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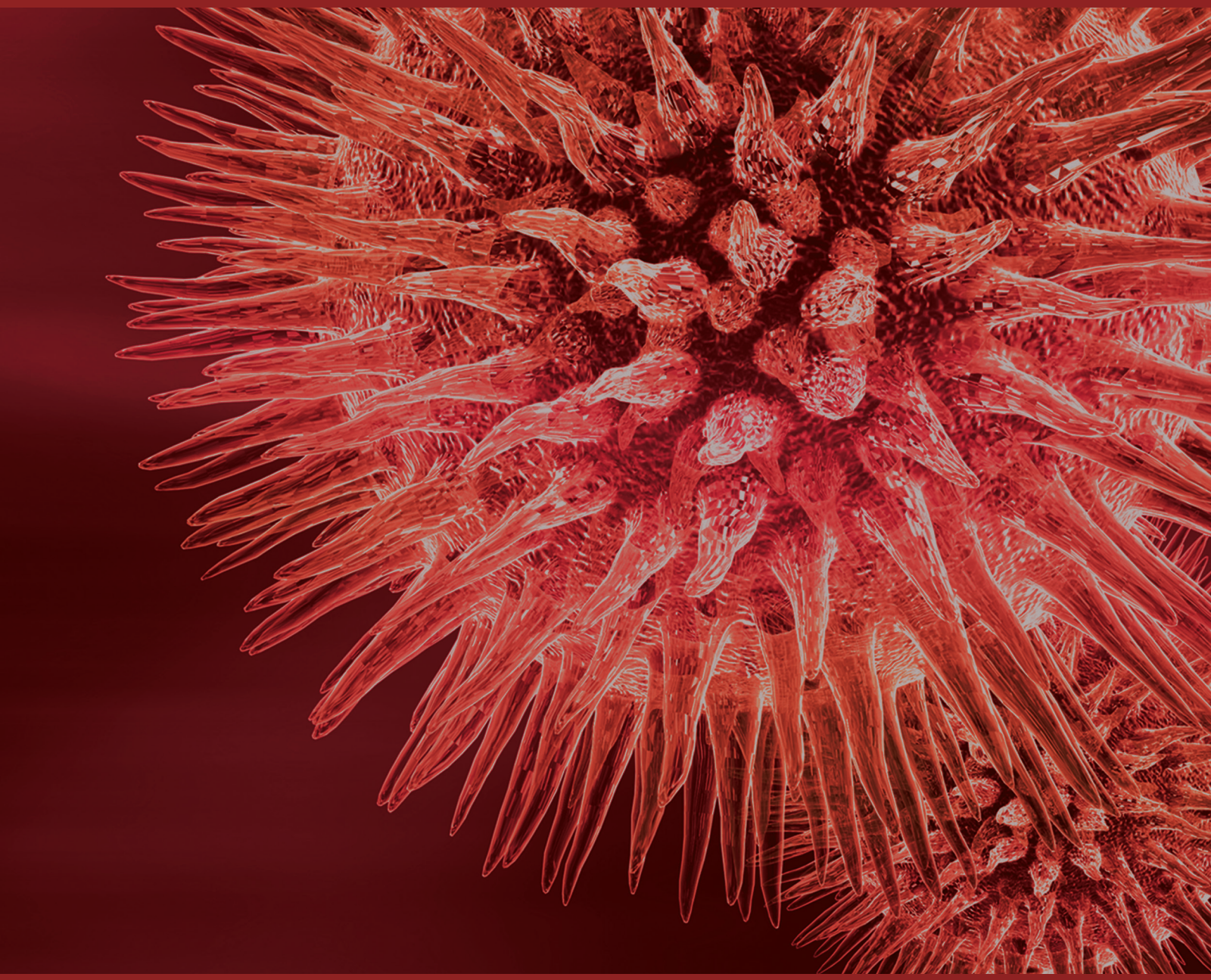
Newly Emerging Parasitic Threats for Human Health: National and International Trends

Guest Editors: Lidia Chomicz, David Bruce Conn, Jacek P. Szaflik, and Beata Szostakowska



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Editorial

Newly Emerging Parasitic Threats for Human Health: National and International Trends

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Parasites impair healthy life and development in hundreds of millions of individuals throughout the world. Various human populations are at serious risk of illness and even death from these pathogens. It should be taken into account that medically important parasites are not only those often infecting human hosts but also species that rarely colonize the human organism in the given region of the world. The latter can induce serious diseases or high mortality rates as they may be not correctly diagnosed or may be left untreated (e.g., dirofilariosis and malaria in Poland). Recently, reemerging parasites and newly emerging species (e.g., free living, facultative parasitic amoebae) have become an increasing worldwide public health problem connected with many mistakes and uncertainties in diagnosis; thus, there is a need for introduction of new diagnostic methods and successive advances in therapeutic management.

The available evidence indicates that there are changes in natural and anthropogenic environments that affect the spread and emergence of these diseases in any given area, including climate change, population growth agricultural intensification, and human encroachment into wildlife habitats. Likewise, increasing environmental pollution and degradation and alterations in microbial and other ecosystems influence the spread, prevalence, and epidemiological dynamics of parasitic infections. This issue addresses the region-dependent, recurrent, and newly emerging health threats generated by the parasites and related pathogens in specific countries. Yet, while the focus of each article in this issue is on specific geopolitical entities, each of these national or regional cases provides a model that can be applied globally to parasitic threats to any country or region. Among different causes, these threats are due to rising numbers of people traveling greater distances, who undertake trips to endemic countries [1].

For example, dengue fever, an emerging vector-borne disease, currently is threatening human populations in different countries of Asia, the Pacific, the Americas, Africa, and Caribbean, some of which are the popular tourist destinations. The World Health Organization and Centers for Disease Control and Prevention reported that the pathogens occur in about 100 countries. Causative agent of the disease is RNA DENV that shows unexpected geographical expansion and an increase in number and severity of outbreaks in the last decades. Dengue infections are transmitted to vertebrate hosts by the external blood-sucking vectors, usually *Aedes* mosquitoes. The geographical range of dengue incidences is expanding; they are much more widespread than has been suggested previously. Climate changes and the human movement between population centers are, among others, important factors of dengue spread. The available data on spread of the viral disease using a novel approach indicates that dengue incidences occur in 128 countries including

those previously classified as dengue-free [2]. At present, dengue is one of the most important mosquito-borne viral diseases in the world that additionally may be transmitted from an infected pregnant mother to her fetus. Although *Aedes aegypti*, the main vector of DENV feeding on human blood, do not remain alive in the winter in temperate zones, not only can some *Aedes* species be active throughout the year in tropical and subtropical zones but they remain alive throughout the winter in the egg stage in temperate climates. This may include invasive species such as *Aedes albopictus,* which has recently colonized vast temperate areas of Europe and North America [3], where it threatens broader transmission of dengue and other diseases such as emerging human dirofilariosis across many new regions of Europe [4]. No diagnostic tests were performed in Poland regarding dengue virus infections until 2005, although, in Europe, sporadic indigenous dengue transmission cases have been detected. The number of Polish travelers to subtropics and tropics, including endemic regions in which dengue fever occurs, increases every year. There was a need to examine and assess a risk of dengue infection during a travel as well as the imported dengue cases. The serological tests performed in the University Centre for Maritime and Tropical Medicine allowed diagnosing the dengue in 149 travelers (19.8% of the investigated group) and indicated a serious risk of dengue virus infection during stays in endemic regions [5].

While these high-profile pandemic and emerging diseases often gain considerable attention from researchers and the popular press, national health ministries, hospitals, and regionally affiliated universities must focus on the health situations and threats that are encountered in their specific areas. Many of these threats come from major neglected diseases that have posed threats to human health for centuries but may be emerging in certain regions or may be emerging within specific groups of patients (e.g., pregnant women), under specific healthcare contexts (e.g., as sequelae to previously treated or cured infections), or under newly emerging situations (e.g., emerging drug resistance). In this issue, some of the most devastating and widespread diseases are presented as they relate to these particular contexts in specific regions of concern. Examples of current health threats in diverse regions and socioeconomic contexts in Asia, North America, South America, and Europe, as well as threats from global migration, are included (e.g., cutaneous leishmaniosis, malaria, Chagas disease, echinococcosis (hydatid disease), and amoebic keratitis). Thus, the issue presents a broad view portraying the intersection of national, regional, and global health. Malaria is among the most prevalent diseases across the globe; its impact on a particular semiurban community in India and within a particular group—pregnant women—is presented; it thus addresses the global public health priority of maternal and child health. Leishmaniosis, which is endemic in many areas, is reported from a nonendemic area in Brazil; the impact of the health-seeking behavior is examined among patients with post-Kala-azar dermal leishmaniosis in Bangladesh.

Besides diverse geographical and biotic situations that affect human health differently in various regions, specific healthcare practices in particular regions may impact the emergence or maintenance of infectious disease. The effective healthcare needs to establish criteria for designating a disease as cured following therapy; methods applied in Brazil and elsewhere and questions of whether it is time to adopt new concepts in dealing with posttherapeutic cure criterion for Chagas disease are critically examined. As a major disease endemic in Brazil and broadly throughout South and Central America, but experiencing a major emergence in both North America and southwestern Europe, this provides an example of how policy in an experienced endemic country might inform better practices in newly threatened regions. The occurrences of *Echinococcus granulosus* sensu stricto infection among Polish patients are studied and analyzed as the important molecular epidemiology question of whether these result from autochthonous transmission or importation from other countries. Emerging threats for human health in Poland due to drug-resistant *Acanthamoeba* keratitis are also studied, among others in terms of adaptive capability of pathogenic strains, causative agents of the serious, vision-threatening disease.

This general theme of international dissemination of infectious diseases is also examined and analyzed in the review; it relates a wide range of viral, bacterial, and parasitic disease threats to the immigration of adoptees and refugees into the United States from countries around the world. Besides their medical and humanitarian importance, both of these raise significant issues regarding national health policies on both domestic and international issues within the respective countries.

In conclusion, it is important to encourage scientists in extension of our knowledge of serious but poorly recognized national/regional health threats, generated by parasites and related pathogens, intensifying during the last decade. It may allow a new appreciation of the importance of the holistic approach that involves *in vivo* and *in vitro* diagnostic techniques and biomolecular detection including advances in control and prevention strategies and therapeutic procedures as well as practical implications for diagnostics, treatments, and prophylaxis for such parasitic infections and diseases.

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David Bruce Conn
Jacek P. Szaflik
Beata Szostakowska

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Research Article

A Study on Health Seeking Behaviors of Patients of Post-Kala-Azar Dermal Leishmaniasis

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Post-Kala-Azar Dermal Leishmaniasis (PKDL) remains a major public health threat in Bangladesh. A cross-sectional study was carried out in Surya Kanta Kala azar Research Centre (SKKRC), Mymensingh, from January 2012 to July 2013 to evaluate the health seeking behaviour and the length of delay of PKDL management. The consecutive 200 diagnosed PKDL cases that got treatment in SKKRC hospital were subjected to evaluation. Most (98%) of the patients were not aware and had no knowledge about PKDL, though 87.5% had a history of history of Kala-azar treatment. Many patients reported first to village doctor (15.5%), the pharmacy shop (10%), or traditional health provider (7.5%) upon recognition of symptom. The time between the initial symptom recognition and first medical consultation (patient delay) ranged from 10 days to 4745 days (13 years) with a median of 373 days (mean: 696; IQR: 138 to 900 days). The time between first medical consultations to definite treatment (system delay) ranged from 0 days to 1971 days (5.4 years), with a median delay of 14 days (mean: 46.48; IQR: 7 to 44 days) that was reported in this study. Age, education, occupation, and residential status had significant association with patient delay ($P < 0.05$). Educational status, occupation, number of treatment providers, and first health care provider had a significant association with system delay ($P < 0.05$). Success in PKDL diagnosis and treatment requires specific behavior from patients and health care providers which facilitate those practices.

1. Introduction

Bangladesh is one of the five countries bearing the major (90%) global burden of visceral leishmaniasis (VL), caused by parasite Leishmania donovani [1]. Twenty million people (18% of the total population) are considered to be at risk for VL and 1000–2000 new cases in Bangladesh are annually identified [1, 2]. Post-Kala-Azar Dermal Leishmaniasis (PKDL), a curious phenomenon believed to be developed after treatment of Kala-Azar [3], presents with wide varieties of skin lesions ranging from hypopigmented marks, to erythematous papules, nodules, and others that appear in individuals. Unlike Kala-Azar, which is ultimately fatal without treatment, it is not usually associated with systemic illness, and patients can remain infectious for years or even decades and it serves as potential reservoir for anthropometric leishmanial transmission [4].

PKDL usually occurs among the poorest segments of rural population. Few data exist on social behavior and economic consequence of PKDL patients. Delay in diagnosis

and treatment increases the risk of transmissibility and morbidity [5, 6]. This is true that sociocultural aspects of care or health seeking behavior particular to Bangladesh are unknown. As humans are the only hosts in Southeast Asia, treatment delay may increase the risk of reservoir for infection. Different social and community factors may play an important role in determining care seeking behavior and the ability to control the disease. Little is known about how individual and community health professionals perceive the disease of PKDL. However, such information is vital because successful control program requires a high level of understanding the pattern of health care seeking behavior.

Evidences revealed that health system, community, family, and other personal issues are influencing factors for the effective health seeking behaviors and case findings [7]. The characteristics of affected patients who reside in endemic area may lead to earlier diagnosis and treatment and thus improve the outcome. The study was designed to investigate PKDL patient care-seeking behavior and the causes of delay of treatment on affected household and identify the risk factors associated with long delay, if any in particular is related to choice of initial health care provider. Delay in receiving treatment for PKDL could be divided into "patient delay" (time from symptoms recognition to initial medical consultation) and "system delay" (time from first medical consultation to definite treatment) [8, 9]. Paucity of any evidence on the magnitudes of delay components (patient delay, health service delay, and total delay) and the factors associated with delay in care- seeking behavior among PKDL patients were among the reasons to conduct this study.

2. Research Design and Methods

2.1. Study Design. This was a cross-sectional study conducted from January 2012 to July 2013. The patient who had confirmed diagnosis of PKDL and got treatment in (Suryia Kanta Kala-Azar Research Centre) SKKRC in Mymensingh was selected successively and interviewed. The pretested questionnaires were used to collect information from the patients. Sample size was determined with a single population proportion formula. Based on the estimated incidence of PKDL (21 cases per 10,000 person-years in 2007), the rising PKDL of the countries studied, a maximum allowed error of 10%, and a 95% confidence interval, the least reliable sample size would have been 150 patients. However, as it was planned to perform bivariate logistic regression analysis, the rule of thumb was used; hence the sample size was calculated at 200 study subjects owing to the large number of studied variables. Logical cross tabulations were analyzed along with Chi-Square test in SPSS 14. Standardized definitions of the different types of delay were followed. Both descriptive and inferential statistics were determined. The rate of presentation to health facility or start of treatment was calculated as Rate = e/d, where e is the number of events occurring over time (such as presentation to health institution or commencement of treatment) and d is the total number of person days (see Table 1 for the specific calculations). The level of significance was set at $P < 0.05$. Frequencies and proportions were used for the descriptive analysis. Differences in proportions were compared for significance using Chi-Square (χ^2) test. The ANOVA test or 2-sample student's t-test and χ^2 test were used to assess differences considering each group is approximately normal. Cross-tabulations and bivariate logistic regression were done to identify the most important predictor variables of PKDL treatment seeking behavior.

2.2. Patient Delay. Time between the initial symptoms recognition by the patient and first medical consultation.

2.3. System Delay. Time between first medical consultations to definite treatment.

2.4. Total Delay. Time from the onset of symptoms until treatment was commenced and was made of above 2 components.

2.5. Focus Group Discussion. Quick participatory methods (Focus Group Discussion) were used to generate information on the myths, knowledge, attitudes, practices, and perceptions of the patients about Post Kala-Azar Dermal Leishmaniasis.

Each FGD involved 10 participants including equal number of both sexes and age ranged from 18 to 60 years. Total two focus group discussions were held in the study area. The informant groups were asked to discuss PKDL, especially the local terminology, knowledge, belief, attitude, and practice about PKDL.

Ethical clearance was obtained from Mymensingh Medical College Hospital and partially funded by DGHS (Director General of Health Services), Bangladesh. After giving all this information, signed written informed consent was obtained from the patient/guardian by signature/finger impressions. In case of subjects below 18 years, assent was taken.

3. Result

3.1. General Characteristics of the Patients. The patient's age ranged between 4 years and 70 years (mean: 24.4 years). Mean BMI was 20.6 ± 4.05 kg. The average monthly family cash income less than 100 US$ (1 US$ = 80 taka) was 76%, whereas only 20% had income >US $150.00/month. Some characteristics of the study participants are shown in Table 1. The median travel time to the nearest public health facility in a single trip was 1 hr (inter quartile range [IQR] = 1.5 hours) and 0.33 hours (IQR = 0.25 hours) for rural and urban patients, respectively, with a statistically significant difference (Mann-Whitney U test = 7.97, $P < 0.001$).

3.2. Clinical Characteristics with Background History. Eighty-seven percent (87%) had history of visceral leishmaniasis (VL) with the median number of years of PKDL presentation and previous treatment for VL was 4.5 years, ranging from 1 to 26 years. Half of the cases (50.6%) were presented in 5 years of VL treatment.

Most of the cases were treated with Sodium Stibogluconate (SSG) for VL (70.5%). Of these, 1/3 had received a total dose that was lower than recommended by the World Health Organisation (28–30 days of Sodium Stibogluconate

Table 1: Formula for calculating specific rates (different components of delay).

Rate	e (numerator)	d (denominator)
Total delay	Number of PKDL treatments	Total person-days of initial presentation in a community
Patient delay	Number of presentations to health provider	Total person-days of presentation before first presentation to a health provider
Health service or system delay	Number of PKDL treatments	Total person-days of skin lesion between commenced first presentation to a health provider and final treatment

(SSG)). Two patients presented after receiving liposomal amphotericin (total 15 mg/kg body weight). Four patients had history of both SSG and miltefosine (2.5 mg/kg for 28 days) treatment for VL in different time period. Thirty-eight percent had family history of VL.

Four patients presented after having received a previous treatment for PKDL with SSG (120 doses). All of those previously treated patients reported to have been cured before it reappeared again. It remains unclear whether those patients were labelled as a relapse or can be considered new case. Four patients were treated with miltefosine for 12 weeks and 3 patients got liposomal amphotericin 5 mg/kg body weight for 6 days without any improvement even after one year.

Symptoms and signs of PKDL were experienced by the patients including hypomelanotic, macular, papular, or nodular rashes (Table 2). Most of the patients (74%) presented with hypomelanotic spot amongst 78.5% considered grade III involving abdomen and limbs [10]. Diagnosis was mainly based on using rapid diagnostic test (RK-39) and skin manifestation. Ninety-seven percent were positive RK-39 tests and slit skin biopsy among 75 cases (out of 140 cases). Significant difference was not observed in time to presentation or initiation of treatment with all the symptoms after examining the symptoms separately or in groups.

3.3. Knowledge and Practice about the Disease. This study revealed that only 7% had heard the name of PKDL (Table 3). Almost all the respondents (98%) were not aware and had no knowledge about the diseases, though 87.5% had history of VL treatment. However 21% believed that the disease is possibly curable. Most of the patients (90%) learned about the disease from hospital. Among the respondents, 70% always used mosquitoes net at bed time but 28% regularly used to sleep at day time. Almost all (96.5%) had sleeping bed. Insecticide spray was done among 87% respondents house in the last year (Table 3). Only nine percent (9%) of the respondents believed that Kala-Azar spreads through sand fly bites.

3.4. Type of Facility First Visited. Two hundred patients consulted with various types of health care providers as the first place for seeking help with most of them visiting private health care providers.

In general, fifteen percent (15.5%) patients reported first to village doctor, 10% to the pharmacy shop, and 7.5% to "Kabiraj" (traditional health provider) upon recognition of symptom. One-third (28.5%) directly went to UZHC (Upazilla Health Complex, Government Hospital) and 15% reported first to the registered doctor. The median total delay from noticing symptoms to commencement of treatment seems higher (417 days) among those patients who reported first to traditional/spiritual healers. Majority (88%) attended at SKKRC as their 2nd- or 3rd-order treatment location. The average number of the different types of health care providers that were visited till the start of definite treatment was 2.43 (median = 3). This was without taking into account the number of repeated visits made to the same health care provider. Most of the patients (86.5%) referred to the SKKRC by registered doctor for definite treatment (Table 4).

3.5. Distribution of Delay

3.5.1. Patient Delay. The rate of presentation to health care providers in this study was 14 per 10000 person-days of symptom recognition in the community (Table 4). Patient delay ranged from 10 days to 4,765 days (13 years) with a median of 373 days (mean: 696, IQR: 138 to 900 days). Patient delay has contributed 91% of the overall total delay to treatment. However, only 32% of patients were able to report within the first six months after developing skin manifestation. The distribution of the different components of delay is summarized in Table 5.

3.5.2. Health System Delay. Health system delay in our context was the time ranging from patient's first contact to any health facility to the date of commencement of definite treatment. This comprises time spent during movement between facilities, diagnosis, and time between diagnoses and start of treatment. Generally, The system delay ranged from 0 days to 54 days, with a median total health system delay of 14 days (mean: 46.48, IQR: 7 to 44 days) was reported in this study, making rate of 447 per 10000 person-days of actively seeking for diagnosis and then treatment (Table 5). Nine percent of total delay was contributed to health system delay.

3.6. Determinants of Delay. The median time between onset of symptoms of PKDL and presentation at the SKKRC was 13.6 months (R: 23 to 4594 days). The patients who came from outside Mymensingh district had longest duration of delay (M: 66 months); in contrast those who were within the district had the shortest delay (M: 9 months). On bivariate analysis, age, education, occupation, and residential status had significant statistical association with patient delay ($P < 0.05$).

Table 2: Baseline characteristics of the study population.

Characteristics (n = 200)	Categories	Number (%)	Patient delay (days)		System delay (days)	
			Mean	Median	Mean	Median
Sex	Male	129 (64.5)	761.70	417	55.74	11
	Female	71 (35.5)	576	293	29.31	13
Age (years)	01–15	63 (31.5)	542.17	290	37.62	13
	16–30	80 (25.6)	670.84	405.50	76	12
	31–45	37 (18.5)	453.76	400	16.62	11
	46–60	15 (7.5)	1171.13	814	12.13	10
	>60	5 (2.5)	446..40	344	10	5
Residential status	Urban	2 (1)	22	22	62	62
	Rural	198 (99)	702.87	380	46.33	12
Address (Upazila)	Gafargaon	80 (40)	579	251	26.06	11
	Trishal	68 (34)	674	417	40.12	12.50
	Baluka	21 (10.5)	537.40	406.31	158	11
	Mymensingh Sadar	9 (4.5)	392	344	17.11	18
	Fulbaria	5 (2.5)	317.50	317.50	25.50	25.50
	Muktagacha	4 (2)	251.30	173	13	11
	Tangail	9 (4.5)	992.67	1174	12.11	6
	Jamalpur	4 (2)	1979.50	1979.50	2	2
Educational status	Illiterate	45 (22.5)	1063	1063	16	16
	Up to 5th classes	108 (54)	769.79	420	52.49	12
	Up to 12th classes	31 (15.5)	223.33	163	24	10
	Graduate or above	16 (8)	87.46	97	16	16
Occupation of the population	Agriculture worker	28 (14)	594.71	600	14.18	11.50
	Business	33 (16.5)	1397..45	1109	11.55	10
	Daily labor	5 (2.5)	1084.80	1600	15	13
	Housewife	31 (15.5)	361	369	42.13	13
	Student	54 (27)	537.48	294	19.13	12
	Unemployed	47 (23.5)	653.85	251	127	13
Household income per month (US$)	<100	152 (76)	630.84	387	43	11
	100–150	8 (4)	1012	89.50	165.25	46.50
	151–200	38 (19)	833.97	277	35.55	13
	>200	2 (1)	767	767	37	37
Housing	Floor and wall made by mud	195 (97.5)	705.30	387	47.19	12
	Floor and wall made by brick and tin	5 (2.5)	335.80	179	18.80	15

Educational status, occupation, number of treatment providers, and first healthcare provider had a significant statistical association with system delay ($P < 0.05$) (Table 6). However, family income could not be significant to remain in any type of delay. Thus, patient's educational status (up to class five) had longer health system delay than patients who graduated.

During the discussion, the participants explained their own perception about PKDL and the risk of PKDL in the community. One male participant said "I never seen such type of skin infection and i have no idea about the disease process and name of the disease. One believed that consumption of arsenic water may play a mechanism of disease process."

PKDL is a mysterious disease, it causes no physical problem. One female said, "Kala azar treatment is not new for me. The last one was the second attack for me. I went directly to the public hospital last time." One female from Trishal Upazila mentioned "People are not aware of the risk of PKDL, probably because it is not fatal disease with clear symptoms and progression."

One male said "I went to the local doctor (drug store) near the village and took some medicine but the medicine did not work. The care giver never asked me about past history of

Table 3: Clinical characteristics and background history of PKDL patients.

Characteristics (n = 200)	Categories	Number	Percentage
Past history of Kala-Azar treatment	Yes	175	87.5
	No	25	12.5
Drug used for KA treatment	Sodium Stibogluconate	141	70.5
	Miltefosine	28	1
	Liposomal amphotericin	2	1
Past history of PKDL	Yes	11	5.5
	No	189	94.5
Drug used for PKDL treatment	Sodium stibogluconate	5	2.5
	Miltefosine	4	2
	Liposomal amphotericin	2	1
Family history of KA	Yes	77	38.5
	No	123	61.5
Types of skin lesion	Hypomelanotic	148	74
	Nodular	11	5.5
	Macular	5	2.5
	Mixed	36	18
Splenomegaly	Yes	14	7
	No	186	93
Hepatomegaly	Yes	2	1
	No	198	99
RK-39 test	Positive	194	97
	Negative	6	3
Skin biopsy for LD body	Yes	75	38.5
	No	65	32.5
	Not done	40	20
Treatment	Miltefosine	146	73
	Ambisome	38	19
	Amphotericin deoxycholate	14	7
	Stibogluconate	2	1

Kala-azar treatment. We didnt have the money to buy more medicine. When my condition was not improved I noticed it to the government hospital. It is often easier (convenient) to go to a private place close by than going to the government hospital, there is usually a long queue there."

One doctor said "There is no local terminology of PKDL. So we need to address the issue to develop BCC materials. Sister from SKKRC suggested the disease may be defined as skin problem after Kala-azar treatment." Another staff mentioned "we need more detail discussion to find out the appropriate terminology of PKDL."

4. Discussion

The study highlights the uncovered duration of infectiousness of PKDL in the community that is a potential source of spread of Kala-Azar. Approaches that take this into account may convey a more appropriate early diagnosis and control of the disease.

It is observed that the number of days of patient delay is much longer among (mean: 696 days) PKDL patients in Bangladesh. The duration of unacceptable level of patient delay observed in this study is consistent with other diseases delay related study [8–14]. A substantial proportion of the total delay of treatment was attributed to patient delay, an important preventable period of infectiousness in the community caused probably by the lack of awareness and knowledge about PKDL.

In this study area, the public health facilities are the common first choice of care for PKDL patients, with more than 60% of individuals presenting to a traditional, alternate, or nonregistered doctor initially due to easy approaches and low cost, similar to other studies specially in case of tuberculosis [12]. However, considerable number of patients contacts the private practitioners (15%) as a first choice, indicating the importance of this sector. In cases, where PKDL was suspected, private provider should refer the patient to public hospital. Significant association was observed between choice of health providers (first contact) and health system delay where visit to traditional healers increased the delay of treatment [15]. Rural residence was a risk factor for late presentation and diagnosis. This may be explained by several factors, including poorer access to health care in rural areas,

Table 4: Knowledge, attitude, and practice of the study patients about PKDL.

Items	Respondents	Number	Patients delay (in days)		System delay (in days)	
			Mean	Median	Mean	Median
Heard the name PKDL	Yes	14 (7)	499.29	97	112.14	38
	No	186 (93)	710.87	380	41.54	11.15
Knowledge about PKDL	Yes	4 (2)	393	393	29	29
	No	196 (98)	702	373	41.45	12
KA is an infectious disease, transmitted by sand fly bite	Yes	18 (9)	393	393	29	10
	No	182 (91)	692.88	373	43.46	12
Complete cure of the disease is possible	Yes	42 (21)	792	402	23	14
	No	2 (1)	680	387	12	12
	Not known	156 (78)	723	320	27	11.15
Frequency of using mosquitoes net	Always	140 (70)	696	383	46.48	12
	Often	32 (16)	732	470	57.80	14
Sleeping bed	Cot	193 (96.5)	670.86	387	47.71	12
	Floor	7 (3.5)	1390	345	12.71	14
Habit of day time sleeping	Regular	56 (28)	756	420	14.41	10.50
	Occasional	118 (59)	740.50	393	25.75	12
	Rarely	26 (13)	361	121	209	20
Insecticide spray in the house in last 1 year	Yes	174 (87)	673.33	344	49.77	12
	No	26 (13)	884	503	24.50	9
First health care provider	Graduate doctor	30 (15)	603.37	238.50	154.90	11
	Union dispensary (Govt.)	8 (4)	377.50	310	19.88	13.50
	Traditional healer	14 (7.5)	604.67	417	33.47	30
	Paramedic	19 (9.5)	661.42	344	19.95	14
	Village doctor	31 (15.5)	815.10	327	15.74	14
	Upazila health complex (UZHC)	57 (28.5)	698	393	14.5	10
	Homeopath	11 (5.5)	731.7	373	64.36	12
	Pharmacy shop	20 (10)	663.40	393.50	14.35	13
	Others	9 (4.5)	1167	600	12	9

Table 5: Distribution of delays and rates to an event throughout the course of health seeking and start of treatment among PKDL patients.

Type of delay	Mean (median)	Rates
Total delay	768.41 (408)	13 per 10000 person-days of skin lesion
Patient delay	696.06 (373)	14 per 10000 person-days of skin lesion
Health system delay	46.48 (14)	447 per 10000 person-days of actively seeking diagnosis

difference in education levels, and lack of supervision of health staff at peripheral level [15].

The unacceptable higher patient delay was possibly due to asymptomatic nature of the disease except skin changes and the fact that community had no knowledge about the nature of PKDL. Unfortunately, most of the patients had past or family history of Kala-Azar and treated with different drugs in different time period. The patients were totally unaware about the linkage between Kala-Azar and PKDL.

