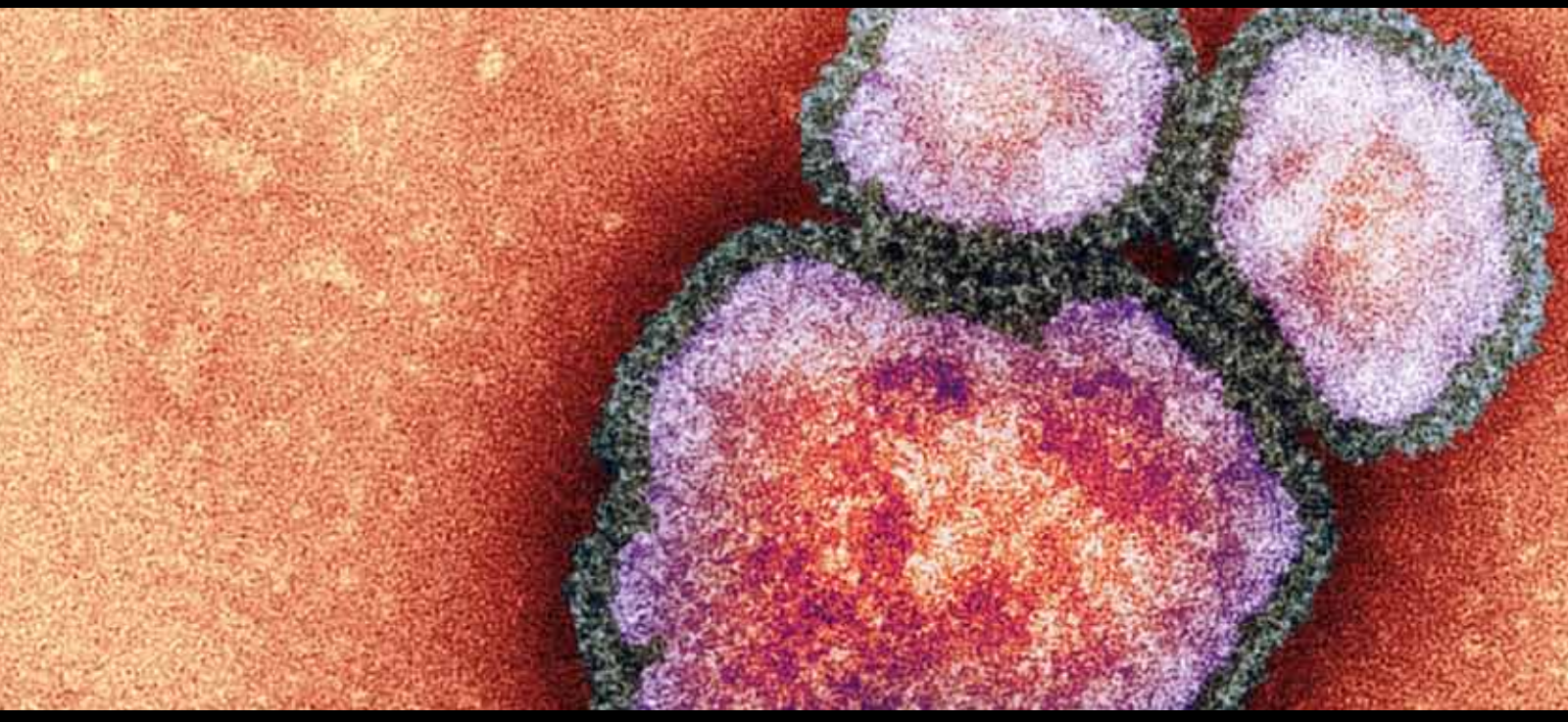
Journal of Tropical Medicine

Endemic Diseases: Globalization, Urbanization, and Immunosuppression

Guest Editors: Maria Aparecida Shikanai Yasuda and Pedro Albajar Viñas



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Editorial

Endemic Diseases: Globalization, Urbanization, and Immunosuppression

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Received 23 December 2012; Accepted 23 December 2012

Globalization and urbanization of endemic diseases represent major challenges in developed and underdeveloped countries. Massive migration within and between the countries was registered particularly since the beginning of this century. As intrinsic part of human history, migration has increased both in number and speed due to economic crises, civil war, and natural disasters. As consequence of the coexistence of transmissible and nontransmissible diseases in urban and periurban centers, several noninfectious chronic diseases occur in association with infectious diseases, contributing synergistically to increase the morbidity and mortality of diseases in urban centers.

In Latin America, the migration from rural area to great cities was registered in the context of socioeconomical disparities and poor basic sanitary infrastructure and low access to preventive medicine. In developed countries, vulnerable groups of migrants have less access to preventive medicine and to health care system bringing new potential for transmission of these diseases by alternative routes.

The globalization of human Chagas disease around the world is discussed in the context of bioecological, sociocultural, and political aspects, including relevant topics as migration and human Chagas disease, international flows to nonendemic countries, vectors and reservoirs movements, Role of the remaining sylvatic cycle *of T. cruzi*, and medical management Chagas disease in a globalised world as a very critical point.

The urbanization of tropical diseases such as malaria and leishmaniasis has been reported in two articles about urban malaria transmission in sub-Saharan Africa and periurban malaria in Lusaka, Zambia and other about canine visceral leishmaniasis in an urban area in Brazil".

New diagnostic methods for dengue virus and the expression of new immunoregulatory molecules by *Trypanosoma cruzi* SSP4 amastigote protein were also discussed in this special number.

In parallel to alternative forms of transmission, the reactivation of neglected tropical diseases under immunosuppressive conditions (HIV infection, immunosuppressive therapy, cytotoxic treatment for cancer and autoimmune diseases, immunobiological drugs for autoimmune disease, transplantation, and graft rejection) represents new challenges in urban centers. The experience in the prophylaxis of *Toxoplasma gondii* myocarditis in heart transplantation is presented and the influence of gender and age in cysticercosis was reported in a case control study.

Finally, in this issue, two articles analyzed the mortality of HIV/AIDS as comorbidity of two tropical diseases: American visceral leishmaniasis and Chagas disease in Brazil.

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Hindawi Publishing Corporation
Journal of Tropical Medicine
Volume 2013, Article ID 789758, 9 pages
http://dx.doi.org/10.1155/2013/789758

Review Article

Human Chagas Disease and Migration in the Context of Globalization: Some Particular Aspects

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Received 25 September 2012; Accepted 20 November 2012

Academic Editor: Maria Aparecida Shikanai Yasuda

Human Chagas disease originated in Latin America, being spread around the world in relation with multiple bioecological, sociocultural, and political factors. The process of the disease production and dispersion is discussed, emphasizing the human migration and correlated aspects, in the context of globalization. Positive and negative consequences concern the future of this trypanosomiasis, mainly in terms of the ecologic and sociopolitical characteristics of the endemic and nonendemic countries.

1. Introduction

In a broad sense, human Chagas Disease (HCD) reflects the bioecological, historic, and social situations of Latin America (LA), presenting a remarkable medical and social impact in the region [1, 2]. In its origin the disease was restricted to rural areas of LA, with a socio-political context strongly marked by poverty, human movements, and very weak productive relations. Emerging from a sylvatic cycle of the protozoan *Trypanosoma (Schizotrypanum) cruzi* (*T. cruzi*), HCD progressively became engaged with the political context of endemic countries and with a low standard of living of their population [3–5]. Following the circulation of the parasite during several thousands of years amongst different mammalian reservoirs and invertebrate vectors, in different ecological sceneries of the continent, the infection reached human beings in the so-called domestic cycle, resulting from man invasion of the natural environment. People migration, very poor conditions of life, and multiple situations of anthropic activities have been considered as the most important epidemiological factors of HCD spreading in LA [6–9]. In the last century, the progressive urbanization and the intensive migration of infected individuals increased the risk of HCD transmitted by blood transfusion and congenital route also in nonendemic regions [5, 6, 10]. In the present work, a general discussion about the role and the main antecedents of migration and other epidemiological aspects of HCD is intended, in the context of globalization.

2. The Medical Impact and the Fight against HCD

Until the last century, HCD affected primordially rural people living in poor dwellings colonized by the vector insect. It has been extremely impacting disease, with expressive mortality among children in its acute phase and severe heart lesions in about 10–20% of chronic adult patients. Its medical and social burdens involve mortality, high hospital and social costs, absenteeism, and labor incapacity [1, 11–13]. For recent years the prevalence of 8-9 million of infected individuals in LA has been estimated, with an expected mortality between 23 and 45 thousand individuals per year, depending on the source of information [14, 15]. The population under the risk of transmission is calculated to be about 30–40 million [10, 16]. The most effective strategies to prevent HCD consist in vector chemical control, housing improvement, and rigorous serologic screening of blood donors. There is no available strategy to prevent congenital and oral transmitted HCD, but early diagnosis and specific treatment of infected individuals are strongly recommended [10]. The specific treatment with the currently available drugs (Nifurtimox and Benznidazole)

is more effective in acute cases, young people, and cases of recent transmission, but now the treatment of indeterminate and initial chronic cases is also being suggested [17–19]. The correct and permanent medical assistance in chronic cases is considered fundamental to improve the quality and quantity of life of infected individuals [20–22]. In the last thirty years, control programs, large scale screening of blood donors, medical care, and the emptying of rural population have contributed to minimizing the social burden of HCD [1, 2]. Nevertheless, it is a mistake to imagine that HCD has been completely conquered; in some countries, regular control programs have not been implemented and, in others, epidemiological surveillance is under the risk of being relaxed. By another side, congenital and oral transmitted cases still exist [1]. The basic challenges of HCD for the next two decades consist of (a) launching adequate programs in those endemic countries without control activities, (b) improving and sustaining the existing programs, mainly in terms of epidemiological surveillance, and (c) implementing and improving adequate medical care for the already infected individuals [16, 23–25].

3. Globalization in Latin America

Social and political changes of the whole society have been very expressive in the last decades, following the process of globalization, all around the world. Among other factors such as the increasing of human migration, globalization has produced an economical effect tremendously unequal among different countries and social groups, as a consequence of economical speculations, international and supranational competition and political affairs [24, 26].

Regarding the overcoming of neglected diseases, Dr. Margareth Chan, Director-General of WHO, said very clearly that "efforts to control neglected tropical diseases constitutes a pro-poor strategy on a grand scale. The logic has changed: instead of waiting for these diseases to gradually disappear as countries develop and living conditions improve, a deliberate effort to make them disappear is now viewed as a route to poverty alleviation that can itself spur socioeconomic development" [16]. In other words, the overcoming of health problems such as HCD depends on the empowerment of state structures and of social commitment of rich and poor countries [3, 24]. Usually, the logic of globalization based on market economy has deeply affected the developing countries, reinforcing social inequalities and making the possibilities of social upgrade for marginal populations very hard [5]. As Professor Aluizio Prata said some years ago, globalization implies domestic deregulation, commercial liberalization, and privatization by means of foreign and volatile capitals, resulting in progressive social inequalities and unstable economy, generating several and complex influences in health sector (Prata, A.R., 2003. Conference about Tropical Medicine in Brazil. Congress of the Brazilian Society of Tropical Medicine. Belém, Pará, Brazil, February, 2003. A*pud* Dias (2007)). Neoliberalism, a main axis of globalization, represents a movement to benefit the great world potencies and the multinational enterprises. Poor countries usually suffer with neoliberal policies, in terms of underemployment, low salaries, inequity, and strong dependency of international capitals and of the global market (http://wikimediafoundation.org/, access in June 27, 2011). Particularly, this situation used to be responsible for the intensive migration process all around the world. Regarding LA, after two decades of Neoliberalism, the World Bank evaluation in 2005 considered that the results (in terms of economic grow) remained much lower than it was formerly awaited [24, 26].

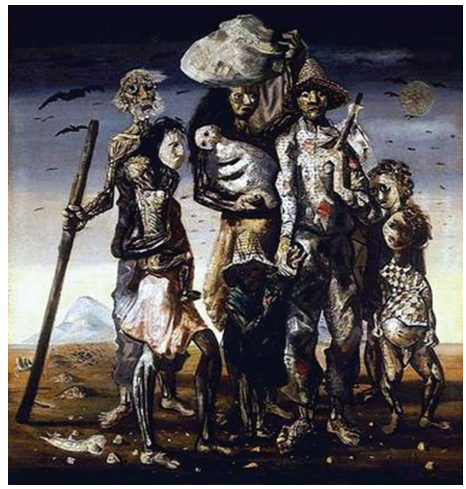

Figure 1: *Rural retirement*. Picture by Candido Portinari (Brazil) *In* ngeladohs.blogspot.com (accessed in Sept. 12, 2012).

4. Globalization and Chagas Disease

In the poor endemic regions of LA, the impact of globalization has been considerable in terms of HCD epidemiology, management, and prevention [27, 28]. The increasing of migration and the progressive changes in rural economy modified the epidemiological patterns of the disease, mainly in terms of its transmission and medical attention. For instance, following the reduction of demographic densities in rural endemic areas, a minor vector infestation can be considered as a "positive" effect. In addition, several areas have been modernized in terms of housing and production aspects; it means a radical change from the classical subsistence way of life to an agroindustrial and large scale economy [7, 24]. By another angle, the expansion of agriculture frontier in some places made possible the spreading of infected individuals and even of vectors [29]. Figure 1 represents the dramatic migration of rural poor people in Brazil, leaving their native land in search of survival abroad.

5. The Global Market, the Role of the State, and HCD

Regarding the social and political evolution of LA, HCD can be considered a reflex of the regional history, particularly in terms of equity and production dealings. By the side of the

infected individuals, globalization and market implications produced bad and good results, spreading the disease and improving the conditions for medical access. In general, the market related with HCD is very weak; the poor chagasic individuals usually depend on public health, especially in poor endemic countries. The main commercial profits (relatively low) concern laboratory diagnostic reagents and insecticides [30]. In addition, private enterprises do not always rely on LA governments, making social projects and the acquisition of products difficult. A correlated situation concerns insecticide prices, with high disparity, in the past, among the different countries [2, 12, 13, 24]. In this aspect, globalization rules have been beneficial, mainly after the emergence of the intergovernmental initiatives against Chagas disease, inducing the equalization of prices [1, 31]. At the side of specific treatment, the development of new drugs by private industries is not stimulant, because of the weak market and commercial risks. For instance, Roche considered its product Benznidazole as "a social drug" some years ago, giving it patent to the Brazilian Government. In terms of electoral benefits, also HCD seems to be inexpressive, because *chagasic* population usually has no political density, being unable to obtain the minimum social gains [13, 24, 28]. Concerning disease prevention, different situations have been observed in LA. First of all, vector control is based basically on insecticides and housing improvement. In this aspect, the poor communities depend almost exclusively on governmental programs, in other words, on political will. Notwithstanding, several observations and mathematical models have shown that vector control can result from the social improvement of the community, without a direct governmental intervention [13, 32]. In this aspect, the governments of LA commonly give no priority to the poorest rural populations, where residual transmission exists [16, 24]. On the positive side, two different situations were affected by globalization: (a) regarding transfusion transmitted HCD, since the 1980 decade, the proportion and the quality blood control have been highly improved, following the global demand for AIDS control [10, 14, 29]; (b) since the 1990s, following the economic and political commitments of globalization, the above mentioned "intergovernmental initiatives" were launched in LA to face HCD in terms of vector and transfusion transmissions, with technical assistance of PAHO and WHO [6, 16, 33]. In general terms, Briceño-León [12], Dias [23], and Schmunis [26] observe that such initiatives are completely integrated in the scenario of retrieval and rescue possibilities of LA, a region that looks for its identity and its better political and social expression.

6. Migration and HCD

It can be said that in the beginning, the spreading of HCD in Latin America was related to human movements, in parallel with progressive rural settlements and the domiciliation of triatomines [5, 8, 29]. Along the history, the total majority of infected individuals have been contaminated in infested dwellings of endemic areas [10, 12]. For instance, in Brazil, Zeni [34] studied 265 seropositive individuals in Paraná state, from whom 255 declared to have been bitten by the vector in rural houses (96,22%). In São Paulo city, Goldbaum [7] detected 232 infected urban workers; all of them immigrated from rural communities of 13 Brazilian states. As an example, the arrival of infected individuals has been registered in Beni and Pando (Amazonic regions of Bolívia), as a consequence of people immigration from endemic areas where mining and agricultural economies have failed [33, 35–37]. International migration waves in LA are also significant, for instance, from Bolivia and Paraguay to Argentina and Brazil, or from Colombia to Venezuela. In other scenery, mainly since the second half part of the last century, the movement of thousands of Latin American citizens towards North America, Europe, Asia, and Oceania was intensified, thus increasing the number of infected individuals living in nonendemic countries [2, 17, 38, 39]. In Belo Horizonte city, amongst 291 seropositive blood donors, Gontijo [40] detected 249 who certainly originate from rural localities (85,6%). This proportion, in Recife, was 95.0% [41]. In 1987, Wanderley [42] pointed out the estimate of 350,000 infected individuals living in the metropolitan area of São Paulo city (Brazil), in the great majority who immigrated from rural zones of Brazil endemic states and from Bolivia. At the Hospital of the Federal University of South Mato Grosso, Pompilio [43] found 88.3% of rural origin amongst 120 infected patients. Studying 57 infected women in Obstetric Unities of São Paulo city, Nisida et al. [44] found 55 who originate from rural endemic areas of Brazil, all of them declaring to know the insect vector.

