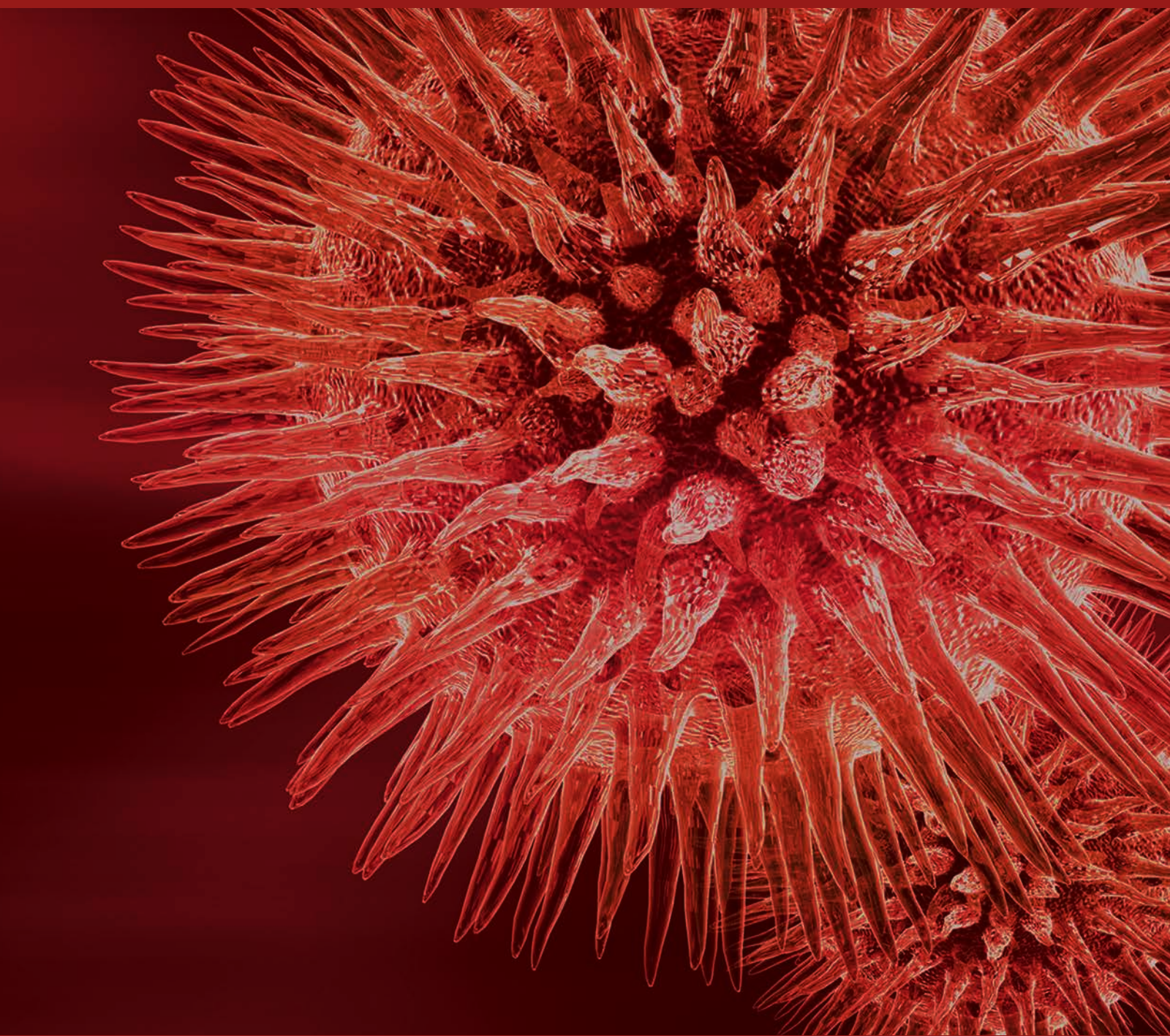
BioMed Research International

# Advances in Emerging and Neglected Infectious Diseases

Guest Editor: Charles Spencer and José Ronnie Vasconcelos



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# *Editorial*
# Advances in Emerging and Neglected Infectious Diseases

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Emerging and neglected infections have proven to be highly lethal with mortality rates ranging from 10 to 80%. However, with a low incidence of disease, less public attention and support have been directed at these infections. Much more research is devoted to infections with a large public health impact, neglecting these potential diseases. Nonetheless, there are numerous infectious diseases waiting for the evolutionary or nefarious alteration that will allow them to spread readily among the human population.

At the time of conception of this special issue, the Zika virus epidemic in the Western Hemisphere has been raging for over a year continually spreading northward from Brazil and Colombia into Latin America, Mexico, and finally the United States of America. Zika has grabbed the public's attention and forced it to pay attention to this emerging disease with over 125,000 cases in 40 countries. However, the Zika virus was originally identified in 1947 with the first human cases documented in 1952 in Uganda. Subsequently, only sporadic human infections were reported until the localized Yap Island outbreak in 2007. Zika virus then receded into obscurity until the recent explosion of Zika-associated microcephaly cases. However, this is just the latest in a string of emerging or neglected diseases to bask in the stage lights of the public health community. Similarly, the Ebola virus outbreak of 2014–2016 is just the latest in a long series of local, regional, and global outbreaks spanning all the way back to 1976. We cannot ignore pathogens with low incidences of disease as these are just the pathogens that could cause the next epidemic.

Therefore, we have assembled a series of manuscripts addressing the topic of this special issue. Manuscripts published in this special issue address topics in bacterial, viral, and parasitic infections ranging from novel treatments and diagnostics to epidemiology and clinical studies.

K. C. Kosinski et al. from the United States of America present a comparative epidemiological study of several assessments indicative of a clinical diagnostic of *Schistosoma haematobium* in Ghanaian children and found agreement between a dipstick self-test and clinical diagnosis of the presence of eggs in the urine.

A. Vina-Rodriguez et al. from Germany describe a novel TaqMan-based quantitative real-time PCR assay for the diagnosis and differentiation of *Venezuelan equine encephalitis virus*. This high throughput assay could greatly facilitate detection and surveillance of VEEV.

S. Petti et al. from Italy present an epidemiological study of healthcare works tending Ebola virus victims during the last outbreak. Despite having proper training and protective equipment, some of these healthcare workers became infected without direct exposure to blood/bodily fluids. Based on their retrospective study, the authors hypothesize that these healthcare workers became infected while working with asymptomatic patients, although they cannot discount unremembered exposure to blood/bodily fluids.

E. Franceschini et al. from Italy have characterized the clinical and microbiological features of an outbreak of visceral leishmaniasis in a province in Northern Italy concluding that despite being a nonendemic area for *Leishmania infantum*, rare diseases must be considered for symptoms with unknown etiology.

D. A. Yones et al. from Egypt compared the antihelminthic activity of edible and ornamental pomegranate extracts with the goal of identifying novel and effective new candidates for the development of drugs potent against *Schistosoma mansoni*.

P. Lou et al. from China present a novel algorithm for predicting and modelling *Brucellosis* epidemics that can be used to developing possible interventions to prevent local outbreaks from developing into epidemics.

M. P. Hernández-Rivera et al. from Mexico present the results of their association study between NRAMP1 polymorphisms and cutaneous leishmaniasis. This study could lead to greater accuracy in treatment of a diverse populace with cutaneous leishmaniasis.

C. L. Hoyos et al. from Argentina present an epidemiological study of the prevalence, magnitude, and risk factors of *Leishmania/Trypanosoma cruzi* coinfection in an endemic area of Argentina.

P. Gundelly et al. from the United States of America present the clinical findings of a group of patients in the USA infected with *Rhodococcus equi* discussing changes in the prevalence and magnitude of infections.

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*Research Article*

# Agreement among Four Prevalence Metrics for Urogenital Schistosomiasis in the Eastern Region of Ghana

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Few studies assess agreement among *Schistosoma haematobium* eggs, measured hematuria, and self-reported metrics. We assessed agreement among four metrics at a single time point and analyzed the stability of infection across two time points with a single metric. We used data from the Eastern Region of Ghana and constructed logistic regression models. Girls reporting macrohematuria were 4.1 times more likely to have measured hematuria than girls not reporting macrohematuria ($CI_{95\%}$: 2.1–7.9); girls who swim were 3.6 times more likely to have measured hematuria than nonswimmers ($CI_{95\%}$: 1.6–7.9). For boys, neither self-reported metric was predictive. Girls with measured hematuria in 2010 were 3.3 times more likely to be positive in 2012 ($CI_{95\%}$: 1.01–10.5), but boys showed no association. Boys with measured hematuria in 2008 were 6.0 times more likely to have measured hematuria in 2009 ($CI_{95\%}$: 1.5–23.9) and those with eggs in urine in 2008 were 4.8 times more likely to have eggs in urine in 2009 ($CI_{95\%}$: 1.2–18.8). For girls, measured hematuria in 2008 predicted a positive test in 2009 (OR = 2.8; $CI_{95\%}$: 1.1–6.8), but egg status did not. Agreement between dipstick results and eggs suggests continued dipstick used is appropriate. Self-reported swimming should be further examined. For effective disease monitoring, we recommend annual dipstick testing.

## 1. Introduction

Schistosomiasis is a neglected tropical disease (NTD). Surveillance and regular mass drug administration (MDA) are essential in highly endemic Sub-Saharan African countries, even as other countries begin to move towards elimination of the disease using additional primary prevention measures [1]. Substantial efforts were made to provide preventive chemotherapy (praziquantel) to at least 75% of school-aged children at risk of morbidity (WHA54.19). However, the goal was not achieved by the target year of 2010 and more work needs to be done to ensure that Sustainable Development Goal 3.3, end the NTD epidemic, is met by the year 2030 [2]. Specifically, it is necessary to have surveillance strategies that are cost-effective and accurate and these surveillance strategies should be used to determine where, when, and among which demographic groups to deploy MDA and water, sanitation, and hygiene (WASH) interventions [1, 3, 4].

For control of *Schistosoma haematobium*, the WHO currently recommends parasitological methods (finding eggs in urine), morbidity measurements (measured hematuria), or self-reported macrohematuria to identify "high-risk," "moderate-risk," and "low-risk" communities [5]. These three metrics are recorded at the level of an individual child and are used to estimate prevalence for a community [5]. However, agreement among these assessment metrics across communities and demographic groups remains to be determined, now that MDA with praziquantel is a widespread practice

in school-aged children. Following MDA, light infections (<50 eggs/10 mL urine) tend to be more common, and these light infections can be difficult to diagnose [6, 7]. In a recent review by Knopp et al. [4], the sensitivity and specificity of a variety of diagnostic tests (questionnaires, reagent strips, egg filtration, polymerase chain reaction (PCR), antibody-based assays, miracidium hatching test, and rapid diagnostic tests for *S. haematobium* infection) were discussed; in the public health literature, sensitivity and specificity are widely used measures of diagnostic test accuracy. Knopp et al. [4] found that questionnaires are not appropriate for diagnosis of light or very light infections; they also concluded that the dipstick test for microhematuria and filtration for *S. haematobium* eggs are appropriate for heavy infections, but only moderately appropriate for light infections.

Identification of endemic communities in resource-poor areas is challenging for a number of reasons. First, schistosomiasis is a focal disease [3, 5, 8–10] and communities that are geographically proximal may not have similar urogenital schistosomiasis (UGS) prevalence levels. Second, screening methods have varying levels of sensitivity and specificity [4, 7, 11, 12] and it is difficult to compare prevalence assessed via different screening tools. Third, the most widely used tools for population-level screening are likely to underestimate UGS prevalence following MDA [4, 6, 7, 13–16]. Finally, healthcare systems incur costs (e.g., labor, supplies, and time) with any screening program, and these costs must be minimized [3, 7, 17, 18].

In practice, identification of endemic communities by national health authorities may rely on expert opinion of healthcare workers, researchers, and officials; knowledge about past endemicity; passive case reporting [5]; nonsystematic observations; large-scale maps; or advocacy on the part of well-connected communities and their leaders. Identification can also be influenced by community accessibility and distance from urban areas. In Ghana, a patient with schistosomiasis symptoms who visits a local health facility is likely to be referred to a district-level healthcare facility that maintains stocks of praziquantel and a lab with diagnostic capability; the case is then reported from that district-level facility, which might be quite distant from where the patient actually lives and where the infection was contracted, providing little to no useful information about UGS transmission sites and endemic communities.

In addition to identifying high-prevalence communities, there is a need to identify communities with consistently low prevalence levels in order to reduce unnecessary treatment of populations that are not at risk [8], minimize community fatigue with MDA campaigns, and reduce the likelihood of drug resistant *S. haematobium*.

It is in the best interest of endemic communities and national health systems, particularly in low-income settings with limited resources, if rapid proxies for UGS can be used that correlate strongly with parasitological and morbidity measurements and with true underlying prevalence. We considered three metrics that are well established and widely used (measured hematuria via dipstick; *S. haematobium* eggs via filtration; self-reported macrohematuria) and the fourth that is not in widespread use (self-reported swimming at water contact sites). Swimming and recreational water contact are often associated with schistosomiasis [3, 19] because these activities often involve long contact times with infectious water bodies and a large percentage of skin exposure [20, 21], but other studies have not found the same relationship [22]. We hypothesized that this fourth metric, self-reported swimming behavior, would correlate with the outcomes of other diagnostics methods such as self-reported macrohematuria, measured hematuria, and parasitological methods [3, 21]. Considering the transmission pathways, agreement between measured hematuria and self-reported macrohematuria/swimming is an important validation of the appropriateness of using the two self-reporting methods in the field. Agreement between the two self-reported metrics could be useful in situations where it is preferable to ask schoolchildren whether or not they swim, rather than asking them to report macrohematuria.

With *S. haematobium* infection, there is high potential for reexposure and reinfection [19, 23]. From a public health perspective, reinfection is of interest for several reasons. First, reinfection suggests that individuals may have repeated behaviors [20] from lack of access to acceptable water infrastructure [22], lack of knowledge about schistosomiasis transmission mechanisms, or risky attitudes and practices such as inability/unwillingness to use existing water infrastructure. To test for temporal stability of infection status at the individual level, it is necessary to have a dataset with matched outcome data at multiple time points, which we used in our analyses.

We analyzed longitudinal datasets collected over four years in five different communities in the eastern region of Ghana to (a) assess agreement among four metrics at an individual level (eggs in urine, measured hematuria, self-reported macrohematuria, and self-reported swimming) at a single time point and (b) analyze the stability (i.e., reproducibility and predictability) of infection across two time points when using a single metric (measured hematuria or eggs via filtration). To achieve both objectives, we used logistic regression to assess the relationship between the outcome of interest and the primary predictor, controlling for age, sex, and town of residence. Our longitudinal data, gathered by tracking schoolchildren across two time points, allowed us to assess stability at the individual level, which is indicative of high potential for reinfection.

## 2. Materials and Methods

*2.1. Study Design.* The study utilized amalgamation of deidentified secondary data collected from previous projects [24]. The data included UGS prevalence data collected at four different time points and involved schoolchildren in five communities in the eastern region of Ghana (Figure 1). Four different metrics were used (*S. haematobium* eggs via filtration, measured hematuria, self-reported macrohematuria, and self-reported swimming) to collect data through the school system in different years (2008, 2009, 2010, and 2012).

There was no previous MDA with praziquantel in the study communities before 2008. Praziquantel was distributed

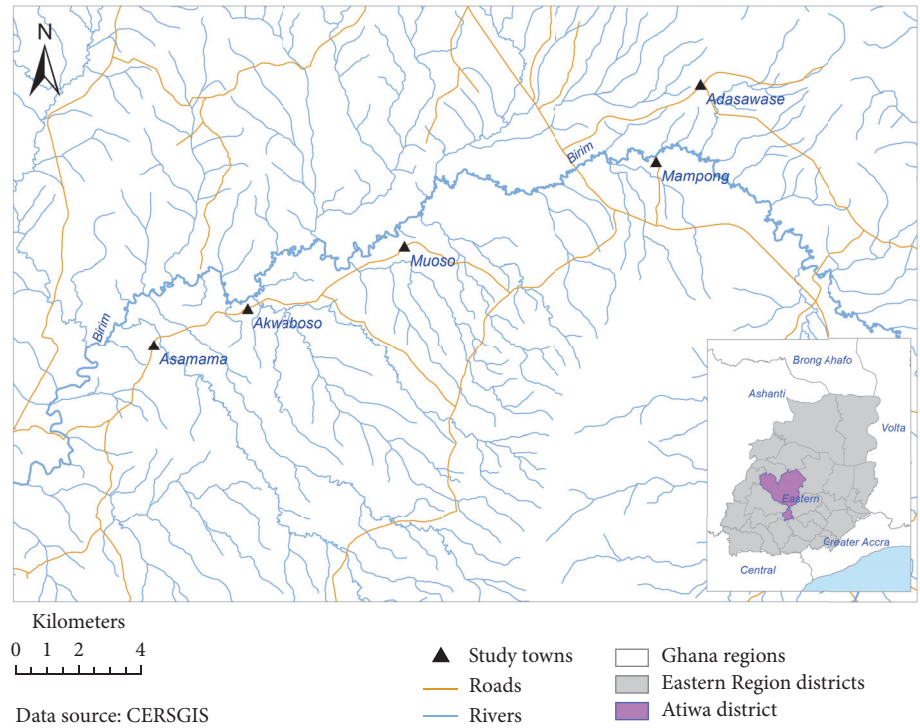


Figure 1: Relative locations of five study communities (black triangles), roads, and rivers; all study communities are located in Atiwa district in the eastern region of Ghana.

after screening for infection in each of the studies from which data was drawn. Treatment distribution methods depended on the prevalence of infection and guidance offered by GHS (Tables S1 and S2; see Supplementary Material available online at http://dx.doi.org/10.1155/2016/7627358). Treatment was also administered by schoolteachers with supervision of GHS nurses at some of the schools in Adasawase, Asamama, Mampong, and Bomaa in the summer of 2011 as part of the national deworming exercise.

*2.2. Study Population and Recruitment.* Data were originally collected during several studies that took place between 2008 and 2012 in five communities (Adasawase, Asamama, Akwaboso, Mampong, and Muoso) located in Atiwa district (Figure 1). In these original studies, screening and treatment took place through schools during the summer (May through July) of each study year (Table S1, Supplemental Material). For the present study, we extracted deidentified data from each of these original studies for the analyses conducted here. The process for obtaining permission, conducting community outreach, and recruiting participants was identical for all study communities and is described elsewhere [24]; the Institutional Review Board at Tufts University, Medford, Massachusetts, approved each of the original studies from which the deidentified data were drawn. Between 47.2% and 97.4% of children who were enrolled in school participated in the various studies, depending on the town and year of the study (Table S2, Supplemental Material).

*2.3. Parasitological and Morbidity Variables.* The parasitological metric used here was identification of eggs in urine. The morbidity measurement was the presence of measured hematuria (either micro- or macro-) via dipstick. All urine samples were tested for measureable hematuria via a semiquantitative dipstick test, regardless of whether they were visibly bloody, and categorized as a binary variable with any blood presence, including "trace," coded as a positive reading [12]. Urine samples were also tested for *S. haematobium* egg presence using filtration methods [24] and categorized as a binary variable with presence of >0.5 eggs/10 mL of urine coded as "1."

*2.4. Self-Reported Variables.* Self-reported macrohematuria and self-reported swimming were collected via one-on-one verbal interviews in a private location by a native Twi-speaker. Individuals were asked if they have seen macrohematuria within the past week and responses were recorded as "yes" or "no"; for teenage girls, the interviewer clarified that the question was not asking about menstrual blood. In order to assess self-reported swimming, participants were asked, "How often do you normally/currently go to the river?" and "What activities do you do at the river?" Participants were given a list of possible activities from which to choose or mentioned additional activities. The following is an exhaustive list of all activities performed (either from the provided list or mentioned by a participant): swim, play, do laundry, bathe, wash dishes, and fetch water to bring home. Each participant was told that "swimming" meant (a) traditional swimming

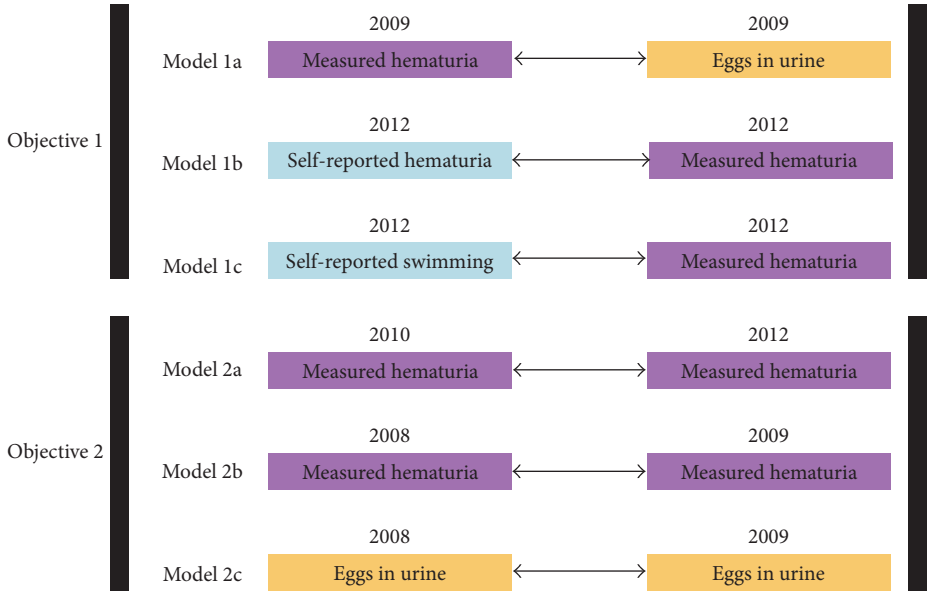


Figure 2: Overall study design: the two study objectives are shown along with the 6 analyses that were performed and the datasets that were used; "eggs in urine" refer to identifying *S. haematobium* eggs in urine samples; "measured hematuria" refers to micro- or macrohematuria via a semiquantitative dipstick; "self-reported macrohematuria" and "self-reported swimming" refer to self-reported presence of macrohematuria and swimming behavior, respectively, by the individual study participant in a private setting and not through hand-raising in a classroom. For logistic regression models, the left column refers to a predictor of interest ($X$) and right column refers to an outcome variable ($Y$).

in which the body is immersed, (b) splashing in the river for recreational purposes, or (c) bathing and occasionally splashing for recreational purposes; Kvalsvig and Schutte [20] similarly found it challenging to distinguish between swimming and washing behaviors. Participants who reported any of these swimming-related activities were recorded as responding "yes" to the question about swimming. During pilot testing of the study questions, we found that, for sociocultural reasons, this series of two questions was more likely to produce an affirmative response about self-reported swimming than simply asking "Do you swim?".

*2.5. Longitudinal Assessment.* For the longitudinal assessments across two time points, among the 324 children who provided a urine sample in 2010, 243 also provided a sample in 2012 (75% retention). Among the 458 children who provided a urine sample in 2008, 306 also provided a sample in 2009 (67% retention). There were no substantial differences in the sex, age, or town distribution in the samples from which data was drawn for longitudinal analyses (individuals who contributed two data points) as compared to the total population (individuals who contributed one data point and were therefore excluded).

*2.6. Data Analysis.* The study had two main objectives (Figure 2): in the first, we assessed the agreement between different metrics of infection at one time point (models 1a, 1b, and 1c) and in the second, we assessed the temporal stability of infection/reinfection over time as determined by two common metrics: eggs and blood in urine (models 2a, 2b, and 2c). A binary variable was defined for each of these four metrics: presence or absence of eggs in urine, presence or absence of measured hematuria, self-reported macrohematuria, and self-reported swimming (see above). Kendall's Tau, chi-square, and logistic regression models were run separately for boys and girls [25, 26]; logistic regression models utilized age category and town as covariates [19, 21]. Logistic regression models were selected given the ability to model a binary outcome variable and to control for factors such as age and town; the models were used to assess correlation without necessarily implying causation. Descriptive statistics of all variables are shown in Table 1.

We categorized the age variable into three groups: 6–10 years, 11–14 years, and 15+ years, and used the youngest group in the regression models as the reference category. The town variable was also categorized into three groups based on the cross-sectional prevalence of UGS at the first screening conducted in the community. Akwaboso, Mampong, and Muoso were in the first group, representing low UGS prevalence (<10%); Adasawase was in the second group (prevalence close to 20%); and Asamama was in the third group (prevalence close to 50%) (Table S2, Supplemental Material). Additionally, Adasawase was placed in group 2 by itself because a water recreation area (WRA) was constructed there and praziquantel was distributed regularly between 2008 and 2012 [27]. Thus, the inclusion of the location category in the model allowed us to control for town-level UGS prevalence.

Chi-square and Kendall's Tau tests were used to conduct exploratory data analysis; data were stratified by sex, age group, and town (Tables S3 and S4, Supplemental Material). For the exploratory analyses, we conducted post hoc power

Table 1: Descriptive statistics of variables used in each model.

| Variables of interest | Boys | | Girls | | All | |
|---|---|---|---|---|---|---|
| | Total (*N*) | *n* (%) | Total (*N*) | *n* (%) | Total (*N*) | *n* (%) |
| Model 1a: agreement between measured hematuria and eggs in urine | | | | | | |
| Measured hematuria in 2009 | 308 | 85 (27.6) | 349 | 105 (30.1) | 657 | 190 (28.9) |
| Eggs in urine in 2009 | 308 | 57 (18.5) | 349 | 86 (24.6) | 657 | 143 (21.8) |
| Age in years in 2009 | | | | | | |
| 6–10 | 308 | 140 (45.5) | 349 | 127 (36.4) | 657 | 267 (40.6) |
| 11–14 | 308 | 116 (37.7) | 349 | 148 (42.4) | 657 | 264 (40.2) |
| 15+ | 308 | 52 (16.9) | 349 | 74 (21.2) | 657 | 126 (19.2) |
| Town | | | | | | |
| Adasawase | 308 | 185 (60.1) | 349 | 226 (64.8) | 657 | 411 (62.6) |
| Asamama | 308 | 123 (39.9) | 349 | 123 (35.2) | 657 | 246 (37.4) |
| Models 1b and 1c: agreement between self-reported macrohematuria and swimming and measured hematuria | | | | | | |
| Measured hematuria | 292 | 41 (14.0) | 344 | 72 (20.9) | 636 | 113 (17.8) |
| Self-reported macrohematuria | 292 | 36 (12.3) | 344 | 77 (22.4) | 636 | 113 (17.8) |
| Self-reported swimming | 292 | 127 (43.5) | 344 | 198 (57.6) | 636 | 325 (51.1) |
| Age in years in 2012 | | | | | | |
| 6–10 | 292 | 116 (39.7) | 344 | 99 (28.8) | 636 | 215 (33.8) |
| 11–14 | 292 | 135 (46.2) | 344 | 162 (47.1) | 636 | 297 (46.7) |
| 15+ | 292 | 41 (14.0) | 344 | 83 (24.1) | 636 | 124 (19.5) |
| Town | | | | | | |
| Akwaboso, Mampong, and Muoso | 292 | 121 (41.4) | 344 | 160 (46.5) | 636 | 281 (44.2) |
| Adasawase | 292 | 84 (28.8) | 344 | 91 (26.5) | 636 | 175 (27.5) |
| Asamama | 292 | 87 (29.8) | 344 | 93 (27) | 636 | 180 (28.3) |
| Model 2a: agreement in measured hematuria in 2010 and 2012 | | | | | | |
| Measured hematuria in 2010 | 119 | 25 (21.0) | 124 | 20 (16.1) | 243 | 45 (18.5) |
| Measured hematuria in 2012 | 119 | 23 (19.3) | 124 | 23 (18.5) | 243 | 46 (18.9) |
| Age in years in 2012 | | | | | | |
| 6–10 | 119 | 30 (25.2) | 124 | 22 (17.7) | 243 | 52 (21.4) |
| 11–14 | 119 | 62 (52.1) | 124 | 76 (61.3) | 243 | 138 (56.8) |
| 15+ | 119 | 27 (22.7) | 124 | 26 (21) | 243 | 53 (21.8) |
| Town | | | | | | |
| Akwaboso, Muoso | 119 | 31 (26.1) | 124 | 39 (31.5) | 243 | 70 (28.8) |
| Asamama | 119 | 88 (73.9) | 124 | 85 (68.5) | 243 | 173 (71.2) |
| Models 2b and 2c: agreement between measured hematuria and eggs in urine in 2008 and 2009 | | | | | | |
| Measured hematuria in 2008 | 130 | 27 (20.8) | 171 | 47 (27.5) | 301 | 74 (24.6) |
| Measured hematuria in 2009 | 130 | 10 (7.7) | 171 | 24 (14) | 301 | 34 (11.3) |
| Eggs in urine in 2008 | 130 | 27 (20.8) | 171 | 43 (25.1) | 301 | 70 (23.3) |
| Eggs in urine in 2009 | 130 | 10 (7.7) | 171 | 29 (17) | 301 | 39 (13) |
| Age in years in 2008 | | | | | | |
| 6–10 | 130 | 38 (29.2) | 171 | 42 (24.6) | 301 | 80 (26.6) |
| 11–14 | 130 | 69 (53.1) | 171 | 90 (52.6) | 301 | 159 (52.8) |
| 15+ | 130 | 23 (17.7) | 171 | 39 (22.8) | 301 | 62 (20.6) |

calculations, restricted to samples collected from boys since this group consistently contained smaller sample sizes than those that involved girls. For the statistical tests conducted on 308 samples, we have sufficient power (>80%) to detect the difference of 6.7% and higher, given that true prevalence is ~25%. Similarly, for the analysis conducted on 292 samples, we have sufficient power to detect the difference of 7%. In measuring an agreement over time, the sample size of 119 and 130 allowed us to detect the difference of 10.5% and 10.1%, respectively. Following the exploratory analyses, we

constructed six logistic regression models (R version 3.1.2) (Figure 2) that were formulated as follows:

$$\text{logit}\,(Y_i) = \beta_0 + \beta_1 X_i + \beta_2 \text{Age}_i + \beta_3 \text{Town group}_i, \quad (1)$$

where $Y_i$ is an outcome and $X_i$ is a predictor of interest for $i$-child (see details below). All models were also run with the outcome ($Y_i$) and predictor of interest ($X_i$) reversed to test for consistency of association.

*2.6.1. Objective 1: Agreement among Diagnostic Metrics.* In model 1a, we assessed the agreement between eggs in urine ($Y_i$) and measured hematuria ($X_i$) for data collected in Adasawase and Asamama in 2009. Adasawase had experienced MDA with praziquantel in July 2008 while Asamama had no systematic distribution of praziquantel in the years immediately prior to the study. In model 1b, we assessed the agreement between measured hematuria ($Y_i$) and self-reported macrohematuria ($X_i$) collected in Adasawase, Akwaboso, Asamama, Mampong, and Muoso in 2012. In model 1c, we assessed the agreement between measured hematuria ($Y_i$) and self-reported swimming ($X_i$) collected in those same towns in 2012. We used odds ratios with 95% confidence intervals (OR, $\text{CI}_{95\%}$) to interpret the association between the main predictor and outcome in each model. A supplemental model was also run to assess self-reported macrohematuria and swimming as a vector of predictors ($X_i$). We extracted model-predicted probability values from this supplemental model (i.e., likelihood of correctly predicting infection status via dipstick using self-reported metrics), estimated as the average predicted probability for all groups of children (branches of the classification tree). We used these predicted probabilities to assess whether each self-reported metric contributed to the model's overall ability to predict measured hematuria.

*2.6.2. Objective 2: Temporal Stability of Infection Status.* In model 2a, we assessed the temporal stability between measured hematuria in 2012 ($Y_i$) and 2010 ($X_i$), collected from Akwaboso, Asamama, Mampong, and Muoso. Data for Adasawase were omitted from the analysis because UGS prevalence was very low in 2010 (<8%), reflecting the presence of an intervention beyond treatment with praziquantel. In model 2b, we assessed the temporal stability between measured hematuria in 2009 ($Y_i$) and 2008 ($X_i$). In model 2c, we assessed the temporal stability between eggs in urine in 2009 ($Y_i$) and 2008 ($X_i$). Data for models 2b and 2c were only available for Adasawase. Similar to Objective 1, statistically significant association between metrics at two time points was assessed using ORs and $\text{CI}_{95\%}$. We also extracted model-predicted probabilities for models 2a, 2b, and 2c to compare predictive ability of past infection status to current infection status.

## 3. Results

*3.1. Agreement among Metrics.* An exploratory analysis of agreement between *S. haematobium* eggs in urine and measured hematuria showed a statistically significant strong relationship for all groups (chi-square tests: Table S3, Supplemental Material; Kendall's Tau: boys = 0.547, $p < 0.0001$; girls = 0.669, $p < 0.0001$). Regression models, which controlled for age and town, also showed a very stable association between measured hematuria and eggs. For girls, logistic regression analysis showed that the Adj.-OR of having eggs in urine increases if the girl tests positive for measured hematuria (Table 2, model 1a).

An exploratory analysis of agreement between measured hematuria and self-reported metrics demonstrated that self-reported macrohematuria and swimming were significant predictors of measured hematuria for girls (Adj.-OR of 3.2 and 2.4 for self-reported macrohematuria and self-reported swimming, respectively) (Table S5, Supplemental Material). For boys, self-reported macrohematuria was not significantly predictive of measured hematuria, but there appeared to be a weak potential association between self-reported swimming and measured hematuria (Table S5, Supplemental Material). The logistic regression models that followed the exploratory analyses controlled for age and town; they showed that girls who self-report macrohematuria are 4.09 times more likely to have measured hematuria than those who do not self-report macrohematuria ($\text{CI}_{95\%}$: 2.1–7.9) (Table 2, model 1b). Girls who self-report swimming are 3.6 times more likely to have measured hematuria than nonswimmers ($\text{CI}_{95\%}$: 1.6–7.9) (Table 2, model 1c). For boys, neither self-reported metric significantly predicted measured hematuria in the logistic regression models (Table 2, models 1b and 1c).

Figure 3 shows model-predicted probability (Table S5, Supplemental Material). In this study, a child had an average probability of 0.18 ± 0.16 to test positive for measured hematuria (0.21 ± 0.20 for girls and 0.14 ± 0.09 for boys). For a girl who self-reported swimming only, the probability of a positive dipstick test was 0.21 ± 0.15; the corresponding probability was 0.45 ± 0.22 if she also self-reported macrohematuria. A girl who answered "no" to both questions had a probability of 0.09 ± 0.08 to test positive. Boys who self-reported swimming only or swimming and macrohematuria had about the same probability of testing positive for measured hematuria: 0.22±0.07 and 0.22±0.06, respectively. Boys who self-reported neither macrohematuria nor swimming had a probability of 0.08 ± 0.03 of testing positive.

*3.2. Infection Status Stability across Time.* When controlling for age and town, girls who were positive for measured hematuria in 2010 were 3.3 ($\text{CI}_{95\%}$: 1.0–10.5) times more likely to be positive in 2012 as well (Table 3, model 2a). A statistically significant association was found between the 2008 and 2009 measured hematuria status for girls (OR = 2.8; $\text{CI}_{95\%}$: 1.1–6.8) (Table 3, model 2b). However, egg status in 2008 was not predictive of egg status in 2009 in the same data set (Table 3, model 2c). For boys, the presence of either eggs or measured hematuria in 2008 is equally predictive of egg or measured hematuria status in 2009. Controlling for age, boys with eggs in urine in one year are 4.8 times more likely to have eggs in urine in the subsequent year ($\text{CI}_{95\%}$: 1.2–18.8) and those with measured hematuria in one year are 6.0 times more likely to

Table 2: Results of logistic regression models showing the agreement between prevalence metrics (models 1a, 1b, and 1c).

| | Boys | | Girls | |
|---|---|---|---|---|
| | Adj-OR | $CI_{95\%}$ | Adj-OR | $CI_{95\%}$ |
| Model 1a: $Y_i$ = eggs in urine | | | | |
| Measured hematuria | **23.84**[d] | **(9.83, 57.80)** | **40.00** | **(18.22, 87.83)** |
| Ages 11–14[a] | 0.94 | (0.42, 2.09) | 0.90 | (0.43, 1.88) |
| Ages 15+ | 0.47 | (0.18, 1.23) | 0.65 | (0.26, 1.62) |
| Town: Asamama[b] | 0.89 | (0.38, 2.11) | 0.79 | (0.37, 1.69) |
| Model 1b: $Y_i$ = measured hematuria | | | | |
| Self-reported macrohematuria | 1.04 | (0.39, 2.74) | **4.09** | **(2.12, 7.89)** |
| Ages 11–14 | 0.74 | (0.35, 1.56) | 1.31 | (0.67, 2.56) |
| Ages 15+ | 1.07 | (0.40, 2.88) | 0.67 | (0.28, 1.62) |
| Town: Adasawase[c] | 1.67 | (0.65, 4.31) | **10.68** | **(4.41, 25.91)** |
| Town: Asamama[c] | **4.30** | **(1.85, 9.99)** | **10.68** | **(4.58, 24.90)** |
| Model 1c: $Y_i$ = measured hematuria | | | | |
| Self-reported swimming | 2.10 | (0.87, 5.05) | **3.60** | **(1.64, 7.94)** |
| Ages 11–14 | 0.77 | (0.36, 1.63) | 0.97 | (0.50, 1.88) |
| Ages 15+ | 1.21 | (0.44, 3.36) | 0.63 | (0.26, 1.49) |
| Town: Asamama[c] | 1.41 | (0.53, 3.75) | **10.91** | **(4.49, 26.49)** |
| Town: Asamama[c] | 2.60 | (0.94, 7.02) | **8.14** | **(3.49, 19.02)** |

[a]Age group 1 (ages 6–10 years) was the reference category for the "age" variable for all models.
[b]Town group 2 (Adasawase) was the reference category for the "town" variable.
[c]Town group 1 (Akwaboso, Mampong, and Muoso) was the reference category for the "town" variable.
[d]Bold indicates statistical significance at the 0.05 level.

Table 3: Results of logistic regression models showing the temporal stability of infection status as assessed by two metrics.

| | Boys | | Girls | |
|---|---|---|---|---|
| | OR | $CI_{95\%}$ | OR | $CI_{95\%}$ |
| Model 2a: $Y_i$ = measured hematuria in 2012 | | | | |
| Measured hematuria in 2010 | 2.0 | (0.65, 5.9) | **3.3** | **(1.01, 10.5)** |
| Age group 2 | 0.84 | (0.27, 2.7) | 0.65 | (0.18, 2.3) |
| Age group 3 | 1.7 | (0.45, 6.3) | 0.64 | (0.13, 3.2) |
| Town group 3 | 1.6 | (0.47, 5.2) | —[a] | — |
| Model 2b: $Y_i$ = measured hematuria in 2009 | | | | |
| Measured hematuria in 2008 | **6.0** | **(1.5, 23.9)** | **2.8** | **(1.1, 6.8)** |
| Age group 2 | 1.1 | (0.18, 6.4) | 1.00 | (0.35, 2.9) |
| Age group 3 | 2.5 | (0.38, 16.2) | 0.56 | (0.14, 2.2) |
| Model 2c: $Y_i$ = eggs in urine in 2009 | | | | |
| Eggs in urine in 2008 | **4.8** | **(1.2, 18.8)** | 1.9 | (0.79, 4.5) |
| Age group 2 | 0.80 | (0.17, 3.7) | 0.95 | (0.37, 2.4) |
| Age group 3 | 0.67 | (0.09, 4.8) | 0.44 | (0.12, 1.6) |

[a]Standard error is too large for town group 3 in model 2a.

have measured hematuria in a subsequent year ($CI_{95\%}$: 1.5–23.9) (Table 3, models 2b and 2c). A similar association was not observed in the 2010 to 2012 analysis (Table 3, model 2a).

Knowing an individual's infection status one or two years before offers additional predictive capacity over just knowing their sex (Figures 4 and 5). In analysis 2a (Figure 4), a child had an average predicted probability of $0.19 \pm 0.12$ to test positive for measured hematuria via dipstick in 2012; this probability was the same for girls and boys ($p = 0.19 \pm 0.16$ and $0.19 \pm 0.07$, resp.). For a girl who was positive by dipstick in 2010, the probability increased to $0.40 \pm 0.21$, as compared to a girl who was negative in 2010 ($p = 0.14 \pm 0.11$). A similar comparison can be made for boys who were positive in 2010 ($p = 0.28 \pm 0.05$) versus negative ($p = 0.17 \pm 0.05$). Lower overall prevalence in 2009 as compared to 2012 contributed to slightly lower probabilities in Figure 5; the associations between prior (in this case one year before) and current measured hematuria status were maintained for boys

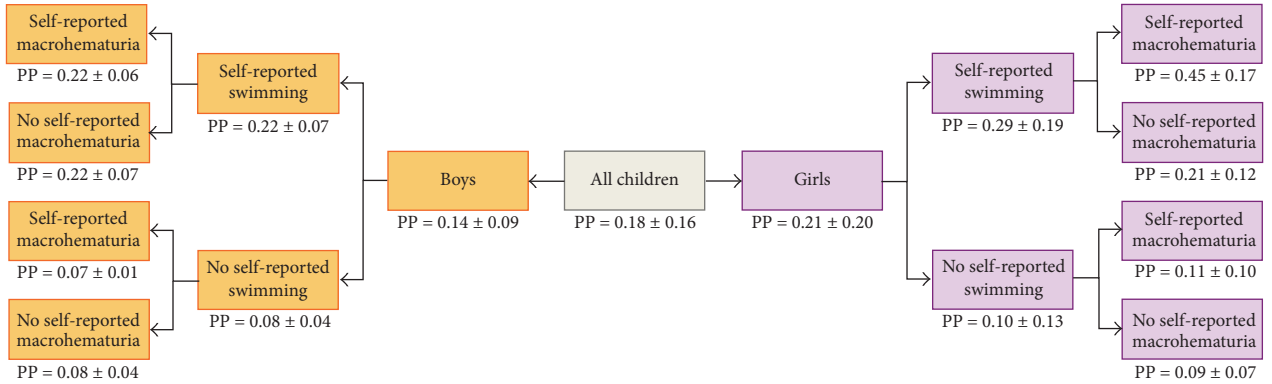


FIGURE 3: Predicted probability (PP) (mean ± SD) of testing positive for measured hematuria given information about self-reported swimming behavior and self-reported macrohematuria.

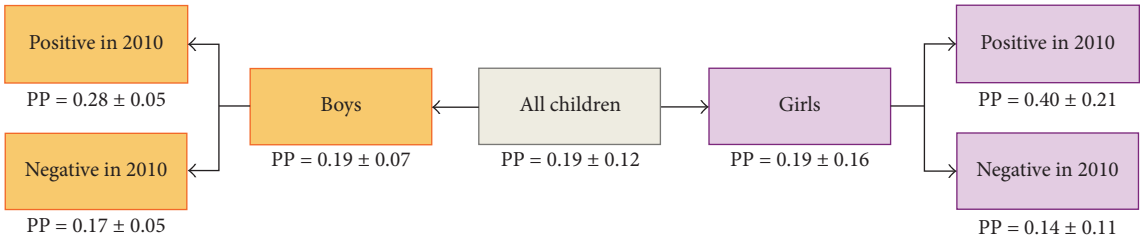


FIGURE 4: Predicted probability of testing positive for measured hematuria in 2012 as a function of measured hematuria status in 2010 (model 2a).

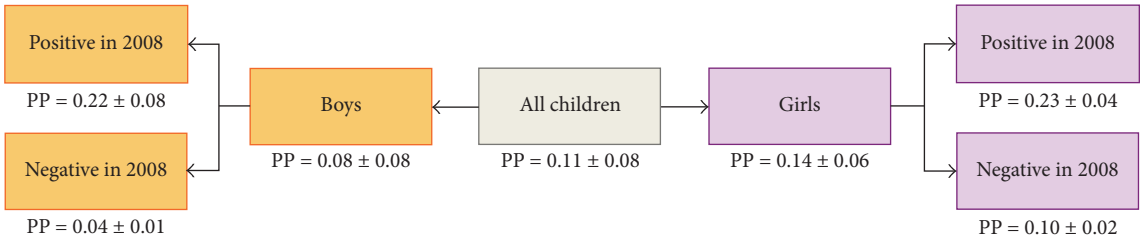


FIGURE 5: Predicted probability of testing positive for measured hematuria in 2009 as a function of measured hematuria status in 2008 (model 2b).

and for girls (model 2b). The analogous figure for model 2c is available in the supplemental material (Figure S1, Supplemental Material).

## 4. Discussion

*4.1. Agreement among Metrics.* Very stable associations between measured hematuria and *S. haematobium* eggs for both boys and girls were detected when these metrics were collected at the same point in time (model 1a). Findings differed somewhat for self-reported metrics. Self-reported data collected from girls showed a statistically significant relationship between self-reported macrohematuria and dipstick and between self-reported swimming and dipstick (models 1b and 1c). For boys, neither of the self-reported metrics significantly predicted measured hematuria via dipstick (models 1b and 1c). This suggests that self-reporting may be sufficient for girls when broadly assessing UGS prevalence, but prevalence among boys may need to be checked using parasitological or morbidity measurements. While this is true in the study communities, an assessment of this trend at a broader scale is needed before widely using self-reporting metrics in areas that regularly experience MDA.

Direct comparison of our results with those of others is challenging. In 1995, the Red Urine Study Group published a report discussing the diagnostic accuracy of methods to identify communities in which individuals are likely to experience high morbidity from schistosomiasis. This was a questionnaire-based approach in which teachers asked schoolchildren to self-report symptoms [8]. This approach differs from the work we report here in that the Red Urine group collected data at the level of the classroom and we considered the correlation between metrics at the level of the

individual. Moreover, the emphases of the two studies were different, with the Red Urine Study Group seeking to identify high morbidity locations [8] and our study assessing low prevalence communities as well. Self-reported swimming was assessed by Hammad et al. [19] as a risk factor for infection, but the outcome variable was *S. haematobium* ova in urine via filtration, and the precise method of filtration was not described clearly enough to compare with our results; the authors' objective was not to assess self-reported swimming as a proxy for infection status. Satayathum et al. [22] found that there was no association between the frequency of water contact and infection status, but the water contact was observed rather than self-reported, and the variable was the amount of contact (>10 contacts versus ≤10 contacts), not necessarily swimming contact, specifically. Rudge et al. [21] assessed correlation between self-reported swimming/playing and infection status as determined via filtration of 10 mL of urine; they found associations for boys and girls during univariate analyses, but these were not significant in multivariate logistic regression models that included distance to water contact sites from a child's home hamlet; the distance variables were much more predictive of infection status than self-reported swimming/playing. As part of a larger study in Tanzania, Knopp et al. [28] assessed the relationship between *S. haematobium* eggs in 10 mL urine samples and reagent strip readings; they found a significant, strong correlation in children aged 9–12 years, which is the age group most comparable to the age groups we studied. Knopp et al. [28] also found a statistically significant relationship between the color of urine samples and the number of *S. haematobium* eggs in a sample.

In our study, the model used to assess whether the two self-reported metrics (self-reported swimming and macrohematuria) each contributed independent predictive ability (Figure 3, Table S5, Supplemental Material) showed that, for girls, knowing both self-reported metrics provides improvement over knowing just one. For boys, knowing only self-reported swimming status might be sufficient; self-reported macrohematuria status does not change predicted probability of infection. Because of the low cost and ease of collecting this type of data during prevalence studies, there are clear advantages to using self-reported metrics. However, the predictive capacity of these two metrics needs to be evaluated in a larger study with sufficient power to detect the desirable change, which is an important direction for future research. Finally, a limitation to self-reported macrohematuria for girls is that macrohematuria could be confused with menstruation.

The ability to consider the exposure location(s), given the focality of the disease, might provide additional insights into disease transmission. In exploring where the exposure may have occurred, we observed that the communities in our study are very small and all surface water contact locations are within walking distance. Some communities have 9+ water contact sites on a single stream, so it would be challenging to determine which site is which based on a child's verbal description and the data quality could be questionable. Furthermore, in many communities, children swim with friends and might vary their swimming location depending on whom they swim with. Thus, we assumed that by asking questions about swimming/playing in water, we might capture a predictive factor of infection; a future study may explore whether the specific locations and/or the number of river access points have a better predictive capacity.

*4.2. Temporal Stability of Infection.* Model 2a showed that girls who were positive for measured hematuria in 2010 were significantly more likely (Adj.-OR = 3.3, $CI_{95\%}$ 1.0–10.5) to be positive again in 2012 than their girl classmates who tested negative for measured hematuria in 2010, although $CI_{95\%}$ is somewhat wide. For boys, no significant association was found for measured hematuria between 2010 and 2012. In contrast to model 2a, model 2b was constructed with data collected only one year apart; measured hematuria status in 2008 was predictive of measured hematuria in 2009 for both girls and boys (Adj.-OR = 2.8, $CI_{95\%}$ 1.1–6.8; Adj.-OR = 6.0, $CI_{95\%}$ 1.5–23.9, respectively), but for boys in particular, $CI_{95\%}$ is wide, indicating some uncertainty. The findings from model 2c showed less temporal stability for girls, with no statistically significant association between *S. haematobium* eggs in 2008 and 2009. For boys, there was a significant association (Adj.-OR = 4.8, $CI_{95\%}$ 1.2–18.8), but again, a wide confidence interval. In Egypt, Hammad et al. [19] found no association between history of infection and an individual's current infection status, but the authors did not explain how history of infection was determined so results cannot be directly compared with ours. Satayathum et al. [22] found that prior hematuria status measured by dipstick was a significant independent predictor of infection status during a 9-year longitudinal study.

The differences in temporal stability over a period of either one or two years could have various causes. First, they could be due to differences in the study population; the 2010–2012 dataset included low prevalence communities and the 2008-2009 dataset did not. While we constructed the models to account for this factor, it is likely that the small number of towns precluded us from fully controlling for this effect. The differences may also stem from the introduction of national MDA in the study area in recent years. MDA was conducted in 2011, almost exactly a year after the 2010 study and a year before the 2012 study. Finally, there are probably gendered differences in the way children interact with surface water that vary in consistency and intensity over time [22]. Swimming is not the only risk factor for UGS [3, 19]; wading through water and consistent fetching of surface water may be more risky than occasional swimming. Domestic water collection and use are also behaviors that are unlikely to change in the presence of education on how to reduce UGS risk, if accompanying infrastructure is not improved or made more accessible. Future studies should seek to assess gender dynamics and additional aspects of surface water use. Finally, the role played by acquired immunity to schistosomiasis should be further assessed, as this could impact the ability to predict current infections based on previous infections [22]. In a practical sense, and with an eye to Sustainable Development Goal 3.3 [2], our results suggest that broad-scale studies in various geospatial and temporal contexts

should be undertaken that follow school-aged children over time in the presence of MDA; it should not be assumed that MDA should be distributed in communities solely because schistosomiasis has historically been endemic or that it is unneeded in communities that have traditionally had low prevalence.

## 5. Conclusions

Our study shows that, in a population of school-aged children where MDA with praziquantel regularly occurs, four different metrics of infection agree for girls and two agree for boys. Thus, where it is affordable and available, the dipstick test can be used for broad-scale prevalence studies with school-aged children, but it is known to underestimate total prevalence unless it is used repeatedly [12], so allowance should be made to offer praziquantel more widely, even to individuals who have not tested positive for measured hematuria. If prevalence data are to be collected via dipstick, we also recommend that data about age, sex, and location be collected; our data shows that performance varies according to these characteristics. The dipstick is a particularly attractive option in comparison with egg filtration, given that cost is generally much lower and it is relatively easy to use. We suggest that self-reported swimming be studied further in broader contexts as a predictor of infection given that it performed reasonably well for girls in our study; if it is found in a larger study to perform well for both boys and girls, this measure could augment self-reported macrohematuria in situations where self-reporting is the only practical option for prevalence assessment. Finally, the observed change in infection status over time is an important tool for disease monitoring; additional large-scale testing should be conducted with both measured hematuria and *S. haematobium* egg data, given that there are differences in how these two metrics perform in boys versus girls and with respect to each other. If these metrics are used to assess the efficacy of disease control strategies, they may be able to provide valuable information about which demographic groups continue to experience transmission over time, so that control measures can be further focused.

## Additional Points

*Author Summary*. Schistosomiasis affects over 200 million people worldwide. Major symptoms of urogenital schistosomiasis include blood and parasite eggs in urine. Many methods can be used for diagnosis, but diagnosing infection is becoming increasingly difficult due to population-based treatment, which causes a high proportion of "light" infections in which it is more difficult to find eggs or blood. We assessed relationships among parasite eggs, blood in urine, and two self-reported metrics (swimming and visibly bloody urine) and we examined the stability of eggs and blood over time in 5 Ghanaian communities. Using logistic regression models, we found a strong relationship between eggs and bloody urine, which was expected. We also found that self-reported metrics performed relatively well for girls but not boys. The results for longitudinal stability of eggs and blood varied for boys and girls, depending on the metric and the gap between the two assessment times. Given the agreement between the blood and eggs, we recommend the dipstick test for blood for broad use. We recommend that self-reported swimming and self-reported blood in urine be examined further prior to being widely used in the field. Finally, the observed change in infection status over time is an informative tool for disease monitoring and annual dipstick testing should be implemented to monitor and inform disease control strategies.

