to drop below 100,000/mm3 and both tended to recover on the 9th day of the disease (Figures 2 and 3).

In this study, the proportion of patients with dengue fever: dengue with warning: severe dengue was 5 : 2.7 : 1, respectively. Among patients with severe dengue include severe plasma leakage and dengue shock (8.1%), severe organ impairment (2.5%), and severe bleeding (0.75%) (Table 3). The mortality rate in this study was 24/2922 (0.8%). Causes of death were shock, severe organ impairment, and severe bleeding. Outcome of dengue fever patients was related to a number of factors such as age and underlying diseases (Table 4).

4. Discussion

Vietnam is a developing country located in an area with tropical climate, where rainfall and temperature are favorable for mosquitoes to develop and spread dengue disease, especially in the South [2]. Large dengue outbreaks were reported elsewhere globally in 2016 [3]. Dengue fever has been recognized as a health problem in Vietnam [9]; however, the results of our study also showed some important issues of the 2017 dengue epidemic as follows.

4.1. Epidemiology and Demographics

4.1.1. Geographically Dispersed. In Vietnam, the first cases of dengue fever were recorded in 1959 in the North and in the South in 1960. Until 1996, the disease was reported to spread to all provinces in Central and Southern Vietnam. However, the disease only occurred in 2/3 of the provinces and cities in the highlands and 15/23 (65%) of provinces and cities in the North which was not common in mountainous provinces [5]. This issue has been assessed by some studies as the spread of dengue fever is associated with climate characteristics in different areas in Vietnam [10, 11]. However, during the outbreak of dengue fever in 2017, dengue fever patients were present in 4/5 of the provinces, cities in the Highlands, and 19/25 (76%) of the provinces, cities in Northern. Dengue fever has occurred in the midlands and northern mountains, such as Son La and Lai Chau, where the economy and transport are growing rapidly. In this study, we identified the living address of patients based on medical records. It should be noted that not only the mosquitoes control but also human activities such as tourism and urbanization are the main factors contributing to the spread of dengue virus [1, 2, 12]. In the process of development of economic and traffic system [13], the movement of patients across regions is the most important factor that facilitates the widespread of dengue in Vietnam.

Table 2: Clinical manifestations.

Clinical	NHTD *n* (%)	HTD *n* (%)	Total *n* (%)
Fever	1715 (98.7)	1116 (94.3)	2831 (96.9)
Skin erythema	1207 (69.4)	831 (70.2)	2038 (69.7)
Myalgia	980 (56.4)	442 (37.3)	1422 (48.7)
Bleed (any hemorrhagic manifestations)	688 (39.6)	725 (61.2)	1413 (48.4)
Generalized body ache/arthralgia	949 (54.6)	131 (11.1)	1080 (37.0)
Nausea and/or vomiting	317 (18.2)	552 (46.6)	869 (29.7)
Anorexia	196 (11.3)	365 (30.8)	561 (19.2)
Abdominal pain	126 (7.3)	369 (31.2)	495 (16.9)
Loose stools	117 (6.7)	255 (21.5)	372 (12.7)
Cough	72 (4.1)	221 (18.7)	293 (10.0)
Sore throat	33 (1.9)	93 (7.9)	126 (4.3)
Retroorbital pain	58 (3.3)	65 (5.5)	123 (4.2)
Nasal discharge	13 (0.7)	76 (6.4)	89 (3.0)
Hepatomegaly	8 (0.46)	53 (4.5)	61 (2.1)
Lymphadenopathy	3 (0.2)	46 (3.9)	49 (1.7)
Expanded dengue syndrome			
Bradycardia	6 (0.3)	43 (3.6)	49 (1.7)
Respiratory failure	11 (0.6)	41 (3.5)	52 (1.8)
Jaundice	4 (0.2)	44 (3.7)	48 (1.6)
Lethargic	11 (0.6)	43 (3.6)	54 (1.8)
Confuse	5 (0.3)	42 (3.5)	47 (1.6)
Convulsions	5 (0.3)	41 (3.5)	46 (1.6)
Other unusual neurological signs	12 (0.7)	40 (3.4)	52 (1.8)

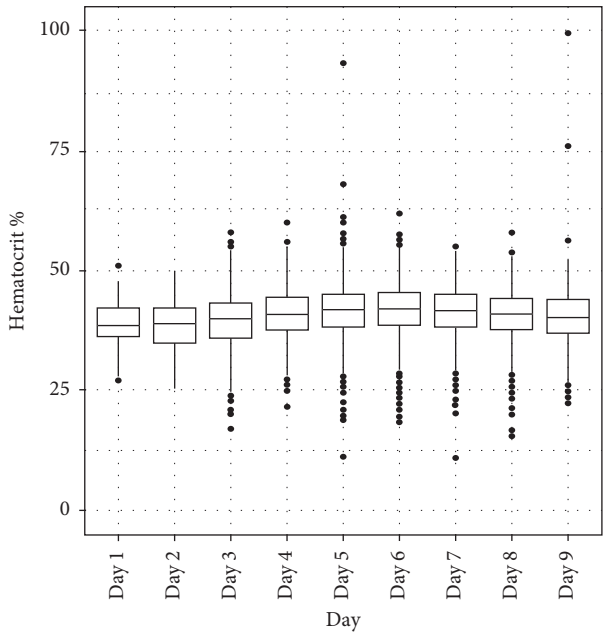

Figure 2: Variation of hematocrit according to days of illness in dengue patients.

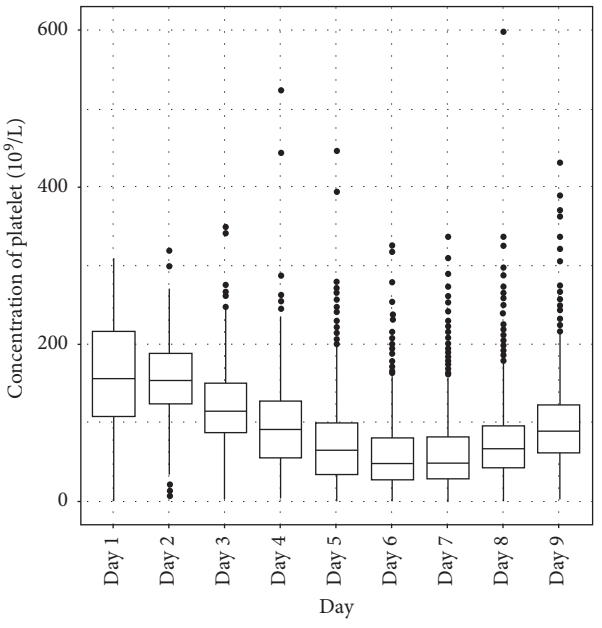

Figure 3: Thrombocytopenia according to days of illness in dengue patients.

4.1.2. Time of Patients Hospitalized in the Year and Age Distribution. Studies have documented dengue patients hospitalized year-round in the South and hospitalized only in the rainy season, from August to November, in the North [9, 11, 14], but in the outbreak 2017, patients with dengue in the North were also hospitalized around the year, including winter months (Table 1). It is possible that climate change with warming trend is a favorable factor for mosquitoes to develop and cause epidemics year-round not only in Southern Vietnam but also in the whole country [11, 13]. Most patients in this study admitted to hospital at around day 4 to day 6 of illness, which could be refer as "critical phase" or "with warning signs" according to Vietnam dengue prevention program guideline. Similarly, some studies have shown that dengue fever was only reported in patients under 60 years of age, focusing mainly aged under

Table 3: Clinical classification.

Clinical classification	NHTD *n* (%)	HTD *n* (%)	Total *n* (%)
Dengue fever	1108 (63.8)	567 (47.9)	1675 (57.3)
Warning signs of dengue	429 (24.7)	485 (41.0)	914 (31.3)
Severe dengue	201 (11.6)	132 (11.1)	333 (11.4)
Severe plasma leakage and dengue shock	129 (7.4)	109 (9.2)	238 (8.1)
Severe organ impairment	58 (3.3)	15 (1.27)	73 (2.5)
Severe bleeding	14 (0.8)	8 (0.68)	22 (0.75)

Table 4: Outcome in dengue cases.

	Mortality	*p*
Gender		
Male	10/1392 (0.72)	0.7
Female	14/1530 (0.92)	
Age		
≤40 years	10/2245 (0.44)	
41–60 years	8/563 (1.4)	0.02*
≥60 years	6/114 (5.3)	
Severe dengue		
With underlying diseases	10/57 (17.5)	
Without underlying diseases	14/276 (5.1)	0.0024

* p value indicates the differences in terms of mortality rate among 3 age groups.

40 due to expose to dengue virus in daily activities [6, 15]. Our findings showed that dengue fever has affected all ages, especially in the age group above 80 years, the oldest being 85-year-old. Along with the aging population, the issue of dengue infection was started to be seen more frequently in aging population in Vietnam.

4.2. Clinical and Laboratory. In this epidemic, the clinical manifestations and laboratory of the disease are similar to previous research results [8]. The most frequent symptom is fever, accounting for 97% patients in this study. The remaining 3% without fever is explained by the lack of fever evidence during disease progression; i.e., no temperature recording was found. However, comparing between the two areas, we found that, in the South, the clinical manifestations of dengue fever was more serious with the signs of hemorrhage, hepatomegaly, abdominal pain, and vomiting (Table 2), and the proportion of patients with warning signs was also higher (41% compared to 24.7%) (Table 3). This can be explained because in the South, patients appeared throughout the year and were infected by all 4 types of dengue viruses [6, 15]. However, the number of severely classified patients between the North and the South was similar (11.6% and 11.1%).

As with the earlier outbreak, hematocrit began to rise and platelets began to drop below 100,000/mm^3 from day 4 of the disease and both tended to recover on day 9. The hematocrit according to illness day was highest in day 5 and day 6 of illness. Thrombocytopenia was found in dengue patients with lowest median platelet count on day 6 [1]. There were no difference between the North and the South of Vietnam in terms of hematocrit and platelet. The change of such two indexes is in accordance with dengue disease progression through 3 phases, as mentioned by many medical literature [1, 2].

The rate of shock was higher than in the South, while in the North, the rate of organ failure was higher (Table 3). There may be an association between organ failure with old age or underlying disease in dengue in adults.

4.2.1. Outcome of Dengue Fever. There is a note in comparison with the previous reports of dengue fever, death mainly in the Southern and common in children [9], and this study shows that elderly people in Northern Vietnam should also be concerned. Results of this study showed that gender was not associated with the prognosis, but at higher ages, the risk of death was also higher ($p = 0.02$). Moreover, the analysis of mortality rate of patients with and without underlying diseases shows that, in patients with underlying disease, the mortality risk is higher than the other group ($p = 0.0024$).

Some studies have also reported that, in adults, the factors associated with severe dengue fever are age over 40 years [16–18], comorbidities [17, 18], and higher alanine aminotransferase (ALT) level [18, 19]. In this study, we could not rule out the relationship between mortality and aging population and underlying diseases. This issue needs a further study in the future. However, we could not differentiate the mortality rate among patients with early admission (before 4 days of illness) and late admission (after 4 days of illness).

4.2.2. Limitations of the Study. First, the primary and secondary infection status of dengue patients was not included in this study. This may explain why there was a substantial difference in number of patients with warning signs in the South. Second, the classification between dengue fever, dengue fever with warning signs, and severe dengue fever sometimes has no clear boundaries. Third, in the context of an article, we have not been able to find out the relationship between the elderly, the underlying disease, and the risk of death. Finally, climate change could be one of the factors contributing to the epidemics; however, we could not discuss this important observation in more detail together with the mosquito index and the temperature of the North during the 2017 outbreak. This is a study conducted in the two biggest hospitals for tropical diseases in Vietnam, so it provides more information for dengue fever situation in Vietnam.

5. Conclusion

Dengue fever is becoming more serious medical issue in Vietnam. The disease affects all age groups and provinces nationwide. Without the dengue vaccination, it is suggested to continuously communicate and educate people about diseases prevention as well as vector control. In the future, more studies are requested to monitor dengue fever surveillance across the whole country and determine the biomarkers associated with prognosis, intervention, and treatment in order to reduce the mortality and morbidity.

Data Availability

The data used to support the findings of this study were supplied by National Hospital for Tropical Diseases (NHTD) in the North and Hospital for Tropical Disease (HTD), Ho Chi Minh City, in the South of Vietnam and so cannot be made freely available. Requests for access to these data should be made to the corresponding author. Data are available for researchers who meet the criteria for access to confidential data.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Research Article

Antibiotic Utilization Trends in Two State Hospitals of Mongolia from 2013 to 2017

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Background. The study aimed to evaluate inpatient antibiotic use in both the State Second Hospital and State Third Hospital in Mongolia, using the WHO developed and standardized ATC/DDD methodology. *Methods*. Data were collected from the State Second Hospital and State Third Hospital which are major public hospitals that provide health care for approximately one fifth of the Mongolian population. Antibiotic utilization was monitored retrospectively for five years (2013–2017) using the ATC/DDD methodology and data were presented in DDD/ admission and DDD/100 bed days. Statistical analysis was performed using a Student's t-test for parametric data. A P value of ≤0.05 was considered to be statistically significant. *Results*. The annual consumption rates in the State Second Hospital were stable over time while in the State Third Hospital consumption rates varied considerately between years. Overall, the total antibiotic consumption rate was very high, but has decreased in both hospitals. The rate of consumption of all antibiotics was approximately twice that in the State Third Hospital (421.7 DDD/100 bed days) between 2013 and 2017 when compared with the State Second Hospital (199.7 DDD/ 100 bed days), $P < 0.001$). The seven most frequently used antibiotics comprised approximately 75% of all DDDs in both hospitals, in the period 2013–2017; being: amoxicillin, cefazolin, cefotaxime, ceftriaxone, clarithromycin, ciprofloxacin, and nitroxoline. However, this was not consistent when considering the individual years, since in 2015 and 2016, these seven active agents represented approximately 50%. *Conclusion*. This is the first hospital-based study of antibiotic consumption rates reported in Mongolia. In addition to very high consumption rates, large differences occurred between the hospitals investigated. Inappropriate and high levels of antibiotic use lead to increased costs and also increased nosocomial infection rates with potentially resistant species. The Government and health professionals need to take more active roles in improving and promoting quality antibiotic use among inpatients.

1. Introduction

Antimicrobial resistance (AMR) is threatening current health care practice. Antibiotic use and especially over-use contributes to increased AMR in society, and WHO recommends every country set up surveillance systems for AMR. Drug utilization research is defined as "an eclectic collection of descriptive and analytical methods for the quantification, understanding and the evaluation of the processes of prescribing, dispensing and consumption of medicines, and for the testing of interventions to enhance the quality of these processes " [1]. Drug utilization research is regarded as essential for rational pharmacotherapy;

Table 1: Hospital demographic data for selected years.

Indicator	2013		2014		2015		2016		2017	
	State second hospital	State third hospital	State second hospital	State third hospital	State second hospital	State third hospital	State second hospital	State third hospital	State second hospital	State third hospital
No of inpatients	7,561	17,064	7,518	17,057	8,189	17,536	8, 518	18,539	9,126	19,491
No of beds per 10000 inhabitants	1.01	2.021	0.998	1.996	0.999	1.997	0.985	1.970	0.973	2.054
Average length of stay per 10000 inhabitants	0.041	0.039	0.041	0.037	0.038	0.036	0.036	0.034	0.033	0.033

it investigates what is being used by evaluating the appropriateness of prescribing and use of medicines [2]. Drug utilization studies of antimicrobial use may create awareness and understanding of the volume of use and potential consequences for AMR from the use of antibiotics in the human and animal health sectors. The use of antibiotics varies substantially among countries [3–6]. Due to different types of health care organizations, variations occur among hospitals within a country, indicating the need for common methodology to facilitate interhospital and longitudinal comparisons. Antibiotic utilization has been monitored frequently at outpatient and inpatient levels in many countries, particularly in high-income countries; however limited data are available from less developed nations. Drug use data have until now not been collected in Mongolia and there is a need to perform such studies, to set a baseline, and when appropriate develop effective interventions towards rational drug prescribing. Recently, the WHO reported antibiotic surveillance of consumption data worldwide, with Mongolia one of the countries with the highest antibiotic consumption (64.4 DDD per 1000 inhabitants per day). However, these data were collected from the records of imported and locally manufactured antibiotics and not at the patient level. These records of imported and locally produced antibiotics were retrieved from an online database, managed by the Center for Health Development, Ministry of Health, Mongolia [7]. Drug utilization studies should be conducted at the end-user level [1]. However, no data regarding medicine utilization statistics are available for any of the hospitals in Mongolia.

This study aimed to evaluate inpatient antibiotic use in both the State Second Hospital and State Third Hospital in Mongolia, using the WHO developed and standardized ATC/DDD methodology.

2. Materials and Methods

2.1. Study Design. A retrospective longitudinal analysis of antibiotic consumption data from two selected state hospitals of Mongolia was completed for the period from 2013 to 2017.

2.2. Setting. In Mongolia, there are 13 central hospitals and specialized centers which provide tertiary level care, 5 Regional Diagnostic Treatment Centers, 16 aimag (province) general hospitals, 12 district general hospitals and health centers, 6 provincial general hospitals provide secondary level care, 39 inter-soum (second level subdivision) hospitals, 273 soum general hospitals, 218 family group practices that provide primary level care. In addition, there are 240 private inpatient hospitals and 1226 outpatient hospitals [8]. The study has included data from two public central hospitals that provide tertiary health care for approximately one fifth of the Mongolian population. Antibiotic utilization was monitored longitudinally for five years (2013–2017) at the Second and Third State Central Hospitals of Mongolia. These two state central hospitals provided health care and comprised of 23 wards covering most branches of medicine with a total of 28,617 inpatients (19.1%) of the 149,648 total number of inpatients admitted to 13 central hospitals and specialized centers in the country (2017). The annual number of in-hospital admissions were 9,126 in the Second Hospital and 19,491 in the Third Hospital in 2017 [9]. Data regarding the bed number and bed days were relatively stable for both hospitals over the selected years (Table 1) [10, 11]. Hospital wards and corresponding bed numbers are provided in Table 2.

2.3. Data Collection. In Mongolia, the pharmaceutical procurement sector is 100% privatized. Medicines are distributed through organizations such as drug wholesalers and retail drug outlets (community pharmacies and revolving drug funds). Medicines are usually supplied to state central hospitals through purchasing by tender from private pharmaceutical companies, wholesalers, as well as distributors.

In this study, reports submitted by the Pharmacy Departments of each hospital to the Statistics Office and hospital purchases from pharmaceutical wholesalers were used to capture the total hospital antibiotic utilization.

As specified in the Law of Mongolia on Procurement of Goods, Works and Services with State and Local Funds [12], medicines and medical devices are procured through an open-tender [12]. The evaluation is based on criteria set by the Drug Therapeutic Coordination Committee of each hospital and the tender with the lowest price is evaluated as "the best" and deemed high value for comparison and estimation to procuring the medical equipment, tools and drugs. A successful wholesaler or tenderer will supply and deliver goods, including medicines to the Hospital Pharmacy Department. Hence, all wards of the two state hospitals receive

Table 2: Name of wards and corresponding bed number in two selected hospitals.

Name of Wards	State second hospital (2013–2017)	State third hospital (2013–2017)
Cardiac surgery	0	40
Cardiology	25	45
Gastroenterology	34	36–40
General surgery	50	45
Infarction	0	20
Intensive care	6	4
Nephrology, Endocrinology	20	35–40
Neurological surgery	0	48
Neurology	30	42
Ocular surgery	0	20
Otolaryngology	0	20–30
Pediatrics	14	0
Pulmonology	15	35–40
Specialized care	5	0
Stroke	0	20
Traditional medicine	16	20
Total number of beds	215	430–454

medicines from pharmacy departments and at the end of each month the Pharmacy Department compiles a report on medicines given to patients. These public pharmacies are called Pharmacy Departments and they are located in the hospitals and they provide medicines only to inpatients during their hospitalization period in Mongolia, [13] hence only data regarding medicine utilization of patients admitted to hospitals were available from the public hospitals. Medicines given while in the hospital are counted as inpatient medication, if patients are to continue at home they will get a prescription and this will be regarded as used as an outpatient. Outpatients seek medication outside a hospital, often from retail pharmacies located nearby or within the hospital. However, in Mongolia, no data are available regarding the utilization of antibiotics for outpatients. Therefore, the study included records of inpatients who were dispensed antibiotics.

Antibiotic data were identified to be all antibacterials for systemic use, i.e., group J01 of the Anatomical Therapeutic and Chemical (ATC) classification and for classes and subclasses of this group [14]. Data collected included unique chemical substance name, generic name, unit strength, pack size, quantity of packs, route of administration, and manufacturer. DDDs were calculated for each product according to the ATC/DDD methodology, ATC/DDD Index 2014 [15]. DDD per 1000 inhabitants per year is only recommended for international comparison.

2.4. Data Management and Analysis. All data were recorded in and analyzed by Microsoft Excel 2016. Data validation was completed by means of ESAC checklist [16] for possible biases, including population and medicine coverage.

As the WHO recommends, if medicine utilization is assessed among inpatients, it should be expressed as number of defined daily doses used either per admission (DDD/admissions, bed days (DDD/100 bed days) and/or per 1000 inhabitants (DID/1000) [2]. In this study, the population prescribed antibiotics were admitted patients staying at least overnight (24 hours).

The definition of a bed day may differ between hospitals or countries. In this study the common definition for the two hospitals selected was: a bed day is a day during which a person is confined to a bed for a day surgery procedure and/or in which the patient stays overnight in a hospital. Moreover, DDD/admission is another indicator that can be used to show antibiotic use according to hospital activity. The day of discharge was also included in the analysis. Both indicators are reported to be important to compare and benchmark in and between hospitals [17, 18].

Census data regarding bed days and hospital admissions were obtained from the Statistical Office of the two state hospitals.

The statistical analysis was performed using Student's t-test for parametric data. A P value of ≤ 0.05 was considered to be statistically significant.

3. Results

3.1. Total Antibiotic Consumption. Total antibiotic consumption differed between the hospitals. The consumption in the State Second Hospital was stable over the five years, while the annual consumption in the State Third Hospital varied considerately between years. Overall, the total antibiotic consumption has fluctuated for the State Third Hospital and decreased in the State Second Hospital, from 2013 till 2017 (Figure 1).

Data regarding DDDs per 100 bed days were calculated for both hospitals in the selected years. First and third generation cephalosporins were the highest consumed antibiotics in the State Second Hospital in 2013. In the State Second Hospital, the 1st generation cephalosporins were the highest with 16.6 DDD/100 bed days in 2013 and it decreased to 5.0 in 2017. Moreover, the use of 3rd generation cephalosporins has increased by 13% (DDD/100 bed days 11.1 versus 13.0) in the State Second Hospital. The consumption of metronidazole (DDD/100 bed days 0.25 versus 0.5) and aminoglycosides (DDD/100 bed days 0.9 versus 1.8) have almost doubled in the study period.

On the other hand, significantly higher DDD/100 bed days for systemic antibiotic classes were reported from the State Third Hospital. DDDs/100 bed days for systemic (J01) antibiotics consumed at the State Third Hospital showed to be decreasing in the study period (2013: 205.3 DDD/100 bed days, 2017: 138.7 DDD/100 bed days). However, a contrary result occurred for macrolides, 3rd generation cephalosporins, aminoglycosides, and combination of penicillins were observed. Increase in consumption of 3rd generation cephalosporins was the highest (DDD/100 bed days 4.2 versus 15.7 DDD/100 bed days) for macrolides were 13.7 in 2013 and they have increased rapidly to 24.7 in 2017 (Table 3).

3.2. Class-Specific Trends. The average consumption of systemic antibiotics was approximately twice in the State

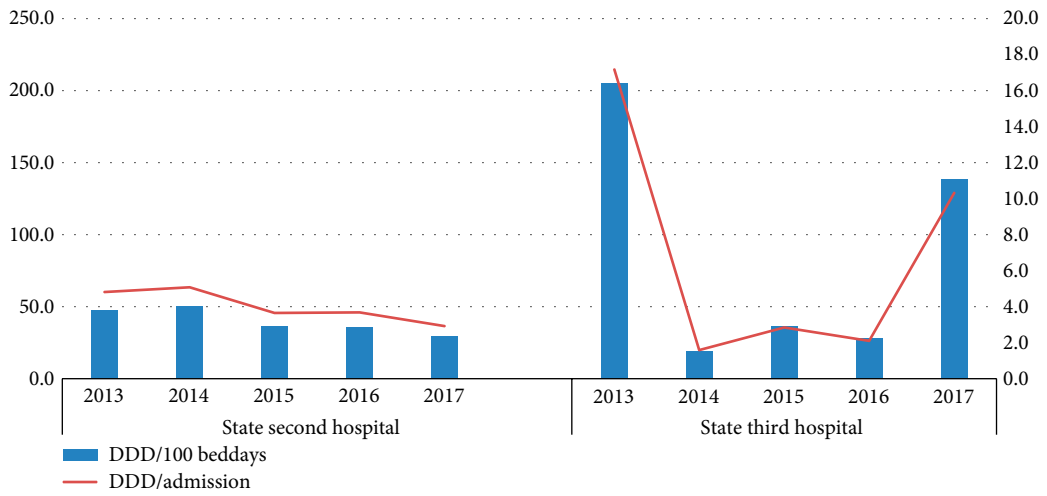

Figure 1: Total use of antibiotics as DDDs/admission and DDD/100 bed days in the two state hospitals in Mongolia (2013–2017).

Table 3: DDDs per 100 bed days in the two state hospitals for 2013–2017.

Antimicrobial class (ATC code)	State second hospital	State third hospital	State second hospital	State third hospital	State second hospital	State third hospital	State second hospital	State third hospital	State second hospital	State third hospital
	2013		2014		2015		2016		2017	
Penicillins with extended spectrum (J01CA)	4.0	28.6	4.2	2.8	1.5	1.0	1.5	0.0	0.6	18.3
Other penicillins (J01CE/J01CR)	1.5	15.0	1.5	0.5	1.7	3.9	1.7	2.9	1.4	6.3
1st gen. cephalosporins (J01CD)	16.6	27.1	14.7	2.1	7.3	0.8	7.1	1.9	5.0	18.1
3rd gen. cephalosporins (J01DD)	11.1	4.2	12.5	0.6	14.5	17.8	14.2	2.3	13.1	15.7
Macrolides (J01FA)	6.6	13.7	8.2	1.0	3.9	0.6	3.8	0.9	3.0	24.7
Fluoroquinolones (J01MA)	4.6	16.3	4.4	1.3	3.5	8.9	3.4	8.8	2.4	15.3
Nitroxoline (J01XX07)	0.6	87.1	0.7	7.0	0.5	0.2	0.5	0.0	0.5	28.3
Metronidazole (J01XA/P01BA01)	0.2	2.8	1.3	1.4	0.8	1.3	0.8	0.0	0.5	0.6
Amphenicols (J01B)	0.5	3.1	0.2	0.2	0.1	0.5	0.1	7.4	0.1	0.0
Other Abs in J01	2.1	7.2	2.9	2.5	2.6	1.5	2.5	3.7	2.7	10.9

Third Hospital (421.7 DDD/100 bed days) period when compared with the DDD/100 bed days in the State Second Hospital (199.7 DDD/ 100 bed days) between the 5-year study [$\chi^2 = 116.240, df = 4, \chi^2/df = 29.06, P(\chi^2 > 116.240) < 0.001$]. Overall, the use of 1st generation cephalosporins was similar in both hospitals (50.7 vs 49.9 DDD/100 bed days); however, comparatively more 3rd generation cephalosporins were used for inpatient care in the State Second Hospital (65.3 DDD/100 bed days) than in the State Third Hospital (40.7 DDD/100 bed days). On the other hand, the use of nitroxoline (2.8 vs 122 DDD/100 bed days) were 44 times higher in the State Third Hospital than in the State Second Hospital.

There has been increased use of cefotaxime (11.1–13.1 DDD/100 bed days and 4.2–15.7 DDD/ 100 bed days) in both hospitals. In addition, use of macrolides were increased (13.7–24.7 DDD/ 100 bed days), as well as the increased use of glycopeptides, vancomycin (72.3–228.6 DDD/100 bed days), and nitrofuran derivates, nitrofurantoin (0.2 versus 0.6 DDD/100 bed days) were observed in the State Third Hospital.

There were also large differences between the hospitals when looking at formulations. When looking at the data in 2017, in the State Second Hospital parenteral formulations were frequently used (81.9%), and in the State Third Hospital oral were most often used (66.4%) (Figure 2).

3.3. Pattern of Use. The consumption pattern of antibiotics differed between the hospitals. In the Second Hospital, three antibiotic groups; 1st and 3rd generation cephalosporins and the macrolides represented around 70% of the antibiotic consumption in a five years period (2013–2017). In the Second Hospital, three antibiotic groups; 1st and 3rd generation

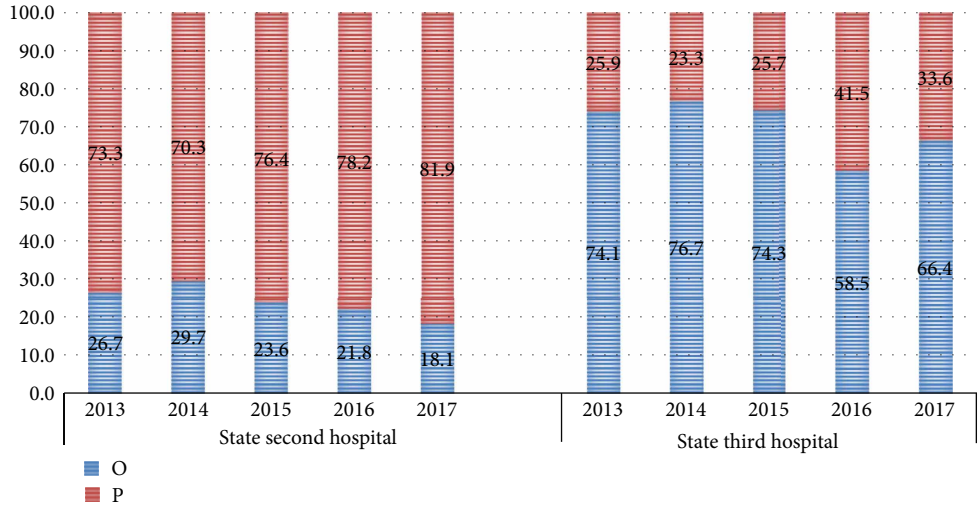

Figure 2: Proportion of oral (O) and parenteral (P) antibiotics in two selected hospitals in Mongolia (2013–2017).

cephalosporins and the macrolides represented around 70% of the antibiotic consumption in a five years period (2013–2017).