In Rio de Janeiro city, amongst 260 infected individuals, Coura verified that all of them originated from endemic rural zones of Brazil (256 individuals), Bolivia, and Paraguay [45]. In Argentina, chagasic individuals studied in Buenos Aires have been contaminated in rural zones of different endemic provinces and of Bolivia [46]. The same rural origin is seen for infected individuals detected in Caracas, in Spain, and in USA [12, 17, 26, 39]. Even in the small towns of endemic areas, the majority of the detected cases come from rural localities, where their contamination occurred during childhood [7, 18, 22, 35].

Departing from the basic rural situation, different flows of individuals and families can be detected along the history of LA: (a) migration inside different rural areas, resulting in vector and reservoir dispersion; (b) migration from endemic to nonendemic rural areas (it is the case of thousands of families in Brazil going to Amazon region); (c) migration from rural localities to urban centres, generally being installed in peripheral sites; (d) the reverse migration from urban to rural areas; (e) the seasonal migration of rural laborers who travel periodically to work in different unskilled labor fronts such as sugar cane crops, petrol exploration, and civil constructions; and (f) migration from endemic to nonendemic countries, departing both from rural and urban situations. Basically, HCD and human migration are correlated with the social process of poor individuals moving themselves to look for better conditions of life [12, 13, 47]. In endemic areas, rural people usually have a poor and very provisional standard of life, depending on a weak familial economy and on a very unstable working situation [7]. Their houses are poor

and provisional; they are not the owners of the land [7, 12, 28, 48]. Migration becomes a question of opportunity and survival. In strict correspondence with globalization, the rural-urban migration was caused by several changes in the productive system, specially industrialization and weakening of the traditional rural economy, a continuous process that is rarely planned or assisted; it emerges as a consequence of the adverse social and economic conditions of the original place [7, 46]. As said by a rural woman in Minas Gerais, Brazil, all the family must be engaged in very poor agricultural activities, first of all to eat and then to sell the rest: "All of us, here, depend of the hoe, of the force of our arms…" and "I dream with health, with a good work… When we are bog down in a hole we dream to leave it… Our husbands must often to leave us, going to São Paulo to earn some money in sugar cane harvesting or in civil constructions…" [48]. Definitive rural emigration generally deals with the process of urbanization, basically resulting in urban peripheral settlements. When these settlements reproduce socially and ecologically the rural environment, housing infestation by means of passively carried triatomines can occur, as it was detected in Cochabamba, Sucre, Tupiza (Bolivia), in Guayaquil (Ecuador), in Tegucigalpa (Honduras), and so forth [35, 37].

Sosa [47] analyzed the migration process in the province of Tucumán, Argentina, in recent years, detecting that the total majority of chagasic individuals left their rural regions ad moved to urban centers of the country because of the lack of employment, due to extensive and semi-industrial sugar cane activities implemented all over the province. Similar situation can be observed in several parts of LA such as San Juan (Argentina), Cochabamba (Bolivia), rural areas of Venezuela, and Jequitinhonha Valley (Brazil) [10, 14, 49].

Analyzing rural emigration in endemic areas, several authors agree that its basic cause is a complex process involving the failure of the classic familial agriculture, the boom of the urban-industrial model, and the new agroindustrial perspectives for massive production. In addition, the lack of a social policy concerning agriculture in Latin America in the last century also stimulated the rural emigration: no prices, no technical assistance, and no proportion between efforts and results [13, 29, 30]. For a long period, the urban reality scared rural immigrants, generally illiterate and not able to work except by means of their physical and few differentiated forces. In 1983, studying chagasic individuals living in Belo Horizonte who immigrated from rural areas, Gontijo verified that the majority of them came to the city pursuing better working conditions, but also declaring their immense desire to go back someday [40]. The same was seen by Dias [18] in the small town of Bambui, Brazil, where all the 87 infected patients followed by 52 years since the acute phase changed from rural to urban places of residence, in order to get better work, health care, and education. The "urbanization" of HCD has been occurring all over LA, mainly after the second half part of the century XX. In the last decades, the disease reached urban areas and nonendemic countries, becoming a new medical and social problem [12, 26, 50]. Only in Brazil, it has been calculated that at least 75% of "chagasic" individuals are now living in urban spaces, a proportion that seems to be lower in other countries such as Bolivia, Guatemala, and Paraguay. In Argentina, the majority of infected individuals are living in the Great Buenos Aires [25, 37, 47, 49]. The presence of infected individuals in urban centres increased the demand for medical and social security assistance, also conveying the risk of congenital, accidental and transfusional transmission [10, 26, 29, 39, 50]. The access to medical attention certainly has been improved in the urban context, particularly in terms of more complex interventions, mostly depending on public health sector. The spreading of HCD to nonendemic countries has been studied by Schmunis, Albajar-Viñas, Storino, Zeledón, and others, receiving particular attention by WHO expert groups [2, 12, 16, 19, 26, 39].

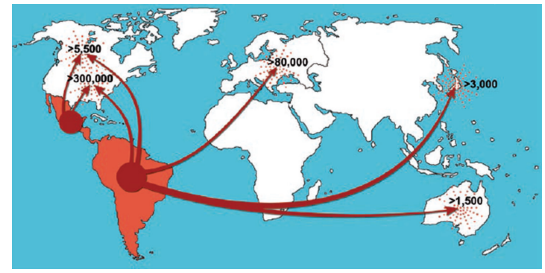

Figure 2: Migration routes from Latin America and estimation of the total number of infected individuals in nonendemic countries.

7. International Flows to Nonendemic Countries

The general situation concerns the migration of infected people from Latin America, particularly since the end of the XIX century. Many authors have been dedicated to this subject, the consensus being that the basic cause involves a survival strategy of poor population in search of employment and better living conditions in more developed countries [12, 14, 19, 26, 39, 50–52]. The principal destination has been to USA, followed by European countries, chiefly Spain (Figure 2 [50]).

Several considerations have been made about this important theme, involving complex social and epidemiological aspects [10, 19, 50]. Concerning the risk of disease transmission, the main possibilities deal with the congenital and transfusion routes, followed by some cases involving organ transplantation. Vector transmission seems to be improbable, in spite of the eventual detection of housing invasion by *T. rubrofasciata* and *Linshcosteus sp.* in Southern Asia [25, 39, 51]. The main preoccupation concerns the demand for medical attention and social security, a problem which has been highly complicated because of the clandestine situation of thousands of LA migrants and the lack of medical expertise in nonendemic countries [17, 50, 52]. In these countries, a network of specialized centers in HCD management should be implemented, aiming to produce standards for disease treatment and control [52].

8. Correlated Aspects Involving Human Movements and the Dispersion of HCD in Endemic Regions

Other different situations must be focused on, not only those related to people displacement. Vectors and reservoirs are also in constant movement, being implied in the whole process of *T. cruzi* dispersion. Climatic and other ecologic factors, but mainly anthropic actions, are naturally involved in such movements. Regarding triatomine vectors, different ecotopes and domiciliation (colonization of human dwellings) depend chiefly on the species and ecologic situations [5, 10, 14, 36, 53]. Among more than 140 species registered, no more than 15 or 20 have the capacity to colonize persistently human dwellings. Adult individuals of some few wild species can eventually invade human dwellings and transmit HCD, independently of colonization, generally attracted by artificial light or searching for food [5, 38]. Originally, all the vectors of HCD were living in sylvatic ambience, associated with birds, small mammals, several amphibian, and reptile species. Human beings entered into this sylvatic cycle much later, modifying the landscape and offering new and appropriate situations to wild vectors, mainly a poor house and an abundant feeding source [10, 35]. The classical example of HCD is its expansion in Bolivian Valleys since the XVII century when the Inca People became socially stable by developing cattle and agriculture activities, attracting wild *Triatoma infestans* to their stone and mud made houses. Once colonized, the species became more and more adapted to human houses, being able to produce enormous indoors colonies. From the first focus to neighbour houses, the vector is able to disperse by means of active or passive migration. From Bolivia, *T. infestans* reached Chile, Peru, Argentina, Uruguay, and Paraguay, mainly at the expense of human displacements due to agriculture expansion and internal wars [35, 37, 38].

The history of *T. infestans* spreading in Brazil since the XIX Century is emblematic, well studied by Silva [8]. The expansion of the international market of coffee induced a tremendous boom of its cultivation, first in São Paulo State, attracting a great amount of migrants coming from other Brazilian states and other countries such as Italy and Japan. Coffee is considered the main reason for S. Paulo development and posterior industrialization. For long years, all the production had to be carried from the farming to the Santos harbour by means of equine transportation. Railways only were implemented in the last decades of 1800, also being involved with triatomine dispersion. Mules should be imported from the Rio Grande do Sul state, a traditional producer of horses, at that time already infested by *T. infestans*, originated from Paraguay and Argentina (Jesuit Missions) and from Uruguay (regular commerce). The troops carried passively the triatomines to S. Paulo during more than 80 years, chiefly arriving in the Sorocaba region, which became the epicenter of *T. infestans* dispersion in S. Paulo. The process of infestation was progressive and continuous, in direction to the west of the state, as well as to the states of Paraná and Minas Gerais, following the expansion of the coffee frontier. Coffee, the so-called green gold, became in São Paulo the basis of a new economic era, impelling immigration, deforestation, urbanization, and industrialization [8, 35, 38]. Later on, with the reverse migration of northeastern workers, the species has been expanded to Bahia, Pernambuco, and Piaui states [52].

Another example refers to *Triatoma dimidiata* being carried passively from wild ecotopes of Costa Rica to human urban and peri urban dwellings by means of firewood transportation, a process that was being reduced when the population changed fire wood cooking to petrol gas and electricity [54].

Passive migration and active displacements of the vector are present in several other reports, including at the international level. For instance, *R. prolixus* seems to have a very complex story of its spreading from Venezuela to Mexico and Central America, involving an international scientific interchange of insect collection (Venezuela > France > El Salvador), the sea commerce between Central and South America, and even, possibly, the dispersion of eggs and nymphs, carried passively in birds migration [55]. Triatomine bugs have been identified outside America in parts of Africa, Middle East, Southeast Asia, and the Western Pacific. Current theories indicate that triatomines detected in Southeast Asia have probably derived from American species passively carried to seaports by sailing ships since the sixteenth century [38, 55]. The construction of railways and roadways has also played a significant role in the dispersion of domestic vectors in endemic countries [28, 38, 51, 54].

Natural reservoirs. In parallel with vectors movement, mammalian reservoirs and their mobilization in the vicinity of human houses play an important role in HCD epidemiology, particularly the so-called synanthropic reservoirs such as the opossums [5, 10, 14]. Besides being a natural source of sylvatic *T. cruzi* strains, wild reservoirs can introduce the parasite in domestic cycle, when they get closer to human dwellings harboured by triatomines. Such reservoir movements many times are a natural consequence of human intervention in natural ecotopes, chiefly when their natural shelters and food sources are destroyed [5, 14, 53]. In general terms, deforestation and massive monocultures have been appointed as the main anthropic activities in natural environments, with direct influence on reservoir and vector mobility. In terms of domestic reservoirs, the ancient culture of rural families to keep dogs, chicken, cats, guinea pigs, rabbits, and so forth very close to the house must be considered one of the most important factors to attract and to maintain domestic triatomine colonies, as well as to maintain (the mammalian ones) the cycle of the parasite [5, 29, 36].

9. The Role of the Remaining Sylvatic Cycle of *T. cruzi*

As a consequence of urbanization and control programs, the wild cycles of the parasite play a particular role in the maintenance of HCD. It is expectable that with the decreasing of domestic cycles in endemic areas, the major risks of

HCD incidence will depend on sylvatic triatomines and wild trypanosome populations in the vicinity of susceptible human beings [5, 53]. The enzootic cycle of *T. cruzi* also has some implications for globalization. Human intensive movements and progressive modifications of sylvatic ambient (macroprojects considering deforestation, monocultures, cattle, and the extensive use of pesticides) are clearly changing the general landscape since America discovery. Particularly the emergence of several acute cases of oral transmitted HCD has been important, especially in Amazon region, basically dependent on the contamination of a series of meals of *T. cruzi* originated from wild triatomines [29, 33, 49]. Nevertheless, in the next decades, domestic cycles of HCD tend to remain in those more isolated and poor rural zones, with lower taxes of social and ambient changes [36, 53, 54]. The future will be marked by the progressive reduction of some classical species such as *T. infestans* and *R. prolixus*, besides a residual peridomestic infestation by ubiquist species (*T. dimidiata, T. pseudomaculata,* and *T. brasiliensis*, etc.). By another way, due to anthropic affairs, wild species such as *P. geniculatus, R. pictipes, T. rubrofasciata,* and *T. picturata* could occasionally invade human dwellings, eventually establishing little colonies and being able to transmit HCD [10, 38, 55]. Probably, in the future, anthropic actions and globalization will be much more associated with the enfeeblement and focalization of the sylvatic cycle of *T. cruzi* than with its exacerbation [23, 29, 56]. Several examples can be remembered, showing strong linkages between HCD and globalization mainly involving the evolution of the productive system, spatial occupation, and human movements, such as [26, 30, 50] the following:

(i) deforestation resulting from a strong wood market and the expansion of agroindustries in endemic areas;

(ii) the extensive use of pesticides in agroindustrial projects;

(iii) the expansion of the use of electricity and industrial machinery, interfering with wild triatomine behavior and influencing the rural demography;

(iv) the progressive reduction of mammal reservoirs of the parasite, resulting from deforestation, pesticides, and extensive monocultures;

(v) progressive changes in the productive model, reinforcing capitalist agroindustries in detriment of the classical strategy of family subsistence;

(vi) in the same logic, the dominant market and large scale economy will overlap the classical microeconomies;

(vii) the modernization of agriculture, specially by means of automatic tools, robotics, and housing improvement, expulsing poor familial economies and hampering triatomine domestic colonization.

10. A Very Critical Point: The Medical Management Chagas Disease in a Globalized World

Presently, all over the world, the increasing life expectancy has been a great tendency of the population, resulting from better medical and social assistance. The medical management of HCD requires new knowledge and practices in terms of disease physiopathology and of the superposition of several other medical problems occurring chiefly in higher age groups, such as hypertension, diabetes, coronary diseases, Parkinsonism and physiological denervation. In such a scenery, the medical management of HCD involves three major challenges, highly depending on the political and social organization all around the world [17, 21, 22, 46, 52, 57]:

(a) the improvement of medical expertise for HCD management in chronic cases all over the world, mainly in terms of the primary health-care level; in corollary, the improvement of drugs and other medical proceedings is highly desirable, considering the elder patients and the superposition of other chronic and degenerative diseases;

(b) the betterment of medical and social security systems in order to assure adequate access and coverage for all infected individuals;

(c) ensuring political and administrative conditions to maintain at least two or three decades more the medical expertise able to manage adequately the infected individuals.

Other predictable situations could be emphasized for HCD management at the medium term, all of them being correlated with globalization. First of all, globalization has been a strong stimulus for the advance of medical security enterprises, able to increase the access of a progressively higher number of infected people to medical attention. The specific treatment is another important question, because it has been more and more indicated for chronic patients, trying to minimize and/or to prevent severe clinical conditions, specially advanced heart disease and sudden death. The basic problems considering this subject lie in diagnosis access, medical expertise, and drug availability, besides a good treatment adherence [2, 15, 17, 20, 57]. In chronological terms, the best moment to improve specific treatment for chronic cases of HCD has been estimated from now until one or two decades more, a time when the number of young infected individuals is still high. After 2020, with the progression of transmission control and the natural aging of infected people, the demand for specific treatment will decrease significantly. In pragmatic terms, globalization tends to facilitate specific treatment, bettering the availability of drugs around the world, chiefly with the assistance of health and humanitarian institutions such as WHO, PAHO, DNDI, and MSF [19, 21, 51].

11. The Future

It is admitted that HCD and other so-called neglected diseases have received and will continue to receive strong influences of the globalization process. Poor, isolated, and marginal areas in LA will continue to exist, as remaining foci of disease prevalence and active transmission. Likewise, the majority of the *chagasic* individuals will continue to be poor, illiterate, and socially excluded. National and international migration certainly will continue, spreading infected individuals in urban spaces all over the world during two or three more decades. The overcoming of HCD will depend on the public sectors (i.e., on political will) and on the reduction of inequity. As a correlated issue, considering developed nonendemic countries such as Canada, the social situation of poor immigrated individuals usually involves personal constraints and severe consequents for their health, in terms of disease management and work safety [58]. Coming back to trypanosomiasis, at the side of universal macropolicies, a recent WHO document stated that, "sustaining the progress made in controlling Chagas disease will depend on political commitment and the retention of public health resources. Resolution WHA 63.20, adopted by the Sixty-third World Health Assembly in May 2010, urges Member States where the disease is both endemic and nonendemic to control all transmission routes (namely vectors, transfusion, organ transplantation and vertical and oral routes) and to integrate the care of patients with all clinical forma of the disease into primary health-care services. WHO has been requested to facilitate networking at the global level and to reinforce regional and national capacities on strengthening global epidemiological surveillance of the disease... to advance intersectoral efforts and collaboration; and to support the mobilization of national and international public and private financial and human resources towards the achievement of these goals" [19].