This is an important preventable period of infectiousness in the community caused by the failure of recognition by the patients. Changes in the skin colour are the only symptom reported by PKDL patients which did not prompt them to seek health care. It is worth mentioning that PKDL is suspected only by the skin changes with past history of Kala-Azar. It might be that more "missed" cases occur among those who present with no background history. Some patients presented with past history of PKDL treatment with different drugs among which two patients again developed skin lesion after initial disappearances and others showed no improvement even after one year (Table 3). There is no good evidence based on treatment of PKDL; even no marker of cure is available at public health facilities.

Most of the patients of PKDL in this study area did not present to health facilities early and/or if they presented,

Table 6: Relationship between sociodemographic factors and patient delay and system delay (bivariate linear regression analysis).

Factor	Patient delay			System delay		
	β coefficient	(95% confidence interval)	(P value)	β coefficient	(95% confidence interval)	(P value)
Age	0.140	(0.091–16.548)	0.048	−0.049	(−2.460–1.192)	0.492
Sex	−0.098	(−449.269–79.479)	0.169	−0.064	(−84.931–31.675)	0.369
Education	−0.139	(−202.397–(−0.180))	0.050	0.145	(−0.993–45.429)	0.041
Occupation	−0.170	(−112.070–(−11.15))	0.017	0.174	(2.849–25.086)	0.014
Residential status	0.253	(64.518–213.598)	0.000	−0.002	(−17.216–16.674)	0.975
Monthly income	0.117	(−0.005–0.056)	0.100	−0.002	(−0.007–0.007)	0.983
First health care provider	NA			−0.169	(−71.421–(−7.090))	0.017
Number of health care providers	NA			−0.147	(−24.501–(−0.681))	0.038

they did not receive treatment on time and thus continued to serve as reservoirs of infection. Even though the short system delay found in this study may be explained by the development of the good general health care, choice of first health care provider can prolong the system delay. We found clear associations between delay and the type of health care provider first visited by the patient. Long system delays were more frequent by the traditional practitioner and least frequent by a registered or government physician. Education on recognition of PKDL by traditional and village practitioner may help prevent long health care provider delays, as may implementation of public-private mix projects in rural areas.

Promotion of a concerted effort to increase awareness of the signs and symptoms of PKDL in the general population in the endemic area and encouraging self-referral to health services is crucial to increase the passive case detection.

The study showed that patients who were from outside Mymensingh district had much longer delay, probably due to lack of well-developed treatment facilities and non-well-concentrated different elimination programme of that area [7].

Socioeconomic indicators are the strong determinants of the health seeking behavior of the patients which is, in turn, the main determinant of patient delay; therefore in-depth analysis was crucial to provide detailed information about the situation. Study showed that PKDL patients are a disadvantaged group in their communities. The illiteracy rates reported were significantly higher. The known association between poverty and PKDL or Kala-Azar was not found in the study though it has been well documented and the diseases have been labelled as a "disease of poverty." Hence poverty reduction would contribute to reduction of the PKDL burden in endemic area [16, 17].

This study reveals that most of the patients are below 45 years (90%) which is consistent with the other study and the size of males is almost double the size of females. Rural people are usually suffering from PKDL as about 99% were from rural area [12, 15]. Ages of the studied patients are considered indicators of the progress in the treatment of the PKDL. Although the majority of households used bed net during sleep, its acceptability and efficacy against KA need to be ascertained [16]. Lack of knowledge about the involvement of humans in the transmissibility and infectious nature of the disease (98%) is a matter of concern for adoption of preventive measures against the disease. Awareness about the signs and symptoms of a disease can prompt patients to seek early treatment. Among the various factors cited earlier which determine treatment-seeking, preexisting health beliefs and perception about illness play an important role (Table 3).

Patients generally choose initial traditional healers; medicine shop or village practitioner resorted to non-prescribed medications from pharmacies. Meanwhile, they would also seek care at government health care provider. This study showed that visiting several health care providers was significantly associated with longer delay. Sociodemographic characteristics proved to be significant predictors of delay in almost all countries [18]. Private health care providers did not have strong linkages with the mainstream public health system. In addition, lack of continuing medical education contributes to poor knowledge and therefore poor ability to immediately diagnose a case. Education and collaboration with private health care providers in all countries are therefore essential to reduce treatment delay. The free services of the Kala-Azar and PKDL treatment should be more widely known to the community.

This study reveals that a large proportion of the community members have no knowledge on PKDL infection. Therefore, there is need to strengthen public awareness efforts against stigmatization with regard to Kala-Azar and PKDL infections.

Conflict of Interests

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

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Research Article

Emerging Threats for Human Health in Poland: Pathogenic Isolates from Drug Resistant *Acanthamoeba* Keratitis Monitored in terms of Their *In Vitro* Dynamics and Temperature Adaptability

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Amphizoic amoebae generate a serious human health threat due to their pathogenic potential as facultative parasites, causative agents of vision-threatening *Acanthamoeba* keratitis (AK). Recently, AK incidences have been reported with increasing frequency worldwide, particularly in contact lens wearers. In our study, severe cases of AK in Poland and respective pathogenic isolates were assessed at clinical, morphological, and molecular levels. Misdiagnoses and the unsuccessful treatment in other ophthalmic units delayed suitable therapy, and resistance to applied chemicals resulted in severe courses and treatment difficulties. Molecular assessment indicated that all sequenced pathogenic corneal isolates deriving from Polish patients with AK examined by us showed 98–100% homology with *Acanthamoeba* genotype T4, the most prevalent genotype in this human ocular infection worldwide. *In vitro* assays revealed that the pathogenic strains are able to grow at elevated temperature and have a wide adaptive capability. This study is our subsequent *in vitro* investigation on pathogenic *Acanthamoeba* strains of AK originating from Polish patients. Further investigations designed to foster a better understanding of the factors leading to an increase of AK observed in the past years in Poland may help to prevent or at least better cope with future cases.

1. Introduction

Amoebae belonging to the genus *Acanthamoeba* are ubiquitous and widely distributed in natural and man-made environments worldwide. *Acanthamoeba* spp. are free-living organisms existing as vegetative mononuclear trophozoites with characteristic acanthopodia and as double-walled dormant cysts, developing after the growth phase as well as under harsh conditions. The protists occur in sea, fresh, tapwater, and drinking water systems and in swimming pools, air conditioning systems, and humidifiers; they also occur in dust and soil, on fruits and vegetables, and in animal bodies.

They have been recognized in the hospital environment as contaminants of surgical instruments and dental irrigation units, as well as in various human cavities and tissues, and on skin surfaces, oral cavities, paranasal sinuses, lungs, and brain [1–4]; trophozoites and cysts of *Acanthamoeba* also have been found by us among the microbiota of periodontal biofilms, accompanying infections with *Entamoeba gingivalis* in patients with systemic diseases [5].

The free-living amoebae complete their life cycles in different external environments, without entering humans or animals, and feed on microorganisms and small organic particles. However, in some circumstances, they are able to enter human bodies from different sources, colonize some organs, multiply within them, and thus exist as opportunistic parasites causing pathogenic effects. Epidemiological, serological, biochemical, and molecular investigations have shown that people may be exposed to pathogenic as well as nonpathogenic *Acanthamoeba* strains [3, 6]. It seems that the amoebae come into the human body relatively frequently, without pathogenic consequences, as indicated by 50–100% of the healthy population having specific antibodies [7–9]. However, *Acanthamoeba* spp. may be causative agents of the rare but usually fatal granulomatous amoebic encephalitis, developing in immunocompromised individuals as an opportunistic infection [4], and of the vision-threatening *Acanthamoeba* keratitis (AK) that occurs mainly in immunecompetent persons. AK was first recognized in 1973 in a Texas rancher [10]. The eye disease symptoms include redness, photophobia, excessive tearing, severe eye pain, and significant deterioration of the visual acuity; without adequate therapy the amoebic infections may lead to blindness [3, 10–17].

The clinical symptoms of AK are nonspecific, similar to those observed in the course of other eye diseases, thus misdiagnosed as viral, fungal, or bacterial keratitis; a mixed keratitis caused by concomitant bacterial, viral, fungal, and *Acanthamoeba* infections is also known. This is why the diagnosis based on clinical symptoms alone is not sufficient to indicate the causative agent of human keratitis. The proper diagnosis needs laboratory identification of the specific pathogen for confirmation. Corneal scrapings are optimal materials for AK diagnosis. The microscopic visualization of amoebae in slides prepared directly from corneal scraping and by *in vitro* cultivation of the amoebic isolates deriving from these samples may be helpful also to verify previous misdiagnoses [3, 17, 18].

For years, *Acanthamoeba* isolates/strains were classified based on morphological criteria, mainly cyst size and structure: three morphological groups and 18 *Acanthamoeba* species were determined [3, 19, 20]. In the recent past, with the development of molecular systematics, PCR techniques and sequence analysis of the 18S rRNA gene have been used for diagnostics and for characterization of clinical and environmental *Acanthamoeba* isolates [4, 21–25].

At present, 19 genotypes are distinguished [17].

The treatment of AK is difficult, and often a resistance to pharmacotherapy develops, among other factors, due to an improper diagnosis leading to delayed suitable therapy. Moreover, the amoeba cysts are highly resistant to chemicals: disinfectants and antimicrobial and antiparasitic drugs; antiamoebic drugs are often efficient in high concentrations, which, however, are toxic for human cells [3, 4, 26–30]. Thus, despite therapeutic advances, the treatment of the keratitis caused by pathogenic *Acanthamoeba* strains continues to be difficult and is often unsuccessful.

In our earlier studies of 2011–2014 [16, 31], several pathogenic *Acanthamoeba* strains acquired from serious AK cases, variable in corneal symptom intensity and in the response to the instituted therapy, were assessed. The corneal strains have been classified as morphological group II and, in genotype identification carried out by us with PCR based on sequence analysis of the 18S rRNA, determined as T4 genotypes. *In vitro* viabilities of particular strains were monitored and compared. Results of the monitoring we analyzed in regard to the survival time of amoebae, AK course as well as therapeutic management difficulties/efficacies [16]. It has been revealed in our preliminary studies that monitoring of *in vitro* viability of pathogenic *Acanthamoeba* strains isolated from the infected eyes may be a useful tool for therapeutic prognosis.

A temperature tolerance of the amoebae, particularly growth/multiplication at high temperature, is often studied and reported, because it is considered as an indirect marker of potential pathogenicity of *Acanthamoeba* strains [3, 17, 32, 33]. However, apart from our preliminary investigations, no studies were undertaken with diagnosed pathogenic *Acanthamoeba* strains originating of AK in terms of their *in vitro* viability in changed temperature conditions.

In the present study, subsequent pathogenic *Acanthamoeba* isolates acquired by us from human *Acanthamoeba* keratitis cases unsuccessfully treated with antibacterial and antifungal medications and poorly responding to topical antiamoebic pharmacotherapy, assessed at clinical, morphological, and molecular levels, were *in vitro* monitored. The corneal isolates diagnosed as pathogenic *Acanthamoeba* strains were examined and compared to one another as well as to the environmental *Acanthamoeba castellanii* Neff strain in terms of their *in vitro* temperature sensibility/tolerance. Moreover, dynamics of these amoebic populations, cultivated parallel *in vitro*, their density, and morphophysiological status of particular developmental stages were assessed.

2. Materials and Methods

2.1. Acanthamoeba Pathogenic Corneal Strains: Isolates and Cultures. The material deriving from twenty-two patients who reported to our hospital in 2010–2014 at different times after first symptoms of keratitis appeared and who were under suspicion of *Acanthamoeba* infection was analyzed. The patients complained of photophobia, pain, excessive tearing, and deterioration of visual acuity. In the clinical diagnosis, noninvasive methods of slit-lamp and *in vivo* confocal microscopy were used. During the laboratory microbiological and parasitological diagnosis, direct microscopic examinations of corneal scraping material and *in vitro* cultures derived from those scrapings were performed to determine etiological agents of the eye deteriorations. Although *Acanthamoeba* infections were confirmed for all

cases, the isolates marked as I-1 up to I-22 showed a high degree of variation.

In ten cases that were properly diagnosed early, 3 to 15 days after first AK symptoms appeared, and thus received early suitable treatment, an improvement was observed relatively quickly. As the isolates showed *in vitro* weak dynamics, trophozoites were dead in the culture medium after 8–10 days, no transformation into cysts was observed, and thus no material from these ten strains was kept for further *in vitro* investigations.

Another two isolates have originated from incidences in which AK has been diagnosed and pathogenic amoebae were isolated; however after moderate improvement, the two patients have not continued therapy and no information on treatment efficacy was available.

For these reasons, finally, ten remaining isolates acquired from the corneal material have been included in this analysis.

The patients, three men, all contact lens wearers, and seven women, six contact lens wearers, one of whom bathed in swimming pool, and one non-contact-lens wearer with a history of swimming in a lake, all 26–42 years old, had different intensities of pathogenic changes in their eyes. All of them had previously been unsuccessfully treated in other ophthalmic units with antifungal and antibacterial pharmaceutics; thus proper diagnosis was delayed ranging from 25 to 45 days after first symptoms appeared.

The initial identification of causative agents was achieved by *in vivo* confocal microscopy. The final diagnosis was made/confirmed by corneal scrapings examinations in the light microscope, first directly and next with enrichment during *in vitro* cultivation; the isolates were assessed at cytological and molecular levels.

The isolates originating from corneal samples of the AK patients, initially examined in wet-mount slides to visualize cysts or/and trophozoites of amoebae, were cultured under bacteria-free conditions for one to three years in sterile 15 mL tubes containing BSC culture medium [34] enriched with 10% calf serum, incubated at 27°C, and subcultured twice each month.

Additionally, environmental *A. castellanii* Neff strain, after years with serial passages in the same growth medium in the Laboratory of the Department of Medical Biology, Medical University of Warsaw, Poland, was used in this study.

2.2. Genotyping of Acanthamoeba Strains. All samples/isolates were also examined by PCR techniques for specific detection of *Acanthamoeba* DNA and to determine genotypes of the particular strains. Extraction of DNA from the samples was performed using commercial Sherlock AX Kit (A&A Biotechnology, Gdynia, Poland). Extraction of DNA from cultured *in vitro* isolates was performed using commercial Genomic Mini Kit (A&A Biotechnology) for routine genomic DNA extraction, according to the manufacturer's instructions. Then, DNA was stored at −20°C. An *Acanthamoeba*-specific PCR following the protocol established by Schroeder et al. [21] amplifying a fragment of the 18S rRNA gene with the primers JDP1 (5′GGCCCAGATCGTTTACCGTGAA3′) and JDP2 (5′TCTCACAAGCTGCTAGGGAGTCA3′) was applied. PCR products were analyzed using GelDoc-IT Imaging Systems (UVP, USA) after gel electrophoresis on agarose gel (Sigma, St. Louis, Missouri) stained with Midori Green DNA stain (Nippon Genetics Europe GmbH, Germany). Cycle sequencing was performed and sequences obtained were compared with data available in GenBank using GeneStudio Pro Software (GeneStudio, Inc., Suwanee, Georgia).

2.3. In Vitro Growth of Acanthamoeba Isolates at Different Temperatures. The population dynamics of the corneal and environmental *Acanthamoeba* isolates cultured *in vitro* in the aforementioned growth medium under bacteria-free conditions at 27°C was systematically monitored in terms of developmental stage status by phase-contrast light microscopy.

For temperature assays, on the second day following subculturing, all cultures were shaken intensively and one mL samples of strains were transferred to 1.5 mL Eppendorf tubes containing culture medium. Next, the samples of the respective cultured strains were exposed to either 20°C, 37°C, or 42°C during 3–7 days following regular subculturing.

In vitro viability and dynamics of each particular strain population were then assessed and compared.

The morphophysiological changes and overall numbers of the amoebae as well as proportion of trophozoites and cysts were microscopically determined in the exponential and stationary growth phases. During exposure to changed temperature, cultures were vigorously shaken and 10 μL samples were successively taken for assessment of each isolate. The changes in overall number of amoebae and number of trophozoites and cysts were counted with the aid of a Burker hemocytometer. The ability of amoebae to multiply *in vitro* was examined; the ranges of four counts calculated for 1 mL of culture medium were compared for particular strains and assays. Results of the investigations were analyzed statistically (ANOVA, Student-Newman-Keuls method, $p < 0.05$).

3. Results

The material assessed in our study was acquired from ten patients with symptoms of *Acanthamoeba* keratitis including redness, photophobia, severe eye pain, excessive tearing, and lid edema, as well as significant deterioration of visual acuity. Active epithelial inflammations, corneal ulcers, and characteristic ringlike stromal infiltration were detected by slit-lamp in the affected eyes (Figure 1).

Keratitis symptoms intensified in different degrees as the disease progressed.

3.1. Effects of Differential Diagnosis. AK was finally confirmed in all ten cases; however, several patients experienced significant delayed proper diagnosis. In the five cases in which patients reported late to their physicians and AK diagnosis was performed more than four weeks after the first keratitis symptoms appeared, hyperreflective objects identified presumably as *Acanthamoeba* cysts by *in vivo* confocal microscopy were revealed (Figure 2).

At the same time, in 3 of five cases, amoebic, bacterial, and fungal coinfections (*P. aeruginosa, E. faecalis*, and *Candida* sp.) were revealed in the microbiological diagnosis (Table 1).

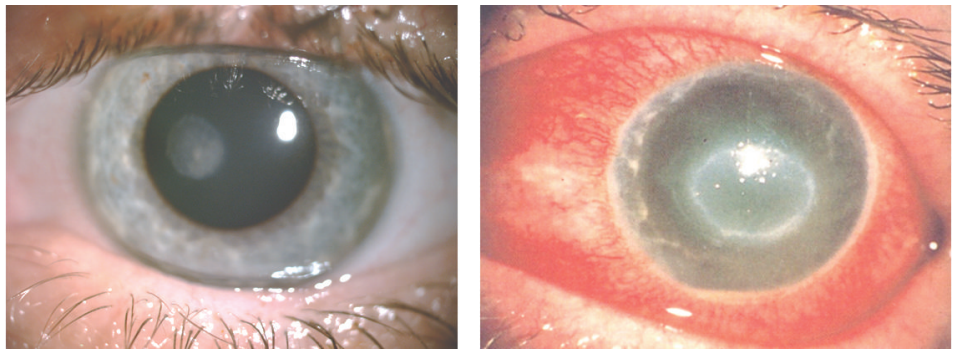

Figure 1: Hyperreflective tissue in corneal ulceration of severe *Acanthamoeba* keratitis cases; slit-lamp photographs.

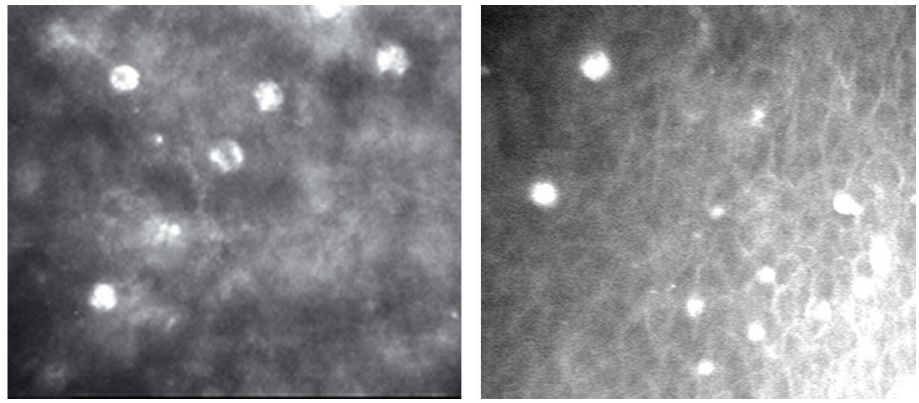

Figure 2: Hyperreflective objects, presumable *Acanthamoeba* cysts; *in vivo* confocal microscopy.

In five remaining cases, in which the duration of symptoms prior to proper diagnosis was somewhat shorter, no cysts were visualized by *in vivo* confocal microscopy.

Finally, in all samples *Acanthamoeba* infection was confirmed by laboratory methods.

In parasitological microscopic examinations, *Acanthamoeba* cysts and trophozoites were found in different materials: some of them were detected immediately in wet-mount slides prepared from corneal scrapings, whereas others were detected after 2–7 days of *in vitro* cultivation of material deriving of these isolates (Table 1).

The results of molecular examinations of the isolates and a comparison of the obtained sequences with those available in GenBank revealed that all sequenced isolates showed 98–100% homology with isolates belonging to the T4 genotype. However, there were differences between particular strains. Respective highest sequence identities were found with strains originating from both environment sources and human ocular infections and from various countries of Europe, Asia, and America.

3.2. Treatment Difficulties. Material included in this analysis originated from AK cases that were previously unsuccessfully treated because of improper diagnosis.

Moderate or severe course of the eye disease needed prolonged therapeutic management. Eyes affected were treated with a topically applied agent, among others, a combination of chlorhexidine (0.02%) and polyhexamethylene biguanide (PHMB, 0.02%), propamidine isethionate (0.1%), and antibiotic neomycin; however the treatment was without clear clinical improvement. Moderate response or a resistance to applied chemicals was observed during the long-term pharmacotherapy; in several cases the treatment difficulties resulted in the necessity for surgical management (corneal X-linking, penetrating keratoplasty).

3.3. Monitoring of In Vitro Dynamics of Pathogenic Corneal Isolates and Their Temperature Adaptability. Successive monitoring of the clinical isolates and comparison to the environmental *A. castellanii* Neff strain cultivated *in vitro* allowed evaluation of morphophysiological features, their associated developmental stages, and changes in their population dynamics.

The living trophozoites with pseudopodia and characteristic protrusions, acanthopodia, were 12–38 μm in diameter, with a nucleus and prominent centrally placed nucleolus. Cysts, 8–24 μm in diameter, with their two cyst walls exhibited a wrinkled ectocyst and a polygonal or round-to-ovoid endocyst (Figure 3). The amoebae detected were identified and classified as belonging to *Acanthamoeba castellanii* morphological group II.

The comparative evaluation of monitored strains that at the beginning were cultivated *in vitro* at 27°C showed that numbers of live amoebae were low in the early adaptive phase and successively increased in the exponential growth phase

Table 1: Compilation of data of *Acanthamoeba* T4 isolates of five severe AK cases with delayed proper diagnosis, resistant to chemicals applied.

Acanthamoeba strain	Probable risk factors	Microorganisms detected	The first-time *Acanthamoeba* detection	
			Stage	Material
I-1	Swimming in a lake	*Acanthamoeba* sp.	Moving trophozoites with acanthopodia	Corneal scrapings
I-12	Contact lens	*Acanthamoeba* sp.	Viable trophozoites, rounded forms	Corneal scrapings
I-13	Swimming pool, contact lens	*Acanthamoeba* sp. *Candida* sp.	Trophozoites, cysts	*In vitro* cultures
I-16	Contact lens	*Acanthamoeba* sp. *P. aeruginosa*	Trophozoites, cysts	*In vitro* cultures
I-19	Contact lens	*Acanthamoeba* sp. *E. faecalis*	Numerous cysts	Corneal scrapings

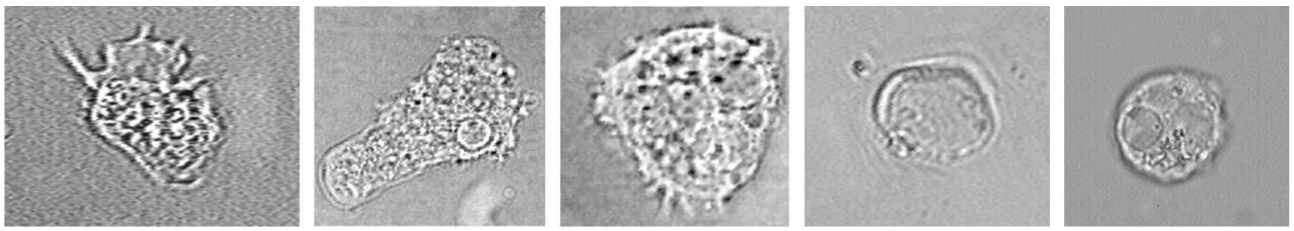

Figure 3: *Acanthamoeba* T4 strains, trophozoites and cysts detected in the *in vitro* cultured corneal scraping; light microscope; unstained preparations.

while the amoebae multiplied and increased population density of particular *Acanthamoeba* strains.

The exposure of parallel cultures of strains to 20°C and 37°C from the 4th day following the subculturing caused clear changes in population dynamics, expressed in the overall number of amoeba cells.

Statistically significant differences were observed between pathogenic strains and the environmental *A. castellanii* Neff strain in terms of number of viable amoebae and thus in population density during the stationary growth phase.

Although numerous living trophozoites were detected in all cultures at 20°C, there was a significantly lower population density of pathogenic isolates cultured *in vitro* than the density of the Neff strain cultures (Table 2).

Contrary to this, the numbers of viable trophozoites in clinical isolates cultured at 37°C (this is near eye and general human body temperature, about 35°C to 37°C) were significantly higher in comparison to that found in the environmental strain. At this temperature, during the stationary growth phase, the highest numbers of amoebae were determined for I-19 pathogenic strain cultured *in vitro*, in range of four counts 44.0–102.2 $\times$ 10^2, that were in comparison to *A. castellanii* Neff strain that were 3.3–7.8 $\times$ 10^2 at the same temperature.

Comparative assessment of isolates exposed to 42°C showed differences between several strains. The pathogenic isolates indicated lower population activity than at 37°C, expressed in somewhat decreasing amoebic numbers.

Table 2: Comparison of T4 strains from AK and *A. castellanii* Neff strain cultured *in vitro* at 20°C, 26°C, 37°C, and 42°C.

Acanthamoeba strain	Range of overall *Acanthamoeba* number ($\times 10^2$) Range of cysts (%)			
	20°C	27°C*	37°C	42°C
I-1	21.1–23.3 0.8–1.5	62.2–138,2 0–0.8	30.0–51.1 0–1.2	17.8–38.2 0–3.5
I-13	20.1–34.4 0–1.7	120.0–207.7 0–1.0	46.6–78.9 0–1.3	12.2–26.7 0–3.0
I-16	25.3–26.1 0–1.5	123,3–164.4 0.9–1.0	52.2–74.4 0–0.8	23.2–29.0 0–3.0
I-19	32.7–34.0 0–0.7	85.5–145.5 0–0.2	44.0–102.2 0.5–0.9	22.0–27.8 0–1.1
Neff	378.1–424.6 0–0.3	102.0–176.7 0	3.3–7.8 0–4.2	2.2–8.2 0

The level of statistical significance was set at $p < 0.05$.
*Data from exponential phase of population growth.

However, in general, high amoebic population density was observed in all examined pathogenic strains during successive days of exposure to 42°C, while the number of amoebae significantly decreased in cultures of the environmental Neff strain.

Comparative data of the environmental and clinical *Acanthamoeba* strains deriving from severe cases of AK cultivated *in vitro* at 20°C, 27°C, 37°C, and 42°C are presented in Table 2.

4. Discussion

Recently, *Acanthamoeba* strains generate a serious human health threat due to their pathogenic potential as facultative parasites. In addition, the amoebae may also act as vehicles/sources/reservoirs of other organisms pathogenic for humans: fungal, protozoan, viral, and bacterial microorganisms which can survive and even multiply within the amoeba cells. For these reasons, epidemiological aspects are included in a majority of studies on severe vision-threatening *Acanthamoeba* keratitis. Investigations on the distribution of the virulent amoebic strains in different soil, air, and water environments have been conducted worldwide, also with the aid of molecular techniques. Potentially pathogenic strains are detected in environmental samples and reported worldwide [18, 25, 35–42].

In Poland, free-living amoebae have been isolated from waters in the vicinity of Poznań [43]. Successively, in further environmental studies on *Acanthamoeba*, potentially pathogenic strains have been detected in Lake Żarnowieckie, the Piaśnica River, and a canal used as a recreational resort in northern Poland; moreover, free-living amoebae have been isolated from natural water bodies including lakes, ponds, rivers, and lagoons of the West Pomeranian and Lubuskie area, in tap water of the water supply system of Szczecin city, in surface water layers, and in water with sediment in northern Poland at the area of the cities Gdańsk, Gdynia, and Sopot, as well as in swimming pools and fountains in western Poland [44–50].

At present, the approach based on genotype identification is more often applied to detect and characterize *Acanthamoeba* strains from environmental and clinical samples below the genus level. There is evidence that among seven genotypes detected in patients with AK about 90% of incidences are linked with the T4 genotype [3, 4, 17, 24]. In our investigations, all pathogenic strains of AK were identified as belonging to the T4 genotype. This is in accordance with the fact that this genotype is considered to be the most common cause of the vision-threatening eye disease. Our previous [16, 31] and this study are the only in that the molecular assessment was performed in diagnosed pathogenic *Acanthamoeba* isolates that originated from Polish patients with drug resistant *Acanthamoeba* keratitis. Complete results of these examinations will be reported in detail in a separate publication.

It is considered that the leading risk factor for *Acanthamoeba* keratitis is contact lens wear. After the first case of AK associated with contact lenses in Central Europe was reported from Germany, more incidences were recognized in different countries and an association between contact lens wear and *Acanthamoeba* keratitis was revealed [51, 52].

Nevertheless, occasionally *Acanthamoeba* corneal infections are also detected in persons not using contact lenses. A corneal epithelial injury, eye surgery, and, especially, an exposure of the eye to water or moist soil in which *Acanthamoeba* forms exist are considered as other important risk factors for acquiring AK [2–4, 17, 53].

Recently, the popularity of contact lens use is rising, and severe AK cases are reported with increasing frequency year after year, particularly in contact lens wearers (85% of all incidences), from various regions of the world, including Poland. Since the first incidences of AK were reported [54], further AK cases have been described in Poland [16, 31, 46, 47, 55].

All patients with keratitis symptoms from whom the corneal isolates were analyzed in our studies at the beginning were assessed to search for/confirm the etiological agent of the disease.

Lorenzo-Morales et al. [17] "the most important step in AK diagnosis is to think of it." In many *Acanthamoeba* cases there is a history of misdiagnoses and improper therapy; also in the ten cases finally included in our study, some diagnostic difficulties and prolonged disease process occurred. The direct detection of etiological agents is considered as the only reliable diagnostic method for AK; simultaneously, cultivation remains the gold standard for *Acanthamoeba* laboratory diagnosis [17]. It is noteworthy that examiners have to be familiar with morphological characteristics of *Acanthamoeba* spp.

Currently, it has been shown that both environmental and clinical *Acanthamoeba* strains/isolates vary in their pathogenicity; they may be virulent, weakly virulent, or nonvirulent [4]. Simultaneously, the ability of *Acanthamoeba* to grow at high temperatures is considered to be correlated with the pathogenicity of *Acanthamoeba* isolates and to be a good indicator of the pathogenic potential of a given isolate. Thermal tolerance examinations and growth at high temperature are used as indirect markers of *Acanthamoeba* virulence/pathogenicity of some environmental samples [4, 17, 32].