In the State Third Hospital, above mentioned three antibiotic groups represented only 31%, while nitroxoline, fluoroquinolones, and penicillins with extended spectrum represented 52% of total DDD/admissions in the same period.

The seven most frequently used antibiotics represented around 75% of all DDDs in both hospitals, in the whole period 2013–2017; these were; amoxicillin (J01CA04), cefazolin, cefotaxime, ceftriaxone, clarithromycin, ciprofloxacin, and nitroxoline. However, this was not true when looking at the separate years, in 2015 and 2016, these substances represented approximately 50% (Table 4).

4. Discussion

To the best our knowledge, this is the first study to report the antibiotic consumption in two selected state hospitals of Mongolia, using the WHO methodology. The changes of antibiotic consumption over a five-year period were quantified in order to inform decision makers, medical professionals, and the community.

Antibiotic consumption is the major driver leading to antibiotic resistance. The study has indicated that the consumption has decreased in both hospitals in the study period. Considering the Government's effort to promote appropriate use of antibiotics and reduce antibiotic resistance, antibiotic use was decreased by almost 30% over the period of study [State Second Hospital: 47.8 DDD/ 100 bed days (2013) vs 29.3 DDD/100 bed days (2013); State Third Hospital: 205.3 DDD/ 100 bed days (2013) vs 138.2 DDD/100 bed days (2017). In addition, this result may be accounted for in part by the greater emphasis on development of recently produced documents, implemented activities [19, 20] and State issued regulations [21–23]. Differences in the consumption levels of antimicrobial agents in the two hospitals were observed in this study and it can be related with various factors, including hospital specialties, patient population, and adherence to clinical guidelines and protocols. The State Third Hospital consists of four centers, 11 wards providing hospital care specializing in Cardiology, Neurology, Diabetes, and Consultancy services. In addition, the Third Hospital provides medical care through 25 departments. The State Second Hospital has about 215 beds, 9 wards, in addition to providing ambulatory and inpatient hospital care for all patients in Mongolia, the hospital provides emergency and planned surgeries of residents in Sukhbaatar district, Ulaanbaatar city. Both hospitals should conduct microbiology testing upon administration; however, due to lack of financial resources, routine testing is not performed.

A previous study conducted in two tertiary hospitals in Mongolia found out that 19% of infections microbiological diagnostics were done and antibiotics were given without sensitivity testing in 92% of cases [24].

The level of antibiotic use was 84.0 DDD/100 bed days [DDD/100 bed days of 29.3 (State Second Hospital) and 138.7 (State Third Hospital)] and it is still exceeding that from other countries, including Croatia (32.9 DDD/100 bed days) [25], Norway (47.5 DDD/100 bed days) [26], and Slovenia (50.4 DDD/100 bed days) [27]. Oral antibiotics ranged from 60.3% to 55.8% and parenteral antibiotics were almost equal throughout the years, ranging from 39.7% to 44.2% in both hospitals combined. However, the individual analysis in this study indicated the State Third Hospital used more oral antibiotics (66.4%) than parenteral medications (33.6%). As for the State Second Hospital, parenteral forms were more utilized starting from 71% in 2013, increasing to 82% in 2017. Differences between consumption of parenteral versus oral forms of antibiotics can be related with hospital specialties, as well as different levels of knowledge and attitude among prescribers practicing in each hospital [28]. Other findings reported the proportion of parenteral DDD being higher (around 70%) than Tajikistan (31.1%) [29], but lower than China (98%) [30].

In general, the findings indicated a decreasing but fluctuating pattern of antibiotic consumption in both hospitals for all medicines. Of the seven most frequent used nitroxoline is the one that is much of interest compared to use elsewhere in the world. The main indication is for urinary tract infections

Table 4: The seven most frequently used antibiotics in two state hospitals of Mongolia, expressed as DDD/100 bed days (2013–2017).

ATC5 name/ DDD 100 bed days	2013		2014		2015		2016		2017	
	State second hospital	State third hospital	State second hospital	State third hospital	State second hospital	State third hospital	State second hospital	State third hospital	State second hospital	State third hospital
Amoxicillin	2.8	27.4	3.5	2.6	1.4	1.0	1.0	0.0	0.0	18.2
Cefazolin	16.6	27.1	14.7	2.1	9.9	0.6	7.1	1.8	0.0	18.1
Cefotaxime	5.9	1.5	6.7	0.0	5.0	0.1	8.4	0.4	0.0	6.6
Ceftriaxone	5.2	2.5	5.2	0.6	3.9	0.0	5.5	1.0	0.0	9.0
Ciprofloxacin	2.9	13.5	1.8	0.8	1.4	0.7	1.4	2.2	0.0	15.2
Clarithromycin	6.5	12.9	7.9	1.0	4.8	0.4	3.8	0.8	0.0	24.3
Nitroxoline	0.6	87.1	0.7	7.0	0.4	0.2	0.5	0.0	0.0	28.3
Total	40.5 (84.7%)	172.2 (83.9%)	40.4 (79.9%)	14.1 (72.7%)	26.8 (77.1%)	3.1 (8.4%)	27.6 (77.7%)	6.3 (22.5%)	0.1 (78.2%)	119.8 (86.3%)

(UTI) which is reported to be one of the prevalent diseases in Mongolia [8]. A recent literature review on efficacy and tolerance of nitroxoline in the treatment of uncomplicated UTI demonstrated equivalent efficacy of nitroxoline with the controls tested (cotrimoxazole, norfloxacin) and justified the use of nitroxoline as one of the first line antibiotics for the treatment of uncomplicated UTI [31]. Uncomplicated UTI is more likely to be prevalent in the ambulatory setting, on the other hand patients preference for inpatient services and the perceived poor quality of outpatient services have been reported previously [13]. Hence the prescribing practice of nitroxoline at these hospitals should be further investigated and justified.

Existing inappropriate use [32], access and availability of essential medicines [33], lack of knowledge among prescribers and dispensers [28], and hospital specialties could be the main contributors to huge differences between hospitals.

Particularly, fluctuations seemed to have occurred dramatically during 2015–2017. This can be somehow related with Health Minister's Order on increasing the Government Budget for medicines and medical devices to public hospitals. Moreover, fluctuations may also have occurred due to outbreaks of infectious diseases and increasing trend in the development of antimicrobial resistance. The local production of some antibiotics (ceftriaxone, cefotaxime), in addition to commercial promotions may also lead to the increased use of cephalosporins. Availability of newer antibiotics on the market, by the pharmaceutical industry, as well as insufficient knowledge of prescribers [29] may also result in the fluctuating results. On the other hand, the procurement and supply of pharmaceuticals and medical devices are still highly dependent on foreign currency [34]. Procurement is also heavily impacted by financial instability, irregular access to and unavailability of funds. Recently approved guidelines and protocols as well as their compliance and treatment outcomes are yet to be assessed and monitored. Together, the quantity of pharmaceuticals, including antibiotics procured and consumed at public hospitals may have varied.

In addition, the consumption of macrolides was 6.6 DDD/100 bed days (5.1 DDD/100 bed days in the State Second Hospital, 8.18 DDD/100 bed days in the State Third Hospital), which is comparable with those reported from Iran (5.8 DDD/100 bed days) [35] and Israel (5.9 DDD/100 bed days) [36]. Moreover, the use of quinolones (6.8 DDD/100 bed days; State Second Hospital: 3.66 DDD/100 bed days, State Third Hospital: 10.12 DDD/100 bed days) was lower when compared with results from Iran (9.3 DDD/100 bed days) [35] and Israel (10.8 DDD/100 bed days) [36].

Carbapenems (0.026 DDD/admission in State Second Hospital, 0.002 DDD/admission in State Third Hospital) and glycopeptides (0.006 DDD/admission in State Second Hospital and 0.005 DDD/admission in State Third Hospital), often required as last-line treatment for multiresistant bacteria, were used at low levels in two hospitals. This is likely because their use requires pre-authorization due to high cost. Nevertheless, data from the State Second Hospital showed that carbapenem consumption increased by 32.9% from 0.019 DDD/admission in 2013 to 0.026 DDD/admission in 2017. This may be in response to the rising prevalence of Extended-Spectrum Beta-Lactamase (ESBL)-producing bacteria, identified in surveillance studies [37]. Also, a five-time rise in glycopeptide, i.e., vancomycin use (from 0.001 DDD/admission in 2013 to 0.006 DDD/admission in 2017) was observed in the State Second Hospital. Similarly, the consumption of vancomycin was increased in the State Third Hospital dramatically from 0.001 DDD/admission in 2013 and 0.005 DDD/admission in 2017.

Increasing rates of MRSA confirmed cases were reported from both hospitals, namely 2% ($n = 369$) in 2014 to 5% ($n = 560$) in 2018 in the State Second Hospital [38] and from 0.9% ($n = 216$) in 2017 to 2% ($n = 436$) in 2018 in the State Third Hospital [39]. A previous study completed in 4 hospitals of Ulaanbaatar showed 3% MRSA in 207 *Staphylococcus aureus* strains investigated [40]. A later study reported that among 251 *Staphylococcus aureus* isolates, methicillin resistance was confirmed in 8.8% of isolates (22/251) [41].

Interestingly, the consumption of beta-lactamase sensitive penicillins, including benzyl penicillin (J01CE01) has declined dramatically from 11.4 DDD/100 bed days in 2013, consequently dropping to 0.2 DDD/bed days in 2014 to no consumption in 2017. Hospital reports indicated that the resistance to these agents and ineffective treatment were the main reasons to stop procuring these antimicrobials.

In addition, aggressive marketing of newer agents and a lack of comprehensive antibiotic-control programs might also have had an effect on consumption rates. Inappropriate use of antimicrobials and increasing number of resistant antimicrobial agents were found to be prevalent in Mongolia [42, 43].

4.1. Limitation. The reports from the Pharmacy Departments in two selected hospitals allowed for precise measurement of total amounts of drugs dispensed at the hospital, including judgement of the total dose, dosage, and volume of each individual prescription. However, due to the unavailability of data on antibiotics dispensed for outpatients which can be obtained with or without prescription from private pharmacies located within hospitals, the extent of antibiotic consumption can be systematically underestimated. The OTC sale of antibiotics has been prevalent in Mongolia and it has been addressed by the Government [23, 44, 45].

5. Conclusion

The first hospital-based study of antibiotic consumption in Mongolia has demonstrated a measurably higher rate of antibiotic consumption in Mongolia than that in other countries. This is a critical first step in planning approaches to limit the emergence of antibiotic resistance through regular data gathering and analysis and the guidance of antibiotic prescribing.

Abbreviations

AMR: Antimicrobial Resistance
ATC: Anatomical Therapeutic Classification
DDD: Defined Daily Dose
ESBL: Extended Spectrum Beta Lactamase
OTC: Over the Counter
UTI: Urinary Tract Infection
STG: Standard Therapeutic Guideline
WHO: World Health Organization.

Data Availability

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

GD, BS, HSB, TS, and MP carried out the study and drafted the manuscript. GS, OT, and BG participated in the design and revised the manuscript. GD performed the statistical analysis and revised the manuscript. BG conceived of the study, and participated in its design and coordination and revised the manuscript. All authors read and approved the final manuscript.

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Review Article

Innovation by Computer-Aided Design/Computer-Aided Manufacturing Technology: A Look at Infection Prevention in Dental Settings

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Recent data indicates limited awareness and compliance on infection prevention procedures by dental offices and by dental laboratories. Guidelines for infection prevention in dentistry have been published by Centres for Disease Control and Prevention since 2003; the section "IX-Special consideration" includes a subsection concerning the prevention in dental laboratories, but it has not been modernised in later versions to fit the needs of traditional and computer-aided technology. Traditional techniques required disinfecting items (impression, chewing waxes, and appliances) with well-suited products, which are also chosen for limiting impression changes or appliance deterioration. Effective procedures are available with difficulties. Some of these contain irritant or non-eco-friendly disinfectants. The transport of impression, to dental laboratories, is often delayed with limited precautions for limiting cross-infection. Gypsum casts are frequently contaminated mainly by bacteria and their antibiotic-resistant strains and even stored for long periods during dental implant supported restoration and orthodontic therapy, becoming a hidden source of infection. Nowadays, computer-aided design/computer-aided manufacturing technology seems to be an interesting way to promote both business and safety, being more comfortable for patients and more accurate than traditional technology. A further advantage is easier infection prevention since, for the most part, mainly digital impression and casts are not a source of cross-infection and the transport of contaminated items is reduced and limited to try-in stages. Nevertheless, a peculiar feature is that a digital electronic file is of course unalterable, but may be ruined by a computer virus. Additionally, the reconditioning of scanner tips is determinant for the optical characteristics and long term use of the scanner, but information for its reconditioning from producers is often limited. This study focuses on some critical points including (a) insufficient guidelines, (b) choice of proper procedure for scanner reconditioning, and (c) data protection in relation to patient privacy.

1. Introduction

For patient and personnel safety in dentistry, one of the main goals is to break the chain of infection transmission. Nevertheless, infection hazards in prosthodontic and orthodontic practice are rather difficult to control [1–4]. Both practices require many items: impression, models, try-in stages, and outsourced different dental appliances (customized bridges, dental implant supported restoration (DISR), different types of orthodontic appliances). In general, traditional procedures suffer from (a) compatibility problems among items and disinfectants and (b) limited compliance, errors, and lapses during infection prevention, which are very frequent in dental offices. In addition, data indicates the limited awareness of infection prevention guidelines and insufficient compliance with infection prevention by most dental laboratories (DLs) during the manufacture of dental prostheses and orthodontic appliances [5–10]. Contaminated items often come and go from the clinic to the DLs and vice versa, and this increases the hazard, the possibility of microbial reservoirs, and the chance of infection transmission [2, 5–13].

The limited compliance with infection prevention is hazardous, taking into account the increasing prevalence of infections by antibiotic-resistant bacteria, killer bugs, or

super spreaders [14], and the growing number of elder dental patients with impaired immune defence system; frequently, they need complex and cutting edge surgical procedures and prosthodontic treatments, which unfortunately also have been associated with incidents of malpractice [15, 16].

Nowadays, computer-aided design/computer-aided manufacturing (CAD/CAM), here indicated as CAD/CAM technology (CCT), is widely used since (a) digital impression is more comfortable for patients (mainly vulnerable aging population or younger ones) [23]; (b) accuracy of prosthetic restorations is equal or higher than conventional restorations [24]; (c) it significantly reduces the costs (about 30% per each crown) and the active working time (90% for final crown) [24, 25]; (d) the exclusive use of aesthetic and more biocompatible materials (i.e., zirconium oxide, lithium disilicate) [15, 26, 26]; (e) the flexibility to manufacture from simple crown to complex DISRs and orthodontic appliances [25–31]; (f) the appeal of virtual technology to promote business; and (g) it improves ecofriendly dentistry [3, 32]. A recent survey shows that restorations by CCT represent a significant innovation adopted by dentists in New Zealand and have been greatly appreciated by dental students [33, 34]. The global dental CAD/CAM & dental prosthesis market is increasing significantly: it was estimated at about 9,400 Mn USD by 2018 growing at a compound annual growth rate of 5.6% over 2024 [35].

Despite the high interest in dentistry on CCT nowadays, gold standard guidelines for infection prevention did not pay attention to it [1, 36]. Concurrently, insufficient notes are available from Laws on health safety and guidelines [37].

Using CCT, the usually reported advantages for infection prevention are the none requirements of impression disinfection and better occupational safety. To our knowledge, this is the first paper that focuses on infection prevention in detail using CCT compared to traditional technology in dentistry. Here, we report three specific problems related to (a) insufficient guidelines, (b) requirements for scanner reconditioning, and (c) data protection and electronic viruses.

2. Materials and Methods

2.1. Information Sources and Search Strategy. The electronic literature search was conducted via the PubMed and Google Scholar databases (from January 2010 up to and including October 2018) using various combinations of the following key indexing terms: (a) CAD/CAM; (b) cross-infection control; (c) infection prevention; (d) disinfection; (e) reconditioning; (f) semicritical items; (g) critical items; (h) cast; (i) digital model; (j) digital impression scanner; (k) dental impression; (l) guidelines; (m) safety precautions; (n) dental laboratory; (o) occupational health; (p) bacterial adhesion; (q) microbial contamination; and (r) biofilm. In addition, manual searches were carried out in the Hindawi Journal database (from 2010 to 2018) using the following key indexing terms: (a) CAD/CAM (n°=237); (b) CAD/CAM dentistry (n°=118), but very few take into account cross-infection or infection prevention according to our topic [38, 39]. Subsequently, bibliographic material from the papers has been used in order to find other or older appropriate sources in relation to specific topics and operative problems. A total of 108 papers and links were found suitable for inclusion in this paper. Only a few papers do not have a DOI or PubMed classification, but the available Internet link and date accessed have been added.

3. Results and Discussion

3.1. Background. Currently, there is increasing interest regarding safety of the dental workplace, personnel, and patients and in particular, on the prevention of infectious adverse events and clinical hazards. Adverse events and outbreaks mainly cause an increase in the cost to society (by productivity loss, additional costs for health care, outbreak investigations by molecular diagnostics), and significant legal claims [40, 41]. In addition, a burning issue is the growing number of susceptible patients (HIV positive, diabetic, the elderly, those under frequent antibiotic treatments or chemotherapy, women in pregnancy, children, teenagers) with an impaired or underdeveloped immune defence system; in addition, other patients show oral lesions or tissue trauma after clinical treatments (i.e., preparation of the cast crown, impression, trying practice of orthodontic band selection, etc.) or gingival inflammation. In all these cases, the chances of infection are expected to increase.

On the whole, dental impressions and appliances from all persons must always be treated as if potentially infectious (by microbes present in saliva, occult blood, dental plaque), since persons could be in an asymptomatic stage (early stage of Hepatitis C infection) and could not know their status, or the infection may be diagnosed late, or undeclared to avoid discrimination (HIV infection). Furthermore, the recommendation to isolate prosthesis of high-risk patients from other laboratory work in dental offices and DLs is nowadays without a rational reason and dated.

Moreover, we have to make every effort to reduce the rate of infection transmission to/from dental offices and to/from laboratories and the chance of there being some microbial reservoirs (impression, dental appliances, etc.). Conventional fabrication methods require considerable human intervention and manipulation of impression, wax and cast, materials and try-in-stage items; as a consequence of these two peculiar features, items exhibit microbial contamination caused by the bioburden of the oral cavity, hand skin, environment, and even by some harmful antibiotic-resistant strains. Here, we focus on some underestimated hazards and operative errors and lapses during infection prevention using traditional technology and CCT.

3.2. Failures in Infection Prevention in Dental Offices Using Traditional Technology. More recent findings indicate insufficient knowledge and very limited awareness by dental healthcare personnel (DHCP) in relation to infection control, taking into account the insufficient use of PPEs, low use of sterilized impression tray (13%), rinsing the impressions with water (37.2%) or brushing away debris (2,6%) before disinfection, blood-contaminated impression (25%),

improper disinfection of impression (about 40%) or of metallic impression trays, denture prosthesis, bite registration and wax, face bole and fork, and lack of communication (24,7%) with DL about impression disinfection in dental offices [2, 11–13, 39, 42–46].

In brief, many opportunistic or nonopportunistic species (i.e., *Staphylococcus*, Methicillin-resistant *Staphylococcus aureus* (MRSA), *Candida, Pseudomonas, Enterobacter cloacae, Escherichia coli*, *Klebsiella oxytoca,* Streptococcus, Actinomyces, Enterobacter, *Klebsiella pneumonia*) have been reported on impressions, dentures, crowns, and wax occlusion rims [47, 48] (Figure 1). Sofou's group reported that seventy-two percent of the impressions were contaminated at a low level (median number of 1.3×10^2 cfu/20 mm^3), while thirteen per cent of the samples yielded at a higher range (10^3- 3.4×10^4 cfu/20 mm^3) [49]. Most of the isolates were non- or α-haemolytic bacteria and presumably low-pathogenic strains. Nevertheless, we would like to note that *Bacillus* strains, mainly nonpathogenic species and well adapted to the human host, have been reported to contribute to latent infections and/or to reactivate others (i.e., HIV, tuberculosis) [50]. In addition, since they are spore forming species, they are difficult to eradicate from stone casts [49–51]. More recently, bacterial contamination, checked by using molecular biology techniques, was also found on the final removable orthodontic appliances (~10^2–10^3 cfu/ml); nevertheless, the contamination derives from the DL rather than from patient's impressions [52].

MRSA is a well-known antibiotic-resistant bacterium with a very low expected infective dose (4 CFU) [14]. Impression material cartridges and handgun dispensers are easily and heavily contaminated with pathogenic agents, including MRSA, during clinical prosthetic procedures [53]. The infective hazard is expected since most invasive dental procedures are performed in dental surgery and prosthetic wards, where patient bleeding is frequent. Nowadays, the use of heat-sterilized hand-pieces and proper water quality during prosthetic tooth preparation, because of the frequent bleeding, is absolutely necessary [1, 36, 54]. On clinical contact surfaces, the MRSA contamination was higher in samples from dental surgery (4.3%) and prosthetic dentistry (3.9%) compared to prosthodontic procedures (1%) that are mostly none invasive [55]. Nevertheless, it is hazardous that the majority of MRSA and *Staphylococcus aureus* isolates, recovered from environmental surfaces, were biofilm producers [14, 22, 55]. The contamination of MRSA is high in conventional impression and gypsum casts: it has been found in 15.4% and 27% of them, respectively [48, 56].

We would like to note some underestimated hazards during traditional prosthetic, prosthodontic, and orthodontic practices as follows:

(a) Concerning the procedure using an addition silicone as impression material, the contamination by the hand microbial flora (including MRSA) [14, 22] is expected during the mixing of the base and its catalyst with ungloved hands. Recently, this problem can be avoided using powder free latex gloves or impression material automatic mixers.

(b) It is a frequent error to touch a cast or contaminated try-in items with gloved contaminated hands [4]. Therefore, it is not a surprise that casts are frequently contaminated mainly by bacteria and MRSA and could be a source of infection [4, 22, 56]. We would like to note that contaminated casts come and go between the dental office and DL and/or are utilized for long periods during DISRs and orthodontic cares; then, they are a hidden source of contamination (Figure 1). Nothing is known about the contamination of articulators, but it is expected to be high.

(c) During partial- or full-denture impressions, it is a frequent error to use the *big* brush of the rubber base adhesive without disinfecting the appliances or the customized tray. We should note that the isolated species from the denture surface are aerobic bacteria, fungi, Enteric rods, *Candida* spp., *Pseudomonas* spp.; they are generally part of the normal oral flora, but could be pathogens for immune-compromised patients, while anaerobic species should colonize the internal porous system of the acrylic resin of the removable appliances [12, 13]. Then, the contamination of the brush and the adhesive are expected, but this should be inconsistent with recognized standards of infection control.

(d) The transport of contaminated impressions, chewing wax, and intermediate tests during prostheses are often carried out without proper precautions against cross-infection, with violation of the national laws, as well as being delayed. Concerning the disinfection of traditional impressions, the instructions for use (IFU) from manufacturers are often insufficient or not usable (i.e., very restrictive use of glutaraldehyde in European Union). The responsibility for ensuring impressions has been cleaned and disinfected before dispatch to the laboratory which lies solely with the dentist.

(e) When at the dental chair, the modification of removable orthodontic and prosthetic appliances should be avoided before try-in and after use by all patients, without their preliminary disinfection. We would like to note that removable prosthodontic appliances received from laboratories are often contaminated by *Bacillus* spp. (57% of the isolates), *Pseudomonads* (22%), *Staphylococci* (13%), and *Candida* species (38%). In addition, acrylic base plates are always contaminated by *Streptococcus* biofilm even after short usage [4, 13]. An option for avoiding the environmental contamination and occupational hazards is to modify appliances inside a closed equipment (usually called dental sandblasting equipment) with dust aspiration.

Concerning specific problems on impression disinfection, we add additional operative details in Section 3.4.

3.3. Contamination of Dental Impression Materials from Manufacturers. Insufficient data exists on the contamination of

(a) (b) (c)

Figure 1: Some examples of cast microbial contamination (see bluish-black hairy colonies probably from Fungus species) due concurrent factors (improper impression disinfection, prolonged conservation inside the plastic bag, presence of alginate residues after manufacture steps in a traditional DL). The casts represent a hazardous reservoir since DHCP hand-touching.

dental impression materials supplied by the manufacturers in sealed containers. They are mainly stored in an anhydrous state and are often hydrophobic, which means the microbial contamination is expectedly low. Extra-mouth contaminants represent only 0.06% of total microbial (aerobic mesophilic bacteria) load of alginate, while after mouth contact, alginate microbial load increases significantly (1600 fold); other powders, from impression material containers and irreversible hydrocolloids received from the manufacturers, were contaminated with viable microorganisms to a substantial amount (90-100% in irreversible hydrocolloids) [57]. It is unclear if dental impression materials themselves can act as vehicles for microorganism transmission or be a hazard for immune-compromised patients [58].

3.4. Focus on Impression and Cast Disinfection Using Traditional Technology. Firstly, the use of all PPEs is always required because of the infective risk and the occupational hazard due to splash in the case of immersion, air contamination in the case of spray, or dryout with compressed air. Studies, among DHCP and dental technicians within different Nations (UK, Pakistan, South Arabia, Iran), indicated a wide variety of chemical solutions and concentrations were used to disinfect impression materials [10, 44, 45, 59–61].This is indicative of the degree of confusion and difficulties in the choice of the proper disinfectant with inadequate recommendations and insufficient knowledge. Data mainly focuses on the effects of disinfectants on impression surface details and dimensional accuracy of items (impressions, master casts, etc.) caused by different reasons [62, 63]. Taking into account the conditions encountered in clinical practice, unfortunately, data is lacking on the effects of procedure delay [64]. Frequently, an alginate impression is placed in plastic bags with moist cotton, but the delayed delivery to the DL of inadequately disinfected impressions could allow for microbial growth during storage. Using conventional technology, it is very important to firstly remove blood and saliva contamination that can alter bacterial adherence capacity, while it is not clear to what extent (0-90%) the preprocedural rinsing of the impression with tap water should significantly remove bacteria and increase the efficacy of subsequent disinfection [5, 49, 57, 65, 66].

In general, the impression disinfection, in a dedicated area near the chair side area, is an ideal way to prevent cross-contamination. Many studies report impression surface disinfection with different commercial products, by spay or immersion and with a contact time of about 5-10 min. Disinfection by soaking in chemical materials has been shown to cover all surfaces of impression materials at one time, while spraying is not capable of disinfecting all surfaces effectively and also cannot cover all undercuts.

It is preferable to avoid the use of irritants (aldehydes, hypochlorite solutions), or non-eco-friendly disinfectants (aldehydes, phenols). Hypochlorite solutions, very effective and cheap products with no or minimal certification, may have corrosive or discoloration effect on prosthesis metal parts as far as occupational hazards [67]. The safer disinfectants specific to this area are based on alcohols, chlorine combination, chlorhexidine ± enzymes, biguanides, and ammonium compounds.

Recently, more ecological approaches have been proposed for dental stone and impression disinfection using microwave and UV radiation [51, 68, 69]; these procedures should avoid the possibility of surface deterioration as they do not involve immersion/spraying of the impression with disinfectant.

3.5. Impression Tray. Before further reconditioning [36, 70], patients' reusable impression trays must be perfectly cleaned of bioburden and of residues of adhesive and impression materials, cements, adhesive, and gypsum, using self-acting products. It is well known that the prolonged immersion of metal trays using specific products may cause some corrosion, mainly of aluminium or chromate trays (Figures 2(c)–2(f)). Careful attention should be given to hazard identification and precautionary statements (indicated in MSDS) of cleaners for alginate and gypsum residues. The preliminary removal of any residues from impression trays is needed since further mechanical action by ultrasonic devices or washer-disinfectors is not able to remove them and would impair further disinfection and sterilization (Figures 2(a) and 2(b)).

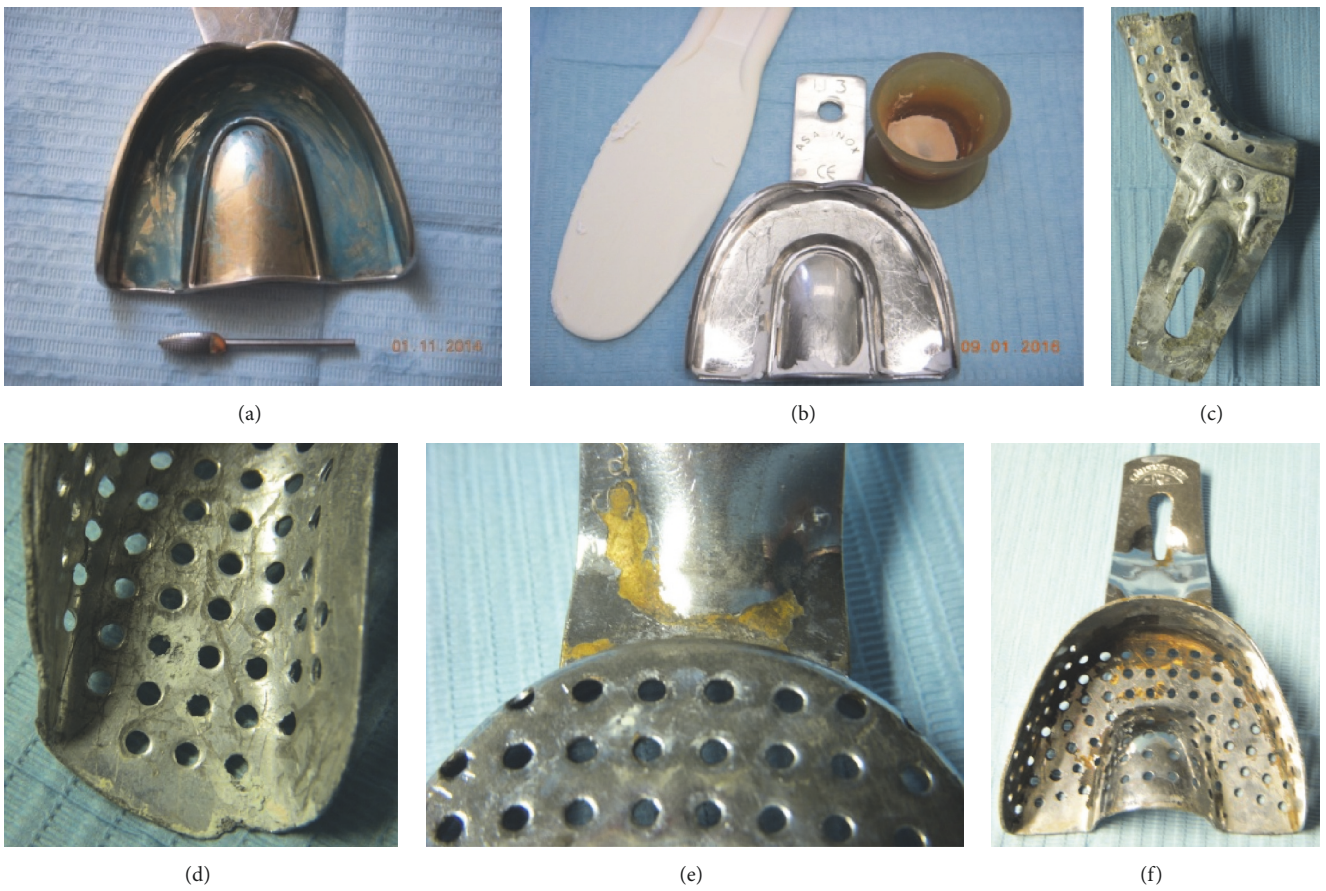

Figure 2: Residues of polyether adhesive, alginate, and autopolymerising acrylic resin on impression trays and other items (alginate spatula, laboratory bur, silicone dappen dish) after treatment by washer-disinfectors (a, b) and corrosion of impression trays by an improper or overly long chemical treatment to remove gypsum (c-f). Tips: (i) remove residues before treatment by washer-disinfectors (a, b) [17]; (ii) these impression trays must be promptly substituted (c-f).