In terms of political strategy, macropolicies and program organization are points to remain in the agenda of WHO and of the governments of endemic countries by the next two or three decades, keeping alive the interest and the priority of HCD and its control. National and regional programs must be adapted to the decentralization of health systems, another universal consequence of globalization [24, 30]. New nongovernmental partners such as Mèdicins Sans Frontiéres appear to be very effective and opportune to face HCD in endemic and nonendemic countries. The recent institution of a global scientific network to face HCD by WHO Neglected Diseases Department is very opportune. For the particular case of LA, it is extremely important to keep Pan American Health Organization in the coordination of the regional intergovernmental initiatives [5, 24, 29].

12. Final Remarks

Chagas Disease has been a concrete and impacting social and medical problem in LA, with multiple aspects associated with social iniquity and globalization. Human migration has been highly dependent on globalization and other social processes, being responsible for disease expansion and for a new epidemiologic situation, in which medical care must be improved. In spite of different financial and political constraints, HCD has been controlled, with remaining two or three more decades of program consolidation and medical attention for all infected people. This is a particular task for LA, because the fight against HCD requires the action of the state, as the basic social provider for the poorest citizens. The main strategies to face HCD have showed to be considerably effective at the medium and long terms, depending on social improvement, transmission control, and medical attention. Considering these points, the persistence of iniquity and other negative aspects of globalization have been hard challenges to be overcome in endemic areas. With the progressive reduction of its transmission and morbidity in the last two decades, the visibility of HCD as well as its political priority tends to decrease. In addition, the emergence of other public health problems such as dengue fever, influenza, and epidemic AIDS is contributing to the deviation of human and financial resources from the existing HCD programs [5, 16, 24].

Also, the transition of health sector all over LA has been slow and complicated, in spite of its highly logical and stimulant theoretical approach. Decentralization of health services and the reduction of vertical programs have been a consequence of globalization, putting the major responsibility of medical care and control programs on peripheral governmental levels [17, 39, 51]. Contextual difficulties and inequities exist but must be overcome by a universal effort involving people, governors, and scientists effectively compromised with poor populations. On the other side, the tremendous advance of medicine must be considered, in which new drugs and new diagnostic approaches are becoming available, thus raising several possibilities to improve the medical attention to HCD. Finally, at the political context, the epidemiological results of the intergovernmental initiatives can represent and stimulate a new and positive moment in the search of the continent political coherence and self-reliance [3, 12, 23, 27].

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Hindawi Publishing Corporation
Journal of Tropical Medicine
Volume 2012, Article ID 956875, 10 pages
doi:10.1155/2012/956875

Research Article

Expression, Purification, and Evaluation of Diagnostic Potential and Immunogenicity of a Recombinant NS3 Protein from All Serotypes of Dengue Virus

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Received 2 June 2012; Revised 14 September 2012; Accepted 18 October 2012

Academic Editor: Maria Aparecida Shikanai Yasuda

Dengue is one of the major public health concerns in the world. Since all the four serotypes are actively circulating in Mexico, there is a need to develop an efficient diagnosis system to improve case management of the patients. There exist few studies evaluating the use of the NS3 protein as a protective antigen against dengue virus (DENV). In this paper we show the expression of a recombinant NS3 protein from all serotypes of dengue virus (GST-DVNS3-1-4) and report a reliable "in-house detection system" for the diagnosis of dengue infection which was field-tested in a small village (Tezonapa) in the state of Veracruz, Mexico. The fusion proteins were immunogenic, inducing antibodies to be able to recognize to antigens up to a 1 : 3200 dilution. The purified proteins were used to develop an in-house detection system (ELISA) and were further tested with a panel of 239 serum samples. The in-house results were in excellent agreement with the commercial kits with $\kappa = 0.934 \pm 0.064$ (95% CI = 0.808–1.061), and $\kappa = 0.872 \pm 0.048$ (95% CI = 0.779–0.965) for IgM and IgG, respectively. The agreement between the NS1 antigen detection versus the rNS3 ELISA, $\kappa = 0.837 \pm 0.066$ (95% CI = 0.708–0.966), was very good. Thus, these results demonstrate that recombinant NS3 proteins have potential in early diagnosis of dengue infections.

1. Introduction

Dengue virus (DENV) infection in America, as in the rest of the world, is increasing dramatically. Currently, Mexico could be considered as an endemic region for dengue since the mosquito vector *Aedes aegypti* is present in more than 85% of the country [1]. Infection can lead to dengue fever (DF), a self-limiting febrile illness. A more severe form of the disease is dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) with fatal consequences. Dengue consists of four, closely related but antigenically, distinct viral serotypes (DENV1–4) [2]. It is well documented that primary infection with one of the four serotypes confers long-lasting immunity to that specific serotype. However, secondary infection with a different serotype is associated with an increased risk of developing DHF where an antibody-dependent enhancement (ADE) of infection is associated with the pathophysiological mechanisms of DHF [3, 4].

The viral genome contains a single open reading frame that codes for a polyprotein of 3391 amino acids, which is processed into 10 individual proteins. Three of these proteins are structural (membrane (M), capsid (C), and envelope (E)) and 7 of them are nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) [5–7]. The cleavage of this polyprotein, which represents an essential step for viral replication, is performed by host enzymes and the

NS3 viral protease. The dengue non-structural 3 (NS3) is a multifunctional protein of approximately 69 kDa, involved in the polyprotein processing, RNA capping, and RNA replication. It contains a serine-protease domain, located at the N-terminal portion, and a helicase [8]. The dengue infection elicits different immune responses towards the viral proteins. Antibodies are generated mainly against the virus surface E protein and the secreted NS1 protein [9–11], while the majority of T-cell epitopes are concentrated within the NS3 protein, the main target for $CD4^+$ and $CD8^+$ T-cell response [11–13]. The E protein may also induce non-neutralizing antibodies involved in the phenomenon of antibody-dependent enhancement (ADE) of DENV infection, which can be associated to the occurrence of increased numbers of DHF in secondary infections [3, 14]. Alternatively, some reports suggest the use of non-structural proteins for dengue vaccines to overcome such problem [15–17]. The NS1 is also highly immunogenic [18]; however antibodies against the NS1 may also cross-react with human proteins, which can be associated to some pathological effects of the dengue infection [19–21]. In contrast, there are only few studies evaluating the use of the NS3 protein as a protective antigen against DENV.

It is has been estimated that there are more than 3.6 billion people at risk of dengue infection with 36 million cases of dengue fever, more than 2 million cases of severe dengue, and more than 21,000 deaths occurring each year [22].

Since all the four serotypes are circulating in Mexico, there is a need to develop an efficient diagnosis system to improve case management of the patients. Until now, the incidence of dengue infection has been underestimated since most cases are not properly diagnosed, especially in small towns or villages where private or state laboratories for diagnosis are lacking [23]. According to this, early diagnosis during acute infection is critical to clinically manage severe disease and to identify potential outbreaks in a timely manner. Dengue infection diagnosis can be achieved by several assays such as RT-PCR [24], virus isolation [25], and NS1 antigen detection [19, 26]. However, the enzyme-linked immune assay (ELISA) has for a while, due to its ease, the routine diagnostic system for the dengue infection serological confirmation [27, 28]. Different kits are commercially available, such as Panbio Dengue Duo IgM and IgG Rapid Cassette test kits and commercial Platelia Dengue NS1 antigen capture ELISA kit. Clearly, the availability of systems for the detection of dengue infections is a public health priority. Therefore, in this study, we show the expression of a recombinant NS3 protein from all four serotypes of dengue virus and we report a reliable "in-house detection system" for the diagnosis of dengue infection that was field-tested in a small village (Tezonapa) in the state of Veracruz, Mexico.

2. Materials and Methods

2.1. Cells and Viruses. *Aedes albopictus* cells (C6/36, ATCC:CRL-1660) were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (DMEM-10% FBS), 0.29 mg/mL L-glutamine, 200 U/mL penicillin, and 0.2 mg/mL streptomycin. DENV-1 (Hawaii), DENV-2 (New Guinea C), DENV-3 (H87) and DENV-4 (H241) were obtained from the State Public Health Laboratory in Veracruz, Mexico. The virus stock was prepared by infecting C6/36 cell monolayer in 75 cm^2 tissue culture flasks at 75%–85% confluence. After 2 h of DENV adsorption, 20 mL of minimum essential medium (MEM) supplemented with 10% FBS was added and the flasks were incubated at 28°C until the cytopathic effect was evident. Cells and supernatant were then harvested by gentle pipetting, clarified by centrifugation (1,200 ×g for 20 min), aliquoted, and stored at −80°C until required.

2.2. Preparation of NS3 Expression Constructs. Viral RNAs were obtained by extracting 2 mL of the clarified culture media with 1 mL of TRIzol LS Reagent (Invitrogen) according to the manufacturer's instructions and used as template for the synthesis of a cDNA. The reaction was performed by reverse transcriptase SuperScript II (Invitrogen) with a primers DENV1–4 NS3 forward (DEN1, 5-GGGGGCGGAGGTAGTGGTGGAGGCGGGTCAGGAGTGCTATGGGACAC-3; DEN2, 5-GGGGGCGGAGGTAGTGGTGGAGGCGGGGCCGGAGTATTGTGGGATGT-3; DEN3,5-GGGGGCGGAGGTAGTGGTGGAGGCGGGTCCGGCGTTTTATGGGACG-3; DEN4, 5-GGGGGCGGAGGTAGTGGTGGAGGCGGGTCAGGAGCCCTGTGGGAC-3) and DEN1–4 NS3 reverse (DEN1, 5-ATCGATGATCATTACCTAAACACCTCGTCCTCAATC-3; DEN2, 5-TAATGGATCCTTACTTTCGAAAGATGTCATCTTCA-3; DEN3, 5-GGCGGATCCTTATGCATTTGTTTGCGCTATTCC-3; DEN4, 5-GGC-GGATCCTTACTT-TCGAAAAATGTCCTCATCC-3; restriction enzyme sites are underlined, DEN1 *Bcl*I, DEN2–4 *Bam*HI) [29]. The samples were subjected as follows: denaturation (94°C for 1 min), primer annealing (NS3-DEN1 at 58°C, NS3-DEN2 at 48°C, NS3-DEN3 at 60°C, and NS3-DEN4 at 55°C for 2 min), and primer extension (72°C for 2 min) followed by 30 cycles with an extension step of 7 min at 72°C. The different amplified products (500–600 bp) were electrophoresed on a 1.8% agarose gel, recovered with gel extraction Kit QIAEX II (QIAGEN, Germany). Four plasmids based on the pCR 2.1-TOPO vector (Invitrogen-Life Technologies) were constructed encoding the NS3pro185 sequence (domain protease). The recombinant vectors were restricted with *Eco*RI, and the fragments were ligated in frame into pGEX-5X-1 vector (Pharmacia) previously digested with this same enzyme.

2.3. Expression, Solubilization, and Purification of GST-DVNS3-1, GST-DVNS3-2, GST-DVNS3-3, and GST-DVNS3-4. Competent *Escherichia coli* strain DH5-α cells were transformed with the parental vector (pGEX-5X-1) as with the recombinant expression vector (pGEX-DVNS3-1, 2, 3, or 4), were inoculated into LB media containing 100 mg/L ampicillin (Sigma, St. Louis, MO, USA), and incubated at 37°C overnight. Fresh LB media was incubated at 37°C with the overnight culture (1 : 100) to an OD_{600} = of 0.5, and protein production was induced by addition of isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 0.1 mM.

After 2 h incubation, cells were harvested and purification of expressed proteins was performed essentially as described by López-Monteon et al. 2003 [30] with the following modifications. Then, pellets were treated to solubilize the inclusion bodies; briefly, the pellets were washed twice with 50 mL of PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, and 1.4 mM KH_2PO_4, pH 7.4), incubated at 37°C under constant stirring for 20 min and centrifuged at 3,046 ×g at 4°C for 10 min. After that, pellets were suspended by vortexing with PBS 1X pH 7.4 containing 2 M urea, the sample was stirred vigorously for 2 min, incubated at 37°C under constant stirring for 30 min, and subsequently, centrifuged at 3,046 ×g for 10 min. The supernatants obtained from the solubilization of the inclusion bodies were dialyzed to remove urea. These supernatants were dialyzed against PBS 1X pH 7.4 overnight at 4°C with constant stirring. The supernatants containing solubilized fusion proteins (GST-NS3DEN1, 2, 3, and 4) were mixed with glutathione-agarose beads (sulfur linkage; Sigma). After adsorption for 30 min, beads were collected and washed by centrifugation. Either GST or GST-DVNS3-1, 2, 3, and 4 (rNS3) were eluted by competition with free glutathione (15 mM glutathione in 50 mM Tris-HCl pH 8.0) and then acetone-precipitated.

2.4. Immunization of Mice with GST-DVNS3-1, 2, 3, 4. Female BALB/c mice (6- to 8-week old) were immunized by intraperitoneal route. All mice were maintained according to the recommendations by our Institutional Animal Care and Use Committee. The mice were immunized with one dose of 100 μg of antigen and two more with 50 μg. First immunizations were performed with the antigen emulsified in complete Freund's adjuvant (CFA), and re-immunizations at one-week intervals were performed with incomplete Freund's adjuvant (Gibco-BRL, Grand Island, NY, USA). The same schedule was used for control group, which received only GST plus adjuvant. At the end of the immunization scheme, animals were bled to obtain immune sera.

2.5. SDS-PAGE and Immunoblotting. Proteins were resolved on 10% SDS-PAGE [31] and visualized by staining with Coomassie brilliant blue or electrophoretically transferred onto nitrocellulose paper for immunoblotting [32, 33]. Pooled sera from each group of immunized mice were used as primary antibodies at serial dilutions (1 : 100–1 : 3200) in TBS-T (150 mM NaCl, 0.05% Tween 20, 2% skim milk, and 10 mM Tris-HCl pH 7.4). Bound antibodies were detected using alkaline phosphatase-conjugated goat anti-mouse IgG (Pierce, Rockford, IL, USA) diluted at 1 : 5000, then developed with NBT and BCIP (Sigma).

2.6. Study Area and Sample Collection. The study was conducted in the municipalities of Tezonapa from the sanitary jurisdiction of Cordoba, in central Veracruz, located at latitude 18°36′ north and longitude 96°41′ west. A total of 10 rural localities were included: Caxapa, El Mirador, El Suspiro, Las Josefinas, La Joya, La Luna, Paraíso La Reforma, Rancho Nuevo, Raya Caracol, and San Agustín del Palmar. These villages are at an altitude of 80–700 m and are located at the junction of the coastal plains of Veracruz on the east and mountains of the Trans-Mexican volcanic belt on the west. In each village, research personnel provided general information on dengue virus and the project to households during open meetings organized in the rural medical units through the IMSS-Opportunities social program. Interested participants were given an appointment for their family at the medical unit for blood draws. On the appointment day, a written informed consent was obtained from each volunteer, and research personnel collected the blood samples in vacutainers tubes. Serum was separated by centrifugation at 1,200 ×g for 10 min and samples were stored at −70°C until used.

2.7. Dengue Diagnostic. A short questionnaire on knowledge of the disease and general clinical signs was also applied to the participants when blood samples were taken, with questions that included whether they have suffered fever higher than 37.5°C, headache, retro-orbital and abdominal pain, vomiting, skin rash, nose or any other type of hemorrhage. A total of 239 serum samples were collected and analyzed for dengue virus infection using four different tests, including Panbio Dengue IgM and IgG Capture ELISA (Panbio Diagnostics, Brisbane, Australia), Platelia Dengue NS1 antigen capture ELISA (Bio-Rad) detection and in-house system (anti-rNS3), and RT-PCR assay specific for a fragment of the protein E from dengue virus.

2.8. Dengue Diagnosis by an In-House System. To test all serum samples, an indirect ELISA method was carried out as follows: 96-well plates were coated with 100 μL of carbonate buffer (pH 9.6) containing the antigen at concentration 2 μg/mL (pool of four recombinant NS3 proteins rNS3). After overnight 4°C incubation, the plates were washed five times with PBS-T (0.05% Tween-20 in phosphate buffer solution), five minutes per wash, and blocked with 5% skimmed milk in PBS for 45 min at room temperature. Serum samples were serially diluted; the dilution that generated an OD value three times higher than that from negative samples was thoroughly used in the rest of the study. All serum samples were diluted 1 : 50, and 100 μL of each one was added to individual wells in triplicate and incubated for two hours at 37°C. Further washing steps were conducted, and a peroxidase-labeled goat anti-human IgG antibody (Pierce, Rockford, IL, USA) was added at a 1 : 8,000 dilution in PBS/0.05% Tween 20 and incubated for 1 hour at room temperature. After eight washes, 100 μL of 2,2,-azino-bis(3-ethylbenzthiazoline)-6-sulphonic acid (Zymed, South San Francisco, CA, USA) was added as substrate and the reaction was allowed to proceed for 20 min at room temperature. The reaction was stopped with 2% sulfuric acid, and absorbance was read at 415 nm with an ELISA microplate reader (Multiscan MS; Labsystems, Vantaa, Finland). The cutoff (0.21) for this assay (at dilution 1 : 100) was established using the average obtained from a sample of 25 apparently healthy human sera plus two standard deviations (SDs). Positive samples were defined as samples with absorbance greater than two SDs above the mean of the negative control.