## Disclosure

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

## Competing Interests

The authors declare that they have no competing interests.

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## References

[1] World Health Organization, *Sixty-Fifth World Health Assembly*, World Health Organization, Geneva, Switzerland, 2012.

[2] United Nations General Assembly, Transforming our world: the 2030 Agenda for Sustainable Development in United Nations General Assembly, ed. 70/1: United Nations, 2015.

[3] T. Handzel, D. M. S. Karanja, D. G. Addiss et al., "Geographic distribution of schistosomiasis and soil-transmitted helminths in Western Kenya: implications for anthelminthic mass treatment," *The American Journal of Tropical Medicine and Hygiene*, vol. 69, no. 3, pp. 318–323, 2003.

[4] S. Knopp, S. L. Becker, K. J. Ingram, J. Keiser, and J. Utzinger, "Diagnosis and treatment of schistosomiasis in children in the era of intensified control," *Expert Review of Anti-Infective Therapy*, vol. 11, no. 11, pp. 1237–1258, 2013.

[5] World Health Organization, *Helminth Control in School-Age Children: A Guide for Managers of Control Programmes*, World Health Organization, Geneva, Switzerland, 2011.

[6] K. M. Bosompem, O. Owusu, E. O. Okanla, and S. Kojima, "Applicability of a monoclonal antibody-based dipstick in diagnosis of urinary schistosomiasis in the Central Region of Ghana," *Tropical Medicine and International Health*, vol. 9, no. 9, pp. 991–996, 2004.

[7] H. Feldmeier and G. Poggensee, "Diagnostic techniques in schistosomiasis control. A review," *Acta Tropica*, vol. 52, no. 4, pp. 205–220, 1993.

[8] Red Urine Study Group, *Identification of High-Risk Communities for Schistosomiasis in Africa: A Multicountry Study*, UNDP/WORLD BANK/WHO Special Prorgramme for Research and Training in Tropical Diseases (TDR), 1995.

[9] A. Kabore, N.-K. Biritwum, P. W. Downs, R. J. Soares Magalhães, Y. Zhang, and E. A. Ottesen, "Predictive vs. empiric assessment of schistosomiasis: implications for treatment projections in Ghana," *PLoS Neglected Tropical Diseases*, vol. 7, no. 3, Article ID e2051, 2013.

[10] L. Van Lieshout and M. Yazdanbakhsh, "Landscape of neglected tropical diseases: getting it right," *The Lancet Infectious Diseases*, vol. 13, no. 6, pp. 469–470, 2013.

[11] J. T. Coulibaly, Y. K. N'Gbesso, N. A. N'Guessan, M. S. Winkler, J. Utzinger, and E. K. N'Goran, "Epidemiology of schistosomiasis in two high-risk communities of south Cote d'Ivoire with particular emphasis on pre-school-aged children," *The American Journal of Tropical Medicine and Hygiene*, vol. 89, no. 1, pp. 32–41, 2013.

[12] K. C. Kosinski, K. M. Bosompem, M. J. Stadecker et al., "Diagnostic accuracy of urine filtration and dipstick tests for *Schistosoma haematobium* infection in a lightly infected population of Ghanaian schoolchildren," *Acta Tropica*, vol. 118, no. 2, pp. 123–127, 2011.

[13] E. Saathoff, A. Olsen, P. Magnussen, W. Becker, and C. C. Appleton, "Patterns of *Schistosoma haematobium* infection, impact of praziquantel treatment and re-infection after treatment in a cohort of schoolchildren from rural KwaZulu-Natal/South Africa," *BMC Infectious Diseases*, vol. 4, article no. 40, 2004.

[14] L. S. Stephenson, M. C. Latham, S. N. Kinoti, and M. L. Oduori, "Sensitivity and specificity of reagent strips in screening of Kenyan children for *Schistosoma haematobium* infection," *The American Journal of Tropical Medicine and Hygiene*, vol. 33, no. 5, pp. 862–871, 1984.

[15] World Health Organization, *Report of the WHO Informal Consultation on Schistosomiasis in Low Transmission Areas: Control Strategies and Criteria for Elimination*, World Health Organization, London, UK, 2001.

[16] World Health Organization, "Report on schistosomiasis. Scientific working group on schistosomiasis," Tech. Rep. TDR/SWG/07, Special Programme for Research and Training in Tropical Diseases (TDR), Geneva, Switzerland, 2005.

[17] C. Lengeler, P. Kilima, H. Mshinda, D. Morona, C. Hatz, and M. Tanner, "Rapid, low-cost, two-step method to screen for urinary schistosomiasis at the district level: the Kilosa experience," *Bulletin of the World Health Organization*, vol. 69, no. 2, pp. 179–189, 1991.

[18] J. Utzinger, E. K. N'Goran, M. Tanner, and C. Lengeler, "Simple anamnestic questions and recalled water-contact patterns for self-diagnosis of *Schistosoma mansoni* infection among schoolchildren in western Côte d'Ivoire," *The American Journal of Tropical Medicine and Hygiene*, vol. 62, no. 5, pp. 649–655, 2000.

[19] T. A. Hammad, N. S. Gabr, M. H. Hussein, A. Orieby, E. Shawky, and G. T. Strickland, "Determinants of infection with schistosomiasis haematobia using logistic regression," *American Journal of Tropical Medicine and Hygiene*, vol. 57, no. 4, pp. 464–468, 1997.

[20] J. D. Kvalsvig and C. H. J. Schutte, "The role of human water contact patterns in the transmission of schistosomiasis in an informal settlement near a major industrial area," *Annals of Tropical Medicine and Parasitology*, vol. 80, no. 1, pp. 13–26, 1986.

[21] J. W. Rudge, J. R. Stothard, M.-G. Basáñez et al., "Micro-epidemiology of urinary schistosomiasis in Zanzibar: local risk factors associated with distribution of infections among schoolchildren and relevance for control," *Acta Tropica*, vol. 105, no. 1, pp. 45–54, 2008.

[22] S. A. Satayathum, E. M. Muchiri, J. H. Ouma, C. C. Whalen, and C. H. King, "Factors affecting infection or reinfection with *Schistosoma haematobium* in Coastal Kenya: survival analysis during a nine-year, school-based treatment program," *The American Journal of Tropical Medicine and Hygiene*, vol. 75, no. 1, pp. 83–92, 2006.

[23] C. F. Hatz, B. J. Vennervald, T. Nkulila et al., "Evolution of *Schistosoma haematobium*-related pathology over 24 months after treatment with praziquantel among school children in southeastern Tanzania," *American Journal of Tropical Medicine and Hygiene*, vol. 59, no. 5, pp. 775–781, 1998.

[24] K. C. Kosinski, M. N. Adjei, K. M. Bosompem et al., "Effective control of *Schistosoma haematobium* infection in a Ghanaian community following installation of a water recreation area," *PLoS Neglected Tropical Diseases*, vol. 6, no. 7, Article ID e1709, 2012.

[25] C. H. King, R. E. Blanton, E. M. Muchiri et al., "Low heritable component of risk for infection intensity and infection-associated disease in urinary schistosomiasis among Wadigo village populations in coast province, Kenya," *American Journal of Tropical Medicine and Hygiene*, vol. 70, no. 1, pp. 57–62, 2004.

[26] R. Ndyomugyenyi and J. N. Minjas, "Urinary schistosomiasis in schoolchildren in Dar-es-Salaam, Tanzania, and the factors influencing its transmission," *Annals of Tropical Medicine and Parasitology*, vol. 95, no. 7, pp. 697–706, 2001.

[27] K. C. Kosinski, J. J. Crocker, J. L. Durant, D. Osabutey, M. N. Adjei, and D. M. Gute, "A novel community-based water recreation area for schistosomiasis control in rural Ghana," *Journal of Water Sanitation and Hygiene for Development*, vol. 1, no. 4, pp. 259–268, 2011.

[28] S. Knopp, B. Person, S. M. Ame et al., "Elimination of schistosomiasis transmission in Zanzibar: baseline findings before the onset of a randomized intervention trial," *PLoS Neglected Tropical Diseases*, vol. 7, no. 10, article e2474, 2013.

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*Research Article*

# A Quantitative Real-Time RT-PCR Assay for the Detection of *Venezuelan equine encephalitis virus* Utilizing a Universal Alphavirus Control RNA

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*Venezuelan equine encephalitis virus* (VEEV) is an *Alphavirus* from the family *Togaviridae* that causes epizootic outbreaks in equids and humans in Central and South America. So far, most studies use conventional reverse transcriptase PCR assays for the detection of the different VEEV subtypes. Here we describe the development of a TaqMan quantitative real-time reverse transcriptase PCR assay for the specific detection and quantitation of all VEEV subtypes which uses in parallel a universal equine encephalitis virus control RNA carrying target sequences of the three equine encephalitis viruses. The control RNA was used to generate standard curves for the calculation of copy numbers of viral genome of *Eastern equine encephalitis virus* (EEEV), *Western equine encephalitis virus* (WEEV), and VEEV. The new assay provides a reliable high-throughput method for the detection and quantitation of VEEV RNA in clinical and field samples and allows a rapid differentiation from potentially cocirculating EEEV and WEEV strains. The capability to detect all known VEEV variants was experimentally demonstrated and makes this assay suitable especially for the surveillance of VEEV.

## 1. Introduction

*Western equine encephalitis virus* (WEEV), *Eastern equine encephalitis virus* (EEEV), and *Venezuelan equine encephalitis virus* (VEEV) are arthropod-borne (arbo) viruses of the genus *Alphavirus* of the virus family *Togaviridae*. To date these viruses are restricted to the Americas but due to worldwide travelling and trade they might be introduced also to other parts of the world in the future. All three equine encephalitis viruses are classified as Category B agent by the Centers for Disease Control and Prevention, Atlanta (https://emergency.cdc.gov/agent/agentlist-category.asp). They are transmitted by sanguivorous mosquitoes within bird (WEEV, EEEV, and epizootic VEEV (epizootic strains)) or rodent populations (VEEV, enzootic strains), respectively. Infections in reservoir hosts do not lead to obvious clinical signs. However, severe diseases can occur when equines and humans are infected with epizootic subtypes by biting mosquitoes. In the last decade 662 equine cases with 302 fatalities of VEE were reported to the OIE and 75,000–100,000 human cases with more than 300 fatalities were counted in the most recent outbreak in Venezuela and Colombia in 1995 [1]. For some epizootic VEEV strains a productive replication followed by successful intra- and interspecies transmission cycles was observed in horses and humans [2–6].

In the time period 2007 to 2012 a total of 1926 EEEV and 3 WEEV associated equine cases were diagnosed in the United States (http://www.oie.int/). In the time period 1964 to 2009 the CDC registered 639 human WEEV and 260 EEEV cases with only few fatalities. It was shown recently in an experimental animal model that all three viruses are transmissible by aerosols [7–9].

In general, PCR-diagnostic for emerging viruses follows different questions: for the surveillance during an epidemic

Table 1: Primers and probes selected for equine encephalitis virus-specific quantitative reverse transcription polymerase chain reaction.

| Target | Primer or probe | Sequence (5′ → 3′) | Genome position | Reference |
|---|---|---|---|---|
| *Eastern equine encephalomyelitis virus* (EEEV) | EEE9391 | ACACCGCACCCTGATTTTACA | 9391–9411 (s) | [10] |
| | EEE9459c | CTTCCAAGTGACCTGGTCGTC | 9459–9439 (as) | |
| | EEE.9414probe | FAM-TGCACCCGGACCATCCGACCT-TAMRA | 9414–9434 (s) | |
| *Western equine encephalomyelitis virus* (WEEV) | WEE10,248 | CTGAAAGTCGGCCTGCGTAT | 10,248–10,267 (s) | [10] |
| | WEE 10,314c | CGCCATTGACGAACGTATCC | 10,314–10,295 (as) | |
| | WEE 10,271probe | FAM-ATACGGCAATACCACCGCGCACC-TAMRA | 10,271–10,293 (s) | |
| *Venezuelan equine encephalomyelitis virus* (VEEV) and synthetic calibrator | AlphaVIR966F | TCCATGCTAATGC*Y*AGAGCGTTTTCGCA | 151–178 (s) | Modified [14] |
| | AlphaVIR966R | TGGCGCACTTCCAATGTC*H*AGGAT | 248–225 (as) | |
| | *INEID-VEEV probe* | FAM-*TGATCGARACGGAGGTRGAMCCATCC*-TAMRA | 193–218 (s) | This study |
| | *VEEV-Coprobe* | VIC-*CTCCGTTCAATAC*-MGB-NFQ* | 180–192 (s) | This study |

The synthetic calibrator RNA is specifically detected by the VEEV-Coprobe in combination with the AlphaVIR966F and AlphaVIR966R primers. Y, H, R, and M are designed for degenerative bases, where Y = C/T, H = A/C/T, R = A/G, and M = A/C. Modifications compared to the original sequence as well as novel sequences were indicated in italic font. *MGB: minor groove binder; NFQ: Nonfluorescent quencher.

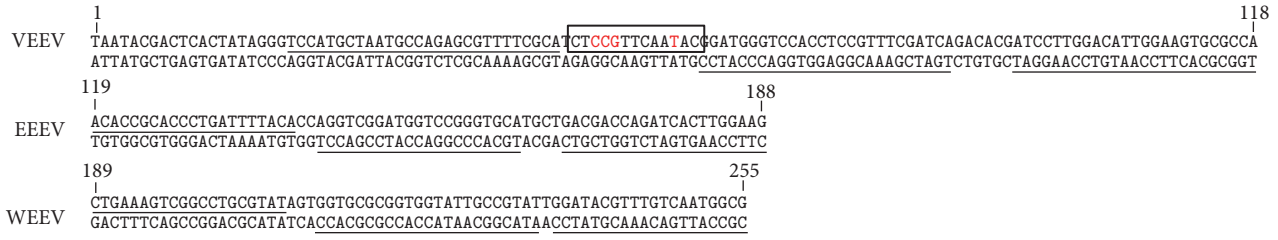
or the confirmation of infections from a known source a PCR specific for some virus variant may be sufficient. In contrast to prevent the introduction of the virus into a corresponding region or country, the use of an assay with an experimentally proven capability is necessary to detect every known virus variant. According to this several conventional RT-PCRs were developed to qualitatively detect VEEV, EEEV, and WEEV genome sequences and real-time reverse transcriptase PCRs (RT-qPCR) for EEEV and WEEV were published [10–13]. However, no RT-qPCR assay was available at the date of our study for the specific detection of different VEEV subtypes. A VEEV diagnosis is presently confirmed mostly by conventional RT-PCR using broad-range primer pairs covering the whole genus *Alphavirus* followed by subsequent amplicon sequencing [14]. Recent publications experimentally demonstrated RT-qPCR assays for detection of the VEEV vaccine strain TC-83 but without proven experimental demonstration of the assay's sensitivity and efficiency regarding other VEEV subtypes [15, 16]. In this study we are introducing a general purpose, rapid, one-step quantitative RT-qPCR assay for the sensitive and specific detection of all VEEV subtypes in combination with an internal calibrator construct which in turn can be used in the quantification of the three equine encephalitis viruses.

## 2. Materials and Methods

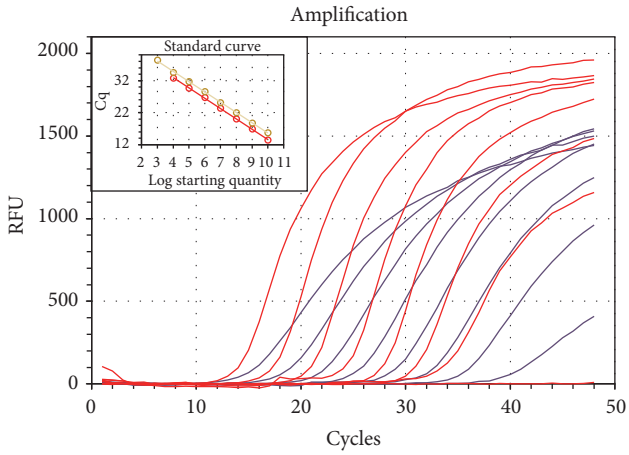
*2.1. Primer Design.* Multiple sequence alignments of VEEV sequences were performed using Vector NTI Advanced v.10 (Invitrogen, Carlsbad, CA, USA) and MEGA Software [17] to reveal primers, as well as a probe. For this purpose, a total of 33 VEEV sequences were retrieved from the GenBank database. Published broad-range primers, which target the nsP1 region of Alphaviruses and previously used within a conventional RT-PCR protocol [14], were modified by the insertion of a degenerated base in each of the forward and the reverse primer and complemented with a FAM- (6-carboxyfluorescein-) labelled probe, which specifically targets VEEV sequences (Table 1) and enables the application of a quantitative real-time RT-PCR protocol.

*2.2. Quantitative Real-Time RT-PCR (RT-qPCR).* RT-qPCR was carried out by using a commercial kit (QuantiTect RT-PCR kit, Qiagen, Germany). After the reverse transcription (50°C for 30 minutes) the DNA was denatured (95°C for 15 min). Amplification cycles included denaturation (95°C for 15 sec), annealing (55°C for 30 sec), and elongation (72°C for 30 sec) steps. Ct values were determined by the CFX96 software (Bio-Rad, USA).

*2.3. Synthetic Calibrator.* To determine the copy number of viral genomes a synthetic calibrator was developed, which comprises a T7 RNA polymerase promoter and the target sequences for the RT-qPCRs of EEEV, WEEV, and VEEV (Figure 1(a)) cloned into the pCR2.1 vector (Eurofins MWG Operon, Germany). The EEEV and WEEV sequences include targets for primer and probes adopted unmodified from the literature [10] (Table 1), but the corresponding probe target sequences were placed on the complementary strand in order to generate a unique (different) amplicon sequence, discriminable from the original virus sequence yet maintaining the same nucleotide composition. In addition, within the VEEV target region the original virus sequence 5′-CTGGCTTCAAAAC-3′ was changed to 5′-CTCCGTTCAATAC-3′ in order to discriminate unambiguously the synthetic RNA from viral RNA and to exclude false positive signals in samples potentially contaminated with synthetic RNA. This specific synthetic RNA sequence section can be detected only by a control probe (Table 1, VEEV-Coprobe). The plasmid was linearized with *Xba*I and subsequently transcribed into RNA

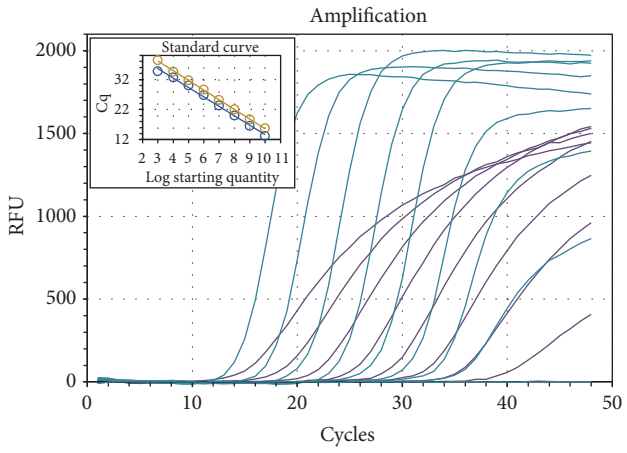



(a)



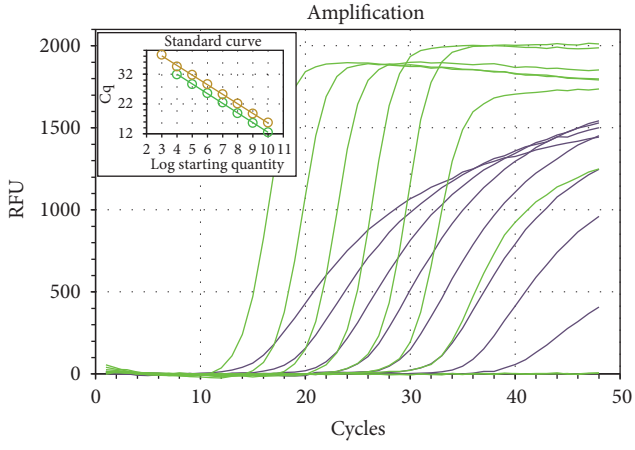

Calibrator (VEEV primer/probe):
$R^2$ = 1,000; slope = −3,226; $E$ = 104,2%
Calibrator (Co probe):
$R^2$ = 0,999; slope = −3,213; $E$ = 104,7%

(b)




Calibrator (EEEV primer/probe):
$R^2$ = 0,995; slope = −3,150; $E$ = 107,8%

(c)




Calibrator (WEEV primer/probe):
$R^2$ = 1,000; slope = −3,244; $E$ = 103,2%

(d)

Figure 1: The nucleotide sequence of the synthetic construct used for calibration of the EEV-specific qRT-PCRs (a). The target sequences (underlined) for the specific qRT-PCRs are cloned into the vector pCR2.1-TOPO. Within the VEEV target region a modified sequence (framed) allows the differentiation of the synthetic calibrator from viral sequences. Nucleotide exchanges are indicated in red. Amplification curves of the qRT-PCRs specific for VEEV (b), EEEV (c), and WEEV (d) using the synthetic calibrator template. Amplification curve of the synthetic calibrator targeted with the control probe is indicated in olive, respectively. Standard curves (see enclosed boxed figures) were obtained by Ct values plotted against the log of the starting quantity. Calculated correlation coefficients ($R^2$), slopes, and amplification efficiencies ($E$) are depicted below the corresponding figures.

Table 2: Sensitivity of RT-qPCR assays for VEEV, WEEV, and EEEV strains and determination of copy number.

| RNA | Dilution | Ct | Copies/$\mu$L |
|---|---|---|---|
| VEEV | $10^{-2}$ | 27,94 | 70200 |
| | $10^{-3}$ | 30,69 | 8840 |
| | $10^{-4}$ | 33,75 | 894 |
| | $10^{-5}$ | 35,51 | 240 |
| | $10^{-6}$ | 37,24 | 66 |
| | $10^{-7}$ | no Ct | 0 |
| WEEV | $10^{-2}$ | 21,76 | 2880000 |
| | $10^{-3}$ | 25,13 | 216000 |
| | $10^{-4}$ | 28,64 | 13780 |
| | $10^{-5}$ | 31,1 | 2060 |
| | $10^{-6}$ | 32,38 | 736 |
| | $10^{-7}$ | no Ct | 0 |
| EEEV | $10^{-3}$ | 29,67 | 12760 |
| | $10^{-4}$ | 32,55 | 1212 |
| | $10^{-5}$ | 34,22 | 316 |
| | $10^{-6}$ | no Ct | 0 |
| | $10^{-7}$ | no Ct | 0 |

and the DNA degraded using the Riboprobe® Combination System, T3/T7 RNA Polymerase (Promega Corporation's, Madison, WI, USA), and the QIAamp Viral RNA Mini Kit (Qiagen) was used for RNA isolation (without carrier RNA). The RNA concentration was estimated with the Quant-It™ RNA Assay Kit, Broad-Range (Invitrogen). The copy number of the synthetic RNA was calculated from the RNA concentration and the molecular mass of the RNA transcript.

## 3. Results and Discussion

In the here presented study, we are introducing a rapid, sensitive, and reliable one-step quantitative RT-PCR assay for VEEV as well as a synthetic RNA construct which can be used as calibrator for the quantification of alpha virus associated equine encephalitis viruses. PCR was carried out on serial dilutions of the synthetic RNA in a one-step RT-qPCR and Ct values were eventually plotted proportionally to the logarithm of the input copy numbers to produce standard quantitation curves. Negative controls were included in each run. The synthetic RNA was concurrently amplified using primer and probes for VEEV, WEEV, and EEEV, respectively (Figures 1(b)–1(d)). In addition the synthetic calibrator was amplified with primer for VEEV and detected by the VEEV-Coprobe. Both assays run independently in a single-plex format. All four standard curves exhibit a correlation coefficient >99% and an amplification efficiency of about 103–107% over a linear range of $10^2$ to $10^{10}$ copies. Based on the corresponding standard curves the sensitivity and viral load for different EEV strains in concurrent runs could be determined: the limit of detection (LOD) corresponded to 66,2 copies per $\mu$L (Ct = 37,24) for VEEV (TC-83 strain), at 736 copies (Ct = 32,38) for WEEV (McMillan strain), and at 316 copies per $\mu$L (Ct = 34,21) for EEEV (New Jersey strain) (Table 2). Since standard curves form part of every run, the copy number from each analysed sample can be determined. Therefore samples with copy numbers above 1 copy per $\mu$L (the theoretical detection limit) are considered to be positive. VEEV specific primer/probe combination did not detect other equine encephalomyelitis viruses (EEEV, WEEV) (Figure 2) or closely related species (Chikungunya virus, Sindbis virus, and Ross River virus; data not shown).

To further assess the performance of the VEEV specific RT-qPCR we used 15 synthetic RNA constructs (sVEEV) encompassing the target region and representing 10 different VEEV subtypes (Figure 3(a)). This included all combinations of observed mutations in the primers and probe target regions. sVEEV were designed as oligonucleotides with a 5′ T7 RNA polymerase promoter sequence and were transcribed *in vitro* as aforementioned. All VEEV subtypes were successfully detected by the novel RT-qPCR assay with a suitable sensitivity and high performance as demonstrated by linear standard curves over 5 logs (Figure 3(b)). $R^2$ values and slope indicate good precision and high efficiency (Table 3). To evaluate the effect of nucleotide changes to the PCR amplification efficiency we applied the relative threshold cycle (RTC) method, which refers to mean Ct-differences (mean $\Delta$Ct) of the corresponding sVEEV template compared to the unmodified template [18]. The data indicate that most nucleotide changes exhibited only small or moderate reduction of the RTC efficiency. Only sVEEV-16, representing one variant of

Table 3: Relative threshold cycle (RTC) amplification efficiencies of synthetic VEEV (sVEEV) RNA constructs.

| Template | sVEEV −1 | sVEEV −2 | sVEEV −3 | sVEEV −4 | sVEEV −5 | sVEEV −6 | sVEEV −7 | sVEEV −8 | sVEEV −9 | sVEEV −10 | sVEEV −11 | sVEEV −12 | sVEEV −13 | sVEEV −14 | sVEEV −15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Dilution | Ct | Ct | Ct | Ct | Ct | Ct | Ct | Ct | Ct | Ct | Ct | Ct | Ct | Ct | Ct |
| $10^{-4}$ | 17,3 | 20,6 | 19,4 | 19,6 | 17,4 | 21,1 | 18,6 | 17,8 | 18,3 | 18,5 | 20,9 | 19,6 | 20,2 | 24,6 | 21,5 |
| $10^{-5}$ | 21,4 | 24,4 | 23,4 | 23,7 | 21,3 | 25,0 | 22,3 | 21,3 | 21,7 | 22,2 | 24,5 | 23,1 | 24,1 | 28,2 | 25,0 |
| $10^{-6}$ | 25,2 | 28,3 | 27,1 | 27,3 | 25,2 | 28,7 | 26,5 | 25,3 | 25,4 | 25,8 | 28,0 | 27,1 | 27,8 | 31,7 | 28,6 |
| $10^{-7}$ | 28,9 | 31,8 | 30,8 | 31,2 | 29,0 | 32,4 | 30,2 | 28,9 | 28,7 | 29,3 | 31,6 | 30,9 | 31,9 | 35,5 | 32,4 |
| $10^{-8}$ | 31,8 | 34,6 | 34,2 | 34,7 | 32,7 | 36,1 | 33,7 | 32,2 | 32,2 | 32,8 | 35,3 | 34,9 | 35,6 | 39,1 | 36,1 |
| Slope $\lvert R^2$ | −2,99\| 0,9969 | −2,91\| 0,9959 | −3,05\| 0,9992 | −3,1\| 0,9995 | −3,16\| 0,9998 | −3,08\| 0,9998 | −3,16\| 0,9992 | −2,98\| 0,9993 | −2,85\| 0,9999 | −2,92\| 0,9999 | −2,95\| 1 | −3,17\| 0,9997 | −3,18\| 0,9999 | −2,99\| 0,9999 | −3,00\| 0,9998 |
| Mean ΔCt | **0,0** | **−3,0** | **−2,1** | **−2,4** | **−0,2** | **−3,7** | **−1,4** | **−0,2** | **−0,3** | **−0,8** | **−3,2** | **−2,2** | **−3,0** | **−6,9** | **−3,8** |
| Mean RTC | **1,0** | **0,12** | **0,24** | **0,19** | **0,87** | **0,08** | **0,39** | **0,89** | **0,80** | **0,57** | **0,11** | **0,22** | **0,12** | **0,01** | **0,07** |

ΔCt is calculated as mean difference of corresponding Ct values compared to unmodified reference template sVEEV-1 across all template dilutions. RTC is calculated according RTC = $2^{\Delta ct}$.

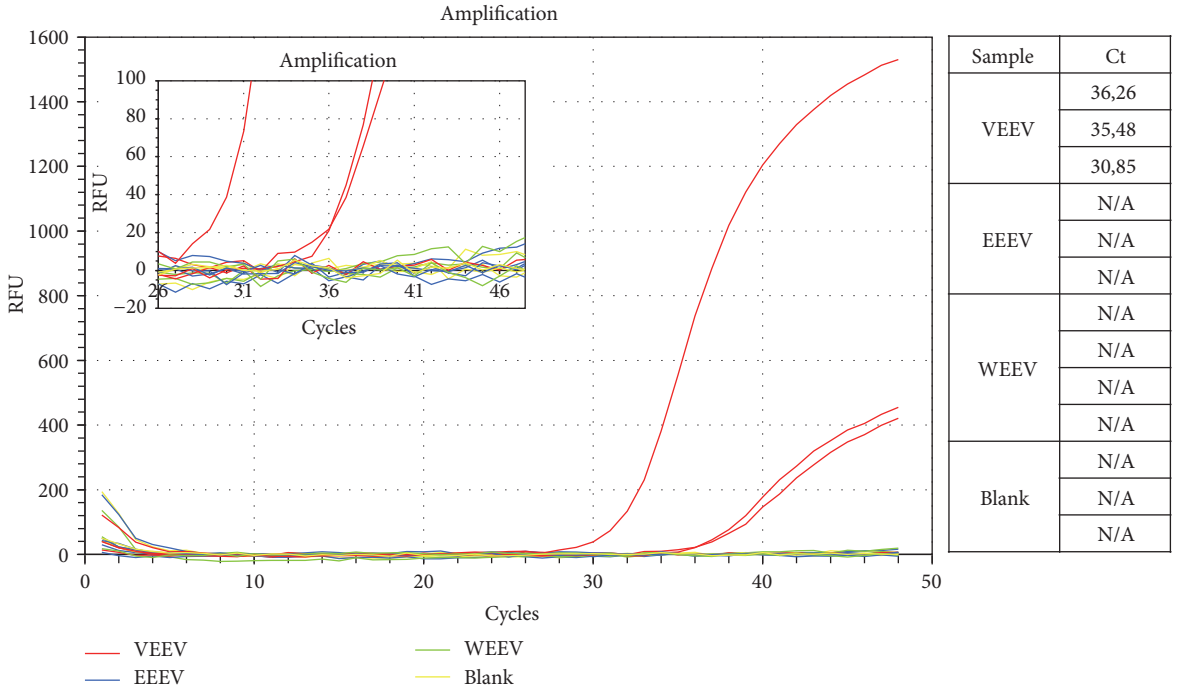



Figure 2: Specificity of the VEEV specific primer-probe combination. Specific amplification of VEEV derived RNA (red) by qRT-PCR. No amplification of EEEV (blue) and WEEV (green) derived RNA. Insert shows the boxed region at higher magnification.

subtype VI, showed a stronger decline in RTC efficiency which is probably caused by 13 nucleotide exchanges compared to the reference template. In summary this assay can be used whenever a sensitive and high-throughput detection or quantification of VEEV RNA is needed, for example, for confirmation of virus presence in patients, during infection experiments or large screening of field probes. But it is particularly useful when a proven application for the detection of all known VEEV variants is required, for example, to prevent the introduction of any virus variant into a so far virus free region or country.

## 4. Conclusions

We report here the first experimental evidence of a quantitative real-time RT-PCR assay for the sensitive and specific detection of all known VEEV subtypes or sequence variants. The synthetic calibrator RNA allows the determination of

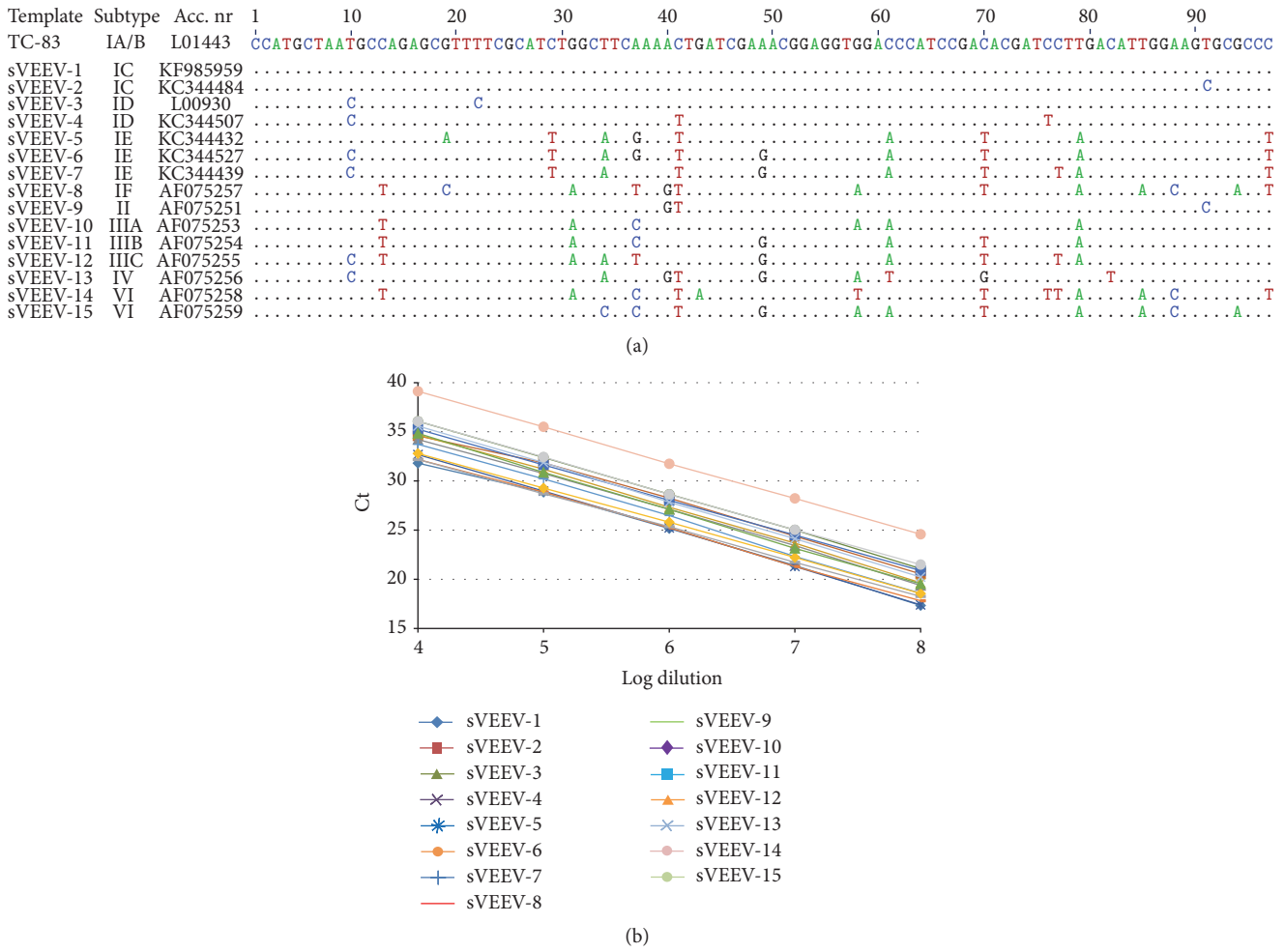



Figure 3: Comparison of the consensus sequences of different VEEV subtypes. (a) Sequences of synthetic RNA constructs (sVEEV) encompass the target region of the VEEV specific qRT-PCR. Nucleotides with mismatch to the reference sequence are indicated. (b) Standard curves of serial diluted sVEEV were obtained by Ct values plotted against the log of diluted template.

viral genome equivalents of VEEV as well as WEEV and EEEV by a one-step RT-qPCR reaction.

## Competing Interests

The authors declare that they have no competing interests.

## References

[1] S. Paessler and M. Pfeffer, "Togaviruses causing encephalitis," in *Encyclopedia of Virology*, B. W. J. Mahy and M. H. V. Van Regenmortel, Eds., pp. 76–82, Elsevier, Oxford, UK, 3rd edition, 2008.

[2] T. E. Walton and M. A. Grayson, "Venezuelan equine encephalitis," in *The Arboviruses: Epidemiology and Ecology*, T. P. Monath, Ed., vol. 4, pp. 203–231, CRC Press, Boca Raton, Fla, USA, 1988.

[3] S. C. Weaver and A. D. T. Barrett, "Transmission cycles, host range, evolution and emergence of arboviral disease," *Nature Reviews Microbiology*, vol. 2, no. 10, pp. 789–801, 2004.

[4] S. C. Weaver, M. Anishchenko, R. Bowen et al., "Genetic determinants of Venezuelan equine encephalitis emergence," in *Emergence and Control of Zoonotic Viral Encephalitides*, C. H. Calisher and D. E. Griffin, Eds., vol. 18 of *Archives of Virology, Supplementa*, pp. 43–64, Springer, Berlin, Germany, 2004.

[5] O. M. Suárez and G. H. Bergold, "Investigations of an outbreak of Venezuelan equine encephalitis in towns of eastern Venezuela," *American Journal of Tropical Medicine and Hygiene*, vol. 17, no. 6, pp. 875–880, 1968.

[6] R. E. kissling, "The arthropod-borne viruses of man and other animals," *Annual Review of Microbiology*, vol. 14, pp. 261–282, 1960.

[7] D. S. Reed, M. G. Lackemeyer, N. L. Garza et al., "Severe encephalitis in cynomolgus macaques exposed to aerosolized eastern equine encephalitis virus," *Journal of Infectious Diseases*, vol. 196, no. 3, pp. 441–450, 2007.

[8] D. S. Reed, T. Larsen, L. J. Sullivan et al., "Aerosol exposure to western equine encephalitis virus causes fever and encephalitis in cynomolgus macaques," *Journal of Infectious Diseases*, vol. 192, no. 7, pp. 1173–1182, 2005.

[9] D. S. Reed, C. M. Lind, L. J. Sullivan, W. D. Pratt, and M. D. Parker, "Aerosol infection of cynomolgus macaques with enzootic strains of venezuelan equine encephalitis viruses," *Journal of Infectious Diseases*, vol. 189, no. 6, pp. 1013–1017, 2004.

[10] A. J. Lambert, D. A. Martin, and R. S. Lanciotti, "Detection of North American eastern and western equine encephalitis viruses by nucleic acid amplification assays," *Journal of Clinical Microbiology*, vol. 41, no. 1, pp. 379–385, 2003.

[11] B. Linssen, R. M. Kinney, P. Aguilar et al., "Development of reverse transcription-PCR assays specific for detection of equine encephalitis viruses," *Journal of Clinical Microbiology*, vol. 38, no. 4, pp. 1527–1535, 2000.

[12] D. J. Johnson, E. N. Ostlund, and B. J. Schmitt, "Nested multiplex RT-PCR for detection and differentiation of West Nile virus and eastern equine encephalomyelitis virus in brain tissues," *Journal of Veterinary Diagnostic Investigation*, vol. 15, no. 5, pp. 488–493, 2003.

[13] K. Grywna, B. Kupfer, M. Panning et al., "Detection of all species of the genus *Alphavirus* by reverse transcription-PCR with diagnostic sensitivity," *Journal of Clinical Microbiology*, vol. 48, no. 9, pp. 3386–3387, 2010.

[14] M. W. Eshoo, C. A. Whitehouse, S. T. Zoll et al., "Direct broad-range detection of alphaviruses in mosquito extracts," *Virology*, vol. 368, no. 2, pp. 286–295, 2007.

[15] S. Qian, B. He, Z. Tu, H. Guo, and C. Tu, "Establishment of a one-step real-time RT-PCR method for the detection of Venezuelan equine encephalitis virus," *Bing Du Xue Bao*, vol. 31, no. 2, pp. 107–113, 2015 (Chinese).

[16] J. G. Julander, R. Skirpstunas, V. Siddharthan et al., "C3H/HeN mouse model for the evaluation of antiviral agents for the treatment of Venezuelan equine encephalitis virus infection," *Antiviral Research*, vol. 78, no. 3, pp. 230–241, 2008.

[17] K. Tamura, J. Dudley, M. Nei, and S. Kumar, "MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0," *Molecular Biology and Evolution*, vol. 24, no. 8, pp. 1596–1599, 2007.

[18] J. A. Sikorsky, D. A. Primerano, T. W. Fenger, and J. Denvir, "Effect of DNA damage on PCR amplification efficiency with the relative threshold cycle method," *Biochemical and Biophysical Research Communications*, vol. 323, no. 3, pp. 823–830, 2004.

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*Review Article*

# Ebola Virus Infection among Western Healthcare Workers Unable to Recall the Transmission Route

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*Introduction.* During the 2014–2016 West-African Ebola virus disease (EVD) outbreak, some HCWs from Western countries became infected despite proper equipment and training on EVD infection prevention and control (IPC) standards. Despite their high awareness toward EVD, some of them could not recall the transmission routes. We explored these incidents by recalling the stories of infected Western HCWs who had no known directly exposures to blood/bodily fluids from EVD patients. *Methodology.* We carried out conventional and unconventional literature searches through the web using the keyword "Ebola" looking for interviews and reports released by the infected HCWs and/or the healthcare organizations. *Results.* We identified fourteen HCWs, some infected outside West Africa and some even classified at low EVD risk. None of them recalled accidents, unintentional exposures, or any IPC violation. Infection transmission was thus inexplicable through the acknowledged transmission routes. *Conclusions.* We formulated two hypotheses: inapparent exposures to blood/bodily fluids or transmission due to asymptomatic/mildly symptomatic carriers. This study is in no way intended to be critical with the healthcare organizations which, thanks to their interventions, put an end to a large EVD outbreak that threatened the regional and world populations.

## 1. Introduction

The West-African Ebola virus disease (EVD) 2014 outbreak was longer and more impactful than previous Central-African outbreaks, covering the largest geographic area, spreading to eight countries across three continents and lasting over 22 months [1]. Healthcare workers (HCWs) bore the brunt of the outbreak with high morbidity and mortality, were more likely to be infected than people in the general population, and accounted for 5% of all EVD cases [2, 3]. Nosocomial transmission was principally due to the incorrect triage of EVD patients and limited or no training on and limited availability and inappropriate use of personal protective equipment (PPE). HCWs thus did not always provide appropriate care to EVD patients, and some were infected [2]. In general, infections occurred because of low compliance with infection prevention and control (IPC) procedures.

The massive intervention of WHO and governmental and nongovernmental healthcare organizations from all over the world, with resources and "train-the-trainer" policies, improved this situation and the infectious risk among all HCWs decreased considerably, thus demonstrating that EVD infections among HCWs were preventable [2, 4, 5].

Nevertheless, some HCWs still got infected and, among them, some HCWs were from Western countries. These subjects likely complied with IPC standards, were aware of the burden of the EVD epidemics, and had also less social contacts with the members of the communities where they were active [2].

Even before the occurrence of the 2014 West-African outbreak, episodes of EVD transmission to HCWs from Western countries had been reported.

In November 1976 the first case of a HCW who developed EVD outside Africa occurred, in a laboratory worker who accidentally pricked his thumb while transferring homogenized liver from a guinea-pig infected with Ebola virus. Following the standard safety protocol, he immediately removed his gloves and immersed his damaged thumb in hypochlorite solution, then squeezing it vigorously. There was no bleeding

and careful examination did not reveal a puncture wound. Nevertheless, he became EVD infected and was treated with convalescent serum and within two weeks he showed symptom remission and Ebola virus was no longer detected in blood, urine, or faeces. Therefore, he was discharged from isolation, but Ebola virus persisted in his semen for months [6].

In November 1989, during an Ebola-Reston virus outbreak among monkeys in the Philippines, some infected animals were imported into the US, where five animal handlers were daily exposed to these animals and four workers became infected without symptoms. One of them recalled cutting his finger while performing a necropsy on an infected animal, but among the remaining infected staff no transmission route was identified [7].

In 1995, during an Ebola virus outbreak in Kikwit (Democratic Republic of the Congo), four Italian missionary nurses developed EVD and three died (http://www.nytimes.com/1995/05/12/world/virus-kills-a-3d-italian-nun-at-zaire-hospital.html). There were no specific EVD-transmission-based precautions in place at that time and transmission through percutaneous exposures to infectious blood was supposed but not confirmed [8].

These episodes suggest that the events that led to Ebola virus infection could not always be known or recalled. Thus, the aim of this study was to report the anecdotal stories of some incidents occurring during the West-African EVD outbreak to HCWs from Western countries who did not recall any obvious contact of broken skin or mucous membranes with blood/bodily fluids of infected people or with surfaces and materials contaminated with these fluids, which are the acknowledged routes of human-to-human Ebola virus transmission (http://www.who.int/mediacentre/factsheets/fs103/en) (http://www.cdc.gov/vhf/ebola/healthcare-us/hospitals/infection-control.html). This study is not a field investigation or an epidemiologic study but was based on the interviews released by the EVD victims and/or the reports provided by the healthcare organizations. This was not a systematic review, and there could be other episodes of infected Western HCWs who could not recall the events that led to transmission; however, these stories could help formulate hypotheses regarding the existence and, possibly, the nature of alternative transmission routes.

## 2. Methodology

In order to collect as much material as possible, we used conventional and unconventional literature searches using the keyword "Ebola" looking for interviews and reports released by infected HCWs and/or the healthcare organizations from Western countries during the West-African outbreak.

An orthodox literature search used the databanks PubMed and Scopus. Full texts of relevant articles, according to titles, abstracts, and keywords, were collected.

An unconventional article search was performed through the Internet search engines (Google and Yahoo) using the search terms "Ebola" and "healthcare worker"/"healthcare provider"/"health worker" or the actual names of the known infected HCWs. Much of the retrieved material had been constructed from interviews with the infected HCWs obtained from the healthcare organizations and from magazines and newspapers.

The list of infected HCWs from Western countries amassed was then reduced to cases of unknown/unrecalled transmission routes in order to shed light over stories that may help provide a direction to the future research regarding Ebola virus transmission, rather than to evaluate EVD risk factors, case-fatality, incidence, or mortality rates.

## 3. Results

The searches provided the stories of fourteen Western HCWs (Table 1). Although the reported material is public, their names along with the names of the healthcare organizations are not displayed.

(i) Three HCWs (A, B, and C) from the USA were infected in Liberia between July and August 2014. HCW-A, a doctor caring for EVD patients, fell ill on 23 July 2014 and EVD was diagnosed four days later. No information regarding potential routes of transmission is available. HCW-B, a nurse who had no direct contact with EVD patients but assisted HCWs with donning and doffing PPE and performing decontamination, was diagnosed with EVD on 27 July (http://www.nbcnews.com/storyline/ebola-virus-outbreak/another-american-doctor-infected-ebola-charity-says-n193911) [9]. HCW-C, an obstetrician working in Liberia, did not care for EVD patients but was delivering babies following EVD-targeted prevention protocols (http://www.dailymail.co.uk/news/article-2745037/Third-U-S-missionary-51-infected-deadly-Ebola-virus-West-Africa-arrives-Nebraska-treatment.html).

(ii) HCW-D, a UK nurse infected in Sierra Leone on 24 August 2014, spent five weeks caring for EVD patients. He and his colleagues could not recall any specific accident that might lead to Ebola virus transmission (http://www.telegraph.co.uk/news/worldnews/ebola/11072041/Ebola-victim-William-Pooley-discharged-from-hospital.html).

(iii) HCW-E and HCW-F from Spain became infected Between August and September 2014 in Sierra Leone and were repatriated to Spain, where they died from EVD on 12 August (HCW-E) and 26 September (HCW-F). No information is provided regarding transmission (http://www.theguardian.com/world/2014/aug/12/ebola-spanish-missionary-dies-madrid-liberia) (http://www.thelocal.es/20140926/madrid-spanish-missionary-dies-from-ebola-spain).

(iv) HCW-G was a nurse infected at a Spanish Hospital complex on 6 October 2014 while caring for one of the above two HCWs (HCW-E and HCW-F). She was the first HCW known to be infected

Table 1: Profiles of HCWs from Western countries infected with Ebola virus during the West-African outbreak who could not recall the route of EVD transmission.

| HCW (country) | HCW category | Country of acquisition/diagnosis | Country of hospitalization | Probable transmission route |
|---|---|---|---|---|
| A (US) | Doctor | Liberia | US | N/A |
| B (US) | Nurse | Liberia | US | Did not care for EVD patients |
| C (US) | Obstetrician | Liberia | US | Did not care for EVD patients |
| D (UK) | Nurse | Sierra Leone | UK | Used the prescribed PPE. Mistakes in precautions due to overwork (colleagues' opinion) |
| E (Spain)[a] | N/A | Sierra Leone | Spain | N/A |
| F (Spain)[a] | N/A | Sierra Leone | Spain | N/A |
| G (Spain) | Nurse | Spain | Spain | Classified as low Ebola risk. Used the prescribed PPE |
| H (Cuba) | Doctor | Sierra Leone | Switzerland | Used the prescribed PPE |
| I (US) | Nurse | US | US | Classified as low Ebola risk (complained ill-prepared hospital staff) |
| J (US) | Nurse | US | US | Classified as low Ebola risk (complained ill-prepared hospital staff) |
| K (US) | Doctor | Guinea (acquisition), US (diagnosis) | US | Used the prescribed PPE |
| L (Italy) | Doctor | Sierra Leone | Italy | Used the prescribed PPE |
| M (UK) | Nurse | Sierra Leone (acquisition), UK (diagnosis) | UK | Used the prescribed PPE |
| N (Italy) | Nurse | Sierra Leone (acquisition), Italy (diagnosis) | Italy | Used the prescribed PPE |

[a]Died from EVD.

outside of West Africa. Paradoxically, during the field investigation, among the 117 HCWs who had participated in the care of the two infected HCWs, HVW-G was classified as a "low-risk contact." In addition, she had always used appropriate PPE such as waterproof long-sleeved clothing covering the feet, waterproof footwear, hood, face mask or goggles, double layer of gloves, and FP3 respirator and did not recall any accident during PPE use. Questioned as to how she may have contracted EVD, HCW-G replied "I really can't say, I haven't the slightest idea"(http://www.independent.co.uk/news/world/europe/spanish-nurse-teresa-romero-ramos-followed-all-protocols-and-has-no-idea-how-she-contracted-ebola-9781373.html) [10].

(v) HCW-H was one of hundreds of Cuban doctors sent as to Sierra Leone. WHO provided rigorous safety training and mentoring to the Cuban Medical Brigade and other foreign medical teams before starting work in the Ebola treatment centres. While working there, HCW-H contracted EVD but declared "I don't know how I got infected. There was no violation of protocols" (http://www.who.int/features/2015/cuban-doctor-survivor/en/).

(vi) HCW-I and HCW-J were US nurses who cared for an EVD patient arriving from Liberia on 28 September 2014 who died from EVD on 8 October in Dallas. HCW-I and HCW-J became infected on 11 and 14 October, respectively, but did not report unprotected exposures with the index patients and had been categorized as low EVD risk. How these two HCWs contracted Ebola virus infection remains unknown [11]. They complained, however, of having being obliged to have worked at the hospital in chaotic surroundings with ill-prepared nurses who received little guidance on how to treat Ebola and protect themselves (http://res.dallasnews.com/interactives/nina-pham/).

(vii) HCW-K was a US doctor, who treated EVD patients in Guinea. On 23 October 2014, nine days after returning to the US, he was diagnosed with EVD. He reported using all the prescribed PPE without any known breach [12]. His charity investigation on the transmission route was inconclusive. HCW-K conjectured that the Ebola virus could be trapped in a wet respiratory mask or that transmission occurred the day he was accidentally poked in his eye by the gloved finger of a hygienist without visible traces of human biological fluids or when he was feeding and cleaning a severely ill patient with EVD (http://nymag.com/daily/intelligencer/2015/06/craig-spencer-after-ebola.html#).

(viii) HCW-L, a doctor from Italy working in Sierra Leone, had an episode of vomiting and diarrhoea with low fever on 20 November 2014, testing positive for Ebola virus four days later. He was

repatriated to Rome, hospitalized, and treated [13]. Both the employer charity investigation and HCW-L reported that he had been observing correct procedures when treating infected patients (http://www.bbc.com/news/world-africa-30180345) (details in Italian at http://www.ilpost.it/2015/02/10/fabrizio-pulvirenti/).