3.6. Failures in Infection Prevention in DLs Using Traditional Technology. When traditional technology is used, the work in DLs comes with many physical, chemical, ergonomic, and biological hazards [6–8, 71]. Despite the lack of contact with patients, there are many opportunities for cross-contamination throughout the manufacture of the appliances.

Data shows the limited awareness on infection prevention and very poor compliance of infection control procedures by most DLs during the manufacture of dental prostheses and orthodontic appliances; in particular, studies show inadequate adoption of standard precautions in terms of the use of PPEs, disinfection of impression and appliances, and vaccinations [5–10]. DTs are exposed to microorganisms via direct contact with nondisinfected items (i.e., impressions) through cuts and abrasions mainly on ungloved hands. A recent study reported that DTs received 95% of blood-contaminated impressions and 15% had encountered blood-filled voids upon trimming back the peripheries of impressions [11]. The risk of cross-infection between the clinical and DL settings seems to be significant; when during '90 yrs, DT adopted very limited infection prevention procedures, and they showed significantly higher exposure to HBV than a comparable population (2.7% vs. 0.76%) [72]. Despite this hazard, the percentage of vaccinated technicians against HBV is unsatisfactory, ranging from 10 to 60% [7, 59].

It is well known that the storage plus transport of impression to DTs takes from 5 to 8 hrs in moist conditions; the influence of humidity on microbial survival is a recently discovered problem; for example, HBV can survive for up to seven days in 42 percent relative humidity. A survey shows that 50% of the responding DTs disinfected all impression partly from uncertainty (no written communication) or inefficiency of disinfection in dental offices [11]. Nevertheless, repeated disinfection has been reported to influence surface detail and the accuracy of the impression. Chorexidine, a disinfectant often used prior to final packaging and dispatch of the custom-made appliances, has been reported to deteriorate the acrylic surface of appliances [5] and recently involved in antibiotic resistance. Furthermore, chemical disinfectants affect the physical properties of the gypsum materials when used as water mixing substitutes; this approach has therefore been discarded by manufacturers [73]. However, the gypsum-based stone model preparation by an exothermic setting reaction may reduce the viable bacterial content on the impression as well as the cast.

In addition, other factors could jeopardize infection prevention: the need to rush a case, the absence of disinfection areas within their dental laboratories, and low awareness of legal responsibility towards occupational risks [5–10].

Only 6.40% of DTs use all PPEs and just 45.6% stated that they clean and disinfect their work surfaces. Astonishingly, 47,8% of DTs only cleaned the rag wheels, brushes, and acrylic burs with water after use, and only 28.26% of them sterilized by heat or chemicals [59]. When polymerizing, grinding, or polishing, the chance of cross-infection is still severe due to heavily contaminated dental pumice, slurry, the brushes, and heated water baths [74–77].

These worrying practices render the rest of the precautions useless because infective agents are able to survive on contact surfaces, air, hand, and work items for several days and then could contaminate already disinfected appliances. Recently, DT behaviour seems to have got worsened as concluded by some authors. Vasquez-Rodriguez's group concluded: "*Substandard cross-contamination practices seem to be a common finding in dental laboratories, which may well compromise the quality of certain dental treatments*" [10], while Diaconu noticed that the majority of technicians were aware of the existence of a real contamination risk, both of the lab surfaces and the personnel; however, the economic crisis has forced them to reduce the lab budget for infection prevention, and vigilance [78].

3.7. Regulation and Recommendations. The recent European Union Regulation n° 745/2017 reported only a vague indication to health safety and some notes on cleanliness and sterility of dental appliances, all classified as medical devices (MDs), placed on the market. There is no specific guidance issued to dental custom-made MDs in contrast with the fact that dental appliances should be free of microbial contamination according to CDC guidelines [1, 36, 37]. The guidelines for dentistry published by CDC *since 2003,* include, as Special Consideration, a subsection called Dental Laboratory, but it has not been updated in later versions [1, 36]. In addition, guideline recommendations or other requirements should reflect what the field regards as good practice, but, in this case, updated instructions from the FDA and Dental Federations (International Dental Federation, American Dental Association) are insufficient [79] or refer to CDC previous guidelines *set in 1993* [70, 80, 81]. CDC guidelines for implementation suggested to "*Consult with manufacturers regarding the stability of specific materials (*e.g., *impression materials) relative to disinfection procedures' including specific information regarding disinfection techniques used (*e.g., *solution used and duration), when laboratory cases are sent offsite and on their return*" [36].

Up to now, there are no disinfection protocols which have been accepted as gold standard for disinfecting dental impressions and dental appliances. Chemical disinfection is still the method of choice since sterilization with heat is not an option for dental impressions and occlusal records.

3.8. Infection Prevention Using CCT Compared to Traditional Technology. The dental service market is always becoming more competitive. Today, the increased ergonomics of the highly complex "human-technical dental office system" are very important in guaranteeing safer and patient-centred dental care concurrent with earning profit [14]. Apart from clinical advantages and limitations already discussed by many authors [6, 8, 10, 15, 29–31, 71, 72, 78, 82–86], CCT seems to be a promising way to prevent cross-infection.

Here, we show the main differences in the case of traditional vs. CCT, mainly focusing on dental offices (Table 1). The biohazard for dental patients and DHCP is greatly reduced using CCT largely due to reduced contamination during digital impression in the dental offices and further digital manufacturing of appliances in closed automated conditions (printing technologies for polymer and metals; metal, zirconia, ceramic, PMMA milling technologies) with mainly environmental contamination. The most modern production process is fully automated and milling machines are equipped with automatic systems for the replacement of tools: this allows, starting from the raw materials, the possibility of arriving at finished dental appliances with limited or without human intervention. The residual biohazard should be prevented by the use of PPEs and adequate infection prevention during the service of rotary cutters, filters, and internal parts of the milling machine, etc. Finally, quality control and appliance disinfection before delivery are easier and automated using CAD/CAM compared to traditional DLs. More attention is needed during the handling of try-in cases and ready dental appliances. These appliances, considered semicritical items, should ideally be sterilized or receive at least intermediate-level disinfection (tuberculocidal claim) before the delivery in a sealed pouch to dental offices.

Other advantages are related to the following:

(i) Better occupational safety for DHCP and DTs by avoiding [87]:

  (a) Skin irritation after extensive use of disinfectants for impressions and dental waxes.
  (b) Silicosis by exposure to airborne particles liberated during the mixing of alginate (dust, lead) in dental offices and melting, grinding, polishing, and finishing procedures in labs.
  (c) Nonexistent or low biohazard due to waste management.
  (d) Higher hazard for the younger DHCP, who are concurrently more exposed to cross-infection, mainly when they are students and in the first years of their dental practice [34, 42, 88].

(ii) Progress towards ecofriendly dentistry by reduction of

  (a) disinfectant use
  (b) waste material (contaminated gypsum and cast) [32]

(iii) Clinical biosafety because

Table 1: Main differences for cross-infection prevention in the case of traditional technology vs. CCT in dental office and DL.

n°	Need for	Traditional Technology	CCT
1	effective communication and coordination between the dental office and laboratory efforts to asepsis	yes	only in the case of intermediate and completed cases
2	written information regarding the methods (e.g., type of disinfectant and exposure time) used to clean and disinfect the material (e.g., impression, stone model, or appliance) and items (articulators, case pans, or lathes) according to the manufacturer's instructions.	during all phases	only in the case of intermediate and completed cases
3	heat-tolerant items used in the mouth (e.g., metal impression tray or face bow fork) that should be heat-sterilized before being used on another patient or single-use plastic impression trays	yes	only for scanner tips
4	clean and disinfected pressure pots and water baths between patients since these are particularly susceptible to contamination by microorganisms	yes	No/ only for positioning wax
5	wearing appropriate PPE (including eyewear!) in both the office or laboratory, when handling contaminated items and until disinfection is completed	yes	only in intermediate and completed cases and after the end of the CAD
6	guarantee that the appropriate and effective cleaning and disinfection procedures are performed in the dental office or laboratory	+++	+
7	use an EPA-registered hospital disinfectant with a tuberculocidal claim, follow IFU and thoroughly rinse item before being handled in the in-office laboratory or sent to an off-site laboratory	yes	no
8	checking IFU and problems regarding the stability of impression and appliance materials during disinfection	yes	no
9	cleaning and disinfection of any items (impressions, prostheses, or appliances) as soon as possible after removal from the patient's mouth before drying of blood or other bioburden that can occur	yes	only in intermediate and completed cases
10	a separate disinfecting, sending, and receiving area should be established to reduce cross-contamination in the dental office	yes	easier and only in intermediate and completed cases
11	identification and reduction of redundancies of procedures since impression materials could be damaged or distorted because of disinfectant overexposure	yes	no

Table 1: Continued.

n°	Need for	Traditional Technology	CCT
12	cleaning, disinfecting, and covering of clinical contact surfaces as a function of the rate of use and contamination of the area	+++	+
13	fabricating stone casts after alginate impression as soon as possible to avoid dimensional changes	yes	no
14	adhesive for impression trays using some impression materials (polyether, polysulfide)	yes	no [18]
15	wastage of impression materials due to the remaking at times of conventional dental impression for inadequate detail production	yes	no
16	wastage of time due to the remaking of dental impression for inadequate detail production	+++	+
17	appliances and prostheses that should be free of contamination delivered to the patient	difficult	easy
18	responsible dental laboratory or dental office staff for the final disinfection process	yes	yes
19	a separate receiving and disinfecting area should be established to reduce contamination in the DL	yes	in intermediate and completed cases
20	waste (gypsum, waxes) management according to national laws	yes	no
21	Appropriated disposal of gypsum and toxic substances (i.e., hydrogen sulphide) when discarded into the environment	yes	no
22	laboratory items (e.g., burs, polishing points, rag wheels, or laboratory knives) which are heat-sterilized, disinfected between patients, or disposable items, or to store items in small quantities (i.e., polishing agents)	yes	low and only to reduce manufacture contamination
23	regulated medical waste and sharp items (e.g., burs, disposable blades, and orthodontic wires) in specific and resistant containers according to national rules	+++	+
24	paper for dentist prescription to DL	yes	no
25	computer antivirus	no	yes

(a) the violation of critical anatomical features is prevented by marginal fit lower than the clinically acceptable value [85]. In particular, the accuracy of DISRs by CCT is determinant in order to avoid microbial niches between prosthesis and connecting elements (implant abutment) [86, 89].

(b) the new dental materials (i.e., PMMA, zirconia), usable only by CCT, show reduced adhesion and decreased biofilm accumulation [90, 91].

(c) DHCP can minimize the risk of osteonecrosis, a rare and unexpected complication during the taking of conventional dental impressions in

patients with predisposed anatomic sites, or the risk of *Candida* transmission in patients with denture stomatitis, a very common condition found among the elderly population [92, 93].

CCT disadvantages are on the prohibition of use on patients with pacemaker and minor occupational hazards (eye safety, extended computer usage, and ultrafine particles and nanosized byproducts) [6, 8, 10, 75, 94].

3.9. Factors Influencing Intraoral Scanning for Digital Imaging. The scanner is a very responsive appliance. Several factors have been reported to influence the accuracy of the intraoral scanning including (a) the learning curve, skills, and scanner usage frequencies in clinical practice; (b) the physical resolution of the scanning system and the postprocessing of the data; (c) the movement of the patient and limited intraoral space; and (d) temperature fluctuation; (e) the presence of moisture, water, saliva and sulcular fluid, and reflective surfaces (metal brackets and implant abutments) [95–101]. It is not known if the presence of *occult* blood in saliva or sulcular fluids or some of their compounds (perhaps hemoglobin, lactoferrin, volatile compounds, glandular mucous) may influence the direct scanning of a tooth prepared subgingivally, for example, or an abutment coupling. In general, scanning technology has to improve (a) the speed of the scanning process (with both hardware and software improvements), (b) the size of the scanner wand and the design of a thinner scanning tip to improve patient comfort, (c) proper devices for a better dry field, and (d) increased resistance to reconditioning and sterilization of the apparatus.

When the powdering procedure is needed to prevent reflections during image capture, there was no way to standardize it for each scan, it is not appreciated by the patient, and the environmental contamination caused by titanium powder nanoparticles is not known.

3.9.1. Unit Hygiene and Scanner Tip Reconditioning. In line with the current minimal requirements for the indication of hazards published [81], the importance and the problems derived from scanner tip reconditioning have not been taken into consideration by other authors [24, 82, 83, 96–98]. We evaluated IFUs indicated for two scanners by TRIOS® and iTero® [19–21] (Table 2). Only iTero® recommends different cleaning and disinfectant commercial products for use for the Scanning Unit and the Base Unit; these disinfectants are often a mixture of different disinfectants (alcohols, Quats plus alcohol, Hydrogen Peroxide), fast acting and with a broad spectrum of activity, and all have clear certifications according to regulations. TRIOS® is rather confusing on IFUs found in two different manuals [19, 20]. IFU mainly contains recommendations for using disinfectants (60-70% alcohol-based ones) to prevent mirror damage and strict prohibition on other types of disinfectants to clean the tip mirror (i.e., ammonia-based or chloride based solutions, acetone, any oxidizing solutions) indicated in the online manual [20]; nevertheless, another IFU allows high-chemical disinfection with Wavicide®-01 and Cidex OPA® solution, if allowed by National rules. To our knowledge, it is a bizarre indication since aldehydes should be avoided on other optical devices (dental curing light) because of their ability to precipitate on optical fiber [102]. TRIOS® does not indicate potential explosion hazards if in the presence of residual flammable products (i.e., alcohol-based disinfectant), except inflammable anaesthetics. Conversely, iTero® uses disposable plastic sleeves for patient scanning. High-level disinfection was possible for 50 and 150 cycles, respectively, for tips with TRIOS® scanner tips with fixed mirror and detectable mirror, while for Carestream®CS 3600 up to 20 cycles [103]. In general, scanner producers always underline not touching the optical surface with gloves, while there are no indications for avoiding the use of powered gloves during reconditioning and the replacement of disposable sleeves.

3.9.2. Some Advices. Finally, we would like to underline some advice for avoiding (a) lint, stains, and dirt on the optical components, (b) damage on optical component, and (c) fast deterioration of the plastic parts of the unit (Table 3).

3.10. Retraction Cord. It is well known that gingival retraction procedures are part of impression procedures; generally, this step is considered "safe" and effective, but also time-consuming, uncomfortable for dental patients, and may delay periodontal tissue repair [104]. The retraction cord is needed, also in the case of CCT, since the difficulty in scanning subgingival margins (>1 mm); in this case, dry retraction cord is used. When wet retraction cord is used during a traditional procedure, retraction cord contamination is expected. In fact, a very frequent lapse is to wet the retraction cord into the solution of topical haemostatic agents (sold in very little bottle, but that is used for long periods) without cross-infection precautions (i.e., use of unsterile College plier).

3.11. Data Protection and Infection Prevention from Computer Viruses Using CCT. Data protection is at the core of the recent EU General Data protection Regulation [105].

All dentists and DTs must pay attention to the health data of their patients, in terms of purpose limitation, data minimization, accuracy, storage limitation, integrity, and confidentiality. Data retention and reuse time must be explicit, while the need to retain the files for defensive dentistry (i.e., medical-legal and insurance reasons) or for future appliance repair is a matter of discussion. Orthodontists can easily backup the digital data and keep them for at least 10 years; meanwhile, gypsum casts could be lost or broken or need space in dental office, in addition to being a hidden source of contamination [106, 107].

The main advantage of CCT depends on the capability of forwarding some images, static or dynamic, coming from different sources (digital camera, CBCT, video, etc.), to a milling centre that will integrate them using Digital Smile Design software. After elaboration and dentist approval, the files will be used by computer-aided design (CAM) software to guide robotic devices which create objects and eventually assemble their parts in a virtual environment. Concerning the safety of the digital workflow, it is highly important to stay protected by installing a robust antivirus program, to

Table 2: IFU according to infection prevention from different manufacturers of scanners [19–21].

	Specific Indications	
Part of the scanner	TRIOS®	iTero®
System or Base Unit [20]	(i) Surface disinfection	(i) Surface disinfection.
Monitor [20]	(i) Do not spray directly with disinfectant.	(i) Do not spray directly with disinfectant. (ii) Use disinfectant wipes for the Scanning Unit and Base Unit.
Handheld scanner [20]	(i) Do not submerge the handheld scanner in any liquids. (ii) Do not place the handheld scanner on heated or wet surfaces. (iii) Surface disinfection.	Not indicated in open source [21].
Medical-grade peripherals (e.g., keyboards and mice) [20]	(i) Easy disinfection.	Not indicated in open source [21].
Scanner tips with fixed mirror or detachable mirror [19]	Immediately after clinical use: (i) Detach the mirror from detachable mirror and go on reconditioning separately for tip and mirror. (ii) Go on reconditioning for tip with fixed mirror. (iii) Clean manually and perfectly using soapy water and a soft dish brush. (iv) Rinse carefully the tip. (v) Inspect the mirror of the tip after cleaning. (vi) Dry the mirror carefully with a paper towel. (vii) Check to make sure it is free of lint, stains, and other kinds of dirt. (a) Wrap the tip using a self-adhesive pouch or heat-sealed pouch. (b) Sterilization using a steam autoclave class B (EN13060) and cycles at 121/134°C with drying. (c) Storage in proper condition.	Not needed.
Disposable plastic sleeve [21]		(i) Dispose of scanner sleeves according to standard operating procedures or local regulations for the disposal of contaminated medical waste.
Type of disinfectant [19]	(i) For optical windows and scanner tips: denatured alcohol (ethyl alcohol or ethanol) – typically 60-70% Alc/Vol. (ii) Mixture free of impurities that can stain the mirror.	(i) Many commercial products. (ii) Follow the disinfectant manufacturers' instructions for appropriate contact time. (iii) Remove residual liquid disinfectant with a lint-free, clean cloth.
Wipe [19, 20]	A soft lint-free nonabrasive cloth.	(i) Disinfectant wipes.
Prohibition on mirror tip of the use of [19]	(i) Ammonia-based or chloride based solutions or acetone on any surface. (ii) Acetone or any oxidizing solutions to clean the tip mirror.	
Disposal of scanner tip	Normally as other clinical waste.	Normally as other clinical waste.

Table 3: Some advices for better scanning.

Target	Actions
To avoid lint, stains, and dirt on the optical components:	Select disinfectants that do not produce faded stains and are nontoxic [4, 22]. Do not allow any solution to dry. Sterilization in wrapped pouches to protect the optical parts and to guarantee the use of sterile tip. Put outside the pouch a type 5 chemical integrators (UNI EN ISO 11140), to avoid the possible interference of their released products. Attention before and during steam sterilization: in particular, it is important: Check water quality, the cleanliness of the steam autoclave camera and trays, autoclave loading, and perfect drying of the wrapped pouches.
To protect optical component from damage:	Put the pouch far from other devices. Use absorbent TNT gauze for protection. It is not known if it is better: (a) to put the mirror tip towards the paper or the plastic side of the barriers, (b) up or down in the autoclave camera.
To prevent fast deterioration of the plastic parts of the unit:	Use single-use wipes soaked with disinfectant, which also act quickly against antibiotic-resistant strains and have good compatibility with optical and plastic parts [4, 22].

protect key functions, applications, and emails and mainly to prevent the copy/deletion/stealing or encryption of the patient's personal and sensitive data. It is obvious that digital dental casts can be controlled more easily by computer cryptographic and pseudonymisation tools, than by paper documents and analogue casts; this feature is expected to prevent clerical errors, involved in the majority of patient safety incidents [18].

4. Conclusion

In every day practice, CCT is one of the most important innovations that support infection prevention compared to traditional technology since it breaks or reduces cross-infection during impression and manufacturing steps. These advantages are expected to balance the higher cost of investments in hardware (scanner in the dental office and CAM in the milling service and dental labs) and software for "digital smiles".

As life expectancy increases, the prevalence of Alzheimer's disease will increase even further. Dentistry seems to be in the first line of prevention and should begin to equip itself with skills, updated knowledge for taking care of the different needs, and demands and aspirations of typically aged and Alzheimer's patients, including innovation through digital dentistry [108].

Unfortunately, guidelines for infection prevention using CCT have not been updated. DHCP needs better IFU and transparency from manufactures. Additionally, the presence of an infection prevention coordinator is necessary to follow IFU, as well as a plan for coordinated infection prevention between dental office, DT, and milling centre.

It is necessary to respect patients' rights in terms of privacy and large data protection.

Abbreviations

ADA: American Dental Association
DHCP: Dental Healthcare Personnel
DISR: Dental Implant Supported Restoration
DL: Dental Laboratory
DT: Dental Technician
CAD/CAM: Computer-Aided Design/Computer-Aided Manufacturing
CCT: CAD/CAM Technology
CDC: Centres for Disease Control and Prevention
HBV: Hepatitis B Virus
FDA: Food and Drug Administration
HIV: Human Immunodeficiency Virus
MD: Medical Device
MRSA: *Methicillin-Resistant Staphylococcus Aureus*
PMMA: Poly(methyl methacrylate)
MSDS: Material Safety Data Sheet
PPE: Personal Protective Equipment.

Conflicts of Interest

Livia Barenghi had a service agreement with KerrHawe and was a consultant for Dental Trey Il Blog

(http://blog.dentaltrey.it/), neither of which gave any input or financial support in the writing of this article. The authors (Alberto Barenghi, Alberto Di Blasio, and Carlo Cadeo) declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

The project started from the Cadeo's thesis entitled "Digital scanning delle arcate dentarie: una innovazione nella formulazione diagnostica e programmazione terapeutica in ortodonzia e gnatologia" discussed at Parma University, in July, 2018 for "Master di II livello in Ortodonzia intercettiva". Livia Barenghi conceived the project, conducted electronic literature search, selected the references, directed the work and wrote the manuscript. Livia Barenghi prepared photos related to cross-infection. Livia Barenghi, Alberto Barenghi, Alberto Di Blasio e Carlo Cadeo, discussed the data. Alberto Barenghi, Alberto di Blasio revised the manuscript.

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Research Article

Prevalence, Species Distribution, and Related Factors of Fish-Borne Trematode Infection in Ninh Binh Province, Vietnam

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Background. Clonorchis sinensis/Opisthorchis viverrini and minute intestinal flukes (MIF) such as *Haplorchis pumilio and H. taichui* are fish-borne trematodes (FBT) that may coexist in regions where local people have a habit of eating raw fish like Vietnam. Responses to FBT should be verified according to the data on the distribution of these flukes. This study aims to explore the prevalence of different species of FBT and related factors among local people in a northern province of Vietnam. *Methods.* A cross-sectional study was conducted in Kim Son and Yen Khanh districts, Ninh Binh province, between March 2016 and March 2017. Four hundred people aged 15 years or older were interviewed and gave stool samples. The FBT eggs in faecal samples were enumerated by modified formalin-ether technique and identified by sequencing of the second internal transcribed spacer (ITS2) region. *Result.* Among the 400 persons, 19.5% were infected with FBT. On univariate analysis, eating raw fish was the main risk factor (odds ratios (OR)) of 6.769 (95% confidence interval (CI) of 2.655–17.259) followed by being of male gender (3.994 (CI95% 2.117–7.536)) and drinking alcohol (2.680 (CI95% 1.440–4.986)), respectively. There was no risk of increased infection among those living at home without hygienic latrines, those living close to rivers or having ponds, or those raising cats or dogs. By multivariate analysis, FBT infection was only related to the consumption of raw fish and gender. Seventy stool samples with a sufficient amount of faecal matter were subjected to DNA extraction, 42.85% of them yielded DNA production, and all were of *Clonorchis sinensis*. *Conclusion*. Results of the study showed the high prevalence of infection of fish-borne trematode, mostly *C. sinensis* among humans in Ninh Binh province. The prevention of FBT should be strengthened with programs detailed according to the distribution of FBT in different endemic areas.

1. Introduction

Digenetic trematodes (digeneans or flukes) are commonly called "flatworms" and a major group of human parasites. They are classified as liver, lung, intestinal, or blood flukes according to the typical microhabitat in which the adult parasite usually resides. Some trematodes such as small liver flukes (SLF) (*Clonorchis sinensis/Opisthorchis viverrini*) and minute

Figure 1: The study site.

intestinal flukes (MIF) (*Haplorchis pumilio; H. taichui*) are of medical importance and public health significance in Asia [1]. Life cycles of FBT involve three types of hosts that are firstly aquatic snail hosts, secondary fish hosts, and definitive hosts including a range of fish-eating mammals and birds [2]. For the same mode of transmission, SLF and MIF infection may coexist in regions where local people have a habit of eating raw fish including Lao PDR, northern Thailand, and Vietnam [3–8]. Although they share many similar biological aspects, SLF and MIF differ in terms of intermediate hosts, time to complete life cycle in the definite host and life expectancy in the human body, response to antihelminth drugs, etc. [9–11]. Thus, control measures have to be appropriately adjusted according to the distribution of different species in endemic areas.

Vietnam is a South East Asian country with the existence of many parasitic zoonoses [12]. Results of epidemiological surveys showed that SLF *(Clonorchis sinensis)* and some MIF (*Haplorchis pumilio, H. taichui, H. yokogawai,* and *Stellantchasmus falcatus*) were endemic in many northern provinces including Ninh Binh province, but there is still controversy over the distribution of these flukes among local people [4, 13–18]. The diagnosis of FBT infection in human has been mostly based on morphological features of small fluke eggs in stool samples [19], but due to the similarity of these eggs, the precise discrimination is nearly impossible [20, 21]. The molecular technique is a rapid and highly sensitive tool to identify FBT eggs but has not been applied in the previous surveys. Studies using molecular tools to identify the species of FBT for adult worms collected from infected persons have revealed inconsistent results [15, 18]. For a long time, much attention has focused on SLF [19, 22] but most of the metacercariae collected from fish in that region were MIF while the prevalence of SLF is very low [17, 23, 24]. With such divergent results, FBT infection among local people should be resituated. The present study was carried out to investigate the prevalence and distribution of FBT species in human as well as factors that affected the transmission of FBT in this endemic area of Vietnam.

2. Material and Methods

2.1. The Site, Sampling, and Examination Procedure. The cross-sectional survey was conducted in four communes, Kim Dong and Kim Tan of Kim Son district and Khanh Thanh and Khanh Thuy of Yen Khanh district, Ninh Binh province (Figure 1). The study site is located around 100 km southeast of the capital, Hanoi. Most residents of the four communes live on rice agriculture, while some residents of Kim Tan commune (Kim Son district)—a coastal commune—work as fish farmers.

The sample size for this study is determined by the standard formula ($n=z^2_{1-\alpha/2}p(1-p)/d^2$) to reach a universal sampling size. At 95 percent confidence interval, absolute precision (d) of 5% and anticipated population proportion (p) of 50%, the desired sample size was 384 [25].

Households in these four communes were randomly selected from the lists provided by local health authorities. In each selected household, all members aged 15 years or older were chosen for the study. About one hundred persons from each commune were involved and a total of 400 participants completed a questionnaire about their demographic features and some habits such as consuming raw fish or drinking alcohol. The respondents were provided with labelled bottles to collect stool samples and required to transport the samples on the same or the following day. The stool samples were placed in dry ice boxes and transported to the laboratory to be examined. Helminth parasite examination was performed on the same day or the next day of receiving the stool samples. One gram of each stool sample was weighed and then tested for helminth eggs using formaldehyde–ether sedimentation technique [26]. All trematode eggs with sizes of less than

Table 1: Prevalence and related factors of small trematode infection.

		N	Infected	%	Univariate analysis OR (CI 95%)	Multivariate analysis OR (CI 95%)
Districts	Kim Son	199	40	20.10	1.079 (0.658 – 1.770)	
	Yen Khanh	201	38	18.91		
Gender	Male	244	65	26.64	3.994 (2.117 – 7.536)	5.088 (1.766 – 14.660)
	Female	156	13	8.33		
Age groups	15 –<30	30	5	16.67		
	30 – <40	70	13	18.57		
	40 – < 50	120	22	18.33		
	50 – < 60	132	30	22.73		
	≥ 60	48	8	16.67		
Eating raw fish	Yes	293	73	24.91	6.769 (2.655 – 17.259)	5.529 (2.066 – 14.798)
	No	107	5	4.67		
Drink alcohol	Yes	267	64	23.97	2.680 (1.440 – 4.986)	0.448 (0.151 – 1.325)
	No	133	14	10.53		
Total		400	78	19.50		

50 μm were considered "small trematode eggs". All the discovered eggs were recorded and the intensity of small trematode infection was categorized as light (< 1.000 eggs per gram (EPG)), moderate (1.000-10.000 EPG), or heavy infection (>10.000 EPG) [27]. One part of each positive sample was diluted in 3 parts by volume of alcohol (70%) and stored in -20°C for further analysis.