2.9. Viral RNA Extraction. To study the molecular typing of DENV, attempts were made to isolate the RNA from all the sera samples as well as from four different DENV serotype strains were performed, which were used as positive control. Using TRIzol-SL according to the manufacturer's protocol isolated viral RNA.

2.10. RT-PCR Serotyping Dengue Virus. In a single tube, viral RNA was converted to a DNA copy (cDNA) prior to enzymatic DNA amplification by the use of reverse transcriptase (RT) and the DENV downstream consensus primer D2-5′-TTGCACCAACAGTCAATGTCTTCAGGTTC-3′ homologous to the genomic RNA of the four serotypes. cDNA was synthesized using SuperScript II kit (Invitrogen) in a 20 μL reaction mixture containing 4 mM dNTPs (Applied Biosystems), 4 mM $MgCl_2$ (Applied Biosystems), 1 U/μL RNase inhibitor, 2.5 U/μL reverse transcriptase (Invitrogen), and 1 μg RNA. The reaction mixture was incubated for 30 min at 42°C and then the transcriptase was inactivated at 70°C for 5 min. 5 μL from obtained cDNA was used as template in a 25 μL reaction mixture containing 1.5 mM $MgCl_2$, 2.5 mM dNTPs, 1X PCR Buffer II, and 2.5 U of AmpliTaq Gold DNA polymerase with 20 pmol dengue virus group-specific consensus primers (D1: 5′-TCAATATGCTAAAACGCGCGAGAAACCG-3′ and D2: 5′-TTGCACCAACAGTCAATGTCTTCAGGTTC-3′). The PCR (511 bp) was carried out under the following conditions: 28 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 2 min, and then an extension step at 72°C for 7 min.

DENV serotyping was conducted by second-round amplification (nested PCR) initiated with 1 μL of diluted material (1 : 40 in sterile distilled water) from the initial amplification reaction. The total 25 μL of reaction mixture was prepared using 1 μL of diluted first PCR products, 1.5 mM $MgCl_2$, 2.5 mM dNTPs, 0.5 U of Taq DNA polymerase, and 20 pmol of primer D1 and 20 pmol of dengue virus type-specific primers (Ts1: 5′-CGTCTCAGTGATCCGGGGG-3′, Ts2: 5′-CGCCACAAGGGCCATGAACAG-3′, Ts3: 5′-TAACATCATCATGAGACAGAGC-3′, and Ts4: 5′-TGTTGTCTTAAACAAGAGAGGTC-3′), as reported earlier [34]. The samples were subjected to initial denaturation (95°C for 3 min) followed by 20 cycles of denaturation (95°C for 30 s), primer annealing (55°C for 1 min), and primer extension (72°C for 2 min) along with final extension (72°C for 7 min). The PCR products were analyzed by running a 1.8% agarose gel stained with ethidium bromide. The sizes of fragments were DENV-1 (482 bp), DEN-2 (119 bp), DENV-3 (290 bp), and DENV-4 (392 bp).

2.11. Data Analysis. All proportion data are presented with a 95% confidence intervals (CIs). Using Fisher's exact tests we compared proportion data, and the kappa index was calculated when applicable. The relationship between age and seroprevalence rate was assessed by chi-square test and by regression analysis. All villages were georeferenced and a spatial database of serologic results was created in ArcView 3.2 (Environmental Systems Research Institute, Redlands, CA, USA) to produce seroprevalence maps.

3. Results

3.1. Expression, Purification and Immunogenicity of Recombinant GST-DVNS3-1, GST-DVNS3-2, GST-DVNS3-3, and GST-DVNS3-4 Proteins. Four DNA fragments were obtained from the cDNA encoding for NS3 domain protease (NS3pro185 sequence) from all serotypes of DENVs by PCR (Figure 1(a)). All fragments (NS3-DEN1/597 bp, NS3-DEN2/596 bp, NS3-DEN3/590 bp, NS3-DEN4/598 bp) (Figure 1(b)) were cloned into pCR2.1TOPO vector and subcloned again in correct open reading frame with orientation into pGEX-5X-1 expression vector (Figure 1(a)). The expression of the recombinant proteins pGEX-NS3DEN1, pGEX-NS3DEN2, pGEX-NS3DEN-3, pGEX-NS3DEN4, and pGEX-5X-1 (parenteral plasmid) was evaluated in *E. coli* DH5α cells upon induction with IPTG at 37°C. The proteins were purified from inclusion bodies. The amount of GST-fusion proteins present in the fractions corresponded to a yield between 1.8 and 5 mg/L culture depending on the recombinant protein; GST-NS3DEN3 protein expression was the lowest (1.8 mg/L). Figure 1(c) shows the fusion proteins obtained after 2 hours of induction and after the inclusion bodies were purified by solubilization with 2 M urea. After purification by affinity chromatography and analysis by SDS-PAGE, results revealed the expression of a novel protein of ~49 kDa (Figure 1(c), lane 1–4) and expression of GST as a 27-kDa protein (Figure 1(c), lane 5). They were detected by western blotting using a polyclonal antibody against GST proteins, and all of them had the predicted weight.

In order to establish that these purified GST dengue proteins can induce humoral immune response, groups of 5 BALB/c mice were immunized with recombinant proteins. Serum samples were analyzed at the end of the immunization scheme. The fusion proteins were immunogenic, inducing antibodies able to recognize antigens up to 1 : 3200 dilution (data not shown).

3.2. Serology and RT-PCR DENV Serotyping. A total of 239 serum samples were collected from an area in Tezonapa, Veracruz (Figure 2), mostly were woman (n = 166, 69.4%). Age of participants ranged from 3 to 65 years. All 239 samples were tested for dengue IgM and IgG antibodies, NS1 antigen detection, and anti-NS3 antibodies (Figure 3(a)). A total of 13 samples were positive for IgM, obtaining a seroprevalence of 5.43% (95% CI = 4.23–6.63%). When analyzing the presence of IgG, a total of 28 samples were positive, obtaining a seroprevalence of 11.7% (95% CI = 10.26–13.16%) (Figure 3(b)). Seventeen samples were positive for NS1 capture was observed 7.11% (95% CI = 5.59–8.73%) (Figure 3(c)). When analyzing the prevalence of anti-DENV antibodies using a pool of recombinant NS3 proteins (rNS3), a total of 26 samples were positive and seroprevalence was observed of 10.87% (95% CI = 9.42–12.32%) (Figure 3(c)). The same samples that were positive to NS1 antigen proved positive PCR for DENV (1 sample for serotype 1 corresponding to Paraíso la Reforma and 16 samples for serotype 2 to San Agustín del Palmar) observing

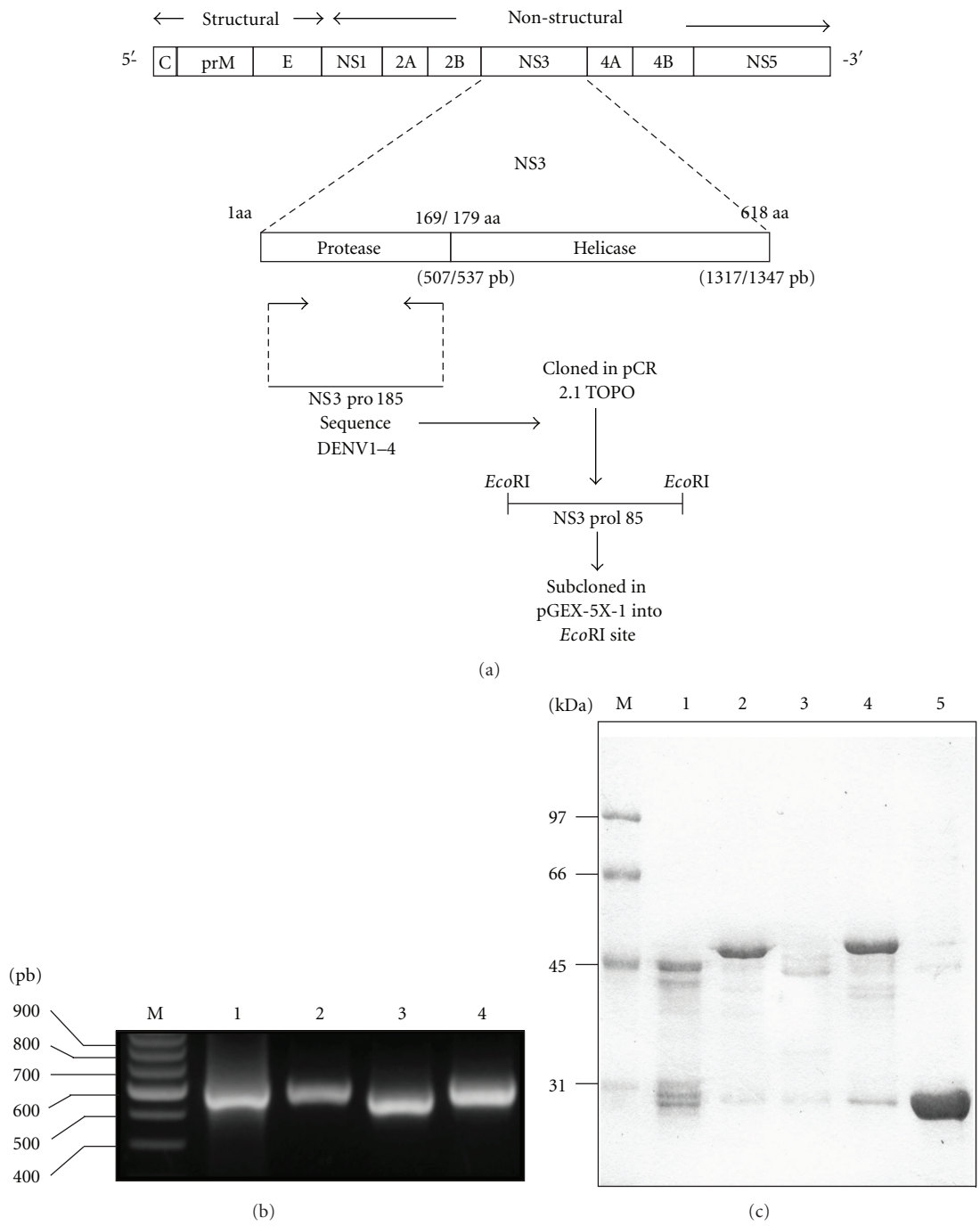

Figure 1: Obtaining recombinant NS3 protein. (a) Cloning strategy for the NS3 domain protease of dengue virus in the cloning vector and in prokaryotic expression vector. (b) Amplification of the NS3 protease gene cloned into pCR2.1TOPO, (M) size marker, Lane 1, NS3-DEN1. Lane 2, NS3-DEN2. Lane 3, NS3-DEN3. Lane 4, NS3-DEN4. (c) Purification of the recombinant protein GSTNS3 of each serotype. (M) molecular weight marker. Lane 1, GST-NS3DEN1. Lane 2, GST-NS3DEN2. Lane 3, GST-NS3DEN3. Lane 4, GST-NS3DEN4. Lane 5, GST.

an overall infection rate of 7.11% (95% CI = 5.59–8.73%) (Figure 3(d)).

3.3. In-House Dengue System Evaluation. Eight samples were positive for the five tests; 7 samples were positive for four tests. The concordance between the three ELISA evaluations was very good. IgM Panbio ELISA versus rNS3 ELISA, $\kappa = 0.934 \pm 0.064$ (95% CI = 0.808−1.061). The value of protein as a possible diagnostic test was evaluated, the observed agreement IgG Panbio ELISA versus rNS3 ELISA, $\kappa = 0.872 \pm 0.048$ (95% CI = 0.779–0.965), was very good (observing an equal number of positive samples in both tests; $P \leq 0.0001$, by Fisher's exact test), a sensitivity of 0.9231 (95% CI = 0.7488–0.9905), a specificity of 0.9812

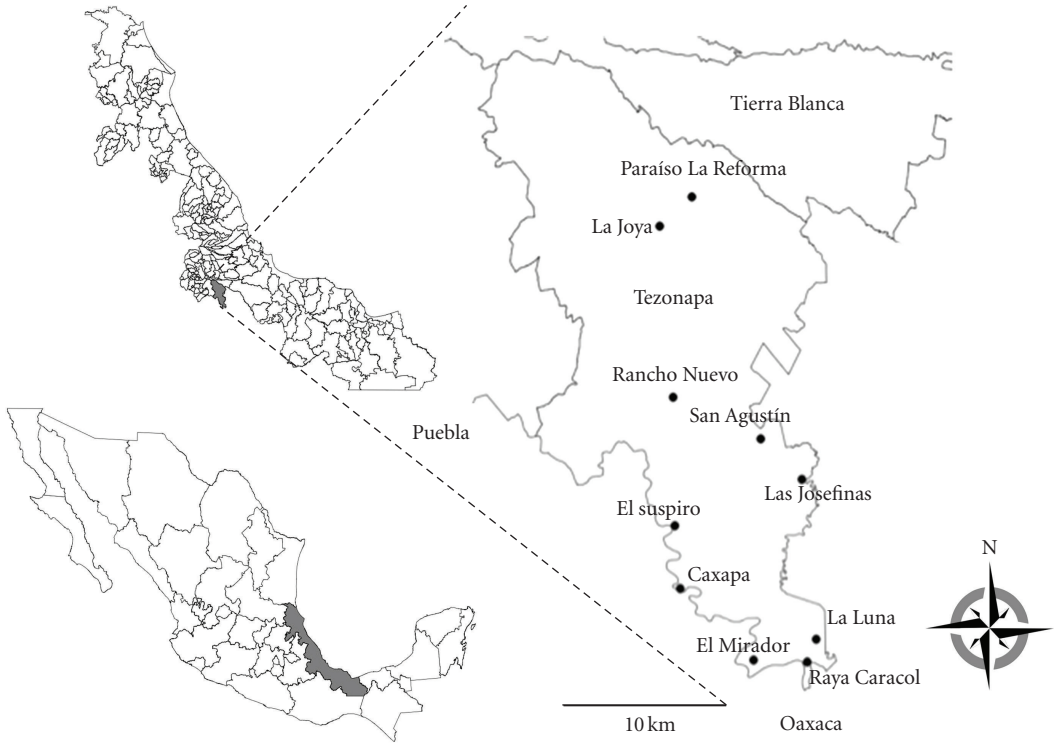

Figure 2: Study area. Mexico (bottom left), the state of Veracruz (center), and the study area (inset), corresponding to municipality of Tezonapa. Black circles show the position of the indicated villages.

(95% CI = 0.9526–0.9949), a positive predictive value of 0.8571 (95% CI = 0.6730–0.9597), and negative predictive value of 0.9905 (95% CI = 0.9661–0.9989). Prevalence rate was not significantly correlated with age ($r^2 = 0.8024$; $P = 0.3761$, by second-order polynomial regression) (data not shown). The agreement between the NS1 antigen detection versus rNS3 ELISA, $\kappa = 0.837 \pm 0.066$ (95% CI 0.708–0.966), was very good, a sensitivity of 1.000 (95% CI = 0.7708–0.9946), a specificity of 0.9595 (95% CI = 0.9219–0.9801), a positive predictive value of 0.6538 (95% CI = 0.4436–0.8206), and negative predictive value of 1.000 (95% CI = 0.9779–0.9996).

4. Discussion

Dengue infection is a growing public health concern in endemic areas all over the world. Hyperendemic geographical areas have been defined as those with continuous presence of multiple viral serotypes and competent vectors, and a large population of susceptible hosts, as it seems to be the case for Mexico [1]. Despite the importance of the DENVs as emerging pathogens, diagnostic tests remain inadequate for efficient and accurate identification of DENV infection. Mexico, currently, has a network of public health laboratories which consists of 30 laboratories that perform confirmation diagnosis of dengue by using immunoassay techniques for detection of viral antigen NS1, IgM antibody, or IgG, depending on the time evolution of the disease when the patient seeks medical care. It is well recognized that there is a high level of underreporting of cases of dengue even in areas where there is an adequate surveillance system. It is expected that the level of underreporting in dispersed rural areas and with poor access to health services is even higher [35]. Our study focused on diagnosis of dengue virus infection as well as on the presence of antibodies in rural areas of central Veracruz state.