(ix) HCW-M was a UK nurse who cared for EVD patients in Sierra Leone. On December 2014, on return home to Scotland, she became ill and was diagnosed with EVD (http://www.theguardian.com/world/2014/dec/30/uk-ebola-patient-named-pauline-cafferkey- scottish-nurse). She was transferred to London for care. A joint investigation performed by the charity and the Centre for Infectious Disease Surveillance and Control, Public Health England concluded that there was no conclusive evidence about when or how she contracted EVD including the possibility of infected social contacts. According to her employing charity, "We will never be 100% sure how HCW-M contracted Ebola" (http://www.savethechildren.org.uk/sites/default/files/images/Significant_Event_Review_Summary_Findings.pdf).

(x) HCW-N was an Italian nurse working in Sierra Leone assisting EVD patients. Two days after his return to Italy he fell ill and, two days later, on 12 May 2015, was diagnosed with Ebola virus infection. In an interview he reported to have no idea on how transmission may have occurred (http://www.sardiniapost.it/cronaca/stefano-marongiu-infermiere-guarito-da-ebola-non-mi-sono-mai-sentito-solo/ in italian)

Of the fourteen reported Ebola virus-infected HCWs from Western countries only two died from EVD. The reason is almost certain: all the infected HCWs were repatriated to USA or Europe, hospitalized, and underwent intensive care treatments [9, 10, 13]. Indeed, according to HCW-K, "whereas in Guinea I took care of 30 patients, in the US 30 doctors took care of me" (http://nymag.com/daily/intelligencer/2015/06/craig-spencer-after-ebola.html#).

## 4. Discussion

The common characteristics of the reported stories of these HCWs from Western countries are that, excluding the single case of HCW-D, whose colleagues suspected that he made mistakes with transmission precautions due to overworking, none of the remaining HCWs recalled any event that might lead to Ebola virus transmission (Table 1) and epidemiologic surveys failed to detect the transmission routes. Astonishingly, some of these Western HCWs, such as HCW-B and C, were not involved in the care of EVD patients. In addition, HCWs were aware, trained, and equipped to prevent Ebola virus infection transmission. We already demonstrated that relatively high disease knowledge and awareness do not necessarily lead to effective disease control and highlighted that this apparent contrast is for reasons beyond the efficacy of control measures but involve social, cultural, and behavioural dimensions [14–17].

The observation that trained and equipped HCWs could not recall the event that led to Ebola virus infection transmission leaves room for two major hypotheses.

Ebola virus transmission could be due to inapparent exposures to infected blood or bodily fluids, an acknowledged transmission route for other blood-borne pathogens, such as HCV and HBV [18, 19].

Ebola virus transmission could be due to caring for asymptomatic or mildly symptomatic EVD carriers, an hypothesis corroborated by several elements. First, Ebola virus may persist at immunologically protected sites in the body including semen, vaginal fluids, sweat, aqueous humor, urine, and breast milk of convalescent patients for months and, indeed, some convalescent patients have transmitted the infection, suggesting an important role of convalescent carriers in EVD persistence within the community [20]. Second, in endemic areas as many as 1–6% apparently healthy individuals show high levels of serum anti-Ebola virus antibodies, while 3–9% close family contacts of EVD patients are infected without developing typical EVD symptoms [21]. Third, mildly symptomatic subjects could also have played a role in infection transmission. Indeed, their prevalence during the West-African outbreak largely increased and most EVD survivors developed nonspecific EVD symptoms rather than haemorrhagic disease [22].

As already reported, this is not an epidemiologic investigation nor an inspection, and therefore, it is not possible to confirm that the EVD transmission route in these individuals was truly through inapparent exposure and/or due to asymptomatic/mildly symptomatic carriers. Even the more accurate investigations during the EVD outbreak failed to identify the exposures in more than one-fourth of infected patients [4] and, more broadly, in all communicable disease outbreaks there is always an important fraction of infected subjects with unknown or unrecalled exposures [23].

Perhaps, knowledge about human-to-human EVD transmission will improve thanks to experimental studies and to observational studies focusing on infected individuals without any obvious exposure [24], but the challenge is exactly this, distinguishing between unrecalled and unknown exposures to EVD.

## Competing Interests

The authors declare that they have no competing interests.

## References

[1] O. A. Makinde, "As Ebola winds down, Lassa Fever reemerges yet again in West Africa," *The Journal of Infection in Developing Countries*, vol. 10, no. 2, pp. 199–200, 2016.

[2] World Health Organization, *Health Worker Ebola Infections in Guinea, Liberia and Sierra Leone*, World Health Organization, 2015.

[3] F. O. Fasina, O. T. Adenubi, S. T. Ogundare, A. Shittu, D. G. Bwala, and M. M. Fasina, "Descriptive analyses and risk of

death due to Ebola Virus Disease, West Africa, 2014," *Journal of Infection in Developing Countries*, vol. 9, no. 12, pp. 1298–1307, 2015.

[4] O. Faye, P.-Y. Boëlle, E. Heleze et al., "Chains of transmission and control of Ebola virus disease in Conakry, Guinea, in 2014: an observational study," *The Lancet Infectious Diseases*, vol. 15, no. 3, pp. 320–326, 2015.

[5] J. Brainard, L. Hooper, K. Pond, K. Edmunds, and P. R. Hunter, "Risk factors for transmission of Ebola or Marburg virus disease: a systematic review and meta-analysis," *International Journal of Epidemiology*, vol. 45, no. 1, pp. 102–116, 2016.

[6] R. T. D. Emond, B. Evans, E. T. W. Bowen, and G. Lloyd, "A case of Ebola virus infection," *British Medical Journal*, vol. 2, no. 6086, pp. 541–544, 1977.

[7] Centers for Disease Control, "Update: filovirus infection in animal handlers," *Morbidity and Mortality Weekly Report*, vol. 39, no. 13, p. 221, 1990.

[8] A. S. Khan, F. K. Tshioko, D. L. Heymann et al., "The reemergence of Ebola hemorrhagic fever, Democratic Republic of the Congo, 1995," *Journal of Infectious Diseases*, vol. 179, no. 1, pp. S76–S86, 1999.

[9] G. M. Lyon, A. K. Mehta, J. B. Varkey et al., "Clinical care of two patients with Ebola virus disease in the United States," *The New England Journal of Medicine*, vol. 371, no. 25, pp. 2402–2409, 2014.

[10] M. A. Lopaz, C. Amela, M. Ordobas et al., "First secondary case of Ebola outside Africa: epidemiological characteristics and contact monitoring, Spain, September to November 2014," *Eurosurveillance*, vol. 20, no. 1, Article ID 21003, 2015.

[11] W. M. Chung, J. C. Smith, L. M. Weil et al., "Active tracing and monitoring of contacts associated with the first cluster of Ebola in the United States," *Annals of Internal Medicine*, vol. 163, no. 3, pp. 164–173, 2015.

[12] K. Yacisin, S. Balter, A. Fine et al., "Centers for Disease Control and Prevention (CDC) (2015) Ebola virus disease in a humanitarian aid worker—New York City, October 2014," *Morbidity and Mortality Weekly Report*, vol. 64, pp. 321–323, 2015.

[13] N. Petrosillo, E. Nicastri, S. Lanini et al., "Ebola virus disease complicated with viral interstitial pneumonia: a case report," *BMC Infectious Diseases*, vol. 15, no. 1, article 432, 2015.

[14] S. Petti and C. Scully, "Oral cancer knowledge and awareness: primary and secondary effects of an information leaflet," *Oral Oncology*, vol. 43, no. 4, pp. 408–415, 2007.

[15] S. Petti, "Why guidelines for early childhood caries prevention could be ineffective amongst children at high risk," *Journal of Dentistry*, vol. 38, no. 12, pp. 946–955, 2010.

[16] S. Petti and C. Scully, "How many individuals must be screened to reduce oral cancer mortality rate in the Western context? A challenge," *Oral Diseases*, vol. 21, no. 8, pp. 949–954, 2015.

[17] S. Petti, "Oral cancer screening usefulness: between true and perceived effectiveness," *Oral Diseases*, vol. 22, no. 2, pp. 104–108, 2016.

[18] M. J. Alter, "Epidemiology of hepatitis C in the west," *Seminars in Liver Disease*, vol. 15, no. 1, pp. 5–14, 1995.

[19] J. Milas, D. Ropac, R. Mulić et al., "Hepatitis B in the family," *European Journal of Epidemiology*, vol. 16, no. 3, pp. 203–208, 2000.

[20] C. R. MacIntyre and A. A. Chughtai, "Recurrence and reinfection-a new paradigm for the management of Ebola virus disease," *International Journal of Infectious Diseases*, vol. 43, pp. 58–61, 2016.

[21] L. Samaranayake, C. Scully, R. G. Nair, and S. Petti, "Viral haemorrhagic fevers with emphasis on Ebola virus disease and oro-dental healthcare," *Oral Diseases*, vol. 21, no. 1, pp. 1–6, 2015.

[22] S. Petti, G. A. Messano, E. M. Vingolo, L. T. Marsella, and C. Scully, "The face of Ebola: changing frequency of haemorrhage in the West African compared with Eastern-Central African outbreaks," *BMC Infectious Diseases*, vol. 15, article 564, 2015.

[23] N. Noah, *Controlling Communicable Disease Berkshire*, Open University Press, Maidenhead, UK, 2006.

[24] S. Judson, J. Prescott, and V. Munster, "Understanding Ebola virus transmission," *Viruses*, vol. 7, no. 2, pp. 511–521, 2015.

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*Clinical Study*

# Clinical and Microbiological Characteristics of Visceral Leishmaniasis Outbreak in a Northern Italian Nonendemic Area: A Retrospective Observational Study

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*Background*. Visceral leishmaniasis (VL) caused by *Leishmania infantum* is endemic in the Mediterranean area. In the last decades a northward spread of the parasite has been observed in Italy. This paper describes a VL outbreak in Modena province (Emilia-Romagna, Northern Italy) between 2012 and 2015. *Methods*. Retrospective, observational study to evaluate epidemiological, microbiological characteristics, and clinical management of VL in patients referring to Policlinico Modena Hospital. *Results*. Sixteen cases of VL occurred in the study period. An immunosuppressive condition was present in 81.3%. Clinical presentation included anemia, fever, leukopenia, thrombocytopenia, and hepatosplenomegaly. Serology was positive in 73.3% of cases, peripheral blood PCR in 92.3%, and bone marrow blood PCR in 100%. Culture was positive in 3/6 cases (50%) and all the isolates were identified as *L. infantum* by ITS1/ITS2 sequencing. The median time between symptom onset and diagnosis was 22 days (range 6–131 days). All patients were treated with liposomal amphotericin b. 18.8% had a VL recurrence and were treated with miltefosine. Attributable mortality was 6.3%. *Conclusions*. VL due to *L. infantum* could determine periodical outbreaks, as the one described; thus it is important to include VL in the differential diagnosis of fever of unknown origin, even in low-endemic areas.

## 1. Introduction

Visceral leishmaniasis (VL) is a protozoan disease caused by members of the *Leishmania donovani* complex and transmitted by phlebotomine sand flies. VL primarily affects the host's reticuloendothelial system and, without treatment, it can be a life-threatening disease. Symptoms include pancytopenia, fever, and hepatosplenomegaly.

VL is endemic in southern Europe in the Mediterranean area, where it is caused by *L. infantum* and the transmission is mainly zoonotic [1]. However, new foci sustained by exotic *Leishmania* species were recently reported in Europe, such as *L. donovani* in Cyprus [2]. Furthermore, two cases of *L. major*/*L. infantum* hybrids in HIV-positive injecting drug users were detected in Portugal [3]. Therefore, monitoring of species circulating in a given region, by either zymodeme analysis or DNA-based typing, should be integrated into an effective surveillance system.

In Italy, the Tyrrhenian littoral, the southern peninsular regions, and the islands have been considered classical endemic zones for VL. Since the early 1990s, however, a northward spread of the disease to previously nonendemic Italian regions has been observed both in humans and in dogs [4–6]. In particular, Biglino et al. showed a seroprevalence of 7.4% in asymptomatic healthy adults in Piedmont Region [5], while Varani et al. reported a little outbreak of autochthonous human VL cases in the Emilia-Romagna region [6].

In this paper we describe a VL outbreak, which took place in the Modena province (Emilia-Romagna, Northern Italy) between 2012 and 2015, focusing on its clinical and microbiological characteristics.

## 2. Materials and Methods

*2.1. Design.* We conducted a retrospective, observational study including all patients with a VL diagnosis referring to Policlinico Modena Hospital from January 2012 to December 2015. We excluded patients living outside Modena province. Epidemiological, clinical, and microbiological features of VL cases were evaluated.

*2.2. Case Definitions.* VL case was defined according to World Health Organization (WHO) criteria that include the presence of VL clinical symptoms (mainly prolonged irregular fever, splenomegaly, and weight loss) with serologic and/or parasitological confirmation (direct microscopy demonstration of *Leishmania* amastigotes, positive *Leishmania* spp. polymerase chain reaction (PCR), or culture) [7, 8].

Hemophagocytic syndrome was defined according to literature criteria [9].

VL recurrence was defined as a new onset of VL signs and symptoms associated with positivity of *Leishmania* spp. PCR after a negative sample in the 6-month follow-up period.

*2.3. Laboratory Diagnostic Methods.* Anti-*L. infantum* antibodies were assayed using an indirect immunofluorescence test (Leishmania Spot-IF; bioMerieux, Marcy l'Etoile, France) and titers were considered significant if equal or above 1 : 80.

DNA in peripheral and in bone marrow blood was investigated using a nested-PCR performed as described elsewhere [10]. Both immunofluorescence assay tests and molecular methods were performed in the Microbiology Laboratory of Policlinico Modena Hospital.

Bone marrow aspirates were inoculated into Evans' modified Tobie's medium. Cultures were maintained at 25°C; the supernatant was examined for parasite growth by light microscopy every three days and subcultured once a week for 4 weeks before they were reported as negative. Positive cultures were transferred to RPMI-1640 supplemented with 10% fetal calf serum for mass culturing. To identify *Leishmania* species, the ribosomal internal transcribed spacers ITS1 and ITS2 were amplified and sequenced as previously described [10]. To each isolate, ITS genotype was assigned according to the number of variable repeats of the 12 microsatellite regions in ITS1 (four sites) and in ITS2 (eight sites). Sequences obtained were aligned using the CLUSTAL W application of BioEdit version 7.0.8.0. and compared with the sequences described by Kuhls et al. [11].

For morphologic assessment, bone marrow smears were stained with May-Grünwald-Giemsa and examined under the light microscope at high magnification. The microscopic evaluation was considered positive in presence of *Leishmania* amastigotes, either into macrophage cytoplasms or dispersed.

*2.4. VL Treatment.* All patients included in the study were treated with liposomal amphotericin B (L-AmB), with a total dose of 40 mg/kg for HIV-infected patients and 20 mg/kg for all the others according to international guidelines [7, 12, 13]. In particular, patients with HIV infection received 4 mg/kg once daily on days 1–5, 10, 17, 24, 31, and 38; HIV-negative patients received 3 mg/kg of L-AmB once daily on days 1–5, 14, and 21. Patients with VL recurrence in the follow-up period were treated with miltefosine 50 mg TID for 28 days. No short course treatment was performed.

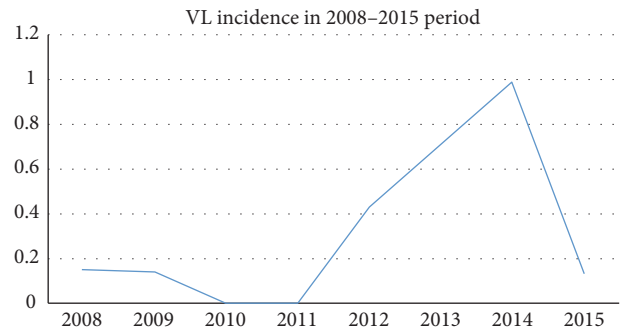


Figure 1: Visceral leishmaniasis incidence per 100,000 inhabitants in 2008–2015 period in the Modena province.

*2.5. Data Collection and Statistical Analysis.* Epidemiological, clinical, microbiological, and treatment characteristics were collected. All patients included in the study had a six-month follow-up period.

Due to the small number of patients enrolled, we performed only a descriptive statistical analysis expressing categorical and continuous variables as frequency (percentage) and median values (range values).

On the basis of calendar date of diagnosis and annual epidemiological data of Modena population [14], incidence rates of VL were calculated.

All statistical analyses have been performed using STATA 13 for Windows (StataCorp ltd., College Station, TX).

*2.6. Ethics Statement.* A corresponding approval from the Modena ethic committee was obtained. The study was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. Due to the retrospective nature of this study the informed consent was waived. Clinical samples were collected during routine diagnosis and/or follow-up, including no additional invasive procedures. Patient records were anonymized prior to analysis.

## 3. Results

From January 2012 to December 2015, 16 new cases of VL occurred in Modena province (3 patients in 2012, 5 in 2013, 7 in 2014, and 1 patient in 2015). The incidence per 100,000 inhabitants was 0.43 in 2012, 0.71 in 2013, 0.99 in 2014, and 0.14 in 2015. Figure 1 describes VL incidence in the calendar years from 2008 to 2015 in the Modena province [15].

The majority of patients were resident in rural and hilly areas. Figure 2 describes VL case distribution. None of the patients have been in endemic areas for human VL in the five years before diagnosis.

81.3% were males (13/16), median age was 63 years (range 33–83 years), and 81.3% had one (9/16) or two (4/16)

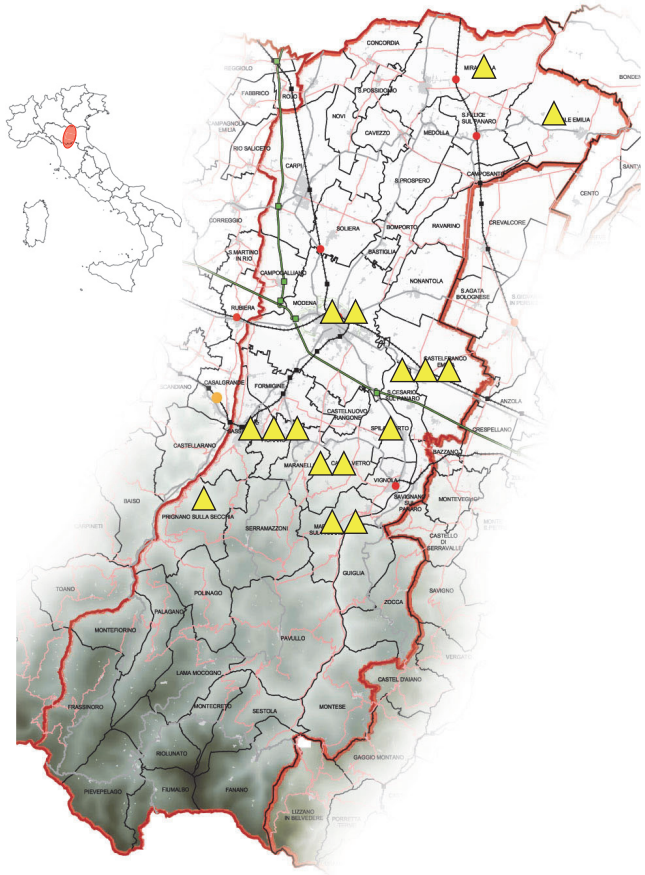


Figure 2: Human visceral leishmaniasis cases distribution map according to residency in Modena province (adapted with permission from www.italomairo.com) from 2012 to 2015. The darkest zones represent the Emilian Apennines.

immunosuppression conditions. In particular, four out of 16 patients (25%) were on chronic steroid therapy, three (18.8%) were HIV-positive, three (18.8%) had a hematologic disease, three (18.8%) had a neoplastic disease, one (6.3%) was an injective drug user, and one (6.3%) was cirrhotic.

Table 1 shows clinical picture at VL diagnosis and microbial tests used for diagnosis of *Leishmania* spp.

The majority of patients (13/16, 81.3%) presented symptoms in autumn/winter. At admission signs and symptoms were fever (15/16, 93.8%), splenomegaly (13/16, 81.3%), asthenia (12/16, 75%), hepatomegaly (11/16, 68.8%), weight loss (6/16, 37.5%), and abdominal lymphadenomegaly (4/16, 25%). Biochemical findings were anemia (16/16, 100%), leukopenia (14/16, 87.5%), thrombocytopenia (13/16, 81.3%), ALT value > 2x upper limit of normal values (7/16, 43.8%), and hemophagocytic syndrome (4/16, 25%). Median baseline creatinine was 1 mg/dL (range 0.8–1.3).

In total, 15 patients performed a serology, 13 patients a peripheral blood PCR, and 12 patients a bone marrow PCR; 6 patients had a culture sample and 11 patients a bone marrow microscopic examination. 11/15 patients (73.33%) had a positive serology and patient 8 had a borderline titer of 1 : 40. Patient 8 was cirrhotic and this could justify the low serologic titer we found. All but one peripheral blood PCR were positive (92.31%), while all the bone marrow PCRs were positive (100%). Three microscopic examination out of eleven (27.27%) demonstrated the presence of amastigotes.

Patient 14 had only a positive serology while no molecular procedures were performed before treatment. The patient was admitted to the hospital with asthenia, anorexia, and hepatosplenomegaly. The biochemical exams showed the presence of pancytopenia and hyperferritinemia. After VL treatment all the signs and symptoms gradually solved as did blood exams. Since the patient had the presence of VL clinical symptoms with serologic confirmation we decided to include her in the analysis (Table 1).

The culture was positive in 3/6 cases. All the isolates were identified as *L. infantum* by the analysis of the 12 microsatellite sites of the ITS1 and ITS2 concatenated sequences [11].

The median time between symptoms onset and hospital admission was 14.5 days (range 4–125 days). The median time between symptoms onset and diagnosis was 22 days (range 6–131 days). The median time between hospital admission and diagnosis was 6 days (range 2–21), with no significant variations during calendar years of the study period.

All patients included in the study were treated with L-AmB as described before. Two patients (12.5%) had a VL recurrence in the follow-up period, both 8 weeks after the first administration of L-AmB. One of the two patients with VL recurrence was on chronic steroid treatment; the other one had a hematological disease. Miltefosine treatment led to clinical and microbiological resolution. One patient died (6.3%) 53 days after symptom presentation, 17 days after diagnosis and starting treatment. He had alcoholic cirrhosis as comorbidity. His death was attributable to VL. L-AmB cure rate was 81.3%.

During treatment and follow-up, hematological and biochemical alterations gradually resolved within two weeks as shown in Figure 3. Of note, we did not observe any significant increase of serum creatinine during the observation period despite the use of L-AmB.

## 4. Discussion

Our study describes an outbreak of VL observed in Modena province (Emilia-Romagna region, Northern Italy) from 2012 to 2015. We collected 16 new cases of VL in a confined area with a peak of 0.99 cases per 100,000 inhabitants in 2014, while the average number of cases per year before 2012 was less than 0.2 cases per 100,000 inhabitants [15]. In 2015 the incidence reduces again.

In the early 1970s a severe outbreak of VL was reported in Emilia-Romagna, with a similar distribution of cases, in particular in the foothills of the Apennines zone. However this focus was considered atypical and no further cases were reported in the following 15 years [16].

Although the southern peninsular Italian regions and the islands have been considered classical endemic areas for VL [4], starting from 1990s, also northern continental Italian regions have become focally endemic for *L. infantum* as shown through active surveillance in dogs and phlebotomine

Table 1: Clinical picture and microbial tests used for diagnosis of *Leishmania* spp.

| Patient number | Fever | Hepatomegaly | Splenomegaly | Asthenia | Weight loss | Abdominal lymphadenomegaly | Anemia | Leukopenia | Thrombocytopenia | ALT > 2NV | Hemophagocytic syndrome | Serology | Peripheral blood PCR | Bone marrow blood PCR | Culture sample | Bone marrow microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | • | • | • | | | • | • | • | • | • | • | − | + | + | N/A | + |
| 2 | • | • | • | • | | | • | • | • | | • | − | + | + | N/A | − |
| 3 | • | | | • | | | • | • | • | | | 1 : 160 | + | + | N/A | + |
| 4 | • | • | • | • | • | | • | • | • | • | • | 1 : 640 | + | + | N/A | − |
| 5 | • | • | • | • | | | • | • | • | | | − | + | + | N/A | N/A |
| 6 | • | • | | • | | • | • | • | • | | | 1 : 160 | + | + | N/A | + |
| 7 | • | • | • | • | • | | • | • | • | • | • | 1 : 160 | + | + | − | − |
| 8 | • | • | • | | | | • | • | • | • | | 1 : 40 | + | N/A | − | N/A |
| 9 | • | • | • | • | • | | • | • | • | • | | 1 : 320 | + | + | + | − |
| 10 | • | | • | • | | | • | | | • | | 1 : 160 | + | + | + | − |
| 11 | • | | • | | | | • | • | | • | | 1 : 160 | N/A | + | + | − |
| 12 | • | • | • | • | • | | • | • | • | | | N/A | N/A | + | − | − |
| 13 | • | | • | | • | | • | • | • | | | 1 : 320 | + | N/A | N/A | N/A |
| 14 | | • | • | • | | | • | • | • | | | 1 : 320 | N/A | N/A | N/A | N/A |
| 15 | • | • | • | • | | • | • | • | • | | | 1 : 80 | − | + | N/A | − |
| 16 | • | | • | • | • | • | • | | | | | 1 : 320 | + | N/A | N/A | N/A |

PCR: polymerase chain reaction; N/A: not available; ALT: alanine aminotransferase; NV: normal values.

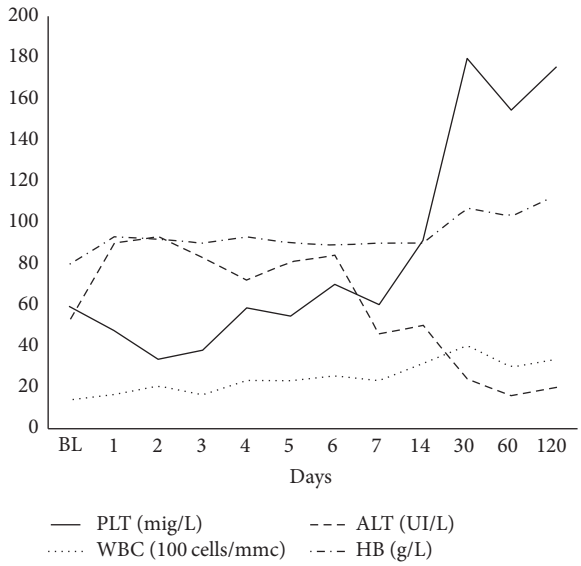


Figure 3: Biochemical trends during visceral leishmaniasis treatment (median values). BL: baseline; PLT: platelets; WBC: white blood cells; ALT: alanine aminotransferase; HB: haemoglobin.

sand flies [17]. With regard to human VL, the Italian Ministry of Health reported 230 VL cases among residents in Northern Italy (10.9% of all Italian cases) from 1990 to 2005, mainly from Lombardy (109 cases), Piedmont (42 cases), and Emilia-Romagna (25 cases) [17].

Modena province is a central area of Emilia-Romagna (North-Eastern Italy) (Figure 2), with a mean population of 704,364 inhabitants during the study period [14]. The province covers an area of 2,688 $km^2$ and approximately half of it, in its northern part, consists of the Po valley plains, while the remaining part of the province is covered by hills and mountains up to 2,165 m above sea level. Climate is subcontinental, with a mean temperature of 12.4°C and mean annual precipitations of 764 mm [18]. The Po plain, which covers the 48% of the study area, is mainly occupied by agriculture and artificial areas. Hilly and mountainous areas cover the remaining 17% and 35% of the province, respectively, with a prevalence of natural and seminatural areas.

Previous entomological surveys have established the presence of two proven vectors of *L. infantum* in Emilia-Romagna: *Phlebotomus perfiliewi* and *P. perniciosus* [16, 17]. Sand flies are mainly distributed in the hilly areas of the region, including the neighbouring area between Modena and Bologna provinces. *P. perfiliewi* represents the most abundant species [19] and it is considered a less efficient vector of *L. infantum* [17] in comparison to *P. perniciosus*. However, Baldelli et al. [20] reported a canine leishmaniasis focus sustained by *P. perfiliewi* and its role in the VL transmission also cannot be excluded.

Our results show an increase of the median number of VL per year starting from 2012 with a decrease in 2015. Anamnestic information, microbiological data, and the presence of competent vectors, combined with previous sporadic reports of the disease in the Modena province [16], suggest the presence of an autochthonous stable focus of *L. infantum* in the study area, characterized by a recent upsurge. During the same period, Varani et al. reported a VL outbreak in the neighbouring Bologna province, Emilia-Romagna, with 14 cases diagnosed in six months [6].

The reasons of the leishmaniasis reemergence in Emilia-Romagna are not definitely known. With regard to dogs, recognized as primary reservoir hosts of zoonotic VL, any important changes in the canine leishmaniasis epidemiology were observed. Results of a multiyear surveillance program carried out in public kennels of Emilia-Romagna showed an increase in canine leishmaniasis seroprevalence from 2010 (1%) to 2012 (2.4%) [19]. Afterwards a slight decrease was observed [21], with prevalence values lower than those observed in traditionally endemic regions of central and southern Italy [4].

Even though climate change might affect leishmaniasis distribution both directly and indirectly, the association between the two has been only surmised, with lack of definitive evidence [17, 22]. It is demonstrated that from 2000 to 2009 the median annual temperature in Italy increased by 0.8 Celsius degrees compared to the previous decade but no more recent data are available [23].

Further investigations involving multidisciplinary researchers should be carried out in Emilia-Romagna in order to address important epidemiological questions. Studies should be focused on environmental and climate factors influencing the exposure to sand fly bites. Considering that clinical VL cases usually represent the tip of an "infection iceberg," the prevalence of asymptomatic human carriers and triggers of clinical disease should also be evaluated.

Even though *L. infantum* is the causative agent of VL in Italy, molecular typing with more discriminant tools and characterization of additional isolates could contribute to better understanding the transmission dynamics between humans, vectors, and animal reservoirs as suggested by Chicharro et al. [24].

Due to the increasing incidence of VL in the last years, we expected to find an improvement in our capability to diagnose VL. In the majority of cases the clinical suspicion is the keystone for a rapid diagnosis and, consequently, a rapid treatment and a better prognosis. Actually, we found no difference in the median time between onset of symptoms and diagnosis in the five years considered in our study.

The clinical characteristics at presentation in our cohort did not differ from the literature, anemia, fever, leukopenia, thrombocytopenia, and hepatosplenomegaly being the most frequent symptoms and signs at diagnosis. Thus, being to include VL in the differential diagnosis of fever of unknown origin.

HIV infection is known to be one of the most important risk factors for VL. Nevertheless, only a small percentage of our patients were HIV-positive while we noticed an important presence of other immunosuppression causes (e.g., chronic steroid therapy, hematologic/neoplastic disease, and cirrhosis), recently associated with the onset of VL [25–27]. With the improvement in treatment strategies for hematological and rheumatological diseases (e.g., patients receiving biological agents [28]), we will probably observe a higher

number of immunosuppressed patients; thus it is important for clinicians to consider also VL in the differential diagnosis of infections.

In our experience, PCR, both on peripheral and on bone marrow blood, is a sensitive technique for diagnosis of VL with 92.3% and 100% of positive samples, respectively. A positive PCR on peripheral blood in a patient with clinical suspicion of VL was sufficient for VL diagnosis and permitted us to avoid bone marrow aspiration in different cases. On the contrary, a negative PCR on peripheral blood in a patient with high clinical suspicion of VL necessitates further investigations in order to exclude VL diagnosis.

Regarding treatment, we followed WHO recommendations for VL due to *L. infantum,* based on regional differences and expert opinions [7, 12]. We did not explore short course, high dose regimens such as single dose L-AmB. In our study, standard doses of L-AmB showed a good cure rate and profile of tolerability.

Our study has some limitations: first of all the limited number of cases did not allow us to derive definitive conclusions on sensitivity or specificity of the single diagnostic approach; second we had only cases in adult patients; thus our findings could not be taken as evidence in children. Finally, the majority of patients in the study presented an underlying immunosuppressive condition; although no one reported anamnestic VL diagnosis, it was not possible to completely exclude reactivation episodes instead of acute illnesses. A routine serologic test for VL in immunosuppressed patients could be implemented.

## 5. Conclusions

VL due to *L. infantum* could determine periodical outbreaks; thus it is important to include VL in the differential diagnosis of fever of unknown origin, even in low-endemic areas, especially if pancytopenia and/or hepatosplenomegaly are present. Since the reasons of leishmaniosis periodic reemergence in the Emilia-Romagna region are not definitely known, multidisciplinary epidemiological and molecular studies are needed, in order to elucidate gaps in epidemiological aspects.

## Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## References

[1] J.-C. Dujardin, L. Campino, C. Cañavate et al., "Spread of vector-borne diseases and neglect of leishmaniasis, Europe," *Emerging Infectious Diseases*, vol. 14, no. 7, pp. 1013–1018, 2008.

[2] M. Antoniou, C. Haralambous, A. Mazeris, F. Pratlong, J.-P. Dedet, and K. Soteriadou, "Leishmania donovani leishmaniasis in Cyprus," *The Lancet Infectious Diseases*, vol. 8, no. 1, pp. 6–7, 2008.

[3] C. Ravel, S. Cortes, F. Pratlong, F. Morio, J.-P. Dedet, and L. Campino, "First report of genetic hybrids between two very divergent Leishmania species: Leishmania infantum and Leishmania major," *International Journal for Parasitology*, vol. 36, no. 13, pp. 1383–1388, 2006.

[4] M. Gramiccia, A. Scalone, T. Di Muccio, S. Orsini, E. Fiorentino, and L. Gradoni, "The burden of visceral leishmaniasis in Italy from 1982 to 2012: a retrospective analysis of the multi-annual epidemic that occurred from 1989 to 2009," *Euro Surveillance*, vol. 18, no. 29, Article ID 20535, 2013.

[5] A. Biglino, C. Bolla, E. Concialdi, A. Trisciuoglio, A. Romano, and E. Ferroglio, "Asymptomatic *Leishmania infantum* infection in an area of Northwestern Italy (Piedmont region) where such infections are traditionally nonendemic," *Journal of Clinical Microbiology*, vol. 48, no. 1, pp. 131–136, 2010.

[6] S. Varani, R. Cagarelli, F. Melchionda et al., "Ongoing outbreak of visceral leishmaniasis in Bologna Province, Italy, November 2012 to May 2013," *Euro Surveillance*, vol. 18, no. 29, Article ID 20530, 2013.

[7] World Health Organization, *Control of the Leishmaniases*, WHO Technical Report Series, World Health Organization, Geneva, Switzerland, 2010.

[8] F. Chappuis, S. Sundar, A. Hailu et al., "Visceral leishmaniasis: what are the needs for diagnosis, treatment and control?" *Nature Reviews Microbiology*, vol. 5, no. 11, pp. S7–S16, 2007.

[9] J.-I. Henter, A. Horne, M. Aricó et al., "HLH-2004: diagnostic and therapeutic guidelines for hemophagocytic lymphohistiocytosis," *Pediatric Blood and Cancer*, vol. 48, no. 2, pp. 124–131, 2007.

[10] H. A. Noyes, H. Reyburn, J. W. Bailey, and D. Smith, "A nested-PCR-based schizodeme method for identifying *Leishmania* kinetoplast minicircle classes directly from clinical samples and its application to the study of the epidemiology of *Leishmania tropica* in Pakistan," *Journal of Clinical Microbiology*, vol. 36, no. 10, pp. 2877–2881, 1998.

[11] K. Kuhls, I. L. Mauricio, F. Pratlong, W. Presber, and G. Schönian, "Analysis of ribosomal DNA internal transcribed spacer sequences of the *Leishmania donovani* complex," *Microbes and Infection*, vol. 7, no. 11-12, pp. 1224–1234, 2005.

[12] S. Sundar and J. Chakravarty, "Leishmaniasis: an update of current pharmacotherapy," *Expert Opinion on Pharmacotherapy*, vol. 14, no. 1, pp. 53–63, 2013.

[13] J. N. Jarvis and D. N. Lockwood, "Clinical aspects of visceral leishmaniasis in HIV infection," *Current Opinion in Infectious Diseases*, vol. 26, no. 1, pp. 1–9, 2013.

[14] Modena Statistiche, Provincia di Modena, Osservatorio demografico, http://www.modenastatistiche.it.

[15] Leishmaniasis in Emilia-Romagna Region, Surveillance system results, http://salute.regione.emilia-romagna.it/documentazione/rapporti/201cleishmaniasis-in-emilia-romagna-region-surveillance-system-results201d.

[16] S. Pampiglione, M. La Placa, and G. Schlick, "Studies on mediterranean leishmaniasis I. An outbreak of visceral leishmaniasis in Northern Italy," *Transactions of the Royal Society of Tropical Medicine and Hygiene*, vol. 68, no. 5, pp. 349–359, 1974.

[17] M. Maroli, L. Rossi, R. Baldelli et al., "The northward spread of leishmaniasis in Italy: evidence from retrospective and ongoing studies on the canine reservoir and phlebotomine vectors," *Tropical Medicine & International Health*, vol. 13, no. 2, pp. 256–264, 2008.

[18] Ministero delle politiche agricole, alimentari e forestali, https://www.politicheagricole.it.

[19] A. Santi, M. Renzi, R. Baldelli et al., "A surveillance program on canine leishmaniasis in the public kennels of emilia-romagna

region, Northern Italy," *Vector-Borne and Zoonotic Diseases*, vol. 14, no. 3, pp. 206–211, 2014.

[20] R. Baldelli, G. Battelli, M. Maroli et al., "A new stable focus of canine leishmaniasis in northern Italy," *Parassitologia*, vol. 43, no. 4, pp. 151–153, 2001.

[21] Istituto Zooprofilattico della Lombardia e dell'Emilia Romagna (IZSLER), *Piano Regionale di Controllo della Leishmaniosi*, 2015, http://www.izsler.it/pls/izs_bs/v3_s2ew_consultazione.redir_allegati_doc?p_id_pagina=1000&p_id_allegato=7369&p_url_rimando=/izs_bs/allegati/1000/Relazione_Piano_Leishmania_2014.pdf.

[22] P. D. Ready, "Leishmaniasis emergence in Europe," *Euro Surveillance*, vol. 15, no. 10, Article ID 19505, 2010.

[23] L'andamento Meteo in Italia-Istituto nazionale di statistica ISTAT, http://www.istat.it/it/archivio/5679.

[24] C. Chicharro, I. P. Llanes-Acevedo, E. García, J. Nieto, J. Moreno, and I. Cruz, "Molecular typing of Leishmania infantum isolates from a leishmaniasis outbreak in Madrid, Spain, 2009 to 2012," *Euro Surveillance*, vol. 18, no. 30, article 20545, 2013.

[25] P. Pagliano, N. Carannante, M. Gramiccia et al., "Visceral leishmaniasis causes fever and decompensation in patients with cirrhosis," *Gut*, vol. 56, no. 6, pp. 893–894, 2007.

[26] F. Di Masi, T. Ursini, M. D. Iannece et al., "Five-year retrospective Italian multicenter study of visceral leishmaniasis treatment," *Antimicrobial Agents and Chemotherapy*, vol. 58, no. 1, pp. 414–418, 2014.

[27] G. Morizot, R. Jouffroy, A. Faye et al., "Antimony to cure visceral leishmaniasis unresponsive to liposomal amphotericin B," *PLoS Neglected Tropical Diseases*, vol. 10, no. 1, Article ID e0004304, 2016.

[28] R. Pereira, P. Lago, R. Faria, and T. Torres, "Safety of anti-TNF therapies in immune-mediated inflammatory diseases: focus on infections and malignancy," *Drug Development Research*, vol. 76, no. 8, pp. 419–427, 2015.

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*Research Article*

# Comparative Evaluation of Anthelmintic Activity of Edible and Ornamental Pomegranate Ethanolic Extracts against *Schistosoma mansoni*

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Due to the development of praziquantel (PZQ) schistosomes resistant strains, the discovery of new antischistosomal agents is of high priority in research. This work reported the *in vitro* and *in vivo* effects of the edible and ornamental pomegranate extracts against *Schistosoma mansoni*. Leaves and stem bark ethanolic extracts of both dried pomegranates were prepared at 100, 300, and 500 $\mu$g/mL for *in vitro* and 600 and 800 mg/kg for *in vivo*. Adult worms *Schistosoma mansoni* in RPMI-1640 medium for *in vitro* and *S. mansoni* infected mice for *in vivo* tests were obtained from Theodor Bilharz Research Institute, Cairo, Egypt. *In vitro* activity was manifested by significant coupled worms separation, reduction of motor activity, lethality, and ultrastructural tegumental alterations in adult worms. *In vivo* activity was manifested revealed by significant reduction of hepatic granulomas number and diameter, decreased number of bilharzial eggs in liver tissues, lowered liver inflammatory infiltration, decreased hepatic fibrosis, and inducible nitric oxide synthase (iNOS) expression. Ethanolic stem bark extract of edible pomegranate exhibited highest antischistosomal activities both *in vitro* and *in vivo*. Therefore, pomegranate showed a good potential to be used as a promising new candidate for the development of new schistosomicidal agents.

## 1. Introduction

Schistosomiasis is one of the world major public health problems, caused by the blood-dwelling fluke of the genus *Schistosoma*. The clinically worldwide relevant species are *S. mansoni*, *S. haematobium*, and *S. japonicum* [1]. In addition to the previously mentioned species, *S. mekongi* and S. *intercalatum* represent the most important pathogenic species for human beings. Depending on the species, the schistosome worms persist in the liver and hepatic portal system or the urinary tract system of humans. Mature schistosomes lay eggs within their host, which often get trapped in the host's tissues, resulting in inflammatory and obstructive diseases of the affected organs [2, 3].

Schistosomiasis is one of the most widely occurring neglected tropical diseases with high levels of incidence in Asia, Africa, and Latin America. Studies have shown approximately 779 million people living at higher risk of infection, and 239 million people are infected with schistosomes [4, 5].

To cure morbidity and prevent the development of severe chronic stage hepatosplenomegaly, praziquantel (PZQ) is the only choice of chemotherapy against all species of *Schistosoma*. Presenting good efficacy and low toxicity, the drug has been widely used for more than three decades and is therefore susceptible to the emergence of praziquantel resistant schistosomes [6].

The study of medicinal plants as a new approach for schistosomiasis treatment is feasible and promising one [7,

8]. The research on medicinal plants is encouraged by the WHO, considering that certain traditional knowledge on curative plants could add up to the development of new pharmaceutical products as well as to the combat against diseases that affects the populations of developing countries [9, 10].

The search for new antischistosomal treatments has led to the study of natural substances such as artemisinin and its derivatives, curcumin, phytol, and pomegranate [11–13].

Pomegranate (*Punica granatum* L.) is a favorite table fruit in tropical countries, belonging to Punicaceae family. This family is unusual in having the sole genus *Punica,* a genus of large shrubs or small trees with two species. One is *P. protopunica* Balf. S., which is found wild in Socotra island, and the other is *P. granatum* L. (edible pomegranate), which is cultivated in tropical and subtropical parts of the world for its edible fruits [14].

*Punica granatum* L. var. *nana* is a dwarf variety of *P. granatum* L. popularly planted as an ornamental plant in gardens. It could well be a wild form with distinct origin. It does not usually produce edible fruits [15].

The peel and seeds of *P. granatum* L. showed various therapeutic applications such as antibacterial, antifungal, antioxidant, antitumor, antiviral, antimalarial, and antimutagenic effects [16, 17]. Edible pomegranate extracts have been reported to have promising results against *S. mansoni* either *in vitro* or *in vivo* [12, 18, 19].

The root, stem bark, and, to lesser extent, the fruit rind of pomegranate have been commonly used as vermifugal or taenicidal agents [20]. Pomegranate has also antiprotozoal activity and it is used in folk medicine for treatment of dysentery [21].

The methanolic extract of *P. granatum* L. var. *nana* leaves exhibited antioxidant activity, nematicidal activity against three root-knot nematode species, and hepatoprotective activity against carbon tetrachloride induced hepatotoxicity [22].

In our locality, many factors, including the high prevalence, wide distribution of schistosomiasis, and drug resistance for the already used treatment, necessitate the need for control of this helminthiasis and impulse the studies for new and more comprehensive alternative therapeutics without adverse effects. Hence, the current study aimed to investigate the *in vitro* and *in vivo* activity of leaves and stem bark ethanolic extracts of both dried edible and ornamental pomegranate against *Schistosoma mansoni* (Egyptian strain).

## 2. Materials and Methods

*2.1. Plant Materials.* 200 g of each of the leaves of edible pomegranate (LEP) and stem bark of edible pomegranate (SEP) (*P. granatum* L.) and similar leaves of ornamental pomegranate (LOP) and stem bark of ornamental pomegranate (SOP) of *P. granatum* L. var. *nana* was locally collected from Faculty of Pharmacy garden of medicinal plants and Experimental Station of Agriculture, Faculty of Agriculture Assiut University, Assiut, Egypt. Test plants were authenticated by Dr. Naeem E. Keltawy, Professor of Ornamental Horiculture and Floriculture, Faculty of Agriculture, Assiut University, Assiut, Egypt. Voucher specimens (number 45) were kept in the herbarium, Pharmacognosy Department, Faculty of Pharmacy, Assiut University.

*2.2. Preparation of Extracts.* Plant samples were allowed to dry at room temperature before being ground to fine powder. Powdered plant materials were extracted with 70% ethanol at room temperature by maceration and then filtered and the filtrates were concentrated under vacuum using rotary evaporator. The obtained solvent-free residue of each plant extract was stored at 4°C for subsequent preparation of the required doses.

*2.3. Dose Preparations of Extracts.* Plant extracts were freshly prepared before usage by suspending 1 g of each extract in 50 mL 3% tween 80 dissolved in 0.9% saline. For *in vitro* antischistosomal testing, 100, 300, and 500 $\mu$g/mL were used, while 600 and 800 mg/kg for *in vivo* assay were prepared [12].

*2.4. Cytotoxicity Assays (CTAs) on Tissue Culture Cells.* CTAs were performed on mouse fibroblast cell BALB/c 3T3 (VACSERA, Egypt) supplemented with 10% bovine calf serum, 4 mM L-glutamine, 100 IU penicillin, and 100 $\mu$g/mL streptomycin (Bioanalyse, Turkey) using the neutral red uptake assay for all pomegranate extracts at the higher concentration [23].

*2.5. Evaluation of Microbial Contamination and Endotoxin Production.* Total aerobic microbial count and total combined yeasts/moulds count were used for quantitative enumeration of mesophilic bacteria or fungi that may grow under aerobic conditions in all pomegranate extracts at the higher concentration and for PZQ using the pour plating technique (EDQM Council of Europe, 2014). The bacterial endotoxin test was performed by the limulus amoebocyte lysate assay (gel-clot technique) as reported by Hussaini and Hassanali [24].

*2.6. Standard Antischistosomal Treatment.* Praziquantel suspension, a product of Egyptian International Pharmaceutical Industries Company (EIPI Co.), was purchased locally. PZQ was used as positive control at concentration of 10 $\mu$g/mL for *in vitro* and 200 mg/kg for *in vivo* experiments [25, 26].

*2.7. Schistosome Parasites and Experimental Infected Hosts.* *S. mansoni* (Egyptian strain) adults were purchased from the experimental animal research unit of the Schistosome Biological Supply Center (SBSC), Theodor Bilharz Research Institute (TBRI), Cairo, Egypt. Swiss albino female mice CD strain, weighing 20–25 g and aged 4 weeks, were individually infected using the tail immersion technique by exposure to a suspension containing 100 *S. mansoni* cercariae (100 cercariae/mouse) from naturally infected *Biomphalaria alexandrina* for 2 h according to the method described by Liang et al. [27]. Mice were bred under environmentally controlled conditions (temperature ~25°C and 12 h light and

dark cycle) and fed with a standard stock commercial pellet diet (containing 24% protein) and water *ad libitum*.

## 3. Experimental Design

### 3.1. Experiment (1)

*3.1.1. In Vitro Assessment of the Antischistosomal Effects of the Prepared Extracts.* For *in vitro* bioassay, *Schistosoma mansoni* adult worm pairs of Egyptian strain were retrieved aseptically from sacrificed infected mice and collected by perfusion of the hepatic portal system and mesenteric veins using citrated saline according to the technique of Stirewalt and Dorsey [28] from mice livers 8 weeks postinfection.

Adult worms were washed three times with the RPMI 1640 (Roswell Park Memorial Institute 1640) culture medium (Invitrogen, Carlsbad, California, USA), which was used for culturing the parasite. The medium was supplemented with L-glutamine, 20% fetal calf serum, and antibiotics (300 $\mu$g streptomycin, 300 IU penicillin, and 160 $\mu$g gentamycin per mL) [12]. After washing, 5 couples of worms were transferred to each well of a 24-well culture plate (TPP, St. Louis, MO) containing the same medium.

Two mL of the tested doses (100, 300, and 500 $\mu$g/mL) from leaves and stem bark extracts was added to each well. The final volume in each well was 2 mL. The plate was incubated at 37°C in a humid atmosphere containing 5% $CO_2$ [29]. The parasites were kept for 12 h and monitored every 2 h. A pure medium and medium with 3% tween 80 in 0.9% saline were used as negative controls, while PZQ (10 $\mu$g/mL) was used as a positive control. All the steps were performed under a sterilized laminar flow chamber. The experiment was carried out in triplicate and repeated three times.

Treated worms were monitored for their mating (pairing) of the worms, motility (worm's motor activity changes), and mortality rate using an inverted optical microscope (Olympus CK2). Worms which did not show motility for one minute were considered dead. Changes in worm's motor activity (motility) of schistosomes were assessed qualitatively and their motor activity reduction was defined as "slight" or "significant" [30].

The effect of the treatment was also assessed with an emphasis on morphological alterations in the tegument which were observed using scanning electron microscopy (SEM) [31]. Observation of adult schistosomes in the *in vitro* experiment was performed at 2 h intervals throughout the 12 h experimental incubation period and the results were reported at 2, 4, 6, and 12 h (the end point of the experiment for the negative control groups).

*3.1.2. Preparation of Adult S. mansoni Worms for SEM.* To observe morphological changes in the tegument of adult parasites, schistosome worms, when they died, and control worms at 12 h, the end point of the experiments, were washed thoroughly with distilled water. The parasites were fixed for 2 h in 4% glutaraldehyde (pH 7.4) and 5% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.2). They were rinsed overnight in cacodylate buffer, dehydrated, dried in a critical point dryer according to Hayat [32], mounted on stubs, and sputter-coated with gold particles in the sputter coating apparatus for 6 minutes. Specimens were processed, examined, and photographed using Jeol-JSM-5400 LV at the Scanning Electron Microscope Unit, Assiut University, Assiut, Egypt.

### 3.2. Experiment (2)

*3.2.1. In Vivo Assessment of the Antischistosomal Effects of the Prepared Extracts.* Fifty-five *S. mansoni* infected female mice were obtained from TBRI, Cairo, Egypt, 8 weeks postinfection. Infected mice were randomly allocated into 11 groups with 5 animals each, at the time of the experiment:

(G1) Infected untreated control mice (negative control 1)

(G2) Infected mice given 3% tween 80 in saline (negative control 2)

(G3) Infected mice treated with 200 mg/kg PZQ (positive control)

(G4) Infected mice treated with 600 mg/kg LEP

(G5) Infected mice treated with 600 mg/kg SEP

(G6) Infected mice treated with 800 mg/kg LEP

(G7) Infected mice treated with 800 mg/kg SEP

(G8) Infected mice treated with 600 mg/kg LOP

(G9) Infected mice treated with 600 mg/kg SOP

(G10) Infected mice treated with 800 mg/kg LOP

(G11) Infected mice treated with 800 mg/kg SOP

Each mouse was given a single oral dose daily for 7 consecutive days using stainless-steel esophageal tube. All mice were sacrificed by cervical dislocation after 7 days of treatment. Assessment of the treatment *in vivo* was performed through histopathological examination of liver tissue for detection of hepatic inflammation, hepatic fibrosis, and schistosomal granulomas formation. The assessment was also done through immunohistochemical analysis of iNOS reactivity in liver tissue. The experiment was repeated three times.

*3.2.2. Histopathological Assessment.* Liver samples of the left lobe of each sacrificed mouse were rinsed with phosphate-buffered saline and fixed in 10% formalin for 24 h. Liver samples were dehydrated in increasing concentrations of ethanol, diaphonized in xylol, and embedded in paraffin wax blocks. Sections of 4 $\mu$m thickness were stained with hematoxylin and eosin [33]. The sections were evaluated using the bright field microscopy to evaluate the degree of inflammation, fibrosis, and granuloma formation followed by image capture and processing using Camidia image manager. All the granulomas found in 10 histologic sections of random fields were counted. Measurement of mean granuloma diameter was performed using an ocular micrometer at magnification of 100x. Only nonconfluent, lobular granulomas containing eggs in their centers were measured (periocular granulomas) [34].