2.2. Molecular Analysis. The extraction of DNA from trematode eggs

Seventy positive samples with a sufficient amount of faecal matter were subjected to DNA extraction. For each stool sample, 1.4 ml of ASL buffer was added to 200 μl faecal liquid, mixed continuously for 1 min or until the stool samples were thoroughly homogenized. After homogenization, the suspension was heated at 95°C for 4 min and then frozen in dry ice for 8 min. The freeze-thaw cycle was repeated twice before incubating at 95°C for 10 min. Then 1.2 ml of supernatant was collected into a new sterile tube. The DNA was extracted from the supernatant using the QIAmp DNA stool mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. At the final step, DNA was eluted with 50 μl of elution buffer.

Primers to amplify the second internal transcribed spacer region (ITS-2) included ITS2-F (5′-CTT GAACGC ACA TTG CGG CCA TGG G-3′) and ITS2-R (5′-GCG GGT AAT CAC GTC TGA GCC GAG G-3′) [28]. PCR reaction was conducted on a thermal cycler (Eppendorf Mastercycler Personal, Germany) in a total volume of 20 μl, including 5 μl template, 10 pmol of each primer and PCR master mix (PCR Master Mix from QIAGEN). The PCR was run 35 cycles of 94°C for 10 seconds, 40°C for 30 seconds, and 72°C for 30 seconds with a final extension step of 15 minutes at 72°C. PCR products were separated by electrophoresis on 1.2% agarose gel and visualized under UV light after staining with ethidium bromide. All yielded PCR products were purified and sequenced using a 3130XL sequencer. The obtained sequences of ITS2 region were aligned with reference sequences retrieved from GenBank using Bioedit 7.0 and MEGA 7 software [29]. Phylogenetic trees were constructed using the neighbor-joining method and significant level was estimated with 1000 bootstrap replicates.

Data were analyzed with the Statistical Package for Social Science (SPSS version 16.0). The comparison between numeric variables was done by student T-test. Univariate analysis of the relationship between prevalence and risk factors (living condition, gender, the habit of eating raw fish or alcohol) was conducted. Factors that showed a significant association with the small trematode egg-positive rate were then subjected to multiple logistic regression analysis. The p-value smaller than 0.05 was considered significant.

Time of the study: interviewing, sampling, and microscopic examination of stool samples were done between March 2016 and March 2017. Molecular analysis was conducted in 2018.

Ethical Consideration. The study was approved by the ethical committee of the National Institute of Malaria, Parasitology and Entomology of Vietnam. Written consent was obtained from all subjects and all persons with positive results of parasite infection were provided free drug treatment at local health care service.

3. Results

Four hundred people with an average age of 46.8 years old were involved in the present study, 61% of them were male and most of them were farmers with a low level of education, none of them having graduated from a university.

Among the participants, 19.5% were infected with FBT. By univariate analysis, persons who were men, eating raw fish and drinking alcohol were at a higher risk of infection (OR = 3.994, 6.769 and 2.680, respectively). By multivariate analysis, only gender and habit of eating raw fish were the risk factors of getting FBT infection (Table 1).

There was no relationship between house characteristics, raising dogs or cats, and FBT infection (Table 2).

Table 2: Some other factors related to small trematode infection.

		Infected	Not-infected	OR (CI 95%)
House with hygienic latrines *	No	15	36	1.892
	Yes	63	286	(0.976 – 3.664)
House nearby rivers**	Yes	57	212	1.408
	No	32	140	(0.812 – 2.443)
House with fish ponds	Yes	46	182	1.106
	No	32	140	(0.669 – 1.827)
Raising dogs	Yes	57	229	1.102
	No	21	93	(0.633 – 1.921)
Raising cats	Yes	48	205	0.913
	No	30	117	(0.549 – 1.520)

*Hygienic latrines: septic tank.
**House nearby rivers: distance less than 1 km from rivers.

Table 3: Intensify of small trematode infection and related factors.

Groups		N (%)	Mean (EPG)	SD	p
Whole			517.06	1103.49	
	Light	68 (87.17)			
	Moderate	10 (12.83)			
	Heavy	0 (0)			
District	Kim Son	40	723.00	1464.42	> 0.05
	Yen Khanh	38	396.84	466.51	
Age groups	15 –<30	5	560.00	689.35	> 0.05
	30 – <40	13	264.62	164.55	
	40 – < 50	22	458.18	545.49	
	50 – < 60	30	853.33	1665.10	
	≥ 60	8	260.00	177.60	
Gender	Male	65	618.46	1199.98	< 0.05
	Female	13	292.31	194.17	
Eating raw fish	Yes	73	587.39	1136.54	< 0.05
	No	5	224.00	186.76	
Drinking alcohol	Yes	47	788.94	1375.68	< 0.05
	No	31	223.23	155.87	

The average density of infection was 517.06 EPG. Most of the infected participants were ranked as light infection (87.17%) and mean of EPG were higher among male and those who ate raw fish and drank alcohol (Table 3).

Identification of trematode: thirty of 70 analyzed stool samples (42.85%) were ITS2-PCR positive with the sizes of 400 bp (Figure 2).

The NJ phylogenetic tree was constructed from typing sentences of 18 representatives from our study (those with subfix NB in the tree) and 9 reference sequences from GenBank using CLUSTAL_X with Kimura's correction. All the obtained sequences in our study were determined as *Clonorchis sinensis* and none were MIF (Figure 3). Some sequences were deposited in GenBank under accession number MK453253, MN128615, MN128616, MN128617, and MN128618.

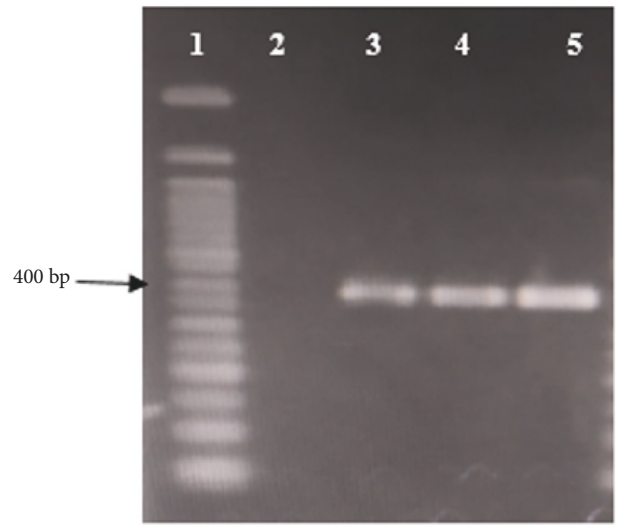

Figure 2: The products of the amplification of the small trematode eggs. Lane 1: 50 bp DNA marker (ThermoFisher), Lane 2: negative control, and Lanes 3-5: positive samples.

4. Discussion

In Vietnam, FBT infection is endemic in the north and the highest prevalences were recorded in Nam Dinh and Ninh

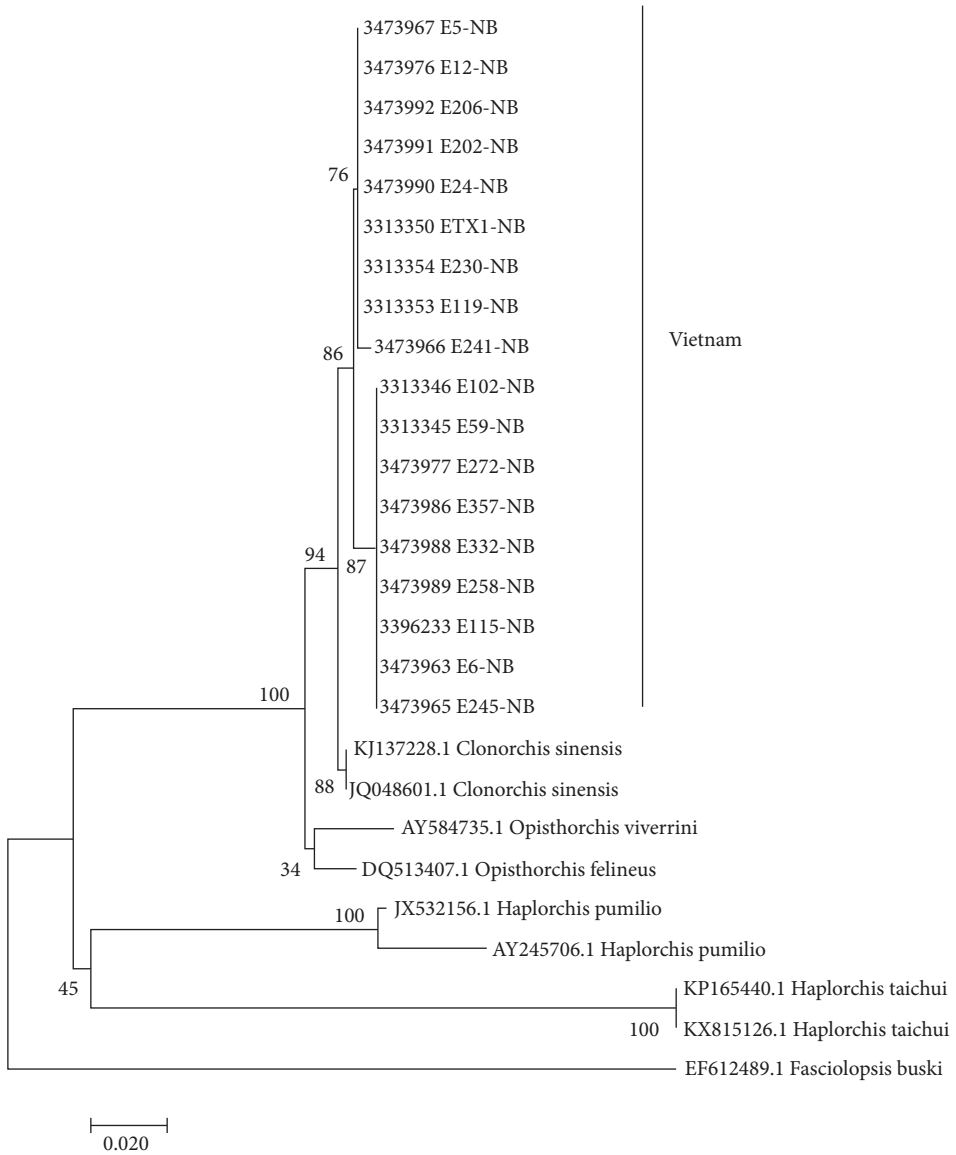

Figure 3: Neighbor-joining phylogenetic tree to identify trematode species.

Binh provinces [19]. The prevalence of FBT in Kim Son and Yen Khanh districts, Ninh Binh province (19.5%), was lower than that of a previous survey carried out in Kim Son district between 1999 and 2000 [15] (26.1%) but higher than a report in Gia Vien, a nearby district in 2015 (16.47%) [30]. The average intensity of infection was 517 eggs per gram which was comparable to that in previous reports in Kim Son district (mean of 504 and 472 EPG) [13, 15]. Almost all (87.2%) cases were light infection and no subjects were heavily infected. This finding was consistent with some observations suggesting that most of the persons infected with small fluke in the community were categorized as light infection [4, 31].

Eating raw fish was the leading factors of FBT infection among local people (OR = 6.769), which was consistent with some other reports [30, 32–37]. Nearly three-quarters of participants usually consumed raw fish which was comparable to the result of Thach et al. (2008) [15] and Vinh et al. (2017) [30]. Some authors stressed that the control of FBT is theoretically very simple by avoiding eating raw or undercooked fish, but it can be extremely difficult in facing of centuries-old traditions [38, 39]. Although many efforts had been made to change the habit of local people, the rate of consuming raw fish was almost unchanged and more works are required to deal with this situation. The prevalence and intensity of FBT infection among males being higher than in women were in line with results of some other studies [14, 32, 40]. By univariate analysis, people who are alcohol drinkers were at 2.680 times higher risk of getting infected than those who did not drink alcohol but by multivariate analysis, drinking alcohol was not related to FBT infection. This may be because it is a common habit of local people to drink alcohol while eating raw fish. This means the main factor for infection is the consumption of raw fish, and drinking is only a confounding factor. However, alcohol drinkers had higher EPG mean

compared to that of nondrinker (Table 3) and there is an experiment showing that alcohol could induce metacercariae excystation, leading to the early development of trematodes in human [41].

There was no risk of increased infection among local people living at home without hygienic latrines, those living close to rivers or having ponds, or those raising cats or dogs. The relationship between these factors and FBT infection has been reported in some studies. Higher prevalence of FBT infection among people living in lowland areas [14, 42]; near freshwater sources [43]; or fish ponds [36] was noted. Nevertheless, there is only a weak relationship between these factors and FBT infection in the above reports and Tesana S et al. (1991) noted a higher prevalence of SLF infection among the people residing far from the rivers than those residing on the banks [44]. Dogs and cats are considered the reservoirs of FBT flukes [45] and the high prevalence of *C. sinensis* in cats and dogs corresponded to high prevalence in humans in South China [46]; therefore controlling flukes in animals may play a role in preventing human infection [12]. However, FBT could not transmit to human by direct contacts with animals, so the absence of a relationship between having dogs or cats and FBT infection was reasonable.

In the present study, molecular techniques were performed to determine the distribution of FBT species among local people and this may be the first report using the molecular technique to discriminate eggs of FBT in Vietnam. Internal transcribed spacer region was selected because it is considered a reliable and precise marker for identification of flukes [44]. Less than half of the analyzed stool samples yielded DNA production. The low sensitivity of molecular techniques is probably related to the low density of eggs in faeces which has been documented by some authors [47, 48]. All yielded DNA products belonged to *C. sinensis* which is consistent with some other reports in northern Vietnam. Dang el al. (2008) found that all adult worms collected from infected people are *C. sinensis* [15]. De and Hoa (2011) showed that among 10 infected persons there were 9 persons infected with *C. sinensis* and 10 persons infected with MIF [18]. Notably, results of the present study contradicted a survey carried out by Dung et al. (2007) which found a high rate of MIF (100%) and lower prevalence of SLF (about 50%) among infected persons [4]. However, previous studies were based on analysis of adult flukes collected from a heavily infected person after taking drugs which meant for intentionally selected persons. Our study is based on molecular analysis of all infected people so that the result would be more representative of the community.

The predominance of *C. sinensis* among people in the present study did not agree with the predominance of MIF (such as *Haplorchis pumilio*) among fish in northern Vietnam [17, 23, 24]. There may be some possible explanation for this contradiction. The first may be the difference between the longevity of two kinds of fluke in humans. In humans, *C. sinensis* can live for 26 years [49] but most MIF only live for one year [50] so the accumulation rate of infection with SLF for such a long time is very high. The second reason is the productivity of different flukes. One SLF can produce up to 4000 eggs per day [51] while the daily egg output of some MIF such as *H. taichui* is very low (estimated as 82 eggs/worm) [52]. This may have led to the difference in intensity of infection, which affected the sensitivity of diagnosis technique based on molecular analysis.

Our findings would be useful in adjusting the response to FBT infection in that area especially measures to reduce the worm burden or morbidity rate by chemotherapy. Praziquantel is the most common means for large-scale or individual treatment [53] and this approach must be based on the results of epidemiological studies [27]. With almost all positive persons infected with *C. sinensis* and the low efficacy of praziquantel on clonorchiasis in the north of Vietnam [9], closely monitoring the efficacy of praziquantel at the community level is needed and a modified dose accordingly may be considered in some selected population.

5. Conclusion

The study aims to explore the prevalence of different species of FBT and related factors among local people in a northern province of Vietnam. The prevalence of infection of fish-borne trematode among human in Kim Son and Yen Khanh district, Ninh Binh province, was still high although most were of a light infection and infected with *Clonorchis sinensis*. The main risk factor of infection was the common habit of eating raw fish by local people. There is a need to strengthen the prevention of FBT in endemic areas including better targeted public education interventions about FBT and people at risk. Applying modern techniques to accurately identify the fluke in community surveys in different areas to get precise information of epidemiology fish-borne disease is very important to adjust response measures to control the infection.

Abbreviations

CI: Confidence interval
DNA: Deoxyribonucleic acid
dNTP: Deoxynucleotide
EPG: Eggs per gram
FBT: Fish-borne trematodes
ITS: Internal transcribed spacer
MIF: Minute intestinal flukes
OR: Odds ratios
PCR: Polymerase chain reaction
SLF: Small liver flukes.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Disclosure

This research is part of thesis work for the fulfillment of Doctor of Literature and Philosophy in Health at National Institute of Malaria, Parasitology and Entomology of

Vietnam. The funder does not have any role in data collection, analysis, and writing of the manuscript.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Immunization Campaigns and Strategies against Human Papillomavirus in Italy: The Results of a Survey to Regional and Local Health Units Representatives

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Objective. The study aimed to assess the impact of HPV immunization campaigns organizational aspects, the characteristics of immunization program (vaccination targets and type of offer), and communicative strategies adopted by four Italian administrative regions on vaccination coverage observed. *Methods.* From November 2017 to March 2018, regional and Local Health Units (LHUs) representatives were invited to complete an online survey including 54 questions evaluating vaccination invite systems, access systems to vaccination centres, reminder and recall systems, and adverse events surveillance. An overall descriptive analysis was conducted. Since observed vaccine coverage (VC) obtained in females (2002-2004 birth cohorts) was lower than objectives fixed by the Italian Ministry of Health, variables were assessed using the national VC mean obtained in the 2003 girls birth cohort as outcome. *Results.* Twenty-six LHUs belonging to 4 Northern and Southern Italian regions participated in the study. Organizational aspects significantly related to VC lower than the national mean were access to vaccine centres without appointment and parents' reservation as appointment planning system. Recall systems for both the first and the second dose, including the appointment in the invitation letter, the availability of regional immunization registry, and education of healthcare workers on universal HPV immunization strategies, instead, were related to higher VC. As regards preadolescent immunization strategies, both VC obtained in girls and boys were far from the Ministerial goals. Only 20% of LHUs introduced multicohort female strategies while all LHUs adopted copayment targeting both men and women. Immunizations strategies targeting subjects at risk were implemented only in half of participating LHUs. *Conclusions.* VC observed in participating LHUs are largely lower than the national objectives in all anti-HPV vaccine targets. Both organizational and educational strategies have to be implemented to improve the VC goals.

1. Introduction

Human papillomavirus (HPV) is considered one of the most common sexually transmitted infections in the world, affecting people especially in developed countries. About 70-80% of the sexually active women and men will acquire an HPV infection during their life [1]. Since HPV was discovered to cause cervical, penile, anal, vaginal,

oropharynx, hypopharynx, and larynx cancers, the development of effective anti-HPV vaccines was considered one of the most important public health goals to be achieved [2–4]. In 2006, the European Medicines Agency (EMEA) approved a quadrivalent vaccine containing antigens against four HPV-types (6, 11, 16, and 18) and, one year later, they approved a second bivalent vaccine against oncogenic HPV-types (16 and 18). Finally, in 2015, a nonavalent vaccine extending antigens to five additional HPV-types (31, 33, 45, 52, and 58) to that contained in the quadrivalent vaccine was approved by EMA [5, 6].

Initially, HPV vaccination has been recommended for adolescent women prior to sexual debut, and it has already been showed to be highly effective in reducing HPV-related lesions, such as genital warts as well as Cervical Intraepithelial Neoplasia (CIN) [7]. Increasing evidence demonstrated the consistent burden of HPV-related diseases also in men, such as anal, head, and neck cancers (4 times or more than women) and penile cancers [8, 9].

Nowadays USA, Canada, Australia, and over 37 out of 53 European (EU) countries have introduced the vaccine into their national routine immunization schedules and several of these both for males and females [10].

In 2014, HPV vaccination coverage in the EU Region accounted for about 50% in the primary target, while African countries reported a mean 88% HPV vaccination coverage (VC) [11]. In Italy, HPV vaccination is free and has been actively offered to all girls during their 12th year of life since 2007, and recently the National Vaccination Plan (PNPV) 2017-2019 established a target vaccination coverage of 95%, within three years of the start of the campaigns [12]. However, despite several promotional activities, VC is largely unsatisfactory, ranging from 30% to 75% among Italian administrative regions for full HPV vaccination in primary cohorts (birth cohort: 2005) of women and from 0% to 65% among primary cohorts of men [13].

In Europe, several studies have previously indicated that common reasons for not receiving or completing the HPV vaccine were the perception of low risk or not needing the vaccine, low perception of HPV vaccination benefits, doubt about the safety and efficacy of the vaccines, fear of side effects, low participation at school seminar on HPV for school-based immunization campaign, lack of physician recommendations, and cost of the vaccine [14–17].

Organizational aspects to improve HPV vaccine uptake were also investigated, in particular the implementation of immunization services' accessibility, the role of reminder systems, and communication technologies. Nevertheless, previous evaluations were rarely comprehensive or specific, thus compromising the estimate of the effect of interventions' interaction and the consideration of HPV vaccine peculiarities (i.e., the variety of involved targets and healthcare professionals, the role of parents, and the range and seriousness of HPV-related diseases, including cancers) [18–29].

Aim of the present study was assessing the different HPV immunization campaigns carried out in four Italian administrative regions, evaluating the impact of organizational aspects, the characteristics of immunization program (vaccination targets, e.g., female, male, categories at risk such as men who have sex with men, etc., and type of offer), and communicative strategies adopted by the regions on vaccination coverage observed.

2. Material and Methods

An online questionnaire was administered through Google Drive platform to 26 Local Health Units (LHUs), belonging to 4 Northern and Southern Italian regions, where the universal preadolescents HPV vaccine recommendation was introduced before the PNPV 2017-2019, covering 25.7% of the national population.

From November 2017 to March 2018, regional and LHUs representatives were invited to complete an online survey including 54 questions evaluating HPV immunization policies targeting preadolescents, adults and subjects at risk, obtained results in different targets, communication and education strategies, and organizational characteristics of vaccination centres. In particular, vaccination invite systems, subjects who invites preadolescents, sending information with invitation letter, subjects who administers the vaccine, access system to vaccination centres, appointment planning system, reminder of the first dose appointment, specific HPV vaccine sessions, recall of subjects who missed the first and the second dose, second dose appointment planning, and AE surveillance within 30 minutes and since the day after were investigated. An overall descriptive analysis was conducted.

Since observed VC obtained in females 2002-2004 birth cohorts were lower than the objectives fixed by the Ministry of Health, VC national mean was used as outcome. In particular, the possible association of organizational aspects with VC obtained in the female 2003 birth cohort higher than the national mean (64.7%) [13] was tested through univariable logistic regression.

VC obtained in boys were not considered as outcome since the objectives fixed by the Ministry of Health in PNPV 2017-2019 refer to the 2006 birth cohort for which data were not available.

After the collinearity assessment, factors resulting statistically significant in univariable comparisons ($p < 0.05$) were included in a multivariable model, by means of a stepwise backward procedure.

Statistical analyses were conducted by the JMP software, version 13.

2.1. Ethical Approval. The study protocol was approved by the Regional Ethic Committee of the Liguria region, Italy (P.R. 162REG2017).

3. Results

3.1. Universal Preadolescents HPV Vaccination Organizational Aspects. The main results concerning universal preadolescents HPV vaccination organizational aspects are shown in Table 1. Almost all LHUs invite preadolescents through letters addressed to parents, which in 76.9% of cases included also informative material. Schools are involved only in 26.9% of cases. The access system to vaccination centre for the first dose administration was by appointment in almost all

Table 1: Universal pre-adolescents HPV vaccination organizational aspects.

Organizational aspects	(N=26) N (%)
Vaccination invite system	
Letter addressed to parents by LHUs	25 (96.2%)
Information given at school	7 (26.9%)
SMS to parents	1 (3.8%)
Smartphone Application	1 (3.8%)
Subject who invites pre-adolescents	
Healthcare workers of immunization centres	26 (100%)
Sending information with invitation letter	20 (76.9%)
Subjects who administers the vaccine	
Healthcare workers of immunization centres	26 (100%)
Access system to vaccination centres	
Free access	5 (19.2%)
Appointment	25 (96.1%)
Appointment planning system	
Included in the invitation letter	19 (76%)
Parents reservation	10 (38.5%)
Reminder of the first dose appointment	14 (53.4%)
Specific HPV vaccine sessions	21 (80.8%)
Recall of subjects who missed the first dose	18 (69.2%)
Letter	14 (77.8%)
Phone call	9 (50.0%)
SMS	1 (5.9%)
Second dose appointment planning	
During the first dose appointment	21 (80.8%)
Invitation letter	4 (15.4%)
Parents reservation	1 (3.8%)
Recall of subjects missing the second dose	16 (61.5%)
Phone call	8 (50%)
Invitation letter	10 (62.5%)
SMS	1 (6.3%)
AE surveillance within 30 minutes	21 (80.8%)
AE surveillance since the day after	
Parents contact to immunization centre	17 (65.4%)
Report	9 (34.6%)
Regional vaccine-vigilance system	21 (80.8%)

LHU=Local Health Unit; SMS=Short Message Service; AE=Adverse Event

LHUs, and the appointment was included in the invitation letter in 76% of cases. The second dose appointment was planned during the first appointment in 80.8% of cases. Only about half of the LHUs adopt reminder systems for the first dose appointment. HPV vaccine is administered in specific vaccine sessions in 80.8% of LHUs and recall systems for subjects who missed the first and the second dose are used in 69.2% and 61.5% of cases, respectively. As regards adverse events surveillance after the HPV vaccine administration, about 20% of LHUs reported no surveillance within 30 minutes and no active regional vaccine-vigilance system.

3.2. Immunization Strategies and Vaccine Coverage. As regards preadolescent immunization strategies, both VC obtained in boys and girls are largely suboptimal in participating LHUs and very far from the goals set by the Ministry of Health in the PNPV 2012-2014 and 2017-2019 (Figures 1(a) and 1(b)). In particular, median VC for complete cycle obtained in 2002, 2003, and 2004 girls birth cohorts were 67.7% (25-75p=59.7%-75.9%), 66.5% (25-75p=57%-77.5%), and 66.5% (25-75p=51.4%-73.1%), respectively. In boys the VC for complete cycle were 26.8% (25-75p=16.3%-45.8%) and 49.7% (25-75p=59.7%-75.9%) in 2003 and 2004 birth cohorts, respectively.

Furthermore, only about 20% of LHUs introduced multicohort female strategies while all adopted copayment targeting both men and women.

Immunizations strategies targeting subjects at risk are implemented only in half of participating LHUs and a multidisciplinary network to identify them is active in 27% of cases. The healthcare workers who recommend the HPV vaccine are various but low in numbers (immunization centres, gynaecologists, infectious diseases specialists, and general practitioners in 41.7%, 25%, 33.4%, and 25%, respectively), while the administration of HPV vaccine is centralized in vaccine centres.

As regards vaccine registries, they are digitized in all participating LHUs but only about the half of them are present on a regional level.

3.3. Communication Strategies. Communication tools were also investigated (Table 2). In particular, informative material available at the immunization centres is prepared locally in the majority of cases and translated in other languages than Italian only in 19.2% of cases. A call centre to discuss about vaccines is active in 53.8% of cases, formative moments such as focus group addressed to preadolescents parents were conducted in 42.3% of cases, and local media were involved in informative campaigns in about 35% of LHUs. The education of healthcare workers on universal strategy was multidisciplinary and it was conducted in almost all the LHUs. The analysis of suboptimal obtained VC and of vaccine hesitancy determinants was conducted only in 57.7% and 34.6% of cases, respectively.

Table 3 shows the results of univariable logistic regression investigating the possible association between organizational aspects and VC obtained in the female 2003-birth cohort. Organizational aspects significantly related to VC lower than the national mean were access to vaccine centres without appointment and parents' reservation as appointment planning system.

Recall systems for both the first and the second dose, including the appointment in the invitation letter, the availability of regional immunization registry, and education of healthcare workers on universal HPV immunization strategies, instead, were related to higher VC.

After multivariable analysis, one variable resulted statistically significant: access without appointment to vaccination centres (p=0.038); recall systems resulted borderline, with p=0.063 (Table 4). In particular, the probability to obtain VC higher than the national mean is equal to 87.6% if a recall of

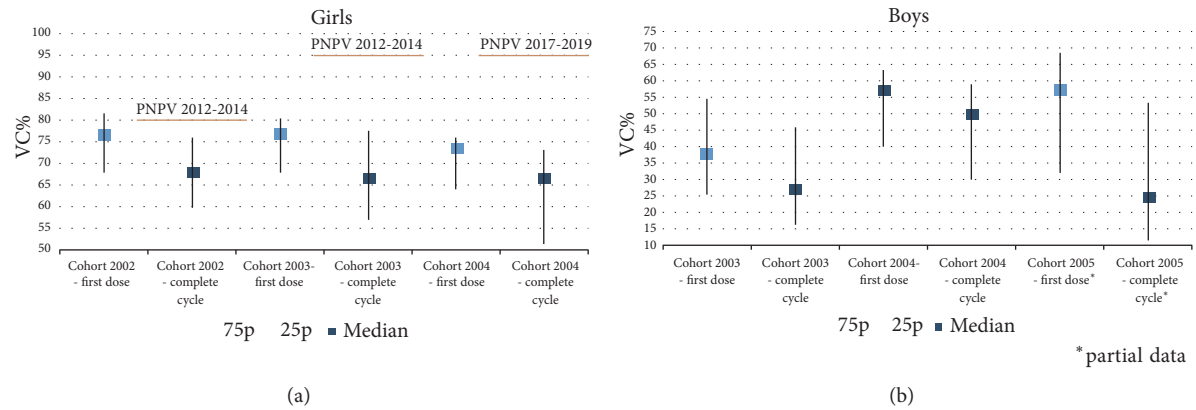

Figure 1: Vaccine coverage (median, 25-75 p) for first dose and complete cycle obtained in preadolescents girls (birth cohorts 2002-2004) (a) and boys (birth cohorts 2003-2005) (b) in participating Local Health Units and vaccine coverage objectives set by the Italian Ministry of Health.

Table 2: Communication strategies about HPV vaccine.

Communication strategies	(N=26) N (%)
Informative material available at immunization centres	
Ministry of Health	2 (7.7%)
Region	11 (42.3%)
LHUs	13 (50%)
Scientific agencies	3 (11.5%)
Pharmaceutical companies	8 (30.8%)
Translation of informative material	5 (19.2%)
Call center	14 (53.8%)
Focus group	11 (42.3%)
Media	9 (34.6%)
Education of HCWs on "universal" strategy	24 (92.3%)
Immunization centres	16 (66.7%)
General practitioners/Pediatricians	14 (58.3%)
Gynecologists	9 (37.5%)
Screening centres	9 (37.5%)
Periodically	12 (50%)
Discussion of obtained vaccine coverages	15 (57.7%)
Analysis of vaccine hesitancy determinants	9 (34.6%)

subjects missing the second dose is active. If the access system to vaccine centre is without appointment, the probability to obtain VC higher than the national mean is only 8.7%. The two organizational aspects are related to a probability of gaining higher VC of about 37%.