Current dengue diagnostic methods have a number of serious limitations. The gold standard for diagnosis of acute DENV infection is viral isolation, but the procedure is costly (US$39.10), time consuming, and technically difficult to perform. Reverse transcriptase PCR (RT-PCR) has been widely adopted as an alternative for viral isolation in the diagnosis of acute infection, but PCR is technically intensive and expensive (US$136.67), and its sensitivity varies from 80 to 90% based on primer sets. An IgM antibody capture enzyme-linked immunosorbent assay (MAC-ELISA) is useful primarily for diagnosing dengue infection in the late acute or early convalescent phase of the illness but is often insensitive for early-acute-phase infections [36]. The distinction between primary and secondary infections is currently assessed by measuring IgM and IgG responses to dengue antigens in paired serum samples taken from a febrile patient in the acute stage of disease and after convalescence [37]. In order to set up a rapid and reliable diagnosis, some laboratories in Mexico use the Panbio Dengue Duo IgM and IgG Rapid Cassette test kits or the ELISA Dengue IgM capture kits. However, it is sometimes difficult to have access to these kits due to stock shortage in the market,

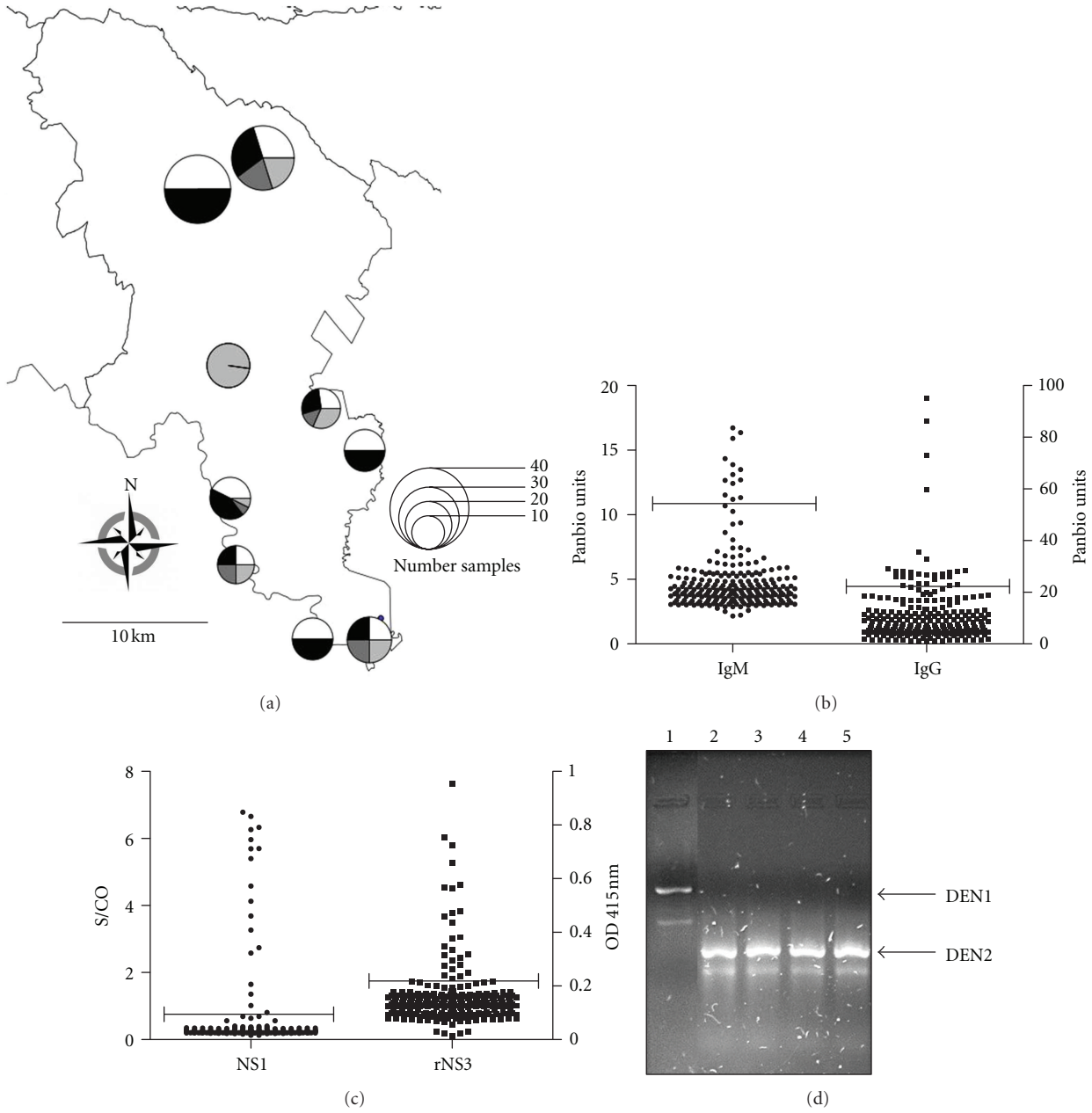

Figure 3: Seroprevalence and serotyping of dengue virus. (a) Geographical distribution of cases in the municipality of Tezonapa; the circles on the map indicate the villages, with their size proportional to the number of samples as indicated on the map, white areas represent IgG-positive samples, dark gray areas represent IgM-positive samples, light gray areas represent NS1-positive samples, and black areas represent NS3-positive samples. (b) Identification of positive samples for IgM and IgG by ELISA (Panbio). (c) NS1 antigen detection (Bio-Rad) and identification of NS3-positive samples by ELISA (in-house detection system). (d) Serotyping of dengue virus by RT-PCR, lanes 1–5 representative samples from rural villages; heat arrow indicates PCR product by DEN 1 and DEN 2.

problems to introduce foreign products, and of course, high cost. Therefore it is necessary to find alternatives for the diagnosis of DENV during the acute phase of disease, because diagnostic tests remain inadequate for efficient and accurate identification of DENV infection. The objective of this work was to study the expression of a recombinant NS3 protein from all four serotypes of dengue virus and report a reliable "in-house detection system" for the diagnosis of dengue infection and compared to commercially available kits (Panbio Dengue Duo IgM and IgG Rapid Cassette test kits and commercial Platelia Dengue NS1 antigen capture ELISA kit). The use of recombinant NS3 protein as a diagnostic method for identifying primary and secondary infections provided very good results, obtaining a good concordance between the tests for both IgM and IgG (Panbio IgM/IgG test versus rNS3 ELISA), and when we perform the study of the predictive ability of a diagnostic test, it was observed a good specificity and sensitivity with ELISA rNS3.

Similarly, very good agreement is observed when comparing the NS1 antigen detection versus rNS3 ELISA. Nonstructural protein 1 (NS1) of DENV has been shown previously to be useful as a tool for the diagnosis of acute dengue infections. NS1 has been detected in the sera of DENV-infected patients as early as the first day of the symptoms and up to 18 days [38]. Our results showed that all samples that were positive for antigen detection NS1 were also positive for the presence of antibodies against the recombinant NS3 protein. These results suggest that the use of ELISA with recombinant NS3 protein may be an alternative method for serological analysis of dengue virus in the acute phase. NS3 protein plays a predominant role in the pathogenesis of the disease and together with its role during the processing of the precursor of the viral polyprotein makes it an interesting protein for evaluating host responses. Some authors have reported a significant antibody response to NS3 in both primary and secondary infections [39–41]. However, Valdes et al., 2000 [40], showed a significant antibody response in secondary cases when the infecting serotype was DEN2. In this case, in-house detection system used an anti-human IgG antibody as second antibody, which allowed to suggest that antibodies present in the sera of patients were IgG but not IgM antibodies. This is consistent with the findings from Valdes et al. [40], as we found that IgG antibodies for a secondary response and virus serotyping results show that the major infecting serotype was DEN2. The conclusion that the use of ELISA with recombinant NS3 protein may be an alternative method for serological analysis of dengue virus in the acute phase results from the fact that there is an excellent concordance when comparing the NS1 antigen detection versus rNS3 ELISA.

It has been reported that dengue is transmitted in rural areas and currently has become condition of urban nature. In addition, migrations of population favor a permanent risk for the spread of dengue-endemic areas.

The characteristics of an "ideal" dengue diagnostic test depend on the purpose for which the test will be used; in case of early diagnosis the dengue test should distinguish between dengue and other diseases with similar clinical aspects (such as malaria, leptospirosis, and typhoid), highly sensitive during the acute stage of infection, provides rapid results, inexpensive, easy to use, and stable at temperatures greater than 30°C for use in rural areas, if necessary [42]. Additionally to this, the study of the predictive ability of a diagnostic test on the in-house dengue system was observed with a good specificity and sensitivity and provides rapid results (3 hours); moreover it is less expensive than commercial tests available in the market. Serological tests have an intermediate price compared to molecular tests. In fact, when quoting a new test, the laboratories indicated that it should be considered a 4–7-fold increase versus the reagent price. In this sense, the cost assessment of "in-house" ELISA and commercial ELISA kits showed that "in-house" ELISA proved to be a cheaper kit than the commercial ones, with a cost of US$8.20, while other kits have higher costs as it is showed next: NS1 antigen detection (US$18.80), IgM (US$22.62), and IgG capture ELISA (US$24.04). This share reflects the actual costs of each test method based on the actual cost of reagents, laboratory supplies, stationery, personnel, and indirect costs as electricity, water, depreciation of equipment, and so forth.

Finally, the identification of the circulation of more than one serotype in the municipality of Tezonapa (DEN1 and DEN2) is a warning sign because it may favor the increase of severe clinical forms of dengue due to the presence of secondary infection by different serotypes, promoted by the phenomenon of increased antibody-dependent infection. We propose the use of recombinant NS3 protein as a detection system as a viable alternative in Mexico, when commercial kits are not available, at least in terms of a first screening. Even if this system needs to be improved by increasing the number of samples, for example, specially from asymptomatic individuals from the endemic regions in Mexico, to increase its accuracy in the dengue virus diagnosis, it is important to keep working on the development of reliable diagnostic tools in order to establish an efficient surveillance system in dengue endemic areas.

Funding

L. M. Álvarez-Rodríguez was recipient of a Ph.D. fellowship from CONACyT, Mexico (215334). This work was supported by Grant REDIV-2007 from DGI, Universidad Veracruzana to A. López-Monteon.

Acknowledgments

The authors would like to thank QFB. Yadira Morán Utrera for technical assistance and Wendy del Rosío Hernández Martínez for paper revision. The authors have declared that no conflict of interests exists.

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Hindawi Publishing Corporation
Journal of Tropical Medicine
Volume 2012, Article ID 829139, 10 pages
doi:10.1155/2012/829139

Research Article

Trypanosoma cruzi SSP4 Amastigote Protein Induces Expression of Immunoregulatory and Immunosuppressive Molecules in Peripheral Blood Mononuclear Cells

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Received 1 June 2012; Revised 25 September 2012; Accepted 2 October 2012

Academic Editor: Maria Aparecida Shikanai Yasuda

The acute phase of Chagas' disease in mice and human is marked by states of immunosuppression, in which *Trypanosoma cruzi* replicates extensively and releases immunomodulatory molecules that delay parasite-specific responses mediated by effector T cells. This mechanism of evasion allows the parasite to spread in the host. Parasite molecules that regulate the host immune response during Chagas' disease have not been fully identified, particularly proteins of the amastigote stage. In this work, we evaluated the role of the GPI anchored SSP4 protein of *T. cruzi* as an immunomodulatory molecule in peripheral blood mononuclear cells (PBMCs). rMBP::SSP4 protein was able to stimulate nitric oxide (NO) production. Likewise, rMBP::SSP4 induced the expression of genes and production of molecules involved in the inflammatory process, such as, cytokines, chemokines, and adhesion molecules (CAMs) as determined by RT-PCR and ELISA. These results suggest that the amastigote SSP4 molecule could play a key role in the immunoregulatory and/or immunosuppressive process observed in the acute phase of infection with *T. cruzi*.

1. Introduction

Chagas' disease is a zoonosis caused by the protozoan parasite *Trypanosoma cruzi*, and it is a major public health problem in most of Latin America and in particular in Mexico. Indeed, the WHO estimates that about 8–11 million persons are infected worldwide [1]. *T. cruzi* infects many cell types, including myocytes, fibroblast, vascular endothelial, and smooth muscle cells among other cells. Since the monocyte is a target cell in *T. cruzi* infection and monocytes play a major role in regulating immune responses, monocyte dysfunction may contribute to host immunosuppression [2]. It has been observed that during the experimental infection with *T. cruzi*, there is an increased expression of proinflammatory mediators, including cytokines [3], chemokines [4], vascular adhesion molecules [5], and nitric oxide synthase [6] among other molecules [7], which promotes the inflammatory process and vascular damage. There is evidence that immune mechanisms are involved in the pathogenesis of many parasitic infections. The initial stages of the disease are generally characterized by the induction of a nonspecific lymphoproliferation, which is believed to disrupt antigen recognition and interfere with protective immune responses. Paradoxically, in most cases, a state of immunosuppression can be evidenced. This hyporesponsiveness to antigen-specific and polyclonal stimuli in chronic parasitic infections could be related to immunosuppressive cytokines secreted by antigen presenting cells and regulatory T cells. A growing list of parasite-derived molecules able to exert immunomodulatory activities on the cells of the

immune system leading to such polarized cytokine secretion has been reported [8].

The intracellular phase of the parasite has been poorly studied, and it is known that *T. cruzi* amastigote surface antigens induce an immune response [9, 10]. However, few such molecules have been thoroughly studied. Recently our group has studied a *T. cruzi* amastigote-specific surface protein (SSP4), that is bound to the plasma membrane by a GPI anchor, which is released to the culture medium by phospholipase C activity [11]. The gene for this protein (cDNA) was cloned and partially characterized [12], obtaining the recombinant protein rMBP::SSP4. We have reported that this protein is a modulator of humoral and cellular immune responses in murine model, inducing low levels of IgA, IgM, and IgG3, but high levels of IgG1, IgG2a, and IgG2b isotypes; moreover, it is able to modulate nitric oxide production, as well as, to modulate the expression of cytokines *in vivo* in murine macrophages after immunization [13], suggesting that rMBP::SSP4 might exert a regulatory influence on macrophages during the immune response against *T. cruzi*. Also, it has been observed that the protein, rMBP::SSP4 activates a population of IL-10/IFN-γ-secreting CD4+ T cells [10], which has been observed to be activated during chronic infections and is responsible for prolonged persistence of parasite and for host protection against severe inflammatory responses [14]. Finally, it was observed that immunization with rMBP::SSP4 protein makes mice more susceptible to trypomastigote infection, with high mortality rates, whereas mice immunized with a eukaryotic expression plasmid containing the rMBP::SSP4 cDNA were able to control the acute phase of infection [15]. This suggests that the SSP4 antigen plays a role in the infection process. It should be noted that parasite molecules that regulate the host (human) immune response during Chagas' disease have not been fully identified, and to date there are few reports about the role of amastigote proteins in the development of the disease; therefore, it is important to characterize parasite molecules and their involvement in the pathology of the disease.

In this work, we analyzed the effect of rMBP::SSP4, a recombinant protein derived from T. cruzi in cursive (a major surface glycoprotein (SSP4) that is bound to the plasma membrane by a GPI anchor) on the induction of nitric oxide (NO), cytokines, chemokines, and adhesion molecules (CAMs) using humans' PBMC.

2. Materials and Methods

2.1. MBP and rMBP::SSP4 Expression and Purification. The conditions of purification of the recombinant protein rMBP::SSP4 were previously reported [13]. Briefly, the TcSSP4 (GeneBank, EMBL, and DDJ databases accession number AF480943) was cloned in the *Eco* RI site of the expression vectors pMAL-C2 (New England BioLabs) resulting in the plasmid pMALSSP4. This plasmid and plasmid pMALC2 were used to transform *E. coli* DH5-α, to obtain the fusion proteins rMBP::SSP4 and maltose binding protein (MBP), which were induced and purified according to the manufacturer. Either MBP or MBP-fusion protein were eluted by competition with free maltose (10 mM maltose in 20 mM Tris-HCl pH 7.4, 200 mM NaCl, and 1 mM EDTA), and then acetone-precipitated. Protein purification was analyzed by 10% SDS-PAGE in reducing conditions and coomassie blue staining [16].

2.2. PBMC Isolation. Heparinized fresh human whole blood (10 IU heparin/mL) was diluted 1 : 2 with PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, and pH 7.4) solution. The PBMC fraction was obtained by Ficoll-Hypaque centrifugation. The cells were then washed in PBS before culture. The PBMCs were cultured for 24 h at 37°C at a density of 1×10^6 cells/well in Dulbecco's Modified Eagle (DMEM) medium supplemented with 10% (v/v) fetal calf serum (FCS). The viability of PBMCs was measured by trypan blue dye exclusion and was consistently greater than 98%. The cells were then suspended in RPMI-1640 (Invitrogen-Life Technologies).

2.3. In Vitro PBMC Stimulation. PBMC were incubated with DMEM containing 10% FCS at 37°C in 5% CO_2 in 24 well plates (1×10^6 cells/mL). Cells were cultured separately in the presence de 10 μg/mL rMBP::SSP4 protein or medium alone. Cells and culture supernatants were collected at different times (12, 24, 48, 72, and 96 h), cytokine and chemokine concentrations and the expression of genes for cytokines, chemokines, adhesion molecules, and metalloproteinases were determined. All experiments were controlled for stimulation with MBP alone.