Results of our earlier investigations [33] on environmental strains of *Acanthamoeba* in terms of their temperature tolerance showed that the amoebae may grow at 37°C, a temperature that is higher than that in which they had been cultured for many months; at the same time, the number of trophozoites of this strain was ~10% lower than at 26°C.

In general, our experimental investigations of ten pathogenic strains revealed the ability of all isolates deriving from AK cases to grow in higher temperature than 27°C at which they had been cultured for many months. The maintenance of metabolic activities was expressed in the relatively high population density that characterized all pathogenic *Acanthamoeba* isolates incubated in parallel at 37°C and 42°C.

It is noteworthy that cultures of the environmental Neff strain exhibited optimal growth at 20°C and weakest growth in temperatures higher than 27°C. Corneal pathogenic isolates developed well also at higher temperatures.

5. Conclusions

Acanthamoeba keratitis is a vision-threatening emerging eye disease caused by the facultative parasite *Acanthamoeba*, ubiquitous in human environments. The leading risk factor for the disease is contact lens wear, steadily rising in popularity; thus, AK is detected with increasing frequency worldwide, including Poland. Awareness of these risk factors

and thus strict hygiene while cleaning and using contact lenses are crucial as preventive measures. The diagnostic and therapeutic difficulties, coupled with severe course of the disease in the cases analyzed in the current study, were due to unspecific clinical symptoms, misdiagnosis, and resulting delay in suitable treatment, as well as resistances to antimicrobial and antiparasitic therapy.

It was shown in our study that the ability of *in vitro* cultured corneal *Acanthamoeba* isolates to adapt to higher temperature was typical for all pathogenic isolates monitored. At the same time, the *in vitro* metabolic activities of these strains were also maintained at 20°C.

In our opinion, the pathogenic strains are not just thermotolerant but rather have a wide adaptive capability.

Our study is the first detailed study from Poland providing evidence of the significant role of adaptability to temperature changes as one of a complex of contributory factors allowing free-living amoebae to exist as parasites, namely, as the causative agents of AK, a serious human eye disease. Nevertheless, from which sources our AK patients acquired their infections remains uncertain, whether they came from natural or from man-made habitats. Also, which mechanisms enable particular strains to grow at a wide temperature range remains unclear, while others do not show this ability. These remaining areas need further studies if we are to understand the epidemiology of these opportunistic infections and to prevent future cases.

Conflict of Interests

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

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Review Article

Revisiting the Posttherapeutic Cure Criterion in Chagas Disease: Time for New Methods, More Questions, Doubts, and Polemics or Time to Change Old Concepts?

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One of the most relevant issues beyond the effectiveness of etiological treatment of Chagas disease is the lack of consensual/feasible tools to identify and certify the definitive parasitological cure. Several methods of distinct natures (parasitological, serological, and molecular) have been continuously proposed and novel perspectives are currently under investigation. Although the simultaneous use of distinct tests may offer better contributions and advances, it also leads to controversies of interpretation, with lack of mutual consent of cure criterion amongst researchers and physicians. In fact, when distinct host compartments (blood/tissues) are evaluated and explored, novel questions may arise due to the nature and sensitivity limit of each test. This short analytical review intends to present a chronological and critical overview and discuss the state-of-the-art distinct devices available for posttherapeutic cure assessment in Chagas disease, their contributions, meanings, and interpretation, aiming to point out the major gaps and propose novel insight for future perspectives of posttherapeutic management of Chagas disease patients.

1. Introduction

Chagas disease, caused by the parasitic protozoa *Trypanosoma cruzi*, is naturally transmitted by triatomine vectors and is endemic in 21 Latin American [1] countries. Currently this disease is present in several other countries of distinct continents (USA, Europe, Asia, and Australia) due to human migration phenomena [2] where its transmission occurs independently of the vectors by other mechanisms such as congenital, blood transfusion, transplants, and common use of syringes. About 6-7 million subjects are estimated to be infected worldwide, mostly in Latin America [1]. After the acute phase, approximately 30% of chronically infected patients will develop cardiac alterations, 10% the digestive alterations (mainly megaesophagus and megacolon), or the mixed clinical form of the disease (association of cardiac and digestive manifestations) [3] which may require complex clinical management and specific treatment. The etiological treatment of Chagas disease is currently based on the use of nifurtimox or benznidazole, developed in the 1960s and 1970s, respectively. Despite their relative proven efficacy to treat hosts during the acute phase of the disease, their effectiveness is very low when administered during the chronic phase of the disease [4]. Moreover, the therapeutic outcome also depends on intrinsic features of distinct *Trypanosoma cruzi* natural resistance [5] or genotypes, even during acute infection [6]. One of the most difficult challenges in Chagas disease treatment is the establishment of a feasible and consensual parasitological cure criterion. In general, the posttherapeutic management of Chagas disease patients involves the use of several methods of distinct natures, focused on the identification of live parasites, host

Table 1: Laboratorial methods used for posttherapeutic cure criteria in human Chagas disease and *Trypanosoma cruzi* infection in experimental models*.

Conventional serological tests (CFR, IHA, IIF, and ELISA)	Nonconventional serological tests (C^oML, FC-ALTA, FC-AFEA, and FC-ATE)	Parasitological test (Direct test, indirect tests, and PCR)	Interpretation
POS	POS	POS	Not cured
NEG	NEG	NEG	Cured (classic criterion)
POS	NEG	NEG	Dissociated/cured (Krettli and Brener criterion)
POS/NEG	POS/NEG	NEG	Oscillating/inconclusive

*Conventional serological tests: CFR (complement fixation reaction); IHA (indirect hemagglutination assay); IIF (indirect Immunofluorescence test), and ELISA (enzyme-linked immunosorbent assay); nonconventional serological tests: C^oML (complement mediated lysis); FC-ALTA (flow cytometry anti-live trypomastigotes antibodies); FC-AFEA (flow cytometry anti-fixed epimastigotes antibodies); FC-ATE (flow cytometry anti-live amastigote/trypomastigotes and fixed epimastigote antibodies); direct parasitological tests: fresh blood examination (FBE), trypomastigotes concentration (buffy coat, microhematocrit, and Strout tests); indirect parasitological tests: xenodiagnosis, hemoculture, and PCR.

immune-mediated biomarkers (specific antibodies and cellular mediated immunological features), or parasite-derived nucleic acid and/or antigens [4, 7]. Distinct serological, parasitological, and molecular tools have been continuously developed over time, leading to subsequent changes on the proposed cure criteria. Despite their incontestable contributions to point out the inefficiency of the current available drugs and clarify several aspects relevant to guide novel rational-guided drug discovery, the use of new approaches has also elicited several doubts and raised controversial ideas amongst researchers and/or physicians. One of the most relevant matters or limitations regarding the establishment of a mutual consent cure criteria relies on the long time required for complete seroreversion of the conventional serology, one of the front-line criteria included in most posttreatment cure management protocol and still considered a relevant biomarker to be adopted as indicator of *T. cruzi* infection cure by several groups in the scientific/medical community. Several studies in humans have demonstrated that the time required for the complete reversion of the conventional serology is directly proportional to the time of patient's infection. It has been expected that seroreversion may take 1–12 months for congenital infections, 1–5 years in children with less than five years of infection, 5–10 years for recent chronic infection (i.e., 12–14 years of infection), and 10–25 years or even more for late chronic infections (i.e., >15 years of infection) [4, 8]. These particularities are, besides the small number of drugs available for Chagas disease treatment and those aspects inherent to genotypic-specific drug resistance, one of the most relevant reasons that discourage physicians from prescribing therapeutic interventions and that also demotivate the patient adherence to treatment.

The purpose of this review is to present the overtime development of novel methods for cure management of Chagas disease patients and also analyze and critically discusses the interpretation of the results obtained with the use of several methodological tolls. In this context we point out the gaps, doubts, and controversial issues and propose novel insight for future perspectives for cure criteria feasible to be applied in follow-up management of treated Chagas disease patients.

2. Laboratorial Methods Available for Posttherapeutic Cure Assessment and Their Meaning

Table 1 summarizes the most relevant proposed methods reported for cure monitoring after etiological treatment of Chagas disease. Two categories of tests, including immunological (conventional and nonconventional serology) and parasitological methods, have been reported with applicability for cure assessment in Chagas disease treated hosts.

2.1. Immunological Methods (Conventional and Nonconventional Serological Approaches). The conventional immunoserological tests, routinely applied for Chagas disease serology in clinical laboratories, comprise those methods based on the detection of IgG anti-*T. cruzi* epimastigote antigens by several techniques, including the complement fixation reaction (now in disuse), indirect hemagglutination assay (IHA), indirect Immunofluorescence test (IIF), and enzyme-linked immunosorbent assay (ELISA). The nonconventional and/or alternative methods, usually applied in reference or research laboratories, include those tests based on the detection of antitrypomastigote, amastigote, or purified/recombinant antigens by several technical fundaments, such as recELISA, immunoblotting, flow cytometry, and immunosensors.

There is a consensus that, after etiological therapeutic intervention in Chagas disease, seronegative results on conventional methods can be considered as cure criterion. However, the seropositivity on these methods is crudely interpreted as indicative of therapeutic failure in Chagas disease.

The first and classical definition of posttherapeutic cure in Chagas disease proposed [8, 9] define as "cured" those treated patients that presented negative conventional serology isolated or associated with negative parasitological tests (xenodiagnosis and/or hemoculture or other tests). Additionally, this "classical cure criterion" defines as "therapeutic failure" the presence of seropositive serology regardless of the results observed in the parasitological methods even when consistently negative (Table 1).

However, since 1982, [10] it has been reported that *T. cruzi*-infected hosts may present dissociation between the results observed in the conventional and nonconventional serology. These authors have proposed that during *T. cruzi* infection the hosts produce two distinct categories of immunoglobulins referred to as "conventional" and "nonconventional" antibodies. The first category, named "conventional antibodies," was useful for diagnosis purposes but may persist after parasitological cure. The second category represents a special type of antibodies, named "nonconventional" antibodies or "lytic antibodies," able to bind live *T. cruzi* trypomastigote forms leading to their complement mediated lysis. These authors observed that this type of "lytic" antibodies are closely associated with the presence of active *T. cruzi* infection but are absent in parasitological cured hosts. Considering these concepts, [10] these authors proposed the inclusion of the complement mediated lysis technique (C^oML) in the context of cure criteria. The C^oML offered an important contribution for posttherapeutic monitoring of Chagas disease, since it was able to detect early seroreversion prior to the conventional serological methods. The C^oML has gained strength for cure assessment of human Chagas disease after an important posttherapeutic follow-up study [11]. According to this proposal, a new concept of cure arises by the introduction of the term "dissociated" patients. By this "novel cure criterion," treated patients presenting with positive conventional serology but negative C^oML should be considered cured (Table 1). The use of the [10] criterion enhances the therapeutic effectiveness from around 9% obtained by the "classic cure criterion" to approximately 37% when including the "dissociated" patients (28%) as cured [11].

Considering the functional nature of the C^oML, the requirement of a fresh complement source (animal or human serum) and the use of live blood trypomastigote (obtained from irradiated mice or tissue cultures) besides the laborious counting system in Neubauer chamber and the low sensibility (using sera dilution 1 : 2 to 1 : 8), this method found restriction for general use and acceptance by the scientific community. In 1995, aiming to overcome some limitations inherent of C^oML, a novel approach for detection of anti-live trypomastigotes antibodies, analogous to "lytic" antibodies, was proposed, based on the use of flow cytometry. This new method, referred to as flow cytometry anti-live trypomastigote antibody (FC-ALTA), was first described [12] and performed in parallel with C^oML. This method overcomes the functional nature of the C^oML, substituting the fresh complement by FITC-conjugated anti-human IgG, in a flow cytometry-based immunofluorescence approach. This innovation contributes to the development of a more sensitive (serum dilution 1 : 256) and reproducible flow cytometric-reading reaction. The FC-ALTA performance led to the same patient categorization obtained by the C^oML, distinguishing "not-treated" (NT) and "treated-not-cured" (TNC), with positive results, from those "dissociated" (DIS) or "cured" patients (TC), with negative results. Later on, the FC-ALTA was validated on a double-blind study, using a larger number of samples [13].

Aiming to overcome the biohazard of using live trypomastigote, a new flow-cytometry methodology using prefixed *T. cruzi* epimastigotes forms was described, easily obtained in large scale by axenic *in vitro* culture in LIT medium [14]. This novel approach was named flow cytometry detection of anti-fixed epimastigote antibodies (FC-AFEA). Both FC-ALTA and FC-AFEA methods are revealed to be able to discriminate "cured" from "not-treated" (NT) and "treated-not-cured" (TNC) patients in a long-term (more than 20 years) follow-up study. However, it has been reported [15] that while FC-ALTA is able to detect early changes in short-term posttherapeutic follow-up (5 years), no changes in the FC-AFEA could be observed.

More recently, a remarkable innovation has been incorporated in the flow-cytometry-based methodology by the introduction of a triplex concept that simultaneously uses the three *T. cruzi* evolutive stage forms amastigote (A) and trypomastigotes (T) from tissue cultures and epimastigotes (E) from axenic LIT culture [16]. This novel methodology, named FC-ATE, applies fluorescent FITC-based discrimination of *T. cruzi* evolutive forms, coupled by a fluorescent phycoerythrin-based development system to detect *T. cruzi* stage-specific antibodies. This method was able to detect "cured" patients with outstanding performance (100%) for anti-amastigote antibodies as compared with anti-trypomastigotes (93%) or anti-epimastigotes (96%) antibodies.

In consonance with the development of nonconventional serological approaches, several novel antigenic preparations, including semipurified/purified/recombinant antigens as well as synthetic peptides, have been used with the purpose of early detection of seroreversion after etiological treatment of Chagas disease [17–19], most of them using ELISA-based tests.

Also, the recELISA using specific *T. cruzi* antigens (cytoplasmic repetitive antigen (CRA) and flagellar repetitive antigen (FRA)) has shown, besides good correlation with the conventional serology (CS) and the ability to anticipate the seroreversion in some patients [20]. Moreover, the use of ELISA-F29 test has revealed negative results within patient with negative or declining conventional serology suggesting that the ELISA-F29 test is useful as an early indicator of negative seroreversion in treated chronic patients [21].

2.2. Parasitological Methods. The parasitological methodologies applied to cure control of Chagas disease include two categories referred to as (1) direct parasitological tests, including fresh-blood-examination (FBE) and trypomastigotes concentration (buffy coat, microhematocrit, and Strout tests) and (2) indirect parasitological tests, including xenodiagnosis and hemoculture [4].

Despite its low sensitivities, a positive parasitological test is considered as definitive evidence of therapeutic failure, even isolated or in association with immunological test since it detects the presence of live parasites in host peripheral blood [4, 8]. On the other hand, a negative parasitological result does not confirm the therapeutic effectiveness or discard the possibility of therapeutic failure. Usually, the direct parasitological tests are useful for cure monitoring of treatment performed during acute and subacute phases.

Table 2: Categories of laboratorial methods and targets available for evaluation of the etiological treatment efficacy/benefits and their interpretation in the context of Chagas disease cure assessment*.

Category	Laboratorial method	Target	Results	Interpretation
Conventional serological tests	CFR, IHA, IIF, and ELISA	Fixed epimastigotes Recombinant antigens	POS	Therapeutic failure (Classic criterion)
			NEG	Cured (Both criteria)
Nonconventional serological tests	C^{o}ML FC-ALTA, FC-AFEA, and FC-ATE	Live trypomastigotes Live amastigotes	POS	Therapeutic failure (Krettli and Brener criterion)
			NEG	Cured (Krettli and Brener criterion)
Direct parasitological tests	FBE, trypomastigote concentration (buffy coat, microhematocrit, and Strout tests)	Blood trypomastigotes	POS	Therapeutic failure (Both criteria)
			NEG	Inconclusive (Both criteria)
Indirect parasitological tests	Xenodiagnosis, hemoculture	Blood trypomastigotes	POS	Therapeutic failure (Both criteria)
			NEG	Inconclusive (Both criteria)
Indirect parasitological tests	PCR, qPCR	Parasite DNA	POS	Therapeutic failure (Both criteria)
			NEG	Inconclusive (Both criteria)
Cellular immunology test#	PBMC/whole blood cultures/flow cytometry	*T. cruzi*-specific INF-γ/IL-10 producing cells	Proinflammatory	Side effect
			Balanced profile	Benefit

*Conventional serological tests: CFR (complement fixation reaction), IHA (indirect hemagglutination assay), IIF (indirect immunofluorescence test), and ELISA (enzyme-linked immunosorbent assay); nonconventional serological tests: C^{o}ML (complement mediated lysis), FC-ALTA (flow cytometry anti-live trypomastigotes antibodies), FC-AFEA (flow cytometry anti-fixed epimastigotes antibodies) and, FC-ATE (flow cytometry anti-live amastigote/trypomastigotes and fixed epimastigote antibodies); direct parasitological tests: fresh blood examination (FBE) and trypomastigotes concentration (buffy coat, microhematocrit, and Strout tests); indirect parasitological tests: xenodiagnosis, hemoculture, PCR, and qPCR; cellular immunology test: peripheral blood mononuclear cell and/or whole blood in vitro cultures in the presence of *T. cruzi*-derived antigens followed by cell surface phenotypic analysis and intracellular cytokine staining by flow cytometry. #Proinflammatory pattern refers to IFN-γ mediated immune response of NK-cells and $CD8^{+}$ T-cells. Balanced profile refers to IL-10 modulated response by monocytes or B-cells.

In general, all parasitological methodologies are employed in parallel with serological tests in order to provide a more precise cure criteria definition. In these cases, a negative parasitological test in consonance with a patent seroreversion phenomenon is considered the final proof of therapeutic success.

2.3. Molecular Methods (PCR and qPCR). In the 1990s, the use of molecular methods has been proposed, mainly to overcome the low sensitivity of most parasitological approaches. In this context, the polymerase chain reaction (PCR) appeared having as target of amplification the variable region of the minicircles of the kDNA [22]. Taking into account its high sensibility, the PCR was immediately used in the context of cure control, shown to be the first to demonstrate the therapeutic failure on a short-term basis and the last to become negative in cases of therapeutic success, as compared to all parasitological methods employed for cure control in humans [23–25] and experimental models treated by the conventional or alternative therapeutic schemes [26, 27]. For this reason, the PCR has become a useful tool for therapeutic failure definition amongst all parasitological cure control methods [23, 28] since the detection of the *T. cruzi* k-DNA in peripheral blood samples eluate was interpreted as indicative of the parasite presence in the host (Table 2). However, later on, several reports have demonstrated that, despite the higher sensitivity of PCR as compared to other parasitological methods, the PCR still presents some sensitivity limitation to detection of parasite DNA in chronic infections, as well as in treated hosts [26, 29]. Therefore, it became mandatory that a negative PCR cannot be interpreted alone as indicative of parasitological cure and also requires, likewise other parasitological test, a parallel seroreversion evidence for conclusive cure report.

Later on, the PCR-based methodologies have evolved towards quantitative methods using fluorescent-based probes q-PCR that represent advantages over the conventional PCR, being more sensitive and able to estimate the parasitemia levels in a quantitative manner which is relevant to monitor patients and host before and after etiological treatment. Two main molecular targets have been explored in the amplification of the parasite DNA by qPCR: the minicircle k-DNA [30] and the satellite DNA [31]. The higher sensitivity of qPCR besides the possibility of estimating the parasitemia levels has been explored as the major immediate and/or permanent advantages of this method in clinical and experimental studies. The good correlation between the qPCR data with

the blood parasitemia and intensity of tissue lesions as well as electrocardiographic alterations have been already reported [32]. Moreover, the applicability of qPCR to monitor patients [33] and animal models [34] after etiological treatment with conventional or alternative new drugs has been also addressed.

It is important to mention that both PCR and qPCR can be used in the analysis of peripheral blood samples and also in several host tissues (biopsy or euthanized experimental animals) and therefore represent an important contribution in the parasitological cure monitoring of animals and humans hosts [34, 35].

2.4. New Insights on Cellular Immune Response Methods. Aiming to improve novel concepts and bring novel insights to the therapeutic efficacy monitoring approaches in Chagas disease, new studies evaluating the *T. cruzi*-specific cellular immune response have been published, trying to surrogate the "old concept" that only seroreversion should be considered as the absolute indicative of cure. In this context, it has been proposed that changes in specific anti-*T. cruzi* T-cell responses, mainly focusing on the IFN-γ production by NK-cells and $CD8^+$ memory T-cells and/or IL-10 produced by B-cell and monocytes should be explored [36, 37]. Some reviews about this theme suggest that despite the difficulty to monitor cellular immunity biomarkers, due to their low frequency and broad antigen specificity, the tracking of theses target biomarkers by phenotypic analysis of *T. cruzi*-specific immune response can represent a promising tool for posttherapeutic evaluation in Chagas diseases hosts. There is a general consensus that, regardless of the fact that parasitological cure cannot be achieved in some cases, the ability of the etiological treatment to shift the immune response towards a mixed and balanced profile, with a IL-10-modulated and IFN-γ-driven-proinflammatory microenvironment, is a benefit to be considered even in not cured patients. Several immunological mediators have also been explored with this purpose [36–38].

3. Discussion, Gaps/Doubts, and Conclusions

The first experimental studies of etiological treatment in Chagas disease were carried out in mice in 1961 [39] and the posttherapeutic evaluation was performed by fresh blood examination (FBE) followed by serological test [39]. However, the first report of nitrofuran compounds use for treatment of human patients with chronic Chagas disease was published in 1969 [40] with posttreatment evaluation performed by xenodiagnosis and conventional serology. The first clinical trial of posttherapeutic evaluation following etiological treatment in human Chagas disease has used the xenodiagnosis and hemoculture, both of low sensitivity, but with an important advantage to clearly demonstrate the therapeutic failure in cases of positive results [4]. Although the xenodiagnosis has been proposed for use in experimental studies and also in humans, the ethical limitations and current controversies regarding this technique must be considered.

The conventional serological tests (CFR, IIF, IHA, and ELISA) for detection of specific antibodies against *T. cruzi* were usually used in parallel with parasitological methods, especially due to their high sensitivity and relative high specificity. The first "classic cure criterion" proposed for posttherapeutic monitoring of humans Chagas disease and *T. cruzi* experimental infection was based on the use of combined parasitological/conventional serology approaches and suggested that treated hosts should be considered cured only when all tests show negative results, indicating parasitological clearance and seroreversion [8, 9]. By using this "classic cure criterion," the initial studies on postchemotherapy follow-up in humans reported the time required for the seroreversion of conventional serology were very long, even when the parasitological methods were persistently negative, leading to resilient dissatisfaction of the scientific community and especially physicians working with human or experimental etiological treatment [41]. Considering the "classic cure criterion" based on the conventional serology (CFR, IHA, IIF, and ELISA) and considering "cure" only the cases of patent seroreversion, most studies revealed that, despite the fact that the therapeutic success during acute/subacute Chagas disease could reach over 90%, the frequency of "cured patients" drops significantly to around 9% when the treatment was performed during chronic infection [8, 9]. It has been proposed that a rapid and intense decrease in conventional serological titers after etiological treatment could be considered an indicative of putative cure [42].

A promising proposal to overcome the long time required for cure assessment by the conventional serology methodologies became available with the detection of anti-live trypomastigote antibodies, initially by the C^oML [10, 11] and later on by flow cytometry (FC-ALTA, FC-AFEA, and FC-ATE) [12, 14–16]. By using these nonconventional serological approaches, a novel "Krettli and Brener cure criterion" has been proposed [10], offering the important advantages, revealing more rapid seroreversion or demonstrating decreasing serological titers of antilive trypomastigotes, even when the conventional serology remained unaltered after treatment. The detection of these special antibody types appears to be associated with the presence of active infection in hosts, both humans and experimental animals [24, 27]. It has been demonstrated that FC-ALTA was negative in the majority of cured patients and in some patients with positive conventional serology [25], indicating the presence of "dissociated" patients, according to the "Krettli & Brener cure criterion" [10]. Despite the contribution of the nonconventional serological approaches anticipating the therapeutic efficacy outcome, these methods have not been effectively introduced in the routine of clinical laboratories for posttreatment evaluation, probably due to technical particularities and methodological difficulties. It is important to mention that the use of semiquantitative serology by flow cytometry is possible to detect decreasing serological titers, even when the anticipation of seroreversion is not yet observed in humans [15, 43] and in isolated cases in murine model [44]. It is important to mention that the standardization, development, and availability of a single, simple, inexpensive, easy to handle, sensitive, and specific method for cure criterion

assessment are absolutely desirable. From this point of view, the FC-ALTA is useful to establish differences between conventional and nonconventional antibodies following Chagas disease chemotherapy. However, this technique still remains restricted to reference laboratories, especially due to the use of live trypomastigotes *T. cruzi* cultures, the requirement of a flow cytometer and trained personnel for standardization, and interpretation of the results.

It is possible that *T. cruzi* genetic variability present in infected hosts could be involved in the time-dependent seroreversion observed in some hosts. Genotypic-specific serology using antigens (whole parasites, purified proteins, or peptides) from distinct *T. cruzi* genetic groups to identify strain-specific infections in humans and experimental models is currently in progress by several teams in order to search and achieve higher specificity in the serological tests. Probably, these methods will be available in a near future for cure assessment in genotype-specific approaches.

It is unquestionable that the use of the "Krettli and Brener cure criterion" [10] enhanced the therapeutic effectiveness from around 9% to approximately 37% when including the "dissociated" patients as cured [11]. Although the acceptation of the "dissociated patients" as parasitological cured patients is still questioned, mainly by the physicians, this concept has increased progressively after the introduction of more sensitive parasitological methodologies in the context of posttherapeutic monitoring of patients. It has been frequently observed that negative PCR results can be associated with negative nonconventional serology even in hosts with positive conventional serology, reenforcing the existence of this category of cured hosts with dissociation between positive conventional serology and negative nonconventional serology.

The introduction of PCR-based methodologies with higher sensitivity, to overcome the gaps of traditional parasitological approaches (xenodiagnosis and hemoculture), offered important contributions in the context of Chagas disease cure control and still supports an improved identification of the parasite persistence after etiological treatment. The questions around whether positive results observed in PCR-based methods in treated Chagas disease patients are really due to the presence of live parasite still remain to be elucidated. Successive follow-up evaluations in humans must be performed in order to better understand the real meaning of positive PCR in the context of cure monitoring, in parallel with other methodologies indicated for posttherapeutic evaluations. However, it is important to mention that, in murine model [45], positive results in PCR of blood eluates can be considered a "definitive indication" for the presence of live parasites, since only intact or recently lysed parasites are able to yield persistent positive results on these methods [46]. Otherwise, PCR of blood eluates may have a short-term positivity, up to two days, when mice are inoculated with purified *T. cruzi* DNA [46]. Therefore, the positivity in PCR-based methods assumed the "gold standard" score for therapeutic failure assessment [46]. On the other hand, there is general consensus that the combination of negative results of PCR-based methods in consonance with negative conventional serology approaches is definitive indication of posttherapeutic cure in Chagas disease hosts [23, 33, 46]. Important contributions have been also observed in the posttherapeutic evaluation in experimental models (mice and dogs) showing good correlation between negative PCR and negative parasitological tests (xenodiagnosis and hemoculture), conventional serology, and nonconventional serology as consensus for cure assessment [26], likewise observed in humans [23]. It is important to mention that although the qPCR has been identified as promising method, this approach, likewise conventional PCR, detects DNA from live and also dead/disintegrated parasites. Moreover, the use of qPCR still remains restricted to specialized laboratories and research centers.

All studies using PCR-based methods for follow-up monitoring of Chagas disease treated patients have pointed out that, likewise the phenomenon observed in the serological approaches, there is a progressive change in the result profiles, starting with oscillating results, interpreted as fluctuant parasitemia, before definitive negative results [23, 25, 33]. However, the time required for negativation of PCR-based methods is not clear yet. After several years, or even decades, of posttherapeutic follow-up by distinct methodologies, several studies have revealed important contradictions amongst PCR and serological results (conventional and nonconventional). It has been demonstrated that when PCR and/or nonconventional serology were negative, oscillating titers in the conventional serology may indicate a tendency of putative cure [44]. These evidences have been shown in a ten-year posttherapeutic follow-up in children and also with experimental infections with the same *T. cruzi* strains [44]. These findings suggested that PCR, likewise nonconventional serology, may take lower time for negativation as compared to conventional serology. In agreement with this proposal, the publications [21, 44] verified that the cure may be confirmed in patients with lowering serological titers after treatment when they are evaluated by ELISA using another antigen.

It has been demonstrated that the targets of the host-immune response mediated by conventional and nonconventional anti-*T. cruzi* antibodies are distinct [47]. While the nonconventional antibodies require the presence of live parasites since they are direct to short-lasting GP160 membrane-bound antigens, the conventional serology antibodies recognize a large range of antigens and can become positive even after mice immunization with soluble *T. cruzi* antigens or dead parasites. Several reasons may explain the long time required for the complete reversion of conventional serology, including mechanisms of autoimmunity.

Moreover, the persistence of parasite antigens in the dendritic and cardiac cells [47, 48], the occurrence of anti-idiotypic antibodies mimicrying parasite epitopes, and the presence of anti-laminin and anti-carbohydrate epitopes antibodies as well as cross-reactivity with other microorganisms such as intestinal or lung bacterias/protozoa may count for the long-term persistence of residual conventional serology after effective treatment of Chagas disease host [47]. Taking into account all these factors, an important question may arise: "*is it really necessary to consider the negativation of the conventional serology as the only way for discrimination between cured and not cured patients?*"

The current knowledge about the different performance of methods comparatively applied for posttherapeutic evaluation of *T. cruzi* hosts (humans and experimental models) leads the group of interdisciplinary experts in Chagas disease chemotherapy [49] to purpose a new protocol for cure monitoring for the evaluation of new compounds in mouse model. Considering the long time required for negativation of conventional serology and the high sensitivity of PCR applicable for cure control, the conventional serological tests have been ruled out of the most recent cure criteria that recommend the use of PCR after immunosuppression, performed 30 days after the end of the etiological treatment, as the "reference standard criteria" for cure assessment.