4. Discussion

This survey allowed us to obtain a detailed picture of a wide range of HPV vaccine offer and promotion strategies and to identify actions adopted in Italian LHUs that are associated with VC higher than the national mean.

In our knowledge, the most studied strategies aimed at gaining adequate HPV VC deal with the evaluation of knowledge, attitudes, and determinants of acceptance and refusal, and they mainly focus on preadolescents girls and their parents [30–41].

Nevertheless, immunization strategies and organizational aspects of vaccine centres also have a relevant role in determining the vaccine compliance.

Some studies investigated interventions to improve HPV vaccine uptake but each of them was evaluated separately [18–23]. Other studies and recommendations on factors influencing VC integrated the evaluation of many interventions but rarely specific for HPV vaccine, that is critical for the variety of targets and healthcare professionals implicated in vaccine counselling, the involvement of parents, and the relevance of indications including cancers [24–27]. In particular, the Community Preventive Services Task Force (CPSTF) supported by the Centers for Disease Control and Prevention (CDC) provides evidence-based findings and recommendations on intervention approaches for increasing vaccination, based on available scientific evidences [28]. Findings are divided into three categories [29], including recommended with strong evidence strategies aiming at enhancing access to vaccination services (e.g., home visits, vaccination programs in schools and organized child care centres, and vaccination programs in women, infants, and children settings), increasing community demand for vaccinations (e.g., client

Table 3: Univariable logistic regression of universal pre-adolescents HPV-vaccination vaccination organizational aspects associated with gaining by LHU of vaccine coverage in 2003 girls birth cohort higher than the national mean.

Organizational aspects and communication strategies	*Cohort 2003* *LHU VC> National mean VC (64.7%)*			
	Yes	No	P-value	OR (95% C.I.)
Access system to vaccination centres				
Free access	1 (5.9%)	4 (44.4%)	0.018	0.08 (0.01-0.87)
Recall of subjects who missed the first dose	14 (82.4%)	4 (44.4%)	0.046	5.8 (1-35.7)
Recall of subjects missing the second dose	13 (76.5%)	3 (33.3%)	0.032	6.5 (1.09-38.63)
Appointment planning system				
Included in the invitation letter	15 (88.2%)	4 (44.4%)	0.017	9.37 (1.3-67.65)
Parents reservation	1 (5.9%)	4 (44.4%)	0.018	0.08 (0.01-0.87)
Immunization registry				
Regional	12 (70.6%)	2 (22.2%)	0.019	8.4 (1.27-55.39)
Education of HCWs on "universal" strategy	17 (100%)	7 (77.8%)	0.043	NA

LHU=Local Health Unit; VC=Vaccine Coverage; HCW=Health Care Worker

Table 4: Organizational aspects selected by multivariate stepwise logistic regression for prediction of gaining by LHU of vaccine coverage in 2003 girls birth cohort higher than the national mean.

Organizational aspects		Recall of subjects missing the second dose (p=0.063)	
		Yes	No
Free access to vaccination centres (p=0.038)	Yes	36.9%	8.7%
	No	87.6%	53.4%

reminder and recall system and vaccination requirements for child care, school, and college attendance), and provider- or system-based interventions (e.g., immunization information systems, provider assessment and feedback, and provider reminders).

Among the abovementioned interventions, the active invitation of eligible subjects represents one of the more effective interventions to increase the VC. In this context, almost all participating LHUs invite adolescents' parents by letter and adopt the appointment as access system to vaccination centres, limiting the free access to 19.2% of cases. In particular, the appointment is included in the invitation letter in 76% of cases.

On the contrary, the access to vaccine centres without appointment and parents' reservation as appointment planning system are significantly related to VC lower than the national mean. Further, recall systems for both the first and the second dose is adopted only in 69.2% and 61.5% of cases, even if recommended with strong evidence by scientific authorities [29] and resulted significantly related to higher VC also in our study.

Among recommended with strong evidence immunization information systems, the digitized vaccine registries could be considered. Even if they are active in all participating LHUs, only about the half reported regional immunization strategies, that resulted significantly related to higher VC. This limits the sharing of immunization data between LHUs and from LHUs to regional authorities, compromising the governance of immunization policies.

Even though adverse events surveillance could not be strictly considered an organizational aspect, we investigated routine system used by LHUs to monitor adverse events as relevant quality and safety standard of immunization policies. Surprisingly, 30 minutes surveillance and regional vaccine-vigilance systems are not conducted in all participating LHUs and thus they should be extended and homogenised.

As regards communication strategies, almost all participating LHUs reported education activities on universal HPV immunization strategy targeting health care workers. Education targets the main professionals involved in HPV vaccine counselling but the proportions are low and the interventions are conducted periodically only in the half of LHUs, highlighting the need of promoting further activities. This is of particular relevance in the field of HPV primary and secondary prevention since a variety of professionals operating in different healthcare settings are involved along a decision path where the immunization centre often represent the point of arrival. Furthermore, available evidences demonstrate the importance of the role of trusted healthcare provider in taking decisions in the field of immunization [25, 26, 33, 40–42]. In particular, the HPV vaccine targets preadolescents of both sexes and subjects at risk; thus paediatricians and general practitioners ease the link between subjects and immunization centre. Parents are also involved in the decision path and they usually identify gynaecologists as reference figure to obtain information on a sexually transmitted infection causing anogenital cancers. Subjects at risk could refer to infectious disease specialists and oncologists but also dermatologists, urologists, and otolaryngology specialists. Thus, the synergy between professionals and their active role in vaccine offer are essential to obtain a good compliance to HPV prevention strategies, including vaccines.

Furthermore, sharing a unique and coherent message among different stakeholders using available communication

tools is crucial to obtain the best results in terms of adherence to HPV vaccine. As healthcare planning activities should include the phase of feedback and report, the discussion of obtained VC and the analysis of vaccine hesitancy determinants among healthcare workers were also investigated, showing a prevalence as low as 57.7% and 34.6%, respectively.

The studied communications strategies directed to target subjects included the availability of informative material at immunization centres that was prepared by LHUs and regions in 50% and 42.3% of cases, respectively, and translated in other languages only in 19.2% of cases. Even if these factors are not significantly related to VC, they could contribute to the circulation of scientifically correct information and compensate the spread of misleading messages by "no-vax". Furthermore, call centres coordinated by immunization centres to obtain information and address doubt about vaccines are available in about the half of participating LHUs. The involvement of media and the conduction of focus group in particular in the school setting could increase the vaccine demand by target when combined with other activities; nevertheless, they are reported in less than half of participating LHUs. This could be due to the suboptimal economic and human resources currently available for prevention in the majority of Italian regions.

Our findings are in line with the most recent evidences on interventions aimed at improving HPV vaccine uptake [42–46]. In particular, recall systems for missed administrations and free access to vaccination centres resulted in the main factors related to VC outcome in preadolescent girls, demonstrating the importance of implementing the accessibility to immunization services and the taking-care process of target subjects by health care professionals.

Of concerns, no participating LHUs gained VC objectives fixed by the Italian Ministry of Health, not only for the recent target represented by preadolescent boys but also for the more consolidated girls target. Furthermore, even if the PNPV 2017-2019 recommend the immunization of male who have sex with men and some regions correctly included among subjects at risk the HIV positive, the implementation of specific strategies is largely suboptimal. These observations are particularly serious from a public health perspective, given the high prevalence of HPV infection and the variety and burden of HPV-related diseases [1–4].

The strengths of our study are represented by the wide spectrum of organizational aspects and communication tools evaluated and the estimate of their role in determining the best outcome in terms of VC. In Italy, from 2010 to 2013 the study "local and evaluation of HPV immunization campaigns against HPV, VALORE" was coordinated by the Ministry of Health and by the *Istituto Superiore di Sanità* in order to improve the compliance to HPV vaccine and provide the regional and LHUs authorities the operational tools to increase VC [47]. Various organizational aspects and communications activities were investigated; nevertheless the boys did not represent the target of HPV vaccine during the study period and no multivariable analysis was conducted to consider diverse independent variables simultaneously.

The main limitations of the study are the difficulty in quantifying and exhaustively evaluating all organizational aspects of immunization centres and communication strategies and the number of participating LHUs that could compromise the representativeness of the national picture. Nevertheless, participating LHUs belong to four among the most populous Italian regions, with heterogeneous sociodemographic characteristics and healthcare systems. In particular, school-based programs were not investigating, even if robust evidences demonstrate their role in increasing the compliance to HPV vaccine. Nevertheless, the paucity of human resources in immunization centres reported in a wide proportion of participating LHUs limits the feasibility of this strategy.

5. Conclusions

In conclusion, our study demonstrated that the majority of Italian LHUs implemented proved actions (e.g., active free offer, invite letter, and recall of subjects who missed vaccine administration) concurrently with some HPV vaccine promotion and communication strategies directed to target subjects and healthcare workers. Nevertheless, VC observed in participating LHUs are largely lower than the national objectives in all HPV vaccine targets and organizational strategies to reach subjects at risk are suboptimal.

Since multicomponent interventions have a synergistic effect, both organizational and educational strategies have to be implemented to improve HPV VC.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

Giancarlo Icardi is the principal investigator of the national multicentre study. The research staff included the fixed-term assistant professor funded by Sanofi Pasteur MSD. The other authors have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Research Article

Duplex TaqMan Hydrolysis Probe-Based Molecular Assay for Simultaneous Detection and Differentiation of *Burkholderia pseudomallei* and *Leptospira* spp. DNA

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Melioidosis and leptospirosis, caused by two different bacteria, *Burkholderia pseudomallei* and *Leptospira* spp., are potentially fatal infections that share a very similar spectrum of clinical features and cause significant mortality and morbidity in humans and livestock. Early detection is important for better clinical consequences. To our knowledge, there is no diagnostic tool available to simultaneously detect and differentiate melioidosis and leptospirosis in humans and animals. In this study, we described a duplex TaqMan probe-based qPCR for the detection of *B. pseudomallei* and *Leptospira* spp. DNA. The performance of the assay was evaluated on 20 *B. pseudomallei* isolates, 23 *Leptospira* strains, and 39 other microorganisms, as well as two sets of serially diluted reference strains. The duplex qPCR assay was able to detect 0.02 pg (~ 4 copies) *Leptospira* spp. DNA and 0.2 pg (~ 25.6 copies) *B. pseudomallei* DNA. No undesired amplification was observed in other microorganisms. In conclusion, the duplex qPCR assay was sensitive and specific for the detection of *B. pseudomallei* & *Leptospira* spp. DNA and is suitable for further analytical and clinical evaluation.

1. Introduction

Burkholderia pseudomallei and *Leptospira* are two important infectious agents for melioidosis and leptospirosis, respectively [1–3]. The Gram-negative *B. pseudomallei* is recognized as CDC Tier 1 select agent and a Category B Priority Pathogen by the National Institute of Allergy and Infectious Diseases (NIAID), in addition to leptospirosis, which has been added to the Emerging Infectious Diseases category (https://www.niaid.nih.gov/research/emerging-infectious-diseases-pathogens). Both organisms are normally found in the soil and freshwater environment [4, 5].

In addition to their ubiquitous habitats, these organisms routinely infect animals, such as cattle, sheep, and horses. Certain animal classes such as rats may asymptomatically carry *Leptospira*. It is generally accepted that animals are responsible for shedding and maintenance of *Leptospira* and *B. pseudomallei* in the environments, through their urines and faeces [5–7]. Human cases are usually associated with interactions with the contaminated environments [6, 8]. To date, increasing cases of melioidosis and leptospirosis have been reported worldwide, especially in the tropical and subtropical regions [1, 6].

Infections by *B. pseudomallei* and *Leptospira* portray a very similar spectrum of nonspecific clinical presentations including fever, headache, myalgia, and pneumonia [4, 8]. In animals, *B. pseudomallei* infections cause pneumonia with lung abscesses, anorexia, and encephalitis [9]. Meanwhile, animal leptospirosis is characterized by abortion, jaundice, and infertility [10]. Several factors, such as bacterial load, underlying medical conditions, and serotypes increase hosts susceptibility to melioidosis and leptospirosis [1, 11–13]. Furthermore, the risk of dual infection is apparent, as several incidences of melioidosis-leptospirosis coinfections were reported previously [14, 15]. It is possible that many cases may be underdiagnosed when only one between the two tests is considered or available [16].

Early detection of melioidosis and leptospirosis could significantly increase the chances of survival and reduce potential economic loss [17]. Current gold standard for detecting *B. pseudomallei* is by the culture method which requires 2-7 days to grow [18]. Meanwhile, leptospiral antibody titer is detected by the microscopic agglutination test (MAT) that usually requires paired sera and is less useful during acute infection [5]. As both diagnostic methods are time-consuming, a more rapid laboratory assay is urgently needed. To date, several molecular assays have been described for detection of individual *B. pseudomallei* and *Leptospira* from the clinical specimens [5, 18]. However, to our knowledge, none of the reported assays is able to simultaneously detect and distinguish *B. pseudomallei* and *Leptospira* within the same reaction tube. In this study, we developed a duplex qPCR that can detect *B. pseudomallei* and *Leptospira* DNA and evaluated the assay on selected clinical and environmental isolates.

2. Materials and Methods

2.1. Microorganism Strains and Growth Conditions. A total of 20 *B. pseudomallei* strains, 23 *Leptospira* strains and 39 other microorganisms isolated from human clinical samples and ATCC strains were used in this study (Table 1). These microorganisms were provided by the Department of Medical Microbiology & Parasitology, School of Medical Sciences, Universiti Sains Malaysia; Makmal Kesihatan Awam Kota Bharu; Universiti Putra Malaysia; and Institute for Medical Research. The bacteria were cultured aerobically in nutrient broth overnight at 37°C on a rotating platform of 180 rpm. Meanwhile, *Leptospira* strains were maintained in EMJH media, incubated at 30°C on rotating platform of 40 rpm, overnight. *Entamoeba histolytica* DNA was obtained directly from School of Health Sciences, Universiti Sains Malaysia.

2.2. Isolation of Genomic DNA. DNA was extracted from pure bacterial culture using NucleoSpin® Tissue DNA Extraction kit (MACHEREY-NAGEL GmbH & Co. KG, Germany). The extraction procedure was carried out according to the manufacturer instructions with a minor modification on the final elution step, in which the column was incubated at room temperature for 10 minutes prior to centrifugation at 11 000 × *g*. Total DNA was quantified using the Eppendorf BioPhotometer (Eppendorf Scientific, Inc., New York, United States) and stored at -20°C until use.

2.3. Duplex Real-Time PCR Parameters. The PCR reaction was prepared in a total volume of 20 μL, containing 10 μL 2× SsoAdvanced™ Universal Probes Supermix, 1 μL PCR grade distilled water, 0.2 μM primers, 0.1 μM probes, and 8 μL DNA template. Sequences of oligonucleotides used are listed in Table 2. The oligonucleotides were designed for amplification of the *orf2* region of *B. pseudomallei* type III secretion system (T3SS) and the *rrs* gene of *Leptospira*.

Amplifications were conducted using Biorad CFX96 Touch Real-Time PCR Detection System. Thermal cycling condition included an initial denaturation at 95°C for 5 minutes, followed by 50 cycles of 95°C for 30 seconds and 61.3°C for 30 seconds. Baseline threshold for the postamplification analysis was set at 50 (for *B. pseudomallei*) and 25 (for *Leptospira*). Any Cq value ≤40 is considered positive. All the amplification in this study was carried out in triplicate, unless specified otherwise.

2.4. Analytical Sensitivity and Specificity. The analytical sensitivity of the assay was carried out using extracted *B. pseudomallei* and *L. interrogans* gDNA, diluted 10-fold ranging from 10 ng/uL to 1 fg/uL. Two microliters of each diluted gDNA were used in the duplex qPCR. Amount of bacterial DNA in each reaction was calculated based on a formula previously described by Aghamollaei *et al.* (2015) [21]. Meanwhile, the assay analytical specificity was determined using 2 μL extracted DNA from other organisms (non-*Leptospira* DNAs and non-*B. pseudomallei*), as listed in Table 1. DNA were extracted using NucleoSpin® Tissue extraction kit.

3. Results and Discussions

Despite the availability of several TaqMan hydrolysis probe-based assays for the detection of either *Leptospira* spp. or *B. pseudomallei*, none of the reported assays are able to simultaneously detect both organisms within the same reaction [18, 22]. Availability of such diagnostic tool that is able to detect and differentiate *B. pseudomallei* or *Leptospira* spp. is crucial as both infections portray similar clinical features and yet require different clinical management. In this study, a duplex qPCR for detection of *B. pseudomallei* and *Leptospira* spp. DNA was evaluated. As shown in Table 3, the developed qPCR was able to amplify 0.02 pg (~ 4 copies) *Leptospira* spp. DNA and 0.2 pg (~ 25.6 copies) *B. pseudomallei* DNA, respectively. The sensitivity of the duplex assay for detection of *Leptospira* DNA is comparable to other reported leptospiral probe-based assays that detected between 1 and 20 DNA

Table 1: List of organism used for analytical specificity test.

Organism	Source	No. tested (*n*)	Results in duplex qPCR
Aspergillus fumigatus	USM, Malaysia	1	Negative
Bacillus subtilis	USM, Malaysia	1	Negative
Burkholderia cepacia	USM, Malaysia	6	Negative
Burkholderia pseudomallei	USM, Malaysia	20	Positive
Burkholderia thailandensis	USM, Malaysia	1	Negative
Campylobacter jejuni	USM, Malaysia	1	Negative
Candida albicans	USM, Malaysia	1	Negative
Citrobacter freundii	USM, Malaysia	1	Negative
Entamoeba histolytica	UNAM, Mexico	1	Negative
Enterococcus faecalis	USM, Malaysia	1	Negative
Klebsiella pneumoniae	USM, Malaysia	1	Negative
Leptospira biflexa serovar Patoc	IMR, Malaysia	1	Positive
Leptospira biflexa serovar Patoc	UPM, Malaysia	1	Positive
Leptospira borgpetersenii Celledoni	IMR, Malaysia	1	Positive
Leptospira borgpetersenii serovar Ballum	UPM, Malaysia	1	Positive
Leptospira fainei serovar Hurtsbridge	IMR, Malaysia	1	Positive
Leptospira fainei serovar Hurtsbridge	UPM, Malaysia	1	Positive
Leptospira interrogans serovar Australis	UPM, Malaysia	1	Positive
Leptospira interrogans serovar Autumnalis	IMR, Malaysia	1	Positive
Leptospira interrogans serovar Bataviae	IMR, Malaysia	1	Positive
Leptospira interrogans serovar Bataviae	UPM, Malaysia	1	Positive
Leptospira interrogans serovar Canicola	UPM, Malaysia	1	Positive
Leptospira interrogans serovar Copenhageni	IMR, Malaysia	1	Positive
Leptospira interrogans serovar Hebdomadis	UPM, Malaysia	1	Positive
Leptospira interrogans serovar Icterohaemorrhagiae RGA	UPM, Malaysia	1	Positive
Leptospira interrogans serovar Javanica	IMR, Malaysia	1	Positive
Leptospira interrogans serovar Pomona	IMR, Malaysia	1	Positive
Leptospira interrogans serovar Pomona	UPM, Malaysia	1	Positive
Leptospira interrogans serovar Pyrogenes	IMR, Malaysia	1	Positive
Leptospira interrogans serovar Pyrogenes	UPM, Malaysia	1	Positive
Leptospira interrogans serovar Tarassovi	IMR, Malaysia	1	Positive
Leptospira licerasiae serovar Varillal	IMR, Malaysia	1	Positive
Leptospira meyeri serovar Semaranga	IMR, Malaysia	1	Positive
Leptospira wolffii	IMR, Malaysia	1	Positive
Plasmodium falciparum	MKA Kota Bharu	5	Negative
Plasmodium knowlesi	MKA Kota Bharu	5	Negative
Plasmodium vivax	MKA Kota Bharu	5	Negative
Proteus mirabilis	USM, Malaysia	1	Negative
Proteus vulgaris	USM, Malaysia	1	Negative
Salmonella Paratyphi A (ATCC 9150)	ATCC, USA	1	Negative
Salmonella Paratyphi B (ATCC BAA 1250)	ATCC, USA	1	Negative
Salmonella Paratyphi C (ATCC 9068)	ATCC, USA	1	Negative
Salmonella Typhi (ATCC 7251)	ATCC, USA	1	Negative
Salmonella Typhimurium (ATCC 14028)	ATCC, USA	1	Negative
Staphylococcus aureus	USM, Malaysia	1	Negative
Staphylococcus saprophyticus	USM, Malaysia	1	Negative

Table 2: List of primers and probes used in this study.

Target	Type	Sequence (5′ ⟶3′)	Source
Leptospira spp.	Forward primer	ACTGAGACACGGTCCATACT	[19]
	Reverse primer	TAGTTAGCYGGTGCTTTAGGYA	
	Probe	FAM-ACGGGAGGCAGC-ZEN-AGTTAAGAATCTTGC-IBFQ	
B. pseudomallei	Forward primer	CCTGGGAGAGCGAGATGTT	[20]
	Reverse primer	GCTGGATGAGAAGAAAGTCC	
	Probe	TexRed-CCACGCACGGCGGAGATTCT-IBRQ	

Table 3: Analytical sensitivity of the duplex qPCR assays.

Copies number	Amount (pg)	Duplex qPCR for *B. pseudomallei*			Copies number	Amount (pg)	Duplex qPCR for *Leptospira* spp.		
		Mean Cq	SD	CV (%)			Mean Cq	SD	CV (%)
2560000	20000	18.41	0.05	0.26	4000000	20000	19.3	0.17	0.86
256000	2000	21.75	0.31	1.43	400000	2000	23.09	0.16	0.67
25600	200	25.34	0.12	0.46	40000	200	27.23	0.04	0.16
2560	20	28.97	0.13	0.44	4000	20	30.67	0.32	1.05
256	2	32.8	0.14	0.44	400	2	34.63	0.08	0.24
25.6	0.2	36.96	1.06	2.88	40	0.2	36.34	0.5	1.37
2.56	0.02	-	-	-	4	0.02	38.59	1.49	3.87
0.256	0.002	-	-	-	0.4	0.002	-	-	-

Table 4: PCR efficiency and linearity of the duplex qPCR assays.

Parameter	Target	
	Leptospira spp.	*B. pseudomallei*
Slope	-3.2776	-3.7006
Efficiency	101.9%	86.3%
Linearity, R^2	0.9837	0.9987

copies per reaction [23–25]. Meanwhile, for the *B. pseudomallei* detection, the sensitivity was slightly lower than the previously reported assays that amplified 5 and 10 DNA copies per reaction [26–28]. In comparison to the corresponding monoplex assay, the duplex assay had comparable sensitivity for *Leptospira*, but had a reduced sensitivity for *B. pseudomallei* target (0.2 pg in duplex *versus* 0.02 pg in monoplex). Reduced performance of multiplex assay as compared to the monoplex assay has been observed in other molecular studies which are associated with primers competition, primer cross hybridization, and template mispriming [29, 30]. When tested on other microorganisms, no cross amplification was observed (Table 1). The *orf2* region is selected because it is only present in *B. pseudomallei* [31]. Meanwhile, for the leptospiral target, the *rrs* gene is used because the gene is present in multiple copies per *Leptospira* genome [32]. As the current panel included limited coverage of organisms, further validation should include *Burkholderia mallei* and other *Burkholderia cepacia* complex (BCC).

As listed in Table 4, the duplex assay had an efficiency of 101.9% for the detection of *Leptospira* DNA, comparable to the monoplex assay (100.5%). However, for the detection of *B. pseudomallei* DNA, the duplex assay had an efficiency of 86.3%, lower than the monoplex assay (95.9%). The suboptimal efficiency may be attributed to the decreased sensitivity of the assay on *B. pseudomallei* target. In an ideal condition, PCR efficiency should be 90% and above [33]. Further optimization is necessary in order to increase the assay efficiency, especially for the *B. pseudomallei* target. Meanwhile, in terms of linearity, the duplex assays (for *Leptospira* and *B. pseudomallei* DNA detection) had R^2 values of close to 1. Noticeably, at low copy number, the CV values ranged between 2.8 and 3.8% (Table 3).

Overall, the establishment of a duplex qPCR assay that can detect and differentiate *B. pseudomallei* and *Leptospira* spp. may help the diagnosis of melioidosis and leptospirosis. However, prior to clinical evaluation, further analytical validation, such as intra- and interassay variation, a wider spectrum of microorganisms for specificity testing, higher number of replicates, and optimization of assays are necessary. In addition, incorporation of internal amplification control should be considered because certain types of clinical samples such as whole blood and urine may cause PCR inhibition.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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Supplementary Materials

The standard curves of the duplex qPCR assays for the detection of *B. pseudomallei* DNA and *Leptospira* spp. DNA are illustrated in Figure S1 in Supplementary Material. *(Supplementary Materials)*

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Research Article

Parents' Attitude about Hepatitis B Disease and Practice of Hepatitis B Vaccination among Children in Ho Chi Minh City, Vietnam

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Introduction. The Expanded Program on Immunization (EPI) in Vietnam for hepatitis B (HepB) among infants has been implemented since 2003. The rates of the birth dose (babies receiving HepB immunization injection within 24 hours after birth) and the later three-dose series were low in 2013-2014. *Objective*. This article evaluated attitudes about the hepatitis B disease and vaccine in relation to the correct practice of vaccination among mothers of 12–24-month-old children in Ho Chi Minh City. *Material and Methods*. The parents of 768 children aged 12 to 24 months, in Ho Chi Minh City, were interviewed and reviewed their vaccination cards from February 2016 to July 2017. *Results*. A total of 768 children had parents of a mean age of 30.8 years, approximately 34% of the children with a mean age of 16.8 months completed all four doses of the hepatitis B vaccine in a timely manner according to the EPI, and only 45.2% of children received the birth dose on schedule within 24 hours. The mother's fears of HepB risk in the community, living in rural areas, and receiving vaccination information from health workers increased the odds of complete and timely HepB vaccination (all $p<0.05$). *Conclusions*. A high rate of children did not receive a complete and timely HepB vaccination in the EPI. Health information strategies should be designed to target urban people and focus on safety of the vaccine, by health workers, to increase the correct practices of hepatitis B vaccination, including the birth dose, and provide education programs that emphasize the high risk for getting hepatitis B.

1. Introduction

Hepatitis B virus (HBV) infection is a major global health problem. The World Health Organization (WHO) in 2017 estimated that 325 million people worldwide are living with chronic HBV [1]. In a study among all Asian regions, East Asia (including Vietnam) had the highest prevalence of HBV infection with little change between 1990 and 2005. Generally, endemicity remains high or close to high in this region [2]. Vietnam has high endemic hepatitis B virus (HBV) infection, with 8.6 million people being identified as hepatitis B-positive. An estimated 8.8% of women and 12.3% of men are chronically infected with hepatitis B and the main mode of transmission of HBV in Vietnam is from mother to child (MTCT) during childbirth or early childhood [3]. A survey of unvaccinated children in Thanh Hoa province in 2003 found that current infection (HBsAg+) rates were 12.5% of infants and 18.4% children [4], but a national survey in 2014 showed that the overall prevalence of HBsAg among 6,949 children was 2.70%, and HBsAg prevalence was significantly higher among children born in 2000–2003 (3.64%) compared to children born in 2007–2008 (1.64%), in which HBsAg prevalence among children with ≥3 doses of hepatitis B vaccine including a birth dose (1.75%) was significantly lower than among children with ≥3 doses of hepatitis B (HepB) vaccine but lacked a birth dose (2.98%) and significantly lower than among unvaccinated children (3.47%) [5]. Based on these results, we found the effect of the HepB vaccine for fully

immunized infants, compared with nonimmunized infants. The same result was among Chinese children aged 0-9 years, the incidence rate decreasing from 15.86/100,000 in 2004 to 6.36/100,000 in 2010, showed that the prevalence decreased after performing the EPI [6]. The EPI in Vietnam for hepatitis B among infants has been implemented since 2003 [3]. The hepatitis B vaccination schedule was a monovalent HepB vaccine birth dose which was recommended to be given within 24 hours after birth from 2005 [7], and the three-dose series was given as part of a pentavalent DPT-Hib-hepatitis B vaccine (commonly known as QUINVAXEM) scheduled at ages 2 months, 3 months, and 4 months [8, 9]. Coverage of the three-dose series was over 90% between 2011-2014, except in 2013 due to adverse events following immunization (AEFIs) with QUINVAXEM, where the rate dropped significantly to 59% [10]. The birth dose coverage rapidly attained approximately 60% within 2 years after its introduction in 2003 and increased from 65% in 2006 to 75% in 2012. However, the birth dose coverage declined to 55% in 2013 and 2014, following media reports of alleged AEFIs associated with the HepB birth dose administration [11, 12], and slightly increased to nearly 70% in 2015-2016 [13]. Several AEFIs occurred involving both the hepatitis B monovalent vaccine used for the birth dose and the pentavalent vaccine used for the 3-dose series (Quinvaxem) in 2013. These events can cause widespread fears over vaccine safety and reduce the rate of vaccination for children [14]. Parents' decisions to delay or refuse vaccines have been shown to be associated with perceived risks for the HBV infection and safety of the vaccine [15, 16]. For the vaccine program to be effective, the procedures must be acceptable to the parents and they must have a strong belief in its effectiveness to reduce the spread of the disease. We used the Health Belief Model (HBM) (Rosenstock 1966 và Becker 1974) as a theoretical framework to evaluate influences of attitudes about hepatitis B disease and vaccine on the correct practice of vaccination among mothers of 12–24-month-old children in Ho Chi Minh City. The HBM was used to interpret differences in compliant and noncompliant parents with regard to childhood vaccinations. It has been used throughout public health to help explain why people adopt behaviors that lead to healthy lives. The five elements of the HBM include (1) perceived susceptibility to HBV infection (likelihood of getting the disease), (2) perceived severity of HBV infection (perception of how serious an outcome or consequence is from the disease), (3) perceived benefits (efficacy of preventive action undertaken), (4) barriers of vaccination (time, effort, inconvenience, pain, and side effects), and (5) cues to action (information to decide the vaccination) [17, 18].

2. Subjects and Methods

2.1. Research Design. The study was conducted using a cross-sectional survey.

2.2. Research Subjects. A total of 768 eligible fathers, mothers, or caregivers and their children between 12-24 months attended 16 community health centers (CHCs), between February 2016 and July 2017. These CHCs were selected by a simple random approach from 24 Dists in Ho Chi Minh City, then choosing a convenient sample of 48 fathers, mothers, or caregivers and their children from each of the 16 CHCs.