2.4. Nitric Oxide Measurement. Nitrite accumulation, an indicator of NO synthesis, was measured in the culture medium by Griess reaction [13]. In brief, human PBMC were stimulated with either rMBP::SSP4 (10 μg/mL), MBP (10 μg/mL), LPS from *Escherichia coli* (0.0111:B4, 4 ng/mL) (Sigma Chemical Co), IFN-γ (100 U/mL) (Genzyme Diagnostic), or LPS plus IFN-γ, respectively. Nonstimulated cells were used as a control. In some cases, N^G-nitro-$_L$-arginine methyl ester (L-NAME; 3 mM) (Sigma Chemical Co) was added separately; similarly 15 μg/mL of Polimyxin B sulphate (PMB) (Sigma Chemical Co.) was added to inhibit the LPS present in the recombinant protein derived from the purification process (data not shown). 100 μL of cell culture medium was mixed with 100 μL of Griess reagent and incubated at room temperature for 15 min. Absorbance at 540 nm was determined, and nitrite concentration was calculated from a sodium nitrite standard curve.

2.5. Determination of Cytokine and Chemokine Pattern by ELISA. Interleukin 1-beta (IL-1β), IL-6, IL-12, TNF-α, IFN-γ, and chemokines CCL3, CCL4, CCL5, CXCL10 (IP-10), CXCL8 (IL-8), and CCL11 were quantified by ELISA in culture supernatants of monocyte under different conditions of stimulation, according to the manufacturer's protocol. Briefly, 96-well flat-bottom plates were coated over night with a capture antibody at a final concentration of 2 μg/mL, and then plates were blocked with 10% PBS-FCS, washed

three times, and incubated with the cell culture supernatant samples or control antigens overnight at 4°C. After washing, plates were incubated with the respective biotinilated anti-cytokine antibodies (R&D System) at 1 μg/mL for 1 h in the dark. Plates were washed and streptavidin-Alkaline Phosphatase at 1 : 2000 was added for 30 min in the dark then washed, and 100 μL of ABTS (2,2,-azino-bis (3-ethylbenzthiazoline)-6-sulphonic acid) (Zymed) was added as substrate and the reaction was allowed to proceed for 20 min at room temperature (RT); the reaction was stopped with 2% sulphuric acid, and absorbance was read at 415 nm by an ELISA reader (Multiscan MS, Labsystem).

2.6. RNA Isolation and RT-PCR. Total RNA from PBMC, cultured in 24-well plates with different treatments for 48 h, was isolated using the TRIzol system (Life Technologies). One microgram of RNA was reverse transcribed to cDNA with an oligonucleotide (poly(dT)16) using the SuperScript II reverse transcriptase (Life Technologies) and the cDNA used as a template for PCR. PCR sequences and PCR conditions used for amplification of GAPDH [17], IL-1β [18], IL-6 [18], IL-12p40 [19], IFN-γ [19], TNF-α [18], CCL3 [19], CCL5 [18], CXCL10 [18], E-selectin [17], ICAM-1 [17], VCAM-1 [17], TNFR-I [20], and TNFR-II [20] were as follows: GAPDH (5′-GGT GAA GGT CGG AGT CAA CGG-3′ and 5′-GGT CAT GAG TCC TTC CAC GAT-3′), IL-1β (5′-ATG GCA GAA GTA CCT AAG CTC GC-3′ and 5′-ACA CAA ATT GCA TGG TGA AGT CAG TT-3′), IL-6 (5′-ATG AAC TCC TTC TCC ACA AGC GC-3′ and 5′-GAA GAG CCC TCA GGC TGG ACT G-3′), IL-12p40 (5′-AAC TTG CAG CTG AAG CCA TT-3′ and 5′-TGA TGT ACT TGC AGC CTT GC-3′), IFN-γ (5′-GAC CAG AGC ATC CAA AAG A-3′ and 5′-CCT TTT TCG CTT CCC TGT TTT A-3′), TNF-α (5′-TTC TGT CTA CTG AAC TTC GGG GT-3′ and 5′-GTA TGA GAT AGC AAA TCG GCT GAC GG-3′), CCL3 (5′-CGC CTG CTG CTT CAG CTA CAC CTC CCG GCA-3′ and 5′-TGG ACC CCT CAG GCA CTC AGC TCC AGG TCG-3′), CCL5 (5′-CGG GAT CCA TGA AGG TCT CCG CGG CA-3′ and 5′-CGG AAT TCC TAG CTC ATC TCC AAA GA-3′), CXCL10 (5′-CCA CGT GTT GAG ATC ATT GCT AC-3′ and 5′-ACA TAG CAC CTC AGT AGA GCT TAC-3′), E-selectin (5′-CTC TGA CAG AAG AAG CCA AG-3′ and 5′-ACT TGA GTC CAC TGA AGC CA-3′), ICAM-1 (5′-TAT GGC AAC GAC TCC TTC T-3′ and 5′-CAT TCA GCG TCA CCT TGG-3′), VCAM-1 (5′-ATG ACA TGC TTG AGC CAG G-3′ and 5′-GTG TCT CCT TCT TTG ACA CT-3′), TNFR-I (5′-TCA GTC CCG TGC CCA GTT CCA CCT T-3′ and 5′-CTG AAG GGG GTT GGG GAT GGG GTC-3′), and TNFR-II (5′- GCT CGC CGG GCC AAT ATG C-3′ and 5′-GGC TTG CAC ACC ACG TCT GA-3′). PCR conditions were as follows: initial DNA denaturation at 94°C for 5 min and 35 rounds of denaturation (95°C for 1 min), annealing (55°C for IL-1β, TNF-α, IL-6, CCL5, CXCL10, ICAM-1, VCAM-1, and E-selectin, 58°C for TNFR-I, and TNFR-II, 59°C for GAPDH, and 60°C for CCL3, IFN-γ, and IL-12p40 for 1 min in each case) and extension (72°C for 1 min). PCR products were electrophoresed on 1.8% agarose gels containing 0.5 μg/mL ethidium bromide and photographed under ultraviolet light. Densitometric analyses were done using the Image J software (Version 1.43 u).

2.7. Statistical Analysis. Statistical analysis was performed with GraphPad Prism (Version 5.0). The results are presented as mean ± standard deviation. Analysis of variance (ANOVA) followed by Tukey's post-hoc test was performed to compare the mean values among various groups. A P value of <0.05 was considered statistically significant.

3. Results

3.1. Induction of Nitric Oxide Production by rMBP::SSP4 in PBMCs. To first test the ability of the protein rMBP::SSP4 to induce nitric oxide production, PBMCs were stimulated with 10 μg/mL of protein *in vitro*. Result showed that rMBP::SSP4 protein is capable of inducing NO production in PBMC after 48 hours of stimulation (Figure 1), and that production is inhibited by the action of the inhibitor L-NAME. Nitrite values obtained by the stimulation of rMBP::SSP4 protein are statistically significant when compared with the values of nonstimulated cells ($P < 0.0001$) or with the values obtained from cells stimulated with MBP. NO production was increased up to 72 h (data not shown).

3.2. rMBP::SSP4 Protein Induces Cytokine and Chemokine Gene Expression. Cytokines play a fundamental role during the acute phase of *T. cruzi* infection [21] and affect the function of all cells types involved in an immune response. To investigate whether rMBP::SSP4 protein altered cytokine expression, RT-PCR analysis was performed in PBMC stimulated *in vitro* with rMBP::SSP4 protein (Figure 2). When PBMC were stimulated with rMBP::SSP4 protein, an increase in the expression of genes for IL-1β, IL-6, IL-12, IFN-γ, CCL3, CCL5, and CXCL10 was observed from 12 to 96 h with low expression at 48 h (Figure 2).

3.3. Cytokine and Chemokine Production by PBMC Stimulated with rMBP::SSP4 Protein. When PBMCs were stimulated with rMBP::SSP4 protein, the production of IL-1β, TNF-α, and IL-6 significantly increased (Figure 3). For IL-1β, the increase was observed at 24–72 h, while for TNF-α, the increase was from 12–48 h and a sustained production of IL-6 from 12–96 h of interaction, with a maximum production at 24–48 h. Likewise, we observed an increase in the production of chemokines, such as IL-8, CCL3, CCL4, CCL5, and CXCL10 in PBMC stimulated with rMBP::SSP4 protein. Production of IL-8 was observed only from 12 to 24 h of interaction, and an increase in the production of CCL3, CCL5, and CXCL10 with a maximum production at 48 h and decreased at 96 h of interaction. CCL4 production was also observed with a peak of synthesis at 72 h (Figure 3).

3.4. rMBP::SSP4 Protein Induces Genes of Adhesion Molecules and TNF-Receptors. To investigate whether rMBP::SSP4 protein was able to induce gene expression of CAMs and TNF receptors, PBMCs were stimulated with recombinant protein. We observed in PBMC an increased expression of

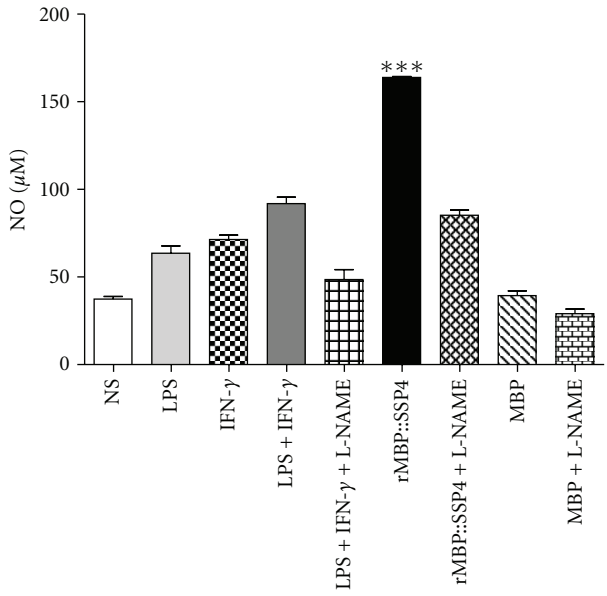

Figure 1: rMBP::SSP4 induces NO synthesis in PBMC. PBMC were stimulated at 48 h with different stimuli. Cells cultured with medium alone were used as controls. Supernatants of cultured cells were harvested, and nitrite concentration was assayed. Data are expressed as means ± SD and are representative of three independent experiments. $^{***}P < 0.0001$ compared with non-stimulated cells and controls. NS: non-stimulated.

gene for ICAM-1 (12–24 h) and an increase in the expression of genes for E-selectin and VCAM-1, with a maximum expression at 96 and 48 h, respectively. Likewise, we observed an increase in the expression of genes for TNFR-I and TNFR-II of the 12 to 24 h (Figure 4).

4. Discussion

Parasitic infections are prevalent in both tropical and subtropical areas. Most of the affected and/or exposed populations are living in developing countries where control measures are lacking or inadequately applied. Although significant progress has been made in our understanding of the immune response to parasites, no definitive step has yet been successfully done in terms of operational vaccines against parasitic diseases [22]. Pathophysiology of Chagas' disease is not completely defined, although innate and adaptive immune responses are crucial. In acute infection, some parasitic antigens can activate macrophages, and this may result in proinflammatory cytokine production, nitric oxide synthesis, and consequent control of parasitemia and mortality [23]. During the acute phase of infection, *T. cruzi* replicates extensively and releases immunomodulatory molecules that delay parasite-specific responses mediated by effector T cells. This mechanism of evasion allows the parasite to spread in the host [10]. The disturbed cytokine-chemokine network could play an important role in the onset of diseases with inflammatory processes [24]. We investigated whether rMBP::SSP4 protein induced NO production and cytokine gene expression in PBMC. The results showed that rMBP::SSP4 protein induced NO production in PBMC from 24 to 48 h (Figure 1). We have previously shown that rMBP::SSP4 protein was able to induce nitric oxide (NO) production by spleen and peritoneal macrophages (pMϕs) and macrophages from immunized mice [13]. Inhibition of NO production by L-NAME in murine Mϕs, results in a down-regulation of *i*NOS expression [25]. Our results showed that NO production was affected when stimulated PBMCs were incubated in the presence of L-NAME, thus indicating that the enzyme *i*NOS was participating in NO synthesis, it is also known that TNF-α regulates NOS expression and/or activity, which exerts direct effects on NO production [26]. According to these observations, and the fact that rMBP::SSP4 protein induces NO production by PBMC, the participation of NO in the suppression of T cell activation has been reported in a number of biological systems. In the case of *T. cruzi*, previous studies have shown that IFN-γ and nonoxidative molecules (TNF-α and NO) could play a role in the control of *T. cruzi* infection in mice [27, 28]. Furthermore, a series of experiments supports the notion that IFN-γ and TNF-α mediated activation of macrophages, which leads to increased production of NO, and in turn suppresses T cell activation. Therefore, it is likely that NO production during the initial phase of acute infections might participate in the clearance of parasites by macrophages, whereas its overproduction during the late phase of acute infection would account for the immunosuppression observed [21].

We investigated the cytokine and chemokine gene expression pattern in these cells as well as the production of these molecules in the culture supernatant. Results showed that this antigen induces the secretion of several chemokines (IL-8, CCL3, CCL4, CCL5, and CXCL10) and cytokines, such as IL-1β, IL-6, IFN-γ, and TNF-α in considerable amounts, whereas IL-12 was produced at a very low level suggesting that SSP4 is an immunomodulatory molecule of *T. cruzi*. Furthermore, IFN-γ is an important Th1 proinflammatory cytokine that could participate in the generation of Tregs cells [29] during acute phase, thus as has been observed in the mouse model using rMBP::SSP4 [10]. In addition, IL-12 has been described to have stimulatory effects on hematopoietic precursor cells and on B lymphocytes. The IL-12 produced during this inflammatory phase, both by direct action and, indirectly, by determining the composition of the cytokine milieu at the site of the immune response, induces differentiation of T helper type 1 (Th1) cells while inhibiting the generation of Th2 cells. Thus, because of its double function of a proinflammatory cytokine and an immunoregulatory factor, IL-12 plays a key role in the resistance to infections, particularly those mediated by bacteria or intracellular parasites, against which phagocytic cell activation and Th1-mediated responses are particularly effective. However, because of the same activities, IL-12 also plays a role in pathological situations, such as septic shock, tissue damage during inflammation, and organ-specific autoimmune diseases [30]. Accordingly, there are reports in animal models showing that inflammatory cytokines play a central role in acute *T. cruzi* infection; upon activation, such cells secrete proinflammatory cytokines and chemokines are

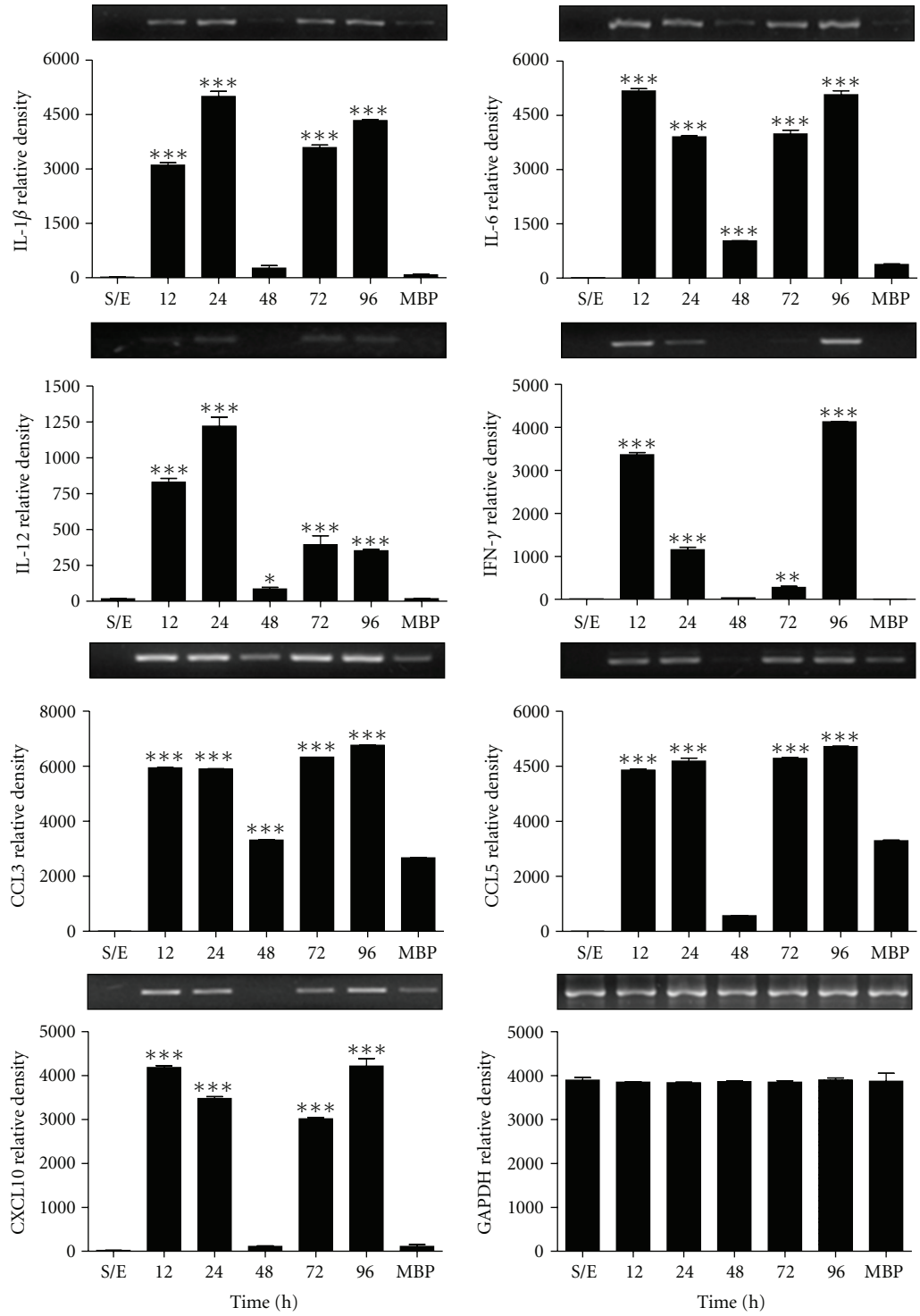

Figure 2: Expression of genes for cytokines and chemokines in PBMC stimulated with rMBP::SSP4. The cells were stimulated with recombinant protein for 12–96 h. The intensities of each band were quantified and plotted from the gels that are on top of each graph corresponding to the expression of genes for cytokines and chemokines. GAPDH was used as control housekeeping gene. $^{*,**,***}P < 0.05$, 0.001, and 0.0001, respectively, versus unstimulated cells.