*3.2.3. Immunohistochemistry for Determination of iNOS Reactivity.* Left lobe liver sections from the previously prepared paraffin blocks, 4 $\mu$m thickness, mounted on glass slides, were kept overnight at 56°C. They were deparaffinized with xylene and rehydrated with decreasing percentages of ethanol and finally with water. For antigen retrieval, slides were heated by microwaving in 10 mM citrate buffer (pH 6.0) for 12 min. Slides were left to cool for 20 min at room temperature and rinsed with distilled water. Surroundings of the sections were marked with a PAP pen. The endogenous peroxidase activity was blocked with $H_2O_2$ for 10 min at room temperature and later rinsed with distilled water and PBS (phosphate-buffered saline). Liver sections were then incubated for 1 h at room temperature with the following antibody: iNOS rabbit Pab (Neomarker, RB-1605-P) antibodies. Antibodies were diluted at 1 : 100. The sections were washed and rinsed with PBS three times for 5 min each. Slides were incubated for 30 min at room temperature with biotinylated goat anti-rabbit antibodies. The streptavidin peroxidase label reagent was applied to the slides after being washed in PBS, for 30 min at room temperature in a humid chamber. After blotting off excess buffer, a universal staining colored product was developed by incubation with AEC (3-Amino-9-Ethylcarbazole) Chromogen (Lab Vision, TA-004-HAC) for 5 min according to manufacturer's instructions. Finally, slides were dehydrated and cleared. The slides were counterstained with Mayer's hematoxylin and mounted in glycerol gelatin after washing in distilled water and mounted with cover slips [35].

*3.3. Ethical Considerations.* A standard protocol was drawn up in accordance with the Good Laboratory Practice (GLP) regulations of the World Health Organization (WHO). The principles of laboratory animal care were duly followed in this study [36]. Ethical animal practices were followed under standard regulations dictated by the animal care committee of Faculty of Medicine, Assiut University. Ethical approval was granted by the Research and Ethic Committee of Faculty of Medicine, Assiut University.

*3.4. Statistical Data Analysis.* The results were analyzed using the SPSS (Statistical Package for the Social Sciences, version 16 for Windows) software (SPSS Inc., Chicago, Illinois, USA). Significant differences were determined by one-way analysis of variance (ANOVA). The values were presented as mean ± standard deviation (SD). Data were analyzed using Student's Tukey's test ($t$-test) which was used to calculate the significance of differences observed between mean values of experimental and control groups in each experiment. $P$ values of less than 0.05, 0.01, or 0.001 were used to indicate statistical significance.

## 4. Results

The preliminary phytochemical screening of both plant extracts showed the presence of volatile constituents, polyphenols glycosides, triterpenes, sterols, flavonoids, anthocyanins, triglycerides, tannins, and alkaloids.

*4.1. Cytotoxicity Assays (CTAs).* The optical density $(OD)_{540}$ of each of the tested extracts was compared with the mean value $OD_{540}$ for the negative control (distilled water). Tested pomegranate extracts fulfilled the mentioned acceptance criteria through absence of cytotoxic effects of the studied extracts. Cell viabilities were more than 70% relative to the negative control for tested extracts at their highest concentrations. Thus, the concentrations in which pomegranate presented the schistosomicidal activity were not associated with cytotoxic effects on fibroblast cell.

*4.2. Evaluation of Microbial Contamination and Endotoxin Production.* Total aerobic microbial count and total combined yeasts/moulds count were negative for the tested pomegranate extracts. Tested pomegranate extracts were endotoxin free.

*4.3. In Vitro Treatment Efficacy of LEP, SEP, LOP, and SOP Extracts on Adults S. mansoni at Different Concentrations.* All the tested extracts influenced the process of natural mating, causing separation of couple schistosomes depending on the concentration used and exposure time. Nearly 95% of the worms had been separated within the first 2 h with the use of 500 $\mu$g/mL SEP, compared to the negative control groups (Table 1). PZQ (10 $\mu$g/mL) caused couple worm separation after the first 2 h of incubation. Negative control groups showed couple separation nearly at 10 h after incubation. Moreover, concentrations which were not 100% lethal to the worms were proven as efficient mating inhibitors, once all of them separated the couples in all samples.

Concerning the motility, a significant reduction in the parasites movements was observed in all concentrations. The percentage of worms that had their motility reduced was directly proportional to the concentration and to the period of incubation. A slight decrease in motor activity was observed after 2 h of incubation for all adult worms exposed to 500, 300, and 100 $\mu$g/mL concentration of SEP. Total motility loss occurred at 4, 6, and 12 h, respectively. No change in motor activity was observed at 4 h interval, while it decreased at 6 h interval and complete loss of motility occurred at 10 h interval in the negative control groups. On the other hand, PZQ (10 $\mu$g/mL) resulted in decrease in motor activity starting from the first 2 h of incubation and complete loss of motor activity in all worms occurred at 4 h interval.

The survival of *S. mansoni* adults exposed to ethanolic extracts of LEP, SEP, LOP, and SOP depended directly on both concentration and incubation period. The 500, 300, and 100 $\mu$g/mL concentrations of SEP caused death of 100% of parasites within 4, 6, and 12 h of incubation, respectively (Table 1). Ethanolic extracts of LEP at 500, 300, and 100 $\mu$g/mL concentrations caused death of 100% worms after 6 and 12 h of incubation, respectively (Table 1). LOP and SOP (500 $\mu$g/mL) caused significant mortality ($P < 0.01$) among schistosome parasites after 6 h of incubation, while 100 and 300 $\mu$g/mL concentrations of the same extracts expressed their mortality effect on adults *S. mansoni* after 12 h of incubation (Table 1). No difference was observed between male and female adult worms in response to different

Table 1: *In vitro* effects of leaves and stem bark ethanolic extracts of edible and ornamental pomegranate on adult worms of *S. mansoni* after 12 h incubation period.

| Group | Conc ($\mu$g/mL) | Incubation period (h) | Number of separated worms (%) | % of worm mortality | % of worms with tegumental alteration | |
|---|---|---|---|---|---|---|
| | | | | | Partial | Extensive |
| SEP | 500 | 2 | 95 | 90 | — | — |
| | | 4 | 99 | 100 | 10 | 90 |
| | | 6 | 100 | 100 | — | — |
| | | 12 | 100 | 100 | — | — |
| | 300 | 2 | 87 | 80 | — | — |
| | | 4 | 99 | 99 | — | — |
| | | 6 | 99 | 100 | 30 | 70 |
| | | 12 | 100 | 100 | — | — |
| | 100 | 2 | 71 | 60 | — | — |
| | | 4 | 80 | 65 | — | — |
| | | 6 | 95 | 90 | — | — |
| | | 12 | 100 | 100 | 50 | 50 |
| LEP | 500 | 2 | 80 | 0 | — | — |
| | | 4 | 85 | 45 | — | — |
| | | 6 | 99 | 100 | 20 | 80 |
| | | 12 | 100 | 100 | — | — |
| | 300 | 2 | 75 | 0 | — | — |
| | | 4 | 85 | 70 | — | — |
| | | 6 | 99 | 90 | — | — |
| | | 12 | 100 | 100 | 15 | 85 |
| | 100 | 2 | 65 | 0 | — | — |
| | | 4 | 80 | 0 | — | — |
| | | 6 | 95 | 90 | — | — |
| | | 12 | 100 | 100 | 60 | 40 |
| LOP | 500 | 2 | 40 | 0 | — | — |
| | | 4 | 75 | 25 | — | — |
| | | 6 | 85 | 100 | 20 | 80 |
| | | 12 | 100 | 100 | — | — |
| | 300 | 2 | 30 | 0 | — | — |
| | | 4 | 65 | 12 | — | — |
| | | 6 | 75 | 34 | — | — |
| | | 12 | 100 | 100 | 50 | 50 |
| | 100 | 2 | 20 | 0 | — | — |
| | | 4 | 55 | 0 | — | — |
| | | 6 | 70 | 0 | — | — |
| | | 12 | 100 | 98 | 70 | 30 |

Table 1: Continued.

| Group | Conc ($\mu$g/mL) | Incubation period (h) | Number of separated worms (%) | % of worm mortality | % of worms with tegumental alteration | |
|---|---|---|---|---|---|---|
| | | | | | Partial | Extensive |
| SOP | 500 | 2 | 20 | 10 | — | — |
| | | 4 | 60 | 45 | — | — |
| | | 6 | 76 | 100 | 30 | 70 |
| | | 12 | 100 | 100 | — | — |
| | 300 | 2 | 18 | 0 | — | — |
| | | 4 | 60 | 0 | — | — |
| | | 6 | 70 | 90 | — | — |
| | | 12 | 100 | 100 | 85 | 15 |
| | 100 | 2 | 15 | 0 | — | — |
| | | 4 | 55 | 0 | — | — |
| | | 6 | 70 | 90 | — | — |
| | | 12 | 100 | 100 | 90 | 10 |
| PZQ | 10 | 2 | 95 | 95 | — | — |
| | | 4 | 100 | 100 | 25 | 75 |
| Control –ve | | 2 | 0 | 0 | — | — |
| | | 4 | 60 | 0 | — | — |
| | | 6 | 70 | 10 | — | — |
| | | 12 | 100 | 100 | 0 | 0 |

Incubation period: 12 h; control –ve in RPMI-1640 and medium with 3% tween 80 in 0.9% saline.

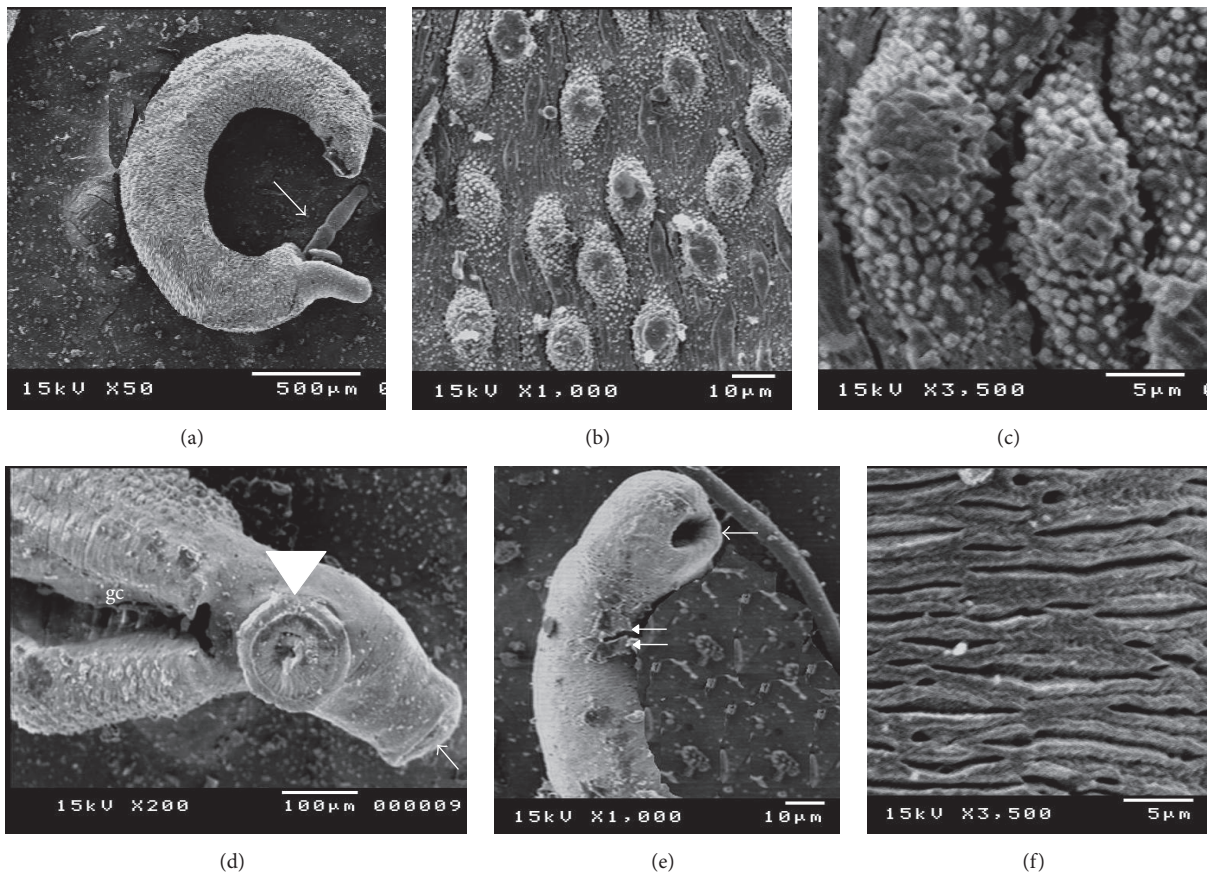



Figure 1: Scanning electron microscopy (SEM) of normal adult *S. mansoni* kept in RPMI-1640 alone or with 3% tween 80; (a) male and female (arrow) in copula; (b) male dorsal surface showing tubercles and spines; (c) higher magnification of the tubercles and spines; (d) male anterior end showing gynecophoric canal (gc), oral sucker (arrow); and ventral sucker (arrowhead); (e) female anterior end showing oral sucker (arrow), and ventral sucker (double arrows); (f) female dorsal surface showing normal appearance of the tegument.

concentrations of the used extracts in either motility affection or survival rates.

The PZQ treated group (positive control) showed total death of the parasites (100%) after 4 h of incubation (Table 1). All the negative control groups were killed at 12 h of incubation which was considered the end point of the experiment.

*4.4. Tegumental Changes of S. mansoni Adult Worms in Response to Exposure to Edible and Ornamental Pomegranate Extracts Visualized by Scanning Electron Microscope (SEM).* Ultramorphological alterations were observed in *S. mansoni* adult males and females after 12 h incubation *in vitro* with the 100, 300, and 500 $\mu$g/mL concentrations of leaves and stem bark of both edible and ornamental pomegranate ethanolic extracts. The parasites exposed to LEP and SEP revealed dose-dependent variable degrees of tegumental morphological alterations, when compared to negative control ones. SEP (500 $\mu$g/mL) induced more morphological destructions than those induced by LEP at the same dose. No tegumental changes in adult worms were observed for the negative control groups (Figure 1). The positive control (PZQ treated group) showed similar tegumental alteration in 100% of schistosome worms.

*Male Worms*. The treatments caused variable degrees of tegumental contractions, tubercles, and spine damage (destruction, peeling of spines, tubercles, and tegument peeling or sloughing) especially on its dorsal surface. The occurrence of bubbles surrounding the morphologically altered tubercles was observed in addition to suckers alteration or destruction (Figures 2(a)–2(d)).

*The Female Worms*. Tegument scaling, wrinkling, and erosion (contraction and peeling of dorsal region) and suckers' alterations or destruction were observed (Figures 2(e) and 2(f)).

Concerning treatment with LOP and SOP extracts, the worms (either male or female) showed similar morphological tegumental changes but of lesser degree to LEP and SEP induced morphological changes.

*4.5. Treatment Efficacy In Vivo of SEP, LEP, LOP, and SOP Extracts*

*4.5.1. Histopathological Assessment.* Microscopical examination of histological liver sections from infected untreated control group revealed pathological chronic granulomatous

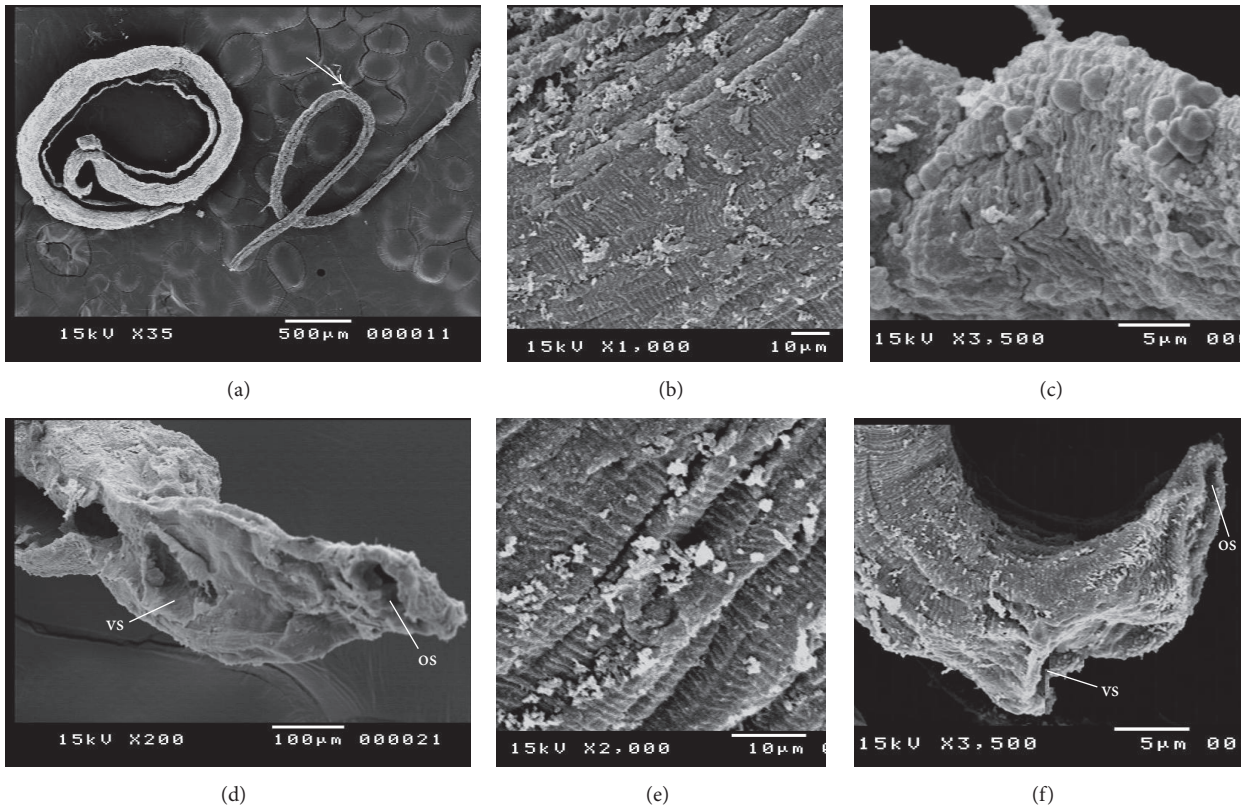



Figure 2: Scanning electron microscopy (SEM) of adult *S. mansoni* worms after their exposure to 100, 300, and 500 $\mu$g/mL ethanolic extracts of leaves and stem bark of edible and ornamental pomegranate. (a) Separated male and female (arrow); (b) male dorsal surface showing tegumental peeling with destruction and peeling of tubercles and spines; (c) male showing bubbles surrounding the morphologically altered tubercles on its dorsal surface; (d) male suckers' alterations or destruction, os: oral sucker and vs: ventral sucker; (e) female showing tegumental scaling, wrinkling, and erosion; (f) female suckers' alterations or destruction, os: oral sucker and vs: ventral sucker.

lesions in the hepatic parenchyma. These lesions formed of numerous bilharzial eggs containing miracidia, surrounded by numerous chronic inflammatory cells in form of epithelioid cells, lymphocytes, plasma cells, macrophages, and eosinophils forming granuloma with severe areas of fibrosis (Figure 3(a)).

Nearly similar observations were detected in the hepatic histological sections from groups treated with 600 mg/kg SOP, LOP, and LEP (Figures 3(b)–3(d)). These inflammatory reactions were less prominent in groups treated with 800 mg/kg LOP and SOP in the form of granuloma with fewer eggs, less fibrosis, and moderate chronic inflammatory cell infiltration (Figures 4(a)–4(c)).

Histological liver sections from groups treated with LEP (800 mg/kg) showed moderate diffuse infiltration of liver parenchyma by chronic inflammatory cells without observed eggs or areas of fibrosis (Figure 4(d)). Similar observations were reported in histological liver sections from groups treated with 600 and 800 mg/kg SEP and PZQ (200 mg/kg). They showed absence of bilharzial eggs and fibrosis with significant reduction of liver parenchyma infiltration by the chronic inflammatory cells. For hepatic granulomas number and diameter, histological liver sections from infected untreated control groups revealed about 121.3 granulomas of average diameter 235.7 ± 16.1 $\mu$m. Livers of different treated groups showed decrease in granuloma size and number with minimal degenerative changes in liver tissues as shown in Table 2.

Oral administration of 800 mg/kg LEP decreased hepatic granulomas number and size to 54.1 and 37.2%, respectively, while administration of 600 mg/kg of the same extract decreased them to 51.3 and 34.5%, respectively. Following oral administration of 600 mg/kg SEP, there was decrease in the mean granuloma diameter to 40.1%, while 800 mg/kg of the same extract showed about 40.9% reduction. Other extracts and PZQ treatments showed variable effects on hepatic granuloma number and diameter (Table 2). Thus, the hepatic granuloma average diameter was significantly smaller ($P < 0.01$) in groups treated with SEP and LEP in comparison to groups treated with SOP and LOP (600 and 800 mg/kg) and control groups. These hepatic granuloma average diameters were nearly similar to positive control group (PZQ 200 mg/kg) with insignificant difference ($P > 0.01$) (Table 2).

*4.5.2. Expression of iNOS Detected by Immunohistochemistry.* In comparison to immunohistochemical expression of iNOS between different groups, iNOS reactivity (cytoplasmic expression) was stronger in the hepatocytes of

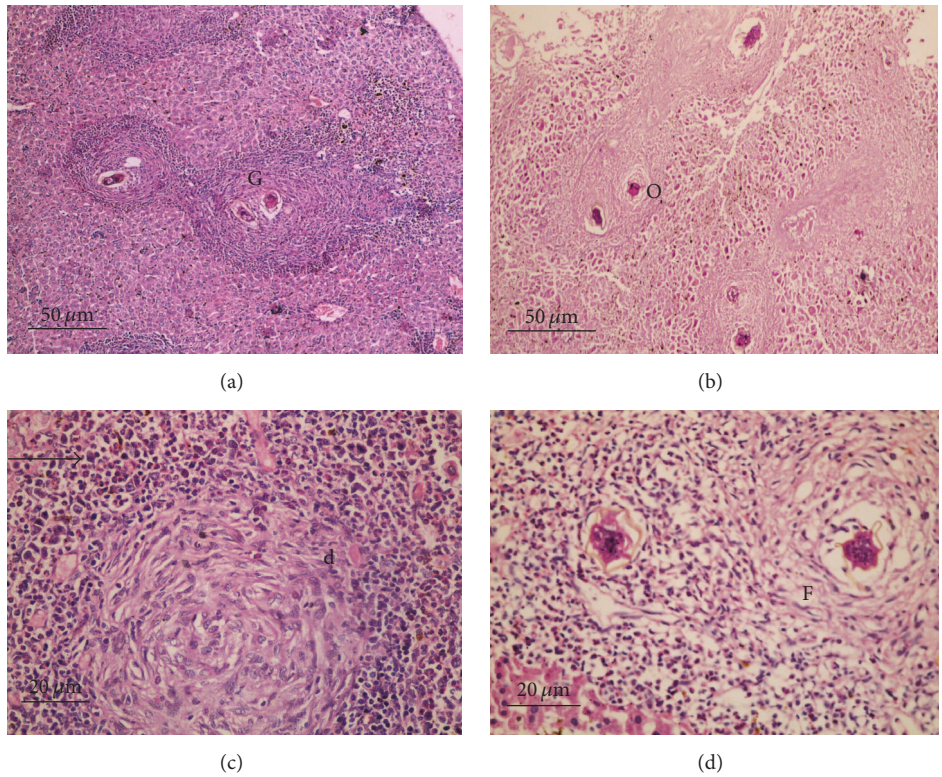



Figure 3: Histological liver sections; H & E staining; (a) infected untreated group showing numerous bilharzial eggs surrounded by numerous chronic inflammatory cells (×200); (b) group treated with 600 mg/kg SOP (×200); (c) group treated with 600 mg/kg LOP (×400); (d) group treated with 600 mg/kg LEP (×400). All showed similar structures: (G) granuloma, (O) bilharzial eggs, and (F) fibrosis and arrow pointed to chronic inflammatory cells.

Table 2: Effect of oral administration of different doses of SEP, LEP, LOP, and SOP extracts *in vivo*.

| Group | Dose (mg/kg) | Granuloma number | | Granuloma diameter (GD) | | Eggs in liver tissue | Immunohistochemical findings | |
|---|---|---|---|---|---|---|---|---|
| | | Mean ± SE | Reduction% | Mean ($\mu$m) ± SE | Reduction% | | iNos hepatocytes | iNos inflammatory cells |
| SEP (G5&G7) | 600 | 51.2** | 57.8 | 141.2 ± 22.1** | 40.1 | Absent | Weakest | Negative |
| | 800 | 45.3** | 62.7 | 139.2 ± 24.5** | 40.9 | Absent | Weakest | Negative |
| LEP (G4&G6) | 600 | 59.1* | 51.3 | 154.3 ± 11.2** | 34.5 | Numerous | Strong | Strong |
| | 800 | 55.7** | 54.1 | 148.1 ± 22.1** | 37.2 | Absent | Weak | Weak |
| LOP (G8&G10) | 600 | 63.2* | 47.9 | 168.1 ± 22.1* | 28.7 | Numerous | Strong | Strong |
| | 800 | 60.8* | 49.9 | 164.5 ± 21.2 | 30.2 | Few | Weak | Strong |
| SOP (9&G11) | 600 | 68.2* | 43.8 | 189.4 ± 24.5* | 19.6 | Numerous | Strong | Strong |
| | 800 | 57.3** | 52.8 | 176.2 ± 25.1* | 25.2 | Few | Strong | Strong |
| PZQ(G3) | 200 | 39.4* | 67.6 | 135.4 ± 20.3 | 42.5 | Absent | Weakest | Negative |
| Infected untreated controls (G1&G2) | | 121.3 | — | 235.7 ± 16.1 | — | Numerous | Strong | Strong |

The difference was significant at $^{*}P < 0.01$ and $^{**}P < 0.001$ compared to infected untreated control group.

infected untreated group, group administered 3% tween 80 (Figure 5(a)), groups treated with 600 mg/kg LEP (Figure 5(d)), 600 mg/kg SOP (Figure 6(a)), 600 mg/kg LOP (Figure 6(b)), and 800 mg/kg SOP (Figure 7(a)) compared to low or weak expression in the hepatocytes of groups treated with 800 mg/kg LEP (Figure 6(c)) and 800 mg/kg LOP (Figure 6(d)) (Table 2). Groups treated with PZQ (Figure 5(c)) and 600, 800 mg/kg SEP (Figure 7(b)) revealed the lowest iNOS expression (reactivity) in the hepatocytes. The iNOS reactivity was stronger around the granulomatous

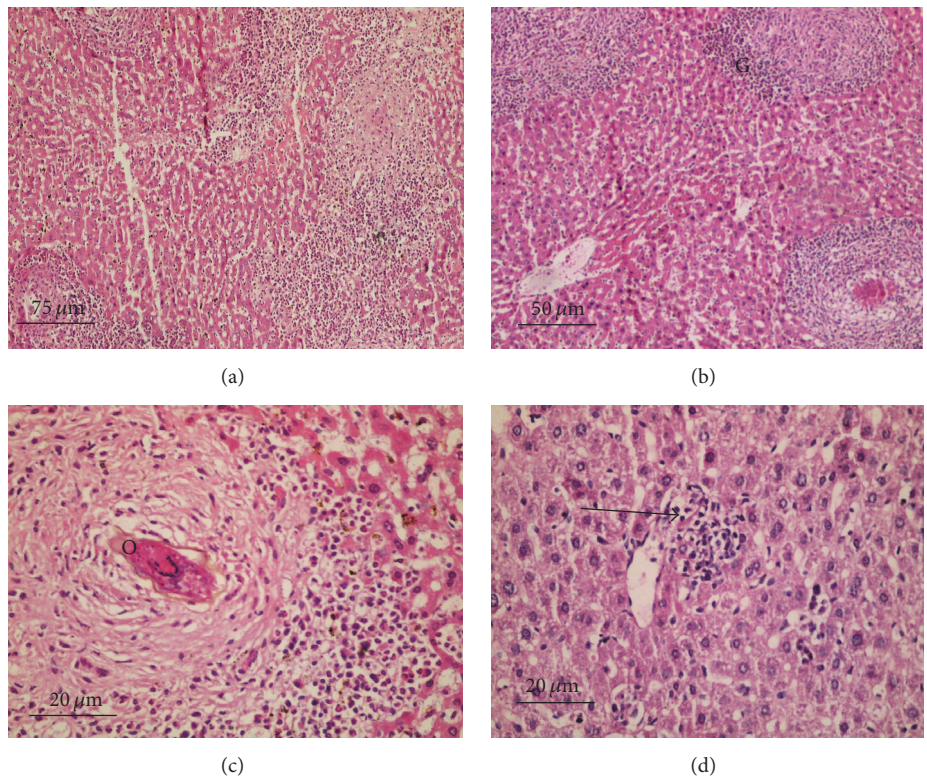



Figure 4: Histological liver sections; H & E staining; (a) group treated with 800 mg/kg LOP (×100); (b) group treated with 800 mg/kg SOP (×200); (c) group treated with 800 mg/kg SOP higher magnification (×400); all showing less prominent inflammatory reactions; (d) group treated with 800 mg/kg LEP showing moderate diffuse infiltration of liver parenchyma by chronic inflammatory cells without observed eggs or areas of fibrosis (×400); (G) granuloma and (O) bilharzial eggs and arrow pointed to chronic inflammatory cells.

lesions. Additionally, the iNOS reactivity reduced simultaneously with decreased granulomatous lesions.

Strong reactivity of iNOS was clearly observed in the cytoplasm of the chronic inflammatory cells in infected untreated group (Figure 5(a)), group administered 3% tween 80 (Figure 5(b)), groups treated with 600 mg/kg LEP (Figure 5(d)), 600 mg/kg SOP (Figure 6(c)), 600 mg/kg LOP (Figure 6(b)), 800 mg/kg LOP (Figure 6(d)), and 800 mg/kg SOP (Figure 7(a)), compared to low or weak expression in inflammatory cells of group treated with 800 mg/kg LEP (Figure 6(a)) (Table 2). Negative reactivity of iNOS was observed in the cytoplasm of the chronic inflammatory cells in groups treated with PZQ (Figure 5(c)) and SEP (600, 800 mg/kg) (Figure 7(b)) (Table 2).

## 5. Discussion

Plants are an important source of biologically active compounds that can provide structures for the development of new drugs [37]. In recent years, an extensive attention to natural products as a treatment of neglected tropical diseases, including schistosomiasis, has been growing. The awareness has stimulated an exertion to improve a new medicine as a substitute method to this parasitosis control [9, 10, 38].

In this study, the effects of different concentrations of leaves and stem bark ethanolic extracts of dried edible and ornamental pomegranate against *S. mansoni* (Egyptian strain) were evaluated *in vitro* and in experimentally infected mice. This study was the first one investigating the efficacy of ornamental pomegranate against *S. mansoni*. Moreover, as a first step, *in vitro* antischistosomal studies were performed on adult worms.

In the present study, the *in vitro* study established the antischistosomal activity of LEP, SEP, LOP, and SOP extracts on *S. mansoni* adult worms concerning (mating, motility, survival time, and tegumental alterations) the worms at different concentrations (100, 300, and 500 $\mu$g/mL). The observed effects were dose-dependent, with 500 $\mu$g/mL being the most effective one in a shorter period of incubation. All the tested extracts caused unpairing of couple worms, slow contractions, motility reduction, and paralysis causing the parasites death most of the time. Noel, in his study, explained that paralysis was associated with important neurotransmitters or neuromodulators such as dopamine, acetylcholine, and/or serotonin [39].

SEP and LEP extracts were more efficient mating inhibitors than SOP and LOP extracts. SEP extract (500 mg/kg) was as effective as PZQ *in vitro*. Earlier studies by Pica-Mattoccia and Cioli [40] informed about the PZQ effects on the worms, causing contractions whenever the parasite was exposed to concentrations 0.1 and 1 $\mu$g/mL. However, it was known that PZQ caused a quick calcium influx followed by contraction, paralysis, and tegument destruction.

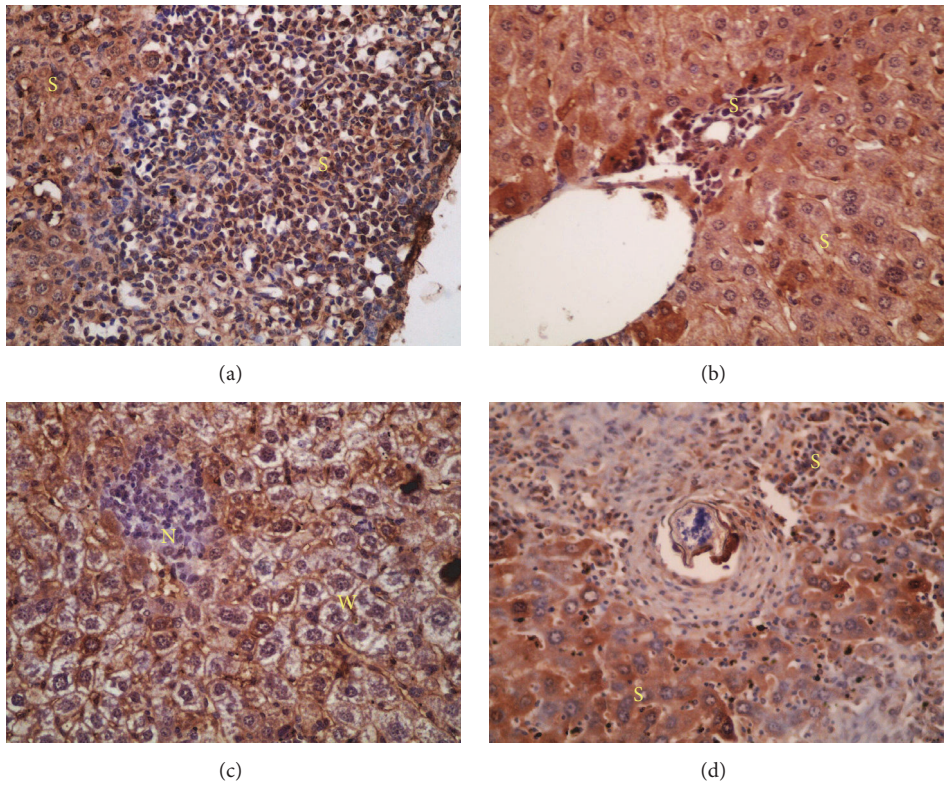
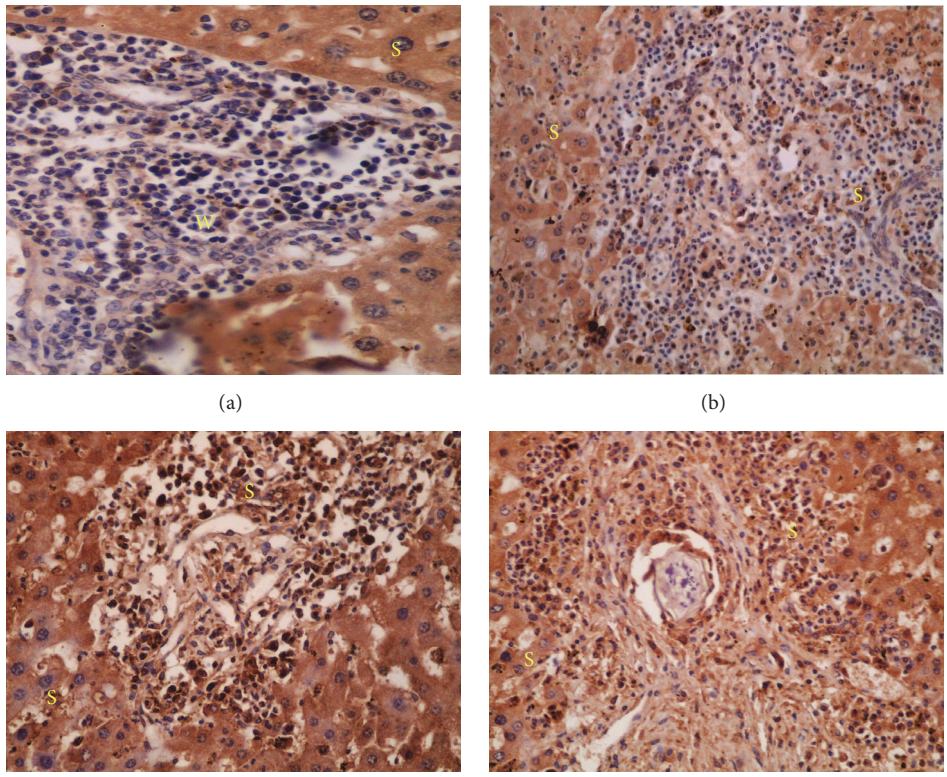



Figure 5: The distribution and intensity of iNOS in liver sections analyzed by immunohistochemistry; (a) infected untreated group; (b) group administered tween 80 showing strong iNOS reactivity (cytoplasmic expression) in the hepatocytes (×400); (c) group treated with PZQ showing lowest iNOS expression (reactivity) in the hepatocytes (×400); (d) groups treated with 600 mg/kg LEP showing similar observation to (a) and (b) (×400); (S) strong, (W) weak, and (N) negative intensity.

The use of inverted optical microscopy did not allow detailing the tegumental changes presented in the parasite; a qualitative analysis to evaluate the tegumental damage of specimens after treatment *in vitro* through the scanning electron microscopy (SEM) was used in this study. SEM had been employed by several authors in order to elucidate the mechanisms of action of drugs/compounds used in the experimental treatment of schistosomiasis [9, 31, 41].

The changes induced by treatments with ethanolic extracts of dried edible and ornamental pomegranate were related to damage in suckers, oral and acetabular in both male and female schistosomes. SEM examinations of adult schistosomes showed that the treatments caused an extensive peeling of the integument especially in the dorsal region, resulting in the exposure of the antigens of this surface. Furthermore, blebs were visible on the tegument of male worms exposed to treatment with pomegranate extracts. Similar results were observed by de Oliveira et al. [9] who evaluated the *in vitro* effect of crude dichloromethane and aqueous fraction extracts of *Baccharis trimera* on *S. mansoni*. It is believed that the morphological changes caused by a drug/compound with schistosomicidal activity over sarcoplasmic membrane and the tegument of the parasite may be accompanied by an increasing of the exposure to antigens on the surface of the worm. These changes were identified and connected with the host immune-response, required to complement the activity of the drug. For this reason, the tegument of schistosomes had been investigated in the development of new antischistosomal drugs since the late 40s to the present days [9, 42].

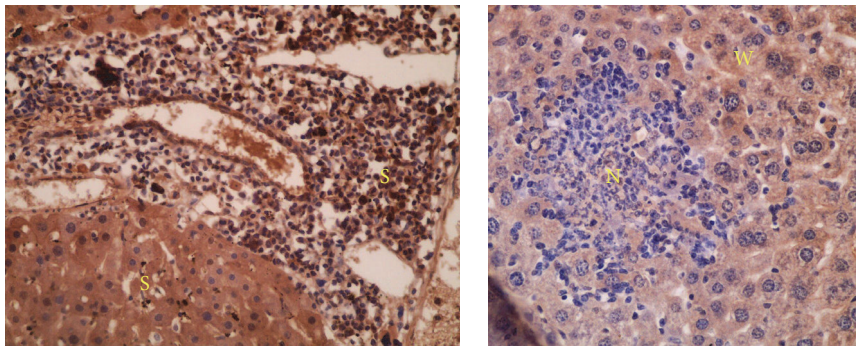
In the present study, the *in vivo* antischistosomal activity of LEP, SEP, LOP, and SOP extracts on *S. mansoni* infected mice was evaluated concerning histopathological changes (hepatic inflammation, schistosomal granulomas affection, and number of eggs in liver tissues) and immunological responses through determination of iNOS reactivity. The tested extracts showed dose-dependent reduction in both granuloma diameter and number, number of eggs in liver tissues, liver inflammatory infiltration, and fibrosis compared to infected untreated control groups. SEP and LEP extracts were more effective than SOP in reducing granuloma number and diameter. SEP extract showed significant reduction in inflammatory liver infiltration and hepatic fibrosis similar to PZQ. LEP showed moderate effect, while SOP and LOP extracts showed less prominent effects. The stem bark extracts (SEP and SOP) were almost better than their corresponding leaf extracts (LEP and LOP). SEP extract at the higher dose showed significant results compared to PZQ in reducing granuloma number and diameter. This reduction in the size of granulomatous inflammation indicated an anti-inflammatory effect of the used extracts. Concerning bilharzial eggs in tissues of infected animals, there was




(a) (b)

(c) (d)

Figure 6: The distribution and intensity of iNOS in liver sections analyzed by immunohistochemistry; (a) group treated with 600 mg/kg SOP (×400); (b) group treated with 600 mg/kg LOP (×200) showing strong iNOS reactivity (cytoplasmic expression) in the hepatocytes; (c) group treated with 800 mg/kg LEP (×200); (d) group treated with 800 mg/kg LOP (×400) showing low or weak expression in the hepatocytes; (S) strong and (W) weak iNOS expression.




(a) (b)

Figure 7: The distribution and intensity of iNOS in liver sections analyzed by immunohistochemistry; (a) group treated with 800 mg/kg SOP showing strong iNOS reactivity (cytoplasmic expression) in the hepatocytes (×200); (b) groups treated with 600 and 800 mg/kg SEP showing lowest iNOS expression (reactivity) in the hepatocytes (×400); (S) strong, (W) weak, and (N) negative iNOS expression.

variable response with different extracts, where LOP and SOP extracts reduced the number of eggs in the liver tissues at the higher dose, while no eggs were found in liver tissues with LEP at the same dose and SEP extracts at the lower dose compared to PZQ. Comparable results were obtained by previous trials *in vitro* and *in vivo* conducted by the other authors, using compounds isolated from *Piper tuberculatum*, 8-hydroxyquinoline derivatives from *Artemisia annua,* and *Baccharis trimera* that have demonstrated activity against *S. mansoni* [8, 9, 43].

Nitric oxide (NO) is an endogenously secreted free radical, formed as a byproduct of conversion of arginine and oxygen into citrulline in an enzymatic reaction mediated by NO synthase (NOS). Three NOS isoforms have been

described to date, inducible NOS (iNOS), which is expressed in response to proinflammatory cytokines. NO production is upregulated in response to parasitic infection. During infection with *S. mansoni*, there is prolonged production of large amounts of NO, so hepatic iNOS is upregulated in *Schistosoma* infected mice, indicating that NO production is a part of an innate immune-response [44, 45]. In our study, iNOS expression was measured by immunohistochemical methods either in hepatocytes or in inflammatory cells. For cytoplasmic iNOS expression in hepatocytes, it decreased with SEP, LEP, and LOP extracts compared to high expression rate in infected untreated control group, while SOP extract failed to reduce iNOS expression. SEP extract produced the highest decrease in iNOS expression at both doses used compared to PZQ group, while LEP and LOP extracts caused weak expression of iNOs at only the higher dose, so the inhibitory effect of the extracts on iNOs expression was dose-dependent. Regarding iNOs expression in inflammatory cells, only SEP and LEP extracts showed inhibitory effect, where SEP abolished iNOS expression at both doses used which was comparable to PZQ group, while LEP at the higher dose caused weak expression of iNOs.

Pomegranate is of a great interest to research in pharmaceutical and new drug development fields because of its distinctive bioactivities, such as hypolipidemic, antiviral, antifungal, antineoplastic, anti-inflammatory, antimutagenic, antioxidant, antibacterial, and antidiarrheal [46–49]. Few studies investigated the effects of edible pomegranate as antischistosomal alternative and reported similar changes in the motility and in the survival rates of the parasite [12, 18, 19]

By reviewing all the available literature, no previous works came across on the use of any ornamental pomegranate extract against *S. mansoni*. Hence, the present work was the first one to prove its antischistosomal activities.

The pharmacological properties of various different parts of this plant have been attributed to its high content of bioactive secondary metabolites, such as polyphenols glycosides, triterpenes, sterols, flavonoids, anthocyanins, triglycerides, tannins, and alkaloids [20, 50].

Pomegranate and its constituents have safely been consumed for centuries without adverse effects. Studies of pomegranate constituents in animals at concentrations and levels commonly used in folk and traditional medicine did not report any toxic effects [51].

## 6. Conclusion

Ornamental and edible pomegranate extracts have *in vitro* and *in vivo* antischistosomal activity against *S. mansoni*. The *in vitro* activity was manifested in couple worm's separation and reduction or complete loss of motor activity and lethality and ultramorphological changes in adult worms. The *in vivo* activity was manifested in reduction of hepatic granulomas number and diameter, decrease of number of bilharzial eggs in liver tissues, less liver inflammatory infiltration, less hepatic fibrosis, and decreased iNOS expression, thus indicating anti-inflammatory effect. Extracts of edible pomegranate were more effective than those of ornamental pomegranate. The highest antischistosomal activity was observed for the ethanolic stem bark extract of edible pomegranate, which gave comparable results to PZQ both *in vitro* and *in vivo*. More studies are needed in order to isolate and identify pomegranate active compounds against the worm and to understand pomegranate mechanism of action on the tegument.

## Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

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## References

[1] N. Cowan and J. Keiser, "Repurposing of anticancer drugs: in vitro and in vivo activities against *Schistosoma mansoni*," *Parasites & Vectors*, vol. 8, article 417, 2015.

[2] B. Gryseels, K. Polman, J. Clerinx, and L. Kestens, "Human schistosomiasis," *The Lancet*, vol. 368, no. 9541, pp. 1106–1118, 2006.

[3] L.-J. Song, H. Luo, W.-H. Fan et al., "Oxadiazole-2-oxides may have other functional targets, in addition to SjTGR, through which they cause mortality in *Schistosoma japonicum*," *Parasites & Vectors*, vol. 9, article 26, 2016.

[4] WHO, "Accelerating work to overcome the global impact of neglected tropical diseases: a roadmap for implementation," 2012, http://www.who.int/neglected_diseases/NTD_RoadMap_2012_Fullversion.pdf.

[5] D. G. Colley, A. L. Bustinduy, W. E. Secor et al., "Human schistosomiasis," *The Lancet*, vol. 383, pp. 2253–2264, 2014.

[6] W. Wang, L. Wang, and Y. S. Liang, "Susceptibility or resistance of praziquantel in human schistosomiasis: a review," *Parasitology Research*, vol. 111, no. 5, pp. 1871–1877, 2012.

[7] S. M. Allegretti, C. N. F. de Oliveira, R. N. de Oliveira, T. F. Frezza, and V. L. G. Rehder, "The use of Brazilian medicinal plants to combat *Schistosoma mansoni*," in *Schistosomiasis*, M. B. Rokni, Ed., chapter 3, pp. 27–70, InTech, Rijeka, Croatia, 2012.

[8] J. de Moraes, C. Nascimento, L. F. Yamaguchi, M. J. Kato, and E. Nakano, "*Schistosoma mansoni*: in vitro schistosomicidal activity and tegumental alterations induced by piplartine on schistosomula," *Experimental Parasitology*, vol. 132, no. 2, pp. 222–227, 2012.

[9] R. N. de Oliveira, V. L. G. Rehder, A. S. S. Oliveira, V. D. L. S. Jeraldo, A. X. Linhares, and S. M. Allegretti, "Anthelmintic activity *in vitro* and *in vivo* of *Baccharis trimera* (Less) DC against immature and adult worms of *Schistosoma mansoni*," *Experimental Parasitology*, vol. 139, no. 1, pp. 63–72, 2014.

[10] J. de Moraes, "Natural products with antischistosomal activity," *Future Medicinal Chemistry*, vol. 7, no. 6, pp. 801–820, 2015.

[11] L. Shaohong, T. Kumagai, A. Qinghua et al., "Evaluation of the anthelmintic effects of artesunate against experimental *Schistosoma mansoni* infection in mice using different treatment protocols," *Parasitology International*, vol. 55, no. 1, pp. 63–68, 2006.

[12] Z. H. Fahmy, A. M. El-Shennawy, W. El-Komy et al., "Potential antiparasitic activity of pomegranate extracts against shistosomules and mature worms of *Schistosoma Mansoni*: in vitro and in vivo study," *Australian Journal of Basic and Applied Sciences*, vol. 3, pp. 4634–4643, 2009.

[13] J. de Moraes, R. N. de Oliveira, J. P. Costa et al., "Phytol, a diterpene alcohol from chlorophyll, as a drug against neglected tropical disease schistosomiasis *mansoni*," *PLoS Neglected Tropical Diseases*, vol. 8, no. 1, Article ID e2617, 2014.

[14] E. Stover and E. W. Mercure, "The pomegranate: a new look at the fruit of paradise," *HortScience*, vol. 42, no. 5, pp. 1088–1092, 2007.

[15] M. M. Mir, I. Umar, S. A. Mir, M. U. Rehma, G. H. Rather, and S. A. Banday, "Quality evaluation of pomegranate crop—a review," *International Journal of Agriculture and Biology*, vol. 14, no. 4, pp. 658–667, 2012.

[16] G. Dipak, P. Axay, C. Manodeep et al., "Phytochemical and pharmacological profile of *Punica granatum*: an overview," *International Research Journal of Pharmacy*, vol. 3, pp. 65–68, 2012.

[17] M. Hajoori, M. Naik, K. Naik et al., "Evaluation of antimicrobial activity of *Punica granatum* peel extracts using different solvent system," *International Journal of Pharmacological Screening Methods*, vol. 4, pp. 26–31, 2014.

[18] K. Abozeid, M. Shohayeb, and A. Ismael, "In vitro tests for efficacy of tannins extracted from pomegranate (*Punica granatum*) against *Schistosoma mansoni* miracidia," *Journal of Science and Technology*, vol. 13, no. 1, pp. 55–65, 2012.

[19] G. Y. Osman, A. H. Mohamed, T. A. Salem et al., "Immunoparasitological effect of *Punica granatum* in *Schistosoma mansoni* infected mice," in *Proceedings of the 10th International Conference on Future Horizon of Environmental Sustainable Development in Arab Countries and Facing the Challenges*, Sharm El-Sheikh, Egypt, December 2013.

[20] C. Prakash and I. Prakash, "Bioactive chemical constituents from pomegranate (*Punica granatum*) juice, seed and peel—a review," *International Journal of Research in Chemistry and Environment*, vol. 1, no. 1, pp. 1–18, 2011.

[21] R. Wang, Y. Ding, R. Liu et al., "Pomegranate: constituents, bioactivities and pharmacokinetics," *Fruit, Vegetable and Cereal Science and Biotechnology*, vol. 4, pp. 77–78, 2010.

[22] A. M. Emam, M. A. Ahmed, M. A. Tammam et al., "Isolation and structural identification of compounds with antioxidant, nematicidal and fungicidal activities from *Punica granatum* L. var. *nana*," *International Journal of Scientific & Engineering Research*, vol. 6, no. 11, pp. 1023–1040, 2015.

[23] G. Repetto, A. Del-Peso, and J. L. Zurita, "Neutral red uptake assay for the estimation of cell viability/cytotoxicity," *Nature Protocols*, vol. 3, no. 7, pp. 1125–1131, 2008.

[24] S. N. Hussaini and H. T. Hassanali, "Limulus amoebocyte lysate assay of endotoxin: a method for visual detection of the positive gel reaction," *Journal of Medical Microbiology*, vol. 24, no. 1, pp. 89–90, 1987.

[25] G. Allam and A. S. A. Abuelsaad, "In vitro and in vivo effects of hesperidin treatment on adult worms of *Schistosoma mansoni*," *Journal of Helminthology*, vol. 88, no. 3, pp. 362–370, 2014.

[26] L. Dong, W. Duan, J. Chen, H. Sun, C. Qiao, and C.-M. Xia, "An artemisinin derivative of praziquantel as an orally active antischistosomal agent," *PLoS ONE*, vol. 9, no. 11, Article ID e112163, 2014.

[27] Y. S. Liang, I. John, J. I. Bruce et al., "Laboratory cultivation of schistosome vector snails and maintenance of schistosome life cycles," in *Proceedings of the 1st Sino-American Symposium*, vol. 1, pp. 34–45, 1987.

[28] M. A. Stirewalt and C. H. Dorsey, "*Schistosoma manonsi*: cercarial penetration of host epidermis at the ultrastructural level," *Experimental Parasitology*, vol. 35, no. 1, pp. 1–15, 1974.

[29] S.-H. Xiao, J. Keiser, J. Chollet et al., "In vitro and in vivo activities of synthetic trioxolanes against major human schistosome species," *Antimicrobial Agents and Chemotherapy*, vol. 51, no. 4, pp. 1440–1445, 2007.

[30] J. de Moraes, A. A. C. Almeida, M. R. M. Brito et al., "Anthelmintic activity of the natural compound (+)-limonene epoxide against *Schistosoma mansoni*," *Planta Medica*, vol. 79, no. 3-4, pp. 253–258, 2013.

[31] R. N. de Oliveira, V. L. G. Rehder, A. S. S. Oliveira et al., "*Schistosoma mansoni*: in vitro schistosomicidal activity of essential oil of *Baccharis trimera* (less) DC," *Experimental Parasitology*, vol. 132, no. 2, pp. 135–143, 2012.