The introduction of qPCR approaches, with additional gain in quantitative analysis of parasitemia associated with high sensitivity level, has offered additional advantages over the original PCR-based methods applied for posttherapeutic evaluation in Chagas disease. The expectation regarding qPCR-based methods is naturally very high, regardless of the few data already available, based on follow-up studies in human. In general, in experimental models the use of qPCR has appeared to have a direct correlation between the level of parasitemia and the intensity of tissue parasitism, inflammatory process, and level of proinflammatory chemokines and fibrosis [34]. Even with all these optimistic features, the same old questions raised to interpret the PCR results remain for qPCR: "*is a positive qPCR result related to the presence of live parasites or dead/disintegrated parasites could also lead to positive results?*" Moreover, it is important to remark that, even considering all the advantages aggregated by PCR and qPCR methodologies, they still have some limit of sensitivity, especially when intended to detect extremely low parasitemia levels like that observed in treated chronic patients. Moreover, as the PCR-based methods are not available yet in most regular primary health care units, even in endemic regions of endemic countries, only physicians that collaborate with research institutions have employed these methods in the posttherapeutic management of Chagas disease patients.

Another question regarding the use of PCR-based methods has been raised by comparative performance of these methods to yield positive results depending on the biological samples used. Surprising and intriguing results have been demonstrated when PCR-based methods are applied to distinct tissues and compared to blood eluate in experimental models for *T. cruzi* infection chemotherapy. The first report for inconsistent positive tissue PCR in mice considered cured by negative conventional serology, hemoculture, and PCR was documented [50]. Afterwards, [27] a more worrying situation of inconsistence in positive tissue PCR even in treated mice presenting negative nonconventional serology (FC-ALTA) was showed. Similar inconsistences were observed between blood and tissue when qPCR methods are applied immediately after the end of the therapeutic intervention, suggesting that, in some cases, the qPCR negativation in blood samples happens earlier than in the heart tissue [34]. Together, these findings may suggest that the negativation of PCR in the blood samples may occur before the parasite clearance in the tissues. Another possibility is that the parasite DNA detected in the tissues may derive from dead or disintegrated parasite and is not indicative of the presence of live parasites in the tissue. In this context, the methods based on immunosuppression have been suggested to be used for cure monitoring assessment in experimental models. This experimental approach may contribute to elucidate these queries. One alternative that has also been proposed for use in experimental protocols during drug development research is the use of immunohistochemistry methodologies in tissue [51] for direct detection of amastigotes or *T. cruzi* antigens in tissue samples in parallel with PCR or qPCR. However, the relative low sensitivity of immunohistochemistry for parasite detection represents important restrictions for its use in humans.

Taken together, in the scenario of all these tolls available for the posttherapeutic monitoring of *T. cruzi* infection in human and experimental models, there is a clear and natural theoretical hypothesis for a decreasing order of negativation as follows: xenodiagnosis/hemoculture > PCR (blood eluate > tissues) > qPCR (blood eluate > tissues) > nonconventional serology > conventional serology.

The goal to be achieved is to disseminate this novel concept of posttherapeutic cure in Chagas disease and to stimulate physicians to pursue these laboratorial methods together in the posttreatment evaluations to undertake a more holistic view for the large benefits underlying the etiological treatment of this disease. To reach this goal, it is necessary to change old concepts. Unfortunately, there is not ethical possibility to determine the treatment efficacy in human *T. cruzi* infection with 100% conviction tissue [36]. It may not be mandatory to unequivocally demonstrate the absence of live parasites in host blood and tissues but to take the whole set of posttherapeutic changes in parasitological (persistent negative xenodiagnosis and/or hemoculture along with negative/oscillating PCR and/or qPCR in blood eluates) and immunological results (negative/decreasing titers in nonconventional and/or conventional serology besides the presence of modulated proinflammatory patterns).

4. What Is Still Necessary to Be Accomplished regarding the Posttreatment Evaluation?

In fact, the extension of human studies during longer period of posttherapeutic follow-up to characterize the behavior of parasitological/molecular/immunological technologies in order to verify changes in the results is still extremely important and necessary. The ideal is that all these evaluations could be taken together to achieve a pattern of biological signatures including parasitological and immunological parameters in parallel, taking the novel concept of systems biology and machine learning tools. It may not be a time to pursue new methodologies but a time to revisit the old or ancient methods for better interpretation of theirs results in the context of integrated parasitological/immunological posttherapeutic management. Meanwhile, there are increasing evidences that the etiological treatment bring benefits to the Chagas disease patients, even for the "classical not cured patients," which evolve with better prognosis.

In this meantime, equivalent approaches of posttherapeutic evaluation should be conducted in experimental models, focusing on novel tools and interpretation approaches, with the advantages that the results can be generated more promptly in a more controlled, precise, and repetitive investigation, besides the possibility of parallel tissue investigation, including tissue biopsy culture to verify the presence of live parasites. Another plausible tool that has been recently applied in possibility in animals is the use of posttreatment immunosuppression to monitor the therapeutic efficacy, in order to verify the presence of live parasites in tissue [49, 52].

5. What Is Desirable?

The standardization, development, and availability of a single, simple, inexpensive, easy to handle, sensitive, and specific method for effective demonstration of the parasite presence in blood (presence of the parasite DNA and/or antigens in blood samples) are absolutely desirable. While this task appears difficult to be accomplished, it is time to change old concepts and accept novel models to look at old or ancient methods in the context of integrated multidisciplinary posttherapeutic management. In the future, an integral concept should include clinical evolution of treated patient, especially in long-term follow-up studies. The posttherapeutic Chagas disease monitoring should not be restricted to laboratorial analysis. It should be extended to an integrated clinical/laboratorial status of treated patients.

It is clear that a first and critical step to address the research and development gap regarding the establishment of an international approved "cure criterion" is to establish consensus on the desirable target product profiles (TPPs) in different conditions of use. To support this process and optimize the development of such tools, the Pan American Health Organization (PAHO), in collaboration with the Drugs for Neglected Diseases initiative (DNDi), Médecins sans Frontières (MSF), and The Special Program for Research and Training in Tropical Diseases (TDR), convened a multidisciplinary group of experts to review the "state of the art" regarding this matter and initiate discussions. The multidisciplinary group has prepared for the development of TPPs, which has been recently reported [53].

6. Conclusions

The etiological treatment of human Chagas disease acts to reduce or eliminate the etiological agent *T. cruzi*, changing the patterns of anti-*T. cruzi* antibodies and remodeling the antiparasitic cellular immune response. Several laboratorial tests of parasitological or immunological nature are available and are usually employed for posttherapeutic cure monitoring. The major dissatisfaction affecting the scientific community and physicians relies on the long time required for persistent seroreversion (10–25 years or even more), which has been classically considered a definitive parasitological cure criterion. Although a range of novel methodologies of distinct and more sophisticated theoretical basis (parasitological, serological, or novel immunological biomarkers) have appeared and applied in the context of posttherapeutic monitoring of Chagas disease, this theme still remains polemical due to controversies between results obtained by distinct tests and/or in different studies. Always when a new test is proposed to be used as a tool for cure monitoring, it faces the lack of a plausible short-term "gold standard" methodology that could be applied to validate the proposed method. Moreover, all initiatives for new or improved tools for cure control must overcome the hard challenge to be sensitive and specific enough to demonstrate the clearance of the live parasite in host tissues. In this context, it seems that more than new methods about what is needed is an urgent change of old concepts. It is time to revisit the methods available and propose better interpretation of their results in the light of an integrated system biology posttherapeutic management, intending to establish a holistic view of the *T. cruzi* hosts as a complex network of biomarkers that could be better assimilated and understood by a multiparametric prognostic equation.

Conflict of Interests

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

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Review Article

Importation and Transmission of Parasitic and Other Infectious Diseases Associated with International Adoptees and Refugees Immigrating into the United States of America

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Each year, hundreds of millions of people travel across international borders or even oceans, and up to 230 million may remain for long periods. Among these, 3–5 million settle permanently in their new homes, with about 1 million migrating permanently to the United States of America. This may result in transport of parasites and other pathogens, which might become established, infecting individuals in the new location. Beyond concern of disease spread, the health of migrants is of concern since the rigors, circumstances, and living conditions surrounding migrations may increase the vulnerability of migrants to infections. International adoptees and refugees are a small subset of these migrants but are of special significance inasmuch as adoptees may be more vulnerable to infection due to their immature immune status, and refugees may be more vulnerable due to substandard living conditions. Both originate from diverse regions, but often from environments of low hygiene and health care standards. This review examines recent examples of infections reported from adoptees and refugees entering the USA through 2010, highlighting the most common origin countries and the diseases most frequently involved, including Chagas disease, *Balamuthia* amebic meningoencephalitis, giardiasis, microsporidiosis, hepatitis, measles, pertussis, tuberculosis, malaria, intestinal helminths, and syphilis.

1. Background

In the past few decades, international adoptions and refugee cases have become increasingly common within the United States of America (USA or US). Between 1989 and 2000, US citizens adopted approximately 18,846 children from China alone, and the annual increase in adoptions is significant [1]. For example, US citizens adopted 20,099 children from 20 countries in 2002 compared with only 7,093 international adoption cases in 1990 [2]. This increase in permanent relocation between countries and cultures introduces a series of significant concerns for the health of both the immigrants and the US public at large. While this appears to be a general trend, however, it is important to note that levels of both international adoption and admission of international refugees are not always positive and fluctuate in response to national and international pressures. In this review, we do not attempt to provide a comprehensive analysis of US immigration policies regarding refugees and adoptees. This report is intended to examine the general issues, highlighting some representative recent examples from specific localities.

It is estimated that approximately 2% of the world's population resides in a nation other than the one in which they were born [3]. Although globalization has several positive consequences, it has also become a great concern as increased technology and economic means have afforded larger populations with access to intercontinental travel, and along with it the capacity to spread diseases rapidly on a global scale [4]. The resulting displaced, foreign-born population composed of immigrants, refugees, and adoptees not only experiences a shift in their personal health experience, but also impacts the health environments of the new communities into which

they come to reside. Two of the most important factors directly relating to the epidemiology of disease due to migrations are the "degree of difference between origin and the destination" and the "size of the mobile population that moves between the different disease prevalence patterns" [4]. Although they comprise relatively small populations, foreign-born adopted children and international refugees may potentially play a significant role in the globalization of some infectious diseases. Not only have their numbers grown considerably in size in the past few decades, but they also represent populations migrating to considerable distances across diverse epidemiological regions, thus making them of increased relevance in the study of infectious disease.

However, it is important to note that the well-being of these legally accepted migrant populations is highly regulated and monitored in the USA, such that data comparing their infections with those of other migrant populations are not readily available at present. These populations are often overrepresented in epidemiological studies of the foreign-born migrants, simply because the administrative and legal requirements of their status generate data. The larger and nationally more significant foreign-born populations (including lawful permanent residents as well as undocumented/unlawful residents) are almost certain to have greater epidemiological impact. Nevertheless, while adoptees and refugees make up a relatively small component of overall US permanent immigrants, to ensure optimal health for them as well as their new neighbors, there is a need for vigilance regarding the pathogens and parasites associated with their migrations.

The United States Centers for Disease Control and Prevention (CDC) defines new and reemerging infectious diseases as "diseases of infectious origin whose incidence in humans has increased within the past two decades or threatens to increase in the near future" [5]. The emergence of an infectious disease is dependent upon the introduction of a disease-causing agent into a new population, reinforced by the disease's establishment and proliferation into the new region [6]. While developed nations have achieved complete or relatively high success in controlling most infectious disease transmission, underdeveloped nations are still struggling with the means to control and treat such diseases, or to provide preventative care. Most developed countries do not routinely perform screening for many nonendemic infections potentially arriving from abroad, so that the probability of spreading of such diseases increases. Foreign children awaiting adoption by parents in another country are often exposed to a variety of infectious diseases due to inferior standards for immunization practices and lack of preventative care within the health care systems of some underdeveloped countries. Within the close confines of an orphanage, transmission of infectious diseases including tuberculosis, hepatitis B, measles, intestinal parasites, bacterial pathogens, and various viruses has been observed between children and caretakers. Studies have shown that "infants and young children who are brought together in groups for care have a higher rate of infection, greater severity of illness, and increased risk for acquisition of resistant organisms" [7]. Furthermore, these same diseases, often untreated before departure from the originating country, are brought into the homes of adoptive parents and caretakers, who thus experience increased risk of exposure to foreign pathogens, potentially leading to new or reemerging cases of infectious disease.

As with international adoptees, international refugees have come to form a relatively small, yet significant, population within the United States. Refugees can be defined as "individuals who are outside their country and cannot return because of a well-founded fear of persecution related to their race, religion, and political or social affiliations" [8]. Like the foreign-born adopted child population, the number of international refugees has also greatly increased in recent decades. In the 1960s, the world refugee population was approximately 1 million, and yet in 2003 it had rapidly grown to include 11.9 million people [9]. The conditions to which this unique population is exposed before departure, during transit, and after arrival in destination camps afford them little or no health care and an increased risk of infectious disease. Between 1979 and 2004, 75,000 refugees settled in the US state of Minnesota alone, and thousands more established residence throughout the United States. All of this has the potential to impact the epidemiology of infectious diseases in the communities in which they have newly settled [9]. The growing refugee population, combined with frequently unsanitary conditions and inadequate hygiene, makes this a group of particular concern.

This review serves to highlight the findings of numerous case studies and reviews in this arena of public health and thus to identify a potential trajectory of dissemination of infectious disease into and across the United States. The goal of this review and analysis is to explore the overall effect international adoption and international refugee cases have on the accidental importation of infectious diseases into the United States. The primary objective was to compile a list of some of the more important infectious diseases known to be introduced through international adoptees and refugees. We also provide a comparison of these two populations with particular emphasis on age and country of origin, using data available through the end of the past decade in 2010.

Internet and literature sources were used to compile a significant pool of data from which general trends were identified. Although treatment is not always sufficiently provided or documented before departure for adopted children or refugees, physical exams and health assessments performed after arrival provide useful data for determining prevalence of specific diseases within these incoming populations. For example, "all refugees to the United States are encouraged to obtain a health assessment at local public health departments within ninety days of arrival" [10]. The documentation of such visits provides a substantial basis from which to gauge accounts of old, latent, active, or previous exposure to infectious diseases.

Compilations of data taken from international adoptees often deal with smaller sample sizes than those for refugees but were still viewed as informative. Specific case studies involving few or even one patient are relevant, as such children could serve as the primary source for a foreign infectious disease within US borders. Such studies serve as indicators of potential emerging infectious diseases.

Together, the compiled series of epidemiological evidence allowed for a limited but representative perspective on the potential risk these two specific populations endure under common circumstances. From this, comparisons were made between the two groups to assess the relative degrees of risk and occurrence of infectious disease.

2. Basic Assumptions

A staggering 700 million annual human migrations affect residents of recipient localities on both a macro and micro level [10]. Globalization has certainly contributed to economic growth and diversification of populations, but perhaps more importantly, it has also opened up numerous avenues for infectious diseases to be transported along with international adoptees, immigrants, refugees, and tourists.

While it may be expected that immigrants and tourists should have access to basic health care, the environments from which most adoptees and refugees come tend to be substandard, and these populations usually stay in the United States for extended periods of time. Orphanages in embattled countries struggling with political unrest and lack of governmental health programs suffer from inferior standards for sanitation, protection, immunization practices, and preventative treatment. For these reasons, this study focuses on the rise of infectious disease due to international adoptees and refugees. In this brief review, it was a basic assumption that exposures to both adoptee and refugee populations serve as sources of transmission for infectious diseases, which potentially leads to an increase in endemic levels of infectious disease in the new home localities. Of the two populations, international refugees were assumed to present a higher risk due to the higher population numbers and the greater chance of inferior health care once displaced into camps. It might be argued that due to lack of extensive study there is a paucity of direct evidence to support these assumptions. In fact, considering the numbers of individuals arriving from locations with greater risks and exposure to infections of low prevalence in the USA, there is only limited evidence of transmission to the host population. Postarrival transmission, when it occurs, frequently may be more common among the foreign-born population [11, 12] but does occur in the receiving population as well [12, 13]. It is important to note that the few studies that have been done have focused on directly communicable diseases, with little information on the more complicated transmission of vector-borne or other indirectly transmitted diseases.

3. Are Infectious Diseases Being Brought into the US?

Several infectious diseases are transmitted by both international adoptees and refugees relocating to the US. Some of these include hepatitis A, hepatitis B, measles, SARS, tuberculosis, syphilis, *Helicobacter pylori*, bacterial gastroenteritis, various intestinal parasites, malaria, and arthropod ectoparasites such as scabies mites and lice. The Yale International Adoption Clinic Experience data demonstrate that adoptees "are at risk for infections well known to be transmitted efficiently within institutional settings" [11]. It would only follow by reason that these same diseases would be efficiently transmitted from infected immigrants within US borders.

The number of international adoptions into the US increased from 1996 through 2010, but the most common countries of origin have remained somewhat stable. The leading country has been China, followed by Russia, Guatemala, South Korea, and Kazakhstan [11]. Thus, infectious diseases endemic in those countries are the most obvious subjects for surveillance and examination.

Hepatitis A has been diagnosed and transmitted within international adoptees and their caretakers. Although often unrecognized because of the exposure's dependency on age, a 2007 report illustrates 5 cases (19%) in adoptees, 2 (7%) in unvaccinated travelers, 13 (48%) in nontraveling contacts of adoptees, and 7 (26%) in contacts of nontraveling contacts of adoptees [12]. Fischer et al. explain this high transmission rate to nontraveling contacts of international adoptees as being a result of asymptomatic hepatitis A present in the children brought into the home of their adoptive parents [12]. Another study, published in 2008, focusing on adopted children from South Korea, identified hepatitis A as a threat to both South Korean children and their contacts within the United States [13]. In 2004, 10 cases were identified in South Korean children of 0 to 9 years of age. Furthermore, 21 cases were confirmed in 2005, and 29 cases were seen in 2006 [13]. This rise in the prevalence of hepatitis A is a cause for concern, as it could potentially impact the epidemiology within the US population with the rising influx of international adoptions.

Hepatitis B has been identified in both internationally adopted children and refugees newly residing in the US. In a study on internationally adopted children, a range of 2% to 5.9% of children from various countries tested positive for active hepatitis B infection, while serological evidence, indicating previous infection or exposure, ranged from 22% to 53% [11]. More specifically, adoptees from South Korea have been identified as having serological markers for hepatitis B [13], and 9% of 164 children adopted from China tested positive for hepatitis B surface antigen, with 28% positive for hepatitis B antibodies [1]. Parents who bring such infected children home are potentially putting themselves and other family members at risk of exposure, and cases of household transmission from such events within the United States have been documented. One report specifically described such hepatitis B transmission from Asian children to their adoptive US families [2].

There also exists significant evidence of international refugees with serologic markers for hepatitis B. Among 12,505 refugees participating in a health study from 1998 to 2001, 70.6% showed active or previous exposure to hepatitis B virus [8]. Also of interest was the fact that 70% of infected refugees were under 30 years of age, and African refugees were 3 times as likely and Asians 2.4 times as likely to be infected compared to Europeans [8].

Hepatitis C infection has also been identified in children from South Korea, but there is much less evidence surrounding the transmission and prevalence. Of concern, however, is the fact that, like most other common diseases in South

Korean children, the number of hepatitis C cases increased between 2004 and 2006 [13]. More recent data are needed to determine the risks and transmission status associated with hepatitis C.

Parasitic and other infectious diseases, such as malaria, *Pneumocystis jiroveci* pneumonia, tungiasis, and leprosy, are also sometimes seen in internationally adopted children [14]. There is also strong evidence to suggest that malaria is entering into the United States through sub-Saharan African refugees, though it is not frequently transmitted naturally in the US due to lower exposure to competent *Anopheles* mosquito vectors. The United States accepts an average of 50,000–70,000 refugees each year, and the percentage of these originating in Africa grew significantly from 9% in 1998 to 39% in 2005 [15]. Studies show that in some cases up to 60% of these refugees have arrived infected with malaria, based on parasitemic blood smears 4 weeks after arrival [15]. This is of great concern mainly because of the severity of the disease and the American population's current lack of immunological resistance to malaria [15]. Symptomatic congenital syphilis, although rarely identified, is of concern in international adoptions, as many of these children are adopted from areas where the prevalence of syphilis within the population is increasing. However, despite this general increase, prevalence levels have remained around 1% or less in international adoptees [11]. This speaks to the success of predeparture screening; however, we should not underestimate the potential opportunity for transmission. Miller and Hendrie report in 2000 details serologic evidence for congenital syphilis in a child adopted from China, the country currently leading in the number of international adoptions [1].

Although not identified in international adoptees, per se, *Balamuthia* amebic encephalitis is of concern as demonstrated by Hispanic Americans in California. Seven cases of amebic encephalitis were identified in California residents as part of the California Encephalitis Project. Almost all of them were fatal, and all patients were immunocompetent and of Hispanic American ethnicity [16]. These data, combined with the US Department of State's 72 visas issued for children adopted from Mexico, make this often undiagnosed disease of interest in the spread of infectious disease. With the US Census Bureau's 2006 census estimating the Hispanic American population to be over 15% as of 2010, the potential influx of *Balamuthia* encephalitis, due to the refugee or the adopted population, could put Hispanic Americans at a heightened risk. However, since *Balamuthia*, like other opportunistic amoebae *Naegleria* and *Acanthamoeba*, is generally acquired from natural environmental sources and is present naturally across the US, concern of spread to new populations is likely to be low, and the infections are likely to remain uncommon.

There is also great concern regarding the reemergence of diseases once almost completely eliminated by the US. Tuberculosis is now making a comeback, especially due to drug-resistant strains of the bacteria, and some studies suggest its reemergence partially through international immigrants. For example, a 2006 report by Stout et al. states that "for the first time since the inception of tuberculosis (TB) surveillance in 1951, foreign-born individuals accounted for most of the active TB cases in the United States" [17]. Internationally adopted children have also been shown to present a risk of tuberculosis introduction. Although the majority of adoptees with evidence of tuberculosis are from Russia and China, tuberculin skin tests are positive in 3–5% of children in most studies, with rates as high as 19% in some cases. In 2007, Varkey et al. reported that refugees are at seven times' higher risk of tuberculosis infection than Americans, and twice as high as other foreign-born persons [9]. Measles is another infectious disease set to make a potential comeback, and outbreaks with endemic transmission in the US and Canada have occurred increasingly even through 2015. In 2001, 14 cases were reported after exposure from the travel and care of an adopted child from China [2]. Pertussis is another infectious disease with potential for reemergence within the United States. Although this infection occurs worldwide, the populations at risk are those who are underimmunized or with low immunity. One report identifies a case of pertussis in an adopted infant from Russia; this child's new family and fellow passengers on the flight back to the United States were exposed [2].

Transmission of *Enterocytozoon bieneusi*, resulting in a form of intestinal microsporidiosis, is an example of an uncommon infection occurring in adopted children. In 2003, several cases were identified in a Thai orphanage, and at the time of the survey, none of the infected children showed any gastrointestinal symptoms [18]. This study, reported by Leelayoova et al. in 2005, is evidence for a problem of asymptomatic children not receiving complete health assessments before departure. The lack of symptoms could allow such children to become major sources of infection once displaced into a new unsuspecting population. This warrants increased surveillance, as various enteric and other microsporidioses are regarded as a newly emerging and understudied infectious disease in many parts of the world [19, 20].

Chagas disease, a parasitic disease also known as American trypanosomiasis, has also been shown to move from endemic countries to developed countries and is now widespread in the United States, especially among recent immigrants from Mexico and Central and South America. Immigrants and refugees seeking safety or political asylum have brought in an estimated 56,028 to 357,205 cases of the etiologic agent for this disease, *Trypanosoma cruzi*, or 8 to 50 cases per 1000 legal immigrants [21]. Although Chagas disease is most commonly transmitted by bloodsucking insects of the triatomine reduviid group (commonly known as kissing bugs or assassin bugs), it is also often contracted through blood transfusion, organ transplants, or congenital infection. It is the latter of these mechanisms that allows *T. cruzi* to proliferate within a developed country where the vector bugs are not common or where human contact with them is limited. This is just another example of a disease that should be identified in newly arriving refugees. Failure to do so only contributes to the spread of this disease into nonendemic areas, especially since the disease, like many others, can be zoonotic in wildlife.

Intestinal parasites, both helminthic (worms) and protistan (eukaryotic microbes), also constitute a significant

number of infections within both internationally adopted children and refugees. Although the rate of prevalence varies between areas of origin, both populations were found to have intestinal protists or helminths within approximately 9% of the population. The most prevalent parasite in both populations was *Giardia lamblia*, though various other parasites were introduced depending on the country of origin. Reports detailed parasites in the stools in a variety of refugee populations, with their prevalence ranging from 9 to 19%; these studies also identified a substantial number of individuals with more than one parasite or helminth [9, 22]. Another common theme within refugee reports is an increase in frequency of intestinal parasites in children under 18 years of age [22]. In a 2003 review, Chen identified intestinal parasites in international adoptees with a prevalence of up to 51%, again varying with country of origin [2]. The highest prevalence rates occurred in children from Romania, Bulgaria, Moldova, Russia, and China [2]. However, the prevalence of intestinal parasites within internationally adopted children averaged approximately 10% in most studies, though China and Guatemala show a relatively low prevalence rate with only 7% and 8%, respectively [1, 23].

If this importation of *Giardia* does not remain in check, the endemic level could be heightened within the United States borders as illustrated in a 2005 analysis by Ekdahl and Andersson, which demonstrated the change in the epidemiology of giardiasis within Sweden. Data taken from the Swedish national database confirmed that 4,151 cases of *Giardia* in newly arrived immigrants and refugees and 455 cases in internationally adopted children were imported and thus disseminated within the country's borders [24]. This substantial transfer of *Giardia* resulted in a substantially greater calculated risk for being diagnosed with giardiasis. According to Ekdahl and Andersson, "in comparable countries, the calculated risk for being notified with giardiasis was 3–30 times higher in immigrant and refugees than in tourists and 2–5 times higher in adopted children than in immigrants and refugees" [24]. Having identified the potential risk of this known parasite as it crosses US borders, it is advisable to call attention to the need to develop public health measures against travelers and immigrants with this disease.

Based on data from the US State Department, Figure 1 shows the geographic distribution of the 12,753 international adoption visas issued by the United States Department of State in 2009 [25]. Figure 2 illustrates the top twenty countries of origin for international adoptees. These data hold great significance for public health officials as they demonstrate the variety of potential epidemiological backgrounds of internationally adopted children. The United States Department of State allocates refugee resettlements into the United States based upon global region. These regions are divided as Africa, East Asia, Europe and Central Asia, Latin America/Caribbean, and Near East/South Asia. Each year, the US Department of State publishes the proposed ceiling for refugee admissions by region for the coming fiscal year and the realized values for the previous one. The breakdown for 2009 is shown in Figure 3. These data are essential to the study of the globalization of infectious diseases. A total of 74,654 refugees entered the United States in 2009, all of

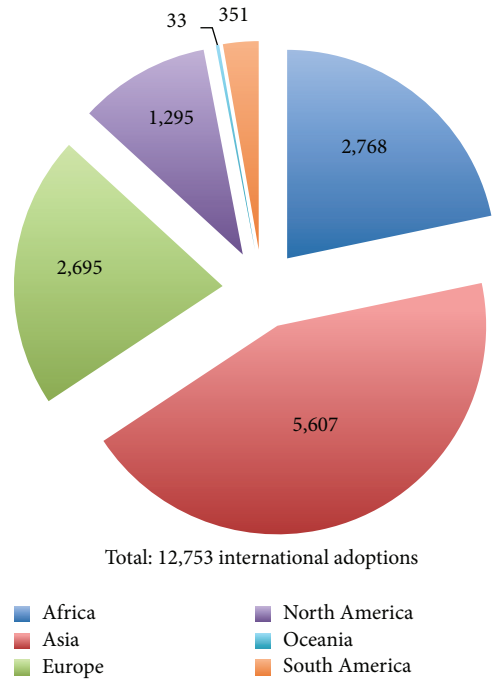

Figure 1: United States international adoption visas issued in 2009 by area of origin. Graphic derived from data provided by US Department of State [25].

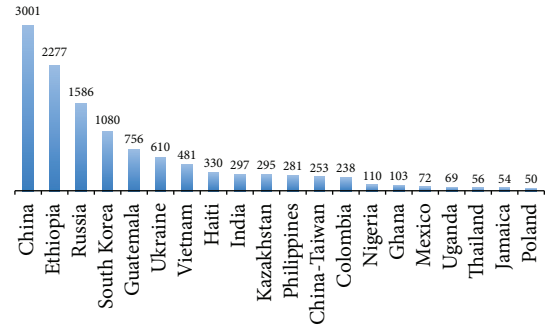

Figure 2: Top 20 most common countries of origin for international adoptees in 2009. Graphic derived from data provided by US Department of State [25].

them from areas with diverse epidemiological fingerprints [26]. Having illustrated the presence and transmission of various infectious diseases within these refugee populations, we recommend increasing vigilance and surveillance among large immigrations from various regions of the world, as these are points of considerable concern in the fight against the spread of infectious disease.

4. Conclusions

This compilation of data shows that both internationally adopted children and international refugees pose significant potential for infectious disease to be brought into the United States. The infectious diseases commonly transmitted between these two populations are very similar, but

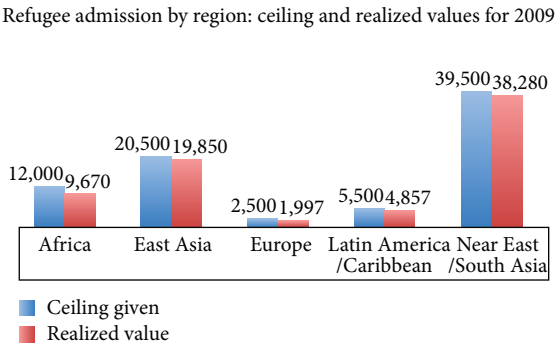

Figure 3: Allotted and realized values of refugees entering the United States by region. Graphic derived from data provided by US Department of State [26].

the sheer difference in the number of international refugees in comparison to foreign-born adopted children makes the most prevalent diseases of the refugees a greater concern. The poor conditions and frequent lack of medical treatment also contribute to the potential for these diseases to persist and spread among these populations, as they often live in close confinement, and therefore allow greater opportunity for transmission. Families providing homes for international adoptees often have greater access to health care and the means to receive medical attention more quickly than their new wards had in the locality of origin. Nevertheless, each member of these two groups of immigrants has the potential to serve as a source of importation of infectious disease.