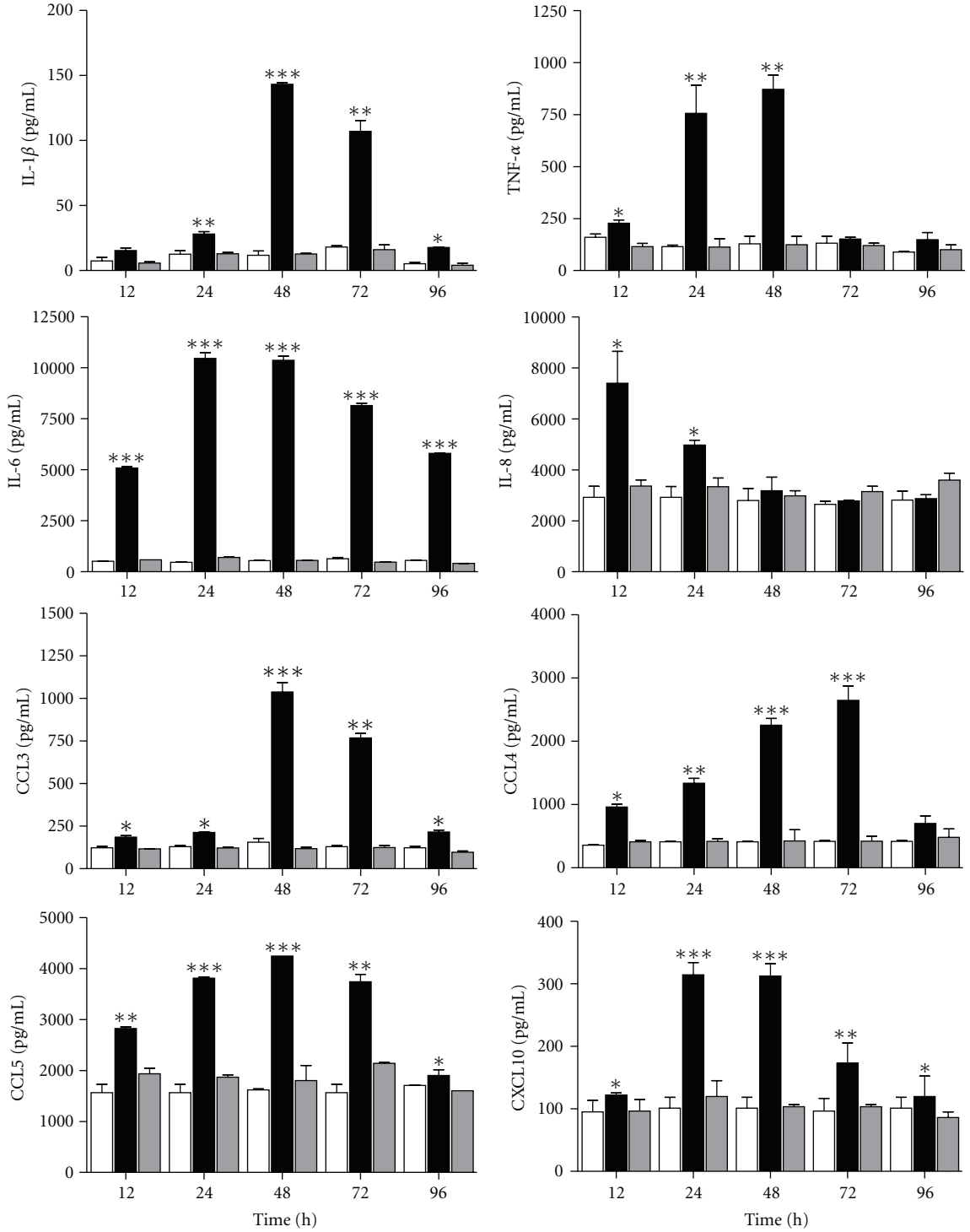

Figure 3: Profile of cytokines and chemokines induced by rMBP::SSP4 in PBMC. PBMC was stimulated with the protein for 12–96 h, and cytokines and chemokines were measured in cells' culture supernatants by ELISA. Unstimulated cells (open bars), cells stimulated with rMBP::SSP4 (full bars), and cells stimulated with MBP (gray bars). Histograms show values in pg/mL (means ± SD) of three experiments run in duplicate. *,**,*** $P < 0.05$, 0.001, and 0.0001, respectively, versus unstimulated cells.

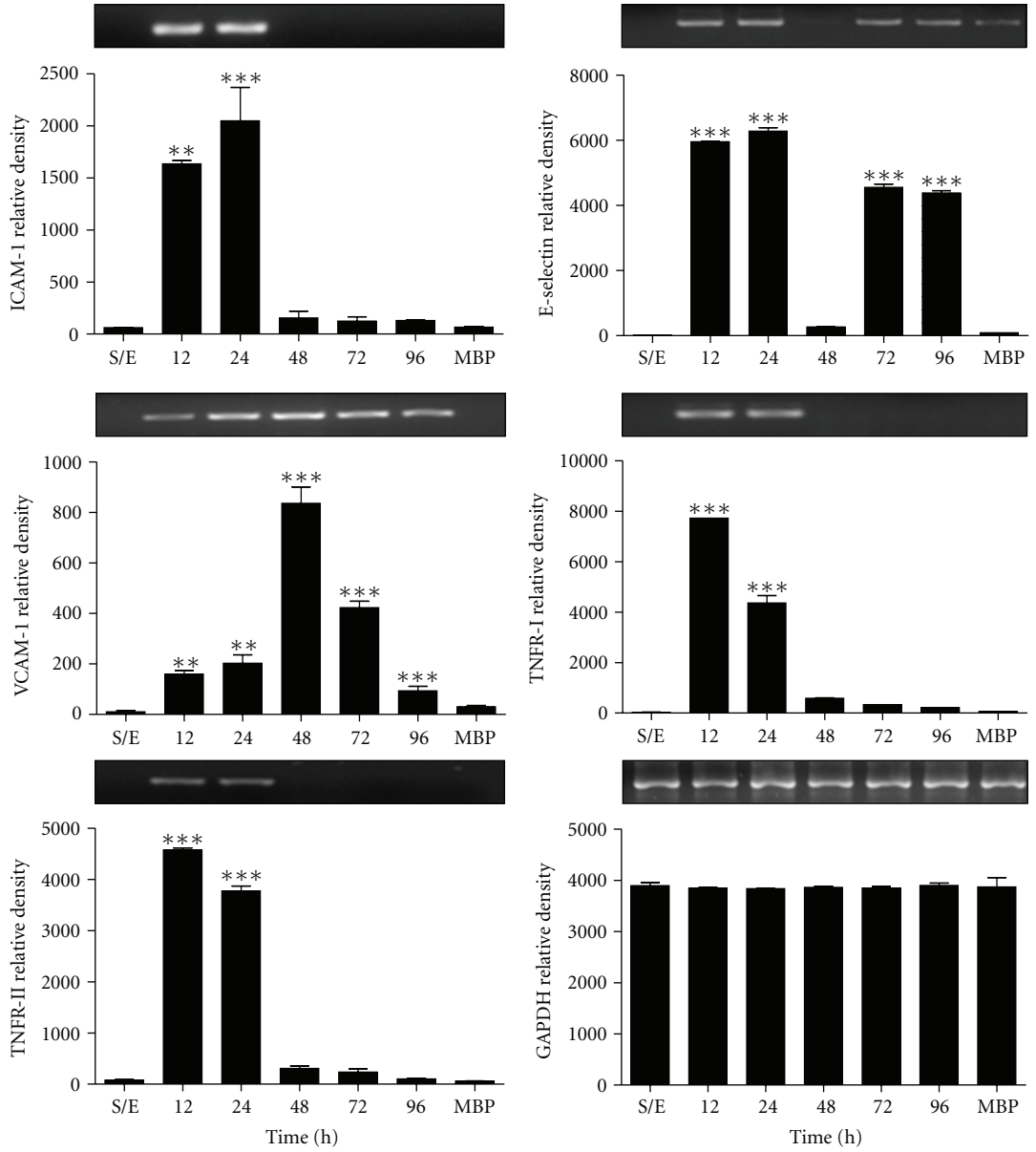

Figure 4: Effect of rMBP::SSP4 on gene expression of CAMs and TNFRs in PBMCs. RT-PCR analysis of CAMs and TNFRs mRNAs in PBMCs was performed as described (see Section 2). PBMCs were stimulated with the protein for 12–96 h, S/E (nonstimulated). The intensities of each band were quantified and plotted from the gels that are on top of each graph corresponding to the expression of genes. GAPDH was used as a control housekeeping gene. $^{**,***}P < 0.001$ and 0.0001, respectively, versus unstimulated cells.

promptly released and further activate other inflammatory cells [31]. This pattern of expression has been observed in the inflammatory responses in cardiomyocytes during *T. cruzi* infection. It was shown that heart tissues collected from *T. cruzi*-infected rats expressed IL-6, IL-1β, TNF-α, and *i*NOS, moreover, hearts of mice infected and cardiomyocytes express the same pattern of cytokines and chemokines [4, 32–34].

SSP4 superficial protein is expressed shortly after trypomastigotes begin to transform into amastigotes, in a phase which is released the amastigote-specific SSP4 protein [12], this protein can interact with and activate PBMC, secreting cytokines, chemokines, NO, and other molecules which might attract leukocytes to the inflammatory site after interaction with specific molecules of the parasite, and that rMBP::SSP4 can significantly increase this effect. Since it has been observed that PBMC recruitment is a rapid and remarkable phenomenon during acute infection with the intracellular protozoan parasite *T. cruzi*, the causative agent of Chagas' disease, the functional capabilities of these cells during the infection, however, are poorly understood [35]. The ability of monocyte-derived macrophages to process and present antigens, produce cytokines, and provide costimulatory signals demonstrate their pivotal role in initiating

immune responses [36]. Activated macrophages exert critical activities in immunity to parasites, playing a key role in the mechanism for halting the acute *T. cruzi* infection. Activation of macrophages by parasite antigens results in proinflammatory cytokine production and consequent control of parasitemia and mortality [24]. On the other hand, it has been observed that this protein induces high production of IL-6 [13, 15], according to our results and because of the pleiotropic character of IL-6 has made it difficult to obtain a clear answer for the role of this cytokine in this model; however, the production of IL-6 observed in PBMCs could possibly modulate the differentiation of T cells infiltrated through the process of chemotaxis toward a Th2 pattern [37] and may later be involved in the maturation process of B cell [13], during polyclonal activation observed in the acute phase of infection [38]. This inflammatory T cell and antibody response leads to control—but not complete elimination—of tissue and blood parasitism.

We showed that the expression of adhesion molecules and TNF receptors are upregulated in PBMC by the stimulation *in vitro* with rMBP::SSP4 protein (Figure 4). Accumulation of leukocytes at the site of local injury or infection of endothelial cells is dependent on the interaction of circulating leukocytes with vascular adhesion molecules, such as E-selectin, VCAM-1, and ICAM-1 [39]. Likewise, it is known that TNF receptors play a role in inflammatory process; TNFR-I may have anti-inflammatory and inflammatory effects, depending on the signaling pathway that is activated. TNFR-II is associated mainly with inflammatory and antiapoptotic processes [40]. Expression of TNFR-II observed in PBMC suggested in the context of infection that parasite probably ensures the survival of the cell to perpetuate the process of infection and their tissue retention, possibly promoted by the action of IL-8 [41].

The soluble parasite factors can elicit a complex series of cellular interactions leading to an immunosuppression state, in addition, may have additional roles in driving early immunological events toward Th2-type or anti-inflammatory responses fully polarized. These raise the distinct possibility that the production of parasite factors that interact with cell surface receptors may be one mechanism, whereby the parasite is able to interfere with the regulation of the induction/initiation phase of the host immune response that may protect the host from excessive inflammation and may potentiate the parasite's own survival [8].

Finally, inflammatory response that follows the infection with *T. cruzi* is essential for host resistance to infection but is also responsible for the diverse pathology observed in Chagas disease [4]. Parasite persistence depends on a combination of factors, including release of molecules that interfere with the immune response. Therefore, suppression induced by parasite molecules is more relevant at the acute phase, when the concentration of such molecules can be fairly high. Although the amastigote stage is considered essentially as the stage of intracellular replication, this form of the parasite is present in the circulation during the acute phase of infection in mice and can enter and develop in both mammalian phagocytic cells (*in vitro*) and nonmammalian phagocytic cells [11].

In conclusion, all these results suggest that the amastigote SSP4 molecule could play a key role in the inflammatory process, modulating the expression and production of inflammatory molecules, which may represent a mechanism participating in the immunoregulatory and/or immunosuppressive processes carried out by *T. cruzi* during the development of the acute phase of Chagas' disease.

Acknowledgments

They would like to thank QFB. The authors as well as L. M. Álvarez Rodríguez for technical assistance. They have declared that no conflict of interests exists. Yadira Morán Utrera was recipient of a Ph.D. fellowship from CONACyT, México (215332). This work was supported by Grant SEP-CONACyT-Básica (49911-Q) to ARL.

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Hindawi Publishing Corporation
Journal of Tropical Medicine
Volume 2012, Article ID 853562, 4 pages
doi:10.1155/2012/853562

Research Article

Toxoplasma gondii Myocarditis after Adult Heart Transplantation: Successful Prophylaxis with Pyrimethamine

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Received 29 May 2012; Revised 24 September 2012; Accepted 2 October 2012

Academic Editor: Maria Aparecida Shikanai Yasuda

Toxoplasma gondii primary infection/reactivation after solid organ transplantation is a serious complication, due to the high mortality rate following disseminated disease. We performed a retrospective study of all cases of *T. gondii* infections in 436 adult patients who had received an orthotopic cardiac transplant at our Institution from May 1968 to January 2011. Six patients (1.3%) developed *T. gondii* infection/reactivation in the post-operative period. All infections/reactivations occurred before 1996, when no standardized toxoplasmosis prophylactic regimen or co-trimoxazole prophylaxis was used. Starting with the 112th heart transplant, oral pyrimethamine 75 mg/day was used for seronegative transplant recipients whose donors were seropositive or unknown. Two patients (33.3%) presented with disseminated toxoplasmosis infection, and all patients (100%) had myocarditis. Five patients (83.3%) were seronegative before transplant and one patient did not have pre-transplant serology available. Median time for infection onset was 131 days following transplantation. Three patients (50%) died due to toxoplasmosis infection. After 1996, we did not observe any additional cases of *T. gondii* infection/reactivation. In conclusion, toxoplasmosis in heart allographs was more frequent among seronegative heart recipients, and oral pyrimethamine was highly effective for the prevention of *T. gondii* infection in this population.

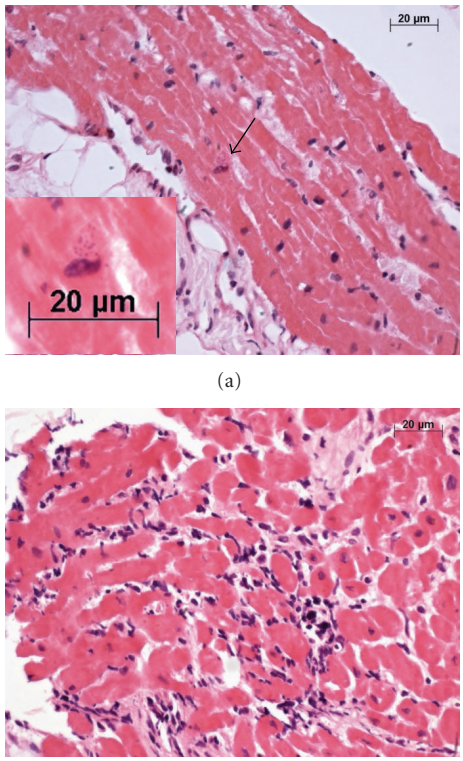
1. Background

Toxoplasmosis is a zoonotic disease of worldwide distribution, with a high incidence in certain countries. Humans are usually infected through ingestion of oocysts that are present in contaminated water or soil, or by eating undercooked meat with tissue cysts. Although the typical course of acute infection is a benign febrile syndrome in healthy patients, severe disease occurs in hosts with immune system impairment, including transplant and AIDS patients. Transplant recipients who are seronegative for *Toxoplasma gondii* are at particular risk of developing a severe toxoplasmosis infection upon receipt of an organ from a seropositive donor [1].