[32] M. A. Hayat, *Principles and Techniques of Electron Microscopy: Biological Applications*, vol. 1, Union New Jersey University Park Press, 2nd edition, 1981.

[33] C. Hirsch, C. S. Zouain, J. B. Alves, and A. M. Goes, "Induction of protective immunity and modulation of granulomatous hypersensitivity in mice using PIII, an anionic fraction of *Schistosoma mansoni* adult worm," *Parasitology*, vol. 115, no. 1, pp. 21–28, 1997.

[34] I. R. B. Aly, M. A. Hendawy, E. A. Ali, E. Hassan, and M. M. F. Nosseir, "Immunological and parasitological parameters after treatment with dexamethasone in murine *Schistosoma mansoni*," *Memorias do Instituto Oswaldo Cruz*, vol. 105, no. 6, pp. 729–735, 2010.

[35] M. R. Hussein, A. K. Haemel, and G. S. Wood, "p53-related pathways and the molecular pathogenesis of melanoma," *European Journal of Cancer Prevention*, vol. 12, no. 2, pp. 93–100, 2003.

[36] G. D. Ruxton and N. Colegrave, *Experimental Design for the Life Sciences*, Oxford University Press, New York, NY, USA, 2nd edition, 2006.

[37] L. R. S. Tonuci, N. I. Melo, H. J. Dias et al., "In vitro schistosomicidal effects of the essential oil of *Tagetes erecta*," *Revista Brasileira de Farmacognosia*, vol. 22, no. 1, pp. 88–93, 2012.

[38] J. de Moraes, "Antischistosomal natural compounds: present challenges for new drug screens," in *Current Topics in Tropical Medicine*, A. J. Rodriguez-Morales, Ed., pp. 333–358, InTech, Rijeka, Croatia, 2012.

[39] F. Noel, "Sistema neuromuscular e controle da motilidade do verme adulto," in *Schistosoma mansoni & Esquistossomose: Uma Visão Multidisciplinar*, O. S. Carvalho, P. M. Z. Coelho, and H. L. Lenzi, Eds., pp. 207–244, FIOCRUZ, Rio de Janeiro, Brazil, 2008.

[40] L. Pica-Mattoccia and D. Cioli, "Sex- and stage-related sensitivity of *Schistosoma mansoni* to in vivo and in vitro praziquantel treatment," *International Journal for Parasitology*, vol. 34, no. 4, pp. 527–533, 2004.

[41] R. El Ridi, H. Tallima, M. Salah et al., "Efficacy and mechanism of action of arachidonic acid in the treatment of hamsters

infected with *Schistosoma mansoni* or *Schistosoma haematobium*," *International Journal of Antimicrobial Agents*, vol. 39, no. 3, pp. 232–239, 2012.

[42] T. Manneck, Y. Haggenmüller, and J. Keiser, "Morphological effects and tegumental alterations induced by mefloquine on schistosomula and adult flukes of *Schistosoma mansoni*," *Parasitology*, vol. 137, no. 1, pp. 85–98, 2010.

[43] G. Allam, A. F. Eweas, and A. S. A. Abuelsaad, "In vivo schistosomicidal activity of three novels 8-hydroxyquinoline derivatives against adult and immature worms of *Schistosoma mansoni*," *Parasitology Research*, vol. 112, no. 9, pp. 3137–3149, 2013.

[44] L. R. Brunet, M. Beall, D. W. Dunne, and E. J. Pearce, "Nitric oxide and the Th2 response combine to prevent severe hepatic damage during *Schistosoma mansoni* infection," *The Journal of Immunology*, vol. 163, no. 9, pp. 4976–4984, 1999.

[45] E. M. Ali, S. M. Hamdy, and T. M. Mohamed, *Nitric Oxide Synthase and Oxidative Stress: Regulation of Nitric Oxide Synthase*, InTechOpen, Rijeka, Croatia, 2012.

[46] S. H. Abdollahzadeh, R. Y. Mashouf, and H. Mortazavi, "Antibacterial and antifungal activities of *Punica granatum* peel extracts against oral pathogens," *Journal of Dentistry*, vol. 8, pp. 1–6, 2011.

[47] S. Das and S. Barman, "Antidiabetic and antihyperlipidemic effects of ethanolic extract of leaves of *Punica granatum* in alloxan-induced non-insulin-dependent diabetes mellitus albino rats," *Indian Journal of Pharmacology*, vol. 44, no. 2, pp. 219–224, 2012.

[48] M. A. Dkhil, "Anti-coccidial, anthelmintic and antioxidant activities of pomegranate (*Punica granatum*) peel extract," *Parasitology Research*, vol. 112, no. 7, pp. 2639–2646, 2013.

[49] J. G. El Diasty, M. M. Hassan, and O. M. Kamal, "Evaluation of some agricultural waste extracts against mosquito larvae, and some types of microorganisms as insecticidal and antibiotic agents," *Egyptian Academic Journal of Biological Sciences*, vol. 6, pp. 1–16, 2014.

[50] M. A. Tantray, S. Akbar, R. Khan, K. A. Tariq, and A. S. Shawl, "Humarain: a new dimeric gallic acid glycoside from *Punica granatum* L. bark," *Fitoterapia*, vol. 80, no. 4, pp. 223–225, 2009.

[51] A. Vidal, A. Fallarero, B. R. Peña et al., "Studies on the toxicity of *Punica granatum* L. (Punicaceae) whole fruit extracts," *Journal of Ethnopharmacology*, vol. 89, no. 2-3, pp. 295–300, 2003.

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*Research Article*

# Modelling Seasonal Brucellosis Epidemics in Bayingolin Mongol Autonomous Prefecture of Xinjiang, China, 2010–2014

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Brucellosis is one of the severe public health problems; the cumulative number of new human brucellosis cases reached 211515 from 2010 to 2014 in China. Bayingolin Mongol Autonomous Prefecture is situated in the southeast of Xinjiang, where brucellosis infection occurs every year. Based on the reported data of newly acute human brucellosis cases for each season in Bayingolin Mongol Autonomous Prefecture, we proposed a susceptible, exposed, infected, and vaccinated (SEIV) model with periodic transmission rates to investigate the seasonal brucellosis transmission dynamics among sheep/cattle and from sheep/cattle to humans. Compared with the criteria of MAPE and RMSPE, the model simulations agree to the data on newly acute human brucellosis. We predict that the number of newly acute human brucellosis is increasing and will peak 15325 [95% CI: 11920–18242] around the summer of 2023. We also estimate the basic reproduction number $R_0 = 2.5524$ [95% CI: 2.5129–2.6225] and perform some sensitivity analysis of the newly acute human brucellosis cases and the basic reproduction number $R_0$ in terms of model parameters. Our study demonstrates that reducing the birth number of sheep/cattle, raising the slaughter rate of infected sheep/cattle, increasing the vaccination rate of susceptible sheep/cattle, and decreasing the loss rate of vaccination are effective strategies to control brucellosis epidemic.

## 1. Introduction

Brucellosis is a contagion-allergy zoonosis, it is caused by Gram-negative bacteria of the genus *Brucella* which includes *Brucella abortus*, *Brucella melitensis*, *Brucella suis*, *Brucella neotomae*, *Brucella ovis*, and *Brucella canis* [1, 2]. British military doctor Bruce was the first to confirm the pathogen of the disease in 1886; hence the disease was named "brucellosis" in order to honor him [3, 4]. Brucellosis primarily affects cattle and sheep, which also infects dogs, elks, swine, horses, and humans. It firstly spreads among animals and then transmits to humans. In animals, brucellosis mainly damages the reproductive system and results in abortion and sterility. In humans, it can lead to the symptoms of fever, wandering arthritis, liver, spleen, and lymph node enlargement, testicular pain and swelling, neuralgia, and so forth. After the mid-20th century, the controlled brucellosis resurrects in most parts of the world, especially in the Mediterranean areas, the Middle East, South and Central America, Asia, and so forth [5]. It has spread so widely that there are 123 countries where brucellosis has occurred around the world. The incidence of brucellosis rose sharply in many countries. At present, approximately 0.5 million new brucellosis cases annually are estimated by the World Health Organization (WHO) to occur globally [6].

In China, the five major pastoral areas of brucellosis infection are located in Inner Mongolia, Xinjiang, Tibet, Qinghai, and Ningxia, among which, Inner Mongolia is the most serious region since 2004 [7]. According to *China Statistical Yearbook*, the numbers of new human brucellosis cases in 2012, 2013, and 2014 were 39151 (2.93/100000), 43468 (3.21/100000), and 57222 (4.22/100000), respectively. And according to *Xinjiang Statistical Yearbook*, the numbers of new human brucellosis cases in 2012, 2013, and 2014 were 2335 (9.80/100000), 4095 (17.51/100000), and 7358 (33.02/100000), respectively. We can infer from the reported data of new human brucellosis cases in China and Xinjiang that brucellosis is becoming increasingly serious.

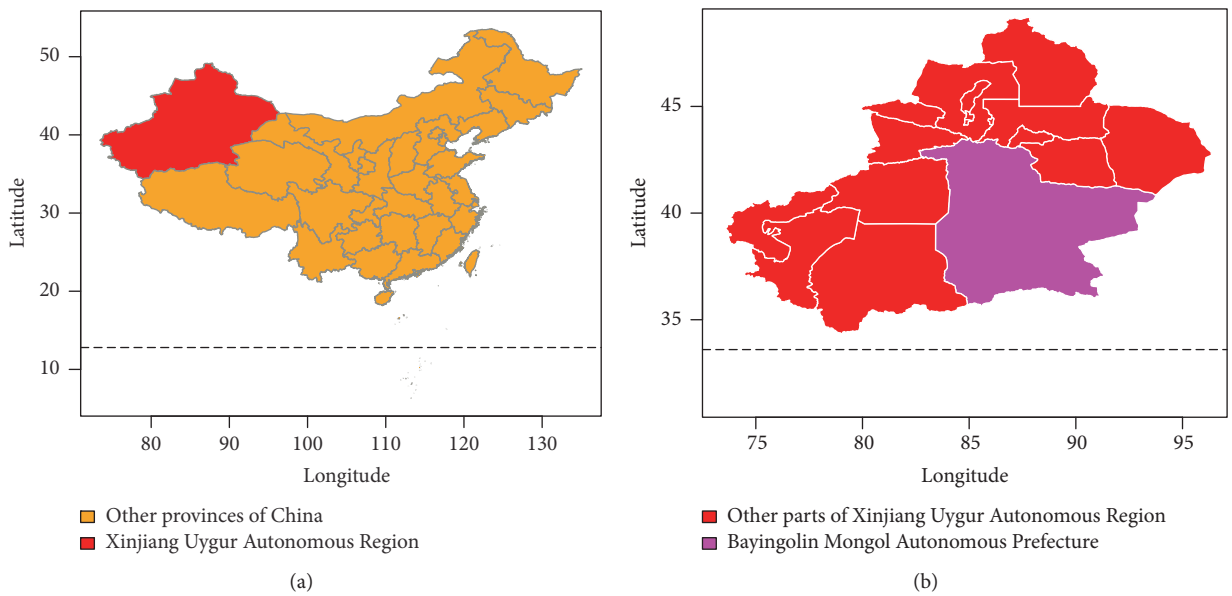


Figure 1: The specific geography location of Bayingolin Mongol Autonomous Prefecture. (a) The map of China. (b) The map of Xinjiang Uygur Autonomous Region.

It is well known that whooping cough, measles, influenza, polio, chickenpox, mumps, and so forth exhibit seasonal fluctuations [8–10]. In China, it is generally acknowledged that from January to March is spring, from April to June is summer, July to September is autumn, and October to December is winter [11]. As we all know that brucellosis has seasonal variations, hence, the numbers of new human brucellosis cases are significantly increasing annually in summer and autumn, while the numbers in spring and winter are relatively decreasing [12].

This paper focuses on the epidemic dynamics of brucellosis in Bayingolin Mongol Autonomous Prefecture. Bayingolin Mongol Autonomous Prefecture belongs to the Xinjiang Uygur Autonomous Region of China, which is one of the major livestock breeding areas, and the specific geography locations are presented in Figures 1(a) and 1(b), where brucellosis infection occurs every year, and even an outbreak during the summer and autumn of 2014 appeared. We utilize the data of newly acute human brucellosis cases in each season from 2005 to 2014 reported by the Center for Disease Control and Prevention of Bayingolin Mongol Autonomous Prefecture in Xinjiang, and then we plot a bar diagram and a boxplot which are presented in Figures 2(a) and 2(b), respectively. In statistics, season exponent $S_j$ $(j = 1, 2, 3, 4)$ reflects a stable relationship between the average number of newly acute human brucellosis cases $\overline{x}_j$ and the average number of total newly acute human brucellosis $\overline{x}$ [13, 14]. If $S_j > 1$, it indicates that $\overline{x}_j$ is higher than $\overline{x}$; if $S_j < 1$, it demonstrates that $\overline{x}_j$ is lower than $\overline{x}$; if $S_j \approx 1$, it manifests that the newly acute human brucellosis does not present an obvious seasonal effect. Eventually, from Table 1 and Figure 3 we can conclude that the numbers of newly acute human brucellosis cases in spring and winter are less than those in summer and autumn. Thus, it can be confirmed that the newly acute human brucellosis cases in Bayingolin Mongol Autonomous Prefecture show a pronounced seasonal fluctuation.

The factors which influence human brucellosis seasonal trend can be shown as follows.

(1) In the early summer and late autumn, the suitable temperature and weather provide a favorable environment for sheep mating and breeding. As a matter of fact, sheep are capable of breeding once or twice a year, mainly in early April and late September, and the average gestation period is around 150 days. The length of estrus and mating period for cattle is approximately from July to September, and the average gestation period is around 285 days; thus breeding season is delayed to the second year starting in May until July. In conclusion, these increase the contact rates between humans and the secretions, abortuses, viscera, skin, fur, and so forth all from the infected sheep/cattle, eating undercooked meat, drinking raw milk, or being exposed to the contaminated environment under the circumstance with absence of protective measures. Consequently, the risk of human infection with brucellosis from infected sheep/cattle in the summer and autumn is enhanced [3, 15].

(2) Temperature has a significant influence on the activity of *Brucella*. Studies have confirmed that under the temperature of 37°C demonstrated the peak breeding activity [16]. Accordingly, the higher temperature in the summer and autumn contributes to the higher activity of *Brucella* compared with spring and winter.

Different mathematical models have been developed to investigate the transmission dynamics of brucellosis among

Table 1: The season exponent of average newly acute human brucellosis cases from 2005 to 2014.

| Season | Average newly acute human brucellosis cases ($\overline{x}_j$) | Season exponent ($S_j$) |
|---|---|---|
| Spring | 38.6 | 0.56 |
| Summer | 91.2 | 1.32 |
| Autumn | 96.1 | 1.39 |
| Winter | 50.8 | 0.73 |
| | $\overline{x} = 69.71$ | |

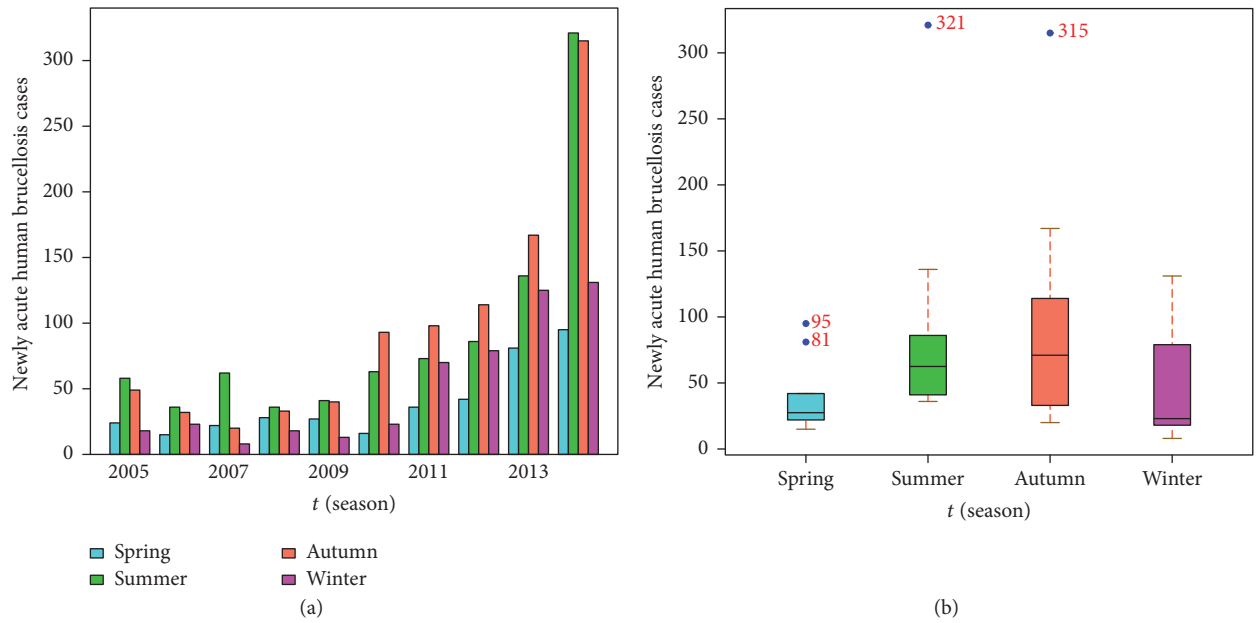


Figure 2: The reported data of newly acute human brucellosis cases in each season from 2005 to 2014. (a) The reported data are described with a bar diagram. (b) The reported data are displayed with a boxplot.

cattle, sheep, elk, and human [17–22]. For instance, Hou et al. [23] proposed an SEIVB dynamic model for the sheep-human transmission of brucellosis considering the impact of *Brucella* in the environment and vaccination for the susceptible sheep on brucellosis transmission and used the model to simulate the brucellosis data in Inner Mongolia of China. Li et al. [24] developed a deterministic model to investigate the transmission dynamics of brucellosis in Hinggan League of Inner Mongolia, China. In addition, they compared the effect of existing mixed cross infection between basic ewes and other sheep on the newly infected human brucellosis cases. Dobson and Meagher [25] employed a simple SIR epidemiological model to describe the population and disease dynamics of brucellosis among bison and elk in the Greater Yellowstone Area. Nevertheless, none of these studies analyzed the brucellosis with seasonal fluctuation.

We refer to the other published articles which are related to the periodic diseases transmission models [26–30]. Particularly, Zhang et al. [29] proposed a SEIRS model with periodic transmission rates to investigate the seasonal rabies epidemics in China and demonstrated that it was more reasonable to regard basic reproduction number $R_0$ rather than the average basic reproduction number $\widetilde{R}_0$ or the basic reproduction number $\widehat{R}_0$ of the corresponding autonomous system as a threshold for the disease. Ma et al. [30] established an $\text{SEII}_e\text{QR}$ epidemic model with periodic transmission rate to study the spread of seasonal HFMD in Shandong Province and analyzed the dynamical behaviors of the model.

The purpose of this paper is to develop a periodic brucellosis transmission model among sheep/cattle and from sheep/cattle to humans in the Bayingolin Mongol Autonomous Prefecture of Xinjiang, China. We firstly use the model to simulate the data of newly acute human brucellosis cases reported by the Center for Disease Control and Prevention of Bayingolin Mongol Autonomous Prefecture from the spring of 2010 to the winter of 2014 and then determine the basic reproduction number and analyze the dynamic behaviors of the model. Some sensitivity analyses of the newly acute human brucellosis cases and the basic reproduction number $R_0$ in terms of some key parameters are carried out. Finally, we explore some effective strategies for the brucellosis in Bayingolin Mongol Autonomous Prefecture.

The article is organized as follows. In Section 2, we introduce the brucellosis transmission model, accounting for

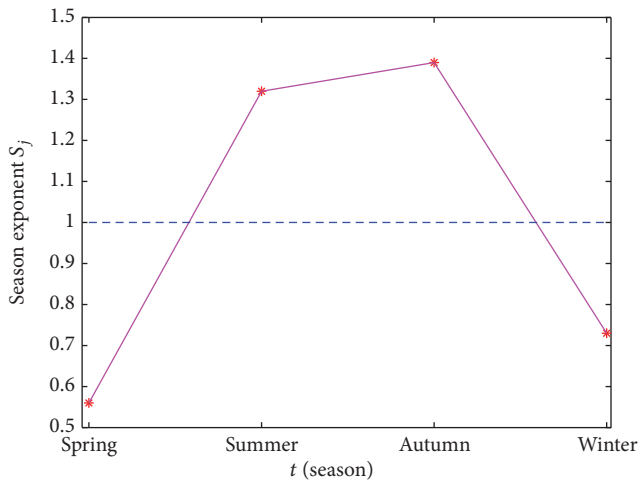


Figure 3: The season exponent of average newly acute human brucellosis cases from 2005 to 2014. The asterisks represent the season exponent values.

the parameters and the functions of periodic transmission rates. In Section 3, the globally asymptotic stability of the disease-free equilibrium is studied. The numerical simulations, prediction of the epidemic trends for the next decades, estimation of the basic reproduction number, and sensitivity analysis of the basic reproduction number and the newly acute human brucellosis cases are presented in Section 4. In Section 5, we put forward various control measures and give a brief discussion.

## 2. Brucellosis Model Formulation and Analysis

In order to establish the brucellosis transmission model between sheep/cattle and humans, we divide the sheep/cattle population into four subclasses: the susceptible, the exposed, the infected, and the vaccinated, denoted by $S(t)$, $E(t)$, $I(t)$, and $V(t)$, respectively. The human population is divided into three subclasses: the susceptible, the acute infected, and the chronic infected, denoted by $S_h(t)$, $I_{\mathrm{ha}}(t)$, and $I_{\mathrm{hc}}(t)$, respectively. The mean incubation period of human brucellosis is about two weeks, infected patients mainly have fever during this period, they only take some medicine for the treatment of fever which was mistaken as the common cold, and after showing clinical symptoms then they would go to hospital for checking. In the meantime, human brucellosis has reached an acute infection status; thus we assume that the susceptible people infected with brucellosis will directly enter into the acute infection compartment. The flowchart of brucellosis transmission is illustrated in Figure 4.

The model is described as the following ordinary differential equations:

$$\begin{aligned}
\frac{dS}{dt} &= A - \beta(t) S (E + I) - (\mu + \nu) S + \delta V,\\
\frac{dE}{dt} &= \beta(t) (S + \epsilon V) (E + I) - (\lambda + \mu) E,\\
\frac{dI}{dt} &= \lambda E - (\mu + f) I,\\
\frac{dV}{dt} &= \nu S - (\mu + \delta) V - \epsilon \beta(t) V (E + I),\\
\frac{dS_h}{dt} &= B - \beta_h(t) S_h (E + I) + \omega_h \eta I_{\mathrm{ha}} - \mu_1 S_h,\\
\frac{dI_{\mathrm{ha}}}{dt} &= \beta_h(t) S_h (E + I) - \omega_h I_{\mathrm{ha}} - \mu_1 I_{\mathrm{ha}},\\
\frac{dI_{\mathrm{hc}}}{dt} &= \omega_h (1 - \eta) I_{\mathrm{ha}} - \mu_1 I_{\mathrm{hc}}.
\end{aligned} \tag{1}$$

All parameters are assumed positive. We need to interpret the parameters that appear in our model. The birth numbers of sheep/cattle and humans per unit time are constants and denoted by $A$ and $B$, respectively. Regarding the parameters for sheep/cattle, $\nu$ and $\epsilon$ are the products of the vaccination rate and the invalid vaccination rate; $\delta$ represents the loss rate of vaccination; the transfer rate from the exposed to the infected can be identified as $\lambda$; the slaughter rate is attributed to being infected with brucellosis and the natural mortality rate which are indicated as $f$ and $\mu$, respectively. For the human population, $\omega_h \eta$ is the cure rate from the acute infection to the susceptible; we assume that all patients who are not healed in acute infection will progress into chronic infection; thus the transfer rate from acute infection to chronic infection is $\omega_h(1 - \eta)$; $\mu_1$ is the natural death rate; since the mortality rate of human brucellosis is quite low, it can be negligible.

The functions $\beta(t)$ and $\beta_h(t)$ can be expressed as $\beta(t) = \alpha[1 + b \sin((\pi/2)t + c)]$ and $\beta_h(t) = \alpha_h[1 + b_h \sin((\pi/2)t + c_h)]$ proposed by Schenzle [32] to describe the transmission rates among sheep/cattle and from sheep/cattle to humans, respectively, where $\alpha$ and $\alpha_h$ are the baseline contact rates, $b$ and $b_h$ are the magnitudes of forcing, and $c$ and $c_h$ are the phase. The above six parameters are constants, which can be estimated by the least-square fitting and bootstrap method in Section 4.

The first four equations are independent of the last three equations in model (1), and thus we can only consider the first four equations:

$$\begin{aligned}
\frac{dS}{dt} &= A - \beta(t) S (E + I) - (\mu + \nu) S + \delta V,\\
\frac{dE}{dt} &= \beta(t) (S + \epsilon V) (E + I) - (\lambda + \mu) E,\\
\frac{dI}{dt} &= \lambda E - (\mu + f) I,\\
\frac{dV}{dt} &= \nu S - (\mu + \delta) V - \epsilon \beta(t) V (E + I).
\end{aligned} \tag{2}$$

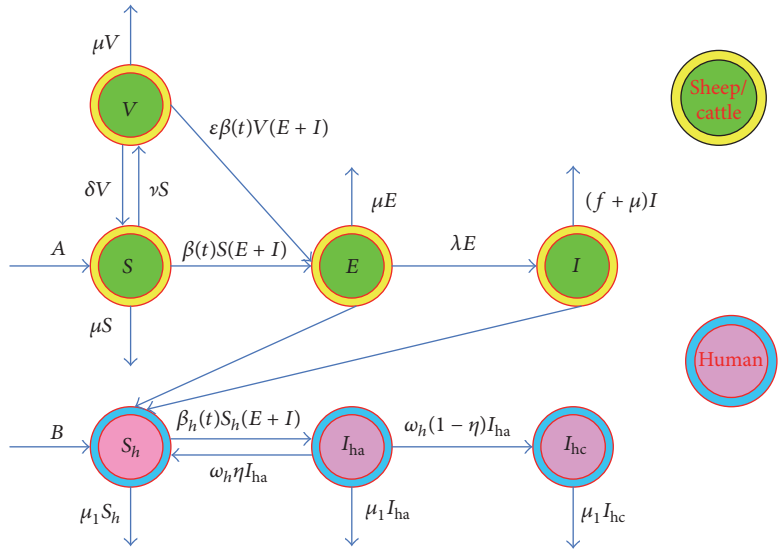


Figure 4: The flowchart of brucellosis transmission among sheep/cattle and from sheep/cattle to humans.

It is easy to see that model (2) has a unique positive disease-free equilibrium $P_0 = (S^*, 0, 0, V^*)$, where

$$S^* = \frac{A(\mu+\delta)}{\mu(\mu+\nu+\delta)},$$
$$V^* = \frac{A\nu}{\mu(\mu+\nu+\delta)}. \tag{3}$$

Consider the following auxiliary equations:

$$\frac{dx}{dt} = A - (\mu+\nu)x + \delta y,$$
$$\frac{dy}{dt} = \nu x - (\mu+\delta)y. \tag{4}$$

**Lemma 1.** *Model (4) has a unique positive globally asymptotically stable equilibrium* $(A(\mu+\delta)/\mu(\mu+\nu+\delta),\ A\nu/\mu(\mu+\nu+\delta))$.

*Proof.* In fact, the Jacobian matrix of model (4) at equilibrium $(A(\mu+\delta)/\mu(\mu+\nu+\delta),\ A\nu/\mu(\mu+\nu+\delta))$ is

$$J = \begin{pmatrix} -(\mu+\nu) & \delta \\ \nu & -(\mu+\delta) \end{pmatrix} \tag{5}$$

and then the corresponding characteristic equation is

$$\Phi(\lambda) = \lambda^2 + (2\mu+\delta+\nu)\lambda + (\mu+\nu+\delta)\mu. \tag{6}$$

By simple calculation, it is easy to obtain that the two roots of $\Phi(\lambda)$ are $\lambda_1 = -\mu$ and $\lambda_2 = -(\mu+\delta+\nu)$. Hence, we obtain that the unique positive equilibrium $(A(\mu+\delta)/\mu(\mu+\nu+\delta),\ A\nu/\mu(\mu+\nu+\delta))$ is locally asymptotically stable. In addition, since model (4) is linear, by the theorems of stability of the differential equations, we obtain that the equilibrium $(A(\mu+\delta)/\mu(\mu+\nu+\delta),\ A\nu/\mu(\mu+\nu+\delta))$ is globally asymptotically stable, which completes the proof. □

Now, we compute the basic reproduction number of model (2) by applying the way given in [33, 34] by Wang et al. Let

$$\mathscr{F}(t,x) = \begin{pmatrix} \beta(t)(S+\epsilon V)(E+I) \\ 0 \\ 0 \\ 0 \end{pmatrix},$$
$$\mathscr{V}(t,x) = \begin{pmatrix} (\lambda+\mu)E \\ (\mu+f)I - \lambda E \\ \beta(t)S(E+I) + (\mu+\nu)S - A - \delta V \\ (\mu+\delta)V + \epsilon\beta(t)V(E+I) - \nu S \end{pmatrix}, \tag{7}$$

where $x = (E, I, S, V)^T$, and then model (2) takes the following form:

$$\dot{x}(t) = \mathscr{F}(t,x) - \mathscr{V}(t,x) \triangleq f(t, x(t)). \tag{8}$$

Obviously, model (8) has a disease-free equilibrium $x^*(t) = (0, 0, S^*, V^*)$.

Next, we set two $2 \times 2$ matrices as follows:

$$F(t) = \left(\frac{\partial \mathscr{F}_i(t, x^*(t))}{\partial x_j}\right)_{1\le i,j\le 2},$$
$$V(t) = \left(\frac{\partial \mathscr{V}_i(t, x^*(t))}{\partial x_j}\right)_{1\le i,j\le 2}, \tag{9}$$

where $\mathscr{F}_i(t, x(t))$ and $\mathscr{V}_i(t, x(t))$ are the $i$th component of $\mathscr{F}(t, x(t))$ and $\mathscr{V}(t, x(t))$, respectively. Then, by simple computations, it follows that

$$F(t) = \begin{pmatrix} \beta(t)\left(S^* + \epsilon V^*\right) & \beta(t)\left(S^* + \epsilon V^*\right) \\ 0 & 0 \end{pmatrix},$$
$$V(t) = \begin{pmatrix} \lambda + \mu & 0 \\ -\lambda & \mu + f \end{pmatrix}. \tag{10}$$

Hence, we easily check that conditions (A1)–(A7) given in [33] are satisfied.

Let $Y(t, s)$ be the $2 \times 2$ matrix solution of the following initial value problem:

$$\frac{d}{dt}Y(t, s) = -V(t)\,Y(t, s) \quad \forall t \geq s,$$
$$Y(s, s) = I. \tag{11}$$

Let $C_\omega$ be the ordered Banach space of all $\omega$-periodic continuous function form $R$ to $R^2$ with the maximum norm $\|\cdot\|$. The positive cone $C_\omega^+ = \{\phi \in C_\omega : \phi(t) \geq 0 \text{ for all } t \in R\}$. Suppose $\phi(s) \in C_\omega$ is the initial distribution of infectious individuals in this periodic environment, then $F(s)\phi(s)$ is the rate of new infectious individuals produced by the infected individuals who were introduced at time $s$, and $Y(t, s)F(s)\phi(s)$ represents the distributions of those infected individuals who were newly infected at time $s$ and remain in the infected compartment at time $t$ for $t \geq s$. Hence, we define a linear operator $L : C_\omega \to C_\omega$ as follows:

$$\left(L\phi\right)(t) = \int_0^{+\infty} Y(t, t-a)\,F(t-a)\,\phi(t-a)\,da \quad \forall t \in R,\ \phi \in C_\omega. \tag{12}$$

The operator $L$ is positive, continuous, and compact on $C_\omega$. Thus, $R_0$ can be characterized by the existence of a nonnegative and nonzero $\phi \in C_\omega^+$ such that

$$\left(L\phi\right)(t) = R_0\phi(t). \tag{13}$$

Now, we define basic reproduction number $R_0$ for model (8) by

$$R_0 = \rho(L), \tag{14}$$

where $\rho(L)$ is the spectral radius of $L$.

Using Theorem 2.2 given in [33], we can obtain the following results on basic reproduction number $R_0$ and the locally asymptotical stability of disease-free equilibrium $P_0$ for model (2).

**Lemma 2.** *(1) On the basic reproduction number $R_0$, one has*

(i) $R_0 = 1$ *if and if only* $\rho(\Phi_{F-V}(\omega)) = 1$;

(ii) $R_0 > 1$ *if and if only* $\rho(\Phi_{F-V}(\omega)) > 1$;

(iii) $R_0 < 1$ *if and if only* $\rho(\Phi_{F-V}(\omega)) < 1$.

*(2) $E^*(t)$ is locally asymptotically stable if $R_0 < 1$ and unstable if $R_0 > 1$.*

Let, for any integer $n > 0$, $R_+^n = \{(x_1, x_2, \ldots, x_n) \in R^n : x_i \geq 0,\ i = 1, 2, \ldots, n\}$. For $u, v \in R^n$, we denote $u \geq v$ if $u - v \in R_+^n$, $u > v$ if $u - v \in R_+^n \setminus \{0\}$, and $u \gg v$ if $u - v \in \operatorname{int} R_+^n$, respectively, where $\operatorname{int} R_+^n$ denotes the interior of $R_+^n$.

Let $B(t)$ be a continuous and $\omega$-periodic $n \times n$ matrix function; we consider the following linear system:

$$\dot{x} = B(t)\,x. \tag{15}$$

Let $\Phi_B(t)$ be the fundamental solution matrix of system (15) with initial condition $\Phi_B(0) = I$, where $I$ is $n \times n$ identity matrix and let $\rho(\Phi_B(\omega))$ be the spectral radius of matrix $\Phi_B(\omega)$.

Further, we assume that $B(t)$ also is cooperative and irreducible; then by the Perron-Frobenius theorem, $\rho(\Phi_B(\omega))$ is the principle eigenvalue of $\Phi_B(\omega)$ in the sense that it is simple and admits an eigenvector $v^* \gg 0$.

**Lemma 3** (see [35]). *Let $B(t)$ be a continuous, cooperative, irreducible, and $\omega$-periodic $n \times n$ matrix function; $\mu = (1/\omega)\ln\rho(\Phi_B(\omega))$. Then there exists a positive $\omega$-periodic function $v(t)$ such that $x(t) = e^{\mu t}v(t)$ is a solution of system (15).*

## 3. Main Result

**Theorem 4.** *The disease-free equilibrium $P_0$ of model (2) is globally asymptotically stable if $R_0 < 1$ and unstable if $R_0 > 1$.*

*Proof.* From Lemma 2, we obtain that if $R_0 < 1$, $P_0$ is locally asymptotically stable and unstable if $R_0 > 1$. Now, we will only prove the attractivity of $P_0$ for the case $R_0 < 1$. From $R_0 < 1$ and conclusion (iii) of Lemma 2, we have $\rho(\Phi_{F-V}(\omega)) < 1$, and then we can choose a small enough constant $\eta > 0$ such that $\rho(\Phi_{F-V+\eta M}(\omega)) < 1$, where

$$M(t) = \begin{pmatrix} \beta(t)(1+\epsilon) & \beta(t)(1+\epsilon) \\ 0 & 0 \end{pmatrix}. \tag{16}$$

By Lemma 1, we obtain that, for above given constant $\eta$, there exists $t_1 > 0$ such that for all $t > t_1$

$$S(t) \leq S^* + \eta,$$
$$V(t) \leq v^* + \eta. \tag{17}$$

From the second and third equations of model (1), we obtain that, for all $t > t_1$,

$$\frac{dE}{dt} \leq \beta(t)\left[S^* + \eta + \epsilon\left(V^* + \eta\right)\right](E + I) - \left(\lambda + \mu\right)E,$$
$$\frac{dI}{dt} = \lambda E - \left(\mu + f\right)I. \tag{18}$$

Consider the following auxiliary system:

$$\frac{d\widetilde{E}}{dt} = \beta(t)\left[S^* + \eta + \epsilon\left(V^* + \eta\right)\right]\left(\widetilde{E} + \widetilde{I}\right) - \left(\lambda + \mu\right)\widetilde{E},$$
$$\frac{d\widetilde{I}}{dt} = \lambda\widetilde{E} - \left(\mu + f\right)\widetilde{I}. \tag{19}$$

For convenience, we will rewrite it as follows:

$$\frac{d}{dt}\begin{pmatrix}\widetilde{E}\\ \widetilde{I}\end{pmatrix} = \left(F(t) - V(t) + \eta M(t)\right)\begin{pmatrix}\widetilde{E}\\ \widetilde{I}\end{pmatrix}. \quad (20)$$

From Lemma 3, it follows that there exists a positive $\omega$-periodic function $q(t) = (q_1(t), q_2(t))^T$ such that $(\widetilde{E}(t), \widetilde{I}(t))^T = e^{\xi t}q(t)$ is a solution of model (19), where $\xi = (1/\omega)\ln(\rho(\Phi_{F-V+\eta M}(\omega)))$.

Denote $J(t) = (E(t), I(t))^T$. We can choose a small constant $\theta > 0$ such that $J(t_1) \le \theta q(t_1)$. Then, from (18) the comparison principle implies that

$$J(t) \le \theta e^{\xi t} q(t) \quad \forall t > t_1. \quad (21)$$

By $\rho(\Phi_{F-V+\eta M}(\omega)) < 1$, it follows that $\xi < 0$, then $\lim_{t\to\infty} J(t) = 0$, and, that is,

$$\lim_{t\to\infty} E(t) = 0,$$
$$\lim_{t\to\infty} I(t) = 0. \quad (22)$$

Moreover, from the equations of model (2), we can get

$$\lim_{t\to\infty} S(t) = S^*,$$
$$\lim_{t\to\infty} V(t) = V^*. \quad (23)$$

Hence, disease-free equilibrium $P_0$ of model (2) is globally attractive. This completes the proof. □

## 4. Model Applications

*4.1. Parameters Estimation and Simulation Results.* In this section, we apply model (1) to simulate the reported data of newly acute human brucellosis cases in Bayingolin Mongol Autonomous Prefecture in each season. We only use the reported data of newly acute human brucellosis cases from 2010 to 2014 (see Figure 5) because of the fact that the reported data from 2005 to 2009 is relatively flat and has not shown a gradual increasing trend. However, it has an influence on the simulation for the rapid growth number of newly acute human brucellosis cases from 2010 to 2014. In the summer and autumn of 2014, the human brucellosis experienced an outbreak; thus the reported number of newly acute human brucellosis cases increased dramatically in that year.

The parameter values of model (1) are listed in Table 2, and we interpret the parameter values as follows.

[A] From *Bayingolin Mongol Autonomous Prefecture Statistical Yearbook 2014*, we obtain the annual birth populations and natural mortality rate and divide them by 4 to derive the birth populations $A, B$ and natural mortality rates $\mu, \mu_1$.

[B] The mean incubation period of brucellosis is almost two weeks [36] and about 1/6 quarter, so we have $\lambda = 6$.

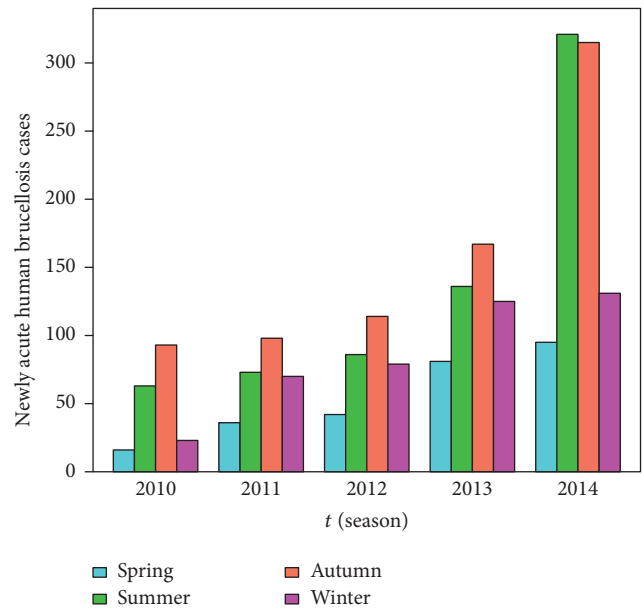


Figure 5: The reported number of newly acute human brucellosis cases from 2010 to 2014.

[C] The acute infection period of human brucellosis is about a half of a year [36] and approximately 2 quarters, so we have $\omega_h = 1/2 = 0.5$.

[D] In Xinjiang, vaccine *B. suis* strain 2 has been used to control brucellosis. The immune duration of vaccine *B. suis* strain 2 is about 2.5 years [23], similar to 10 quarters, so we have $\delta = 1/10 = 0.1$.

[E] We did not acquire the deterministic information of sheep/cattle invalid vaccination rate $\epsilon$ in Bayingolin Mongol Autonomous Prefecture. However, vaccine *B. suis* strain 2 can protect 82% of sheep/cattle from *Brucella* annually in Inner Mongolia [37], and we use it as a substitute for $\epsilon = (1 - 0.82)/4$.

We assume that the slaughter rate of infected sheep/cattle $f$ and the vaccination rate of susceptible sheep/cattle $\nu$ in Bayingolin Mongol Autonomous Prefecture are approximate to the whole Xinjiang region, respectively. Thus we can suppose that $f = 0.0983$ and $\nu = 0.0412$ [38]. In humans, the cure rate from the acute infection to the susceptible $\eta = 0.6$ and the transfer rate from the acute infection to the chronic infection $1 - \eta = 0.4$ are given by [31].

We need the initial values to perform the numerical simulations of model (1). We determine both initial values $S(0) = 944500$ and $S_h(0) = 292710$ from the *Bayingolin Mongol Autonomous Prefecture Statistical Yearbook*. The initial value $I_{ha}(0) = 16$ is obtained from the Center for Disease Control and Prevention of Bayingolin Mongol Autonomous Prefecture in Xinjiang. We can derive the initial value $V(0)$ from $S(0)$ by vaccination rate $\nu$ and deduce the initial value $E(0)$ from the initial values $S(0)$ and $V(0)$ by the parameters $\epsilon$, $\beta(t)$. Similarly, we can derive the initial value $I(0)$ from $E(0)$ by the parameter $\lambda$ and deduce the initial value $I_{hc}(0)$ from

Table 2: The parameter values and interpretations.

| Parameter | Unit | Value | Comments | Reference |
|---|---|---|---|---|
| $A$ | season$^{-1}$ | 188900 | Sheep/cattle birth population | [A] |
| $\mu$ | season$^{-1}$ | 0.05 | Sheep/cattle natural mortality rate | [A] |
| $f$ | season$^{-1}$ | 0.0983 | The slaughter rate of infected sheep/cattle | Assumption |
| $\delta$ | season$^{-1}$ | 0.1 | Sheep/cattle loss of vaccination rate | [D] |
| $\nu$ | season$^{-1}$ | 0.0412 | The vaccination rate of susceptibility sheep/cattle | Assumption |
| $\epsilon$ | season$^{-1}$ | 0.0450 | Sheep/cattle invalid vaccination rate | [E] |
| $\lambda$ | season$^{-1}$ | 6 | Transfer rate from exposed to infected | [B] |
| $B$ | season$^{-1}$ | 3305 | Human birth population | [A] |
| $\eta$ | season$^{-1}$ | 0.6 | The cure rate from acute infection to susceptible | [31] |
| $1-\eta$ | season$^{-1}$ | 0.4 | Transfer rate from acute infection to susceptible | [31] |
| $\omega_h$ | season$^{-1}$ | 0.5 | The leave rate from acute infection | [C] |
| $\mu_1$ | season$^{-1}$ | 0.0010 | Human natural mortality rate | [A] |

Table 3: Parameter values for point estimation and 95% interval estimation.

| Parameter | Point estimation | 95% confidence interval |
|---|---|---|
| $\alpha$ | $1.2507 \times 10^{-7}$ | $[1.2290 \times 10^{-7}, 1.2821 \times 10^{-7}]$ |
| $b$ | 5.9830 | [1.7072, 9.5677] |
| $c$ | 1.14029 | [0.83036, 1.5590] |
| $\alpha_h$ | $6.1790 \times 10^{-8}$ | $[3.8848 \times 10^{-8}, 1.0290 \times 10^{-7}]$ |
| $b_h$ | −0.8115 | [−1.0351, −0.5770] |
| $c_h$ | −6.1382 | [−6.3661, −4.9694] |

$I_{\mathrm{ha}}(0)$ by the parameter $\omega_h(1-\eta)$. Above initial values are $E(0) = 3000$, $I(0) = 2500$, $V(0) = 377800$, $I_{\mathrm{hc}}(0) = 7$.

For the functions of both periodic transmission rate functions $\beta(t) = \alpha[1 + b\sin((\pi/2)t + c)]$ and $\beta_h(t) = \alpha_h[1 + b_h\sin((\pi/2)t + c_h)]$, we use the least-square fitting and bootstrap sampling method to estimate the six parameters $\alpha$, $b$, $c$, $\alpha_h$, $b_h$, and $c_h$ and the 95% confidence interval for each parameter which are listed in Table 3. Hence, two periodic transmission rates functions are expressed as $\beta(t) = 1.2507 \times 10^{-7}[1 + 5.9830\sin((\pi/2)t + 1.14029)]$ and $\beta_h(t) = 6.1790 \times 10^{-8}[1 - 0.8115\sin((\pi/2)t - 6.1382)]$. According to the bootstrap estimate value for each parameter, we plot the frequency distribution histogram and the probability density curve which are presented in Figure 6.

We take the spring of 2010 as the start time of simulation, and the numerical simulations of the model on the number of newly acute human brucellosis cases in each season are presented in Figure 7. Moreover, under the same conditions which include parameter values and initial values, the cumulative numbers of newly acute human brucellosis cases and fitted curve are presented in Figure 8. At the same time, we estimated the 95% confidence interval for fitted curves by the bootstrap sampling method with salmon areas which are presented in Figures 7 and 8, respectively. It indicates that our model provides good matches to the reported data from Figures 7 and 8.

Table 4: Criteria of MAPE and RMSPE.

| MAPE and RMSPE | Forecasting power |
|---|---|
| <10% | Highly accurate forecasting |
| 10–20% | Good forecasting |
| 20–50% | Reasonable forecasting |
| >50% | Inaccurate forecasting |

The mean absolute percentage error (MAPE) and the root mean square percentage error (RMSPE) are critical evaluation indicators, which are used to assess the fitting effect and the precision of our established model. The MAPE and the RMSPE are defined as

$$\begin{aligned} \mathrm{MAPE} &= \left(\frac{1}{n}\sum_{q=2}^{n}\left|\frac{W(q)^* - W(q)}{W(q)^*}\right|\right) \times 100\%, \\ \mathrm{RMSPE} &= \sqrt{\frac{\sum_{q=2}^{n}\left[\left(W(q)^* - W(q)\right)/W(q)^*\right]^2}{n-1}} \\ &\quad \times 100\%, \end{aligned} \tag{24}$$

where $W(q)^*$ is the real value at time $q$ and $W(q)$ is its fitting value and $n$ is the number of data used for prediction. The criteria of MAPE and RMSPE are shown in Table 4 [39, 40]. We use model (1) to simulate the number of newly acute human brucellosis cases in each season, where MAPE = 18.07% and RMSPE = 20.89%. When we simulate the cumulative number of newly acute human brucellosis cases, the values of MAPE and RMSPE are 2.55% and 4.03%, respectively. Comparing with the criteria of MAPE and RMSPE, the real data and the fitted curve match quite well by using our model.

*4.2. Model Predication.* We can not only fit the real data by using our model but also predict the fluctuation tendency in the next 35 years about 140 quarters which are presented

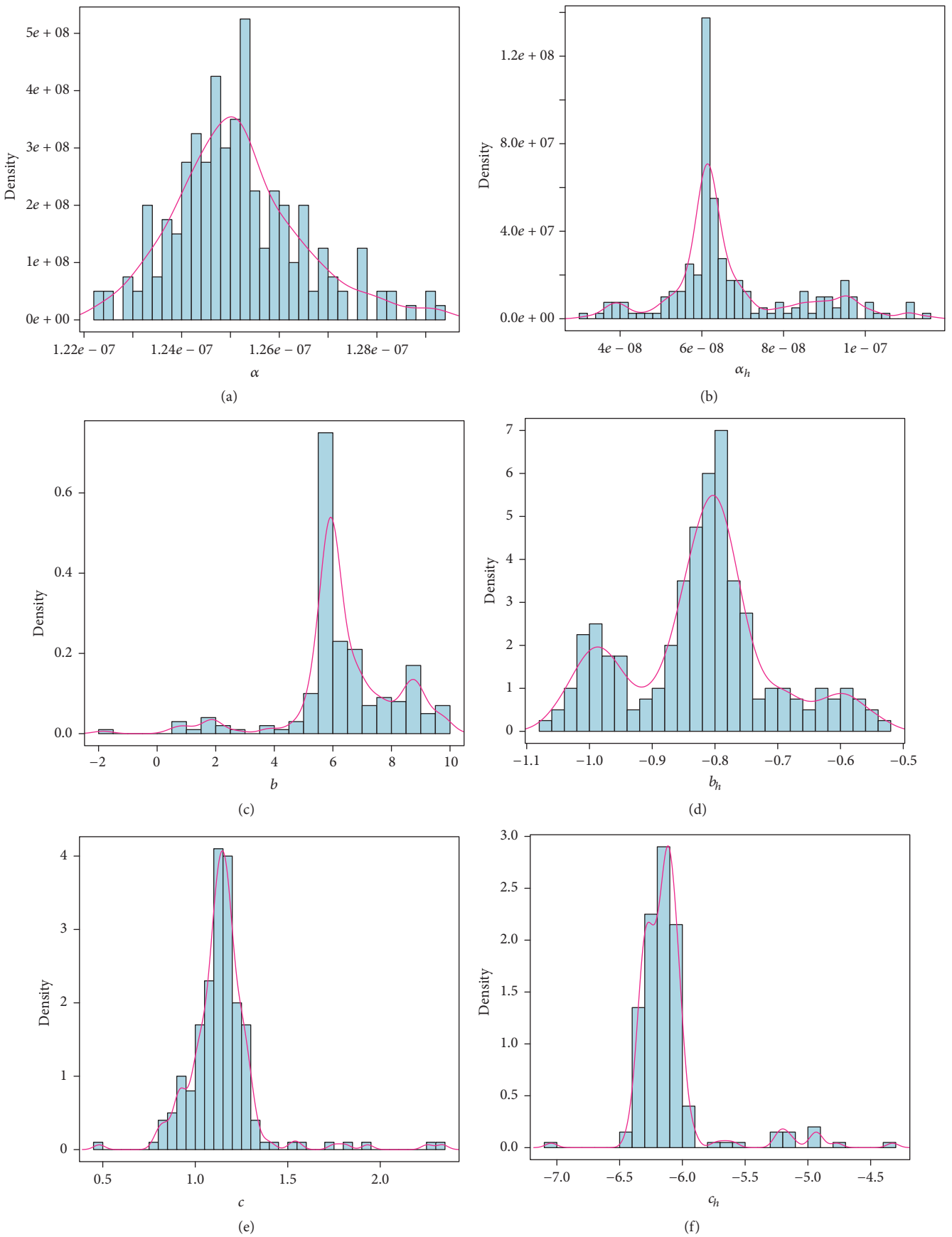


FIGURE 6: Frequency distribution histograms and probability density curves of the estimated parameters $\alpha, b, c, \alpha_h, b_h, c_h$. The blue bars represent frequency distribution histograms and pink lines represent probability density curves.