The origin of international refugees and internationally adopted children often reflects the political climate of the region as well as the country's social and economic conditions [27]. Despite mandates that children up for adoption must be subjected to predeparture health screening, Hostetter et al. demonstrated in 1989 that 54% of the adopted children they screened once arriving to the United States had an undiagnosed infectious disease at the time of visiting a practitioner; 63% were diagnosed with an unsuspected medical problem regardless of country of origin [28]. Since their study was published, the number of international adoptions and the number of international refugees given admission into the United States have increased, and unfortunately, the data presented in this review do not demonstrate that predeparture detection methods of infectious disease have improved proportionally. However, it is also important to note that not all refugees arrive from refugee camps, and, for those that do, frequently camp medical services, access to care, and predeparture treatment may exceed the levels of local health services in their country of origin. This is clearly the case for many refugees bound for the USA, where CDC-directed treatment programs screen and treat several infections and infestations of public health importance prior to departure.

Finally, it is important to note that zoonotic and other parasitic diseases are frequently emerging in human populations in various parts of the world from which adoptees and refugees regularly migrate to the United States and other countries where those parasites have not yet been recorded but possess characteristics that would allow their colonization of these new localities [29]. An example is the current rapid emergence and spread of canine-associated dirofilariasis caused by *Dirofilaria repens*, throughout Eurasia, and especially in Eastern Europe [30, 31]. The *Aedes* mosquito vectors of this disease and diverse canid reservoirs are present in areas of the United States to which adoptees and refugees might move. The same mosquitoes, currently within the US, are also able to transmit such viral pathogens as dengue, Chikungunya, Zika, and yellow fever, so that health officials as well as entomologists and vector biologists in both the origination and recipient countries should be alert to the possibility of such dissemination.

Conflict of Interests

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

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Research Article

Identification and Biological Characterization of *Leishmania (Viannia) guyanensis* Isolated from a Patient with Tegumentary Leishmaniasis in Goiás, a Nonendemic Area for This Species in Brazil

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The aim of this study was to characterize clinical field isolates of *Leishmania* spp. obtained from patients with American Tegumentary Leishmaniasis (ATL) who live in Goiás state, Brazil. The presumed areas of infection were in Goiás, Tocantins, and Pará states. Three isolates of parasites were identified as *L. (Viannia) braziliensis* and one as *L. (V.) guyanensis*. The *in vitro* growth profiles were found to be similar for all parasites. Nevertheless, in C57BL/6 mice, *L. (V.) guyanensis* infection was better controlled than *L. (V.) braziliensis*. Yet in C57BL/6 mice deficient in interferon gamma, *L. (V.) guyanensis* lesions developed faster than those caused by *L. (V.) braziliensis* isolates. In BALB/c mice, the development of lesions was similar for isolates from both species; however, on the 11th week of infection, amastigotes could not be observed in macrophages from *L. (V.) guyanensis*-infected mice. Thus, *L. (V.) guyanensis* can be circulating in Goiás, a state where autochthonous cases of this species had not yet been reported. Considering the difficulties to differentiate *L. (V.) guyanensis* from *L. (V.) braziliensis* at the molecular, morphological, and clinical (human and murine models) levels, the presence of *L. (V.) guyanensis* infections is possibly underestimated in several regions of Brazil.

1. Introduction

Leishmaniases are protozoan diseases caused by more than 20 *Leishmania* species, which are transmitted by about 30 species of phlebotomine sand flies. Human infections cause three strikingly different clinical presentations and numerous clinical varieties ranging from asymptomatic to disfiguring forms of tegumentary and potentially fatal visceral leishmaniasis. American Tegumentary Leishmaniasis (ATL) presents a spectrum of clinical manifestations characterized by cutaneous (CL), mucosal (ML), disseminated (DL), and diffuse cutaneous leishmaniasis (DCL) [1, 2]. Brazil together with other nine countries accounts for 70–75% of estimated CL cases in the world [3]. A report of the Brazilian Secretary's Office of Surveillance in Health showed a geographic expansion of ATL during the 1980s from the Northern

towards the Southern region, and, in 2003, all Brazilian states registered autochthonous cases [4]. In 2013, the distribution per Brazilian region was reported as follows: Northern region with 8,407 new cases (49.5 cases per 100.000 population); Central Western region with the second highest prevalence, 2,922 new cases (19.5 cases per 100.000 population), Northeastern region with 5,355 new cases (9.6 cases per 100.000 population), Southeastern region with 1,150 new cases (1.4 cases per 100.000 population), and Southern region with 296 new cases (1.0 case per 100.000 population) [5, 6].

Three main *Leishmania* species are responsible for ATL in Brazil: *L. (Viannia) braziliensis*, *L. (Leishmania) amazonensis*, and *L. (Viannia) guyanensis*. Besides, *L. (Viannia) lainsoni*, *L. (Viannia) naiffi*, and *L. (Viannia) shawi* have also been identified as new agents of ATL in the Northern region. The species *L. (V.) braziliensis* presents wider geographic distribution than the other species in Brazil (it is reported in all Brazilian states) whereas *L. (V.) guyanensis* is believed to be restricted to the Northern region [7, 8]. The distribution of *Leishmania* species depends on the vectors, animal reservoirs, and hosts as well as the ecology of the endemic areas. As *L. (V.) braziliensis* is widely distributed in South America, this species may be transmitted by several different sand flies species and different animal species can be the reservoirs in distinct ecologic and geographical areas, which increases the molecular diversity of the parasites [9, 10]. In Brazil, *L. (V.) braziliensis* is commonly transmitted by *Lutzomyia whitmani* (Northeastern, Central Western, and Southeastern regions), *L. wellcomei* (Northern region), *L. intermedia* (Southeastern region), and *L. neivai* (Southern region). Besides, *L. umbratilis* has also been suggested as vector for *L. (V.) braziliensis* in Mato Grosso state (Central Western region). The latter species is the main vector for *L. (V.) guyanensis*, which is also transmitted by *L. anduzei* and *L. whitmani* [1, 9, 11, 12]. In Goiás state (Central Western region), *L. intermedia* and *L. whitmani* have been associated with ATL [13, 14]. Mammal reservoirs of *L. (V.) braziliensis* can be found among numerous species of forest animals, especially rodents, whereas *L. (V.) guyanensis* is more frequent in sloths, anteaters, and opossums [8].

L. (V.) braziliensis and *L. (V.) guyanensis* are associated with the same clinical manifestations of ATL as localized cutaneous, disseminated, and mucosal leishmaniasis can be ascribed to both species [15–18]. Therefore, determining the *Leishmania* species causing disease in a patient cannot rely on clinical criteria and parasite identification is essential to prescribe the best species-specific therapeutic regimen [15, 17]. Furthermore, genetic heterogeneity and clonal diversity, which leads to variability in parasite virulence, are also common among *L. (Viannia)* spp. parasites [10, 19, 20].

In the present study, we characterized four *Leishmania* spp. isolates obtained from patients with ATL assisted at the Tropical Disease Hospital of Goiânia, Goiás, Brazil, a reference center for leishmaniasis diagnosis and treatment in Goiás state (Central Western region). The leishmaniasis cases from Northern and Central Western Brazil are referred to this hospital. The patients in our study were probably infected in Goiás (Central Western region), Tocantins, or Pará (Northern region) states. A comprehensive knowledge of the species and the characteristics of the parasites are very important for controlling the disease, mainly when patients migrate to other regions/states with different ecosystems and increase the threat of new *Leishmania* foci.

2. Materials and Methods

2.1. Mice. Female C57BL/6 (wild-type [WT]) or C57BL/6 IFN-gamma knockout (IFNγ KO C57BL/6) and BALB/c mice, six to eight weeks old, were obtained from the breeding animal facilities of the IPTSP/Federal University of Goiás, Goiânia, Brazil. All the animal handling and procedures were approved by the Ethical Committee from Clinical Hospital of the Federal University of Goiás on the ethical handling of research animals.

2.2. Patients. Four patients were assisted at the Tropical Disease Hospital (Anuar Auad, Goiânia, Goiás, Brazil) with the diagnostic hypothesis of leishmaniasis. All of them live in Goiás, but the presumed areas of infection were Goiás, Tocantins, and Pará/Maranhão border. Diagnosis of ATL was confirmed by epidemiological, clinical, and laboratory analyses. The patients' data are presented in Table 1. The protocols on this investigation relative to human patients and animals were approved by the Ethical Committee from Hospital das Clínicas, Universidade Federal de Goiás, and all patients signed an informed consent form.

2.3. Parasite Isolation and Cultures. A fragment of the cutaneous lesion biopsy was macerated and inoculated in mice footpads (IFNγ KO C57BL/6) or directly cultured at 26°C in Grace's Insect Cell Culture Medium (Gibco-BRL Life Technologies, Grand Island, NY, USA) containing 20% heat-inactivated fetal bovine serum (Cripion, Andradina, SP, Brazil), 2 mM glutamine, penicillin (100 U/mL), and streptomycin (100 μg/mL) (supplements and antibiotics were purchased from Sigma Chemical Co., St. Louis, MO, USA). After 1-2 months draining lymph nodes from the animals were processed as described [21] and cultured in complete Grace's medium. After being expanded in culture, the parasite isolates were cryopreserved in liquid nitrogen. The parasite isolates were thawed and expanded once in complete Grace's medium before use in all experiments. All these procedures were previously described [21]. The parasite isolates were coded as WWS5 (MHOM/BR/2005/WSS5), UAF5 (MHOM/BR/2005/UAF5), HPV6 (MHOM/BR/2006/HPV6), and PLR6 (MHOM/BR/2006/PLR6). The isolates WWS5, UAF5, and HPV6 were previously identified as *L. (V.) braziliensis* [22, 23].

2.4. Molecular Characterization: Polymerase Chain Reactions (PCR). The identification of the isolates was based on three strategies: (1) small subunit ribosomal RNA (SSU rDNA) was sequenced as previously described, using primers S12/S4 [24]. Positive control reactions were performed using a reference genomic DNA purified from axenic cultures of *L. (L.) amazonensis* MHOM/BR/1973/M2269, *L. (V.) braziliensis* MHOM/BR/1975/M2903, *L. (L.) chagasi* MHOM/BR/1972/LD, *L. (V.) guyanensis* MHOM/BR/75/M4147, or *L. (V.) shawi*

Table 1: ATL patients from whom the isolates were obtained[a].

Patients	HPV	UAF	WSS	PLR
Sex[b]	M	M	M	M
Age[c]	46	29	22	19
Clinical form[d]	CL	CL	CL	CL
Number of lesions	2	1	1	3
Type of lesions	Ulcerated	Ulcerated	Ulcerated	Ulcerated
Time of lesion[e]	3 m	2 m	8 m	2 m
Lesion site	Upper limbs	Lower limb	Lower limb	Lower limbs
Satellite adenomegaly	No (lymphangitis)	No	No	No
Leishmanin skin test	No reaction	5 mm	NR[f]	5 mm
Histopathological analysis	Presence of amastigotes	Presence of amastigotes	NR	Presence of amastigotes
Treatment	Pentavalent antimonial	Pentavalent antimonial (two cycles)	No treatment	Pentavalent antimonial
Indirect immunofluorescence reaction	No reaction	No reaction 80 (after treatment)	NR	160
Clinical outcome	Clinical cure	Clinical cure	No treatment	NR
Presumed place of infection	Tocantins (TO)[g]	Tocantins (TO)	Goiás (GO)[h]	Pará[i]/Maranhão (MA)[j] (PA)

[a]Patients were assisted at Anuar Auad Tropical Disease Hospital, Goiânia, Goiás (2005-2006).
[b]F = feminine, M = masculine, [c]age in years, [d]clinical form CL = cutaneous localized, [e]time of lesion = in months, and [f]NR = patient did not return. [g]Tocantins (TO) is a state of the Northern region, [h]Goiás (GO) is a state in Central Western region, [i]Pará (PA) is a state of the Northern region, and [j]Maranhão (MA) is a state of the Northeastern region (border with Pará).

MCEB/BR/84/M8408, while in negative controls no genomic DNA was added. The amplified product was analyzed in a 2% agarose gel electrophoresis stained with ethidium bromide. The nucleotide sequence of the 520 bp fragment was obtained directly by automatic sequencing using an ABI Big-Dye kit as described [23]. (2) Sets of primers were used in PCR assays to discriminate *L. (V.) braziliensis* (G6PD-ISVC and G6PD-ISVB) from other organisms of the *Viannia* subgenus (G6PD-ISVG and G6PD-LVF) as described before [25]. PCR reactions were prepared in 50 μL final volume containing 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 0.2 mM of each deoxyribonucleotide, 15 pmol of each primer, 2.5 U of Taq DNA polymerase (Gibco), and 100 ng of template DNA. (3) The ribosomal internal transcribed spacer (ITS) was amplified using primers IR1 and IR2 [26]. The approximately 1 kb PCR amplified product was digested with *Hae* III as described [27] and analysed by gel electrophoresis. The amplified product was also purified from agarose gels using the QIAquick PCR purification kit (Qiagen, Valencia, USA) and cloned in pGEM-T easy (Promega Corporation, Madison, WI, USA). The nucleotide sequence of three independent positive clones, confirmed previously by restriction analysis, was determined as described above using pUC/M13, IR1, IR2, 5.8F (5′ GCAGTAAAGTGCGATAAGTGG 3′), and 5.8R (5′ GGAAGCCAAGTCATCCATC 3′) primers. Nucleotide sequence analyses were performed using Lasergene Software (DNASTAR) and Clone Manager 9.0 Software. Phylogenetic analysis was performed using RAxML [28].

2.5. In Vitro Growth of Leishmania Isolates. The parasite isolates extracted from draining lymph nodes of infected IFNγ KO C57BL/6 mice were cultured at an initial concentration of 5×10^5/mL in 24-well culture plates (TPP, Techno Plastic Products, Trasadingen, Swizerland) in complete Grace's medium at 26°C. Samples of parasites were counted daily for 13 days in a hemocytometer after dilution in 2% formaldehyde solution in PBS, under light microscopy.

2.6. Infection of Mice. Groups of four mice were injected subcutaneously (s.c.) with 5×10^6 live promastigotes (50 μL) in stationary phase of growth into the left hind footpad. Lesion development was followed by measuring the thickness of the infected paw with a dial caliper at weekly intervals and expressed by the arithmetic mean and standard error mean (SEM) of the net thickness increase (infected minus control contralateral paw thickness). Following ethical procedures, when the paw lesion reached 5 mm in thickness or presented ulceration, the mice were euthanized.

2.7. Tissue Processing for Optical Microscopy. To analyze the local inflammatory reaction and presence or absence of parasites, footpads were removed postmortem on the 5th or 6th (IFNγ KO C57BL/6) or 11th (C57BL/6 WT, BALB/c) week after infection, excised, and prefixed with 10% formalin, followed by fixation in Bouin solution (picric acid 75%, glacial acetic acid 5%, and formaldehyde 10%) prior to paraffin embedding. Five μm sections from the material were stained

with hematoxylin and eosin (H&E) and examined under light microscopy.

2.8. Statistical Analysis. Data are presented as mean ± standard error of the mean (SEM). Two way ANOVA/Bonferroni was used to compare the data, and the differences were considered significant when $P < 0.05$.

3. Results

3.1. Patient Profiles. The age of patients varied from 19 to 46 years and they had one to three cutaneous lesions located in the limbs that appeared from two to eight months before the diagnosis. All patients were diagnosed with ATL, presenting the cutaneous localized clinical form (LCL) according to clinical and laboratory analyses. In all patients, the lesions were ulcerated. Patients' data are presented in Table 1.

3.2. Molecular Characterization of Leishmania Isolates. SSU rDNA amplification was performed on four clinical field isolates and controls, using primers S12/S4 and the PCR products were analyzed by automatic sequencing. The nucleotide sequence of the four isolates identified HPV6, UAF5, WSS5, and PLR6 as species of the *Viannia* subgenus (data not shown). All samples were also analyzed by PCR of the *G6PD* gene with primers specific for *L. (V.) braziliensis* or "non-*braziliensis*" *Viannia* species. This analysis confirmed the identity of HPV6, UAF5, and WSS5 as *L. (V.) braziliensis* and of PLR6 as a "non-*braziliensis*" isolate (data not shown).

The identification of the PLR6 isolate was based on the analysis of ribosomal ITSs 1 and 2. Approximately 1 kb fragment was amplified and digested with *Hae* III. The analysis of restriction fragment polymorphisms indicated that PLR6 displayed a pattern compatible with *L. (V.) guyanensis* (Figure 1). This was confirmed by nucleotide sequencing of the 1 kb fragment encompassing ITS1, 5.8S rDNA, and ITS2. The sequence obtained (Genbank number AJ000299.1) showed 99% identity with *L. (V.) guyanensis* (MHOM/BR/75/M4147).

3.3. Behavior of the Isolates in In Vitro Culture. Replication rates of the four isolates were similar in complete Grace's medium at 26°C during 13 days. The growth curves exhibited typical logarithmic and stationary phases. The parasites formed large clumps at the stationary phase (data not shown). The maximum number of parasites occurred within 4 to 6 days, ranging from around 5×10^7 to 1×10^8/mL (Figure 2). After 10 days of culture, parasites of all isolates began to die.

3.4. Course of Infection in Mice. In order to compare the outcome of infection caused by all isolates, stationary-growth-phase promastigotes (the 6th day of culture) were inoculated into C57BL/6 WT and BALB/c mouse footpads. Infection was successfully established for all *L. (V.) braziliensis* isolates in C57BL/6 WT and BALB/c mice and the lesions increased to a size of approximately 1.0–1.5 mm (Figures 3(a), 3(b), and 3(c)). The infection with the *L. (V.) guyanensis* PLR6 isolate caused a lesion more severe in BALB/c mice than in C57BL/6 ($P < 0.05$), which completely controlled the infection by 11 weeks (Figure 3(d)).

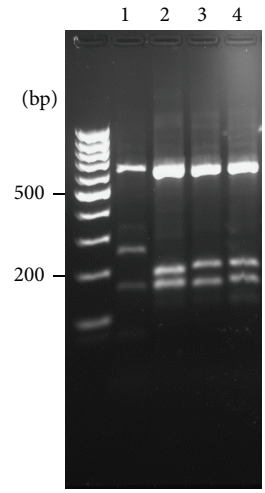

Figure 1: Identification of *Leishmania* PLR6 isolate by ITS1 and 2 amplifications from genomic DNA. The PCR amplified products of approximately 1 kb were digested with *Hae* III and restriction fragment analysis was evaluated in an ethidium bromide stained 2% agarose gel. 1: *L. (L.) amazonensis*, 2: *L. (V.) braziliensis*, 3: *L. (V.) guyanensis*, and 4: PLR6 isolate.

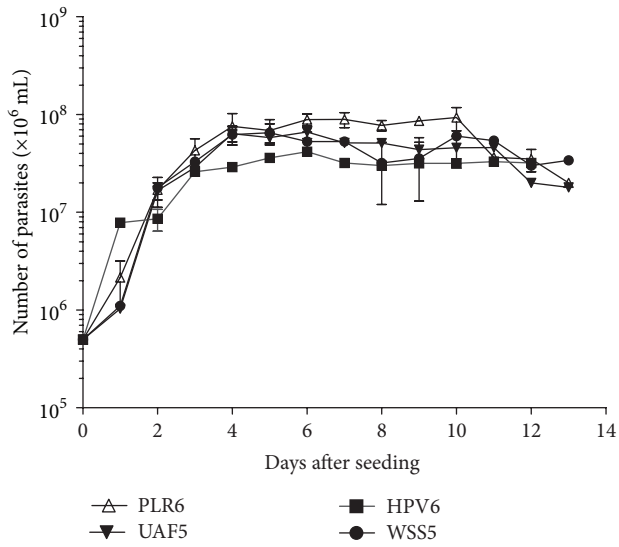

Figure 2: *In vitro* growth curves of *Leishmania (V.) braziliensis* (HPV6, UAF5, and WSS5) and *Leishmania (V.) guyanensis* (PLR6) in complete Grace's medium. Parasites were seeded in 5×10^6/mL and cultured during 13 days, at 26°C in BOD. The data represent mean ± SEM of two-to-three independent experiments performed in triplicate.

In the absence of IFNγ (IFNγ KO C57BL/6), progressive lesions developed rapidly, except for the *L. (V.) braziliensis* WSS5 isolate (Figure 4(a)). Both *L. (V.) braziliensis* and *L. (V.) guyanensis* caused ulcerated lesions in IFNγ KO C57BL/6 (Figure 4(b)). In IFNγ KO C57BL/6 mice inoculated with *L. (V.) guyanensis* PLR6 isolate, lesions developed faster

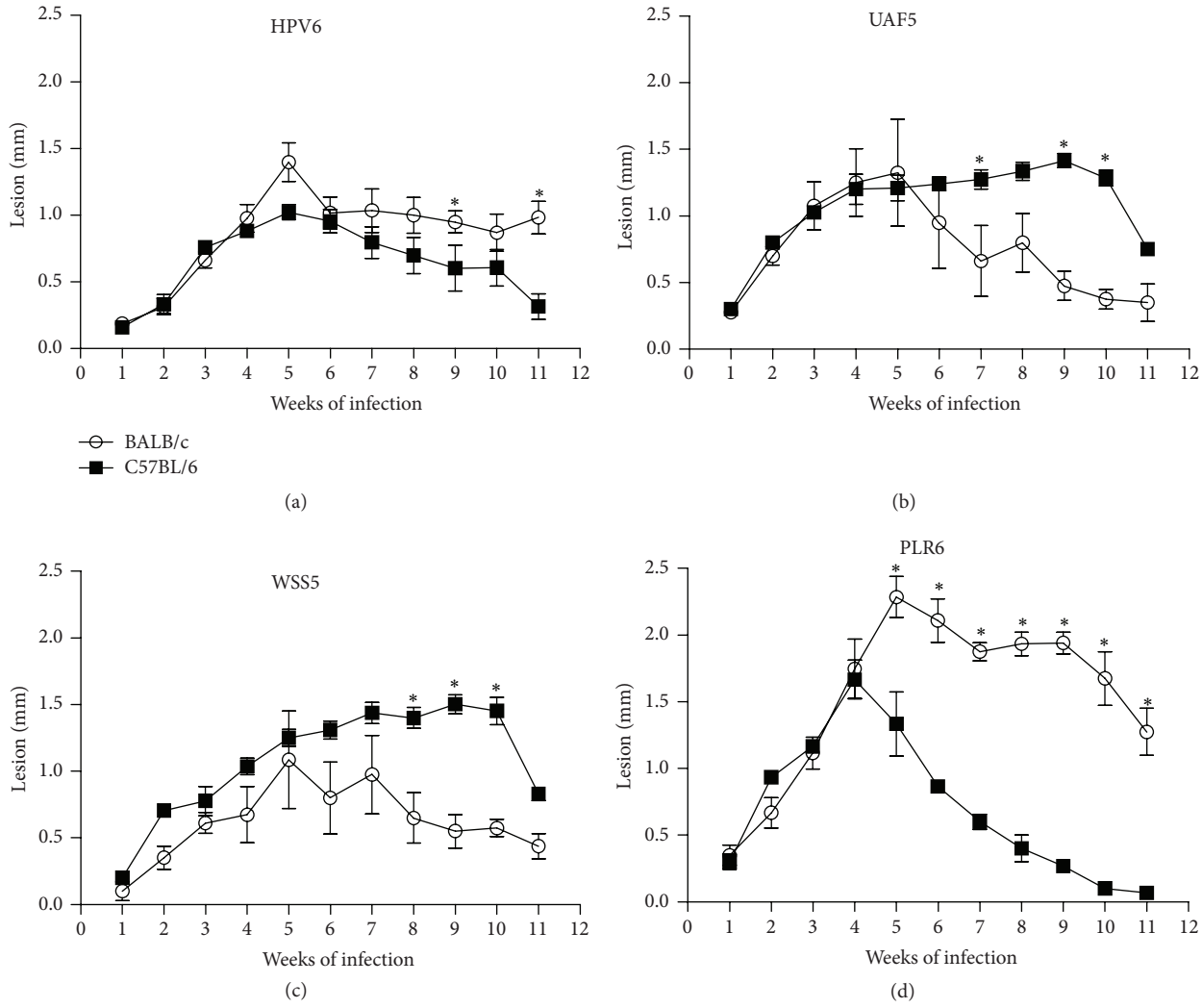

Figure 3: Time course of lesion development in C57Bl/6 WT (■) and BALB/c (○) mice infected with *Leishmania (V.) braziliensis* (HPV6, UAF5, and WSS5) or *Leishmania (V.) guyanensis* (PLR6). Mice were infected with promastigotes in stationary phase of growth (5×10^6 parasites) into hind footpads. The lesion size was expressed in mm (infected minus control contralateral paw thickness). Data represent mean ± SEM of results from two or three independent experiments (4–14 animals): HPV6 (a), UAF5 (b), WSS5 (c), and PLR6 (d); $^*P < 0.05$.

than in mice of the same strain inoculated with *L. (V.) braziliensis* (Figure 4(a)) and dissemination of parasites to the contralateral paw (increased thickness) was apparent on the 6th week after infection when the infected footpad began to ulcerate (data not shown).

3.5. Histopathological Analysis. Sections of the footpads obtained on the 11th week after infection with *L. (V.) braziliensis* WSS5 or *L. (V.) guyanensis* PLR6 were examined (Figures 5 and 6). On histological examination, the lesions in C57BL/6 footpads infected with either *Leishmania* isolate were characteristic of the late phase of tissue repair, with hypertrophic scar formation and marked fibrosis in the dermis with few mononuclear inflammatory cells; the epidermis was intact with hyperplasia of epidermal cells (Figures 5(a) and 5(b)). No parasites could be seen at higher magnification (1000x, data not shown).

In comparison, BALB/c mice infected with *L. (V.) braziliensis* also presented intact epidermis and superficial dermis but, in the deep dermis, a mononuclear inflammatory infiltrate rich in vacuolated macrophages was located close to and infiltrating the muscle bundles (Figure 5(c)); most macrophages were parasitized with *L. (V.) braziliensis* WSS5 (Figure 5(e)). On the other hand, in mice inoculated with *L. (V.) guyanensis* PLR6, hypertrophic scar and accentuated fibrosis were seen in the dermis and a mononuclear inflammatory infiltrate (Figure 5(d)) with vacuolated macrophages free of intact parasites was observed in the deep dermis (Figure 5(f)).

A marked difference in the histology was seen in IFNγ KO C57BL/6 on the 6th week after inoculation with the same isolates (Figure 6). These mice, inoculated with the *L. (V.) braziliensis* WSS5 isolate, did not have epidermal ulceration of the paw and from the plantar to dorsal side of

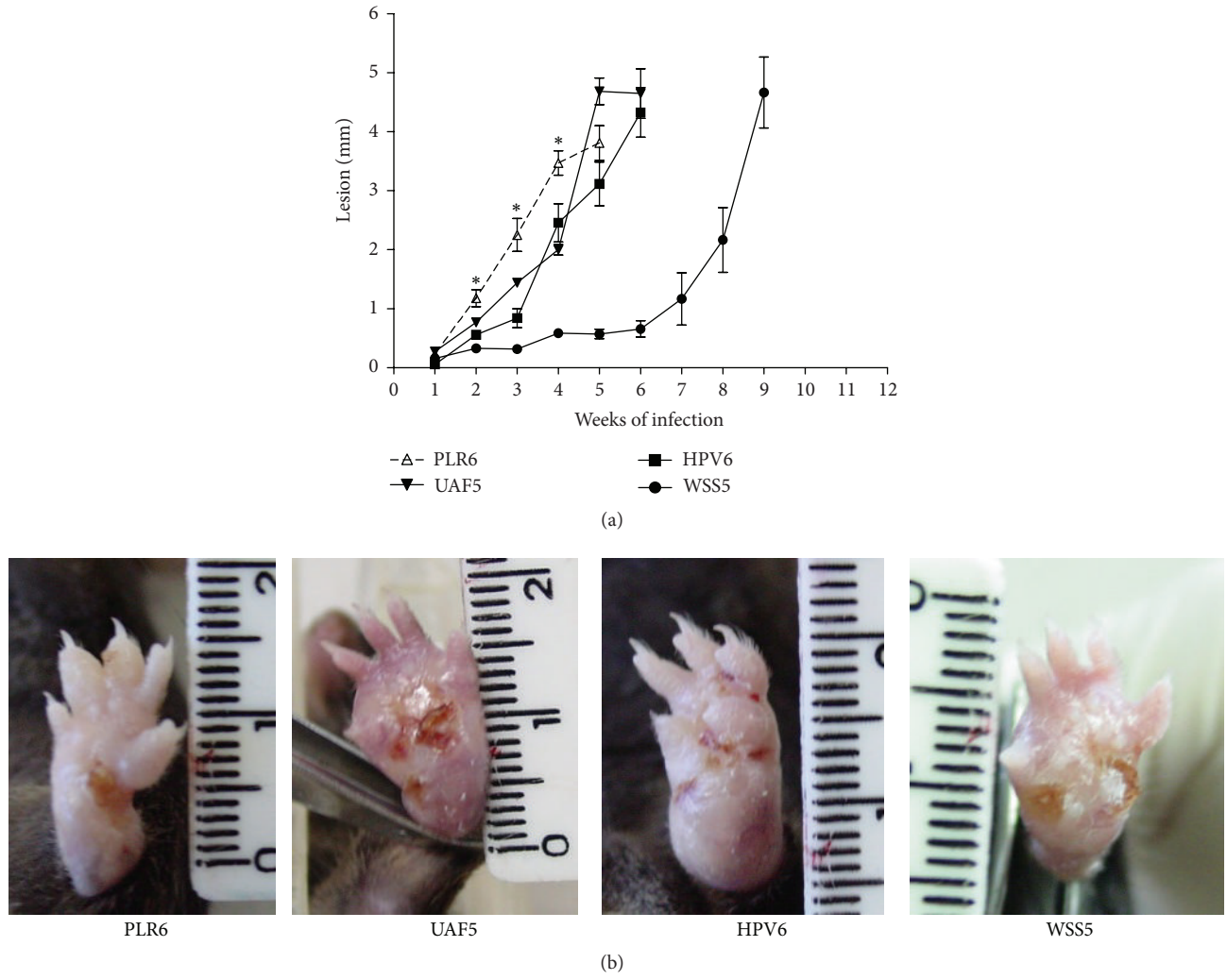

Figure 4: Time course of lesion development in IFNγ-deficient C57BL/6 mice infected with *L. (V.) braziliensis* (HPV6, UAF5, and WSS5) or *L. (V.) guyanensis* (PLR6). Lesion size was expressed in mm (infected minus control contralateral paw thickness). Data represent mean ± SEM (3–10 animals, (a)), $^{*}P < 0.05$. In (b), lesions caused by *L. (V.) braziliensis* (HPV6, UAF5, and WSS5) or *L. (V.) guyanensis* (PLR6)

the paw there was an infiltration of mononuclear cells with many parasite-laden macrophages (Figures 6(a) and 6(c)). In contrast, the footpads of mice inoculated with the *L. (V.) guyanensis* PLR6 isolate, as also the other *L. (V.) braziliensis* isolates, presented a visible ulceration in the plantar surface of the footpad; the inflammatory infiltrate was predominantly mononuclear with areas of necrosis and fibrin deposition near the base of the ulcer (Figures 6(b) and 6(d)); large numbers of parasite-laden macrophages were scattered in the whole dermis (Figure 6(d)).