2.3. Inclusion Criteria. Research subjects included fathers, mothers, or caregivers and their children aged 12-24 months and a consensus that was approved by their fathers, mothers, or caregivers.

2.4. Exclusion Criteria. Parents who did not directly take care of their children were excluded; parents/caregivers and their children were excluded from this study if they did not have a vaccination card.

2.5. Data Collection Procedures. A structural questionnaire included three sections. The first section was baseline characteristics of parents/caregivers such as age, gender, residence location, occupation, education, income, members in the household, having infected friends with HBV, attending a health education session on HBV and information of HepB vaccine, and baseline characteristics of children included age, gender, and status of hepatitis B vaccination. The second section assessed parents/caregivers' attitude about the hepatitis B disease and vaccination; a questionnaire included the fifth components of the HBM, combined with Bigham's questionnaires, which was tested for validity and keywords from our preliminary qualitative study [19, 20]. The third section assessed the practice of hepatitis B vaccination among fathers/mothers/caregivers based on vaccination records of their children. The instrument was pretested and subjected to an assessment of construct validity by five experts on immunization in the EPI and public health fields.

The fathers, mothers, or caregivers were interviewed when they took their children aged 12–24 months to the CHCs. Participants were assured that the data collected would remain anonymous.

2.6. Variable Definitions. Questions were designed so that each response choice represented an answer a respondent might give if asked the question [21]. In evaluation of attitude about hepatitis B disease and vaccination, for each sampled father/mother/caregiver, we defined the correct attitude when they answered "strongly agree" or "agree" for their attitude about susceptibility to HBV infection, severity to HBV infection, benefits and barriers, and cues to action. Parents were also asked to respond to questions relating to side effects of the vaccine with answers to slight side effects, moderate side effects and serious side effects having a "Yes" response. With regard to the evaluation of practices of hepatitis B vaccination, for each sampled child, we evaluated the vaccination status based on their vaccination records. The binary dependent variables were "timely vaccination" for the hepatitis B vaccine. This was defined as a child received hepatitis B vaccination doses within the schedule proposed by the WHO [22] and recommended by the EPI in Vietnam [9], which was a birth dose and the three-dose series for children aged 2 months (59–88 days old), 3 months (89–118 days old), and 4 months (119–148 days old). In order to measure these, the "time span" for the given vaccination (date of birth

subtracted from the date of immunization) was calculated, and timely vaccination was duely concluded if the "time span" fell within the recommended schedule or zero (otherwise). Complete vaccination was defined as a child received full 4 doses of hepatitis B vaccine. Finally, the correct practice was defined if a child was vaccinated both complete and timely hepatitis B vaccine.

2.7. Data Analysis. All our estimates were computed using Stata13 and Epidata 3.0 software. Continuous variables were estimated as mean (standard deviation) and discrete variables as frequency and percentage. Comparisons of estimates between 2 groups were performed using the t-test for continuous variables and Chi-square or Fisher's exact test for discrete variables. Multivariate analysis for the binary variable as the practice of correct hepatitis B vaccination was performed using logistic regression with selected variables with significant levels <0.20 in the binary analysis. Statistical p value was defined as <0.05.

2.8. Ethical Approval. All subjects agreed and gave informed consent before taking part in the study. This study was approved by the Ethics Council, University of Medicine and Pharmacy at Ho Chi Minh City, Vietnam (protocol number 125/UMP-BOARD).

3. Results

3.1. Baseline Characteristics of Parents and Children. A total of 768 parents recruited in the study were mainly in the age group from 25 to 40 years old (85.0%), public workers (35.1%), in high school level education (55.0%), and living in urban areas (73.7%), with only 11.7% parents attending a health education session on hepatitis B. Their children had a mean age of 16.8 months, 51.7% boys, among 65.9% received full 4 doses of the HBV vaccine; only 45.2% of infants received the birth dose on schedule in 24 hours and 33.9% of them got the full and timely vaccination (Table 1).

3.2. The Association between HepB Vaccination Status of Children and Baseline Characteristics of Parents and Children. As depicted in Table 2, baseline characteristics of parents/guardians and children receiving both full and timely vaccination were similar to those not getting the full or timely vaccination.

However, children who were living in the rural areas were receiving full and timely vaccinations at a higher rate than children who were living in the urban areas (56.9% versus 25.5%, p<0.001). Children who were living with parents were vaccinated fully and on schedule at a higher rate than those who were living with grandparents (37.0% versus 29.3%, p<0.05) and those whose parent heard about HBV information were vaccinated fully and on schedule at higher rate compared with those whose parents did not (35.1% versus 19.0%, p<0.05) (Table 2).

3.3. The Association between HepB Vaccination Status of Children and Parents' Attitude about Hepatitis B Disease. The questionnaire included fourteen items about attitude, three items about prior contact, and two items to estimate vaccination status. Results of responses were summarized in Table 3. We found a statistically significant relationship between the status of hepatitis B vaccination and parent/caregivers' attitude to susceptibility to HepB disease, vaccination information from health workers, and observing serious side effects caused by vaccines (all p<0.05) (Table 3).

Table 4 summarizes results of the logistic regression model of factors associated with getting full and timely HepB vaccine to infants. When adjusted for all other model variables, there was a 2.14-fold increase in the odds of HBV correct immunization for a 1-unit increase in "rural location" (AOR 2.14, 95% CI: 1.77 – 2.59, p<0.001), and there was a 1.24–fold increase in the odds of correct immunization for a 1-unit increase in "thought that their child is at high risk for HBV" (AOR 1.24, 95% CI: 1.01–1.53, p<0.05). In addition, children's parents/caregivers received vaccination information from health workers; their children had a higher rate of full and timely vaccination (AOR 1.31, 95% CI: 1.10 – 1.58, p<0.05).

4. Discussion

4.1. Low Rate of Full and Timely HepB Vaccination. The results of the study in Ho Chi Minh City found that 65.9% of the 768 children had received four doses of the HepB vaccine, but only 33.9% of children had completed all four doses of the HepB vaccine on schedule and 45.2% received the birth dose in 24 hours according to the EPI. This also indicated that timely birth dose coverage was low. It was similar to Dao Thi Minh An' study on children under five in Vietnam had "timely immunization completion", among seven vaccines used in the EPI in 2000, 2006, and 2011; hepatitis B dose 1 had the lowest at 17.5%, 19.3%, and 45.5%, respectively [23]. However, it was lower than those in a national survey reported in 2014 and 2016 in Vietnam, the rate of receiving the birth dose with 55.0% and almost 70%, respectively [13]. This could be explained due to the study being conducted in 2016-2017 and included children aged 12-24 months old. Therefore, it correlated with Hep B vaccine coverage in 2014-2015. Therefore, the period of our study was close to the period of the AEFIs in 2013 and the drop in hepB vaccination was consistent.

4.2. Factors Associated with the Full and Timely Hepatitis B Vaccination. Our study showed parents in rural areas practiced correct immunization for their children at a higher rate those parents in urban areas (AOR 2.14, 95% CI: 1.77 – 2.59, p<0.001) (Table 4). A similar finding was obtained in a study by Smith (2009), children whose parents neither delayed nor refused vaccination were 1.5 times more likely to live in rural areas (OR 1.5, 95% CI: 1.0 – 2.4, p<0.05) [24]. In addition, parents who received vaccination information from health workers had a higher rate of correct HBV vaccination than those who did not receive it (AOR 1.31, 95% CI: 1.10 – 1.58, p<0.05). This result was also similar to Bigham's study that HBV immunization was significantly associated (p<0.001) with a recommendation

Table 1: Baseline characteristics of parents/caregivers and children (n=768).

Baseline characteristics of parents	N(%)
Gender (Female)	621(81.0)
Residence location (n=767)	
Urban	565(73.7)
Rural	202(26.3)
Education	
< Primary school	116(15.1)
Secondary school	229(29.9)
> High school	422(55.0)
Occupation	
Government officer/Staff	142(18.5)
Housewife	265(34.6)
Seller/ Retail	70(11.7)
Worker	270(35.2)
Age (years) (M ± SD)	30.8 ± 5.1
<25	68(8.9)
25- 40	652(85.0)
≥40	47(6.1)
Gross household income (n=727)	
Poor, near-poor	36(4.9)
Moderate	691(95.1)
Number of children in the household	
1	375(48.9)
2	318(41.5)
≥ 3	74(9.6)
Members in the household	
Parents	314(40.9)
Grand-parents	454(59.1)
Having infected friends with HBV	138(17.9)
Attended a health education session on HBV	90(11.7)
Having information about HBV	710(92.5)
Baseline characteristics of children	
Gender (Male)	397(51.7)
Age (months) (M ± SD)	16.8 ± 4.2
Status of HepB vaccination	
Birth dose vaccination	347(45.2)
Full dose vaccination	506(65.9)
Full and timely vaccination	260(33.9)

for HB immunization from a healthcare professional [19]. Therefore, parents in rural areas were more likely to follow the advice of health commune staff and consequently; their children had a higher rate of correct vaccination over children with parents in urban areas. Health information strategies should be focusing on the safety of the vaccinations and this needs to be delivered by health commune staff to increase the rate of HepB correct vaccination, including the birth dose. Although media influence played a major role in the drop in HepB vaccination in Vietnam, our study did not find a statistically significant difference between groups with and without receiving information via mass media. Our results found a statistically significant relationship between the attitude of parents/caregivers who thought that their children could get sick if they were not vaccinated and the rate of complete and timely HepB vaccination. Parents of the full and timely HBV immunized children had a more active attitude towards high risk for HBV infection than parents of children who did not receive full and timely immunization (AOR 1.24, 95% CI: 1.01–1.53, $p<0.05$) (Table 4). Yousafzai et al. (2014) also showed a statistically significant relationship between attitudes of HepB disease threats of medical staff and the practices of HepB vaccination. For example, perceived disease threats after exposure to blood and body fluids was a significant predictor of complete HepB vaccination [25]. Smith's study (2009) had similar results showing that parents

Table 2: The association between HepB vaccination of children and baseline characteristics of parents and children (n=768).

	Full and timely HepB vaccination		p –value*
	Yes (260)	No (508)	
Baseline characteristics of parent			
Gender of parents/ caregivers			
Male	48(18.5)	98(19.3)	0.80
Female	211(81.5)	410(80.7)	
Residence location			
Urban	144(55.6)	421(82.9)	*<0.001*
Rural	115(44.4)	87(17.1)	
Education			
< Primary school	43(16.6)	73(14.4)	0.706
Secondary school	77(29.7)	152(29.9)	
> High school	139(53.7)	283(55.7)	
Occupation			
Government officer/Staff	55(22.1)	87(17.8)	0.534
Housewife	88(35.3)	176(36.1)	
Seller/ Retail	30(12.1)	59(12.1)	
Worker	76(30.5)	166(34.0)	
Age (years)			
<25	24(9.3)	44(8.7)	0.960
25- 40	219(84.5)	433(85.2)	
≥40	16(6.2)	31(6.1)	
Gross household income			
Poor, near-poor households	8(3.2)	28(5.9)	0.111
Moderate	243(96.8)	448(94.1)	
Number of Children in household			
1	119(45.9)	256(50.4)	0.470
2	115(44.4)	203(40.0)	
≥ 3	25(9.7)	49(9.6)	
Having infected friends with HBV (*Yes*)	51(19.6)	87(17.2)	0.402
Attended a health education session on HBV (*Yes*)	34(13.1)	56(11.1)	0.408
Members in the household			
Parents	168(64.9)	286(56.3)	*0.022*
Grand-parents	91(35.1)	222(43.7)	
Having information about HBV (*Yes*)	249(95.8)	461(90.8)	*0.013*
Baseline characteristics of children			
Gender			
Male	127(48.9)	270(53.2)	0.259
Female	133(51.1)	238(46.8)	
Age (Mean ± SD)	17.0 ± 4.3	16.6 ± 4.1	0.352**

*Chi-square and **t-test used to compare with and without getting full and timely HepB vaccination groups, excluding missing data.

who delayed and refused vaccines were significantly less likely to believe that their child might get a disease (71.0% versus 90.0%), and their children also had significantly lower vaccination coverage [24]. Therefore, health workers need to inform everyone that all people were susceptible to HBV and their children were also at high risk of getting the disease and need to be fully vaccinated.

5. Study Limitations

This study has limitations that should be considered when unvaccinated children were not included in our sample. Therefore, the findings may not be generalizable to all parents with children from 12-24 months of age living in the region. There is also the possibility of social desirability bias; however, interviewers encouraged parents to express their opinions freely. Future studies could be conducted on unvaccinated children.

6. Conclusions

The study showed that a high number of children did not receive a full and timely HepB vaccination in the EPI. Health information strategies should be designed to target urban people and focus on the safety of the vaccine. This message needs to be delivered by health workers to increase the rate of the full and timely hepatitis B vaccination, including the birth dose, and provide education programs that emphasize the high risk of getting hepatitis B in the community, including urban areas.

Table 3: Percentages of respondents answering, "strongly agree" or "agree" to Health Behaviour Conceptual Questions and "yes" to prior contact questions, stratified by HepB Vaccination Status of Children (n=768).

Concepts	Full and timely vaccination		p -value*
	Yes (n=260)	(n=508)	
Susceptibility to HepB			
My child is at high risk for HepB	156 (60.0)	233 (45.9)	*<0.001*
I think my child will get HepB in future	218 (83.8)	384 (75.6)	*0.009*
Severity of HepB			
HBV is a serious disease	249 (95.8)	477 (93.9)	0.280
My child could be severity if s/he got HepB	209 (80.4)	429 (84.5)	0.155
I'm afraid to even think about my child getting sick with HBV	227 (87.3)	445 (87.6)	0.908
Benefits and barriers of vaccination			
Immunization will prevent HBV	244 (93.9)	484 (95.3)	0.399
By getting HepB vaccine, child will not spread HBV to others	189 (72.7)	379 (74.6)	0.567
Need to be HepB vaccination on schedule	228 (87.7)	463 (91.1)	0.132
The HepB shot can be painful	215 (82.7)	418 (82.3)	0.888
Vaccination can cause AEFIs	315 (62.01)	177 (68.08)	0,097
It is a convenient time to take my child in for vaccines	215 (82.7)	406 (79.9)	0.356
It is a convenient location to take my child in for vaccines	217 (83.5)	420 (82.7)	0.784
Cues to action			
Vaccination information from health workers	109 (41.29)	166 (32.68)	*0.011*
Vaccination information from mass media	117 (45)	200 (39.37)	0.134
Prior Contact (n=667) (yes) *missing 101*			
Observed slightly side effect	89 (34.2)	187 (36.8)	0.481
Observed serious side effect	25 (9.6)	24 (4.7)	*0.009*
My child had side effect after vaccination	44 (27.3)	151 (29.8)	0.542

∗ Chi-square tests used to the comparison between with and without getting the full and timely hepatitis B vaccine groups, excluding missing data.

Table 4: Results of logistic regression factors associated with the full and timely hepatitis B vaccination.

	OR	AOR	95% CI	p-value
Residence location (rural)	2.23	2.14	1.77 – 2.59	*<0.001*
Vaccination information from health workers	1.29	1.31	1.10 – 1.58	*<0.05*
My child is at high risk for hepatitis B	1.46	1.24	1.01 – 1.53	*<0.05*
I think my child will get HBV in future	1.43	1.24	0.94 – 1.64	0.12
Observed serious side effect	1.56	1.25	0.92 – 1.69	0.12

Data Availability

The primary data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

This work was carried out in collaboration between all authors. Huynh Giao, Pham Le An, and Bui Quang Vinh designed the study, was responsible for acquisition of the data, wrote the protocol, and wrote the first draft of the manuscript. Huynh Giao and Nguyen Huynh Tam Lang were the contributors to the analysis and interpretation of the data. All authors read and approved the final manuscript.

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Research Article

Knowledge, Preference, and Willingness to Pay for Hepatitis B Vaccination Services among Woman of Reproductive Age in Vietnam

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Background. Hepatitis B virus (HBV) vaccine is a critical approach to prevent HBV transmission from mother to child. However, despite high HBV prevalence, evidence about the preference of women of productive age for HBV vaccine in Vietnam was constrained. This study aims to explore the preference and willingness to pay (WTP) for the HBV vaccine in Vietnamese women in productive age. *Methods*. A cross-sectional study was conducted in Hanoi in April 2016. A structured questionnaire was used to collect information about respondents' socioeconomic status and knowledge about HBV vaccination. A contingent valuation approach was employed to measure the WTP for the HBV vaccine. Logistic and interval regressions were used to determine the associated factors. *Results*. Among 807 women, 80.8% were willing to have the vaccine injected which had the average price of 108,600 VND (95% CI, 97,580 VND–119,570 VND). Participants not suffering any diseases during pregnancy were more likely to be willing to pay for the HBV vaccine (OR = 3.41, 95% CI = 1.73–6.70). Not having the antenatal examination at central hospitals and working as farmers/workers were positively correlated with willingness to pay for this vaccine, while the number of children of respondents had a negative correlation with WTP. *Conclusions*. Our sampled women expressed a high willingness to pay for the vaccine. The price people were willing to pay for the vaccine, however, is equal to half of the actual price. These findings implied needs for better targeted public education interventions about HBV and the involvement of local medical staffs and the media in providing information. Efforts to reduce the price of the vaccine should also be warranted for scaling-up the coverage of this vaccine.

1. Introduction

Hepatitis B (HBV) is a viral infection that has been well-recognized as a serious global health concern [1]. The World Health Organization (WHO) estimated its death tolls to be about 887,000 per year, while roughly 257 million people worldwide are currently disease carriers [2]. Vietnam is an HBV epidemic country as the prevalence of positive HB surface antigen (HbsAg+) in two metropolitans ranges from 9 to 14% [3]. A recent study forecasted that the number of chronic HBV cases in Vietnam will amount to 8 million by 2025 with HBV–related annual mortality reaching 40,000 [4].

One of the major HBV transmission routes is vertical transmission or mother-to-child [5]. In countries where HBV is an endemic problem, it was estimated that 50% of the HBV positive cases got their infections from either perinatal or early childhood; the chance of such infection developing into chronic Hepatitis B was 70% to 90% [6, 7]. Even when most young children were sufficiently HBV vaccinated, the mother-to-child transmission still resulted in a 40% to 50% of the new HBV positive cases [5]. Thus, effective prevention of this vertical HBV transmission, especially through treating the potentially infected mothers and would-be mothers, can be said to be crucial in decreasing the burden of HBV.

Table 1: Study settings and sample size.

Level	Settings	Total woman fitting the research criteria	Sample size
District (rural)	Phu Son Commune	220	207
	Thuy An Commune	200	200
District (urban)	Trung Tu Commune	465	200
	Phuong Lien Commune	410	200

Current vaccines were found to be remarkably effective against chronic HBV infection with the rate of prevention ranging from 94 to 98% [8]. However, limited success has been reported for the vaccination program targeting women of reproductive ages, due to a number of constraints [9]. Some studies have showed that insufficient attendance of antenatal care and poor knowledge on vaccinating pregnant women would have adverse impact on the efficiency of maternal health care providers in developing countries, while others pointed to a shortage of practical knowledge about immunization programs in younger, poorly educated, and illiterate mothers [10, 11]. Access barriers to the vaccination program and a lack of adherence to standard infection control precautions have also been possible causes [12].

Despite high HBV prevalence, researches looking into HBV prevention in women of productive age in Vietnam have been scarce. This study attempts to partially fill this gap in the literature, exploring several aspects of HBV vaccination in Vietnamese mothers: their awareness of HBV vaccine, willingness to be vaccinated and to communicate about vaccination, and potential influencing factors, employing a contingent valuation approach.

2. Material and Methods

2.1. Survey Design and Sampling Procedure. A cross-sectional study was conducted in two districts of Hanoi, including the Dong Da district and Ba Vi district from April 1 to April 30, 2016. Two communes in each district were selected for the survey. In Dong Da, Trung Tu commune and Phuong Lien commune were selected. In Ba Vi, Thuy An commune and Phong Van commune were selected. The subjects of the survey were women living in the selected site. The eligibility criteria also included (1) being pregnant or having children under 1 year of age; (2) living at research site for at least 6 months; (3) agreeing to join the survey; (4) not suffering from HBV disease before.

A formula to estimate a population proportion with specified absolute precision was used to calculate the sample size. With the expected proportion of women being willing to pay for HBV vaccine = 0.5 (for maximizing the sample size) and absolute precision d = 0.07, the sample for each commune was 196 women. We added 10% for compensation rate; the final sample size per commune was 216 women. We listed all women who met our inclusion criteria in each commune with the support of local authorities. Then, we randomly selected participants using computer software. "If one individual did not accept to participate, we selected the one who was next to them in the list. Detailed information was presented in Table 1.

2.2. Measures and Instruments. We conducted face-to-face interviews by well-trained staffs and students from Ba Vi district health center and Hanoi Medical University. A structured questionnaire was used to gather data about respondents' socioeconomic status and knowledge about and willingness to pay for Hepatitis B vaccination.

2.2.1. Socioeconomic Information and Self-Rated Health. Data about age, pregnancy status, occupation, education, internet usage, and health facilities in which the antenatal examination took place and average income were self-reported. Self-rated health status was also collected by asking participants to rate their health in four categories: "Very good," "Good,","Normal," and "Weak."

2.2.2. Source of Vaccine Information. The indicators to measure the source of vaccine information were as follows: information resources, the types of vaccine information that respondents wanted to know, preference on the channel of communication about vaccination, and the awareness of Hepatitis B vaccine price.

2.2.3. Willingness to Have Hepatitis B Vaccine Injected. To elicit willingness to have Hepatitis B vaccine injected, double-bounded dichotomous-choice questions were utilized to ask the respondents whether they were willing to be vaccinated. In this study, 200,000 VND (~ US$ 9, 2016 exchange rate) was used as a starting bid based on the actual price of this vaccine in the clinics. Firstly, they were asked whether they were willing to pay 200,000 VND per vaccine injection, and then they would move to the double bid for "Yes" response and the half bid for "No" response. Finally, the respondents had to answer an open-ended question about the maximum price they would be willing to pay to have Hepatitis B vaccine injected. The bidding process was described in Figure 1.

2.3. Statistical Analysis. The descriptive statistical analysis was used to describe the sociodemographic characteristics and knowledge about HBV vaccine of respondents (including information resources, the types of vaccine information that respondents wanted to know about, and preference on the channel of communication about vaccination). The significance level was set at $p < 0.05$. Logistic regression was performed to determine the factors related to willingness to

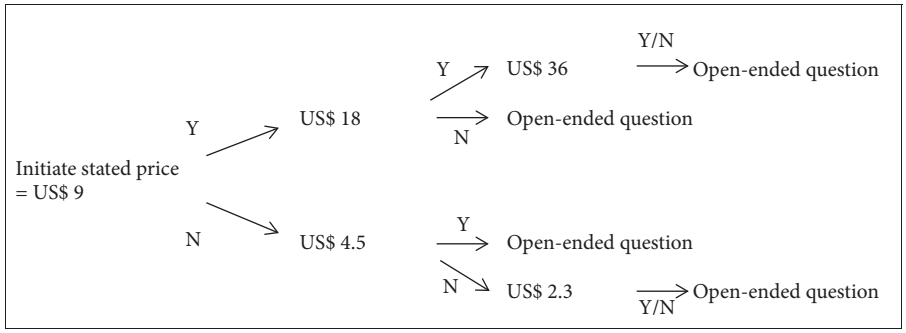

Figure 1: Bidding process.

get vaccinated and communicate about Hepatitis B vaccination, combined with a backward stepwise selection strategy. Interval regression was used to measure the amount of WTP.

2.4. Ethical Approval. The study protocol was approved by the Institutional Review Board (IRB) of Hanoi Medical University. The data collection at study sites was approved and supported by Dong Da and Ba Vi District Health Centre. Written informed consent was obtained from all participants after clearly introducing the survey. Respondents were informed that they could refuse to participate or withdraw from the study at any time they want.

3. Results

Majority of participants were having a baby under 1 year of age (76%), over 25 years old (82.6%), having finished high school or higher education (82.7%), working as workers/farmers/public servants (61.8%), and reportedly in good health (64.2%). Private/international hospital was the most popular place of choice among respondents for having antenatal examination (35.3%), followed by central hospital (28.9%). The average number of the antenatal examination was 8 times (Table 2).

91.5% of respondents have heard about Hepatitis B vaccine, mostly via television (55.6%) and from medical staffs (55.5%). Most of the participants wanted to communicate about the vaccine (89.7%), with three most commonly requested topics of information being benefits of vaccine (64.8%), schedules of vaccination (37.5%), and consequences of nonvaccination (32.8%). A most preferred channel of communication was the health worker's advice (29.3%). 87.3% of those interviewed were not aware of the vaccine price (Table 3).

Among participants, 80.8% were willing to have the vaccine injected which had the average price of 108,600 VND (SD=142,700). Willingness to vaccinate was highest in farmers (93.2% of them would accept vaccination), people under 25 years old (90.3%), those with an education level of high school and lower (83.0%-88.9%), and those without health insurance (82.8%). However, willingness to pay was the lowest in these exact subgroups, with willingness to pay the average prices of 79,000 VND; 87,000 VND; 73,100–100,700

Table 2: Demographics of respondents.

Characteristics	*N*	%
Pregnancy status		
Pregnant	190	24.0
Having a baby under 1 year of age	602	76.0
Age group		
Under 25 years old	138	17.5
From 25 to 30 years old	374	47.3
Above 30 years old	279	35.3
Education attainment		
Lower Secondary school	137	17.3
High school	230	29.2
College	178	22.6
University and above	244	30.9
Occupation		
Homemaker	108	13.7
Farmer/ Workers	267	33.8
Public servants	221	28.0
Others	194	24.6
Facilities where having an antenatal examination		
Commune/ward medical station	174	21.9
District/province hospital	197	24.8
Central hospital	229	28.9
Private/International hospital	280	35.3
Others	15	1.9
No Answer	76	9.6
Current health status		
Very good	31	4.4
Good	455	64.2
Normal	244	34.4
Weak	10	1.4
Suffered from disease during pregnancy	58	7.4
	Mean	SD
The number of antenatal examination	8.0	4.7
Average Income per month (mil VND)	12.8	9.3
The number of children of the respondent	1.7	0.8

Table 3: The knowledge about HBV vaccine of respondents.

	N	%
Heard about Hepatitis B vaccine	*720*	*91.5*
Information resources		
At school	40	5.0
Via television	441	55.6
Via listening radio	160	20.2
By reading a newspaper, magazines	174	21.9
Internet	323	40.7
Medical staff	440	55.5
Friends, relatives	177	22.3
Communication about vaccine	*695*	*89.7*
The information of the vaccine they want to know		
Benefits of vaccine	514	64.8
Schedules of vaccine	297	37.5
Vaccine location	158	19.9
Consequences on non-vaccination	260	32.8
Free-of-charge vaccines and location	145	18.3
The price of vaccines	217	27.4
The type of vaccines	226	28.5
Other	33	4.2
Preference on a channel of communication about vaccination		
TVs	116	14.9
Radios	73	9.4
Newspaper/magazine	16	2.1
Posters/ Leaflets	26	3.3
Cell phone	44	5.7
Health worker's advice	228	29.3
A guideline in the vaccination booklet	29	3.7
Direct talks	98	12.6
Integrate with local meetings	9	1.2
Internet	112	14.4
Other	11	1.4
Know the price of hepatitis B vaccine		
Yes	47	6.0
No	690	87.3
Free	53	6.7

VND; 87,400 VND, respectively. Meanwhile, business managers were willing to pay the highest price for the vaccine (197,000 VND; SD=246,200) (Table 4).

Participants who did not suffer from the disease during pregnancy were more likely to demand vaccination (OR = 3.41, 95% CI = 1.73–6.70). Not having the antenatal examination at central hospitals and working as farmers/workers were positively correlated with willingness to be vaccinated, while the number of children of respondents displayed a negative correlation with willingness. People who had the antenatal examination in places other than central or private/international hospitals were more likely to want more information about the vaccine, whereas those working as public servants or currently in good health were less likely to demand communication about a vaccine, compared to stay-at-home mothers and people in very good health status. The number of children of respondents was also negatively associated with the willingness to communicate (Table 5).

4. Discussion

This study found that despite a high rate of awareness of HBV vaccine, a majority of participants were still willing to know more about the vaccine, especially about its benefits. Willingness to be vaccinated was generally high; however, the average price respondents were willing to pay for vaccination was just half of the offered price. Sociodemographic characteristics of those interviewed were found to influence their preferences toward vaccination.

Although a majority of respondents were found to know about HBV vaccine, their more in-depth knowledge regarding the vaccine may still be lacking, as most of them were willing to acquire rather basic information such as the vaccine's benefits. This finding is comparable to a study on Chinese pregnant women that reported the high rate of HBV vaccine awareness (65%-92% of respondents knew HBV could be prevented by vaccination) but low rate of awareness on HBV transmission mechanism [13]. Other studies on women with children in other developing countries reported a substantially lower level of HBV knowledge; 34.7% of those were interviewed in Ghana and 12.2% in Kenya [14, 15]. Thus, it can be said that in Vietnam, some success has been achieved in communication about the disease and vaccine to the public, especially via mass media. According to the study, television was one of the most common channels from which information about the HBV vaccine was obtained. On the other hand, health workers' advice was the most preferred vaccination communication channel, probably due to the higher level of trust people generally have for health workers regarding health topics, compared to other sources of information. This highlighted the importance of medical staffs, especially those at a local level and more remote areas, in providing sufficient knowledge of diseases and cures to the community [16].

The high rate of willingness to take HBV vaccination discovered in the study was in line with the finding from a Ghana study showing that 93.8% of pregnant women surveyed would take medication to prevent HBV [17]. In contrast, a Chinese study stated that only 16.5% of participants would accept HBV preventive drugs during pregnancy [18]. Lack of HBV knowledge, which lead to, among others, unnecessary worry about the safeness of the vaccine to the child, was cited by the author of that study as one of the causes of such low willingness level. Our study found instead that people not being examined at central hospitals and who have enjoyed healthy pregnancy were more willing to have the vaccine injected, which indicated a level of trust participants have in preventive medication. Such a view was shared by a study by Guo Na et al (2017) in China which found both living

Table 4: The willingness to be vaccinated with Hepatitis B vaccine.