Table 5: The predicted number of newly acute human brucellosis cases from the spring of 2015 to the winter of 2034 and 95% confidence interval.

| Year | Spring | Summer | Autumn | Winter |
|---|---|---|---|---|
| 2015 | 181 [131–246] | 575 [487–652] | 533 [478–604] | 282 [211–373] |
| 2016 | 340 [247–469] | 1118 [928–1287] | 1004 [887–1163] | 521 [377–735] |
| 2017 | 659 [482–919] | 2195 [1818–2543] | 1943 [1697–2276] | 998 [700–1458] |
| 2018 | 1281 [938–1784] | 4193 [3464–4863] | 3750 [3258–4292] | 1921 [1319–2775] |
| 2019 | 2393 [1749–3239] | 7388 [5866–8439] | 6903 [6023–7943] | 3572 [2404–4762] |
| 2020 | 4064 [2957–5341] | 11290 [8706–12826] | 11431 [10142–13223] | 6057 [4085–7929] |
| 2021 | 5935 [4283–7929] | 14361 [10993–16722] | 16113 [12757–18557] | 8839 [4684–11153] |
| 2022 | 7244 [5179–9825] | 14415 [11556–17446] | 18959 [13194–21629] | 10786 [4582–13018] |
| 2023 | 7526 [5339–10324] | 15325 [11920–18242] | 19191 [12443–22493] | 11260 [4198–13130] |
| 2024 | 7008 [4864–10170] | 12669 [10336–15563] | 17674 [11259–21278] | 10606 [3758–12407] |
| 2025 | 6175 [4253–9571] | 10878 [8868–13556] | 15574 [10089–18989] | 9489 [3367–11094] |
| 2026 | 5350 [3707–8676] | 9365 [7607–11835] | 13572 [9104–16576] | 8349 [3053–9760] |
| 2027 | 4657 [3270–7775] | 8186 [6609–10483] | 11908 [8332–14458] | 7365 [2813–8590] |
| 2028 | 4114 [2891–6989] | 7297 [5857–9463] | 10605 [7749–12739] | 6578 [2635–7625] |
| 2029 | 3702 [2603–6349] | 6634 [5291–8708] | 9612 [7319–11391] | 5968 [2504–6861] |
| 2030 | 3393 [2388–5842] | 6141 [4856–8151] | 8862 [7003–10349] | 5504 [2408–6267] |
| 2031 | 3160 [2408–6267] | 5771 [4524–7739] | 8297 [6770–9546] | 5152 [2337–5821] |
| 2032 | 2985 [2104–5142] | 5490 [4276–7430] | 7867 [6596–8926] | 4883 [2283–5484] |
| 2033 | 2851 [2010–4903] | 5271 [4065–7192] | 7536 [6431–8443] | 4676 [2239–5224] |
| 2034 | 2746 [1937–4715] | 5097 [3904–7028] | 7276 [6272–8087] | 4514 [2202–5019] |

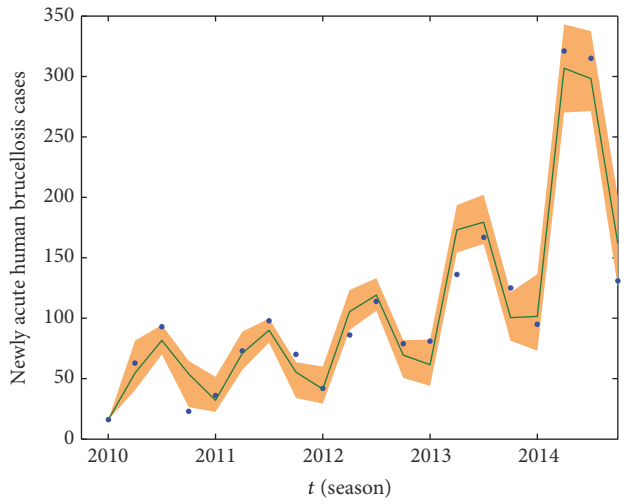


Figure 7: The number of newly acute human brucellosis cases and fitted curve. The blue dots represent the real data while the dark green solid curve is fitted by using our model, and the salmon area represents the 95% confidence interval around model fitted.

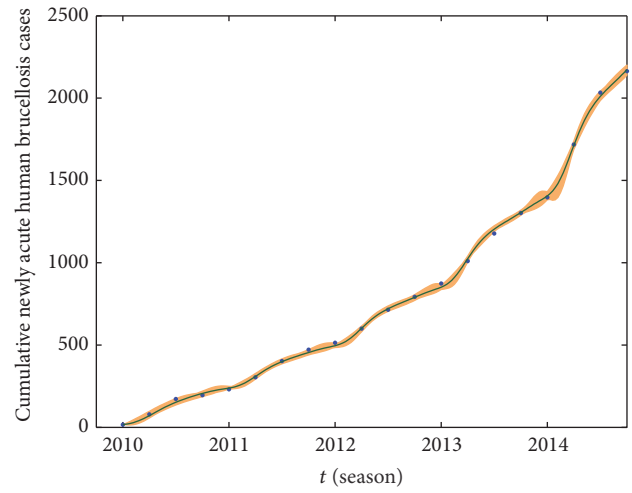


Figure 8: The cumulative number of newly acute human cases and fitted curve. The blue dots represent the real data while the dark green solid curve is fitted by using our model, and the salmon area represents the 95% confidence interval around model fitted.

in Figure 9(a). The predicted values and 95% confidence interval for each season from 2015 to 2034 are listed in Table 5. Combining Figure 9(a) with Table 5, we find that human brucellosis increases sharply from the spring of 2015 to the winter of 2023 and reaches the peak 15325 [95% CI: 11920–18242] in the summer of 2023. Shortly afterwards, the number of newly acute human brucellosis starts to gradually reduce during the period of 2023 to 2040 and maintains its equilibrium level after 2040. Figure 9(b) describes the predicted tendency of cumulative number of newly acute human brucellosis from 2015 to 2049.

*4.3. The Calculation of Basic Reproduction Number.* According to the method for basic reproduction number with periodic coefficients (see [33, Theorem 2.1]), we can calculate the basic reproduction number $R_0 = 2.5524$ [95%

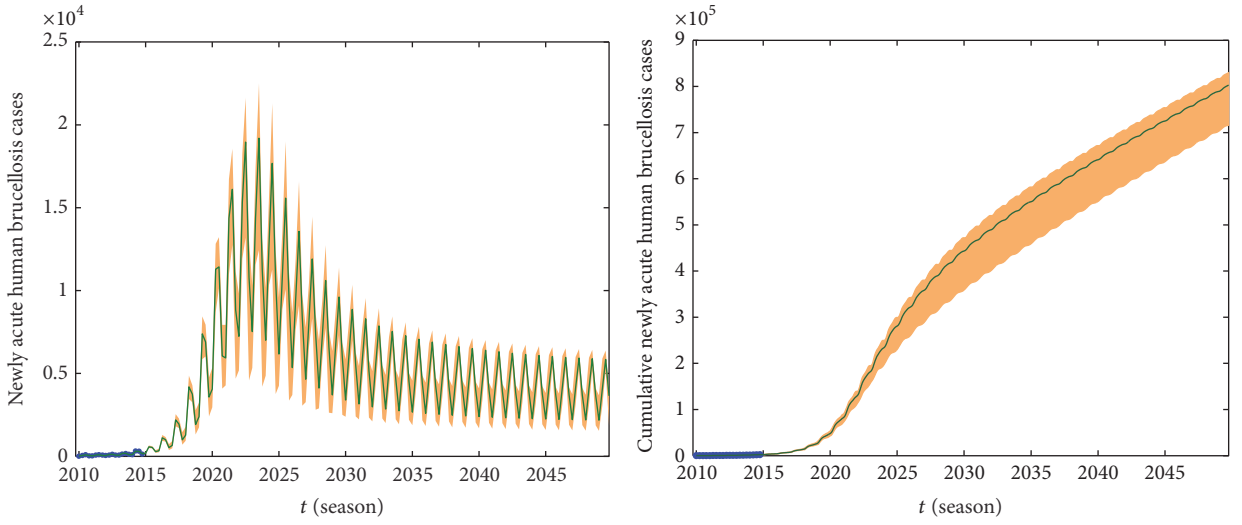


FIGURE 9: Prediction of newly acute human brucellosis in 35 years from 2015 to 2049. (a) The number of newly acute human brucellosis cases. (b) The cumulative number of newly acute human brucellosis cases.

CI: 2.5129–2.6225] which means that human brucellosis in Bayingolin Mongol Autonomous Prefecture persists under current circumstances. When model (2) degenerates into an autonomous case with $\overline{\beta} = (1/4)\int_0^4 \beta(t)dt$, we obtain

$$F = \begin{pmatrix} \overline{\beta}(S^* + \epsilon V^*) & \overline{\beta}(S^* + \epsilon V^*) \\ 0 & 0 \end{pmatrix}, \quad V = \begin{pmatrix} \lambda + \mu & 0 \\ -\lambda & \mu + f \end{pmatrix}. \tag{25}$$

Using the method given by van den Driessche and Watmough given in [34], we obtain basic reproduction number

$$\widetilde{R}_0 = \frac{\overline{\beta} A (f + \lambda + \mu)(\delta + \mu + \epsilon\nu)}{\mu (f + \mu)(\lambda + \mu)(\delta + \mu + \nu)}, \tag{26}$$

which is called the average basic reproduction number. Meanwhile we also estimate $\widetilde{R}_0 = 2.5723$ [95% CI: 2.5671–2.6393]. The boxplots for $R_0$ and $\widetilde{R}_0$ are presented in Figure 10. We can see that $\widetilde{R}_0$ is slightly higher than $R_0$, and it implies utilizing average method to calculate $\widetilde{R}_0$ which overestimates the risk of epidemic of human brucellosis. Moreover, we demonstrate that the periodic basic reproduction number $R_0$ is a threshold, which determines whether or not brucellosis persists in the population. From Figures 11(a) and 11(b), it is clear that when $R_0 < 1$, the number of newly acute human brucellosis tends to zero. On the contrary, when $R_0 > 1$, the number of newly acute human brucellosis tends to be a stable periodic solution. Human brucellosis cases increase with the raise of the basic reproduction number $R_0$.

*4.4. Sensitivity Analysis and Disease Control.* We use Latin hypercube sampling (LHS) and partial rank correlation coefficients (PRCC) [41] to examine parameters which have a significant influence on the number of newly acute human brucellosis cases. We choose the sample size $n = 1000$ and $n = 2000$, respectively, parameters interested as the input variables, and the number of newly acute human brucellosis cases as the output variable. The accurate PRCC values and $p$ values of each parameter with sample size $n = 1000$ and $n = 2000$ are listed in Table 6, respectively. Figure 12 depicts the PRCC values of each parameter, we assume the significance level $\alpha = 0.05$, and parameters with star above the bar are the significant ones. Combining Table 6 with Figure 12, we find that there is no significant difference between the PRCC values and $p$ values when comparing the sampling size

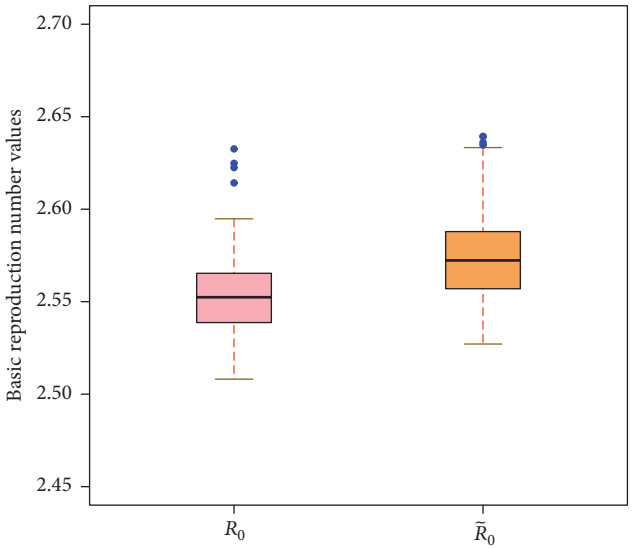


FIGURE 10: The boxplots of estimated basic reproduction number $R_0$ and average basic reproduction number $\widetilde{R}_0$.

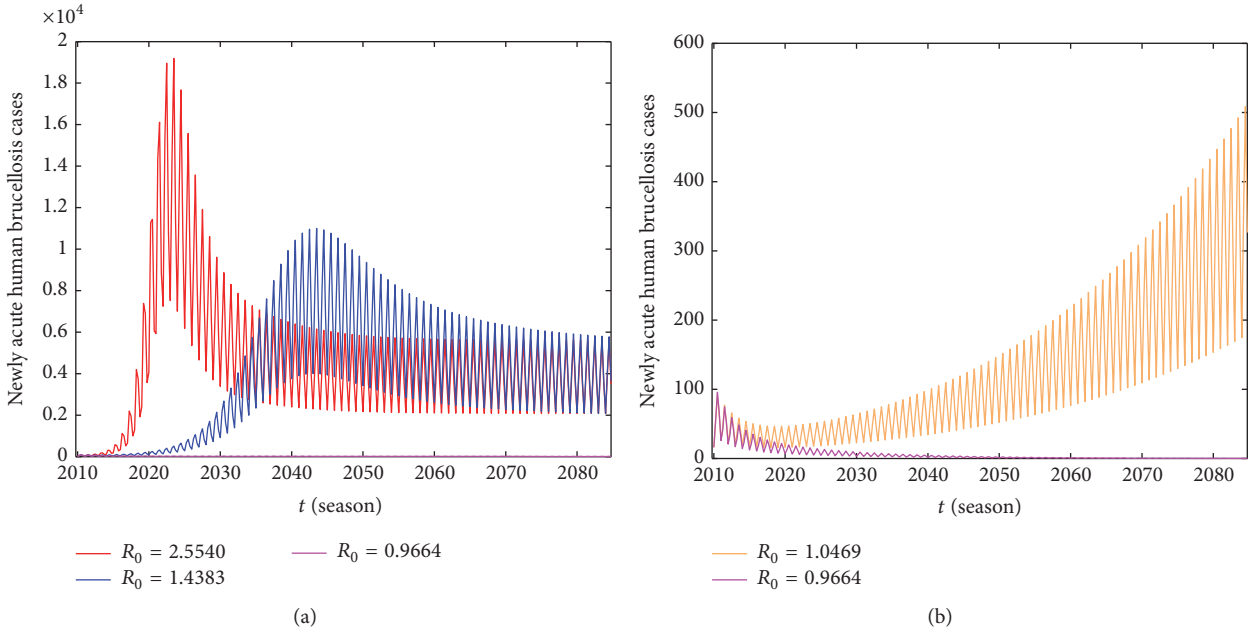


Figure 11: The variations of the newly acute human brucellosis cases for different values of $R_0$. (a) When $\nu = 0.0412, 0.206$, and $0.412$, $R_0 = 2.554, 1.4383$, and $0.9664$, respectively. (b) When $\nu = 0.36$, $R_0 = 1.0469$. Other parameters are as in Tables 2 and 3.

Table 6: Partial rank correlation coefficients (PRCC) for newly acute human brucellosis cases in each season and each input parameter variable.

| Input parameter | 1000 samples | | 2000 samples | |
|---|---|---|---|---|
| | PRCC | $p$ value | PRCC | $p$ value |
| $A$ | 0.1208 | $1.426 \times 10^{-4}$ | 0.1541 | $5.0505 \times 10^{-12}$ |
| $f$ | −0.8482 | $7.538 \times 10^{-274}$ | −0.8577 | 0 |
| $\delta$ | 0.4172 | $8.2104 \times 10^{-43}$ | 0.4712 | $2.623 \times 10^{-110}$ |
| $\nu$ | −0.5313 | $6.4607 \times 10^{-73}$ | −0.5273 | $1.4028 \times 10^{-142}$ |
| $\epsilon$ | 0.0601 | 0.0590 | 0.0288 | 0.19967 |
| $\eta$ | 0.0318 | 0.3184 | 0.003 | 0.8854 |
| $B$ | 0.0249 | 0.4347 | −0.002 | 0.9146 |
| $\lambda$ | 0.2469 | $3.6347 \times 10^{-15}$ | 0.2279 | $0.8100 \times 10^{-25}$ |
| $\omega$ | −0.004 | 0.8790 | 0.001 | 0.9620 |
| $\alpha$ | −0.0522 | 0.1012 | −0.0159 | 0.4764 |
| $b$ | 0.9895 | 0 | 0.9913 | 0 |
| $c$ | 0.5856 | $7.734 \times 10^{-92}$ | 0.5897 | $2.5675 \times 10^{-186}$ |
| $\alpha_h$ | −0.0866 | 0.0647 | −0.0183 | 0.4151 |
| $b_h$ | 0.0151 | 0.6349 | 0.003 | 0.8779 |
| $c_h$ | 0.0532 | 0.0961 | 0.001 | 0.9560 |

$n = 1000$ with $n = 2000$. The larger the PRCC in absolute value, the more important the parameters in responding to the change of newly acute human brucellosis cases; therefore we can confirm that parameters $A, \delta, \lambda, b, c$ have positive impact on the number of newly acute human brucellosis cases. On the contrary, $f$ and $\nu$ have negative impact. We do not take the other parameters into account due to the reason that PRCC values are small and $p > 0.05$.

Through the above mentioned analysis, we demonstrate that parameters $A, \delta, \lambda, b, c, f, \nu$ have significant impact on the number of newly acute human brucellosis cases, so it is necessary to study the influence of parametric modification

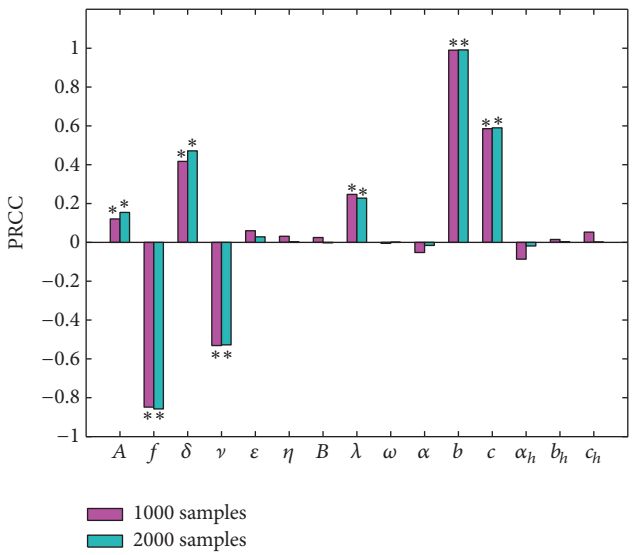


Figure 12: Partial rank correlation coefficients (PRCC) results for the dependence of newly acute human brucellosis cases in each season on each parameter. ∗ denotes the value of PRCC which is not zero significantly, where the significance level is 0.05.

on the human brucellosis epidemic which are presented in Figure 13. We can see that the effects of parameters $A, f, \nu$ are stronger and other parameters have little impact on the newly acute human brucellosis cases; moreover, we find that the parameter changes can influence not only the number of newly acute human brucellosis cases but also the time of peak for newly acute human brucellosis cases. As Figures 13(a) and 13(c) illustrated when fixing other parameters at constant, the number of newly acute human brucellosis cases falls substantially with a decrease in $A$ and $\delta$, respectively. And the peak of initial outbreak will be postponed. As similar as above, we investigate the impact of parameters $f$ and $\nu$ on newly acute human brucellosis cases which are presented in Figures 13(b) and 13(d), respectively. We observe that newly acute human brucellosis cases decrease with an increase in $f$ and $\nu$, and the peak of initial outbreak will be postponed. From Figure 13(e) we can see that parameter $b$ does not have such effects; it only controls the magnitudes of forcing. We do not consider the influence of parameters $\lambda$ and $c$ on newly acute human brucellosis cases for the reason that $\lambda$ is expressed as the transfer rate from exposed to infected class in sheep/cattle, and $c$ denotes phase.

Finally, in order to find better control strategies for brucellosis transmission, we carry out some sensitivity analysis to confirm the influence of parameters $A, f, \delta, \nu$ on $R_0$.

We show variations of $R_0$ for different values of $A$ in Figure 14(a), which illustrates that parameter $A$ has a great impact on $R_0$, the values of $R_0$ increase as $A$ is rising, and there appears a linear relationship. When $A$ is less than 74100, $R_0 < 1$, the disease can die out. Nevertheless, at present, the birth number of sheep/cattle can achieve 188900 in Bayingolin Mongol Autonomous Prefecture in each season. This indicates that, in order to eradicate human brucellosis, herdsmen should reduce the birth number of sheep/cattle without affecting the economic benefit.

It is well known that vaccination for the susceptible sheep/cattle is an effective measure to control brucellosis. The influence of $\nu$ on $R_0$ is given in Figure 14(b). It can be observed that the value of $R_0$ decreases as $\nu$ is increasing. Moreover, Figure 14(b) shows that when vaccination rate $\nu$ is higher than 0.375, $R_0 < 1$, the disease can be eradicated. Thus, government should strengthen the vaccination rate of susceptible sheep/cattle and improve the herd immunity level. Figure 14(c) reflects that reducing the sheep/cattle loss of vaccination rate is also an approach to decrease $R_0$. However, $R_0$ cannot become less than one even if the sheep/cattle loss of immunity rate $\delta$ is zero. In other words, implementing this measure alone cannot eliminate brucellosis.

In fact, the common ways to dispose the infected sheep/cattle are slaughter and then bury. Figure 14(d) represents the relationship between the slaughter rate $f$ and $R_0$. The value of $R_0$ decreases as $f$ is increasing. When $f$ is higher than 0.32, $R_0 < 1$, the disease can be eliminated. In real life, a large number of infected sheep/cattle are not culled, and the slaughter rate only reaches 0.0983 since enhancing the slaughter rate of infected sheep/cattle can inflict the most economic damage on the herdsman.

The above analysis demonstrates that human brucellosis can be controlled with three strategies: reducing the seasonal crop of newborn sheep/cattle, increasing the vaccination rate of susceptible sheep/cattle, and raising the slaughter rate of infected sheep/cattle.

## 5. Conclusion and Discussion

As a zoonotic disease, brucellosis is one of the biggest public health threats which cannot be ignored in China. Despite its acknowledgment as an important economic and health problem and the availability of proven control measures, it continues to occur with a relatively high frequency.

In this article, in order to explore effective control and prevention measures, by using the seasonal newly acute human brucellosis cases from 2010 to 2014 in Bayingolin Mongol Autonomous Prefecture of Xinjiang, we proposed an SEIV model with periodic transmission rates to investigate the spread of brucellosis. The model describes the transmission of brucellosis among sheep/cattle and from sheep/cattle to humans.

We estimated the basic reproduction number $R_0 = 2.5524$ [95% CI: 2.5129–2.6225] and the average basic reproduction number $\tilde{R}_0 = 2.5723$ [95% CI: 2.5671–2.6393]. It shows that the average basic reproduction number $\tilde{R}_0$ overestimates the risk of brucellosis infection, and brucellosis persists with the current prevention and control measures in Bayingolin Mongol Autonomous Prefecture of Xinjiang. Hou et al. [23] estimated the basic reproduction number $R_0 = 1.8$ in Inner Mongolia from 2005 to 2010. Li et al. [24, 31] calculated the basic reproduction number $R_0 = 1.9789$ in Hinggan League of Inner Mongolia from 2001 to 2011. Nie et al. [22] obtained the basic reproduction number $R_0 = 1.1987$ from 1987 to 1998 and $R_0 = 2.1327$ from 1998 to 2005 in Jilin Province.

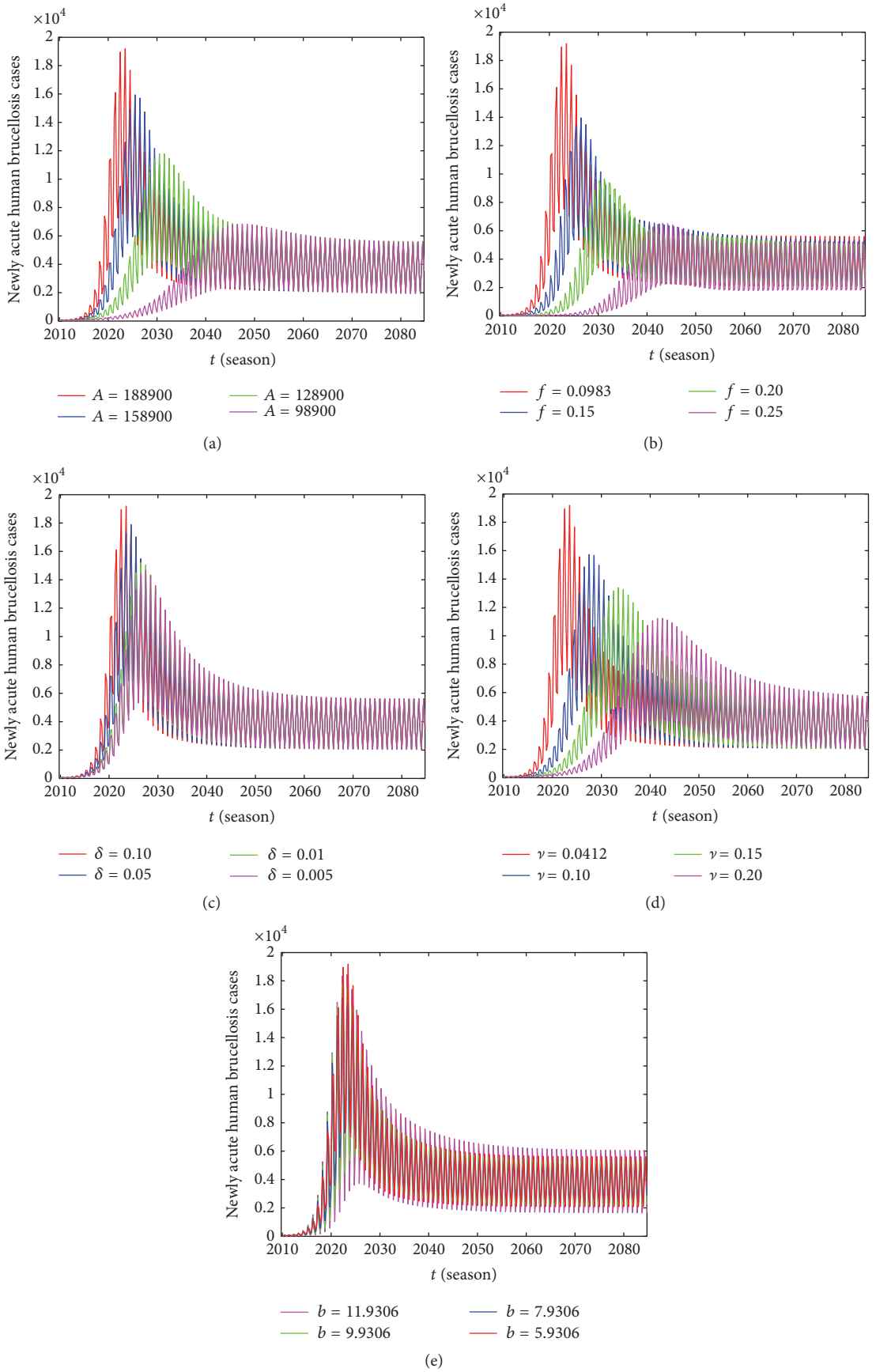


FIGURE 13: The influence of different parameters on the number of newly acute human brucellosis cases $I_{ha}(t)$: (a) different values of $A$; (b) different values of $f$; (c) different values of $\delta$; (d) different values of $\nu$; (e) different values of $b$.

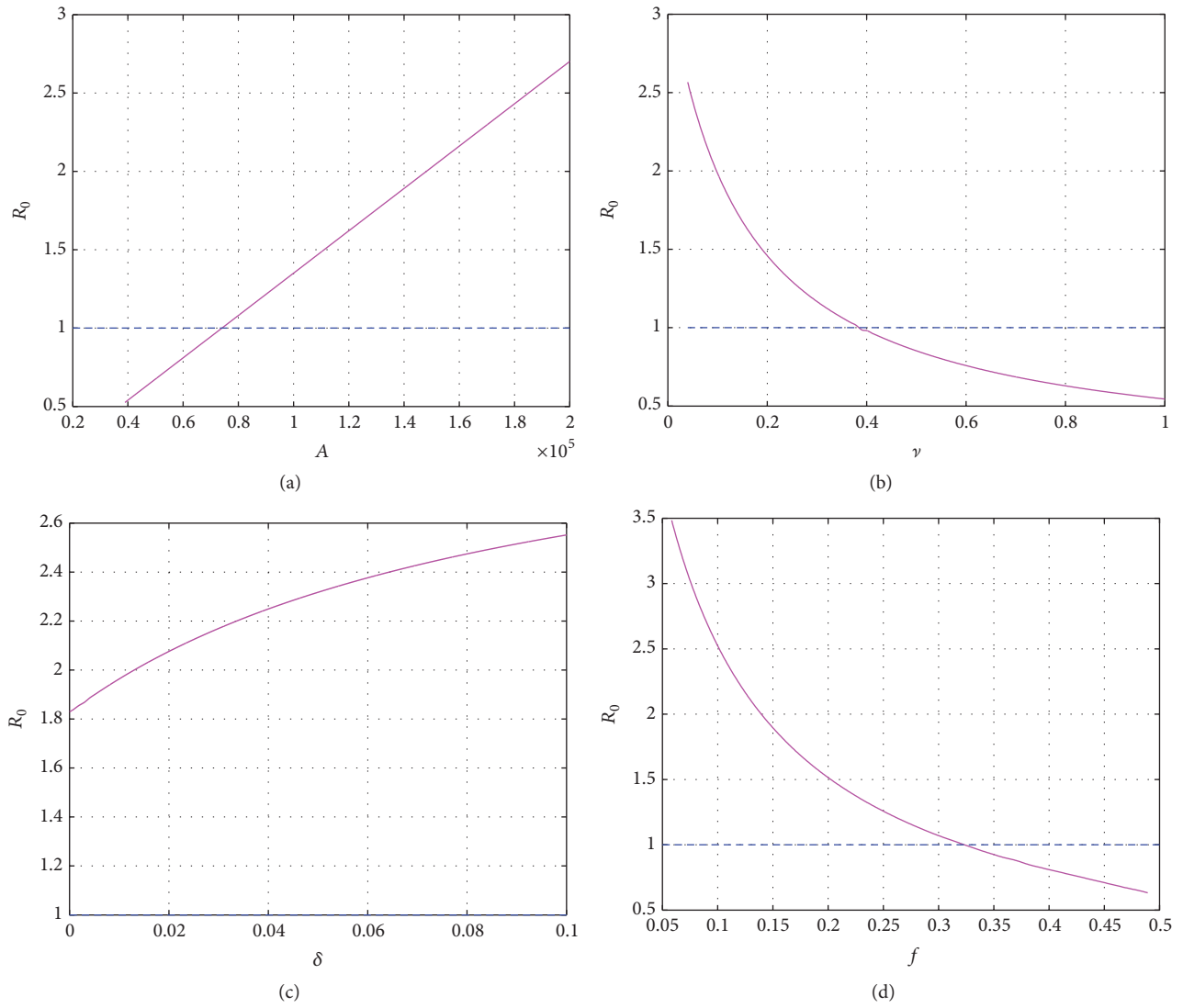


FIGURE 14: The influence of parameters on $R_0$: (a) versus A; (b) versus $\nu$; (c) versus $\delta$; (d) versus $f$.

That is to say, the dynamics of brucellosis in Inner Mongolia, Xinjiang, and Jilin of China are serious in these years. We need to control the disease spreading by carrying out effective measures.

Then we used our model to simulate the number of seasonal newly acute human brucellosis cases and predicted the general tendency of disease in Bayingolin Mongol Autonomous Prefecture. Next, we carried out some sensitivity analysis of newly acute human brucellosis cases and the basic reproduction number $R_0$ in terms of the model parameters which have significant influence on the number of newly acute human brucellosis cases by partial rank correlation coefficients (PRCC).

Finally, there are some limitations in this research. Firstly, the influence of *Brucella* in the environment was not taken into account, and it maybe affects the whole dynamic model for brucellosis transmission. Secondly, other common animals also can transmit the brucellosis to humans such as dogs, pigs, and horse. We leave these for further research.

## Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## Acknowledgments

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## References

[1] A. S. Dean, B. Bonfoh, A. E. Kulo et al., "Epidemiology of brucellosis and Q fever in linked human and animal populations in northern togo," *PLoS ONE*, vol. 8, no. 8, article e71501, 2013.

[2] M.-T. Li, G.-Q. Sun, Y.-F. Wu, J. Zhang, and Z. Jin, "Transmission dynamics of a multi-group brucellosis model with mixed cross infection in public farm," *Applied Mathematics and Computation*, vol. 237, no. 11, pp. 582–594, 2014.

[3] Y. B. Zhou and X. L. Liu, "The research progress in terms of prevalence, incidence reason and control strategies of brucellosis," *Liaoning Medical University*, vol. 1, no. 1, pp. 81–85, 2010.

[4] F. B. Zhang, Z. W. Li, X. L. La et al., "Multiple-locus variable-number tandem-repeat analysis of Brucella isolates from patients in Xinjiang, China," *International Journal of Clinical and Experimental Medicine*, vol. 8, no. 9, pp. 15716–15723, 2015.

[5] G. Pappas, P. Papadimitriou, N. Akritidis, L. Christou, and E. V. Tsianos, "The new global map of human brucellosis," *The Lancet Infectious Diseases*, vol. 6, no. 2, pp. 91–99, 2006.

[6] G. Pappas, N. Akritidis, M. Bosilkovski, and E. Tsianos, "Brucellosis," *The New England Journal of Medicine*, vol. 352, no. 22, pp. 2325–2367, 2005.

[7] Y. W. Wang and H. Sun, "Brucellosis epidemics situation and the countermeasures and suggestions in China," *Disease Surveillance*, vol. 24, no. 7, pp. 475–477, 2009.

[8] O. N. Bjørnstad, B. F. Finkenstädt, and B. T. Grenfell, "Dynamics of measles epidemics: estimating scaling of transmission rates using a time series SIR model," *Ecological Monographs*, vol. 72, no. 2, pp. 169–184, 2002.

[9] S. F. Dowell, "Seasonal variation in host susceptibility and cycles of certain infectious diseases," *Emerging Infectious Diseases*, vol. 7, no. 3, pp. 369–374, 2001.

[10] W. P. London and J. A. Yorke, "Recurrent outbreaks of measles, chickenpox and mumps. I. Seasonal variation in contact rates," *American Journal of Epidemiology*, vol. 98, no. 6, pp. 453–468, 1973.

[11] R. X. Ren and P. Zhang, "Luni-solar calender," *Journal of Pengcheng Vocational University*, vol. 18, no. 3, pp. 105–107, 2003.

[12] Y. Li, X. Yu, and T. He, "An analysis of brucellosis epidemic situation in humans from 2004 to 2010 in China," *Chinese Journal of Control of Endemic Diseases*, vol. 27, no. 1, pp. 18–20, 2004.

[13] Q. H. Liu, "A forecast model of monthly electric power demand based on season exponent and grey forecast," *Journal of Nanjing Institute of Technology (Natural Science Edition)*, vol. 4, no. 1, pp. 1–6, 2006.

[14] W. M. Wang, H. Y. Zhou, J. Cao et al., "Analysis of malaria seasonal prevalent regulation in Jiangsu Province in 2003–2007 by using trend season model," *China Tropical Medicine*, vol. 10, no. 2, pp. 153–154, 2010.

[15] J.-P. Gorvel, "Brucella: a Mr 'Hide' converted into Dr Jekyll," *Microbes and Infection*, vol. 10, no. 9, pp. 1010–1013, 2008.

[16] Z. M. Yan, "The observation of difference temperatuer impact on the brucella growth," *Inner Mongolia Medical Journal*, vol. 4, no. 35, pp. 236–238, 1984.

[17] J. Zinsstag, F. Roth, D. Orkhon et al., "A model of animal-human brucellosis transmission in Mongolia," *Preventive Veterinary Medicine*, vol. 69, no. 1-2, pp. 77–95, 2005.

[18] F. Xie and R. D. Horan, "Disease and behavioral dynamics for brucellosis control in elk and cattle in the greater yellowstone area," *Journal of Agricultural and Resource Economics*, vol. 34, no. 1, pp. 11–33, 2009.

[19] P. C. Cross, E. J. Maichak, J. D. Rogerson et al., "Estimating the phenology of elk brucellosis transmission with hierarchical models of cause-specific and baseline hazards," *The Journal of Wildlife Management*, vol. 79, no. 5, pp. 739–748, 2015.

[20] T. Yamamoto, T. Tsutsui, A. Nishiguchi, and S. Kobayashi, "Evaluation of surveillance strategies for bovine brucellosis in Japan using a simulation model," *Preventive Veterinary Medicine*, vol. 86, no. 1-2, pp. 57–74, 2008.

[21] G.-Q. Sun and Z.-K. Zhang, "Global stability for a sheep brucellosis model with immigration," *Applied Mathematics and Computation*, vol. 246, pp. 336–345, 2014.

[22] J. Nie, G.-Q. Sun, X.-D. Sun et al., "Modeling the transmission dynamics of dairy cattle brucellosis in Jilin Province, China," *Journal of Biological Systems*, vol. 22, no. 4, pp. 533–554, 2014.

[23] Q. Hou, X. D. Sun, J. Zhang, Y. J. Liu, Y. M. Wang, and Z. Jin, "Modeling the transmission dynamics of sheep brucellosis in Inner Mongolia Autonomous Region, China," *Mathematical Biosciences*, vol. 242, no. 1, pp. 51–58, 2013.

[24] M. Li, G. Sun, J. Zhang et al., "Transmission dynamics and control for a brucellosis model in Hinggan League of Inner Mongolia, China," *Mathematical Biosciences and Engineering*, vol. 11, no. 5, pp. 1115–1137, 2014.

[25] A. Dobson and M. Meagher, "The population dynamics of brucellosis in the Yellowstone National Park," *Ecology*, vol. 77, no. 4, pp. 1026–1036, 1996.

[26] L. J. Liu, X.-Q. Zhao, and Y. C. Zhou, "A tuberculosis model with seasonality," *Bulletin of Mathematical Biology*, vol. 72, no. 4, pp. 931–952, 2010.

[27] J. Liu, "Threshold dynamics for a HFMD epidemic model with periodic transmission rate," *Nonlinear Dynamics*, vol. 64, no. 1-2, pp. 89–95, 2011.

[28] Y. T. Zhu, B. Y. Xu, X. Z. Lian, W. Lin, Z. M. Zhou, and W. M. Wang, "A hand-foot-and-mouth disease model with periodic transmission rate in Wenzhou, China," *Abstract and Applied Analysis*, vol. 2014, Article ID 234509, 11 pages, 2014.

[29] J. Zhang, Z. Jin, G.-Q. Sun, X.-D. Sun, and S. G. Ruan, "Modeling seasonal rabies epidemics in China," *Bulletin of Mathematical Biology*, vol. 74, no. 5, pp. 1226–1251, 2012.

[30] Y. Ma, M. Liu, Q. Hou, and J. Zhao, "Modelling seasonal HFMD with the recessive infection in Shandong, China," *Mathematical Biosciences and Engineering*, vol. 10, no. 4, pp. 1159–1171, 2013.

[31] D. L. Wang and S. Y. Zhang, "Research progress for the treatment of acute brucellosis," *Chinese Journal of Control of Endemic Diseases*, vol. 16, no. 2, pp. 94–95, 2001.

[32] D. Schenzle, "An age-structured model of pre- and post-vaccination measles transmission," *Mathematical Medicine and Biology*, vol. 1, no. 2, pp. 169–191, 1984.

[33] W. Wang and X.-Q. Zhao, "Threshold dynamics for compartmental epidemic models in periodic environments," *Journal of Dynamics and Differential Equations*, vol. 20, no. 3, pp. 699–717, 2008.

[34] P. van den Driessche and J. Watmough, "Reproduction numbers and sub-threshold endemic equilibria for compartmental models of disease transmission," *Mathematical Biosciences*, vol. 180, pp. 29–48, 2002.

[35] F. Zhang and X.-Q. Zhao, "A periodic epidemic model in a patchy environment," *Journal of Mathematical Analysis and Applications*, vol. 325, no. 1, pp. 496–516, 2007.

[36] C. E. Sun, H. Z. Ma, and S. S. Yin, *Brucellosis*, Ningxia People's Press, 1985.

[37] T. Z. Sun, Z. R. Wu, and X. Y. Pang, "Prevention measures and countermeasures on brucellosis in Inner Mongolia," *Neimenggu Preventive Medicine*, vol. 1, no. 37, pp. 136–139, 2000.

[38] H. Muhtar, H. B. He, X. P. Tai, X. Chen, S. X. Tong, and Z. Wang, "Analysis of surveillance data and epidemic situation of human brucellosis in Xinjiang, 2013," *Chinese Journal of Vector Biology and Control*, vol. 26, no. 1, pp. 86–88, 2015.

[39] S. A. DeLurgio, *Forecasting Principles and Applications*, Irwin/McGraw-Hill, 1998.

[40] C. D. Lewis, *Industrial and Business Forecasting Methods: A Practical Guide to Exponential Smoothing and Curve Fitting*, Butterworth Scientific, London, UK, 1982.

[41] S. Marino, I. B. Hogue, C. J. Ray, and D. E. Kirschner, "A methodology for performing global uncertainty and sensitivity analysis in systems biology," *Journal of Theoretical Biology*, vol. 254, no. 1, pp. 178–196, 2008.

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*Research Article*

# NRAMP1 Polymorphisms like Susceptibility Marker in Mexican Focus of Cutaneous Leishmaniasis

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Cutaneous leishmaniasis (CL) is endemic in Campeche state, Mexico. Host and parasite factors are involved in the establishment and development of CL. Host factors include immune response and genetic background. NRAMP1 (Natural Resistance Associated Macrophage Protein 1) is important in innate immunity. Polymorphisms in *NRAMP1* have been associated with susceptibility or resistance to infectious and autoimmune diseases. To study the association of *NRAMP1* mutations with CL in patients from Calakmul, Campeche, samples from 115 CL patients and 69 samples of healthy people from the same area were evaluated. Five regions in *NRAMP1* were amplified and digested, looking for mutations in the promoter region (−524G/C), exon 3 (274C/T), exon 8 (823 C7T), and exon 15 (G/A) and deletion of 4 bp in the 3′UTR region. We found a statistical association between polymorphisms in 3′UTR region and exon 8 and CL [$\chi^2 = 13.26$; $p < 0.05$; OR = 17.00; IC of 95% (2.24–128.99)]. Some patients who needed more than 40 doses of Glucantime® to heal injuries presented mutations in exons 3, 8, and 15. Multiple or ear lesions were not associated with *NRAMP1* polymorphism.

## 1. Introduction

Leishmaniasis is zoonotic disease, caused by different species of the genus *Leishmania*, protozoa transmitted to human and animal reservoirs by the bite of infected phlebotomine sandflies (genus *Lutzomyia*, in the new world) [1–3]. Leishmaniasis is endemic in the most tropical regions of the world; furthermore several factors like climate change, urbanization, deforestation, increased travel for tourist and work-related reasons, immigrations from endemic countries, and military operations can lead to an increase in risk of contracting the diseases in new areas [4]. In Mexico, four clinical forms of leishmaniasis are found, localized cutaneous leishmaniasis (LCL), diffuse cutaneous leishmaniasis (DCL), mucocutaneous leishmaniasis (ML) caused by *L. (L.) mexicana*, and visceral leishmaniasis (VL) [5, 6]; furthermore there are reports of *L. (V.) braziliensis* [5, 7] and *L. (L.) chagasi* [8]. The sylvatic region of the Yucatan Peninsula is an endemic focus of leishmaniasis; most of the recorded cases are LCL with few well limited ulcers located mainly on the ear, head, neck, and upper limb [9]. DCL is characterized by a large number of nodular skin lesions and MCL is a severe form with inflammations with progressive destruction of the mucocutaneous tissues. Both forms are rare in Mexico.

In murine models, the earliest phases and resistance to the infection with different species of *Mycobacterium* and other intracellular microorganisms like *Leishmania* are under control of NRAMP1 (Natural Resistance Associated Macrophage Protein 1) encoded by *NRAMP1* gene, previously called *Bcg*/*Lsh*/*Ity* (*Mycobacterium bovis* BCG, *Leishmania donovani*, and *Salmonella typhimurium*) which has 88% identity with the human formerly called *NRAMP1* [10, 11] and now called *SLC11A1* (solute carrier family 11 member 1 protein). NRAMP1 is expressed in the membrane fraction in late endosome and lysosomes in macrophages and polymorphonuclear leukocytes as a 90–100 KDa phosphoglycoprotein

[12] and acts as a pH-dependent divalent cation carrier. This protein has been found in close association with natural resistance to infection with some intracellular pathogens, which plays a critical role in early innate macrophage responses to intracellular infection. Polymorphisms of human *SLC11A1* are associated with susceptibility to several inflammatory autoimmune disorders, as well as infectious diseases in African and Asian populations, including leprosy, tuberculosis, visceral leishmaniasis, and human immunodeficiency virus [12, 13]. Polymorphisms have also been suggested to modulate expression or alter the functional capacity of SLC11A1 to transport divalent cations, respectively [14]. In the case of leishmaniasis diseases, polymorphism of *SLC11A1* coupled with the expression of other genes has been associated with susceptibility to some forms of this disease in a specific Mexican endemic area [15] and specifically with CL in Brazil [16] and VL in Sudan [17] and Morocco [18].

Over five years of work in Campeche state, located in the Yucatan Peninsula, specifically in the municipality of Calakmul, endemic area for LCL, we have observed different behaviors of the clinical expression of CL and we found people living in this area who have never developed any form of CL to people who present with the disease more than once, all of them living under the same conditions. On the other hand, we have noticed that there is a variable response to Glucantime [19]. Some patients resolved CL with 15 to 20 doses and others required 40 to 100 doses to get the same effect. The host genetic background and immune response are important in the evolution of the infection. The purpose of this study was to evaluate the correlation between susceptibility or resistance to leishmaniasis with polymorphisms in NRAMP1 in patients with CL from Calakmul, Campeche.

The polymorphisms analyzed in this research were four single nucleotide polymorphisms (SNPs) identified in the promotor region (−524G/C), in exon 3 (274C/T), in exon 8 (823C/T), and in exon 15 (D543N) and one deletion in the 3′UTR region (1729 + 55del4). All of them related to resistance to intracellular microorganisms in humans.

## 2. Materials and Methods

*2.1. Blood Samples.* Blood samples were collected in the municipality of Calakmul, Campeche, from 115 patients with clinical and parasitologic diagnosis of CL. Patients, male or female over 10 years old, were included. *Leishmania* species was identified by PCR amplification. *Leishmania (L.) mexicana* was the causative agent in most of the cases, with few caused by *Leishmania (V.) braziliensis*. All the cases in the current study responded to antileishmanial therapy with Glucantime. Control group was defined as healthy individuals ($n = 69$), who never had the disease, are without scars or injuries related to CL, and are male or female over 10 years old. Each participant signed a letter of informed consent, and inclusion of young children consent was obtained from parents or guardians. The research was approved by the ethical committee of the Escuela Nacional de Ciencias Biológicas from Instituto Politécnico Nacional and with authorization from the municipality of Calakmul, Campeche, with the participation of its Public Health Department, in agreement with the International Ethic Guidelines for Biomedical Research Involving Human Subjects and the Norma Oficial Mexicana de Salud: NOM-003-SSA 2-1993, for sampling blood from human beings for diagnosis and therapeutics. The study was registered in the "Libro de Actas del Cabildo" with date May 24, 2002.

Table 1: Primers used in this study.

| Polymorphisms | Primers |
|---|---|
| −524G/C | 5′-AAC AAC TCT GAG AAG GGA CA-3′ |
| | 5′-TGT GCC CCA CAA CAC ATC TG-3′ |
| 274C/T | 5′-TGC CAC CAT CCC TAT ACC CAG-3′ |
| | 5′-TCT CGA AAG TGT CCC ACT CAG -3′ |
| 823C/T | 5′-CTT GTC CTG ACC AGG CTC CT-3′ |
| | 5′-CAT GGC TCC GAC TGA GTG AG-3′ |
| D543N | 5′-GCA TCT CCC CAA TTC ATG GT-3′ |
| 1729 + 55del4 | 5′-AAC TGT CCC ACT CTA TCC TG-3′ |

*2.2. DNA Extraction.* DNA was obtained from venous blood samples, and DNA extraction was performed using the Gentra Puregene Kit (QIAGEN, Germany).

*2.3. NRAMP1 Genotyping.* Polymerase Chain Reaction-Restriction Fragment Length Polymorphisms (PCR-RFLP) methods were used for genotyping four polymorphic regions (SNPs) of the NRAMP1 gene: substitution −524 G/C in the promoter region, substitution 274 C/T in exon 3, substitution 823 C/T in exon 8, amino acid substitution D543N G/A in exon 15, and one deletion 1729 + 55del4 (TGTG) in the 3′ untranslated region (UTR). The methodology used was as described by Saiki and colleagues [20], using sequence specific oligonucleotides which were previously described (Table 1) [21–24] and performed in a Gene Amp PCR Systems 9700 thermocycler (Applied Biosystems), using 0.20 mM of each dNTP, 50 pM of each primer, 1.5 mM $MgCl_2$, 10 mM Tris pH 8.4, 50 mM KCl, 0.01% gelatin, and 1.25 U of *Taq* polymerase (Invitrogen Life Technologies, CA, USA) in a 50 $\mu$L reaction. The PCR conditions for the five polymorphisms were the same: one cycle of 96°C for 5 min, 60°C for 60 sec, and 72°C for 60 sec, followed by 35 cycles of denaturation at 93°C during 45 sec, annealing at 60°C for 60 sec and extension at 72°C for 60 sec and one elongation step at 72°C for 10 minutes. The amplicons were analyzed by horizontal electrophoresis on 2% agarosa gels in TBE buffer at 110 V for 1 hour. The gels were stained with ethidium bromide (1 mg/$\mu$L) and visualized using UV illumination system. RFLP were made using 10 $\mu$L of amplicon in 20 $\mu$L of reaction with one of the following restriction endonucleases: *Ava II*, *Fok I*, *Hinf I*, *Mnl I*, and *Nar I* for exon 15, 3′UTR, promoter region, exon 3, and exon 8 amplicons, respectively. According to the manufacturer's instructions (New England Biolabs and Invitrogen Life Technologies), restriction enzyme digestion products were resolved using a vertical electrophoresis, on 8% acrylamide gel, and were stained with ethidium bromide (1 mg/$\mu$L) and visualized under UV light.

Table 2: Details of the polymorphisms genotyped and identified by PCR-RFLP in *NRAMP1*.

| Polymorphisms | Localization | PCR product size | Restriction enzyme | Restriction enzyme products |
|---|---|---|---|---|
| −524G/C | Promoter region | 585 bp | *Hinf I* | Allele 1 (G): 43 (2), 61, 62, 79, 297 bp<br>Allele 2 (C): 43, 61, 79, 105, 297 bp<br>G/C: 43, 61, 62, 79, 105, 297 bp |
| 274C/T | Exon 3 | 216 bp | *Mnl I* | Allele 1 (C): 12, 37, 65, 102 bp<br>Allele 2 (T): 12, 37, 167 bp<br>C/T: 12, 37, 65, 102, 167 bp |
| 823C/T | Exon 8 | 234 bp | *Nar I* | Allele 1 (C): 99, 135 bp<br>Allele 2 (T): 234 bp<br>C/T: 99, 135, 234 bp |
| D543N | Exon 15 | 240/244 bp | *Ava II* | Allele 1 (Asp): 39, 79, 122/126 bp<br>Allele 2 (Asn): 39, 201/205 bp<br>Asp/Asn: 39, 79, 122/126, 201/205 bp |
| 1729 + 55del4 | 3′UTR | 240/244 bp | *Fok I* | Allele 1 (+TGTG): 33, 211 bp<br>Allele 2 (−TGTG): 240<br>+/−: 33, 211, 240 |

*2.4. Statistical Analysis.* Frequency in genotype differences between patients and control subjects was examined using the Chi-square test with two degrees of freedom and was considered statistically significant when $p < 0.05$. Further the previous analysis included odds ratio test, with a confidence interval of 95% to quantitatively assess the degree of association between polymorphisms in NRAMP1 gene and CL.

## 3. Results

*3.1. NRAMP1 Polymorphisms.* −524G/C; 274C/T; 823C/T; D543NG/A; and 1729 + 55del4 (TGTG) were evaluated by PCR-RFLP. Table 2 shows details of the polymorphisms genotyped from 115 blood samples from patients with CL and 69 samples healthy controls, collected in the Calakmul municipality in Campeche state, Mexico.

823C/T polymorphism was significantly associated with CL infection ($p < 0.05$), a silent nucleotide substitution (C to T). The genotype C/T and the minor allele (T/T) were expressed in CL group while its expression is absent in the control group. This mutation was found related to the presence of the CL ($p < 0.05$, OR [CI = 95%] = 17.0) (Table 3). 3′UTR (1729 + 55del4) was significantly associated with infection of CL either; the mutated alleles del/del and TGTG+/del are present in CL patients and in minor proportion in the control group ($p < 0.05$, OR [CI = 95%] = 2.76).

In this study no association was detected with other polymorphisms analyzed when comparing patients with control group.

*3.2. Polymorphism in NRAMP1 and Requirements of Glucantime Doses.* The potential relationship between polymorphisms and requiring of higher dosages of Glucantime than the average dose of 25 ampules used in CL patients of the same region [25] was evaluated in this study. Association with polymorphism 274C/T in exon 3 of NRAMP1 was found. 11 of 13 samples presented the mutated alleles (Table 4), and two samples had the original allele. Association with polymorphisms 823C/T in exon 8 and D543NG/A in exon 15 of NRAMP1 was also found. Only 4 samples for exon 8 and 3 samples for exon 15 had mutated allele in homozygous way. It was not finding the heterozygous mutation in both cases. Analysis in the 3′UTR region shows no association. The correlation between the number of lesions and polymorphisms was also analyzed (results not shown) but no significant association was found.