4. Discussion

This report characterizes the *Leishmania (Viannia)* species isolated from skin biopsies of four patients assisted at the Tropical Diseases Hospital (Anuar Auad, Goiânia, Goiás), with a diagnosis of localized cutaneous leishmaniasis. In our previous studies, three of these isolates were identified as *L. (V.) braziliensis* (data not shown and [29]) and one remained unidentified. Here, characterization of the ribosomal ITS allowed identification of the latter isolate as *L. (V.) guyanensis*. It is important to stress here the difficulties in correctly identifying this isolate as *L. (V.) guyanensis*. To achieve this characterization, we have used three strategies: small subunit ribosomal RNA (SSU rDNA) was sequenced [24]; sets of primers for G6PD were used in PCR assays to discriminate *L. (V.) braziliensis* from other organisms of the *Viannia* subgenus [25]; and ITS was amplified and cloned and the nucleotide sequence of three independent positive clones was phylogenetically analyzed [28]. It is crucial to identify the *Leishmania* species in order to define which parasites are circulating in a geographic area, to establish the transmission cycles of ATL and to implement the best possible treatment. These points, especially the last one, together with our results, indicate the need of more suitable molecular techniques to define the *Leishmania* species in the diagnosis of ATL.

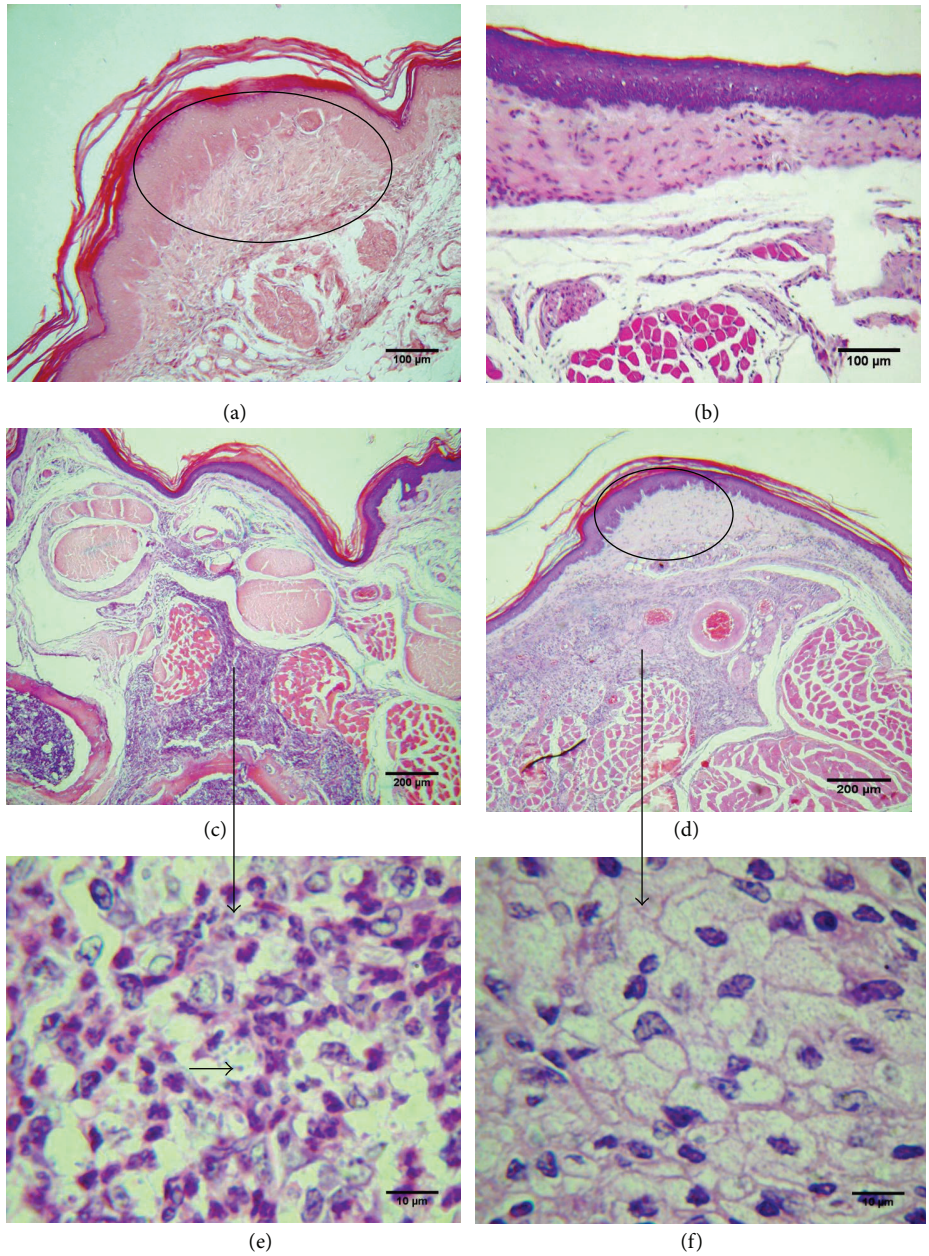

Figure 5: Photomicrographs of nonulcerated lesions obtained from wild-type C57BL/6 and BALB/c mice infected with *L. (V.) braziliensis* WSS5 or *L. (V.) guyanensis* PLR6. Mice were inoculated with 5×10^6 parasites at stationary phase of growth, and 11 weeks after infection the histopathology of footpad lesions was evaluated after H&E staining. (a) C57BL/6 WT mouse infected with *L. (V.) braziliensis* WSS5, (b) C57BL/6 WT mouse infected with *L. (V.) guyanensis* PLR6, (c) BALB/c mouse infected with *L. (V.) braziliensis* WSS5 showing inflammatory infiltration in deep dermis, (d) BALB/c mouse infected with *L. (V.) guyanensis* PLR6 showing inflammatory infiltrate in deep dermis, (e) BALB/c mouse infected with *L. (V.) braziliensis* WSS5 (horizontal black arrow indicates the parasite; vertical black arrow indicates the infiltrate of deep dermis in (c) that contains macrophages and parasites), and (f) BALB/c mouse infected with *L. (V.) guyanensis* PLR6 (vertical black arrow indicates the mononuclear cell infiltrate in deep dermis in (d) that contains vacuolated macrophages without intact parasites). Areas of hypertrophic scar formation are indicated by the circles in (a) and (d).

In this study, all ATL patients resided in Goiás, but the presumed geographic areas of patient infections for the three *L. (V.) braziliensis* isolates obtained were Goiás and Tocantins states. The patient infected with *L. (V.) guyanensis* reported having travelled to a forest zone in the boundary of the states Pará (Northern region) and Maranhão (Northeastern region) two months prior to the appearance of lesions. It is known that *L. (V.) braziliensis* is the parasite largely responsible for ATL in all five Brazilian regions, including Central Western, whereas *L. (V.) guyanensis* is prevalent only

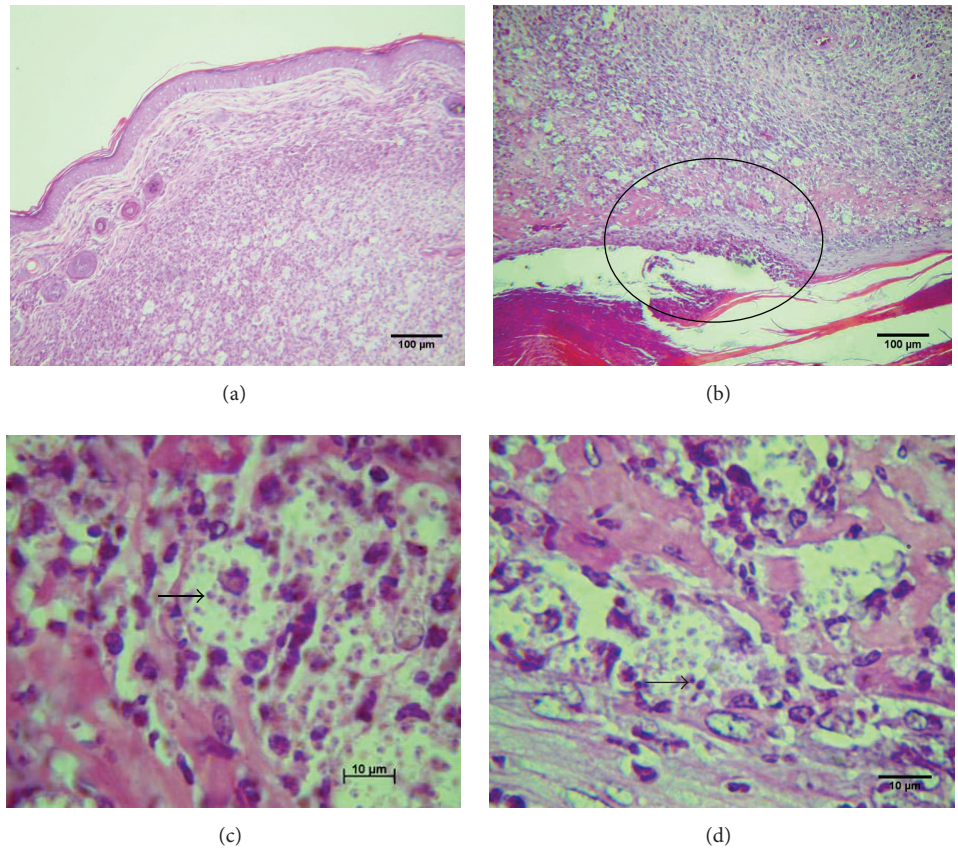

Figure 6: Photomicrographs of lesions caused by *L. (V.) braziliensis* WSS5 and *L. (V.) guyanensis* PLR6 in IFNγ-deficient C57BL/6 mice. Mice were inoculated with 5×10^6 parasites in stationary phase of growth, and six weeks after infection the histopathology of footpad lesions was evaluated after H&E staining. (a) Nonulcerated lesion in mouse infected with *L. (V.) braziliensis* WSS5 isolate, (b) ulcerated lesion in mouse infected with *L. (V.) guyanensis* PLR6 (the circle shows part of the ulceration that contains mononuclear cells and parasites showed in (d)), (c) mononuclear cell infiltrate with presence of parasites of *L. (V.) braziliensis* WSS5 (black arrow), and (d) mononuclear cells infiltrate with presence of parasites of *L. (V.) guyanensis* PLR6 (black arrow).

in the Northern region of Brazil, and there are no reports about autochthonous cases of ATL caused by this species in Goiás state or another Brazilian regions [7, 8]. Thus, our findings confirm the fact that there is a high probability of *L. (V.) guyanensis* being introduced in Goiás state due to the migratory behavior of patients infected with these parasites from the Northern to Central Western region. This possibility is reinforced by the fact that the Tropical Disease Hospital/Anuar Auad assists several patients from the states of the Northern region (around 25% of the assisted patients, *personal communication*), pointing out the need of parasite species identification in ATL patients.

The distribution of *Leishmania* spp. is dependent on vectors and reservoir hosts present in a geographic area. Thus, in Goiás, there are 47 different species of phlebotomine sand flies [14], with a predominance of *L. intermedia* and *L. whitmani* [8, 13, 14] which are vectors of *L. (V.) braziliensis* in Goiás, Tocantins, Pará, and Maranhão states. For *L. (V.) guyanensis*, *L. umbratilis* is the main vector in the Brazilian Northern region; it has not been reported in Goiás [8, 13, 14]. However, *L. umbratilis* is also *L. (V.) braziliensis* vector in Mato Grosso (Central Western region); and *L. whitmani*, which is associated with a great variety of vegetation, including Amazonian forest, Cerrado (savanna, predominant in Goiás), and Caatinga (Northeastern savanna), also transmits *L. (V.) guyanensis*. Moreover, *L. flaviscutellata*, present in Goiás, was found to transmit *L. (V.) guyanensis*. Besides, anteaters and opossums, considered as reservoir of *L. (V.) guyanensis*, are present in Goiás [1, 8, 14, 30–34].

Our findings prompted us to closely evaluate *L. (V.) guyanensis* which has so far been poorly investigated in Brazil. The clinical findings were similar between patients infected with *L. (V.) braziliensis* and with *L. (V.) guyanensis*. The patient infected with *L. (V.) guyanensis* presented three ulcerated lesions and received the treatment but did not return for a follow-up examination. The similarity between clinical manifestations in LCL caused by *L. (V.) braziliensis* and *L. (V.) guyanensis* has been reported, but the response to antimonial treatment can be different [15, 17]. The isolate *L. (V.) guyanensis* PLR6 has not been tested for antimonial

susceptibility, but the other isolates *L. (V.) braziliensis* HPV6, UAF5, and WSS5 were uniformly susceptible *in vitro* to meglumine antimoniate and amphotericin B [22].

Corroborating the difficulties in identifying the *Leishmania* species relying on the clinical findings, our results did not show any difference in the monophasic-culture replication rates among the different isolates. In these cultures, *Leishmania* species were morphologically similar, and the *in vitro* growth profiles were similar to those previously described for *L. (V.) braziliensis* [34].

Our group has previously confirmed that the isolate *L. (V.) braziliensis* HPV6 is able to infect C57BL/6 mice and the J774 murine macrophage cell line [33]. In the present study, we confirmed the infection capacity of this isolate in C57BL/6 and BALB/c mice. All four isolates infected C57BL/6 and BALB/c mice. In contrast to C57BL/6 WT mice infected with *L. (V.) braziliensis* isolates, those infected with *L. (V.) guyanensis* PLR6 showed fast regression of the lesion, which almost disappeared after 11 weeks. BALB/c mice also showed nonulcerative skin swelling when infected with all isolates. However, in the deep dermis of *L. (V.) guyanensis* PLR6-infected mice, no parasites were detected inside macrophages whereas in *L. (V.) braziliensis* WSS5-infected footpads we observed a large number of parasites. The size of lesions caused by (*L. (V.) braziliensis*) HPV6, UAF5, and WSS5 was similar to those described by Pereira et al. [35] but larger than the size found in murine models of infection with this species [36–38]. de Moura et al. [39] reported that inoculation of *L. (V.) braziliensis* into the ear dermis of BALB/c mice leads to the development of an ulcerated lesion. The discrepancies with our results could be related to the site of inoculation or the virulence of the parasite strain. Considering the size and time course of the infection caused by *L. (V.) guyanensis* PLR6, our results were similar to those obtained by Sousa-Franco et al. [40], and like these authors we did not find parasites inside macrophages after 11 weeks of infection.

In this study, high susceptibility of IFNγ KO C57BL/6 mice to all four isolates confirms a close association between resistance and production of Th1 cytokines (IFNγ) during the course of *L. (Viannia)* spp. infection as has been described by de Souza-Neto et al. [41] in one *L. (V.) braziliensis* mouse model. The infection of IFNγ KO C57BL/6 showed macroscopical and microscopical differences between infected footpads of mice inoculated either with *L. (V.) braziliensis* or *L. (V.) guyanensis*. The development of the lesion caused by *L. (V.) guyanensis* PLR6 was faster than those caused by *L. (V.) braziliensis* isolates and PLR6 caused cutaneous metastatic lesions that could be observed in the contralateral footpad. The histopathology analysis showed parasites in the ulcerative area in lesions from the 5th week after infection. Secondary cutaneous metastatic lesions induced by *L. (V.) guyanensis* have also been reported in hamsters [42]. On the other hand, on the 5th week of infection, the whole extension of *L. (V.) braziliensis* WSS5-infected footpad consisted of an intense inflammatory infiltration full of parasites, without signs of ulceration; however, after nine weeks of infection, this parasite induced the same ulceration as the other *L. (V.) braziliensis* isolates. As C57BL/6 WT mice presented better control of the *L. (V.) guyanensis* infection and the development of the lesion caused by this species was faster than that caused by *L. (V.) braziliensis* in IFNγ KO C57BL/6 mice, it can be suggested that *L. (V.) guyanensis* can present higher susceptibility to leishmanicidal mechanisms induced by IFNγ than *L. (V.) braziliensis*. The highest susceptibility of IFNγ KO C57BL/6 to *L. (Viannia)* spp. led us to use this mouse strain for the process of parasite isolation from lesions of ATL patients [21, 43].

ATL has been considered as a social and economic problem of the poor population which has resulted largely from an intense migratory movement into rural areas and the forested hillsides that are close to the outskirts of the urban centers. However, due to the intense international travel and the large contingents of displaced and migratory populations, Tegumentary Leishmaniasis has to be considered as a diagnosis of nonhealing indolent ulcers also in nonendemic areas. Moreover, infection by *L. (V.) guyanensis* has been diagnosed in Europe in a soldier who denied travelling to endemic areas or having blood transfusions, raising some uncommon possibilities of contagion [44].

Nowadays, migration among Brazilian regions has largely increased, both inside forested areas and in urban areas. The finding of infection with *L. (V.) guyanensis* in a patient residing in the state of Goiás, where species other than *L. (V.) braziliensis* and *L. (L.) amazonensis* had not been previously described [7, 8, 45], improves the knowledge about ATL spreading pattern and reinforces the need for surveillance, control, and prevention of new ATL foci in Brazil. The difficulties to differentiate *L. (V.) guyanensis* from *L. (V.) braziliensis* at several levels, such as molecular, morphological, and clinical ones, draw attention to the possible underestimated prevalence of *L. (V.) guyanensis* in different Brazilian regions. Besides this contribution, our results also increased the knowledge on *L. (V.) guyanensis* infectivity in murine infection models, suggesting that IFNγ can be more relevant for controlling *L. (V.) guyanensis* than *L. (V.) braziliensis*.

5. Conclusions

We have isolated and characterized three clinical field isolates of *Leishmania* spp., from patients probably infected in Goiás, Tocantins, and Pará states, Brazil, as *L. (Viannia) braziliensis* and one as *L. (V.) guyanensis*. The latter species had not yet been described in Goiás. Infection of mouse strains BALB/c, C57BL/6 wild-type, and C57BL/6 lacking gamma-interferon (IFNγ KO C57BL/6) showed differences in lesion development among the *Leishmania* strains. In addition, better infection control of *L. (V.) guyanensis* than *L. (V.) braziliensis* was observed in mice in the presence of IFNγ but not in the absence of this cytokine. Molecular identification of *L. (V.) guyanensis* in a patient resident in Goiás stresses the importance of correct species identification and suggests that the presence of this species is possibly underestimated in several areas of Brazil.

Conflict of Interests

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

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Research Article

Prevalence of Malaria Infection and Risk Factors Associated with Anaemia among Pregnant Women in Semiurban Community of Hazaribag, Jharkhand, India

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The escalating burden, pathogenesis, and clinical sequel of malaria during pregnancy have combinatorial adverse impact on both mother and foetus that further perplexed the situation of diagnosis, treatment, and prevention. This prompted us to evaluate the status of population at risk of MIP in Hazaribag, Jharkhand, India. Cross-sectional study was conducted over a year at Sadar Hospital, Hazaribag. Malaria was screened using blood smear and/or RDT. Anaemia was defined as haemoglobin concentration. Pretested questionnaires were used to gather sociodemographic, clinical, and obstetrical data. The prevalence of MIP was 5.4% and 4.3% at ANC and DU, and 13.2% malaria was in women without pregnancy. Interestingly, majority were asymptomatically infected with *P. vivax* (over 85%) at ANC and DU. Peripheral parasitemia was significantly associated with fever within past week, rural origin of subjects, and first/second pregnancies in multivariate analysis, with the highest risk factor associated with fever followed by rural residence. Strikingly in cohort, anaemia was prevalent in 86% at ANC as compared to 72% at DU, whereas severe anaemia was 13.6% and 7.8% at ANC and DU. Even more anaemia prevalence was observed in MIP group (88% and 89% at ANC and DU), whereas severe anaemia was 23% and 21%, respectively. In view of observed impact of anaemia, parasitemia and asymptomatic infection of *P. vivax* during pregnancy and delivery suggest prompt diagnosis regardless of symptoms and comprehensive drug regime should be offered to pregnant women in association with existing measures in clinical spectrum of MIP, delivery, and its outcome.

1. Introduction

Malaria in tropical regions, which is caused by the protozoan parasites *Plasmodium falciparum* and *Plasmodium vivax*, is responsible for 515 million clinical cases [1] and 1 to 3 million deaths annually [2]. *Plasmodium vivax*, the most widespread parasite causing human malaria, is responsible for estimated 130–435 million infections annually and is the major cause of malaria in most of Asia and Latin America [3]. Although *P. vivax* infection is commonly considered to

be much more benign than *Plasmodium falciparum* infection, historical evidence suggests significant mortality associated with *P. vivax* malaria in the preantimalarial era [4], and death caused by *P. vivax* malaria has been increasingly recognized over the past few years [3, 5]. Hazaribag, the region under investigation, was primarily dominated by *P. vivax* whereas some buffering, bordering, and adjoining regions have lower prevalence of *P. falciparum* and mixed infection. The other human infecting *Plasmodium* parasites, like *P. ovale*, *P. malariae*, and *P. knowlesi,* are the rarest in Indian isolates and these parasites neither were observed during our investigation nor have been reported previously from Jharkhand.

The emergence and spread of drug resistance to commonly used chemotherapeutics are major factors contributing to this increasing burden and most of the mortality and morbidity are borne by children and pregnant women. Pregnant women and their infants are susceptible to common and preventable infectious diseases including malaria but are woefully left unscreened and untreated. According to an estimate, approximately 125 million pregnant women worldwide are exposed to the risks of malaria in pregnancy (MIP) each year, resulting in 200,000 infant deaths [6]. Every year, in India, 28 million pregnancies take place with 67,000 maternal deaths (Registrar General of India, Sample Registration System, Special Bulletin on Maternal Mortality in India, 2004-06), with 1 million women left with chronic ill health and 1 million neonatal deaths [7]. Pregnancy is an event of immunologic tolerance, whereby a woman accepts the implantation of the fetal allograft in her uterus; initiating a gestation phase becomes physiologically susceptible and vulnerable to malaria infection. Pregnant women with relatively lower levels of previously acquired immunity are particularly at high risk of the most severe complications of malaria during pregnancy, such as cerebral malaria, severe malaria anaemia, abortions, intrauterine fetal death, premature delivery, stillbirths, and maternal and infant mortality [6, 8, 9]. In malaria endemic areas, pregnant women are more susceptible to *Plasmodium* infections than their nonpregnant peers. The adverse outcomes of these infections are primarily felt by primigravidae [10, 11], although, in areas of low or unstable transmission, women of all gravidities may be equally at risk [11]. Pregnant women are 3 times more likely to suffer from severe disease as a result of malarial infection compared with their nonpregnant counterparts and have a mortality rate from severe disease that approaches 50% [12, 13].

In spite of severe and fatal consequences of malaria during pregnancy for the mother, foetus, and newborn child, the harmful effects can be substantially prevented and reduced [14] either by using available interventions or through appropriate treatment upon early and stringent diagnosis [15–17]. Because malaria infection during pregnancy is often asymptomatic, the most common control strategy is intermittent preventive treatment during pregnancy (IPTp), designed to clear any malaria infection present at the time of treatment and also to provide posttreatment prophylaxis to prevent infection for a period of weeks. However, increasing concern of widespread resistance of commonly used antimalarial drugs [18, 19] over the globe has opened the avenues for alternative and effective interventions. The diagnosis of malaria during pregnancy is complicated by several factors, including multistage pregnancy terms lacerated with diminished immunity, increased susceptibility of severe diseases, various obstetric complications, splenic and placental sequestration of parasites, various forms of anaemia, and variation in patient presentation. Thus, development of prompt and accurate diagnosis is an important goal of MIP research.

P. falciparum malaria during pregnancy is a well-known cause of maternal and fetal morbidity and mortality. Although *P. vivax* infection has received less attention than *P. falciparum* infection, it is clearly an important contributor to both maternal anaemia and low birth weights [20–23] where they frequently coexist. However, of 50 million pregnancies occurring each year in countries where malaria is endemic, approximately one-half occur in areas where *P. vivax* malaria is endemic [14]. Although *P. vivax* infection during pregnancy has been recognized for many years [20], the impact of such infection during pregnancy has been assessed only recently. In series from Thailand and India, women with *P. vivax* infection were more commonly anaemic and delivered lower birth weight neonates, compared with uninfected women, but the effects were less pronounced than those associated with *P. falciparum* infection [21, 22]. In both studies, *P. vivax* infection was most common during the first pregnancy, and the prevalence of such infection peaked early during the second trimester.

Limited and past MIP studies in India have demonstrated the important contribution of malaria to maternal and neonatal morbidity and mortality [21, 23, 24]. Although preliminary results from earlier studies carried out primarily in central India suggest that both *P. falciparum* and *P. vivax* are associated with adverse pregnancy outcomes, these studies primarily focused on symptomatic pregnant women infected with *vivax* [21, 25]. Relatively little information is available from India about *vivax* associated malaria during pregnancy, particularly from Jharkhand, an understudied and tribal dominant region with perennial malaria transmission zone where malaria is rampant and causing sizable annual malaria deaths, second to Orissa in India as per the latest observations published by Dhingra et al. [26] and Hussain et al. [27], which reflects the importance of the area and its necessity of undertaking extensive investigation in terms of malarial pathology concerned and by Hamer et al. [23] reflecting the malaria during pregnancy associated with an increased risk of neonatal and infant mortality.

Thus, in view of the limited information on asymptomatic and *vivax* infection during pregnancy in India, it prompted us to investigate with an objective to better define the estimate of MIP, the prevalence of asymptomatic malaria, and the relative contribution of *P. falciparum* and *P. vivax* during pregnancy and at delivery. To the best of our knowledge, such profile, epidemiological association, and clinical correlation have not been investigated before on isolates of malaria in pregnancy from Hazaribag, Jharkhand, among malaria endemic regions of India. Most significantly, our investigation will be the first report attempting to evaluate the interplay among anaemia, pregnancy, and asymptomatic malaria, stratified according to clinical groups in adult population residing in a perennial

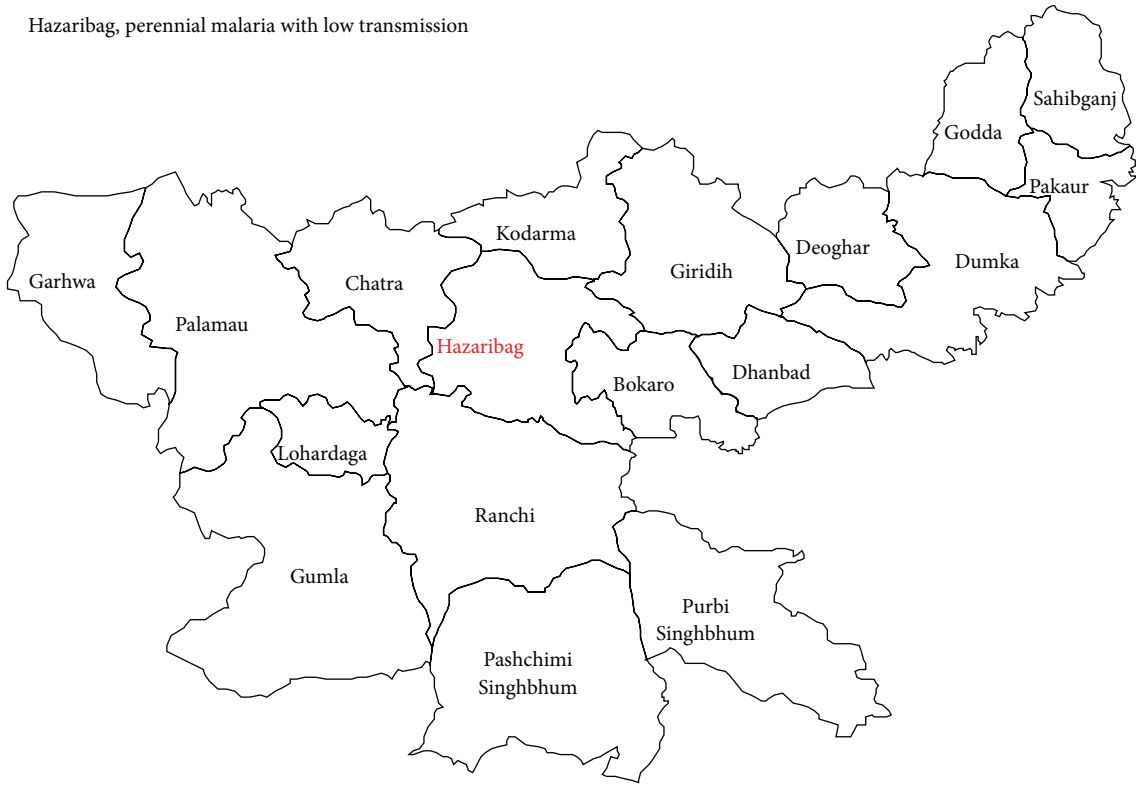

Figure 1: Map of Jharkhand with study site, Hazaribag shown in red.

transmission zone with a codominance of *P. vivax* and *P. falciparum* prevalent region. Thestudy was conducted at Hazaribag in the state of Jharkhand in east India, with the ultimate goal of enhancing the development of evidence-based policies to reduce the burden of disease due to MIP in this region of India.

2. Methods

2.1. Study Sites/Design and Population. This study consisted of cross-sectional surveys conducted in three units, that is, antenatal care units (ANCs), delivery units (DUs), or the inpatient antepartum ward of Sadar Hospital in Hazaribag districts of Jharkhand, India (Figure 1). Jharkhand had a yearly average slide positivity rate (SPR) for symptomatic individuals of 6.8% over the last three years with *P. falciparum*, *P. vivax*, and mix infection accounting for 44%, 44%, and 7% of the cases, respectively [28]. The province of Jharkhand in eastern India is one such area where malaria is rampant. The complexity and magnitude of malaria in the central eastern part of India deserve special mention and attention as the central eastern state contributes 15–20% of total malaria cases in the country as per the Draft on National Policy on Tribals by Government of India, 2005. The investigation is conducted in the Jharkhand state emphasizing tribal dominant area (total population according to 2001 census is 31 463 866), and the state of Jharkhand is selected to represent an endemic with stable transmission of malaria, with a total of 230 686 malaria cases reported in 2009, of which 39.53%, 52.64%, and 7.83% were due to *P. falciparum*, *P. vivax*, and mix infection, respectively [29]. The present study was carried out in Hazaribag district, considered to be a malaria endemic area in the state of Jharkhand.