	N	Willingness to vaccinate		Price of willingness to pay for the vaccine	
		N	%	Mean	SD
Total	*805*	*650*	*80.8*	*108.6*	142.7
Occupation					
Homemaker	110	87	79.1	87.9	108.1
Farmer	221	206	93.2	79.1	83.3
Public servants	222	169	76.1	144.3	171.2
Workers	57	49	86.0	84.3	139.5
Self-Business	136	95	69.9	103.5	119.1
Business Manager	9	7	77.8	197.0	246.2
Others	47	34	72.3	176.0	240.6
Age group					
Under 25 years old	144	130	90.3	87.0	106.1
From 25 to 30 years old	376	304	80.9	117.3	151.6
Above 30 years old	283	215	76.0	109.2	145.9
Education attainment					
Illiteracy/Primary	27	24	88.9	100.7	76.0
Secondary school	116	103	88.8	87.7	101.1
High school	235	195	83.0	73.1	92.3
College	180	142	78.9	102.9	127.5
University	220	166	75.5	162.6	201.4
Post graduated	23	16	69.6	145.9	149.5
Having Health insurance					
Yes	508	403	79.3	122.6	161.6
No	290	240	82.8	87.4	105.0

Table 5: Factor associated with willingness to inject and communicate about Hepatitis B vaccination.

	Willingness to have Hepatitis B vaccines injected		Wanting to communicate about vaccines	
	OR	95% CI	OR	95% CI
Pregnant / Having a baby under 1 year of age (Vs. Yes)	1.94**	(1.11 - 3.38)	2.07*	(0.96 - 4.48)
Health facilities where having the antenatal examination				
Central hospital (No vs. Yes)	3.21***	(1.99 - 5.18)	2.39***	(1.30 - 4.40)
Private/International hospital (No vs. Yes)	1.47*	(0.94 - 2.31)	2.30***	(1.30 - 4.09)
Current health status (Ref - Very good)				
Good	1.50*	(0.98 - 2.30)	0.39***	(0.22 - 0.72)
Week	5.36	(0.60 - 48.00)		
Suffered from disease during pregnancy (No vs. Yes)	3.41***	(1.73 - 6.70)		
Occupations (Ref - Homemaker)				
Farmer/ Worker	2.63***	(1.47 - 4.73)		
Public servants	1.45	(0.89 - 2.34)	0.26***	(0.13 - 0.55)
Others			0.48*	(0.22 - 1.03)
The number of children of respondent	0.68***	(0.52 - 0.91)	0.55***	(0.39 - 0.79)
Constant	0.33**	(0.12 - 0.93)	19.09***	(5.73 - 63.56)

*** <0.01, ** <0.05, * <0.1

in urban areas and having higher income level would likely mean better knowledge on HBV vaccine and were found to be positively correlated with the acceptance to get vaccinated [19].

The average price that respondents of this study deemed affordable for HBV vaccine was lower than clinics' listed price, though the willingness to pay varied among different subgroups. Younger mothers without jobs or with lower paying ones and those without health insurance were willing to pay just about a third of the offered price. Such low willingness to pay may be due to the financial difficulties of these participants but may also be the result of insufficient knowledge of the danger of the disease and benefits of getting vaccinated. Researches had indeed found a correlation between lack of comprehensive understanding regarding the importance of vaccination and low willingness to pay for the vaccine in adults [20]. Nonetheless, low willingness to pay would pose a threat to increase the coverage of HBV vaccination in Vietnam. Lowering the vaccine price would be immensely challenging in Vietnam context, one may argue, as the price currently offered has already reflected subsidizations benefited from the support of Global Alliance for Vaccines and Immunization (GAVI) [21]. Therefore, providing the public with appropriate information on the disease and life-saving benefits of vaccination would be a more probable and effective solution.

In this study, we found that women with lower levels of education had high rate of willingness to vaccinate, but this association was not statistically significant when being adjusted in regression model. Previous studies in China were in line with us when it was found that education was negatively correlated with the need of HBV vaccine and willingness to pay for it [16, 22]. The reason of this phenomenon is unclear. However it may be explained by the lack of familiarity with HBV vaccines among participants with low education, or in other words, the percentage of people being aware of HBV vaccine was higher among those with higher education level (data not shown). Indeed, contingent valuation method uses hypothetical scenarios to describe HBV vaccine that would be offered in the future; thus, participants may not know about the HBV vaccine before hearing from interviewers. In literature, insufficient familiarity with the proposed HBV vaccines could result in hypothetical bias that may lead to overestimating participants' WTP [23].

The current study also indicated that people who were farmers/workers were more likely to be willing to pay for the vaccination. Farmers/workers are among those who are most at risk of HBV infection. A study in Iran revealed that HBV prevalence among farmers was the highest compared to other occupations [24]. Another nationwide study in Lao PDR depicted that 71% of HBV infected mothers were farmers [25]. Therefore, we assumed that farmers/workers in our study were aware of their risk and more likely to be willing to pay for the vaccine than other people.

Few implications can be drawn from this study. Education campaigns covering topics of HBV transmission mechanism, especially from mother-to-child, essentialism of vaccination, and how paying to get vaccinated would work as a cost-effective solution with lifelong effect should be developed and ran more frequently, targeting younger and poorer women of reproductive age. Media should be continuously used as the main channel via which information reaches the public, while the active participation of medical staffs at the less central level in this educational effort should be encouraged. In addition, although it seems challenging, efforts should be made by the government to look for ways of reducing the vaccine price, possibly through encouraging additional contribution from the private sector via corporation social responsibility program or from philanthropists. The government also should enforce the coverage of health insurance, especially to the lesser fortunate group.

This study has several limitations. Its cross-sectional, self-reported setting would allow only a 'snap-shot' of information at the time of study with potential inaccuracy resulting from recalling errors of the participant. Though efforts were made to include a relatively large number of respondents from diverse backgrounds, the sample of this study cannot be said to be representative of the population concerned. Moreover, further researches are encouraged to incorporate the rate of vaccination among participants or explore the influencing factors of respondents' willingness to pay for the vaccine which are topics that had not been covered in this study.

5. Conclusion

The study aimed to explore several aspects of HBV vaccination among woman of reproductive age in Vietnam. Although those interviewed demonstrated high awareness of the vaccine's existence, further knowledge regarding the benefits and price of the vaccine was limited. Participants however expressed high willingness to communicate about the disease as well as to be vaccinated. The price people were willing to pay for the vaccine, however, was on average just half of the often-quoted price. These findings implied the need for better targeted public education regarding the danger and solution of HBV, active participation from local medical staffs and the media in providing information, and efforts to reduce the listed price of the vaccine.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Changing Sources of Stigma against Patients with HIV/AIDS in the Rapid Expansion of Antiretroviral Treatment Services in Vietnam

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Stigmatization against HIV/AIDS greatly hinders efforts to increase the accessibility and utilization of HIV/AIDS services to meet the 90-90-90 goal. This study assessed the stigmatization and discrimination experienced by people living with HIV (PLWH) across multiple social settings such as family, community, and healthcare facilities in Vietnam. A total of 1,016 patients (63.8% males, mean age = 35.4) participated in a cross-sectional study using a culturally tailored HIV stigma measure in three HIV-epidemic-concentrated cities in Vietnam. Zero-inflated Poisson models were used to examine factors associated with the number of types of stigma that patients experienced. 86.2% PLWH reported experiencing stigma against HIV/AIDS, more frequently from their community (62.8%) and family (30.2%) than from health care facilities (8%). The level of stigma from community reported by PLWH is associated with socioeconomic status (e.g., income, occupation). The poor and middle economic classes and unemployed patients reported more stigmatization and discrimination from the community. Across all settings, PLWH experienced fewer stigmatization over the course of ART indicating the benefits of rapidly expanded ART programs. PLWH reported more stigmatization and discrimination at the provincial level of the health administration. Those with the history of drug injection reported significantly less stigmatization from healthcare setting. More culturally tailored interventions to reduce stigmatization overall to improve the quality of life and health outcomes of PLWH should be warranted to achieve the 90-90-90 goal. Improving HIV-related knowledge of the general population and providing opportunities for PLWH to be reintegrated into should be considered. Using mass media with positive messages and images would also foster positive attitudes towards HIV/AIDS among the population and could potentially change social values. Continuous training of health staffs' attitude could minimize the occurrence of stigmatization and discrimination at healthcare facilities.

1. Introduction

Stigma encompasses any stereotypes, prejudices, and unfair treatments of individuals perceived or associated social status, value, or label [1]. It is not naturally occurring but is rooted deeply in culture and is driven by personal and social values. Stigma is a fundamental determinant of health that directly affects patient quality of life and disease treatment outcomes, especially with HIV/AIDS [2]. It has been found in numerous literature that HIV/AIDS-related stigma is the main obstacle to seeking HIV-related services due to feeling shame and the fear of discrimination. Stigma exists in various forms and at different levels such as family, community, and health care sector [3–5]. People living with HIV (PLWH) usually experience self-blaming, social isolation, physical or verbal abuse, mistreating, and political discrimination. This decreases the likelihood of HIV status disclosure, causes adverse health effects such as depression or anxiety, and

impacts treatment adherence [6–8]. Injecting drug users (IDU), female sex worker (FSW), and men who have sex with men (MSM) experience double stigma due to their HIV status and their considerably "illegal and immoral" behaviors. HIV/AIDS-related stigmatization experiences differ across cultures and communities. Thus, it is important to understand stigma within a cultural context.

In Vietnam, HIV epidemic concentrated on IDU (9.53%), FSW (2.39%), and MSM (7.36%) [9]. The Government of Vietnam adopted UNAIDS 90-90-90 target which states that 90% of PLWH will know their HIV status, 90% of people with diagnosed HIV infection will receive ART, and 90% of those received treatment will be virally suppressed by 2020 [9]. To respond to this goal, the Vietnamese government has established 401 ART clinics for 124,000 patients by 2018. With the rapid scale-up of ART services, it promises substantial benefits to HIV/AIDS patients in Vietnam. However, HIV/AIDS-related stigma is widespread and remains to be the greatest challenge for LHW to disclose HIV status and seek ART services, which in turn challenges the 90-90-90 goal.

HIV/AIDS-related stigma in Vietnam is perpetuated by the collectivism culture and conservative social values associated with PLWH [10–13]. In the Vietnamese society, people live together as a community and believe in the majority's opinion despite true or false information. From previous national campaign, the negative images of illicit drug users, sex workers, and PLWH had embedded into people's mind as "social evils" [5, 14, 15]. When the majority of people, even health workers, have the prejudice against "social evils" and the fear of infection, PLWH is forced to hide their status and feel ashamed. Their family would receive backlash for having HIV-infected family members. Under social pressure and the fear of infection, HIV-infected individuals are abandoned and expelled from the home [16–20]. Thus, PLWH in Vietnam is afraid of disclosing their HIV status or associating with any HIV/AIDS-related matters due to society's discrimination and stigmatization. As a result, PLWH hardly seeks prevention, treatment, and care services, which make 90-90-90 goal more difficult to achieve.

There have been several studies examining stigma against PLWH in Vietnam in different settings and target groups, including community and family [4], health facility [12], or other social groups [4, 20–22]. However, these studies did not examine the different levels within the health service delivery system. For measurements of stigma, there have been many instruments developed for measuring HIV-related stigma, for example, the PLWH Stigma Index which has been used in many countries, including Vietnam [23, 24]. However, the HIV Stigma Index has a wide breadth of measurement with a long list of items that required intensive resources and patients' collaboration for data collection. In addition, the HIV Stigma Index asked about patient's perception and experience in the past 12 months that might not be effective for use in clinics to monitor patient's well-being over the course of ART. Third, the structure of the HIV Stigma Index was more patient-focused rather than indicating the levels or places in which the stigma exists to facilitate specific interventions to be implemented. Since Vietnam has limited resources, it is important to identify the greatest sources of stigma for targeting with stigma reduction programs; a program may not be been needed in all sectors.

There have been several conceptual frameworks for understanding stigma at multiple levels, including the sociocognitive models at the micro/individual level to the structural models at the macrolevel [25]. In the design of this study, we referred to the theory of structuration by Giddens [26] in which time and space are critical in understanding the agents and structures given the fact that agents' actions will vary in accordance with their contexts [25]. Since stigma against HIV has significantly changed over the past decades as a result of synergic efforts in expanding the coverage of effective interventions, we were interested in identifying the types of stigma experienced by PLWH at different social levels and settings. Therefore, this study assessed stigmatization experienced by PLWH across multiple social domains such as family, community, and levels of healthcare provision. We anticipated that the findings might provide information to assist the Government of Vietnam on how to develop more effective policies, and strengthen efforts to reduce stigma, improve quality of life and health outcomes of PLWH as well as achieve the 90-90-90 goal.

2. Materials and Methods

2.1. Ethics Statement. The study was approved by the Authority of HIV/AIDS Control. Before enrolling in the study, participants were informed about the objectives, anonymity, and confidentiality of this research. Researchers obtained written consent forms from all participants involved in the study. The confidentiality of the subject information was maintained at all time during the study. Dataset and questionnaire were securely stored.

2.2. Settings. In 2012, we conducted the HIV Services Users Survey, which was a cross-sectional study in three cities which have highest HIV prevalence in Vietnam, including Ha Noi, Hai Phong, and Ho Chi Minh City, with the population of 6 million, 1.8 million, and 8 million people, respectively. Three cities were chosen to represent the different geographical areas where has remained the highest HIV prevalence. Ha Noi is the capital city where approximately 18,000 PLWH reside. Hai Phong is a port city in northern Vietnam which has 6,930 PLWH. Ho Chi Minh City is the largest southern metropolitan city with the largest PLWH population, approximately 46,507 people [27]. This survey explored multiple dimensions of the access, utilization, and outcomes of HIV services from the perspective of the patient. Using the same dataset, we have previously published on patient's satisfaction, quality of life, and health care costs [2, 28, 29].

2.3. Study Design. ART patients were conveniently selected and invited for an interview when they visited the clinic. A total of 1,016 patients were interviewed; 201 patients (20%) from central level, National Hospital for Tropical Diseases; 406 patients (40%) from provincial level, Dong Da, Viet Tiep, and Ho Chi Minh City Tropical Diseases Hospital; and 409

patients (40%) from district level, Tu Liem, Le Chan, and Binh Tan Health Center.

2.4. Study Instrument and Data Collection. Data were collected by researchers and students at the Hanoi Medical University. We did not involve any clinic staff to avoid potential social desirability bias. In addition, we invited patients to a private counseling room to ensure that their information was confidential. During the interview, the researchers collected information using a structured questionnaire. It included socioeconomic status (e.g., age, gender, marital status, education level, occupation, and household monthly income) and self-reported clinical status (e.g., HIV/AIDS stages, Asymptomatic/Symptomatic/AIDS/Unknown, CD4 cell count, history of drug use, and duration of ART). Household monthly income was stratified into five quintiles for analysis purpose.

2.5. Measuring Stigma and Discrimination by PLWH. In this study, we developed a contextualized measure to evaluate the stigma and discrimination towards PLWH. First, we reviewed the scope of stigma and discrimination against HIV/AIDS from previously published studies to construct a list of items for measurement. Second, we conducted four focus group discussions with PLWH, their family members, health care workers, and public health researchers for cultural validation and feasibility. Final, the measure was shortened to 20 items. Patients responded "Yes" or "No" for each type of stigma and discrimination that they experienced.

2.6. Statistical Analysis. Stata 14.0 software was used for analyzing data. Sociodemographic and HIV-related characteristics of respondents were described using frequency, mean, and standard deviation (SD). Cronbach's alpha was employed to estimate the internal consistency reliability of the stigmatization measures. Factor analysis was utilized to explore the construct of the scale by determining factors and restructuring the items into appropriate factors to increase the interpretability of the measure. Polychoric correlation matrix was applied because each item included in the factor analysis was binary variables. We extracted the factors using the principal component analysis (PCA) with the eigenvalue of 1.0 as a threshold for flattening out the eigenvalue curve and used Orthogonal Varimax rotation with Kaisers' normalization to reorganize the items. We used a value of 0.4 as a cut-off point for factor loadings. Multiple Zero-inflated Poisson regression models were constructed to examine the correlates of the rate of numbers of types of stigma that patients experienced. Candidate independent variables included socioeconomic, drug injection, and HIV-related characteristics of respondents. Statistical significance was defined when the p-value was less than 0.05.

3. Results

A total of 1,016 participants were interviewed (63.8% were male). The mean age was 35.4 (SD=7.0). Majority of participants attained education below high school (54.7%) and lived with a spouse or partner (64%). 52.6% of participants were freelancers, and 20.4% had stable jobs. Half of the participants were symptomatic, and one-third of participants (37.6%) had been diagnosed with AIDS. Majority of participants had low CD4 cells count. Only 13.6% participants had CD4 cell count greater than 500 cells/mL. The majority had received ART (88.8%), and 55.3% of those has been treated for at least two years. Most participants reported that they had never used drugs (53.9%).

Types of a stigma that the participants have ever experienced from community, family, or healthcare system or due to HIV status disclosure are presented in Table 1. A majority of participants (82.3%) had told their family members about their HIV status. One-third of the participants (34.5%) reported losing jobs or profits/income due to their HIV status. 34.4% of the participants experienced feared to get HIV infected from them by others. 14.6% of the participants reported being blamed or criticized because they had HIV. 3.6% of the participants perceived receiving poor health care services. 3.1% of the participants reported having been discriminated against by health workers. A majority of participants reported experiencing at least one type of stigma due to HIV disclosure (86.2%); 62.8% from the community, 30.2% from family, and 8% from health care system.

The factors associated with the number of types of HIV/AIDS-related stigmatization that participants have ever experienced are presented in Table 2. These factors are presented across the four domains of community, family, health care system, and HIV disclosure. We found that the number of types of stigma and discrimination by the community was more frequently experienced by patients who were poorer, employed, initiating ART and who were attending clinics at lower levels within the healthcare system.

HIV-related symptoms were also factors that increased the likelihood of experiencing stigma and discrimination by PLWH in families. Symptomatic patients experienced more stigma from family than asymptomatic patients (Coef.=0.69, CI=0.03; 1.36). Meanwhile, those with the history of drug injection experienced less stigmatization from healthcare workers than nonusers (Coef.=-1.92, CI= -2.97; -0.87). In addition, PLWH receiving ART treatment for longer durations experienced less stigmatization and discrimination across settings. For instance, those taking ART between 4-7 years reported less stigma from healthcare workers than those has not taken ART (Coef.=-0.95; CI=-1.99; 0.10).

4. Discussion

Our findings contribute the existing literature on how cultural and social values directly affect HIV/AIDS-related stigma and HIV/AIDS care [3, 4, 25, 26]. Overall, the expansion of ART services and the enrollment of HIV patients have reduced stigmatization and discrimination within the community, family, and healthcare settings. However, the stigmatization from the community reported by PLWH remains high, which were associated with different socioeconomic status, employment, ART treatment stage, HIV-related symptoms, and levels of health administration. Interestingly, we found that HIV patients with the history of drug injection

Table 1: Factor loading, reliability, and measurement of Stigma.

Items	% Yes	Factor Loadings			
		Community	Family	Healthcare	HIV disclosure
Having told family members you have HIV	82.3%				0.46
Losing jobs or profits	34.5%	0.62			
Others feared to get HIV infected from you	34.4%				
Having told friends you have HIV	28.8%				0.81
Others gossiped about your HIV status	28.2%	0.67			
Relatives, friends have grown more distant with you	25.8%	0.58			
Being excluded from community events because of HIV	25.6%	0.61			
Afraid that others disclose your HIV status	15.6%				0.44
Being blamed, criticized because you have HIV	14.6%	0.56			
Devalued, disrespected by others because of HIV	14.3%	0.65			
Having told neighbors, you have HIV	11.0%				0.78
Being abandoned by your spouse	9.2%		0.65		
Hurt, annoyed, or offended by others because you have HIV	8.8%	0.49			
Discriminated by family members	7.9%		0.69		
Dismissed or unable to rent an accommodation because of HIV	6.9%		0.47		
Losing heredity because of HIV	6.9%		0.59		
Health workers feared of getting HIV-infected	5.2%			0.65	
Being threatened	4.5%				
Perceived poorer health service quality	3.6%			0.77	
Discriminated by health workers	3.1%			0.76	
Number of stigma types reported (mean (SD))		1.59 (1.80)	0.30 (0.70)	0.12 (0.46)	1.41(0.99)
% patients reported at least one type of stigma	93.3%	62.8%	20.2%	8.0%	86.2%
Reliability (Cronbach's alpha)		0.75	0.56	0.66	0.65

Table 2: Factors associated with the number of stigma types against HIV/AIDS that patients experienced (zero-).

VARIABLES	Community		Family		Healthcare		HIV disclosure	
	Coef.	95%CI	Coef.	95%CI	Coef.	95%CI	Coef.	95%CI
Income per capita (vs Poorest)								
Poor	0.28**	0.03; 0.54	0.12	-0.66; 0.90	0.73	-0.28; 1.74	-0.02	-0.19; 0.15
Middle	0.20	-0.09; 0.50	-0.04	-0.64; 0.57	-0.25	-1.22; 0.72	-0.06	-0.22; 0.10
Rich	0.13	-0.14; 0.39	0.29	-0.22; 0.80	0.58	-0.48; 1.64	-0.03	-0.19; 0.13
Richest	0.10	-0.19; 0.38	-0.19	-0.75; 0.37	-0.45	-1.52; 0.62	0.03	-0.13; 0.20
Occupation (vs Unemployed)								
Free lancer	-0.28**	-0.53; -0.04	-0.18	-0.63; 0.28	-0.10	-0.82; 0.62	-0.02	-0.16; 0.13
Stable Jobs	-0.42***	-0.70; -0.15	-0.58	-1.28; 0.11	0.16	-0.69; 1.02	-0.05	-0.22; 0.12
Other	-0.14	-0.43; 0.15	0.14	-0.64; 0.92	-0.52	-1.46; 0.43	-0.02	-0.20; 0.16
Gender (Male vs Female)	-0.04	-0.22; 0.15	-0.13	-0.55; 0.28	-0.08	-0.80; 0.64	-0.07	-0.19; 0.05
Education (>= High school vs < High school)	0.06	-0.13; 0.24	-0.02	-0.46; 0.42	-0.30	-0.96; 0.36	0.03	-0.08; 0.14
Marital status (vs Single)								
Live with spouse/partner	0.16	-0.11; 0.42	-0.20	-1.03; 0.63	-0.39	-1.11; 0.33	0.01	-0.15; 0.16
Widow/Divorce/Separate	0.16	-0.16; 0.48	0.08	-0.91; 1.06	-0.87*	-1.80; 0.06	0.15	-0.04; 0.33
Ever inject drug (Yes vs No)	0.04	-0.14; 0.23	0.28	-1.46; 2.02	-1.92***	-2.97; -0.87	0.09*	-0.01; 0.20
HIV stage (vs Asymptomatic)								
Symptomatic	-0.01	-0.30; 0.28	0.69**	0.03; 1.36	-0.14	-1.29; 1.01	0.23**	0.05; 0.41
AIDS	-0.15	-0.44; 0.14	0.42	-0.20; 1.03	-0.06	-1.00; 0.88	0.12	-0.06; 0.31
Duration of ART (vs. Not yet)								
<=1 yr	0.24*	-0.04; 0.52	-0.06	-0.55; 0.42	-0.12	-1.12; 0.89	0.01	-0.20; 0.21
1; <=2 yr	0.03	-0.26; 0.32	0.15	-0.41; 0.70	-0.40	-1.56; 0.77	0.10	-0.08; 0.29
2; <=4 yr	-0.13	-0.40; 0.14	-0.98**	-1.78; -0.17	-0.90	-2.04; 0.24	-0.09	-0.27; 0.10
4; <=7 yr	-0.05	-0.33; 0.24	-0.18	-0.70; 0.33	-0.95*	-1.99; 0.10	-0.04	-0.23; 0.14
CD4 cell count (<= 200)								
200< CD4 <= 350	0.12	-0.10; 0.34	0.47	-1.53; 2.48	-0.55	-1.99; 0.89	0.00	-0.12; 0.13
350< CD4 <= 500	-0.04	-0.31; 0.24	0.26	-1.99; 2.51	-1.17*	-2.39; 0.05	-0.07	-0.22; 0.08
> 500	0.27**	0.00; 0.55	0.56	-1.62; 2.74	-0.66	-1.59; 0.26	-0.12	-0.29; 0.06
Level of health administration (vs Central)								
Provincial	0.39***	0.13; 0.65	0.53	-0.27; 1.32	0.77	-0.91; 2.45	0.12	-0.03; 0.27
District	0.35***	0.09; 0.62	0.81**	0.08; 1.54	1.15	-0.56; 2.86	0.22***	0.06; 0.37
Constant	0.37	-0.11; 0.85	-1.34	-3.67; 0.98	0.78	-2.17; 3.74	0.05	-0.26; 0.35

Robust ci in parentheses

*** p<0.01, ** p<0.05, and * p<0.1.

reported significantly less stigmatization in the healthcare setting.

For the past decade, the Government of Vietnam has successfully controlled the HIV epidemic and improved quality of life of HIV patients by providing free treatment services [18–20, 28]. Those who initiate ART have to disclose HIV status and will be more likely to feel discriminated by a community when others see them walking to the clinics [24, 25, 30]. As treatment course progresses, HIV patients often obtain better health and are able to manage their life. As a result, ART does not only increase self-efficacy and self-esteem but also improves health outcomes and reduces stigmatization from the community experienced by PLWH [28, 29, 31]. Similarly, because of a collection of effective policy and technical interventions by the Government of Vietnam, with the expansion of Methadone Maintenance Treatment (MMT) facilities and numbers of trained staffs, drug users with or without HIV are receiving additional services. Specifically, those in the sample of this study received various counseling sessions since HIV testing in addition to peer-education and support. Thus, the stigma against drug users and patients with HIV/AIDS has been significantly decreased. Contrary to existing literature, after adjusting to potential confounders in multiple regression models, we found that HIV patients with the history of drug injection experienced less stigmatization in healthcare settings. In previous research conducted in Canada, nurses who constantly worked with illicit drug users developed more positive attitudes and compassion towards this population. Therefore, the more well-trained and specialized staffs may help to reduce stigmatization and make patients feel welcomed which in turn improving treatment adherence and health outcomes of patients.

Despite existing efforts, stigmatization and discrimination remain higher at lower levels of health administration. Staffs at the central level of health administration who receive training more frequently, may perceive and treat HIV patients better. However, at the provincial and district health centers, the staffs, especially general health workers, have less training resulting in poor HIV knowledge that perpetuates prejudicial attitudes towards PLWH. In addition, medical students who have high knowledge and are trained to treat patients professionally reported some misconception regarding HIV transmission and prevention [32]. Some medical students also showed stigmatizing attitudes towards HIV/AIDS by avoiding HIV cases. HIV patients report experiencing nonverbal or verbal discrimination and unfair treatments [11, 33]. For instance, surgeons have refused operating surgery because of the fear of HIV infection, and staffs used different bedding for HIV patients or burned beddings used by HIV patients [10, 33, 34]. These experiences impact HIV patients' decision in seeking medical care in the future.

At the beginning of HIV epidemic era, there were antiprostitution and anti-illicit drug use campaigns that used negatives images to criminalized prostitution and illicit drug use in order to educate the public and stop the spread of HIV/AIDS [7]. However, the campaigns provided insufficient knowledge to cause bias, negative attitudes and fear of HIV transmission. This tactic has caused communities to label PLWH as "social evils," criminal, failure and immoral [7, 11, 21]. In the context of Vietnamese culture, people believe the evils deserve consequences for their immoral behaviors. Some people believe that PLWH deserves to suffer in poverty and to have a difficult life.

Our findings indicate a relationship between household economic status and stigmatization, that PLWH who were poorer experienced stigmatization from the community. From previous research, PLWH experienced difficulty in finding jobs and maintain their employment due to their HIV status [11, 21]. PLWH often lose their jobs due to their employers' negative perception about HIV and the fear of being infected through casual contact; those who are self-employed lose customers or business partners [7, 24]. As a result, it is difficult for PLWH to earn money for their living. Moreover, poorer PLWH experienced more stigmatization from the community because they might be causing the financial burden or failing to fulfill the expectation as the bread-maker of the family [11, 21, 23]. Unlike the previous study which found that unemployed HIV-infected individuals reported moderate to severe level of felt-stigma, our result indicates those with stable jobs experienced more stigmatization from the community as these people would have a larger social network and in turn, experience more discrimination from this large network. Regardless of their employment and financial status, PLWH in Vietnam experience multiple stigmatizations from the community due to the perception that they committed a "social evil" and deserve the consequences.

HIV patients also receive more stigma from their family due to HIV-related symptoms. In Vietnamese culture, the family remains the sole means of support for most people. When symptomatic HIV patients disclose HIV status to family members, HIV patients often do not usually receive support, but stigmatization and discrimination [7, 11]. Family members might blame PLWH for contracting the "social evil" disease and having HIV symptoms. Moreover, family members often feel shameful and distressed by the community due to the spreading of rumors and gossips about the PLWH; thus, they keep distant, avoid contact, treat HIV-infected individuals differently or even expel them from the home [11, 24]. Losing family support due to HIV status has negative impacts on treatment adherence and health outcomes [11]. Altogether, stigma creates an adverse effect on HIV patients.

5. Implications

Previous authors have analyzed the barriers and facilitators of stigma intervention in various settings [33]. Even though our findings indicate that stigma exists in community and family more often than in health care, it is necessary to have interventions at all levels to eliminate stigma. This principle is also supported by findings from a systematic review by Stangl et al. on interventions to reduce the stigma that highlighted the limitation of current practices which only focused on a single socioecological level and a single domain of stigma [30]. Stigma within the community, family, and health care begin with the negative perception of HIV disease. Therefore, it is necessary to provide correct knowledge and

address attitudes towards HIV. Unlike using negative images like the previous propaganda, recommended interventions include public education, posters, and campaigns with the positive message displaying where people gather the most or on multiple media outlets (e.g., TV, radio and social media). Interventions that aim to bring HIV population closer to the community should be considered such as inviting HIV patients to participate in talk shows, sharing stories about stigma to create the emotional connection with the community. One suggestion is to use the messages in the Prevention Access Campaign's called "U=U" (undetectable = untransmittable), which could help to reduce the self-stigma among PLWH and their relatives/partners, as well as increased testing and treatment access [34–36]. Furthermore, instead of excluding PLWH from a community, they need to be reintegrated into the community through opportunities such as jobs or vocational training. It must start with mandating laws to abolish stigmatization and discrimination against PLWH in any forms at the workplace. This does not only protect PLWH from stigmatization but also provides a friendly environment where PLWH can work to secure their income. In addition, by providing vocational training to PLWH, they have the skills to work on their own instead of suffering from unemployment and depending on the financial support of others.