## 4. Discussion

Several studies have established that *NRAMP1* is a gene coding for an important regulatory element in the tracks of macrophages activation and differentiation [24, 26]. *NRAMP1* gene has been associated with susceptibility and resistance to diseases caused by intracellular pathogens, such as the protozoan *Leishmania*. In this study, we analyzed five polymorphic regions of *NRAMP1* in samples of patients with CL form and control people (without CL), four single nucleotide polymorphisms, and one deletion of four nucleotides in the 3′UTR region, in order to know their effect on the risk to develop CL after infection in an endemic area. Bellamy [25] has suggested a strong relationship between some polymorphisms in *NRAMP1* (5′(CA)n, INT4, D543N, and deletion in 3′UTR region) and the increased risk of acquiring tuberculosis in west area of Africa [26, 27]. However, other studies which involved patients from Korea, Japan, Brazil, and Denmark suggested no association between polymorphisms in *NRAMP1* and the risk of acquiring tuberculosis. Although it is the same disease, the results are different because the patients belong to different ethnic groups with different genetic background and clinical status [27]. Similar results were found with other diseases caused by intracellular pathogens in different populations. In *Leishmania* infection, *NRAMP1* polymorphisms could play an important role in the pathology of the disease, referring specifically to CL and VL susceptibility in an endemic area in Chiapas state, Mexico [15]. Therefore, we consider the study of the polymorphisms present in three exons in *NRAMP1* relevant if they could have high possibility of affecting the NRAMP1 protein function. In addition, the promoter region plays a decisive role in the

Table 3: Genotype frequency of the NRAMP1 polymorphisms.

| Genotypes | CL (%) | Controls (%) | $\chi^2$ value | OR (95% CI) |
|---|---|---|---|---|
| *Promoter region −524* | | | | |
| Promoter region −524 G/G | 115 (100) | 69 (100) | Not statistically significant | |
| Promoter region −524 G/C | 0 | 0 | | |
| Promoter region −524 C/C | 0 | 0 | | |
| *Exon 3 274* | | | | |
| Exon 3 274 C/C | 48 (41.73) | 35 (50.72) | Not statistically significant | |
| Exon 3 274 C/T | 51 (44.34) | 27 (39.13) | | |
| Exon 3 274 T/T | 16 (13.91) | 7 (10.14) | | |
| *Exon 8 823* | | | | |
| Exon 8 823 C/C | 92 (80) | 68 (98.55) | 13.26 | 17.00 (2.24–128.99) |
| Exon 8 823 C/T | 18 (15.65) | 1 (1.44) | | |
| Exon 8 823 T/T | 5 (4.34) | 0 | | |
| *Exon 15 D543N* | | | | |
| Exon 15 D543N G/G | 75 (65.21) | 52 (75.36) | Not statistically significant | |
| Exon 15 D543N G/A | 40 (34.78) | 16 (23.18) | | |
| Exon 15 D543N A/A | 0 | 1 (1.44) | | |
| *3′UTR* | | | | |
| 3′UTR TGTG/TGTG | 65 (56.52) | 54 (78.26) | 9.6 | 2.76 (1.40–5.46) |
| 3′UTR TGTG/del | 3 (2.6) | 0 | | |
| 3′UTR del/del | 47 (40.86) | 15 (21.73) | | |

$\chi^2$ value = Chi-square value with $p < 0.05$ in the presence of the mutation and CL.
OR = odds ratio (95% confidence interval) presence of mutation is a risk for CL.
UTR = untranslated region. del = deletion.

Table 4: Genotype frequency of the *NRAMP1* polymorphisms in patients requiring high doses of Glucantime (HDG).

| Genotypes | CL-HDG (%) $n = 13$ | CL (%) $n = 115$ | $\chi^2$ value | OR (95% CI) |
|---|---|---|---|---|
| Exon 3 274 C/C | 2 (12.5) | 48 (41.73) | 22.69 | 3.94 (0.83–18.59) |
| Exon 3 274 C/T | 2 (12.5) | 51 (44.34) | | |
| Exon 3 274 T/T | 9 (69.23) | 16 (13.91) | | |
| Exon 8 823 C/C | 9 (69.23) | 92 (80) | 15.43 | 1.77 (0.50–6.28) |
| Exon 8 823 C/T | 0 | 18 (15.65) | | |
| Exon 8 823 T/T | 4 (30.76) | 5 (4.34) | | |
| Exon 15 D543N G/G | 10 (76.92) | 75 (65.21) | 31.75 | 0.56 (0.14–2.16) |
| Exon 15 D543N G/A | 0 | 40 (34.78) | | |
| Exon 15 D543N A/A | 3 (23.07) | 0 | | |
| 3′UTR TGTG/TGTG | 8 (61.53) | 65 (56.52) | Not statistically significant | |
| 3′UTR TGTG/del | 0 | 3 (2.6) | | |
| 3′UTR del/del | 5 (38.46) | 47 (40.86) | | |

$\chi^2$ value = Chi-square value with $p < 0.05$ in the presence of the mutation and requirement of high doses of Glucantime.
OR = odds ratio (95% confidence interval). UTR = untranslated region. del = deletion.

expression of the protein, to change the receptor of transcription factors. And finally, a 4 bp deletion in an untranslated region plays a crucial role in posttranscriptional regulation gene expression. All of the above explain the observation made in the municipality of Calakmul, Campeche state, where some people acquire CL, while others, living in the same area, under similar conditions, never developed the disease. Also, we expected to find a correlation between the polymorphisms in *NRAMP1* and the tolerance presented in some patients, who need high doses of medication to heal CL.

In the present, no mutation in the promoter region (−524 G/C) (Table 3) was found, and 100% of the control people and patients with LCL presented the original allele (G/G). However, Donninger and colleagues [22] found that this

polymorphism creates a site which binds a transcription factor and then the overexpression of NRAMP1, so this mutation has been associated with protection to infection due to hyperactivity of macrophages, but in this study, this mutation was absent.

Mutation in exon 3 274 (C/T) place was analyzed, and no significant differences in the genotype frequencies obtained were found between both groups, so mutation in exon 3 could not be associated with disease. Nevertheless Ortiz-Flores and colleagues [15] found an association between allele 274 C/C in exon 3 and susceptibility to developing CL in Mexican patients from two communities in Chiapas state, Mexico. The authors addressed that their finding differs from other studies realized in Sri Lanka and Brazil for the same diseases form, where no relation was found between this mutation and susceptibility to developing CL. The explanation for this could be that these two endemic areas in Mexico, Campeche and Chiapas states, are geographically widely separated, with different ethnic groups, and affected by different *Leishmania* species and probably by different variants of the same parasite species.

When exon 8 (823C/T) was analyzed, our results suggested that there is a significant association between this polymorphism and CL after applying Chi-square test ($\chi^2 = 13.26$; $p < 0.05$) and the same with odds ratio test (OR = 17.00; CI = 95%), and we conclude that this mutation is a risk factor to develop CL when this mutation is present. This is because about 20% of patients with CL presented differences in this exon, unlike the control group, which merely happens in approximately 1.5% of the people of this group. Nucleotide changes in exon 3 and exon 8 in *NRAMP1* gene are considered silent mutations, since the change of a nucleotide or amino acid generates a change but may actually cause disease by altering regulatory elements affecting exon RNA splicing [28, 29]. In the regular edition of RNA, the primary transcript of a gene contains sequences encoding amino acids (exons) and long noncoding sequences (introns) that should not be present in the final mRNA. In each exon there are short nucleotide sequences called Exonic Splicing Enhancer (ESE), which function as enhancers of RNA splicing, indicating the editing complex, where there are the ends of exons [30, 31]. The binding of regulatory proteins of the edition to enhancer sequences in the primary transcript makes the spliceosome directed towards both ends of the intron and cleave the primary transcript before splicing the ends of exons. Synonymous changes in a nucleotide sequence of an exon could become invisible to the enhancer sequences to protein complex of the spliceosome (due to less affinity), so an entire exon could be excluded from the final RNA. Then the secondary structure of the mRNA is affected as the stability of mRNA [30, 32]. Additional studies suggested that decreased enzymatic activity due to reduced protein expression mediated by changes in the mRNA structures could explain the differences in the phenotype [32, 33]. Over 50 human diseases have been associated with synonymous mutations and, in a recent survey, of 21,429 polymorphisms associated with human disease, nonsynonymous and synonymous variations were determined to have a similar probability of disease association (1.46% and 1.26%, resp.) [34].

Although the polymorphisms present in exon 3 and exon 8 have the same effect on NRAMP1 protein, in our study only mutation in exon 8 showed an association with CL. These findings agree with the association found in Morocco with VL infections, while the same polymorphism seems not to be associated with CL infection caused by *Leishmania (V.) braziliensis* in Brazil [16].

In relation to polymorphism in exon 15 (D543N), the polymorphism consisting in the change of G for A, which generates a substitution of amino acid (aspartic acid/asparagine), affecting protein function, has been associated with susceptibility to tuberculosis in populations of West Africa and East Asia [27, 29]. When exon 15 polymorphism was analyzed in samples from CL patients, 65.2% presented the original allele and 34.8% presented both alleles. With samples from the control group, 75% presented original allele, 23% presented both alleles, and 1.4% presented mutated allele. After statistic analysis, no significant differences between groups were found, referring to genotype frequency.

Finally, polymorphisms in 3′UTR region of NRAMP1 gene were analyzed to find any association between CL susceptibility and deletion of four nucleotides in this region (−TGTG). 78.26% of samples from control people presented original allele and 21.73% were homozygous for the mutated allele. In the case of patients with CL 56.52% who had the original allele, 2.6% presented both alleles and 40.86% had mutated allele. After statistic analysis significant association was found between this polymorphism and CL after applying Chi-square test ($\chi^2 = 9.6$; $p < 0.05$) and with odds ratio test (OR = 2.76; CI = 95%); then there is a risk factor to develop CL when this mutation is present. This is because about 43% patients with CL presented differences in the 3′UTR region, unlike the control group, which merely occurs in about 22%. The physiological effect of polymorphism in this region is not known exactly, but it is known that this region is considered gene regulatory elements [23] and its association with tuberculosis susceptibility in populations from Eastern Asia and Africa has been shown [35]. It is noteworthy that, unlike other regions in this study, patients with CL presented allele mutated in 40.86% and this allele was homozygous. In other regions, where we found a mutation, most were heterozygous. Some authors have mentioned that homozygous mutation has high relevance because the stability is greater [36] and draws attention to the high proportion of patients with mutated allele. Other authors have related the best transporter activity of NRAMP1 and its ability to generate an intraphagosomal ionic concentration (which allow survival and reproduction of microorganisms) and the presence of mutated allele and even heterozygote alleles [37]. This mutated allele also causes an appropriate level of $Fe^{+2}$ that allows development of microorganisms because it has been suggested that affected NRAMP1 reduces iron release from macrophages. Then iron accumulates in the liver and spleen when erythrophagocytosis happens and when there are changes in level of iron and other metals, ROS- (Reactive Oxygen Species-) regulated signaling is altered, in the same way as inflammatory pathway activation, a very important antimicrobial mechanism [38].

In some studies, where macrophages with a mutation in NRAMP1 protein were infected with mycobacteria, the ability to process antigens was loose, affecting the CD4 lymphocytes activity and therefore the Th1 response, promoting a Th2 profile and the progression of tuberculosis [27, 35]. For the case of leishmaniasis diseases, a similar model is proposed, due to be an intracellular pathogen. Susceptibility to CL has been associated with several polymorphic genes, including *NRAMP1 (SLC11A1)* in Brazil. Another study realized in a Mexican endemic area (Chiapas state, Mexico) with the object related to polymorphism of different genes, including *NRAMP1*, with different forms of leishmaniasis diseases, showed that, for this population and endemic area, none of *SLC11A1* alleles analyzed (they chose seven polymorphisms of this gene) had no relation with susceptibility to CL, except for one genotype (C/C) in exon 3 (274C/T) which was associated with CL susceptibility [15]. This differs from results obtained with patients from Campeche state, where exon 3 polymorphisms in *NRAMP1* gene and CL susceptibility were not related. Maybe as mentioned before, the differences in the ethnic group, *Leishmania* species and geographical environment, may be the explanation for the results from Calakmul foci.

When the analysis was made with samples from patients with CL which needed higher doses of Glucantime than the mean of 25 ampules used by Vargas-Gonzalez [39], the association between polymorphisms (homo- and heterozygote mutation) in exon 3 caught our attention; 11 samples of 13 patients, which needed high doses of Glucantime, had a mutation in exon 3 and presented the allele (274C/T). We made a statistically important relationship between the presence of this allele in a patient and the need for high doses of Glucantime; the relative risk was found (OR = 3.94) (Table 4). Two samples had the original allele. Analysis of exon 8 and exon 15 shows association with risk of needing large doses of medication, but with OR values lower than those obtained in exon 3. When the UTR region for the same samples was realized, there was no association between high doses of treatment and polymorphisms in this region. Some samples showed an extra mutation in some other exons in addition to those existing in exon 3.

The correlation between the number of lesions and the amount of drug administered was also analyzed [results not shown] because patients who required high doses of Glucantime were the patients with many lesions, then 25 samples were analyzed and 22 presented, at least, one mutation in any of five regions of this study, and 3 patients had no mutation. Of 22 patients, 13 presented mutation in exon 3 and 2 patients presented mutation in exon 8; 3 patients had mutation in exon 15 and 2 presented mutation in 3′UTR region, unfortunately without statistical significance. There is clearly an alteration in NRAMP1 protein, affecting innate immunity, so the patient does not stop the growth of the pathogen and develops the disease again. Similarly, patients who had a single lesion were analyzed, and results showed that this group of patients at least had one mutation in some of the regions of this study, but no significant association was found.

## 5. Conclusions

In the case of leishmaniasis diseases, to date, there are no effective chemoprophylaxis measures as in malaria. Several groups are working to produce protective vaccines; others are looking for better treatment, less toxication, and more accessible. In Mexico there are no mechanisms for the prevention and control of leishmaniasis in short term. Unfortunately, new cases of CL arise in farming communities, who are looking for new lands to work in the jungle, where the life cycle of this parasite has prevailed. And as it was demonstrated in the population of this study, the probabilities of developing CL are higher if a person is susceptible due to mutation in exon 8 of *NRAMP1* or a deletion in 3′UTR.

Other polymorphisms of *NRAMP1* remain to be investigated in the same community and extend the study to other endemic areas in Mexico and what relation it has with CL expression. In the same way, genetic causes of the different response to drug must be investigated in depth, looking for the pharmacological activity protein genes.

## Disclosure

Amalia Monroy-Ostria was fellow of CONACyT, EDI, COFAA, Mexico. Mario Eugenio Cancino-Díaz is a fellow of SNI, COFAA, EDI, Mexico. Mirsha Pamela Hernández-Rivera was fellow of CONACyT, Mexico.

## Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

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## References

[1] I. Cruz, A. Millet, E. Carrillo et al., "An approach for interlaboratory comparison of conventional and real-time PCR assays for diagnosis of human leishmaniasis," *Experimental Parasitology*, vol. 134, no. 3, pp. 281–289, 2013.

[2] J. Alvar, I. D. Vélez, C. Bern et al., "Leishmaniasis worldwide and global estimates of its incidence," *PLoS ONE*, vol. 7, no. 5, article e35671, 2012.

[3] M. D. J. Rovirosa-Hernández, L. Cortes-Ortíz, F. García-Orduña et al., "Seroprevalence of *Trypanosoma cruzi* and *Leishmania mexicana* in free-ranging howler monkeys in southeastern Mexico," *American Journal of Primatology*, vol. 75, no. 2, pp. 161–169, 2013.

[4] I. Kevric, M. A. Cappel, and J. H. Keeling, "New world and old world leishmania infections: a practical review," *Dermatologic Clinics*, vol. 33, no. 3, pp. 579–593, 2015.

[5] O. Velasco Castrejon, "Leishmaniasis in Mexico," *Revista Latinoamericana de Microbiología*, vol. 29, no. 2, pp. 119–126, 1987.

[6] O. Hernandez-Montes, A. Monroy-Ostria, S. McCann, and D. C. Barker, "Identification of Mexican *Leishmania* species by analysis of PCR amplified DNA," *Acta Tropica*, vol. 71, no. 2, pp. 139–153, 1998.

[7] G. Sanchez-Tejeda, N. Rodríguez, C. I. Parra, O. Hernandez-Montes, D. C. Barker, and A. Monroy-Ostria, "Cutaneous leishmaniasis caused by members of *Leishmania braziliensis* complex in Nayarit, State of Mexico," *Memorias do Instituto Oswaldo Cruz*, vol. 96, no. 1, pp. 15–19, 2001.

[8] A. Monroy-Ostria, O. Hernandez-Montes, and D. C. Barker, "Aetiology of visceral leishmaniasis in Mexico," *Acta Tropica*, vol. 75, no. 2, pp. 155–161, 2000.

[9] F. J. Andrade-Narváez, A. Vargas-González, S. B. Canto-Lara, and A. G. Damián-Centeno, "Clinical picture of cutaneous leishmaniases due to *Leishmania* (*Leishmania*) *mexicana* in the Yucatan peninsula, Mexico," *Memórias do Instituto Oswaldo Cruz*, vol. 96, no. 2, pp. 163–167, 2001.

[10] J. M. Blackwell, S. Searle, T. Goswami, and E. N. Miller, "Understanding the multiple functions of Nramp1," *Microbes and Infection*, vol. 2, no. 3, pp. 317–321, 2000.

[11] J. M. Blackwell, T. Goswami, C. A. W. Evans et al., "SLC11A1 (formerly NRAMP1) and disease resistance," *Cellular Microbiology*, vol. 3, no. 12, pp. 773–784, 2001.

[12] J. R. Forbes and P. Gros, "Divalent-metal transport by NRAMP proteins at the interface of host-pathogen interactions," *Trends in Microbiology*, vol. 9, no. 8, pp. 397–403, 2001.

[13] A. Awomoyi, G. Sirugo, M. J. Newport, and S. Tishkoff, "Global distribution of a novel trinucleotide microsatellite polymorphism (ATA)n in intron 8 of the SLC11A1 gene and susceptibility to pulmonary tuberculosis," *International Journal of Immunogenetics*, vol. 33, no. 1, pp. 11–15, 2006.

[14] N. S. Archer, N. T. Nassif, and B. A. O'Brien, "Genetic variants of SLC11A1 are associated with both autoimmune and infectious diseases: systematic review and meta-analysis," *Genes and Immunity*, vol. 16, no. 4, pp. 275–283, 2015.

[15] A. Ortiz-Flores, G. De la Rosa-López, B. Zavaleta-Villa et al., "Association of leishmaniasis with TNF alpha promoter and SLC11A1 gene polymorphisms in patients of two endemic areas in Mexico," *Microbes and Infection*, vol. 17, no. 5, pp. 387–394, 2016.

[16] L. Castellucci, S. E. Jamieson, E. N. Miller et al., "*CXCR1* and *SLC11A1* polymorphisms affect susceptibility to cutaneous leishmaniasis in Brazil: a case-control and family-based study," *BMC Medical Genetics*, vol. 11, article 10, 2010.

[17] H. S. Mohamed, M. E. Ibrahim, E. N. Miller et al., "SLC11A1 (formerly NRAMP1) and susceptibility to visceral leishmaniasis in the Sudan," *European Journal of Human Genetics*, vol. 12, no. 1, pp. 66–74, 2004.

[18] R. Ejghal, M. Hida, M. L. Idrissi, A. E. Hessni, and M. Lemrani, "SLC11A1 polymorphisms and susceptibility to visceral leishmaniasis in Moroccan patients," *Acta Tropica*, vol. 140, pp. 130–136, 2014.

[19] M. P. Hernandez-Rivera, O. Hernandez-Montes, A. Chinas-Perez et al., "Study of cutaneous leishmaniasis in the State of Campeche (Yucatan Peninsula), Mexico, over a period of two years," *Salud Pública de México*, vol. 57, no. 1, pp. 58–65, 2015.

[20] R. K. Saiki, D. H. Gelfand, S. Stoffel et al., "Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase," *Science*, vol. 239, no. 4839, pp. 487–491, 1988.

[21] J. Liu, T. M. Fujiwara, N. T. Buu et al., "Identification of polymorphisms and sequence variants in the human homologue of the mouse natural resistance-associated macrophage protein gene," *American Journal of Human Genetics*, vol. 56, no. 4, pp. 845–853, 1995.

[22] H. Donninger, T. J. Cashmore, T. Scriba, D. C. Petersen, E. Janse van Rensburg, and V. M. Hayes, "Functional analysis of novel SLC11A1 (NRAMP1) promoter variants in susceptibility to HIV-1," *Journal of Medical Genetics*, vol. 41, no. 4, article e49, 2004.

[23] Y. S. Yang, S. J. Kim, J. W. Kim, and E.-M. Koh, "NRAMP1 gene polymorphisms in patients with Rheumatoid Arthritis in Koreans," *Journal of Korean Medical Science*, vol. 15, no. 1, pp. 83–87, 2000.

[24] K. Ouchi, Y. Suzuki, T. Shirakawa, and F. Kishi, "Polymorphism of SLC11A1 (formerly NRAMP1) gene confers susceptibility to Kawasaki disease," *Journal of Infectious Diseases*, vol. 187, no. 2, pp. 326–329, 2003.

[25] R. Bellamy, "The natural resistance-associated macrophage protein and susceptibility to intracellular pathogens," *Microbes and Infection*, vol. 1, no. 1, pp. 23–27, 1999.

[26] M. J. Maliarik, K. M. Chen, R. G. Sheffer et al., "The natural resistance-associated macrophage protein gene in African Americans with sarcoidosis," *American Journal of Respiratory Cell and Molecular Biology*, vol. 22, no. 6, pp. 672–675, 2000.

[27] W. Zhang, L. Shao, X. Weng et al., "Variants of the natural resistance-associated macrophage protein 1 gene (NRAMP1) are associated with severe forms of pulmonary tuberculosis," *Clinical Infectious Diseases*, vol. 40, no. 9, pp. 1232–1236, 2005.

[28] I. D'Souza, P. Poorkaj, M. Hong et al., "Missense and silent tau gene mutations cause frontotemporal dementia with parkinsonism-chromosome 17 type, by affecting multiple alternative RNA splicing regulatory elements," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 10, pp. 5598–5603, 1999.

[29] C. L. Lorson, E. Hahnen, E. J. Androphy, and B. Wirth, "A single nucleotide in the SMN gene regulates splicing and is responsible for spinal muscular atrophy," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 11, pp. 6307–6311, 1999.

[30] J. V. Chamary and L. D. Hurst, "The price of silent mutations," *Scientific American*, vol. 300, no. 6, pp. 46–53, 2009.

[31] Y. Lee and D. C. Rio, "Mechanisms and regulation of alternative Pre-mRNA splicing," *Annual Review of Biochemistry*, vol. 84, pp. 291–323, 2015.

[32] Z. E. Sauna and C. Kimchi-Sarfaty, "Synonymous mutations as a cause of human genetic disease," in *eLS*, John Wiley & Sons, Chichester, UK, 2013.

[33] L. Cartegni, S. L. Chew, and A. R. Krainer, "Listening to silence and understanding nonsense: exonic mutations that affect splicing," *Nature Reviews Genetics*, vol. 3, no. 4, pp. 285–298, 2002.

[34] R. C. Hunt, V. L. Simhadri, M. Iandoli, Z. E. Sauna, and C. Kimchi-Sarfaty, "Exposing synonymous mutations," *Trends in Genetics*, vol. 30, no. 7, pp. 308–321, 2014.

[35] J. Jin, L. Sun, W. Jiao et al., "SLC11A1 (formerly *NRAMP1*) gene polymorphisms associated with pediatric tuberculosis in China," *Clinical Infectious Diseases*, vol. 48, no. 6, pp. 733–738, 2009.

[36] P. M. Altrock, A. Traulsen, and F. A. Reed, "Stability properties of underdominance in finite subdivided populations," *PLoS Computational Biology*, vol. 7, no. 11, article e1002260, 2011.

[37] S. J. Meisner, S. Mucklow, G. Warner, S. O. Sow, C. Lienhardt, and A. V. S. Hill, "Association of NRAMP1 polymorphism with leprosy type but not susceptibility to leprosy per se in west Africans," *The American Journal of Tropical Medicine and Hygiene*, vol. 65, no. 6, pp. 733–735, 2001.

[38] M. Wessling-Resnick, "Nramp1 and other transporters involved in metal withholding during infection," *The Journal of Biological Chemistry*, vol. 290, no. 31, pp. 18984–18990, 2015.

[39] A. Vargas-Gonzalez, S. B. Canto-Lara, A. G. Damian-Centeno, and F. J. Andrade-Narvaez, "Response of cutaneous leishmaniasis (chiclero's ulcer) to treatment with meglumine antimoniate in southeast Mexico," *American Journal of Tropical Medicine and Hygiene*, vol. 61, no. 6, pp. 960–963, 1999.

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*Research Article*

# Epidemiology of American Tegumentary Leishmaniasis and *Trypanosoma cruzi* Infection in the Northwestern Argentina

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*Background*. Endemic areas of tegumentary leishmaniasis (TL) in Salta, Argentina, present some overlap zones with the geographical distribution of Chagas disease, with mixed infection cases being often detected. *Objectives*. The purpose of this study was to determine the magnitude of *Leishmania* sp. infection and potential associated risk factors, the serologic prevalence of *T. cruzi,* and the presence of *T. cruzi-Leishmania* sp. mixed infection in a region of the northwest of Argentina. *Methods*. Cross-sectional studies were conducted to detect TL prevalence and *T. cruzi* seroprevalence. A case-control study was conducted to examine leishmaniasis risk factors. *Results*. Prevalence of TL was 0.17%, seroprevalence of *T. cruzi* infection was 9.73%, and mixed infection proportion—within the leishmaniasic patients group—was 16.67%. The risk factors associated with TL transmission were sex, age, exposure to bites at work, staying outdoors more than 10 hours/day, bathing in the river, and living with people who had lesions or were infected during the study. *Discussion*. The endemic pattern of TL seems to involve exposure of patients to vectors in wild as well as peridomestic environment. Cases of *T. cruzi* infection are apparently due to migration. Therefore, a careful epidemiological surveillance is necessary due to the contraindication of antimonial administration to chagasic patients.

## 1. Introduction

Tegumentary leishmaniasis (TL) is caused by protozoan parasites belonging to the genus *Leishmania*. Clinical manifestations of this disease include single, multiple, disseminated cutaneous, and mucocutaneous forms [1]. The species *Leishmania (Viannia) braziliensis*, *L. (V.) guyanensis*, *L. (L.) amazonensis,* and *L. (V.) panamensis* have been reported from northern Argentina [2–4], although the vast majority is caused by *L. (V.) braziliensis*. In this region, *Leishmania* spp. are transmitted by the bite of the female sandfly belonging to the genus *Lutzomyia* (Diptera, Psychodidae, and Phlebotominae). *Migonemyia migonei* (França 1920), *Evandromyia cortelezzii* (Brèthes 1923), *Evandromyia sallesi* (Galvão & Coutinho 1940), *Psathyromyia shannoni* (Dyar 1929), *Psathyromyia punctigeniculata* (Floch & Abonnenc, 1944), and *Nyssomyia neivai* (Pinto 1926) have been reported in northern Argentina [5, 6]; and particularly *Ny. neivai*, *Mg. migonei,* and *cortelezzii* complex have been proposed as potential vectors [7, 8].

The described scenarios of TL transmission in Argentina include four cycles' patterns: (a) wild cycle with transmission in primary or residual vegetation, (b) with eventual peridomestic transmission due to wild or secondary vegetation

changes, (c) with peridomestic transmission in contiguous domiciles with the residual vegetation, and (d) peridomestic cycle in rural, ruralized periurban, or urban-rural interface environment [9]. However, the potential existence of urban transmission has been reported, which represents an important change in the transmission pattern paradigm of this disease at the regional scale [10]. Oran and San Martin departments (Salta province) are the areas with the greatest risk of transmission in the country, which reported the highest number of cases to the overall TL incidence in Argentina [10, 11].

In several areas of Latin America (including northern Argentina) the geographical distribution of TL overlaps with transmission areas of American trypanosomiasis (Chagas disease). The World Health Organization estimates that 8 to 10 million people are infected worldwide, mostly in Latin America where the disease is endemic [12]; this is caused by *Trypanosoma cruzi* and it is transmitted by several species of triatomine insects, *Triatoma infestans* being the most important in Argentina. In the last century, the progressive urbanization and the intensive migration of infected individuals increased the risk of transmission by blood transfusion and congenital route in nonendemic regions [13]. In restricted areas located in the east and northeast of Salta province, corresponding to the Gran Chaco ecoregion, vectorial transmission of *T. cruzi* still occurs but not in the rain forest ecoregion (Yunga ecoregion).

Mixed infections due to *Leishmania sp.* and *T cruzi* have been reported in patients showing clinical symptoms of TL, ranging between 12% and 70% [2, 14–16]. Prevalence of *Leishmania sp.* and *T. cruzi* mixed infection is unknown for northern Salta. Cross-reactivity between *T. cruzi* and *Leishmania* sp. infections has been reported when some serological tests were evaluated [2, 16, 17], possibly due to the close phylogenetic relationship between these parasites. The occurrence of *Leishmania* sp. and *T. cruzi* mixed infections also has therapeutic implications. Antimonial drugs used to treat leishmaniasis have potential cardiac toxicity [18–20], which is an important concern in patients infected by *T. cruzi* because about 30% of people infected by this parasite develop chronic cardiomyopathy [21].

In the present study, we examined the prevalence of single infections by *Leishmania* sp. and *T. cruzi* as well as the proportion of mixed infections due to both parasites in people living in the northern of Argentina. Demographic, behavioral, and environmental variables have also been studied as potential risk factors associated with transmission of cutaneous leishmaniasis. The epidemiologic pattern here observed can occur in several countries in Latin America and this research can provide information to optimize global and local prevention measures of public health.

## 2. Materials and Methods

*2.1. Area and Population Studied.* Hipólito Yrigoyen (Orán Department, Salta, Argentina) is located at 23°14′S, 64°16′W, 323 m.a.s.l. in large-scale farming and forest exploitation area, with subtropical climate. It is within the perimeter of the Pedemontana rain forest. This urban place has a population of 10 363 inhabitants (Primary Health Care Record, 2009). The study population included people who lived in Hipólito Yrigoyen between 2001 and 2011.

*2.2. Study Design.* This research included two cross-sectional studies, a tegumentary leishmaniasis case-control study, and the report of mixed infection for *Leishmania* sp. and *T. cruzi*.

A cross-sectional study was conducted in 2009 to determine the prevalence of *Leishmania* sp. (LP) taking into account the active cases of TL (ACTL) and total population of Hipólito Yrigoyen (HYTP: population censed by the PHC):

$$\mathrm{LP} = \frac{\mathrm{ACTL}}{\mathrm{HYTP}} \times 100 = \frac{\mathrm{ACTL}}{10363} \times 100. \quad (1)$$

The seroprevalence of *T. cruzi* infection (TCSP) corresponding to 2009 was also calculated through a cross-sectional study. The sample size was 113 people. It was calculated considering an expected prevalence 5% with 4% of accuracy and confidence level of 95% using the Epidat software v3.1 (Epidat Xunta de Galicia, Santiago de Compostela, Spain and Pan-American Health Organization, Washington, DC). Seventy-nine quasi-randomly selected households were studied (Figure 1(b)). Of all household members, only those who wanted to participate voluntarily were selected (one person or more per household). The seroprevalence (TCSP) was calculated as the number of seropositive people for *T. cruzi* infection (TCP) over the sample size (SS):

$$\mathrm{TCSP} = \frac{\mathrm{TCP}}{\mathrm{SS}} \times 100 = \frac{\mathrm{TCP}}{113} \times 100. \quad (2)$$

Also, the mixed infection proportion within group of patients with TL in 2009 was calculated (Figure 2).

An unmatched case-control study was conducted to identify risk factors associated with TL cases. The TL cases included in the case-control study were patients living in Hipólito Yrigoyen and diagnosed between 2001 and 2009. The control population was selected within the cross-sectional sample used to calculate the *T. cruzi* seroprevalence infection (Figure 2). A survey was conducted in both cases and on the control people through a structured epidemiological questionnaire. After each person was interviewed, 6 mL of venous blood was drawn by clinical laboratory technicians and allowed to clot at room temperature. The sera were obtained by centrifugation at 3500 rotations per min for 5 min, then aliquoted into 1.5 mL tubes, and stored at −20°C until tested.

The variables registered were age, sex, occupation (exposure to vector bites at work), recreational habits (staying outdoors for more than 10 h and bathing in the river), personal preventive measures, household data (i.e., location, construction material, proximity to sites of possible development of sandflies, distance from crop fields and primary vegetation, knowledge about and application of preventive measures), living with people who were infected or had lesions, and aspects indicating knowledge about TL [22–24]. The data from the questionnaire were managed using the application EpiData Entry version 3.1 [25]; the resulting database was exported to the R statistical software for respective analysis.

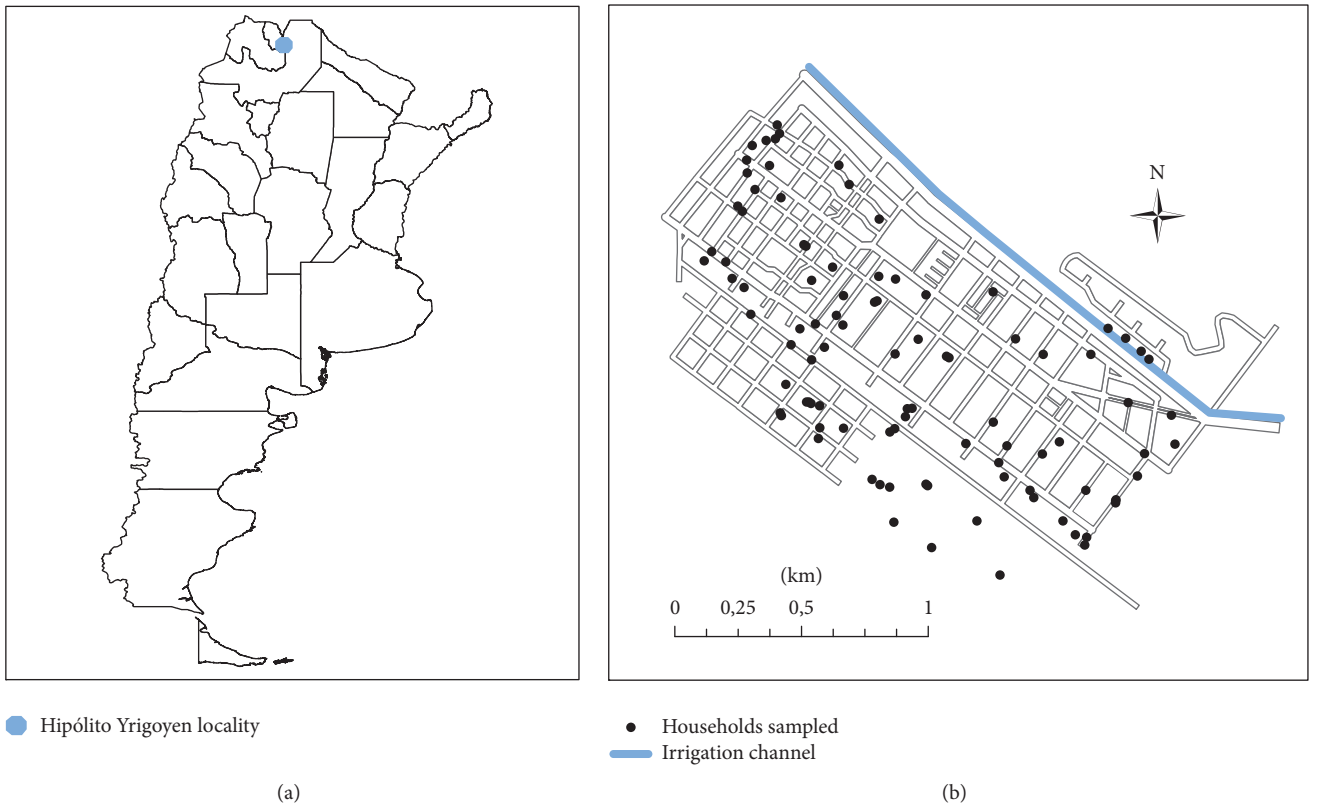


Figure 1: (a) HY within Argentina. (b) The black dots indicate households' distribution, where the sampling was conducted in the HY locality.

The people included in each study group mentioned above were defined according to the diagnostic criteria described below.

*2.3. Diagnostic Procedures.* The patients were evaluated in the field by the personnel at Instituto de Investigaciones en Enfermedades Tropicales (IIET) at Universidad Nacional de Salta, the San Vicente de Paul Hospital in Orán, and Eva Peron Hospital in Hipólito Yrigoyen.

The sera and blood samples collected in the field were transported to the IIET for processing. The parasitological diagnostic of *Leishmania* was made in the IIET and the patients were derived from Hipólito Yrigoyen Hospital. Both diagnostic procedures such as serological, parasitological, and molecular techniques were performed following the protocols established in previous studies. The commercial kits were applied according to manufacturer's instructions.

*2.4. Diagnostic Criteria.* Diagnostic criteria included the following.

*2.4.1. Leishmaniasis Cases.* Included were individuals who had lesions clinically compatible with TL and visualization of amastigotes of *Leishmania* sp. in Giemsa-stained smears and/or positive reaction of serum samples by enzyme-linked immunosorbent assay (ELISAg) homogenate protein of *L (V.) guyanensis* [15] and/or positive reaction to leishmanin skin test [4, 5].

*2.4.2. T. cruzi Infection.* The subjects were considered infected with *T. cruzi* when serum samples by ELISA and Indirect hemagglutination (IHA) tests (Wiener Lab, Argentina) were reactive. The samples with discordant results between ELISA and IHA were examined by recombinant ELISA 3.0 (Wiener Lab, Argentina) and Immunofluorescence test [26] or Polymerase Chain Reaction (PCR) [27]. The recombinant ELISA 3.0 was reported as specific test for *T. cruzi* infection detection without cross-reaction with Leishmania [17, 28].

*2.4.3. Mixed Infections.* Included were patients with TL and positive results for at least two tests for *T. cruzi* infection, mentioned above.

*2.4.4. Controls in Case-Control Study.* Included were individuals living in Hipólito Yrigoyen who were not grouped as leishmaniasis cases and/or *T. cruzi* infected.

*2.5. Data Analysis.* The prevalence of *Leishmania* sp., seroprevalence of *T. cruzi* infections, and mixed infection proportion with 95% Confidence Intervals (CI) were calculated using the EPIDAT software version 3.1.

In the case-control study, the independent continuous and discreet variables were, respectively, categorized or dichotomized. Univariate and multivariate logistic regression (LR) analysis were carried out. The Odds Ratios (OR) and 95% CI were calculated to assess the link between the TL cases and potential risk factors. The variables with OR > 1

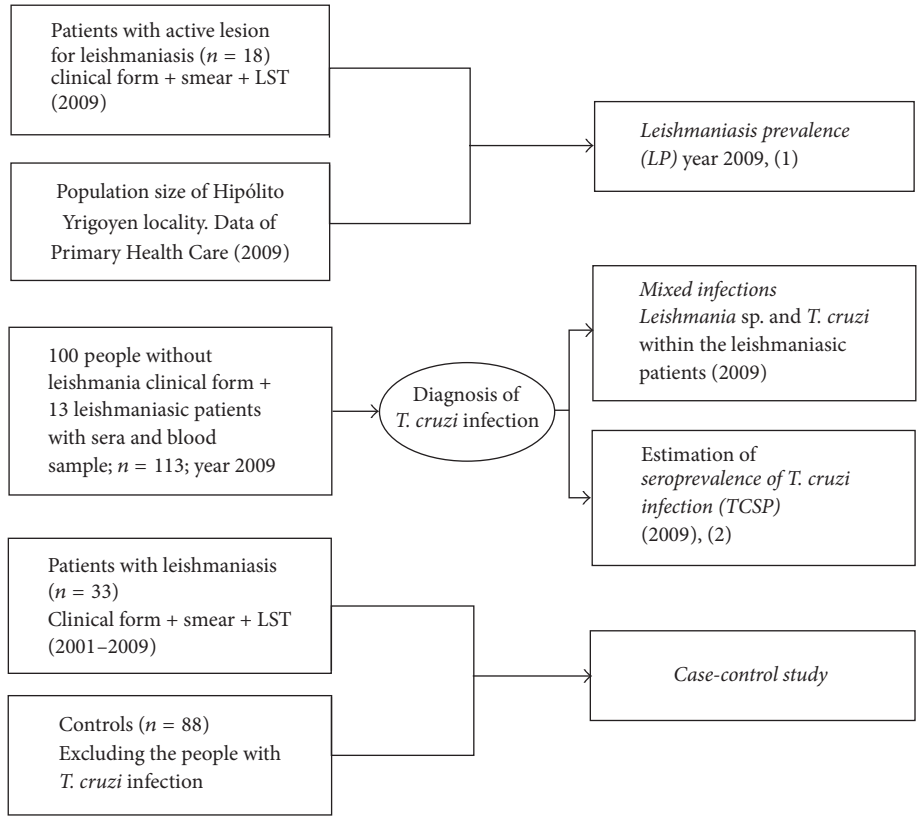


Figure 2: Flow chart showing the groups of patients and noninfected people included in prevalence (LP) and case-control study of leishmaniasis and *T. cruzi* seroprevalence (TCSP) infection. Mixed infections detected (*T. cruzi-Leishmania*) from 13 patients with active leishmaniasis ulcer in 2009 are also shown. Diagnostic of *T. cruzi* infection applied to the 100 people to detect both seropositive people to calculate *T. cruzi* seroprevalence and select the negative control people for the case-control study. People positive for ELISA-leishmaniasis or leishmanin skin test (LST) may have been exposed to the *Leishmania* parasite. The cross-reaction of both ELISA and leishmanin skin test with chagasic infection does not distinguish whether the person was exposed to *Leishmania* parasite or is infected with *T. cruzi*. Due to this, the people positive for Chagas lab tests were not included as controls in the case-control study.

and $p < 0.05$ in univariate logistic regression analysis were tested in a multivariate analysis to establish a model involving the least number of variables that best explains the dependent variable (TL cases).

The final model was obtained using the stepwise technique, a procedure that combines forward method (it starts from a model only with the constant or independent variable, followed by progressive introduction of variables in the equation, provided they are significant) and backward method (all the variables are initially considered in the model, and those lacking significance are then progressively eliminated) [29]. The Akaike Information Criterion (AIC) was used as selection criterion. AIC calculation is based on minimization of the loss of information function, penalizing for the number of variables introduced that seeks the model that best adjusts to the data with the minimum number of possible variables, thus producing simpler models [30, 31]. The model that has been chosen minimizes the AIC. Data were considered statistically significant if $p < 0.05$. All statistical analysis for case-control study was performed using R software version 2.15.2 [32].

*2.6. Ethical Approval.* All the people included in the study agreed to participate by signing an informed consent form (ICF). The project and ICF were approved by the Ethics Committee of the School of Health Sciences at the National University of Salta and the "Fundación Huesped."

## 3. Results

In 2009, only 18 cases of TL were diagnosed in Hipólito Yrigoyen, which represented a TL prevalence of 0.17% (CI 0.09–0.26). The age range of study patients was 7–69 years with an average of 35.45 ± 16.69 (SD).

Of the 113 samples analyzed to detect *T. cruzi* infection, 67 (59%) corresponded to females and 46 (41%) to males. Their age ranged between 7 and 74, with an average of 37.5 ± 17.3 (SD) years. There was no statistically significant difference in prevalence between males and females ($p = 0.73$). The seroprevalence for this infection was 9.73% (CI 3.83–15.64) in 2009. The frequencies of cases according to age and sex are summarized in Table 1.

Table 1: Number of individuals included in the study, percentage (%) of individuals infected with *T. cruzi* according to sex and age group. [a]Indication of the overall prevalence in Hipólito Yrigoyen for 2009. *i*: infected individuals according to sex and age; *n*: total sample in seroprevalence of *T. cruzi* study.

| Sex | Female *i*/*n* (%) | Male *i*/*n* (%) |
|---|---|---|
| Total infected | 5/67 (7) | 6/46 (13) |
| Age | *n* | Infected (%) |
| 10–35 | 62 | 4 (6.45) |
| 36–55 | 28 | 3 (10.7) |
| >55 | 23 | 4 (10.4) |
| Total | **113** | **11 (9.73)**[a] |

Positive results were obtained for *T. cruzi* infection in three patients with TL in 2009 (Table 2), which represents a mixed infection proportion of 16.67% CI (3.58–41.42) within the group of 18 patients with TL.

The samples for the case-control study included 33 cases of TL (18 actives cases in 2009 and 15 cases registered in the 2001–2008 period) and 88 controls (Figure 2). The control group consisted of individuals without positive diagnosis for leishmaniasis and Chagas diseases according to criteria above described.

The variables that showed significant association ($p < 0.05$) with the presence of TL (*case*) in univariate LR analysis were *sex*, *age*, exposure to vector bites at work, staying outdoors for more than 10 h (*so*), bathing in the river, and living with people who were infected or had lesions during the study period (Table 3). These variables were included in a multivariate logistic regression analysis and final model obtained using the stepwise method. This model includes only 3 predictive variables (*sex*, *age,* and *so*) that explained the occurrence of TL cases (Table 3). The AIC value was 122.2

$$\text{The selected model: } CASE = -2.82 + 1.80 * SEX + 2.04 * AGE + 1.42 * SO.$$

## 4. Discussion

In the north of Salta province, TL levels are hyperendemic in some sites and periods [33, 34]. This situation is worsened by the presence of cases of Chagas disease, which may further generate cases of mixed infections, causing a synergistic problem for the health care system. In the locality Hipólito Yrigoyen, TL prevalence values of 0.17, 0.79, and 0.18% were previously reported [33, 34]. The prevalence calculated in the present study (0.17%; CI 0.08–0.26) is similar, which indicates a level of active transmission that persists over time. However, high incidence foci are likely to occur in short exposure periods in this area [5].

In the case-control study, the associated variables would be reflecting the existence of a complex pattern of transmission. Male sex and staying outdoors for more than 10 hours would be indicators of a sylvatic mode of transmission, facilitated by labour, subsistence, or recreational activities (in rural environments and/or deforestation areas, or hunting and fishing activities), as it is indicated in regions where TL is endemic [9, 24, 33]. The significantly higher proportion of infected children compared to that of adults found in this analysis (OR = 7.73; CI: 2.05–29.16) suggests the existence of other patterns of transmission in Hipólito Yrigoyen. The incidence of TL in children has been cited as an indicator of peridomiciliary transmission, especially in localities adjacent to primary and/or secondary vegetation [4, 22–24, 33].

In addition, a high density of sandflies has been detected in the vegetation near to the irrigation channels located in the outskirts of the city (Figure 1) [35], showing a species diversity similar to that found in a nearby place where there was a high rate of infection [5, 36]. Many families go to these sites for recreational purposes in times of high temperature and risk leishmaniasis transmission (7 pm to 10 pm approximately) with the consequent risk of being bitten by infected sandflies and contracting leishmaniasis, as reported in a study of spatial distribution of TL cases [37].

The presence of active TL cases among elderly people that remain mostly in their houses and of sandflies in the center of the town [35] offers another plausible epidemiologic situation of disease transmission (but with low probability) in urban environments because lower abundance of sandflies was recorded here [35]. Indeed, in Hipólito Yrigoyen, house courtyards have vegetation patches that can be colonized by sandflies from the periphery, according to the characteristics of metapopulation dynamics [10].

On the other hand, in the study area, the possibility of vector-borne transmission of *T. cruzi* has been discarded, because no insects or indicators of their presence have been found in the annual activities of entomological surveillance of triatomines carried out by Primary Health Care System in recent and historical monitoring. Thus, cases of *T. cruzi* infection in Hipólito Yrigoyen would be associated with migratory processes (movement principally of rural populations from Argentina and Bolivia of the Gran Chaco ecoregion where Chagas disease is endemic) [13]. The *T. cruzi* seroprevalence found in this work is low compared with the prevalence value observed in rural populations of endemic areas (25%) [38], and it is high compared with other regions without endemic transmission [39]. In turn, infected children may have acquired infection by congenital transmission, as reported in previous studies of this type of transmission in the province of Salta [40].

The proportion of *T. cruzi-Leishmania* sp. mixed infection within the group of patients with TL reported in the north of Salta reaches 30 and 40% [2, 14, 15] and does not show differences from the percentage obtained in this study. Knowing the level of this condition in the population allows us to explore the factors involved in the origin and persistence of mixed infections. In addition, because antimonials are cardiotoxic, a careful diagnosis and implementation of alternative treatments are needed to avoid further complications.

The complex situation in Hipólito Yrigoyen in reference to TL is aggravated by the coexistence of *T. cruzi* infection. The transmission pattern involves mainly natural areas, but the possibility of a peridomiciliary transmission in the outskirts of the city cannot be ruled out. This situation

Table 2: Results of samples with mixed infection. SMEAR: visualization of amastigotes of *Leishmania* sp. in Giemsa-stained smears; ELISAg: ELISA based on homogenate protein of *L (V.) guyanensis*; LST: reaction to leishmanin skin test; ELISA Rec: recombinant ELISA Kit; IHA: indirect hemagglutination; TIF: test of Immunofluorescence; PCR: Polymerase Chain Reaction. nd: no data (samples were not evaluated with TIF).

| | | *Leishmania* infection tests | | | *T. cruzi* infection tests | | | |
|---|---|---|---|---|---|---|---|---|
| | | Smear | ELISAg | LST | ELISA Rec. | IHA | TIF | PCR |
| Patient code | HI 54 | + | + | + | + | + | + | − |
| | HI 55 | + | + | + | − | + | nd | + |
| | HI 58 | + | + | + | + | + | nd | + |

Table 3: Crude OR and adjusted OR for the factors associated with the presence of tegumentary leishmaniasis. OR, Odds Ratio; CI, Confidence Interval. [a]Significant association at $p < 0.05$; [b]highly significant association at $p < 0.01$; [c]highly significant association at $p < 0.001$.

| Logistic regression | OR (CI 95%) univariate analysis | OR (CI 95%) multivariate analysis |
|---|---|---|
| *Sex* | | |
| Female | 1 | 1 |
| Male | 5.47 (2.21–13.54)[a] | 6.05 (2.2–16.64)[c] |
| *Age (years)* | | |
| >16 | 1 | 1 |
| 7–15 | 4.37 (1.39–13.8)[b] | 7.73 (2.05–29.16)[b] |
| *Exposure to vector bites at work* | | |
| Unexposed | 1 | |
| Exposed | 2.68 (1.14–6.3)[a] | |
| *Permanence > 10 hours outdoors (SO)* | | |
| No | 1 | 1 |
| Yes | 4.67 (1.23–17.78)[a] | 4.16 (0.97–17.77)[a] |
| *Bathing in the river* | | |
| No | 1 | |
| Yes | 3.89 (1.59–9.54)[a] | |
| *Living with infected people* | | |
| No | 1 | |
| Yes | 3.17 (1.23–8.15)[a] | |

demands the involvement of different stakeholders to control the magnitude of disease incidence, implementing prevention strategies and taking into account biogeographical and sociocultural characteristics, as well as human-induced environmental changes and situations that pose a risk.

The present work provides epidemiological information of potential determinants of TL occurrence in Hipólito Yrigoyen, its magnitude, and situation of *T. cruzi* infection in the same area. This information is useful for the local health system because it may contribute to a better planning of the surveillance systems and to the design of prevention strategies in the area. These epidemiological patterns of mixed infections can occur in other countries were *T. cruzi* transmission does not exist or was interrupted and tegumentary leishmaniasis is endemic.

## Competing Interests

The authors declare that they have no competing interests.

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## References

[1] A. J. Krolewiecki, H. D. Romero, S. P. Cajal et al., "A randomized clinical trial comparing oral azithromycin and meglumine antimoniate for the treatment of American cutaneous leishmaniasis caused by *Leishmania (Viannia) braziliensis*," *The American Journal of Tropical Medicine and Hygiene*, vol. 77, no. 4, pp. 640–646, 2007.

[2] F. M. Frank, M. M. Fernández, N. J. Taranto et al., "Characterization of human infection by *Leishmania* spp. in the Northwest of Argentina: immune response, double infection with *Trypanosoma cruzi* and species of *Leishmania* involveds," *Parasitology*, vol. 126, no. 1, pp. 31–39, 2003.

[3] J. D. Marco, P. A. Barroso, T. Mimori et al., "Polymorphism-specific PCR enhances the diagnostic performance of American tegumentary leishmaniasis and allows the rapid identification of *Leishmania* species from Argentina," *BMC Infectious Diseases*, vol. 12, article 191, 2012.

[4] F. M. Locatelli, S. P. Cajal, P. A. Barroso et al., "The isolation and molecular characterization of *Leishmania* spp. from patients with American tegumentary leishmaniasis in northwest Argentina," *Acta Tropica*, vol. 131, no. 1, pp. 16–21, 2014.

[5] A. J. Krolewiecki, J. F. Gil, M. Quipildor et al., "Restricted outbreak of American tegumentary leishmaniasis with high microfocal transmission," *American Journal of Tropical Medicine and Hygiene*, vol. 88, no. 3, pp. 578–582, 2013.