Hazaribag (total population according to 2011 census is 1,734,005) is selected to represent a rural-cum semiurban district with low but perennial transmission of malaria. Hazaribag had a yearly average SPR of 7.3% for symptomatic individuals over the last three years, with *P. falciparum*, *P. vivax*, and mix infection accounting for 14%, 73%, and 13% of the cases, respectively [30]. The majority of the indigenous population is mix of tribals, schedule caste, schedule tribes, and other castes, exceptionally typical social stratification having gender disparity. Moreover, the district and state lie in the tropical zone with an annual rainfall of 1234.5 mm with favorable geoclimatic and ecological conditions conducive for perennial malaria transmission. The climatic conduciveness of the investigated district can be best visualized in the self-explanatory Supplementary Figure-1A (see Supplementary Material available online at http://dx.doi.org/10.1155/2015/740512). Most interestingly, with the monthly climatic temperature when compared with monthly malaria episode, we observed significant correlation between ambient temperature and subsequent rise and fall in malaria episode as shown in Supplementary Figure-1B.

The recent (2010–2012) data on malaria epidemiology has been analyzed during investigation in this project and we observed the increasing trend of malaria episodes as shown in Supplementary Figure-2A-C, despite consistent interventions and preventive measures implemented by various national and international bodies.

Thus, the selected study district is meant to provide a representation of typical conditions that would be found in malaria endemic districts of Jharkhand.

The District Level Household and Facility Survey conducted between December 2007 and April 2008 revealed that 56% of women had at least one antenatal clinic (ANC) visit and 18% overall had institutional deliveries including 59% in urban areas but only 13% in rural settings [31]. Sadar Hospital, the district hospital for Hazaribag district, serves a predominantly rural population and has a separate obstetric unit with 40 beds, with a high volume of annual deliveries ranging from an average of 4800 to 5500 per year in 2010 to 2013. The Sadar Hospital also has a high volume of ANC visits including an average of 5200 to 6600 per year from 2010 to 2013.

2.2. Screening and Enrollment. The study had two components with recruitment targeted to all the women presenting to antenatal care unit (ANC) and delivery units (DUs). For the ANC component, pregnant women aged ≥17 years who reported to the study site for routine care were screened and enrolled; those were willing and consented to participate in our study. For the DU component, women aged ≥18 years who presented for delivery and were willing to provide written informed consent were enrolled. Inclusion in the study protocol was based on the considerations like residency and availability status in the study region, no history of hereditary diseases and/or no known severe disease at the time of conceiving and/or at first ANC attendance, voluntary and consented participation in our study, and no immediate illness due to other infectious diseases or malaria in preconception and/or during present pregnancy at the time of first attendance at ANC. Exclusion from study was based on either refusal to give signed consent or unwilling for sampling, clinically suspected or identified cases of HIV and hepatitis B infection, and stringently those who are apparently and at first sight so weak due to unknown reason compounded by pregnancy that may not sustain sampling stress and may lead to undesired complications.

Detailed strategy of enrollment, sampling procedures, and broad groups were as described; recruitment and enrolment took place from September 2012 to December 2013. Of 1890 pregnant women screened during their ANC visits, 1746 were willing to understand our study protocol, out of which 1715 consented and agreed on peripheral sampling and 31 refused to participate in the study. Thus, we enrolled 1715 subjects, interviewed by trained technical staff, and, upon pregnancy screening report and based on other clinical investigations, divided them into the two broad groups, that is, pregnant and nonpregnant women group consisting of 1271 and 444 subjects, respectively. The nonpregnant group was subdivided into women with malaria and healthy women without malarial complications, consisting of 227 and 217, respectively. In the delivery unit, 870 pregnant women were screened and enrolled as shown in schematic flow chart in Supplementary Figure-3. All the women at each attendance underwent clinical investigations, parasite slide examination, and measurement of auxiliary body temperature before enrollment and we found 68 and 37 MIP cases at ANC and DU, respectively. In ANC, we found 59, 3, and 6 cases of *P. vivax*, *P. falciparum*, and mix infection, respectively, whereas at DU, we found 32, 2, and 3 cases of *P. vivax*, *P. falciparum*, and mix infection, respectively, at Sadar Hospital, Hazaribag (Table 1). The controls for malaria in pregnancy were malaria in women without pregnancy group in addition to healthy women; those are without pregnancy having no known diseases including malaria at the time of sampling.

Table 1: Parasitaemia, reported fever, and anaemia among pregnant women attending antenatal clinics and delivery units.

	Antenatal clinics $n = 1271$ N (%)	Delivery units $n = 870$ N (%)
Peripheral parasitaemia		
Overall	68 (5.4)	37 (4.3)
Falciparum	3 (0.23)	2 (0.22)
Vivax	59 (4.6)	32 (3.67)
Mixed	6 (0.47)	3 (0.34)
By gravidity		
Primigravid	21/423 (4.9)	11/338 (3.2)
Secundigravid	38/578 (6.6)	15/209 (7.1)
Multigravid	9/270 (3.3)	11/323 (3.4)
Report of fever within 1 week	167 (13.1)	93 (10.6)
Anaemia	1093 (86)	626 (72)
Severe anaemia	148/1093 (13.6)	49/626 (7.8)

2.3. ANC Procedures. Trained study personnel interviewed the enrolled women and collected information on sociodemographic characteristics (i.e., date of birth, socioeconomic status, and literacy), reproductive history including gravidity, history of fever and antimalarial drug use, and use of antimalarial prevention measures. A complete physical examination including the determination of gestational age was assessed by palpation of uterine fundus height combined with information on last menstrual period; measurement of auxiliary temperature with digital thermometer and other vital signs was also performed. Peripheral venous blood (3–5 mL) was collected from all the attendees for malaria blood film preparation, rapid diagnostic test (RDT), and haemoglobin determination apart from other biochemical and molecular investigations. Women with positive RDT results or who were anaemic were referred immediately to the hospital physician for treatment. The hospital staffs were informed of additional parasitaemic individuals identified through blood smears so that they could be appropriately treated.

2.4. DU Procedures. Pregnant women enrolled at the DUs were interviewed, with data collection focused on sociodemographic and anthropometric characteristics, obstetric

complications, history of fever and antimalarial use during pregnancy and the use of antimalarial prevention measures, birth outcome, and mode of delivery. Peripheral venous blood (3–5 mL) was collected after delivery for malaria blood film preparation and/or rapid diagnostic test (RDT) and haemoglobin determination apart from other biochemical and molecular investigations. Women with positive RDT or blood smear results were referred for treatment. Apart from malaria prevalence study in DU, we have also collected clinical and demographic data and samples based on the mode of delivery, that is, normal, caesarean, and stillbirth delivery, and further on the mode of birth/delivery outcome, that is, preterm, postterm, and term delivery; details were presented in Supplementary Table-1. To assess the gestational age, we mainly adopted the simplest method, that is, symphysis-pubis fundal height (SFH) measurement (also known as palpation of uterine height measurement), most widely used method over the globe especially in resource poor settings like ours. Assessments were performed by trained nurses followed by gynaecologist. However, in case of any undesired measurement or dought over positioning of foetus, they were confirmed by ultrasound to record the gestational ages.

2.5. Laboratory Procedures. Thick and thin smears prepared from peripheral blood of ANC and DU subjects were Giemsa-stained and examined under high power. The parasite density was evaluated by counting the number of asexual forms of parasites for every 200 leukocytes, assuming a leukocytes count of 8000 leukocytes/μL of blood [32]. The thin film was used to identify the *Plasmodium* species. All slides were cross-checked using stringent diagnostic criteria to diagnose *Plasmodium* infection with our trained technical staff. The commercial (RDT kit) First Response Malaria pLDH/HR2 combo test kits (Premier Medical Corporation, Mumbai, India) were also used as per the manufacturer's guideline as a screening tool for diagnosing malaria in pregnant women. We have used the PCR technique also to diagnose malaria but in selective samples not in all the samples due to budgetary constraint. The selective samples were all the MIP positive samples at ANC and DU verified by PCR, those subjects who were disputed on microscopy and RDT also verified by PCR, and clinically most suspected cases with strong sign and symptoms but microscopically negative samples were also verified by PCR.

2.6. Haemoglobin Concentration. Haemoglobin (Hb) levels were recorded at the first ANC and DU visit. Determining the concentrations of haemoglobin (Hb) was performed in peripheral blood samples using a portable HemoCue haemoglobinometer (HemoCue AB, Ängelholm, Sweden) as stated by the manufacturer. The concentration of Hb was recorded on the study questionnaire and double-checked by the laboratory technician. Women were classified as anaemic (Hb < 11 g/dL) and then categorized as being moderately to severely anaemic, with haemoglobin <8 g/dL and <7 g/dL, respectively, as the primary outcome, and being mild to nonanaemic (Hb ≥ 9 g/dL) according to [33, 34].

2.7. Study Definitions. Severe malaria was defined as a malaria attack associated with any of the following: cerebral malaria, severe anaemia, renal failure, pulmonary oedema, hypoglycaemia, shock, spontaneous bleeding, or repeated convulsions [35]. Maternal height and weight were taken at the first visit to ANC and DU; based on this information, the body mass index (BMI) was calculated as weight (kg) divided by the squared height (meters); a low BMI was defined as a BMI < 22.0 kg/m^2. A documented fever was defined as an auxiliary temperature ≥37.5°C.

2.8. Ethics Statement and Subject Consent. All human blood samples used in this study were collected after obtaining written consent from the study participants under protocols activities approved by the Institutional Ethics Committee (IEC) of the Vinoba Bhave University, Hazaribag, Jharkhand, and human ethical guidelines as reflected in the guidelines of the Medical Ethics Committee, Ministry of Health, Government of India. Present study does not involve any minor/children. Thus, signed and written approval was given by adult subject herself. All study participants were included only after informed consent. The study protocol and consent proposal are approved from IEC, VBU, having memo number VBU/R/888/2012, dated 05-06-2012.

2.9. Data Management and Analysis. All clinical, demographic, and anthropometric information were carefully checked for correctness and inconsistencies were resolved before analysis. Data were entered in MS-Excel and analyses were performed using SPSS version 16 (SPSS Inc., Chicago, IL, USA) and Graphpad Prism version 5.0 (GraphPad Software, Inc., CA, USA). For comparisons of means between two groups of subjects, Student's t-test was used for evaluating significance for normally distributed data and when data were not normally distributed; nonparametric tests (Mann-Whitney U) test were used to analyze the data. Categorical data are presented as frequency counts (percent) and compared using the Chi-square or Fisher's exact statistic as appropriate. Continuous data are presented as means (± standard error) and compared using the t-test or analysis of variance as appropriate. The age of the recruited subject was between 18 and 37 years, whereas mean age was 26.7 years. We have presented participants' ages in ranges based on their responses (Supplementary Table-1). Risk factors for either *P. falciparum* or *P. vivax* parasitemia were evaluated by univariate analysis and then adjusted for significant predictors in multivariate analysis. Simple and multiple logistic regressions were used to analyze potential risk factors associated. Precisely, to investigate the association between the various independent variables (selecting only strong epidemiological and biological plausibility for association) and malaria parasitemia, we began by performing simple logistic regressions with each independent variable. Next, we applied multiple backward logistic regression models and all covariables present in univariate were kept in model, independent of their significance, in univariate analysis due to their possible relevance in the final results; thus, we could analyze their possible influence when considered together with the other

variables. Similar strategies were followed for factors associated with haemoglobin and anaemia during pregnancy and malaria in pregnancy; risks were assessed using haemoglobin or anaemia as dependent variables and all other factors as independent variables. The differences were considered statistically significant when the p value obtained was <0.05.

3. Results

3.1. Antenatal Clinics. Most pregnant women attending ANC were in the 18 to 38 years of age range and had some level of formal education (Supplementary Table-1). The vast majority of participants were Hindi speaking (97.6%) and nonsmoking (98.7%). Most owned their own home (75.4%) and were engaged in household work (76.7%) with a small proportion involved in farming (12.3%). They had attended a median of one ANC visit (range 0–9) during their current pregnancy and almost one-third of the attendees were primigravidae (33.3%). Slightly more than half of participants presented to the ANC in the latter half of pregnancy whereas 44.6% presented prior to 20 weeks. Less than half of the participants reported taking iron/folate supplements (46.3%) while 33.2% were taking multivitamins. In terms of malaria prevention activities, most pregnant women reported having untreated bed nets in their homes and using them recently, but very few had ITNs (Supplementary Table-2). Similarly, only 9 of the women were taking prophylaxis for malaria and most of them (7/9, 78%) were unable to identify the drug they were taking and the rest (two), who were able to identify the drug, were taking chloroquine.

A positive diagnostic test for malaria was obtained in 5.4% (68/1271) of the total cohort (Table 1). Blood smears for malaria were positive in 4.3% of pregnant women while an additional 14 (1.1%) women had positive RDTs. The mean density of parasitemia in the 54 women with positive blood smears was 63,236 asexual forms/μL (range 600–489,000). *P. falciparum* was identified in 4.4% of parasitaemic individuals while *P. vivax* was found in 86.8% and 8.8% of infections were mixed. Peripheral parasitemia was over four times more likely among women living in rural areas when compared with those from urban or semiurban subjects (OR 4.36, 95% CI 2.48–7.32) and among primigravidae and secundigravidae relative to multigravidae (OR 4.23, 95% CI 2.15–8.82). Parasitaemia was more commonly encountered in pregnant women who had a history of fever within the week prior to enrollment or were febrile at the time of the study visit (4.2% versus 2.3%, $p = 0.02$). The majority of positive malaria tests occurred from July to January with the greatest number in between August and October, corresponding to the monsoon season. Further multivariate analysis was performed in order to identify the association between specific demographic, socioeconomic, and malaria prevention activities and the risk of parasitemia. Among pregnant women attending ANCs, first/second pregnancies, fever in the past week, and residence in rural areas were significantly associated with peripheral parasitemia as shown in Table 2.

3.2. Delivery Units. Like the ANC cohort, most pregnant women attending DUs were aged 20–36 years and had some level of formal education (Supplementary Table-1). All were nonsmokers (100%) and nearly all spoke Hindi (97.2%). Most owned their own home (73.9%) and were involved in household work (84.3%); a minority engaged in farming (14.6%). Study participants had attended a median of three ANC visits (range 0–9) and about slightly less than two-thirds were primigravidae and secundigravidae (Supplementary Table-1). The majority of pregnant women reported having untreated bed nets in their homes and using them recently but ITN ownership was uncommon (Supplementary Table-2). Only three women were taking chemoprophylaxis for malaria and none knew the name of the medication that they were taking. Only 4.3% of the women enrolled at the DUs had peripheral parasitemia (either a positive blood smear or RDT). *P. falciparum* was identified in 5.4% (2/37), *P. vivax* in 86.5% (32/37), and mixed infection in 8.1% (3/37). The mean density of parasitemia in the women with positive blood smears was 16,395 asexual forms/μL (range 870–65,000). The peripheral parasitemia density was significantly higher in primigravid women than in those who had one or more prior pregnancies (mean $\pm$ SD of $36,600 \pm 9,743$ versus $7,532 \pm 4623$ asexual forms/μL, resp.; $p = 0.002$). Pregnant women with peripheral parasitemia were more likely to have either a self-reported fever or fever measured at enrollment than those who were aparasitaemic (36.4% versus 9.2%, $p = 0.005$). A sizable proportion of women presenting to the rural origin were parasitaemic as compared to semiurban and urban origin and this difference was significant (OR 4.36, 95% CI 2.48–7.32, and $p = 0.0001$) (Table 2). Primigravidae and secundigravidae also were more likely to be parasitaemic, and difference was significant (OR 4.23, 95% CI 2.15–8.42, and $p = 0.0001$). Asymptomatic malaria infections were present in 70% of women with peripheral parasitemia (26/37) as compared to 30% symptomatic infection (11/37). Pregnant women with peripheral parasitemia were more likely to have either a self-reported fever or fever measured at enrollment than those who were aparasitaemic (28.3% versus 9.2%, $p = 0.004$).

As observed in the ANC participants, most episodes of parasitemia occurred in July to September during the monsoon season. For DU participants with peripheral parasitemia, 83.7% had anaemia as compared to 47.6% of those who did not have parasitemia ($p = 0.004$). More women with peripheral parasitemia had severe anaemia (5.7%) than those without parasitemia (2.6%) and the difference was significant ($p = 0.02$).

Multivariate analysis revealed a significant association between peripheral parasitemia and primigravidae and secundigravidae, fever within the last week, and semiurban and rural residency status as shown in Table 2.

3.3. Association between Pregnancy and Asymptomatic P. vivax with Haemoglobin. Anemia is the most prominent hematological manifestation of malaria infection. Hemoglobin concentration is the best characterized method and well accepted indicator for diagnosis of anemia and assessment of severity. In addition to this, it is regarded as one of the most serious global public health problems which prompted us to investigate the status of hemoglobin and severity of

Table 2: Factors associated with peripheral parasitemia during malaria in pregnancy using univariate and multivariate analysis.

	Peripheral parasitemia % (Positive/total)	Adjusted OR (95% CI)	p	Adjusted OR (95% CI)	p
Factors at ANC					
1st/2nd pregnancies	6.3 (64/1001)	4.45 (2.32–9.61)	0.0001	4.23 (2.15–8.42)	0.0001
3rd or greater pregnancies	1.4 (4/270)	1		1	
Age < 20	7.2 (12/166)	1.43 (0.34–3.76)	0.052	1.31 (0.26–2.84)	0.076
Age ≥ 20	5.0 (56/1105)	1		1	
Fever within past week	16.1 (27/167)	4.42 (3.64–8.21)	0.002	4.62 (3.73–9.83)	0.001
No fever within past week	3.7 (41/1104)	1		1	
Bed net use*	7.6 (42/563)	1.12 (0.27–2.47)	0.072	1.37 (0.48–3.24)	0.084
No bed net use	6.7 (26/374)	1		1	
Rural	7.1 (61/857)	4.21 (1.53–5.21)	0.003	4.36 (2.48–7.32)	0.0001
Not rural	1.7 (7/414)	1		1	
Tribal caste	6.3 (23/363)	1.26 (0.64–2.96)	0.054	1.42 (0.81–3.75)	0.12
No tribal caste	4.9 (45/908)	1		1	
No formal education	6.1 (22/357)	1.22 (0.42–2.46)	0.065	1.34 (0.68–3.92)	0.084
Formal education	5.0 (46/914)	1		1	
Factors at DU					
1st/2nd pregnancies	5.8 (32/547)	3.9 (0.97–11.56)	0.004	3.62 (0.94–7.83)	0.001
3rd or greater pregnancies	1.5 (5/323)	1		1	
Age < 20	8.2 (28/109)	2.32 (1.32–9.37)	0.062	2.47 (1.17–10.63)	0.14
Age ≥ 20	3.6 (9/761)	1		1	
Fever within past week	13.9 (13/93)	4.47 (1.25–12.42)	0.0001	4.43 (1.38–11.57)	0.0001
No fever within past week	3.1 (24/777)	1		1	
Bed net use*	5.3 (27/503)	1.97 (0.83–7.62)	0.084	1.62 (0.58–6.39)	0.27
No bed net use	2.7 (10/367)	1		1	
Rural	7.1 (29/405)	4.22 (0.41–4.51)	0.003	3.87 (0.78–13.62)	0.0001
Not rural	1.7 (8/465)	1		1	
Tribal caste	5.5 (14/251)	1.51 (0.56–3.92)	0.053	1.74 (0.83–5.38)	0.59
No tribal caste	3.7 (23/619)	1		1	
No formal education	5.3 (17/321)	1.46 (1.23–3.17)	0.57	1.62 (0.87–4.63)	0.21
Formal education	3.6 (20/549)	1		1	

*ITN use was not evaluated in this model since ITN were very rarely used and because of quite lesser awareness about ITN among women.

anemia in Jharkhand population, as anaemia is particularly high for women with no education (74%), women from the scheduled tribes (85%), and women in the two lowest wealth quintiles (over 70%). The prevalence of anaemia among adults in Jharkhand is higher than in almost all other states in India (national family health survey, NFHS-3 India, 2006). Anaemia was prevalent among ANC participants whereas severe anaemia was reasonably observed in the investigated cases (Supplementary Table-1). More than two-thirds of the DU participants were anaemic whereas 7.8% had severe anaemia (Table 1). Of these ANC and DU participants, the prevalence of mild, moderate, and severe anaemia is shown in Figures 2(a)–2(d).

3.4. Association of Asymptomatic Infection with Malaria during Pregnancy at ANC and DU Subjects. Clinical malaria cases are suspected and investigated on the basis of malaria associated sign and symptoms in various studies including community based epidemiological studies; and based on the prevalence of sign and symptoms, we interestingly observed in our study that 70.6% (48/68) of the positive cases of malaria in pregnancy subjects at ANC were asymptomatic with peripheral parasitemia compared to 29.4% symptomatic MIP cases, whereas 75.7% were asymptomatic cases with peripheral parasitemia compared to 24.3% symptomatic infection during malaria in pregnancy at DU. Based on the data collected on sign and symptoms from the pregnant women attendees at ANC and DU subjects, we performed positive predictive value (PPV) (Table 3) and multivariate (Table 4) analysis to further consolidate our observation and to explore the association between symptoms and malaria infection during pregnancy. For positive predictive value (PPV), fever, history of fever, body pain, headache, dizziness, vomiting, and convulsions were evaluated at ANC and DU

Table 3: Positive predictive value (PPV) of clinical signs and symptoms for *Plasmodium vivax* infection.

	N	Observed value (OV) (%)	Positive predictive value (PPV) (%)	95% CI in proportion of PPV (%)
Sign/symptoms at ANC				
Fever	54	4.2	26	23.5–28.4
History of fever	167	13.1	45	42.2–47.7
Headache	114	8.9	32	29.4–34.5
Body pain	15	1.2	18	15.8–20.1
Dizziness	29	2.3	21	18.7–23.2
Vomiting	22	1.7	23	20.3–25.3
Convulsions	13	1.1	12	10.2–13.7
Sign/symptoms at DU				
Fever	43	4.9	36	32.8–39.1
History of fever	93	10.6	47	43.6–50.3
Headache	172	19.7	33	29.8–36.1
Body pain	23	2.6	26	23.1–28.9
Dizziness	19	2.2	19	16.3–21.6
Vomiting	31	3.5	27	24.1–29.9
Convulsions	14	1.6	21	18.2–23.7

Table 4: Association between signs/symptoms and malaria infection using multivariate analysis.

		n/*N* (%)	OR (95% CI)	*p* value
Sign/symptoms at ANC				
Any symptoms	No	937/1203 (77.8)	1	0.14
	Yes	20/68 (29.4)	1.3 (0.9–1.9)	
Fever	No	1138/1203 (94.5)	1	0.0003
	Yes	11/68 (16.1)	2.9 (1.6–5.4)	
History of fever	No	1031/1203 (85.7)	1	0.14
	Yes	14/68 (20.5)	1.4 (0.8–2.3)	
Headache	No	1076/1203 (89.4)	1	0.13
	Yes	11/68 (16.1)	1.5 (0.8–2.6)	
Dizziness	No	1168/1203 (97.1)	1	0.16
	Yes	4/68 (10.1)	1.9 (0.7–5.5)	
Vomiting	No	1178/1203 (98)	1	0.21
	Yes	3/68 (4.4)	2.1 (0.6–6.8)	
Sign/symptoms at DU				
Any symptoms	No	623/833 (74.7)	1	0.9
	Yes	9/37 (24.3)	0.9 (0.5–1.7)	
Fever	No	784/833 (94.1)	1	0.01
	Yes	5/37 (13.5)	2.7 (1.2–6.1)	
History of fever	No	770/833 (98.6)	1	0.01
	Yes	7/37 (18.9)	2.5 (1.2–5.1)	
Headache	No	655/833 (98.6)	1	0.46
	Yes	6/37 (16.2)	0.7 (0.3–1.5)	
Dizziness	No	792/833 (95.1)	1	0.12
	Yes	4/37 (10.8)	2.1 (0.8–5.6)	
Vomiting	No	775/833 (93.1)	1	0.12
	Yes	5/37 (13.5)	1.9 (0.8–4.5)	

n = observed, *N* = total considered subjects, and OR= odds ratio.

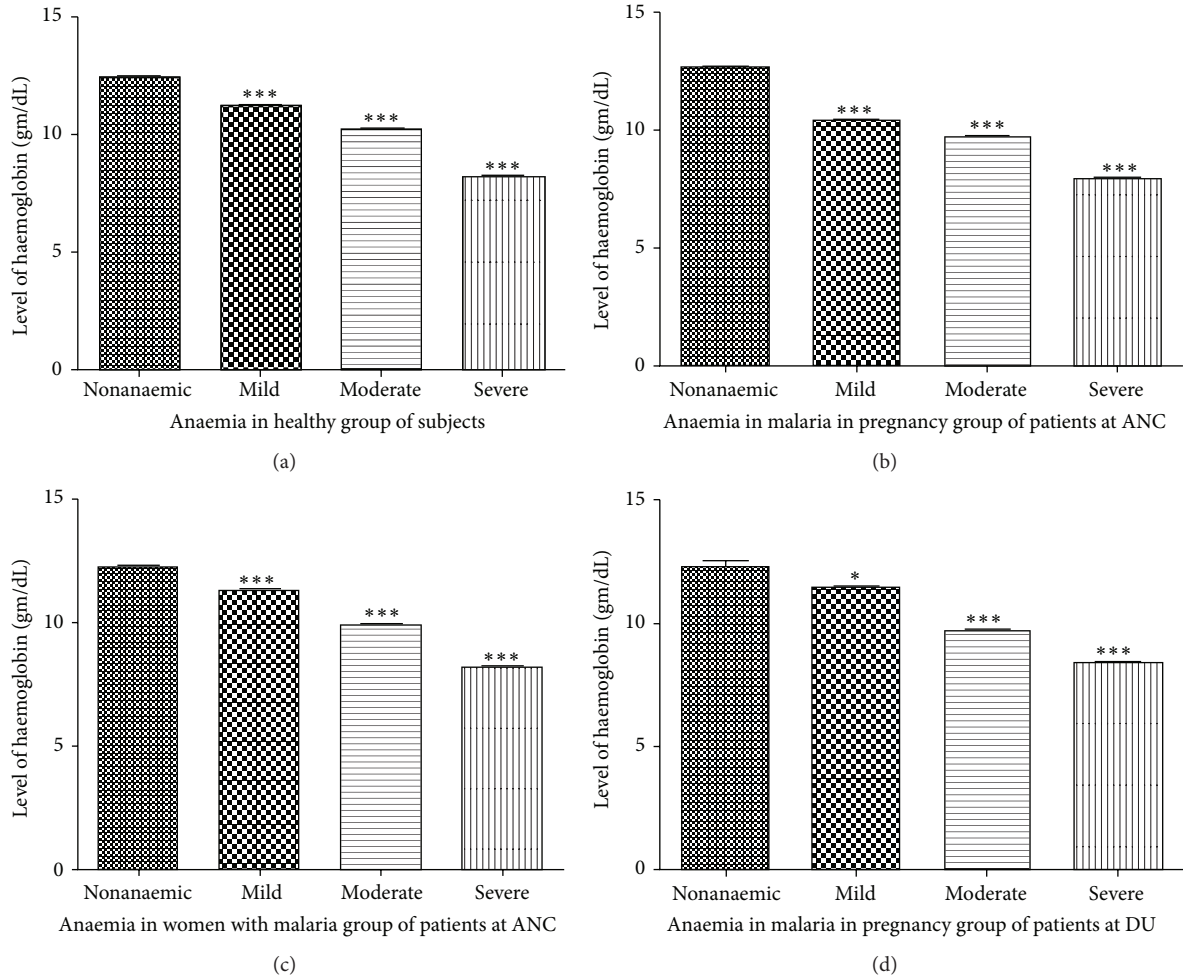

Figure 2: Level of haemoglobin as classified anaemia in malaria infected subjects screened at antenatal care (ANC) unit and delivery unit (DU) in stratified group as (a) anaemia in healthy group of subjects, (b) malaria in pregnancy group of patients among ANC attendees, (c) women with malaria group of patients without pregnancy, and (d) malaria in pregnancy group of patients among DU attendees. Data is presented as mean and error bar represents the plus or minus SE $^{*}P \leq 0.01$, $^{**}P \leq 0.001$, and $^{***}P \leq 0.0001$ compared with nonanaemic women using paired t-test through Graphpad Prism version 5.0.

as shown in Table 3. Almost all the predictive values for respective symptoms were observed to be very much low except for history of fever, which is relatively higher than the others only, despite being the highest among all at both ANC and DU. However, the positive predictive value for history of fever at DU was slightly higher than ANC. None of the predictive value for any sign and symptoms was neither nearly 50% or even above (Table 3). The prevalence of observed values (%) and frequencies (N) for all the signs and symptoms were also presented in Table 3. Further, in applying multivariate model, we analysed any symptoms, fever, history of fever, headache, dizziness, and vomiting at ANC and DU as shown in Table 4. We observed that presence of any symptoms, history of fever, headache, dizziness, and vomiting were not significantly associated with incidence of malaria during pregnancy at ANC, whereas only fever was found to be significantly associated at ANC as shown in Table 4. However, in case of DU subjects, all the symptoms were not significantly associated except fever and history of fever which were significantly associated with incidence of malaria as shown in Table 4. Thus, based on the observation and analysis, we can infer that majority of the sign and symptoms have not been shown or trended to be significantly associated, except fever and/or history of fever that have some degree of significant association with malaria in pregnancy at ANA and DU in multivariate analysis. The absence of higher percentage of positive predictive value for all the symptoms as well as lower prevalence of observed value and frequency can also be regarded as an indicative of nonassociation of sign and symptoms with incidence of malaria during pregnancy at both ANC and DU. As majority of subjects were infected with *vivax* strain as described earlier (Table 1) both at ANC and DU, thus, view of nonassociation of sign and symptoms with the incidence of malaria during pregnancy can be coined and corroborated with asymptomatic *Plasmodium vivax* infection in the present study both at ANC and DU.