However, providing only economic developmental opportunities is not sufficient; it is essential to provide support and motivation to optimize the outcomes [34–36]. This will not only allow them to receive better treatments and improve health outcomes but also strengthen the bond between individuals and family, friends and society. With the goal of better physical and mental well-being and integrate into the community [37], interventions such as family day could help to bring family and HIV patients together could be implemented; family day is when family members are invited to learn about HIV and inform how well patients are doing in an ART program. This could also involve the family's involvement in the patient's treatment plan to improve adherence.

Last but not least, there is a need for additional policies and intervention to address HIV-related stigma and discrimination at all levels of the health care system. Li et al. report on the effectiveness of using popular opinion leaders in reducing HIV-related stigma and improving HIV testing, treatment, and care in the healthcare setting in China [27]. In Senegal, stigma impact mitigation in combination with increased service linkages has also proved to be effective in delivering services [32]. There is a need for training and workshops for health workers at provincial and district treatment facilities on how to communicate and treat patients fairly to avoid HIV-related stigma and discrimination. This is crucial for HIV-infected individuals to feel welcomed and motivated to come to treatment facilities and receive appropriate health care services.

6. Strength and Limitation

This study described in-depth HIV/AIDS stigma across multiple social domains including the community, family, and health care system. The study population was recruited from three epicenters to represent geographic difference and to understand stigma across the nation.

There are limitations to the study that need to be considered when interpreting the results. It was a cross-sectional study and there may have been social desirability bias due to sensitive topics. The perceived fear and discrimination could be exaggerated or underestimated. Moreover, the study only measured HIV patients' perspectives. Future study needs to look at community, family, and healthcare workers' perspectives about HIV and HIV patients, as well as opinions from stakeholders such as HIV-related nongovernment organizations or local authorities. Furthermore, we did not address the issue of homosexuality in this study, which should be warranted in further studies. In addition, despite the acceptable reliability, in order to apply this tool in the common practice, the instrument should be improved to ensure the appropriateness regarding the context, language and logical issues. Lastly, since HIV information is confidential due to Law on HIV, the community-based sampling is not feasible. Therefore, we captured only those accessed health services. Therefore, the convenient sample in this study affects the generalizability. This sample was also limited to patients who discontinue their drug use and hence were provided ART. This group is likely to be less stigmatized than active drug users.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Disclosure

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Prognostic Factors of Mortality among Adult Patients on Antiretroviral Therapy in India: A Hospital Based Retrospective Cohort Study

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Introduction. HIV related deaths still continue to occur in large numbers in spite of good quality drugs being freely available in India. This study was therefore done to assess the prognostic factors of mortality among people living with HIV (PLHIV) on antiretroviral therapy (ART). This would help in planning strategies for further improving their survival. *Materials and Methods*. Record based data from baseline and follow-up visits of a cohort of patients aged above 14 years on ART was retrospectively reviewed over a seven-year period. The Kaplan-Meier models were used to estimate life time survival probability, and Cox proportional hazard regression model was used to determine independent prognostic factors of death, among patients, after initiation of ART. *Results*. Mean age of the total 285 patients enrolled in this study was 45.8±9.7 years. Mean duration of treatment on ART was 1127±611.8 days. During the follow-up period, 44/285(15.4%) patients died, resulting in incidence density of death rate as 3.12 per 100 person years. Good adherence with treatment was reported by 267(93.7%) patients. Nearly half of the deaths, i.e., 21(47.7%), occurred within three months of them starting ART. The mean survival time after initiation of ART was 2084.0±55.3 days (95% CI is 1975.5-2192.5). The presence of opportunistic infections (OIs) and tuberculosis before and poor/average adherence to ART and alcohol usage after starting ART were independent prognostic factors of mortality among patients. *Conclusion*. Several prognostic factors influencing mortality among adult HIV patients receiving treatment were identified in this study. Screening efforts is essential in early detection and management of OIs among PLHIV. Good counselling and monitoring is recommended to improve adherence and also to prevent alcohol usage after initiation of ART. Such measures would help in further reducing mortality among HIV patients in the settings.

1. Introduction

India stands third with respect to HIV epidemic in the world. About 2.1 million people are estimated to be living with HIV in India. The number of deaths due to AIDS related illnesses in India was estimated to be around 62,000 people in the year 2016 [1].

Free antiretroviral therapy (ART) is being provided by National AIDS Control Organization (NACO) of India since 2004. With the introduction of Highly Active Antiretroviral Therapy (HAART), there has been a sizable reduction of HIV associated mortality in developing countries [2, 3]. In India, a 54% decline in AIDS related deaths was reported between 2007 and 2015 [4]. However, reasons for the occurrence of so many deaths in 2016 require investigation.

Mortality at an early stage has been reported among patients soon after initiation of ART which is unlikely to be related to drug efficacy [5–7]. Hence there are several other factors which indirectly contribute to mortality among PLHIV (people living with HIV) which are often poorly understood [8]. Factors such as poor therapy adherence, drug toxicity, and presence of substance abuse and addiction, limit the success of HAART and affect the quality of self-care among PLHIV [9].

Identification and rectification of these modifiable factors during routine clinical care might help in prolonging survival thereby preventing more deaths among PLHIV. This study therefore aims to identify prognostic factors influencing mortality among a cohort of HIV infected adult patients on ART.

2. Materials and Methods

This retrospective cohort study was conducted in May 2017 in a major tertiary care hospital affiliated to a medical college in the city of Mangalore of Karnataka state in southern India. The approval to do this study was obtained from the Institutional Ethics Committee. Then, the permission to do the study at the setting was taken from the medical superintendent of the respective hospital.

The secondary data of all confirmed patients of HIV started on ART over a seven year follow-up period from January 2010 till December 2016 was collected by the investigators. The information regarding sociodemographic details of the patients, age at diagnosis of HIV, age at starting ART, functional status of patients, substance usage, associated OIs, WHO clinical staging of the patient, haematological reports, height, weight of the patient, and health status of the spouse were noted down in a predesigned content validated pro forma. The number of follow-up visits to the treatment centre, type of ART regimen, adherence to treatment, chemoprophylaxis regimen, and outcome of management whether survived or died was also recorded. No personal identifier was stated on the data collection form.

The cohort constituted patients aged above 14 years and who underwent at least two visits to the treatment centre.

Transferred out patients and lost to follow-up patients whose outcome following treatment was not known were censored. Patients who did not return for follow-up visits for more than three consecutive months were considered as lost to follow-up [10].

Patients who had already started treatment with ART before the period of observation of this study and those aged below 15 years were excluded.

The most recent laboratory results before starting ART was used as a base line value. In patients where this information was unavailable, the results obtained within a month of ART initiation were recorded as baseline values [11].

Body mass Index (BMI) was categorized as per Asian classification. Adherence to ART more than 95% was considered good, between 85 and 94% as average and less than 85% as poor adherence with treatment [11].

The survival time was calculated in days between the date of treatment initiation and the date of the event (death) or date of censoring [12]. Data were censored on 26th May 2017.

Data were entered and analyzed using SPSS Inc., Chicago, IL, USA, version 16.0.

Descriptive statistics were used to describe the characteristics of the cohort. Person years of observation (PYO) and incidence rates were also calculated for various periods. The Kaplan-Meier curves were generated to estimate life time survival probability of patients.

Univariate analysis was done using Chi square test, unpaired t test, and Analysis of Variance. All the risk factors significant at 0.15 level qualified entry in the multivariable model and were excluded using a backward stepwise elimination procedure to identify the true predictors. Cox proportional hazard regression model was used to calculate the adjusted hazard rate and to determine independent prognostic factors of mortality among patients after initiation of ART. $p \leq 0.05$ was considered statistically significant association.

3. Results

Mean age of the 285 patients enrolled in this study was 45.8±9.7 years with age ranging from 18 years to 76 years (Table 1). Mode of transmission of HIV in most of the patients was by hetero sexual contact 278(97.5%). The other modes of transmission were by intravenous drug usage in 3, blood transfusion in 2, and homo sexual contact and from mother to child transmission in one case each. Type of virus involved was HIV 1 in most patients 282(98.9%) and HIV 2 among the remaining 3 (1.1%) patients.

Median BMI (Kg per sq.mtrs) of patients was 20.1(IQR 17.7-22.9) before the start of ART (n=257), 20.7(IQR 18.4-23.6) after 6 months (n=249), 21.2(IQR 18.6-23.7) after 12 months (n=242), 21.1(IQR 18.6-23.1) after 24 months (n=178), 21.3(IQR 18.2-23.5) after 36 months (n=124), 21.4(IQR 18.3-23.2) after 48 months (n=105), and 21.5(IQR 18.2-23.6) after 60 months (n=67) of starting ART.

Around 3% and 7% increase of BMI from baseline median level was observed after 6 months and 60 months of ART, respectively.

Median CD4 count (cells per cu.mm) of patients was 364(IQR 201-498.5) before the start of ART (n=285), 410(IQR 314-574.5) after 6 months (n=254), 494(IQR 355-656.5) after 12 months (n=241), 562(IQR 401-728) after 24 months (n=175), 584.5(IQR 432-770) after 36 months (n=130), 575.5(IQR 418-754.5) after 48 months (n=106), and 563.5(IQR 450-722.5) after 60 months (n=62) of starting ART. The CD4 count before the start of ART was <200 cells per cu.mm of blood in 71(24.9%) patients.

13.6% and 59.4% increase of CD4 count from baseline median level were observed after 6 months and 48 months of ART, respectively.

The median CD4 count of patients (cells per cu.mm) at the time of death (n=44) was 210(IQR 128-375.5).

During the follow-up period, CD4 count remained the same or was less than the baseline values among 67(23.5%) patients, yielding an immunologic treatment failure rate of 41.5/100 person years of observation (PYO).

Death was reported among 6(85.7%) patients with CD4 count less than 100 cells per cu.mm of blood in comparison to 38(13.7%) patients with CD4 count ≥100 cells per cu.mm of blood (X^2=27.1, p<0.001).

Mean haemoglobin values (gms per dl) before start of ART were 12.1±2.4 among patients.

Mean age of patients at the start of ART was 42.9±9.6 years (Table 1). Mean duration of treatment on first line drugs was 396.5±174.3 days.

Table 1: Demographic and clinical characteristics of HIV patients.

Demographic characteristics	Number	Percentage
Age group (years)		
18-25	6	2.1
26-35	28	9.8
36-45	111	39.0
46-55	94	33.0
56-65	40	14.0
>65	6	2.1
Gender		
Male	176	61.8
Female	109	38.2
Marital status		
Unmarried	46	16.1
Married	177	62.1
Divorced	9	3.2
Widow/ widower	53	18.6
Educational status (n=278)		
Illiterate	25	9.0
Primary school	129	46.4
Secondary school	81	29.1
College and above	43	15.5
Occupation (n=269)		
Unemployed	87	32.3
Unskilled	54	20.1
Semi-skilled	70	26.0
Skilled	32	11.9
Semi-professional	7	2.6
Professional	19	7.1
Monthly income (n=239)	Median INR 5000 (IQR 3000- 8000)	
Health condition of the spouse (n=203)		
Healthy	102	50.2
Chronically ill	61	30.1
Died	40	19.7
Place		
Urban	243	85.3
Rural	42	14.7
Clinical characteristics	Number	Percentage
Type of ART		
First line	266	93.3
Second line	19	6.7
Mean age at diagnosis of HIV (years)	41.1±10.3	
Age at starting ART (years)		
≤20	4	1.4
21-30	20	7.0
31-40	91	31.9
41-50	112	39.3
51-60	51	17.9
>60	7	2.5

Table 1: Continued.

Time between diagnosis and initiation of ART (days)	Median 55 (IQR 7 - 910)	
Duration of taking ART (days)	Mean 1127.0±611.8	
Time between initiation of ART and death (days) (n=44)	Median 110 (IQR 25.2 - 780)	
Cotrimoxazole prophylaxis		
Yes	73	25.6
No	212	74.4
Isoniazid prophylaxis		
Yes	43	15.1
No	242	84.9
Adherence to ART		
Good	267	93.7
Average	14	4.9
Poor	4	1.4
Side effects of ART		
Present	35	12.3
Absent	250	87.7
Mean number of visits to ART centre	30.4±16.5	
Outcome of treatment		
Survived	241	84.6
Died	44	15.4
Total	285	100.0

Good adherence with ART was seen among 238(95.2%) patients without any side effects with ART compared to 29(82.9%) among those with side effects (X^2=7.9, p=0.005).

Extra-pulmonary and pulmonary tuberculosis were the most common OI before and after starting ART, respectively (Table 2).

Cause of death was known in 13 patients. It is comprised of cardiac causes in 3, pneumonia in 6, and tuberculosis, and encephalitis in 2 patients each.

Majority of deaths, i.e., 21(47.7%), occurred within three months of starting ART. The proportion of patients who died was 7.4%, 1.1%, 1.1%, 1.9%, 2.4%, and 2.4% at <3 months, 3-6 months, 6 months to 1 year, 1-2 years, 2-3 years, and ≥3 years of treatment with ART, respectively. In terms of person years it was 3.2, 0.4, 0.2, 0.1, 0.1, and 0.1, respectively, during these periods of treatment with ART.

During the follow-up period, a total of 44/285(15.4%) patients died. The proportion of patients who died was 4.9%, 1.5%, 1.1%, 2.3%, 1.2%, 0%, and 5.5% at <3 months, 3-6 months, 6 months to 1 year, 1-2 years, 2-3 years, 3-4 years, and ≥4 years, respectively, following the diagnosis.

The death rate was 44 deaths/880.79 person years of treatment X 100 = 4.99 per 100 person years of treatment with ART. The death rate was 44/1407.8 person years since diagnosis X 100 = 3.12 per 100 person years.

The time between diagnosis of HIV and death was ≤100 days in 14(31.8%), 101 days to 1 year 8(18.2%), 1 year to 5 years in 12(27.3%), and more than 5 years in 10(22.7%) patients.

Out of the 44 deaths reported, the most recent CD4 count (cells per cu.mm) before death was <100, 100-199, 200-299, 300-399, 400-499, and ≥500 in 6(13.6%), 15(34.1%), 8(18.2%), 6(13.6%), 3(6.9%), and 6(13.6%), respectively.

The median duration of taking ART among the patients who died (n=44) was 110 (IQR 25.2-780) days.

The mean duration of taking ART (days) was 36.5, 335.3, 392.9, 490.2, 550, and 1207.5 among patients whose most recent CD4 count (cells per cu.mm) before death was <100, 100-199, 200-299, 300-399, 400-499, and ≥500, respectively (F=3.345, p=0.013).

Therefore patients who died with a low CD4 count were on ART for a much shorter time than those who died with a higher CD4 count. This implies that deaths at low CD4 count were caused by patients starting ART, too late to benefit.

The median survival time among the patients who died (n=44) was 380.5 (IQR 44-1756.2) days.

The mean survival time (days) following diagnosis with HIV was 401, 584.7, 1235.2, 757.8, 2719, and 1606.2 among patients whose most recent CD4 count (cells per cu.mm) before death was <100, 100-199, 200-299, 300-399, 400-499, and ≥500, respectively (F=2.494, p=0.048).

The mean survival time (days) following diagnosis with HIV was 1401±1393.8 days (or 46.7 months) and median survival time was 790 days (IQR 148-2667) for patients whose most recent CD4 count before death was ≥200 cells per cu.mm.

Table 2: Distribution of various characteristics before and after start of antiretroviral therapy (ART) among patients.

Characteristics before starting ART			Characteristics after starting ART		
	Number	Percentage		Number	Percentage
Functional status (n=285)			Functional status (n=240)		
Ambulatory	51	17.9	Ambulatory	1	0.4
Bedridden	6	2.1	Bedridden	1	0.4
Working	228	80.0	Working	238	99.2
Pattern of substance usage (n=285)			Pattern of substance usage (n=285)		
Tobacco chewing	13	4.6	Tobacco chewing	14	4.9
Smoking	32	11.2	Smoking	15	5.3
Alcohol consumption	62	21.7	Alcohol consumption	22	7.7
Opportunistic infections present (n=285)			Opportunistic infections present (n=285)		
Extra pulmonary tuberculosis	34	11.9	Pulmonary tuberculosis	15	5.3
Pulmonary tuberculosis	28	9.8	Extra pulmonary tuberculosis	9	3.2
Candidiasis	5	1.7	Cytomegalovirus infection	2	0.7
Pneumocystis jiroveci pneumonia	4	1.4	Toxoplasmosis	2	0.7
Cryptococcal infections	2	0.7	Others**	2	0.7
Toxoplasmosis	2	0.7			
Others*	3	1.0			
BMI (Kg per sq.mtrs) of patients before starting ART (n=257)			BMI of patients after 60 months of starting ART (n=67)		
Under weight	87	33.9	Under weight	19	28.4
Normal weight	112	43.6	Normal weight	23	34.3
Over weight	25	9.7	Over weight	16	23.9
Obese	33	12.8	Obese	9	13.4
Clinical staging of HIV patients before starting ART (n=285)			Clinical staging of HIV patients after 60 months of starting ART (n=90)		
Stage 1	172	60.3	Stage 1	75	83.3
Stage 2	18	6.3	Stage 2	6	6.7
Stage 3	27	9.5	Stage 3	5	5.6
Stage 4	68	23.9	Stage 4	4	4.4
Mean CD4 count (cells/cu.mm) before starting ART (n=285)	381.4±235.9		Mean CD4 count (cells/cu.mm) after 60 months of starting ART (n=62)	596±216.7	

*Cytomegalovirus infection 1, recurrent respiratory infections 1, and cellulitis 1.

**Cryptococcal infections 1 and skin rashes 1.

Univariate analysis found those patients whose baseline functional status was nonworking ($p<0.001$), those who were tobacco users before starting ART ($p=0.019$), those who were alcoholics before ($p=0.003$) and after ($p=0.001$) starting ART, those with OIs and tuberculosis before ($p<0.001$) and after starting ART ($p<0.001$), patients who were in WHO Clinical Stage 4 of HIV disease before initiation of ART ($p<0.001$), those who had ≤15 visits to treatment centre ($p<0.001$), those who took ART for ≤2 years ($p<0.001$), those whose adherence to ART was either average or poor ($p=0.03$), and patients from urban areas ($p<0.001$) were significantly associated with mortality status. There was no association between age ($p=0.602$), gender ($p=0.537$), marital status ($p=0.287$), educational status ($p=0.598$), occupation ($p=0.06$), history of smoking before starting ART ($p=0.582$), tobacco chewing after starting ART ($p=0.903$), smoking after starting ART ($p=0.817$), health status of the spouse ($p=0.125$), clinical staging of HIV at different intervals during the observation period after starting ART (p= ns), type of ART ($p=0.54$), age at starting ART ($p=0.627$), monthly income ($p=0.588$), BMI ($p=0.624$), and time since diagnosis to start of ART ($p=0.133$) with mortality status among patients in this study.

Mean CD4 count (cells per cu.mm) before the start of ART among patients who survived (n=241) was 402.5±235.3 in comparison to 266±206.1 among those who died (n=44). This difference in the mean CD4 counts was significant using unpaired t test, $t=3.602$, $p<0.001$.

There was no significant difference in the mean values of base line total leucocyte count ($p=0.7$) and base line haemoglobin levels ($p=0.454$) between patients who survived and died.

To calculate unadjusted hazard ratio, WHO clinical stage 1 of HIV was taken as reference value. Cox's regression was applied to the mortality data using variables significantly associated with it and the output is presented as Table 3.

A patient with any OI before the start of treatment was 2.251 times more likely to die in comparison to patient without any OIs. Similarly, patients with tuberculosis before starting treatment, unsatisfactory (poor/average) adherence to ART, and those with history of alcohol usage during treatment were at 2.22, 2.564 and 1.348 times, respectively, at greater risk of dying when compared with the rest. The confidence interval of these observation does not contain 1, indicating statistical significance (Table 3).

The cumulative proportion surviving was plotted against the survival times. This resulted in a stepped survival curve presented as Figure 1. The mean survival time after initiation of ART was found to be 2084.0±55.3 days (95% CI is 1975.5-2192.5).

The mean survival time for patients after detection of HIV in this study was 5583.5±249.3 days (95% CI is 5094.8-6072.2).

The hazard function h(t) which is the conditional probability of dying at time t is shown in Figure 2.

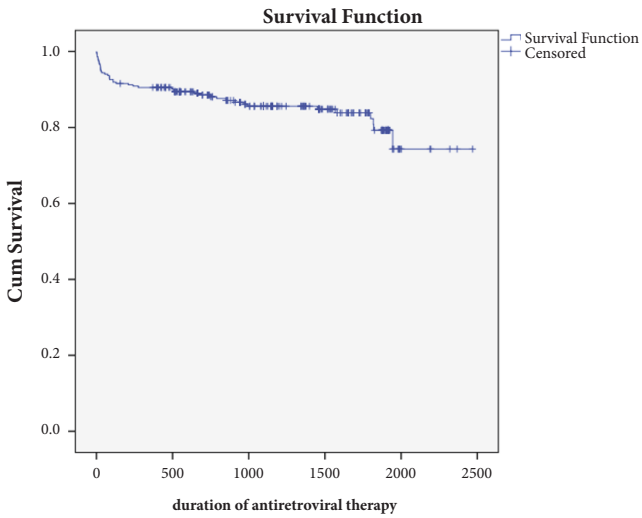

Figure 1: Kaplan-Meier survival curves illustrating cumulative survival probability of people living with HIV after initiation of ART (n=285).

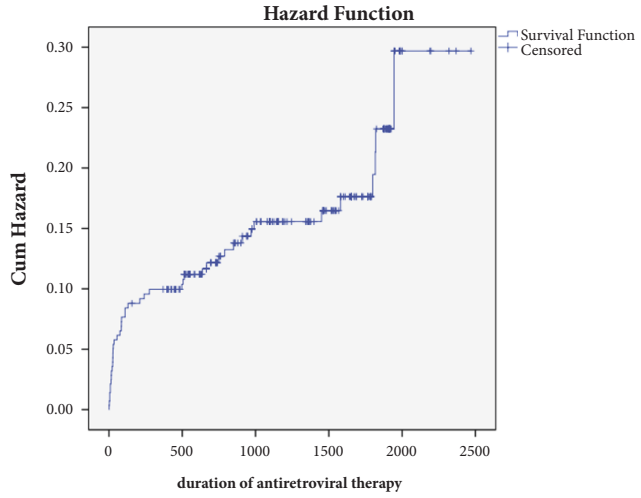

Figure 2: Cumulative hazard function of people living with HIV after starting ART (n=285).

4. Discussion

This study was done to identify the prognostic factors influencing mortality among a cohort of HIV infected adult patients on ART. It was observed that the presence of OIs and tuberculosis before and poor/average adherence to ART and alcohol usage after starting ART were independent prognostic factors of mortality among these patients.

The incidence density of death rate in this study was 3.12 per 100 PYO compared to other studies, where it was 1.75 [12], 2.03 [3], 3.4 [13], 3.8 [8], 5.1 [11], 7.6 [10], and 20.2 [14] PYO, respectively.

The proportion of patients who died in this study was 15.4% in comparison to two other studies done in Ethiopia, where it was reported as 9.4% [13] and 11.1% [11]. The proportion of patients who died was highest four or more years following diagnosis with HIV in the present study, as also observed in other studies [11, 15]. The proportion of deaths during this phase was 14.1% [11] and 21% [10, 15] in

Table 3: Multivariable Cox regression analysis of prognostic factors of mortality among HIV patients after starting ART (n=285).

Characteristics	Unadjusted HR	95% CI of unadjusted HR		P value	Adjusted HR	95% CI of Adjusted HR		P value
		Lower	Upper			Lower	Upper	
Presence of opportunistic infections before starting ART	3.504	1.691	7.26	<0.001	2.251	1.047	4.839	0.038
Presence of tuberculosis before starting ART	6.872	3.31	14.266	<0.001	2.22	1.074	4.592	0.031
Functional status at baseline	4.06	2.037	8.093	<0.001	0.644	0.442	0.936	0.021
WHO clinical staging of HIV at baseline	4.624	2.295	9.314	<0.001	1.266	0.965	1.66	0.089
Unsatisfactory adherence to treatment	3.013	1.067	8.511	0.03	2.564	1.001	6.571	0.05
History of alcohol usage after starting ART	4.51	1.795	11.332	0.001	1.348	1.02	1.781	0.036

comparison to 5.5% observed in this study. The wide variation in these observations as reported in different studies could be due to varying time of enrolment into ART care.

This study observed that the mean survival time was 46.7 months, among patients with the most recent CD4 count before death, as ≥200 cells per cu.mm. This was more than 44.2 months reported in a study done in Hyderabad, India [10]. This infers that the survival probability of patients in the present settings was better than that reported elsewhere. This may be due to their better access to ART and good follow-up visits to treatment centres.

Majority of deaths in this study occurred within 3 months of starting ART, as also observed in previous studies [8, 16, 17]. However other studies reported majority of deaths during first 4 months [7], 5 months [15], 6 months [10, 18–20], and within 1 year [3, 12] of starting ART. Early mortality among patients on treatment may be due to undiagnosed OIs [21]. Other reasons cited for this could be, late diagnosis leading to delay in starting ART at a time when the disease has progressed to the later stages [5]. These observations emphasize the importance of early diagnosis and initiation of ART for all PLHIV's. This needs to be supported with careful monitoring of patients during the early phase of treatment.

In this study, good adherence to ART was reported in 93.7% patients in comparison to 27.4% [20], 94.2% [15], and 97.5% [8] reported in other studies. Medication adherence is very essential to utilize maximum benefit of ART. Nonadherence increases risk of drug resistance and early treatment failure. The side effects of treatment which were associated with unsatisfactory adherence among patients in the present study need timely management to ensure good compliance with treatment.

In a study done in Ethiopia, an 8 percentage gain in weight was observed in the first 6 months of treatment [11]. However, minimal change in median weight was noticed in the subsequent months. In comparison, in this study there was notable increase in BMI after 6 months of ART, indicating good response with continual treatment.

Progressive change in CD4 count after initiation of ART was noticed in this study. However the increase in median CD4 count level from baseline values over the first 6 months of ART was lesser compared to 76.2% reported in an Ethiopian study [11].

Multivariable analysis in the present study showed that the presence of OIs, including tuberculosis before starting ART, to be associated with greater risk of mortality. Therefore, early identification of OIs will be helpful in identifying patients who need more intense follow-up for treatment of specific OI.

Similarly, the presence of tuberculosis infection as an independent prognostic factor of mortality was reported by previous studies [8, 11, 12, 15]. It is a known fact that mycobacterium tuberculosis can cause tuberculosis in HIV patients at any stage of the disease, irrespective of the severity of immunosuppression. It also features among the leading cause of death in HIV infection which might be the reason for this observation.

Another important independent prognostic factor of mortality observed in this study was poor/average pattern of adherence to ART, which was also supported by other studies [15, 22]. Careful follow-up of patients with unsatisfactory adherence, timely management of side effects related to ART, and providing them drug counselling for compliance are crucial to improve survival of HIV patients on treatment.

Further, history of usage of alcohol after starting ART was also an important prognostic factor of mortality. This was probably because it causes poor adherence with treatment. Another Ethiopian study too observed that patients reporting substance usage, with significant independent risk of mortality [12]. Avoidance of alcohol during course of treatment by counselling constitutes additional supportive measures for improving survival of PLHIV.

Previous studies using Cox regression analysis identified male gender [14, 15, 19, 23, 24], single marital status [11, 15], bedridden functional status [3, 11–13, 23], advanced WHO clinical stage [3, 8, 11–15, 22–25], underweight [11, 13, 14, 19], low CD4 count [3, 11, 14, 19, 22, 23], severe anaemia [8, 11, 14, 19, 22, 25], and older age [13, 15, 19, 23] as independent risk factors of mortality among PLHIV which was not seen in this study. There was no hazard difference for low and high CD4 count in this study, as three-fourth of the patients had CD4 count ≥200 cells per cu.mm. Also no hazard difference between early and advanced stage disease in this study supports the importance of early initiation of ART therapy irrespective of the clinical stage of the disease.

The proportion of deaths was minimal 4 years after diagnosis of HIV in this study when compared with previous studies. Similarly, the survival probability of patients was better than that reported in other settings, probably due to acceptable level of adherence to treatment and good number of follow-up visits by the patients. The CD4 count and BMI were found to increase among them, as soon as 6 months of treatment. However, majority of deaths were reported within 3 months of starting ART. This could be due to delayed diagnosis of HIV and associated OIs. Added to this, side effects of ART need to be attended to during follow-up visits. Therefore intensive monitoring of HIV patients on ART with various factors identified in multivariable analysis such as presence of OIs before the start of treatment and counselling for the alcoholics and those with poor/average adherence to treatment (probably due to drug side effects) is a must for prolonging the survival of the HIV affected patients in this settings.

5. Conclusion

This study highlights the mortality statistics among PLHIV and the factors influencing it in an urban tertiary care setting of coastal India. It was observed that clinical and simple laboratory data available to health care providers can predict patients on treatment who are at higher risk of mortality. Screening efforts need to be intensified for early detection and management of OIs among HIV infected patients both before and after the start of ART. Other measures like good counselling and periodic monitoring would improve adherence and might also prevent alcohol consumption after treatment with ART. Monitoring and frequency of visits need

to be more frequent in the first 3 months of starting ART, during which the proportion of deaths was found to be the most. These measures would help a long way in reducing mortality among PLHIV in the setting.

6. Limitation

This being a record based study has the limitation of availability of information, as stated in the records. There is also a possibility of deaths in this study which may have occurred due to causes not directly related to HIV/AIDS. The retrospective study design which limits analysis of the role of social and psychological factors and a single centric study which limits generalizability of information are other shortcomings of this research study.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Disclosure

This was a students' research project conducted during their research postings in the Department of Community Medicine, Kasturba Medical College, Manipal Academy of Higher Education, Mangalore, India. It was a self-funded study. The investigators of this study did not receive any financial support from the employers. Further no funder was involved in the manuscript writing, editing, approval, or decision to publish this research work.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Nitin Joseph is guarantor of this research work, concept, design, literature search, tool preparation, and manuscript preparation. Ushasti Sinha contributed to data collection, data analysis, statistical analysis, and interpretation of data. Nishtha Tiwari contributed to data collection, data entry, literature search, manuscript preparation, and manuscript editing. Pritha Ghosh contributed to data collection, data entry, and manuscript editing. Patneedi Sindhu contributed to data collection and manuscript editing. All authors approved the final manuscript before submission.

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