[6] O. D. Salomón, M. L. Wilson, L. E. Munstermann, and B. L. Travi, "Spatial and temporal patterns of phlebotomine sand flies (Diptera: Psychodidae) in a cutaneous leishmaniasis focus in northern Argentina," *Journal of Medical Entomology*, vol. 41, no. 1, pp. 33–39, 2004.

[7] D. de Pita-Pereira, C. R. Alves, M. B. Souza et al., "Identification of naturally infected *Lutzomyia intermedia* and *Lutzomyia migonei* with *Leishmania (Viannia) braziliensis* in Rio de Janeiro

(Brazil) revealed by a PCR multiplex non-isotopic hybridisation assay," *Transactions of the Royal Society of Tropical Medicine and Hygiene*, vol. 99, no. 12, pp. 905–913, 2005.

[8] E. Córdoba-Lanús, M. L. De Grosso, J. E. Piñero, B. Valladares, and O. D. Salomón, "Natural infection of *Lutzomyia neivai* with *Leishmania* spp. in northwestern Argentina," *Acta Tropica*, vol. 98, no. 1, pp. 1–5, 2006.

[9] O. D. Salomón, P. W. Orellano, M. G. Quintana et al., "Transmission of tegumentary leishmaniasis in Argentina," *Medicina*, vol. 66, no. 3, pp. 211–219, 2006.

[10] J. F. Gil, J. R. Nasser, S. P. Cajal et al., "Urban transmission of american cutaneous leishmaniasis in Argentina: spatial analysis study," *The American Journal of Tropical Medicine and Hygiene*, vol. 82, no. 3, pp. 433–440, 2010.

[11] O. D. Salomon, J. R. Rosa, M. Stein et al., "Phlebotominae (Diptera: Psycodidae) fauna in the Chaco region and Cutaneous Leishmaniasis transmission patterns in Argentina," *Memórias do Instituto Oswaldo Cruz*, vol. 103, no. 6, pp. 578–584, 2008.

[12] World Health Organization, "Research priorities for chagas disease, human african trypanosomiasis and leishmaniasis," WHO Technical Report Series 975, World Health Organization, Geneva, Switzerland, 2013.

[13] J. C. P. Dias, "Human chagas disease and migration in the context of globalization: some particular aspects," *Journal of Tropical Medicine*, vol. 2013, Article ID 789758, 9 pages, 2013.

[14] M. G. Chiaramonte, F. M. Frank, G. M. Furer, N. J. Taranto, R. A. Margni, and E. L. Malchiodi, "Polymerase chain reaction reveals *Trypanosoma cruzi* infection suspected by serology in cutaneous and mucocutaneous leishmaniasis patients," *Acta Tropica*, vol. 72, no. 3, pp. 295–308, 1999.

[15] J. F. Gil, C. L. Hoyos, R. O. Cimino et al., "Role of three ELISA tests using promastigote homogenates of *Leishmania braziliensis, L. amazonensis* and *L. guyanensis* in the diagnosis of tegumentary leishmaniasis," *Medicina*, vol. 71, no. 5, pp. 420–428, 2011.

[16] A. F. Vega Benedetti, R. O. Cimino, P. S. Cajal et al., "Performance of different *Trypanosoma cruzi* antigens in the diagnosis of Chagas disease in patients with American cutaneous leishmaniasis from a co-endemic region in Argentina," *Tropical Medicine & International Health*, vol. 18, no. 9, pp. 1103–1109, 2013.

[17] J. F. Gil, R. O. Cimino, I. Lopez Quiroga et al., "Reactivity of GST-SAPA antigen of *Trypanosoma cruzi* against sera from patients with Chagas disease and leishmaniasis," *Medicina*, vol. 71, no. 2, pp. 113–119, 2011.

[18] S. D. Lawn, M. Armstrong, D. Chilton, and C. J. M. Whitty, "Electrocardiographic and biochemical adverse effects of sodium stibogluconate during treatment of cutaneous and mucosal leishmaniasis among returned travellers," *Transactions of the Royal Society of Tropical Medicine and Hygiene*, vol. 100, no. 3, pp. 264–269, 2006.

[19] L. F. Oliveira, A. O. Schubach, M. M. Martins et al., "Systematic review of the adverse effects of cutaneous leishmaniasis treatment in the New World," *Acta Tropica*, vol. 118, no. 2, pp. 87–96, 2011.

[20] L. F. Gamboa Latorre and J. A. Becerra Mateus, "Cardiotoxicidad por antimoniales," *Revista Salud Bosque*, vol. 2, no. 2, pp. 69–74, 2012.

[21] L. H. Malik, G. D. Singh, and E. A. Amsterdam, "The epidemiology, clinical manifestations, and management of chagas heart disease," *Clinical Cardiology*, vol. 38, no. 9, pp. 565–569, 2015.

[22] S. Sosa-Estani, E. L. Segura, A. Gomez et al., "Cutaneous leishmaniasis in Northern Argentina: identification of risk factors in a case-cohort study of three municipalities in Salta," *Revista da Sociedade Brasileira de Medicina Tropical*, vol. 34, no. 6, pp. 511–517, 2001.

[23] Z. E. Yadon, L. C. Rodrigues, C. R. Davies, and M. A. Quigley, "Indoor and peridomestic transmission of American cutaneous leishmaniasis in northwestern Argentina: a retrospective case-control study," *American Journal of Tropical Medicine and Hygiene*, vol. 68, no. 5, pp. 519–526, 2003.

[24] F. D. A. Pedrosa and R. A. D. A. Ximenes, "Sociodemographic and environmental risk factors for American cutaneous leishmaniasis (ACL) in the State of Alagoas, Brazil," *American Journal of Tropical Medicine and Hygiene*, vol. 81, no. 2, pp. 195–201, 2009.

[25] J. M. Lauritsen and M. Bruus, *EpiData Entry, Version 3.1. A Comprehensive Tool for Validated Entry and Documentation of Data*, The EpiData Association, Odense, Denmark, 2005.

[26] M. Alvarez, J. A. Cerisola, and R. W. Rohwedder, "Immunofluorescence test in the diagnosis of Chagas' diseases," *Boletín Chileno de Parasitología*, vol. 23, no. 1, pp. 4–8, 1968.

[27] C. Britto, M. A. Cardoso, C. M. Monteiro Vanni et al., "Polymerase chain reaction detection of *Trypanosoma cruzi* in human blood samples as a tool for diagnosis and treatment evaluation," *Parasitology*, vol. 110, no. 3, pp. 241–247, 1995.

[28] Z. C. Caballero, O. E. Sousa, W. P. Marques, A. Saez-Alquezar, and E. S. Umezawa, "Evaluation of serological tests to identify *Trypanosoma cruzi* infection in humans and determine cross-reactivity with *Trypanosoma rangeli* and *Leishmania* spp.," *Clinical and Vaccine Immunology*, vol. 14, no. 8, pp. 1045–1049, 2007.

[29] S. Domínguez-Almendros, N. Benítez-Parejo, and A. R. Gonzalez-Ramirez, "Logistic regression models," *Allergologia et Immunopathologia*, vol. 39, no. 5, pp. 295–305, 2011.

[30] V. Chongsuvivatwong, *Analysis of Epidemiological Data Using R and Epicalc*, Epidemiology Unit, Prince of Songkla University, Songkhla, Thailand, 2008.

[31] M. M. Rodríguez del Águila and N. Benítez-Parejo, "Simple linear and multivariate regression models," *Allergologia et Immunopathologia*, vol. 39, no. 3, pp. 159–173, 2011.

[32] R Core Team, "R: A language and environment for statistical computing," 2012, R Foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org/.

[33] S. Sosa-Estani, E. L. Segura, O. D. Salomón et al., "Tegumentary leishmaniasis in Northern Argentina: distribution of infection and disease, in three municipalities of Salta, 1990–1992," *Revista da Sociedade Brasileira de Medicina Tropical*, vol. 33, no. 6, pp. 573–582, 2000.

[34] O. D. Salomón, S. S. Estani, L. Canini, and E. C. Lanus, "Tegumentary leishmaniasis in an area with epidemic levels of transmission, Salta, Argentina, 1998," *Medicina B: Aires*, vol. 61, no. 3, pp. 284–290, 2001.

[35] M. M. Chanampa, *Monitoring sandflies of Lutzomyia genus and associated environmental variables, in the town of Hipólito Yrigoyen [M.S. Biologist thesis]*, Biology School. Facultad de Ciencias Naturales de la Universidad Nacional de Salta, Salta, Argentina, 2012.

[36] M. G. Quintana, O. D. Salomon, and M. S. De Grosso, "Distribution of phlebotomine sand flies (Diptera: Psychodidae) in a primary forest-crop interface, Salta, Argentina," *Journal of Medical Entomology*, vol. 47, pp. 1003–1010, 2010.

[37] J. C. Rosales, H. M. Yang, A. Ibarra, and R. Barraza, "Análise da distribuição espacial dos casos de leishmaniose tegumentar americana em Hipólito Yrigoyen, Orán, Salta, Argentina, no período 2005-2006," *Boletim Epidemiológico Paulista*, vol. 5, pp. 4–9, 2008.

[38] P. Diosque, A. M. Padilla, R. O. Cimino et al., "Chagas disease in rural areas of Chaco Province, Argentina: epidemiologic survey in humans, reservoirs, and vectors," *The American Journal of Tropical Medicine and Hygiene*, vol. 71, no. 5, pp. 590–593, 2004.

[39] N. Klein, I. Hurwitz, and R. Durvasula, "Globalization of Chagas disease: a growing concern in nonendemic countries," *Epidemiology Research International*, vol. 2012, Article ID 136793, 13 pages, 2012.

[40] O. S. Negrette, M. C. Mora, and M. Á. Basombrío, "High prevalence of congenital *Trypanosoma cruzi* infection and family clustering in Salta, Argentina," *Pediatrics*, vol. 115, no. 6, pp. e668–e672, 2005.

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*Review Article*

# Differences in *Rhodococcus equi* Infections Based on Immune Status and Antibiotic Susceptibility of Clinical Isolates in a Case Series of 12 Patients and Cases in the Literature

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*Rhodococcus equi* is an unusual zoonotic pathogen that can cause life-threatening diseases in susceptible hosts. Twelve patients with *R. equi* infection in Kentucky were compared to 137 cases reported in the literature. Although lungs were the primary sites of infection in immunocompromised patients, extrapulmonary involvement only was more common in immunocompetent patients ($P < 0.0001$). Mortality in *R. equi*-infected HIV patients was lower in the HAART era (8%) than in pre-HAART era (56%) ($P < 0.0001$), suggesting that HAART improves prognosis in these patients. Most (85–100%) of clinical isolates were susceptible to vancomycin, clarithromycin, rifampin, aminoglycosides, ciprofloxacin, and imipenem. Interestingly, there was a marked difference in susceptibility of the isolates to cotrimoxazole between Europe (35/76) and the US (15/15) ($P < 0.0001$). Empiric treatment of *R. equi* infection should include a combination of two antibiotics, preferably selected from vancomycin, imipenem, clarithromycin/azithromycin, ciprofloxacin, rifampin, or cotrimoxazole. Local antibiograms should be checked prior to using cotrimoxazole due to developing resistance.

## 1. Introduction

*Rhodococci* are aerobic, Gram positive, pleomorphic, and nonmotile bacteria, which can be detected in soil and grow well on simple nutrients provided by herbivore manure. They also grow well in the intestines of grazing animals. Among the organisms making up the genus *Rhodococcus*, *R. equi* is the most common isolate and has been well recognized as an important pathogen in veterinary medicine. It can cause bronchopneumonia, especially in foals [1]. *R. equi* was first isolated from foals with bronchopneumonia in 1923 [2]. The genus *Rhodococcus* is now known to be distinct from the other closely related species of acid-fast or modified acid-fast organisms of the genera *Gordonia*, *Nocardia,* and *Mycobacterium* [3].

The vast majority of human infections with the *Rhodococcus* spp. are caused by *R. equi* [4], whereas there have been case reports of human infection by the other species [5–7]. The first human case of *R. equi* infection was described by Golub et al. in 1967 in a 29-year-old male with plasma cell hepatitis on chronic steroid therapy who presented with cavitary pneumonia [8]. Frequency of infection with *R. equi* increases in immunocompromised individuals such as those with AIDS and organ transplants [9]. Since the first case of *R. equi* infection in an AIDS patient in 1986 [10], there have been increasing numbers of infections with this bacterium reported in AIDS patients [9]. Emergence of resistance to macrolides and rifampin has been well documented in *R. equi* isolated from animals. However, there is no systematic information available regarding antibiotic resistance of *R. equi* isolates from humans. In addition, there is no standard antibiotic susceptibility testing panel for *R. equi* in humans. The main objective of the present study is to summarize disease characteristics and antibiotic susceptibility testing results in 12 cases of *R. equi* infection at a tertiary care center in Central Kentucky and compare with the cases of the infection with this pathogen in the United States and Europe reported in the literature to generate systematic information on clinical diseases associated with *R. equi* infection and antibiotic susceptibility of the bacterium isolated from the patients.

## 2. Methods

*2.1. Identification of Patients with R. equi Infection at the University of Kentucky.* Following IRB approval, the microbiology laboratory database was used to identify subjects from whom *R. equi* was isolated in the period from January 1998 to December 2013. A SUNQUEST Epi report was generated searching for *Rhodococcus equi* in the result field. Other information collected in this report included subject name, medical record number, specimen source, date of specimen collection, and antibiotic susceptibility test results for the isolate. A total of 14 subjects were identified from whom *R. equi* was isolated. Among these isolates, the results of antibiotic susceptibility testing were available for 11 subjects. Chart review was performed on 12 of the 14 subjects to gather age, gender, occupational exposure to animals, HIV status, viral load, CD4 count, clinical and radiographic features, and antibiotic treatment and outcomes. Two subjects were from a referral hospital and their medical records were not available for review.

Identification of *R. equi* was performed by BD Phoenix™ Automated Microbiology System (Becton, Dickinson and Company, New Jersey). Screening of antibiotic susceptibility for these clinical isolates was performed by Epsilometer-test (E-test) (BioMérieux, Durham, NC). The interpretive criterion for determining susceptibility to antibiotics followed the CLSI Guidelines in the M100 document for *Staphylococcus aureus*, with the inclusion of results for vancomycin and rifampin [17]. Break points for ciprofloxacin and levofloxacin were interchangeable. Clarithromycin was used as a class representative for newer macrolides.

*2.2. Literature Search on Cases of R. equi Infection.* PubMed search was performed using key words "*Rhodococcus equi*" and "*Corynebacterium equi*" (an older, now obsolete name for the organism). The articles containing these key words were then filtered by using additional key words "Humans" and "English language". Abstracts of these studies were reviewed and any study describing at least 10 cases was included in this study. There were four European studies [11–13, 16] and two United States (US) studies [14, 15] that satisfied this requirement. Antibiotic susceptibility data were not available in one of the two US studies [15].

*2.3. Statistical Analyses.* Statistical significances of the differences in clinical features between groups of patients and in antibiotic susceptibility of clinical isolates were determined by Fisher's exact test. In the analyses of the differences in antibiotic susceptibility, "corrected" $P$ values were calculated by multiplying each $P$ value by the number of antibiotics studied [18].

## 3. Results

*3.1. Case Series at the University of Kentucky.* There were twelve cases in this series including six patients positive for human immune deficiency virus (HIV), two with organ transplant, and four immune competent individuals (Table 1). Two of the immune competent patients had wound infection with *R. equi* and the other two had pneumonia. All eight immunocompromised patients had pneumonia. *R. equi*-infected patients presented with varying symptoms depending on organ system involved (Table 1). Most common symptoms were fever, cough, dyspnea, anorexia, and weight loss in patients diagnosed with pneumonia. *R. equi* was isolated from respiratory specimens in 7 patients and from the peripheral blood from 5 patients. *R. equi* was also isolated from wound cultures from two immunocompetent individuals.

Among the 10 cases with *R. equi* pneumonia, bacteremia was noted in five patients, one immunocompetent and four immunocompromised. A cavitary lesion was noted in radiographic imaging in 4 of the 10 patients. Three of these patients had upper lobe cavitary lesions and one had lower lobe cavitation. The presence of the cavitary lesion did not correlate with the presence of bacteremia. All six patients with HIV infection were not on antiretroviral therapy at the time of diagnosis of *R. equi* infection; 5 of them were antiretroviral therapy (ART) naïve and the other patient stopped ART a few months prior to diagnosis of *R. equi* infection. ART was initiated in all the patients after the diagnosis of *R. equi* infection at outpatient follow-up. One patient was consistently nonadherent with ART and other medications and finally succumbed to infection after one year of initial diagnosis of *R. equi* pneumonia.

In the two organ transplant patients with *R. equi* infection, one had received a lung transplant and the other had received a renal transplant. Both the transplant patients were treated successfully with prolonged course of antibiotics and they did not have any recurrence of infection.

In the immunocompetent patients, treatment of the two individuals with wound infection was successful. The other two individuals with *R. equi* pneumonia did not follow up in the university health system. Based on social security death index data, one patient died after 6 months and the other patient expired after 15 months, both from unknown causes.

*3.2. Review of Clinical Characteristics of Infection with R. equi in This Study and Cases in the Literature.* The epidemiological and clinical characteristics of subjects infected with *R. equi* were analyzed for four European studies [11–13, 16] and three US studies including two studies in the literature [14, 15] and the present study.

In the four European studies, all 113 patients were immunocompromised with HIV infection. Among these patients, 107 subjects (95%) had pulmonary disease with or without extrapulmonary *R. equi* infection, and six subjects had extrapulmonary involvement only (Table 2). In the three US studies including the present study, 21 immunocompromised subjects (95%) had pulmonary diseases with or without extrapulmonary involvement and one subject had extrapulmonary involvement only (Table 2). Therefore, there is no significant difference in the organ system involvement in infection with *R. equi* in the immunocompromised patients between the European and US subjects.

In contrast, in the cases of *R. equi* infection in immunocompetent individuals in the US, only 5 of 14 patients had pulmonary diseases with or without extrapulmonary involvement, and the other 9 patients had extrapulmonary

Table 1: Demographic and clinical characteristics of *R. equi* infection at the University of Kentucky*.

| | Patient data | | | Clinical data | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Year | Case/age/gender | Occupational/environmental exposure to livestock | Specimen source | Clinical symptoms | Immune status | CD4 (%) | Viral load copies/mL | ART | Radiography | Treatment duration (months) | Surgery | Relapse/outcome |
| 2001 | 1/40/m | Unknown | Sputum, blood | Fever, cough, nausea | HIV | 27 (6) | 56784 | No | RUL infiltrate with cavitation and LUL infiltrate | 3 | No | Cured |
| 2001 | 2/32/m | Unknown | Sputum, blood | Fever, nausea, weight loss | HIV | 43 (4) | <400 | Yes | LUL and LLL pneumonia | 12 | No | Relapsed†/expired |
| 2003 | 3/33/m | Lawn care worker | Sputum | Fever, cough | HIV | 60 (8) | 257927 | No | LUL cavitary lesion | 9 | No | Cured |
| 2009 | 4/40/m | Unknown | Sputum | Cough, dyspnea | HIV | 7 (3) | 354000 | No | Right lung infiltrate | 36 | No | Cured |
| 2010 | 5/37/m | Horses | Sputum | Cough, dyspnea | HIV | 59 (22) | 276000 | No | LLL cavitary lesion | 24 | No | Cured |
| 2013 | 6/48/m | Horses, donkey | Blood pericardial fluid | Fever, cough, dyspnea | HIV | 10 (5) | 143000 | No | Multiple bilateral nodular lesions | 12 | Pericardial drain | Cured |
| 2008 | 7/62/m | Unknown | Sputum | Anorexia, dyspnea, nausea | Lung tx | NA | NA | No | Right pleural effusion | 7 | Pericardial drain | Cured |
| 2012 | 8/53/m | Cattle, pigs | Blood | Fever, cough, dyspnea | Renal tx | NA | NA | No | RUL dense consolidation | 15 | No | Cured |
| 2003 | 9/25/f | Motor vehicle accident with soil debris | Tissue | Left foot wound | NA | NA | NA | No | NA | 3 weeks | Amputation | Cured |
| 2005 | 10/53/m | Unknown | Sputum | Fever, cough, dyspnea, weight loss | NA | NA | NA | No | RUL cavitary lesion | Unknown | No | Expired‡ |
| 2007 | 11/79/f | Unknown | Blood | Fever | NA | NA | NA | No | LUL mass lesion, bilateral infiltrates | Unknown | Central line removed | Expired§ |
| 2011 | 12/42/m | Unknown | Tissue | Right great toe wound | NA | NA | NA | No | NA | 1 | Amputation | Cured |

*HIV: human immune deficiency virus; tx: transplant; ART: antiretroviral therapy; RUL: right upper lobe; LUL: left upper lobe; LLL: left lower lobe; NA: not applicable.

†*R. equi* bacteremia recurred after 9 months and the patient expired.

‡Lost to follow-up and expiredafter 15 months from unknown causes.

§Lost to follow-up and expired after 6 months from unknown causes.

Table 2: Organ systems involved in infection with *R. equi* in immunocompromised and immunocompetent patients in Europe and the United States.

| Immune status | Europe* | | United States† | |
|---|---|---|---|---|
| | Pulmonary ± extrapulmonary | Extrapulmonary only | Pulmonary ± extrapulmonary | Extrapulmonary only |
| Immunocompromised | 107/113‡ (95%) | 6/113 (5%) | 21/22 (95%) | 1/22 (5%) |
| Immunocompetent | 0 | 0 | 5/14 (36%) | 9/14 (64%)§ |

*From Arlotti et al. [11], Donisi et al. [12], Topino et al. [13], and Torres-Tortosa et al. [16].
†From Scott et al. [14], Verville et al. [15], and the 12 cases in Central Kentucky reported in the present study.
‡Number of patients with the organ involvement/total number of patients.
§$P = 0.0002$ when compared to immunocompromised patients in the US and $P < 0.0001$ when compared to immunocompromised patients in Europe.

Table 3: Comparison of organ system involvement, sites of clinical isolation, and radiographic findings in *R. equi* infection in the seven studies under analysis.

| Studies | Number of patients | Site of isolation* | | | Organ system involved | | Radiographic findings | |
|---|---|---|---|---|---|---|---|---|
| | | Respiratory specimen (%) | Blood (%) | Other† | Pulmonary ± extrapulmonary (%) | Extrapulmonary only (%) | Pneumonia on imaging | Cavitary lesion (%) |
| Europe | | | | | | | | |
| Donisi et al. [12] | 12 | 4 (25) | 10 (63) | 2 (12) | 9 (75) | 3 (25) | 9 | 5 (56) |
| Arlotti et al. [11] | 24 | 21 (51) | 13 (32) | 7 (17) | 24 (100) | 0 (0) | 24 | 18 (75) |
| Torres-Tortosa et al. [16] | 67 | 64 (52) | 34 (27) | 26 (21) | 65 (97) | 2 (3) | 65 | 45 (49) |
| Topino et al. [13] | 10 | 5 (38) | 8 (62) | 0 (0) | 9 (90) | 1 (10) | 9 | 6 (67) |
| Subtotal | 113 | 94 (48) | 65 (34) | 35 (18) | 107 (95) | 6 (5) | 107 | 74 (69) |
| United States | | | | | | | | |
| Verville et al. [15] | 12 | 8 (53) | 5 (33) | 2 (13) | 8 (67) | 4 (33) | 8 | 6 (75) |
| Scott et al. [14] | 12 | 6 (35) | 7 (41) | 4 (24) | 8 (67) | 4 (33) | 8 | 7 (88) |
| University of Kentucky | 12 | 7 (47) | 5 (33) | 3 (20) | 10 (83) | 2 (17) | 10 | 4 (40) |
| Subtotal | 36 | 21 (45) | 17 (36) | 9 (19) | 26 (72) | 10 (28) | 26 | 17 (65) |
| Total | 149 | 115 (48) | 82 (34) | 44 (18) | 133 (89) | 16 (11) | 133 | 91 (68) |

*Total numbers of specimens are more than numbers of patients as *R. equi* was isolated from a variety of specimens.
†Bone, joint fluid, abscess, wound, pleura and pericardial fluid, liver, brain, cerebral spinal fluid, stool, and skin.

involvement only (Table 2). Extrapulmonary sites of infection included central nervous system, bone, blood, eyes, and soft tissue. The frequency of extrapulmonary involvement only in the immunocompetent patients (9/14 cases) was significantly higher when compared to the frequencies of those in the immunocompromised patients in the US (1/21) and those (6/113) in European studies ($P = 0.0002$ for US and $P < 0.0001$ for European studies; Table 2).

In patients with pneumonia, among the 107 subjects in the European studies, 74 (69%) had cavitary lesions in the lungs (Table 3). Similarly, 17 of 26 subjects (65%) in the US studies who were diagnosed with pneumonia presented with cavitary lesions (Table 3).

The outcome of *R. equi* infection in HIV-positive patients depended strongly upon treatment of HIV with highly active antiretroviral therapy (HAART). Among the total 113 HIV-positive subjects in the European studies, 93 were diagnosed in the pre-HAART era and 20 were diagnosed in the HAART era. Mortality was 56% (52/93) in pre-HAART era while it is only 5% (1/20) in HAART era (Table 4, $P < 0.0001$). The outcome in two additional subjects diagnosed with *R. equi* infection in pre-HAART era was unknown. Therefore, HAART significantly improved survival in HIV-positive patients with *R. equi* infections. A similar tendency was observed in the US studies. Among 12 HIV-positive patients diagnosed with *R. equi* infection in pre-HAART era, 7 of them (58%) died, whereas mortality was only 1 of 6 patients (17%) diagnosed in HAART era (Table 4). However, the difference in the mortality between pre-HAART and HAART era in the US studies did not reach statistical significance most likely due to the small number of patients available in the analysis. When the data from the European and US studies are combined, mortality in *R. equi*-infected HIV patients was higher in the pre-HAART era (56%, 59/105) than in HAART era (8%, 2/26) ($P < 0.0001$).

*3.3. Review of Antibiotic Susceptibility of R. equi Isolated from Patients in the Present Study and in the Literature.* The method of antibiotic susceptibility testing and minimum inhibitory concentration (MIC) breakpoints assessment are not described in all of the 6 studies that were analyzed. E-test was used in the study by Arlotti et al. [11] and the present study at the University of Kentucky. There were significant differences in the panel of antibiotics chosen for susceptibility

Table 4: Comparison of mortality in *R. equi*-infected HIV$^+$ patients in pre-HAART and HAART era in the seven studies under analysis*.

| Studies | Total HIV patients | Diagnosed before 1997 (pre-HAART era) | Diagnosed in or after 1997 (HAART era) | Patients died (%) (pre-HAART era) | Patients died (%) (HAART era) |
|---|---|---|---|---|---|
| Europe* | 113 | 93 | 20 | 52 (56) | 1 (5) |
| United States† | 18 | 12 | 6 | 7 (58) | 1 (17) |
| Total | 131 | 105 | 26 | 59 (56) | 2 (8) |

*From Arlotti et al. [11], Donisi et al. [12], Topino et al. [13], and Torres-Tortosa et al. [16].
†From the cases in the Central Kentucky reported in the present study, Scott et al. [14], and Verville et al. [15].

testing as these studies span over two decades. Antibiotics for which susceptibility testing was performed in most studies included vancomycin, erythromycin/clarithromycin, cotrimoxazole, rifampin, penicillin, ciprofloxacin, and gentamicin. Twenty-one antibiotics were selected for which susceptibility data were available for at least 10 isolates of *R. equi*. These are listed in the order of higher susceptibility in Table 5. At least 85% of *R. equi* isolates were susceptible to 10 of the 21 antibiotics evaluated. These ten antibiotics are clarithromycin, vancomycin, rifampin, amikacin, erythromycin, teicoplanin, imipenem, netilmicin, gentamicin, and ciprofloxacin. In contrast, *R. equi* isolates were consistently resistant to penicillin, oxacillin, ampicillin, cephalothin, and clindamycin, and their susceptibilities to these antibiotics were lower than 15% (Table 5).

In most of the 21 antibiotics listed in Table 5, susceptibility of the bacterium was similar among 6 studies including the present study. This strongly suggests that methodological differences in testing antibiotic susceptibility of *R. equi* isolates in these 6 studies did not significantly affect the outcome of the tests. However, there is a marked difference in susceptibility of the clinical isolates to cotrimoxazole between European and US studies. One hundred percent (15/15 isolates) of *R. equi* from the US were susceptible to cotrimoxazole, whereas only 35 of 76 isolates (46%) from Europe were susceptible to this compound ($P < 0.0001$, corrected $P < 0.0018$). A similar tendency was also observed in susceptibility of the bacterium to tetracycline. Nine of the 10 isolates (90%) from the US were susceptible to tetracycline, whereas only 17 of 42 (43%) of isolates from Europe were susceptible to this antibiotic ($P = 0.0127$). However, this difference did not reach statistical significance when corrected for the number of variables tested (corrected $P = 0.229$). The difference in susceptibility of the *R. equi* isolates to cotrimoxazole was not due to differences in the sites of isolation of the bacterium from the patients in the European and US studies, because a majority of the isolates were from either the respiratory specimen (48% in Europe and 45% in the US) or the peripheral blood (34% in Europe and 36% in the US) (Table 3).

*3.4. Empiric Treatment of R. equi Infection.* Based on antibiotic susceptibility of the clinical isolates shown in Table 5, *R. equi* infection in immunocompromised patients and the serious infection in immunocompetent individuals should receive intravenous therapy with 2 or 3 drugs that include vancomycin, imipenem, clarithromycin/azithromycin, rifampin, aminoglycoside, or ciprofloxacin/levofloxacin [9, 19]. Most patients require a minimum of 2 weeks of intravenous therapy at which point oral therapy with 2 or 3 drugs that include vancomycin, rifampin, or ciprofloxacin/levofloxacin may be substituted based on clinical improvement [9, 20]. Immunocompetent patients with mild to moderate diseases can be treated with two active oral agents [19]. Vancomycin and imipenem are bactericidal agents that help decrease high bacterial burden and rapid clearance of bacteremia [9, 16]. Clarithromycin, rifampin, ciprofloxacin, and vancomycin achieve good intracellular concentrations and can be very effective on intracellular pathogens [15, 19]. Cotrimoxazole is an option for both intravenous and oral therapy but is recommended for empiric therapy in the US but not in Europe due to the high frequency of resistance of the *R. equi* isolates to this antibiotic in Europe as described earlier.

## 4. Discussion

*R. equi* has been known to cause pneumonia in immunocompromised individuals since it was first reported in 1967 [8]. In the literature, 95–100% of immunocompromised patients infected with the bacterium presented with pulmonary involvement [11, 16]. In agreement with these findings, the cases identified in Kentucky also presented with pulmonary involvement for all eight immunocompromised subjects. This was not the case for immunocompetent patients, however. Only two of four (50%) immunocompetent subjects in the currently described patient series had pulmonary involvement. A similar tendency can be seen in other studies in the US, in which pneumonia was observed only in 3 of 10 (30%) infected immunocompetent patients [14, 15]. Therefore, it is important to recognize that immunocompetent subjects may not present with pneumonia when infected with *R. equi* but instead may have localized infections such as cutaneous wounds or osteomyelitis.

Since pulmonary involvement was observed in the majority of immunocompromised but not of immunocompetent patients infected with *R. equi*, insufficient immune responses appear to be a significant cause that makes individuals susceptible to pulmonary infection with this bacterium. In immunocompetent individuals, a local exposure of organ(s) such as skin or a bone to the materials, possibly soil, highly contaminated with *R. equi* may have caused these individuals to be susceptible to the infection.

Table 5: Comparison of antibiotic susceptibilities of *R. equi* isolated from patients in Europe and the United States in the six studies under analysis[*].

| Antibiotic | Europe | | | | | United States | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Donisi et al. [12] | Arlotti et al. [11] | Torres-Tortosa et al. [16] | Topino et al. [13] | Subtotal (%) | Scott et al. [14] | U of Kentucky | Subtotal (%) | *P* value | Total (%) |
| *Clarithromycin* | NA | NA | NA | 6/6[†] | 6/6 (100) | NA | 6/6 | 6/6 (100) | NS | 12/12 (100) |
| *Vancomycin* | 9/9 | 21/21 | 60/60 | 8/9 | 98/99 (99) | 9/9 | 10/10 | 19/19 (100) | NS | 117/118 (99) |
| *Rifampin* | 1/2 | 21/21 | 47/48 | 6/8 | 75/79 (95) | NA | 6/7 | 6/7 (86) | NS | 81/86 (94) |
| *Amikacin* | NA | 9/9 | 22/22 | 4/6 | 35/37 (95) | 9/9 | 6/7 | 15/16 (94) | NS | 50/53 (94) |
| *Erythromycin* | 6/9 | 24/24 | 53/58 | 6/6 | 89/97 (92) | 9/9 | 5/5 | 14/14 (100) | NS | 103/111 (93) |
| *Teicoplanin* | 8/8 | 9/9 | NA | 6/8 | 23/25 (92) | NA | NA | NA | NA | 23/25 (92) |
| *Imipenem* | 2/2 | 17/21 | 41/42 | 7/7 | 67/72 (93) | NA | 4/6 | 4/6 (67) | NS | 71/78 (91) |
| *Netilmicin* | 8/8 | 8/9 | NA | 4/5 | 20/22 (91) | NA | NA | NA | NA | 20/22 (91) |
| *Gentamicin* | NA | 15/15 | 40/47 | 5/7 | 60/69 (87) | 9/9 | 5/5 | 14/14 (100) | NS | 74/83 (89) |
| *Ciprofloxacin* | 6/9 | 11/19 | 47/50 | 8/9 | 72/87 (83) | 9/9 | 6/6 | 15/15 (100) | NS | 87/102 (85) |
| *Chloramphenicol* | NA | NA | 12/18 | 6/6 | 18/24 (75) | 9/9 | NA | 9/9 (100) | NS | 27/33 (82) |
| *Ceftriaxone* | 4/5 | NA | NA | 4/8 | 8/13 (62) | NA | 3/6 | 3/6 (50) | NS | 11/19 (58) |
| *Cotrimoxazole* | 2/7 | 15/24 | 17/38 | 1/7 | 35/76 (46) | 9/9 | 6/6 | 15/15 (100) | <0.0001[‡] | 50/91 (55) |
| *Tetracycline* | 0/8 | 6/18 | 15/22 | 2/5 | 23/53 (43) | 9/9 | 0/1 | 9/10 (90) | 0.0127[§] | 32/63 (51) |
| *Amox-Clav* | NA | 5/5 | 12/33 | 0/4 | 17/42 (40) | NA | 3/6 | 3/6 (50) | NS | 20/48 (42) |
| *Cefotaxime* | NA | NA | 13/32 | 1/2 | 14/34 (41) | NA | NA | NA | NA | 14/34 (41) |
| *Ampicillin* | NA | 3/18 | 7/41 | 0/5 | 10/64 (16) | 0/9 | NA | 0/9 (0) | NS | 10/73 (14) |
| *Clindamycin* | 1/10 | NA | 6/33 | 0/2 | 7/45 (16) | 0/9 | NA | 0/9 (0) | NS | 7/54 (13) |
| *Cephalothin* | 0/4 | 2/20 | NA | 0/3 | 2/27 (7) | 2/9 | NA | 2/9 (22) | NS | 4/36 (11) |
| *Penicillin* | 0/4 | 1/23 | 2/40 | 0/7 | 3/74 (4) | 0/9 | 1/3 | 1/12 (8) | NS | 4/86 (5) |
| *Oxacillin* | 0/8 | 0/15 | NA | 0/7 | 0/30 (0) | 0/9 | NA | 0/9 (0) | NS | 0/39 (0) |

[*]NA: not applicable; NS: not significant.

[†]Number of clinical isolates susceptible to the antibiotic/total number of clinical isolates tested.

[‡]Comparison of the antibiotic susceptibility between Europe and the US, corrected $P < 0.0018$.

[§]Comparison of the antibiotic susceptibility between Europe and the US, corrected $P = 0.229$.

In a review of the literature, cavitary lesions were detected on radiologic imaging in 40–88% (mean 68%) of subjects diagnosed with *R. equi* pneumonia. In the case series at the University of Kentucky, 3 of 4 (75%) of patients with cavitary lesions had the lesions of an upper lobe. Similar tendencies were observed in other case series describing radiologic findings in *R. equi* pneumonia [21, 22]. Therefore, the presence of upper lobe cavitary lesions appears to be an important differential feature of *R. equi* infection by imaging.

In this review, mortality of HIV patients infected with *R. equi* is 56% in the pre-HAART era while it was only 8% in HAART era. The study by Torres-Tortosa et al. [16] showed improved survival in patients in whom HAART was started. These results strongly suggest that the protective immune responses are crucial, in addition to antibiotic treatment, to eradicate *R. equi* and prevent mortality.

Studies using animals infected with *R. equi* have demonstrated the importance of both cell-mediated and humoral immune responses in clearing the bacterium [23]. It is well recognized that innate immune cells such as neutrophils and macrophages are crucial to control primary infection [24]. Th1-type immune responses (IL-2, IL-12, IFN-$\gamma$, and TNF-$\alpha$) are important for resistance to *R. equi*. Both CD4 and CD8 T cells produce IFN-$\gamma$ which is necessary to activate macrophages to eliminate *Rhodococcus* from intracellular locations. *Rhodococcus*-specific antibodies opsonize the extracellular organisms to promote phagocytosis and enhance bacterial killing [25]. This antibody-mediated protective mechanism is relevant because *R. equi* is a facultative intracellular bacterium. For example, newborn foals are exposed to *R. equi* infection soon after birth as it is ubiquitous in soil. However, most of them do not develop clinical infection until 6–12 weeks after birth, which coincides with decreases of maternal antibodies in these animals.

In agreement with the importance of the protective immunity to control *R. equi* in animal models, immunocompetent patients with *R. equi* infection treated with antibiotics have better outcomes in general when compared to infected immunocompromised individuals [15, 26–29]. However, there are case reports of deaths in immunocompetent patients diagnosed with *R. equi* pneumonia [30, 31]. In the current case series, two immunocompetent patients with *R. equi* pneumonia died after six and fifteen months of discharge from hospital, respectively, although the cause of death in these patients is unknown as they were lost to follow-up. Antibiotic treatment is essential to eliminate *R. equi* infection not only in immunocompromised but also in immunocompetent individuals [30, 31]. Virulent strains of *R. equi* have been shown to contain a plasmid, which encodes seven related virulence associated proteins (Vaps) including the immunodominant surface-expressed protein Vap A that is essential for intracellular growth of the bacterium in macrophages [32, 33]. These virulence proteins may contribute to reducing the efficiency of the protective immunity to control the bacterium.

The present study made an interesting observation with regard to antibiotic susceptibility to cotrimoxazole between the US and Europe. All clinical isolates in the US were susceptible to cotrimoxazole, whereas more than half of the isolates in Europe were resistant. This is the first time that this difference has been reported. This difference in susceptibility to cotrimoxazole may suggest that the US and Europe have different strains of *R. equi*. It is unclear why and how the bacterium in Europe acquired resistance to cotrimoxazole. It is possible that differences in usage of antibiotics in domestic animals between these two continents have resulted in the disparity in susceptibility to the antibiotic.

Based on the consensus findings for antibiotic susceptibility for clinical isolates, an empiric antibiotic treatment regimen for *R. equi* infection would include 2-3 intravenous agents (vancomycin, imipenem, clarithromycin/azithromycin, rifampin, an aminoglycoside, ciprofloxacin/levofloxacin, or cotrimoxazole), administered for at least 2 weeks if the patient is immunocompromised [9, 19], or 2 oral agents (clarithromycin/azithromycin, rifampin, ciprofloxacin/levofloxacin, or cotrimoxazole) if the patient is immunocompetent with mild or moderate diseases [19]. Final antibiotic choice should be determined based on *in vitro* antibiotic susceptibility testing results. Monotherapy for *R. equi* infection is not recommended, as it is often ineffective. This infection should be treated with at least two agents with activity against *R. equi*. Parenteral therapy is generally recommended for administration during the first few weeks of a 6-month or longer course of treatment. One of these agents should be bactericidal given the frequency with which it is isolated from blood [34, 35] and one agent should be active against intracellular organisms. *R. equi* is usually susceptible to erythromycin and extended spectrum macrolides, rifampin, fluoroquinolones, aminoglycosides, glycopeptides, and imipenem [36]. Macrolides, fluoroquinolones, glycopeptides, and rifampin have the additional advantage of achieving good intracellular drug concentration [37]. *In vitro* studies showed combinations of gentamicin with erythromycin or rifampicin had antagonistic effects on killing compared to either drug alone, while combinations of erythromycin with rifampicin or penicillin had synergistic effect [38].

In this case series at the University of Kentucky, one in eight clinical isolates was resistant to rifampin. Recent studies have shown emergence of macrolide and rifampin resistant *R. equi* strains in horse breeding farms because of widespread use of these antibiotics for prophylaxis and treatment [39]. Rifampin resistant *R. equi* strains were isolated from HIV patients from northern Thailand [40]. Rifampin resistance is associated with mutations in rpo B gene. In view of this, a combination of vancomycin and macrolide as initial empiric therapy for *R. equi* infections is suggested. Aminoglycosides have good activity against *R. equi*. However, they are falling out of favor because of their toxicity profile. Among carbapenems, more data is available for imipenem than for meropenem [11, 13, 16].

The duration of therapy depends on the site(s) and extent of infection, underlying immunocompetence of the host, and the clinical response to therapy. A minimum of 6 months of antibiotic therapy is typically required for immunocompromised patients with pulmonary, bone and joint, or central nervous system infections [9]. Secondary prophylaxis with an oral agent may be considered in patients who are on immune suppressive agents or HIV patients with low CD4 count [9].

Any improvement in the immunocompetence of the host should be pursued as a therapeutic adjunct in treating *R. equi* infections, either through curtailment of immunosuppressive medications or through aggressive antiretroviral therapy.

In the present study, twelve patients with *R. equi* infection in Kentucky were compared to 137 cases reported in the literature. In both the cases in Kentucky and those in the literature, lungs were the primary sites of infection in immunocompromised patients, whereas extrapulmonary involvement only was more common in immunocompetent patients. In *R. equi*-infected immunocompromised patients with HIV, HAART improves prognosis in these patients. Based on antibiotic susceptibility of the bacterium isolated from the patients, the empiric treatment of *R. equi* infection should include a combination of two antibiotics, preferably selected from vancomycin, imipenem, clarithromycin/azithromycin, ciprofloxacin, rifampin, or cotrimoxazole. Local antibiograms should be checked prior to using cotrimoxazole due to developing resistance.

## Competing Interests

The authors declare that they have no competing interests.

## References


[1] J. F. Prescott, "*Rhodococcus equi*: an animal and human pathogen," *Clinical Microbiology Reviews*, vol. 4, no. 1, pp. 20–34, 1991.

[2] H. Magnusson, "Spezifische infektioese Pneumonie beim Fohlen. Ein neuer Eitererreger beim Pferd," *Archiv fur Wissenschaftliche und Praktische Tierheilkunde*, vol. 50, pp. 22–37, 1923.

[3] M. Goodfellow and G. Alderson, "The actinomycete-genus *Rhodococcus*: a home for the 'rhodochrous' complex," *Journal of General Microbiology*, vol. 100, no. 1, pp. 99–122, 1977.

[4] F. Al Akhrass, I. Al Wohoush, A.-M. Chaftari et al., "*Rhodococcus* bacteremia in cancer patients is mostly catheter related and associated with biofilm formation," *PLoS ONE*, vol. 7, no. 3, article e32945, 2012.

[5] P. Lalitha, M. Srinivasan, and V. Prajna, "*Rhodococcus ruber* as a cause of keratitis," *Cornea*, vol. 25, no. 2, pp. 238–239, 2006.

[6] S. R. Bagdure, M. A. Fisher, M. E. Ryan, and F. A. Khasawneh, "*Rhodococcus erythropolis* encephalitis in patient receiving rituximab," *Emerging Infectious Diseases*, vol. 18, no. 8, pp. 1377–1379, 2012.

[7] H. Vosoghi, C. E. Lyle, and J. C. Fleming, "Recurrent *Rhodococcus gordoniae* eyelid infection after combined septoplasty and blepharoplasty," *Ophthalmic Plastic and Reconstructive Surgery*, vol. 26, no. 2, pp. 127–129, 2010.

[8] B. Golub, G. Falk, and W. W. Spink, "Lung abscess due to *Corynebacterium equi*. Report of first human infection," *Annals of Internal Medicine*, vol. 66, no. 6, pp. 1174–1177, 1967.

[9] D. M. Weinstock and A. E. Brown, "*Rhodococcus equi*: an emerging pathogen," *Clinical Infectious Diseases*, vol. 34, no. 10, pp. 1379–1385, 2002.

[10] J. H. Samies, B. N. Hathaway, R. M. Echols, J. M. Veazey Jr., and V. A. Pilon, "Lung abscess due to *Corynebacterium equi*. Report of the first case in a patient with acquired immune deficiency syndrome," *The American Journal of Medicine*, vol. 80, no. 4, pp. 685–688, 1986.

[11] M. Arlotti, G. Zoboli, G. L. Moscatelli et al., "*Rhodococcus equi* infection in HIV-positive subjects: a retrospective analysis of 24 cases," *Scandinavian Journal of Infectious Diseases*, vol. 28, no. 5, pp. 463–467, 1996.

[12] A. Donisi, M. G. Suardi, S. Casari, M. Longo, G. P. Cadeo, and G. Carosi, "*Rhodococcus equi* infection in HIV-infected patients," *AIDS*, vol. 10, no. 4, pp. 359–362, 1996.

[13] S. Topino, V. Galati, E. Grilli, and N. Petrosillo, "*Rhodococcus equi* infection in HIV-infected individuals: case reports and review of the literature," *AIDS Patient Care and STDs*, vol. 24, no. 4, pp. 211–222, 2010.

[14] M. A. Scott, B. S. Graham, R. Verrall, R. Dixon, W. Schaffner, and K. T. Tham, "*Rhodococcus equi*—an increasingly recognized opportunistic pathogen: report of 12 cases and review of 65 cases in the literature," *American Journal of Clinical Pathology*, vol. 103, no. 5, pp. 649–655, 1995.

[15] T. D. Verville, M. M. Huycke, R. A. Greenfield, D. P. Fine, T. L. Kuhls, and L. N. Slater, "*Rhodococcus equi* infections of humans: 12 cases and a review of the literature," *Medicine*, vol. 73, no. 3, pp. 119–132, 1994.

[16] M. Torres-Tortosa, J. Arrizabalaga, J. L. Villanueva et al., "Prognosis and clinical evaluation of infection caused by *Rhodococcus equi* in HIV-infected patients: a multicenter study of 67 cases," *Chest*, vol. 123, no. 6, pp. 1970–1976, 2003.

[17] CLSI, *Susceptibility Testing of Mycobacteria, Nocardiae, and Other Aerobic Actinomycetes; Approved Standard*, CLSI Document M24-A2, Clinical and Laboratory Standards Institute, Wayne, Pa, USA, 2nd edition, 2011.

[18] B. W. Brown Jr. and M. Holander, *Statistics: A Biomedical Introduction*, John Wiley & Sons, New York, NY, USA, 1977.

[19] A. V. Yamshchikov, A. Schuetz, and G. M. Lyon, "*Rhodococcus equi* infection," *The Lancet Infectious Diseases*, vol. 10, no. 5, pp. 350–359, 2010.

[20] L. L. Van Etta, G. A. Filice, R. M. Ferguson, and D. N. Gerding, "*Corynebacterium equi*: a review of 12 cases of human infection," *Reviews of Infectious Diseases*, vol. 5, no. 6, pp. 1012–1018, 1983.

[21] S. Wicky, F. Cartei, B. Mayor et al., "Radiological findings in nine AIDS patients with *Rhodococcus equi* pneumonia," *European Radiology*, vol. 6, no. 6, pp. 826–830, 1996.

[22] J. H. MacGregor, W. M. Samuelson, D. C. Sane, and J. D. Godwin, "Opportunistic lung infection caused by *Rhodococcus (Corynebacterium) equi*," *Radiology*, vol. 160, no. 1, pp. 83–84, 1986.

[23] T. R. M. Y. Dawson, D. W. Horohov, W. G. Meijer, and G. Muscatello, "Current understanding of the equine immune response to *Rhodococcus equi*. An immunological review of *R. equi* pneumonia," *Veterinary Immunology and Immunopathology*, vol. 135, no. 1-2, pp. 1–11, 2010.

[24] I. R. Tizard, "Dendritic cells and antigen processing," in *Veterinary Immunology: An Introduction*, pp. 89–100, Saunders, Philadelphia, Pa, USA, 8th edition, 2008.

[25] J. Cauchard, C. Sevin, J.-J. Ballet, and S. Taouji, "Foal IgG and opsonizing anti-*Rhodococcus equi* antibodies after immunization of pregnant mares with a protective VapA candidate vaccine," *Veterinary Microbiology*, vol. 104, no. 1-2, pp. 73–81, 2004.

[26] M. J. Linares, A. López-Encuentra, and S. Perea, "Chronic pneumonia caused by *Rhodococcus equi* in a patient without impaired immunity," *European Respiratory Journal*, vol. 10, no. 1, pp. 248–250, 1997.

[27] A. Tünger, F. Özkan, F. Vardar, D. Burhanoğlu, M. A. Özinel, and A. Tokbaş, "Purulent meningitis due to *Rhodococcus equi*. A case of posttraumatic infection," *Acta Pathologica, Microbiologica, et Immunologica Scandinavica*, vol. 105, no. 9, pp. 705–707, 1997.

[28] E. Sigler, A. Miskin, M. Shtlarid, and A. Berrebi, "Fever of unknown origin and anemia with *Rhodococcus equi* infection in an immunocompetent patient," *The American Journal of Medicine*, vol. 104, no. 5, p. 510, 1998.

[29] K. Bouchou, P. Cathebras, J. M. Dumollard et al., "Chronic osteitis due to *Rhodococcus equi* in an immunocompetent patient," *Clinical Infectious Diseases*, vol. 20, no. 3, pp. 718–720, 1995.

[30] M. R. Jones, T. J. Neale, P. J. Say, and J. G. Horne, "*Rhodococcus equi*: an emerging opportunistic pathogen?" *Australian and New Zealand Journal of Medicine*, vol. 19, no. 2, pp. 103–107, 1989.

[31] R. D. Walsh and B. A. Cunha, "*Rhodococcus equi*: fatal pneumonia in a patient without AIDS," *Heart and Lung*, vol. 23, no. 6, pp. 519–520, 1994.

[32] S. Jain, B. R. Bloom, and M. K. Hondalus, "Deletion of vapA encoding Virulence Associated Protein A attenuates the intracellular actinomycete *Rhodococcus equi*," *Molecular Microbiology*, vol. 50, no. 1, pp. 115–128, 2003.

[33] W. G. Meijer and J. F. Prescott, "*Rhodococcus equi*," *Veterinary Research*, vol. 35, no. 4, pp. 383–396, 2004.

[34] R. M. Rouquet, D. Clave, P. Massif, N. Moatti, and P. Leophonte, "Imipenem/vancomycin for *Rhodococcus equi* pulmonary infection in HIV-positive patient," *The Lancet*, vol. 337, no. 8737, p. 375, 1991.

[35] P. Chavanet, B. Bonnotte, D. Caillot, and H. Portier, "Imipenem/teicoplanin for *Rhodococcus equi* pulmonary infection in AIDS patient," *The Lancet*, vol. 337, no. 8744, pp. 794–795, 1991.

[36] M. M. McNeil and J. M. Brown, "Distribution and antimicrobial susceptibility of *Rhodococcus equi* from clinical specimens," *European Journal of Epidemiology*, vol. 8, no. 3, pp. 437–443, 1992.

[37] M. Barcia-Macay, C. Seral, M.-P. Mingeot-Leclercq, P. M. Tulkens, and F. Van Bambeke, "Pharmacodynamic evaluation of the intracellular activities of antibiotics against *Staphylococcus aureus* in a model of THP-1 macrophages," *Antimicrobial Agents and Chemotherapy*, vol. 50, no. 3, pp. 841–851, 2006.

[38] J. F. Prescott and V. M. Nicholson, "The effects of combinations of selected antibiotics on the growth of *Corynebacterium equi*," *Journal of Veterinary Pharmacology and Therapeutics*, vol. 7, no. 1, pp. 61–64, 1984.

[39] A. J. Burton, S. Giguère, T. L. Sturgill et al., "Macrolide- and rifampin-resistant *Rhodococcus equi* on a horse breeding farm, Kentucky, USA," *Emerging Infectious Diseases*, vol. 19, no. 2, pp. 282–285, 2013.

[40] N. Asoh, H. Watanabe, M. Fines-Guyon et al., "Emergence of rifampin-resistant *Rhodococcus equi* with several types of mutations in the rpoB gene among AIDS patients in Northern Thailand," *Journal of Clinical Microbiology*, vol. 41, no. 6, pp. 2337–2340, 2003.