3.5. Risk Factors Associated with Anaemia in Overall Study Cohort and Malaria in Pregnancy at ANC and DU Subjects. Multivariate logistic regression showed that malaria infection, ferritin, iron, haemoglobin, and formal education were significantly associated with a higher risk of anaemia in overall cohort ($N = 1271$ at ANC and $N = 870$ at DU) as well as in malaria in pregnancy at ANC ($N = 68$) and DU ($N = 37$) subjects as presented in Table 5. The highest (adjusted odds ratio in multivariate analysis) risk factor associated with anaemia was observed with haemoglobin level, followed by presence of malaria infection in both malaria in pregnancy and overall study cohort at ANC and DU as shown in Table 5. However, ANC subjects have shown relatively higher risk ratio association of anaemia with haemoglobin and malaria compared to DU subjects (Table 5). Comprehensive results of univariate and multivariate analysis are shown in Table 5.

4. Discussion

The estimate of malaria in pregnancy continues to be grave concern for community reproductive health care management across the tropical region including India, up to the level of pacifying the concept of healthy mother and healthy baby of National Family Welfare Programme. In fact, situation is much more aggravated in developing countries like India, where poverty, illiteracy, geographical diversity, socioeconomic disparities, and multiple pregnancies take their toll of mother's health.

Among the prominent findings of the present study, we found 5.4% and 4.3% malaria during pregnancy at ANC and DU, respectively, as compared to only 1.8% and 1.7% at ANC and DU, respectively, reported by Hamer et al. [23] from the series of cross-sectional and multicentric study in Jharkhand. However, our study design is slightly broader than the earlier investigation from Hamer et al. [23] in terms of subject stratification, as we have also taken into account women with malaria without pregnancy, and the prevalence of malaria was found to be 13.2%, which itself reflects the importance of the investigated region and population under malaria sensitive zone. However, our study lacks the difference of investigating placental malaria. The pondering difference in the prevalence of malaria during pregnancy between our investigations, though we have selected only one centre in one district, that is, Hazaribag, Jharkhand, as compared to three centres from two districts, that is, Ranchi and Gumla of Jharkhand by Hamer et al. [23], may be attributed to various other reasons but primarily linked to the selection of study sites. As Ranchi is an urbanized capital with lots of high-tech development in and around the city, local and buffering populations are much more educated, aware of practicing healthy life style and various diseases prevention strategies including malaria, having high socioeconomic status, excellent with a choice of health facility compared to the rest of the districts of Jharkhand state, and most importantly less malarious than almost 20 other districts of Jharkhand as far as malarial epidemiology is concerned in last ten years [29]. Thus, selected site by Hamer et al. [23] may not be the true representation of the malaria scenario and rather burden of malaria during pregnancy in Jharkhand but absolutely true as far as the outcome of the project is concerned. However, our results of higher prevalence of malaria in pregnancy are in accordance with the earlier observations (ranging from 1.7% to 20%) across India [21, 23, 36, 37]. Most of these studies focused on pregnant women with selective approach, tend towards screening for mostly febrile, or had a recent history of fever cases and thus may have had a selection bias towards expecting higher malaria rates. This approach, targeting malaria diagnostic and treatment for symptomatic pregnant women, is consistent with India's National Vector Borne Disease Control Programme guidelines [38]. In contrast, all pregnant women were evaluated in the current study regardless of classical symptoms and, interestingly, we observed well that over 70% of the pregnant women in ANC and DU had asymptomatic malaria during pregnancy, which suggests the region specific intervention. The broader spectrum of screening strategies was in accordance with earlier investigation in this region [23], though our observations are notably varied from their observations as far as asymptomatic malaria during pregnancy is concerned. Infection from *P. vivax* in pregnancy has conventionally been regarded less severe as compared to *P. falciparum* malaria. Interestingly, we reported that majority were infected with *P. vivax* infection during malaria in pregnancy. This observation may be attributed to the lack of placental sequestration in *P. vivax* infection and the parasite tropism for reticulocytes accounting for a milder form of anaemia [39, 40].

The higher prevalence of malaria in women without pregnancy and with pregnancy, irrespective of ANC and DU attendees' location of residence, that is, rural, urban, and semiurban, suggests that Hazaribag and its buffering zone have perennial rate of malaria transmission. Therefore, populations of all age groups including pregnant women are at potential risk of getting malaria infection even irrespective of transmission season, though peak was observed in post-monsoon season. Apart from this, there is significant lack of education, general awareness towards health issues, congenial environmental factors for vector growth and survival, and most importantly sizable population lack access to vector control methods or limited access to antimalarial drugs. People residing below poverty line linking to malnutrition and anaemia may be plausible reasons for various opportunistic infectious diseases including malaria.

Interestingly, insecticide residual spray (IRS) of home, which is usually conducted by government agencies, was reported more in rural areas as compared to urban and semiurban zone of Hazaribag, though its seasonal usage of IRS in those areas regarded as perennial transmission may be suggestive of vector resistance and subsequent higher prevalence of disease. Our observations warrant the potential need to enhance the IRS and distribution of ITNs in and around the investigated district.

We report that *P. vivax* is associated with a high burden of anaemia and remarkable severe anaemia during pregnancy and malaria in pregnancy in endemic population of Hazaribag.

Overall, there was significant burden of anaemia among women in Jharkhand and particularly during pregnancy [23]. Our observations regarding anaemia are in accordance with

Table 5: Risk factors for anaemia in pregnant women (PW) and malaria during pregnancy (MIP) using univariate and multivariate analysis.

	PW Number	PW Crude OR (95% CI)	PW p	PW Adjusted OR (95% CI)	PW p	MIP Number	MIP Crude OR (95% CI)	MIP p	MIP Adjusted OR (95% CI)	MIP p
Factors at ANC										
Malaria**										
No	1203	1	0.0001	1	0.0002	9*	1	0.0001	1	0.0001
Yes	68	2.7 (1.4–3.8)		2.8 (1.2–3.4)		59¥	3.1 (1.7–5.3)		3.4 (1.9–6.5)	
Ferritin (ng/mL)										
<50	117	1	0.002	1	0.001	5	1	0.0001	1	0.0004
≥50	1154	1.8 (1.3–2.3)		2.2 (1.6–3.5)		63	2.1 (1.6–3.3)		2.4 (1.7–4.1)	
Iron (μg/dL)										
≥40	236	1	0.006	1	0.003	10	1	0.001	1	0.0001
<40	1035	1.7 (1.6–2.7)		1.9 (1.8–3.4)		58	2.2 (1.5–3.3)		2.3 (1.2–2.7)	
Haemoglobin (Hb) (g/dL)										
Hb > 11	217	1	0.0001	1	0.0003	19	1	0.0001	1	0.0002
Hb < 11	1054	3.8 (2.3–8.7)		4.2 (2.1–8.8)	0.0002	49	4.8 (1.7–8.1)		5.4 (1.6–8.6)	
Education										
NFE	357	1	0.003	1	0.001	13	1	0.0001	1	0.0001
FE	914	2.1 (1.4–2.9)		2.3 (1.7–3.9)		55	2.2 (1.6–3.5)		2.6 (1.3–3.4)	
Factors at DU										
Malaria**										
No	833	1	0.0001	1	0.0002	5*	1	0.001	1	0.0001
Yes	37	2.1 (1.2–2.5)		2.4 (1.6–3.8)		32¥	2.2 (1.4–3.1)		2.8 (1.7–4.7)	
Ferritin (ng/mL)										
<50	205	1	0.004	1	0.0003	7	1	0.0001	1	0.0002
≥50	665	1.4 (1.2–1.7)		1.7 (1.4–2.3)		30	2.1 (1.8–3.8)		2.3 (1.5–3.5)	
Iron (μg/dL)										
<40	683	1	0.002	1	0.0002	31	1	0.006	1	0.0003
≥40	187	1.9 (1.6–3.1)		2.2 (1.3–2.9)		6	2.3 (1.5–3.5)		2.5 (1.3–3.3)	
Haemoglobin (Hb) (g/dL)										
Hb > 11	173	1	0.0001	1	0.0001	8	1	0.001	1	0.0001
Hb < 11	697	3.1 (1.8–5.6)		3.6 (2.1–7.6)		29	4.2 (1.9–7.8)		4.8 (1.6–7.7)	
Education#										
NFE	321	1	0.004	1	0.0002	11	1	0.002	1	0.0001
FE	549	1.6 (1.1–1.8)		1.8 (1.4–2.6)		26	1.7 (1.5–2.6)		2.1 (1.7–3.6)	

PW = pregnant women, MIP = malaria in pregnancy, ANC = antenatal care unit, and DU = delivery unit; #NFE = no formal education, FE = formal education. **In case of MIP, the comparison is between *P. vivax* versus *P. falciparum* and mix infection; *$P. falciparum$ + mix infection, ¥$P. vivax$.

the findings from other studies in Jharkhand [23], across India [41, 42], and most relevant study by Nosten et al. [43] in which they have demonstrated that women who had malaria at any time were more likely to be anaemic than women without malaria. Among multifactorial involvement in malarial anaemia are included haemolysis of parasitized erythrocytes and increased clearance of nonparasitized ones as well as an inadequate bone marrow response [44]. It has been suggested that pregnancy has also confounding association with anaemia and malaria [43, 45] and *P. vivax* has shown 2-fold higher risk of moderate anaemia than uninfected subject [46, 47].

Thus, regardless of transmission level and the level of prepregnancy immunity against malaria, maternal anaemia remains the most frequent adverse consequences of malaria during pregnancy [48]. The symptoms and complications of malaria in pregnancy vary according to malaria transmission intensity in the given geographical area and the individual's level of acquired immunity. In low-transmission settings, where women of reproductive age have relatively little acquired immunity to malaria, MIP is associated with anaemia, an increased risk of severe malaria. This may lead to spontaneous abortion, stillbirth, prematurity, and low birth weight [49, 50]. In such settings, malaria affects all pregnant women, regardless of the number of times they have been pregnant. In pregnant women, additional sequestration of malaria infected erythrocytes occurs in the placenta. Pregnant women therefore suffer disproportionately from severe anaemia as a result of infection [14]. Our observation is also substantiated by the fact that the majority of malaria infections in pregnancy remain asymptomatic or paucisymptomatic and yet are a major cause of severe maternal anaemia and low birth weight, especially in the first and second pregnancies [22, 23]. In areas with stable but low transmission like our investigated area and certainly in areas with unstable and exceptionally low transmission, infections can become severe in all gravidae groups because most women of childbearing age in these regions have low levels of prepregnancy and pregnancy-specific protective immunity to malaria [14].

High prevalence of anaemia was observed and strongly correlated with asymptomatic *P. vivax* infection. This prevalence is similar to that reported by Brutus et al. [47] and Douglas et al. [46, 51]. Recent work has shown that, in Papua New Guinea and Papua, Indonesia, mixed infection causes more severe haematological impairment than infection with either species alone [52–54]. The impact of *Plasmodium vivax* infection on haemoglobin concentration varies from negligible to dramatic [52, 55–57]. The clinical consequences of the reduction in haemoglobin depend on the haemoglobin concentration prior to infection. Although the spectrum of anaemia seen with *vivax* infection is reasonably well documented, the clinical, developmental, and socioeconomic consequences are largely unknown. Population-based estimates of mortality in severely anaemic individuals with *vivax* malaria have not been established but recent studies from Latin America, New Guinea, and the Indian subcontinent have identified deaths in patients with severe *vivax* anaemia [52, 55, 58, 59]. However, authors did not establish the extent to which anaemia contributed to those deaths.

The very low rate of ownership of insecticide treated bed nets (ITNs) and awareness suggests that this component of the enhanced malaria control programme (EMCP) has not effectively reached this vulnerable population although it was encouraging to find that many households had bed nets and that they were used on a regular basis. However, our investigation suggests that approaches for ITN distribution and enhancing community awareness about the importance of their use need to be addressed as similarly observed and proposed by earlier investigation in adjacent region by Hamer et al. [23].

Despite the change in drug policy in 2008 in the studied state (Jharkhand), the availability and implementation of combination therapy, that is, artesunate plus sulfadoxine pyrimethamine, are a major concern. It has been well documented that chloroquine resistance has been rising in India [60–63]; this drug was recommended for malaria prophylaxis in pregnant women in high risk areas as reported by Hamer et al. [23], though it has been discontinued since recommendation. Presently, quinine sulphate was recommended for malaria prophylaxis in pregnant women in the investigated area irrespective of gestational age. However, this is partly in accordance with The Directorate of National Vector Borne Disease Control Programme (NVBDCP) and current WHO guidelines suggesting prophylaxis for trimester based treatment of malaria during pregnancy as quinine for first trimester and subsequently ACTs in the second and third trimester of pregnancy (http://www.nvbdcp.gov.in/Doc/Diagnosis-Treatment-Malaria-2013.pdf). Since the intensity of transmission and the prevalence of malaria in pregnant women in Jharkhand are comparatively lesser than in many areas in sub-Saharan Africa, notably, sulfadoxine pyrimethamine was commonly used in Africa as intermittent preventive treatment of pregnant women (IPTp) [15], which may not be presently suggestive priority for Jharkhand to implement IPTp though it may be considered as an alternative to the priority failure strategy. The top priority for Jharkhand should be on preventive measures like improved availability, awareness and uses of ITNs by pregnant women, and well organised IRS system. In addition, we recommend much more stringent and frequent screening and diagnosis using conventional and RDTs irrespective of classical malaria symptoms to pregnant women in all the trimesters. Most importantly, in view of sizable prevalence based on hospital study and potential risk for population at large in the investigated region, we are also suggestive of dedicated active and passive surveillance for MIP at the community level like regular malaria surveillance under India's NVBDCP. This strategy alone could potentially reduce the burden of MIP while limiting the potential for antimalarial resistance to develop due to the widespread use of drugs for chemoprophylaxis. The present study shows two important findings; that is, the observed predominant prevalence of asymptomatic infections differs from that of symptomatic disease and marked alteration in haematological indices during *P. vivax* infection with pregnancy synergistically

contributes to maternal anaemia in a low and perennial malaria transmission setting.

One major limitation of this study is that we were unable to access the placental malaria due to limitation of our study design. Although the study was restricted to women delivering in the hospital, a sizable number of (more than 60%) women give birth outside Sadar Hospital, Hazaribag.

Further, a longitudinal study instead of cross-sectional one would have provided better estimate of MIP in this region and probably our study design may have given underestimate as compared to actual risk population. This has also been apprehended and suggested by Hamer et al. [23]. Despite these limitations, this study provides important data on the epidemiology and clinical implications of *vivax* malaria during pregnancy and delivering at Hazaribag district Sadar Hospital. In spite of restricted and facility based study, we preferentially covered marginalized, tribes, and remote population of the investigated rural-cum semiurban district, Hazaribag. The majority of the districts and particularly malaria endemic districts in Jharkhand have similar geographical, socioeconomic, demographic, literacy, and basic amenities including health facility and awareness. Thus, our observation may be utilized for baseline information for further comprehensive and multicentric study design, in strengthening MIP associated preventive measures and screening methods within the state of Jharkhand.

5. Conclusion

As the global control and elimination of malaria progress, *P. vivax* is set to become the dominant *Plasmodium* species [64]; yet, the health, developmental, and socioeconomic consequences of *vivax* malaria and *vivax*-associated anaemia have received very little attention. Salient findings of this study are as follows:

(i) There is high prevalence of anaemia during pregnancy and in delivering women in the malaria endemic population of Hazaribag, Jharkhand.

(ii) Prevalence of anaemia is significantly associated with *Plasmodium vivax* infection during pregnancy and in delivering women.

(iii) The most significant observation was the high prevalence of asymptomatic *P. vivax* infection at both ANC and DU.

Taken together, these observations are quite indicative and emphasize the need to actively diagnose and treat malaria infection during ANC visit in the areas of perennial transmission. Additionally, in view of the sizable population at risk in this malaria endemic region of India, we are suggestive of few priority practice amendments and reorientation of policies for MIP prevention strategies:

(i) There is an urgent need to enhance the ITN availability, use, and awareness both in population and health worker.

(ii) Distribution of ITNs at first ANC visit will be lucrative alternative for preventive strategy.

(iii) There should be priority consideration of early case detection and management of asymptomatic pregnant women through restructuring the need of active and passive surveillance strategy in endemic as well as in nonendemic zone.

(iv) In view of the asymptomatic prevalence of coinfection, we need to further strengthen and emphasize the robust screening strategies, curative attention, and safe treatment facilities at the community level health centres.

Further, integrated investigation is desperately needed to understand the magnitude and prevalence of asymptomatic malaria infection linking as an important infected reservoir to continue malaria transmission. Precisely, our finding highlights the public health importance of integrated genus-wide malaria control strategies using diagnostic tests including RDTs and ensuring the availability of safe and effective drugs for the treatment of pregnant women in areas of *Plasmodium* coendemicity.

Conflict of Interests

Sneh Lata is employed in respective government organization, which is directly providing health services to the community; however, this does not alter the authors' adherence to policies on sharing data and materials. The authors have declared that no competing interests exist.

Authors' Contribution

Mohammad Sohail and Mohammad Raziuddin conceived and designed the experiments. Mohammad Sohail, Shayan Shakeel, Shweta Kumari, Aakanksha Bharti, and Faisal Zahid performed the experiments. Mohammad Sohail, Ajay Kumar Sharma, Shadab Anwar, Krishn Pratap Singh, and Mazahirul Islam analyzed the data. Mohammad Sohail, Shayan Shakeel, Shweta Kumari, Aakanksha Bharti, and Sneh Lata designed the clinical studies and collected samples. Mohammad Raziuddin, Ajay Kumar Sharma, Vahab Ali, Mazahirul Islam, Tridibes Adak, Pradeep Das, and Sneh Lata contributed reagents/materials/analysis tools. Mohammad Sohail, Mohammad Raziuddin, and Krishn Pratap Singh wrote the paper. Mohammad Sohail, Shayan Shakeel, Shweta Kumari, and Aakanksha Bharti equally contributed to the paper.

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Research Article

Cases of *Echinococcus granulosus* Sensu Stricto Isolated from Polish Patients: Imported or Indigenous?

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The cases of nine Polish patients with diagnosed cystic echinococcosis (CE) were examined. A total of nine isolates obtained postoperatively were investigated using PCR and sequencing. The mitochondrial region of *nad1* gene was amplified. This PCR and sequencing analysis revealed the presence of *Echinococcus canadensis* G7 in seven patients and *E. granulosus* G1 in two patients. These data demonstrate that *E. canadensis* is the predominant causative agent of human cystic echinococcosis in Poland. *E. granulosus* G1 detection in Polish patients suggests that the parasite was imported; however it does not exclude the possibility that these cases could have been of Polish origin.

1. Introduction

Cystic echinococcosis (CE) is caused by the metacestode stage of the *Echinococcus granulosus* complex belonging to the family Taeniidae. CE is considered as one of the most important zoonotic parasitic diseases worldwide. The life cycle of *Echinococcus* species is mostly domestic, involving dogs as the definitive host, in which the adult worm lives in the small intestine. Wild canids (dingoes, wolves, jackals, coyotes, red foxes, etc.) can also serve as definitive hosts in the transmission cycle. The hydatid cyst metacestode develops in the internal organs of an intermediate host that acquires the infection through accidental ingestion of the tapeworm eggs. The intermediate hosts can be sheep, goats, swine, cattle, and humans. The cysts in humans develop mainly in the liver (70%), lungs (20%), and other organs like the brain, heart, and bones [1]. The cysts usually may develop asymptomatically for years and clinical symptoms occur when the cysts press on the surrounding tissues or organs. CE can be life-threatening when the cysts rupture into the peritoneal cavity causing anaphylaxis or can cause secondary CE. Echinococcosis is regarded as an emerging disease occurring worldwide with the highest prevalence in parts of Eurasia, north and east Africa, Australia, and South America [2–6].

Analysis of mitochondrial and nuclear genes of different *Echinococcus* species has led to taxonomic revisions and the genotypes G1–G3 are now grouped as *E. granulosus* sensu stricto, G4 is as *Echinococcus equinus*, G5 is as *Echinococcus ortleppi*, G6–G10 are as *Echinococcus canadensis*, and the "lion strain" is as *Echinococcus felidis* [7–11]. Among these strains, *E. granulosus* sensu stricto has a broad geographical distribution with a wide host range and is the major causative agent of human cystic echinococcosis.

In Poland, identified CE human cases have not been common; 260 patients have been treated in the Department of Zoonoses and Tropical Diseases within 10 years. The main *Echinococcus* strain, detected in Polish domesticated animals (pigs, dogs), is *E. canadensis* (G7) [12]. Molecular studies of the cysts isolated from humans revealed the presence of the G7 genotype [12, 13]. *E. canadensis* G7 strain has also been identified in humans in Ukraine [12], the Slovak Republic [12, 14], Turkey [15], and Austria [16]. Recently the first human cases of G7 infection in Mongolia [17], South Africa [18], and China [19] were found.

In our report we aim to characterize species of *Echinococcus* causing CE among cases of patients who underwent surgery to remove a cyst.

2. Materials and Methods

2.1. Materials. Fragments of cysts were collected from 9 patients postoperatively, carried out between January 2010 and May 2015 in the Department of Zoonoses and Tropical Diseases of the Medical University of Warsaw, Poland. Some patients treated with albendazole due to having cystic echinococcosis have been under medical supervision for many years. On account of some complications the cysts were removed surgically. The patient data collected included age, gender, and city. Six patients were females and three were males. The age of the patients ranged from 26 to 77 years. The examined samples represented by part of a cyst were stored frozen at −20°C or fixed in 70% ethanol prior to molecular analysis.

2.2. DNA Extraction and PCR. The samples of examined isolates and *E. canadensis* G7 positive control (isolates JX266793 and JX266824) were rinsed with phosphate-buffered saline (PBS) several times to remove any ethanol and centrifuged at 5000 ×g for 10 min. Each pellet was dissolved in 100 μL PBS and genomic DNA was extracted using a NucleoSpin kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. Two mitochondrial regions were amplified by PCR using the prepared DNAs as templates. Part of the NADH dehydrogenase 1 (*nad1*) gene was amplified using primers JB11 (5′-AGATTCGTAAGGGGCCTAATA-3′) and JB12 (5′-ACCACTAACTAATTCACTTTC-3′) [20]. The 50 μL reactions were comprised of 1 μL of DNA template, 50 pM of each primer, 0.2 mM of each dNTP, 1x PCR buffer containing 2.5 mM $MgCl_2$, and 1 U of Taq DNA polymerase (Qiagen, Hilden, Germany). The following PCR was performed in a PTC-200 thermal cycler (MJ Research, Waltham, USA) in conditions: 3 min at 95°C followed by 35 cycles of 1 min at 95°C, 1 min at 50°C, and 1 min at 72°C. The PCR products were separated by electrophoresis on a 2% agarose gel (MetaPhor, FMC BioProducts, Philadelphia, USA) and then stained with ethidium bromide and observed on a UV transilluminator. The *nad1* gene fragments were purified and then directly sequenced in both directions using a BigDye Ready Reaction Cycle Sequencing kit and an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, USA). Chromatograms were manually checked and edited using Chromas 2.0. The obtained sequences were aligned with others retrieved from NCBI GenBank using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2).

3. Results

A total of 9 samples were obtained from patients who underwent surgery to remove a cyst located in the liver (6 cases of women and 2 men) and in the hip muscle (1 case of a man). The patients came mostly from Warsaw, other cities and villages of central Poland, where they lived in either urban (40%) or rural areas (60%), and most of them had close contact with dogs. Eight patients suffered from primary and one patient from secondary cystic echinococcosis. The majority of the cysts ranged in size from 3 to 7 cm in diameter, and the largest one was 9 cm; 6 out of 9 cases were active (mostly type CE2), whereas the remaining cysts were sterile (type CE3) (Table 1). DNA extracted from all 9 samples and positive controls were used as the template in separate PCRs to amplify region of the mitochondrial NADH dehydrogenase 1. Each PCR produced a single band upon agarose gel electrophoresis. All isolates and controls were diagnosed as positive by amplification of *nad1* fragment (~500 bp). The *nad1* fragments were sequenced and compared with sequences of *Echinococcus* genotypes available in NCBI GenBank. The sequences of 7 isolates showed 100% identity to that of the pig strain G7, designated *E. canadensis* (Table 1). The sequences of 2 other isolates were identical to the sheep strain *E. granulosus* G1 (Table 1). All *nad1* sequences were deposited in GenBank with accession numbers KT780293–KT780301.

4. Discussion

E. granulosus sensu lato is a complex of genetic variants with genotypic and phenotypic differences such as intermediate host specificity or development. Recent molecular studies on the taxonomy of *E. granulosus* sensu lato have revealed that it is a complex of five independent species [7–11]. The genetic identification has been significant in understanding the transmission of the parasite between definitive and intermediate hosts, including humans. The cosmopolitan strain of *E. granulosus* is the G1 (sheep) genotype distributed worldwide and occurring particularly in areas of extensive sheep farming. It is the predominant strain infecting humans, but the other genotypes are also known to be infective. The presence of other *Echinococcus* strains in humans has been confirmed in Argentina, Kenya, Egypt (G1, G2, G5, and G6), Poland, Ukraine, the Slovak Republic, Turkey, Austria, South Africa (G7), Russia, and Mongolia (G10) [12–18, 21–25]. Transmission of *E. canadensis* G7 is considered to be restricted to certain areas in central, southern, and eastern Europe, Spain, Poland, Lithuania, the Slovak Republic, Ukraine, Romania, and Italy [12–16, 26–30], where the most common intermediate host is the pig. Recently, *E. canadensis* G7 has also been identified in pigs from Mexico and Brazil [31–33]. It has often been assumed that *Echinococcus* strains isolated from pigs of European origin are a separate strain and have low infectivity for humans and domestic ungulates [34]. Genetic investigations based on *nad1* sequence alignment showed the presence of *E. canadensis* G7 in Polish patients [12, 13], although another pig strain identified in human was designated G9, closely related to G6 (based on *its1* region analysis) and to G7 (*nad1* sequence analysis) [35].

In this report, we used PCR and sequencing to characterize *Echinococcus* cysts isolated from the liver and hip muscle of patients who underwent surgery to remove the cyst during the period of the last 5 years. In this analysis samples from 9 patients were examined. The patients were diagnosed as having cystic echinococcosis, following histopathological examination, imaging techniques, and/or immunological tests. Most of these patients have been under medical supervision for many years and treated with albendazole for months and due to some complications (bleeding inwards, detachment of internal capsule, and strong abdominal pain) it was decided that the cyst should be removed. Of these

Table 1: The characterization of nine CE cases, including patient data, diagnosis, and species. F: female, M: male.

Case number	City/region	Age in years, sex	Diagnosis details	Species
1	Warsaw (central Poland)	55 F	Liver single cyst (~6 cm), type CE2, removed with albendazole administration.	*E. canadensis* G7
2	Central Poland	77 M	Liver single cyst (3.5 cm), type CE2, removed with albendazole administration.	*E. canadensis* G7
3	Central Poland	48 F	Three cysts in the liver (size 5–7 cm), type CE2, removed with albendazole administration.	*E. canadensis* G7
4	Warsaw (central Poland)	31 F	Single liver cyst (~5 cm), type CE3, treated with albendazole for months and then removed. ELISA and Western blot positive.	*E. canadensis* G7
5	Ostrołęka (northeast of central Poland)	26 F	Single liver cyst (~9 cm), type CE2. When bleeding occurred, the cyst was removed with albendazole administration.	*E. canadensis* G7
6	Warsaw (central Poland)	28 F	Single cyst (6.5 cm), type CE2 with two cysts inside (1.4 and 2.2 cm), located at the surface of liver, not treated pharmacologically, Western blot positive. When severe abdominal pain occurred, cyst was removed. Patient spent one week in Turkey 2 years before CE was diagnosed.	*E. granulosus* G1
7	Central Poland	53 F	Single liver cyst (~5 cm), type CE3, removed with albendazole administration.	*E. canadensis* G7
8	Warsaw (central Poland)	60 M	Three cysts (each ~4 cm) in the hip muscle, type CE2. The cysts were detected during CT scan of colon cancer diagnosis and then removed. In 2001, the primary cyst from hip muscle was removed in Kazakhstan.	*E. granulosus* G1
9	Central Poland	48 M	Single liver cyst, removed with albendazole administration.	*E. canadensis* G7

9 samples, we were able to diagnose 7 cases as liver CE caused by *E. canadensis*, predictably as it was described already in a group of 30 patients in Poland [13].

The *nad1* sequences from 7 samples corresponded to the *E. canadensis* G7 strain. Taking into consideration the fact that *E. canadensis* G7 has already been confirmed in Poland and pigs are the major intermediate host, the G7 strain plays a significant role being the aetiological agent of human cystic echinococcosis in Poland. Interestingly, 2 other samples were identical to *E. granulosus* G1. This is the first report revealing *E. granulosus* sensu stricto hydatids isolated from humans carried out in Poland. These isolates involved a female and a male. The female patient (case number 6) with diagnosed CE of the liver, resembling sheep strain in computer tomography, was treated with albendazole. The cyst had two daughter cysts and after albendazole treatment, detachment of the capsule and parasite death were observed. A year later the patient suffered from very strong abdominal pain and the deceased hydatid was surgically excised. In the interview with the patient it was denoted that she travelled to Turkey a few years ago and spent a week there before CE was diagnosed, suggesting possibility of infection with G1 strain abroad. The Polish male patient (case number 8) was operated on in 2001 in Kazakhstan on account of the cyst found in the muscle of the left hip but the diagnosis of CE was not confirmed by histopathological or any other study. At present, after resettlement to Poland, colon cancer has been diagnosed and during routine tomography small cysts have been localized in the left hip muscle. The tumor and the cysts were removed and sent for confirmation. The preliminary identification of *E. granulosus* G1 in this case, considering the patient's history, implies the case has been imported from Kazakhstan where G1 strain is predominant. This fact does not exclude the possibility of infection of different hosts by *E. granulosus* G1

or other strains in Poland. The knowledge of the distribution of *Echinococcus* genetic variants in all parts of the country has not been ascertained yet.

Genotypes *E. granulosus* G1 and *E. canadensis* G7 are morphologically and genetically different; however both can develop in humans and pigs, generating threats to the public health and the animal breeding. Therefore, it is necessary to examine the pathology and etiology of CE based on molecular identification to assess the genotype. Still many aspects remain inadequately determined such as the factors establishing host specificity and developmental diversity among different strains of *Echinococcus*.

5. Conclusion

Cystic echinococcosis is an important zoonotic emerging disease occurring worldwide which is caused by the hydatid cyst metacestode stage of different *Echinococcus* species. In Poland, human CE is considered to be rather infrequent and mainly induced by the G7 pig strain of *E. canadensis*. In this report we identified *E. canadensis* G7 in seven human liver cysts; however in two cases (liver and hip muscle) the hydatids were diagnosed as *E. granulosus* G1. As it follows, *E. canadensis* has been the predominant strain in Poland, but revealing G1 strain in two patients remains unclear if these have been imported cases or maybe indigenous ones.

Conflict of Interests

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

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