In this study, we studied six cases of *T. gondii* myocarditis following heart transplantation before routine use of pyrimethamine prophylaxis in our institution.

2. Patients and Methods

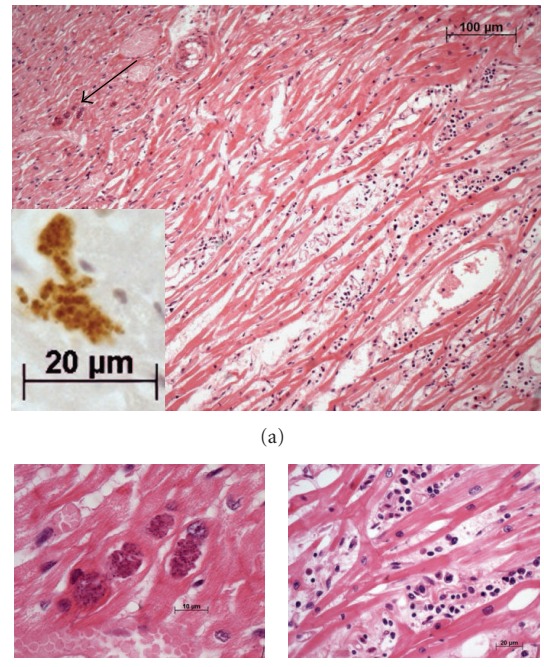
We performed a retrospective study of all cases of *T. gondii* infections in adult patients who had received an orthotopic cardiac transplant at our institution from May 1968 to January 2011. The Heart Institute (Incor-HCFMUSP) is a tertiary cardiology care hospital in São Paulo, Brazil, with a heart transplant program that was founded in 1964. Until 1996, there was no standardized toxoplasmosis prophylactic regimen. Starting with the 112th heart transplant, oral pyrimethamine 75 mg/day was used for seronegative transplant recipients whose donors were seropositive or unknown. This regimen was given from the first day after transplant and was maintained until the 100th day after transplant. *Pneumocystis jiroveci* pneumonia prophylaxis with cotrimoxazole was not used following heart transplant.



(a)

(b)

Figure 1: Photomicrographs of the endomyocardial biopsy. (a) In this region of the myocardium, a small *Toxoplasma* pseudocyst was noted in one cardiomyocyte (arrow). The inset shows amplification of this area, as indicated by the arrow. The presence of round corpuscles consistent with *T. gondii* tachyzoites near the cardiomyocyte nucleus was noted. (b) In another region of the myocardium within the same endomyocardial biopsy sample, there were moderate amounts of mononuclear infiltrates with aggression and destruction of the cardiomyocyte. This led to the misdiagnosis of rejection, even though it was subsequently determined that this case of myocarditis was caused by *T. gondii*. ((a) and (b) Hematoxylin and eosin stain with objective ×40 in all images and digital zoom at the inset).



(a)

(b) (c)

Figure 2: Photomicrographs of the myocardium in the necropsy. (a) In panoramic view, moderate to intense myocardial inflammation with edema and mononuclear cells is visible. Large *Toxoplasma* pseudocysts, indicated by the arrow, existed in a region without inflammatory infiltrate. This region is amplified in image (b). The inset shows amplification of the pseudocyst, with antibody specific for *T. gondii* highlighting the round corpuscles, which is consistent with the tachyzoites form of *T. gondii*. (b) The tachyzoites are still closed and hidden intracellularly. (c) A high magnification of the myocardium in an area of myocarditis. The infiltrate was composed of lymphocytes, plasma cells and histiocytes ((a), (b) and (c) Hematoxylin and eosin stain with objective ×10, ×100 oil and ×40, respectively; objective ×40 with digital zoom at the inset).

We diagnosed toxoplasmosis by analyzing the histology of endomyocardial biopsies and/or necropsies, in which *Toxoplasma* infection was determined by morphology using hematoxylin and eosin, and immunohistochemistry using commercially available polyclonal rabbit antibody against *T. gondii* (produced by Chemicon International, Inc., Temecula, CA, USA). Patient medical charts from all histologically confirmed *T. gondii* infections were reviewed.

3. Results

During the study period, 436 adult heart transplants were performed, and six patients (1.3%) developed *T. gondii* infection/reactivation in the postoperative period. All infections/reactivations occurred before 1996, and none of the patients received pyrimethamine prophylaxis. Demographic and clinical information are described in Table 1, and examples of histological examination are in Figures 1 and 2. All patients were seronegative for Chagas' disease. Two patients (33.3%) presented with disseminated toxoplasmosis infection, and all patients (100%) had myocarditis. Five patients (83.3%) were *Toxoplasma* seronegative before transplant and one patient did not have before transplant serology available. Median time for *Toxoplasma* infection onset was 131 days (mean = 69 days, range 21–534) following transplantation. No seroconversion was detected. Three patients (50%) died due to toxoplasmosis infection. After 1996, we did not observe any additional cases of *T. gondii* infection/reactivation.

4. Discussion

T. gondii primary infection/reactivation after solid organ transplantation is a serious complication, due to the high mortality rate after disseminated disease. Following transplant, the parasitic infection is clearly related to the type and duration of suppressive therapy [1]. In a multicenter,

Table 1: Clinical and laboratorial data for six cases of *Toxoplasma* infection after cardiac transplantation without toxoplasmosis prophylaxis occurring before 1996.

Patient sex/age (years)	Toxoplasmosis serostatus D/R—IgG	Time from transplant to toxoplasmosis diagnosis (days)	Source of diagnosis	Signs/symptoms	Treatment (days)	In-hospital lethal outcome*
M/52	NA/Neg	21	EMB	Heart failure	S + P	Yes
F/61	NA/Neg	534	EMB	None	S + P	No
M/44	NA/Neg	70	EMB	Heart failure	S + P	No
M/66	NA/NA	69	Necropsy (CNS and heart)**	Heart failure and low-level consciousness	—	Yes
M/49	NA/Neg	25	EMB	Heart failure	S + P	No
F/49	NA/Neg	69	Necropsy (CNS, heart and lungs)	Heart and respiratory failure and low-level consciousness	—	Yes

Note: M: male; F: female; D/R: donor/recipient; NA: not available; Neg: negative; EMB: endomyocardial biopsy; CNS: central nervous system; S + P: sulfadiazine plus pyrimethamine.

*Survival after discharge not analyzed.

**The EMB was misdiagnosed as a rejection, and the patient subsequently received methylprednisolone 1 g/d for 3 days. At necropsy, the EMB was reviewed and the diagnosis of toxoplasmosis was determined.

matched-case control study [2], the frequency of toxoplasmosis was significantly higher in heart recipients compared to the kidney and liver recipients. A negative serostatus prior to transplantation was the only independent risk factor for toxoplasmosis (odds ratio = 15.12 [95% confidence interval, 2.37–96.31]; $P = .004$). The central nervous system is mostly affected by this infection in AIDS patients [3]. Patients receiving heart transplants have a higher risk of developing *T. gondii*-induced myocarditis when compared with other solid organ transplants. Campbell et al. reviewed tissue-invasive toxoplasmosis in noncardiac solid organ transplant recipients (kidney, liver, small bowel, and pancreas) and found that in 52 cases, 44 (85%) patients had disseminated disease, while only 19% developed myocarditis [4]. Patients with toxoplasmosis after heart transplantation have myocarditis that may cause allograph dysfunction in 75%; disseminated disease occurs in nearly 45% of them [5]. Mortality due to *Toxoplasma* infection is also high. In a kidney transplant patient cohort with 31 cases of Toxoplasmosis, 20 patients (64.5%) died [6]. In our study, we observed 50% mortality among the six infected patients. Death in most reported cases has been related to a delay in diagnosis and targeted treatment [2].

Endomyocardial biopsy (EMB) is a reliable way to identify and track rejection following heart transplantation, and in some cases, EMB may show the presence of infectious organisms, including *Toxoplasma* pseudocysts. Moreover, *Toxoplasma* is one of the most commonly opportunistic organisms diagnosed on EMB [7]. *Toxoplasma*-related myocarditis has a fugacious exudative or neutrophilic component occurring when the myocyte ruptures following *Toxoplasma* proliferation. This fugacious component becomes mononuclear shortly after rupture, which results in an increasingly difficult differential diagnosis of rejection. Therefore, in some cases, the lymphoid infiltrate may not be caused by transplant rejection. In addition, the pseudocysts are often present in non-inflamed myocardial tissue, that is, when there is no myocyte rupture and the microorganism remains hidden from the immune system and may go unnoticed, even by a very experienced pathologist. This occurred in one case in our series of patients, where the review of a biopsy showed a small *Toxoplasma* pseudocyst present in a non-inflamed area of the myocardial sample. The use of *T. gondii* immunohistochemistry could help with the differential diagnosis of seronegative patients with suspected acute rejections (Figures 1 and 2). Consequently, we would like to caution the pathologist to be careful in their interpretation of tissue samples, considering the difficulty in visualizing *Toxoplasma* pseudocysts, as well as the difficulty in differentiating the diagnosis of rejection when inflammation is induced. In our series, five of six *Toxoplasma* infection presented as cardiac failure without fever. In the first months following cardiac transplant, this clinical situation could be misdiagnosed as acute rejection, resulting in increased immunosuppression and inadvertent favor of *T. gondii* infection.

Although the combination of pyrimethamine and sulfadiazine is the most effective regimen in the prevention of *T. gondii* infection, monotherapy with pyrimethamine has been suggested after heart transplantation for prophylaxis [8, 9]. Other transplant programs that have been successful in preventing *Toxoplasma* infection include the use of trimethoprim/sulfamethoxazole (cotrimoxazole) during the post transplant period [10–12]. Cotrimoxazole is systematically used following kidney or liver transplants to prevent opportunistic infections, such as pneumocystosis, but there is no consistent evidence for its efficacy following heart transplantation. In a review of two transplant programs in

the USA, Baran et al. found no evidence of toxoplasmosis postheart transplantation and proposed that *P. jiroveci* prophylaxis with cotrimoxazole is sufficient to prevent toxoplasmosis infection/reactivation [13]. Our heart transplant program does not recommend routine prophylaxis with cotrimoxazole for pneumocystosis prevention. In areas with a high prevalence of toxoplasmosis (50%–80% in adults), such as in Brazil [14], there is a low number of seronegative patients receiving heart transplants, and the prophylaxis could be individualized. Considering the adverse events related to universal *P. jiroveci* prophylaxis with cotrimoxazole (i.e., skin rashes or nephrotoxicity) and the low risk of pneumocystosis, we adopted the use of pyrimethamine as *Toxoplasma* prophylaxis in seronegative recipients. The reactivation of *T. gondii* within seropositive recipients is rare following heart transplantation [15]. We did not observe any incidents of *T. gondii* reactivation (IgG-positive recipients) in 43 years of cardiac transplant experience.

In conclusion, toxoplasmosis in heart allographs was more frequent among seronegative heart recipients, and oral pyrimethamine was highly effective for the prevention of *T. gondii* infection in this population.

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Hindawi Publishing Corporation
Journal of Tropical Medicine
Volume 2012, Article ID 819563, 10 pages
doi:10.1155/2012/819563

Review Article

Factors Contributing to Urban Malaria Transmission in Sub-Saharan Africa: A Systematic Review

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Received 29 June 2012; Revised 17 September 2012; Accepted 17 September 2012

Academic Editor: Maria Aparecida Shikanai Yasuda

Sub-Saharan Africa suffers by far the greatest malaria burden worldwide and is currently undergoing a profound demographic change, with a growing proportion of its population moving to urban areas. Urbanisation is generally expected to reduce malaria transmission; however the disease still persists in African cities, in some cases at higher levels than in nearby rural areas. *Objective*. This paper aims to collate and analyse risk factors for urban malaria transmission throughout sub-Saharan Africa and to discuss their implications for control. *Methods*. A systematic search on malaria and urbanisation was carried out focusing on sub-Saharan Africa. Particular interest was taken in vector breeding sites in urban and periurban areas. *Results*. A variety of urban vector breeding sites were catalogued, the majority of which were artificial, including urban agriculture, tyre tracks, and ditches. Natural breeding sites varied according to location. Low socioeconomic status was a significant risk factor for malaria, often present in periurban areas. A worrying trend was seen in the adaptation of malaria vector species to the urban environment. Urban malaria is highly focused and control programs should reflect this. *Conclusion*. As urbanisation continues and vector species adapt, continued monitoring and control of urban malaria in sub-Saharan Africa is essential.

1. Background

Despite recent declines in *Plasmodium falciparum* malaria transmission, largely due to increased distribution of long-lasting insecticide-treated nets (LLINs) and a switch to artemisinin-based combination therapy (ACT) drugs, sub-Saharan Africa still suffers greatly from the disease. According to World Health Organization (WHO) estimates, in 2010, of the 655,000 deaths attributed to malaria worldwide, 91% of these were in Africa [1]. At the same time, Africa's demography is rapidly changing, with an increasing number of people moving to urban areas. In West Africa, the population growth rate for urban areas is estimated at 6.3%, which is more than double the total population growth rate [2], and it is predicted that, by 2035, the urban population of sub-Saharan Africa will outnumber the rural one [3]. As Africa becomes increasingly urbanized, factors contributing to urban malaria will become more relevant.

The general consensus is that urbanization will lead to decreased malaria transmission. One recent modelling study predicts a 53.5% reduction in malaria transmission by 2030, largely due to expected demographic changes [4]. It is thought that urbanization leads to improved infrastructure, better-quality "mosquito-proof" housing, increased access to healthcare, and a reduction in vector breeding sites. Malaria vector species are known to prefer clean water for breeding, which is difficult to come by in polluted urban areas, and the higher ratio of humans to mosquitoes is also thought to lead to a decreased human biting rate [5].

However, despite these encouraging factors, malaria transmission persists in African cities and, in some cases, at even higher levels than in surrounding areas [6]. Indeed, there are African cities experiencing entomological inoculation rates (EIRs) greater than 80 infective bites per person per year [7]. A variety of factors may contribute to this, including socioeconomic status, urban agricultural practices

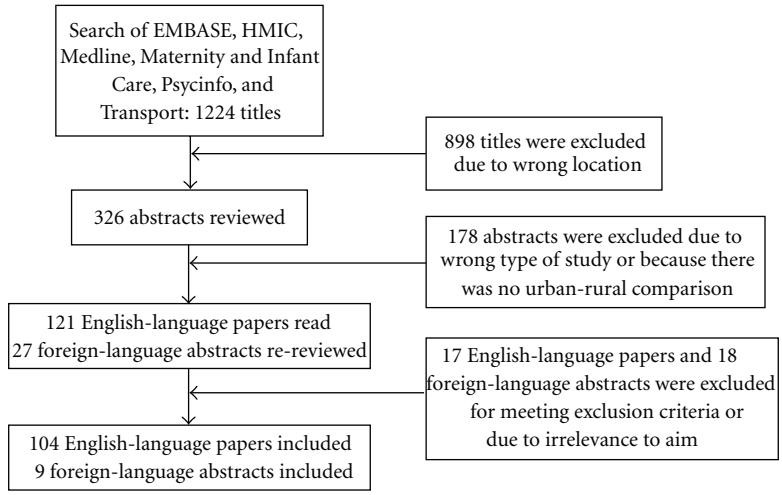

Figure 1: Flow chart of study selection process.

and poorly-monitored land use [8]. Uncontrolled urban expansion can lead to increased malaria transmission as town planners are unable to keep up with sprawling city boundaries and rural practices, which are conducive to vector breeding sites and incorporated into the urban fringes. Furthermore, areas of low socioeconomic status, often at the periphery of cities, are at particular risk. Here, poor-quality housing, unpaved roads, and reduced access to healthcare provide little protection against the disease [9].

A number of systematic reviews have investigated the impact of urbanization on malaria transmission in sub-Saharan Africa [10–12], dividing transmission into urban, periurban, and rural settings. Annual EIRs compiled across dozens of African cities show a strong tendency for transmission to increase in a gradient from urban to periurban to rural areas—in the most recent meta-analysis, the average EIRs were 18.8, 63.9, and 126.3 infective bites per year, respectively [10]. However, urban malaria transmission varies according to a number of additional factors such as location (e.g., altitude, proximity to a sea, river, or floodplain), climate, land use, human movement patterns, socioeconomic factors, local vector species, vector breeding sites, waste management, and local malaria intervention programs. This paper aims to identify the important factors in urban malaria transmission in sub-Saharan Africa, to better understand their interactions, and to discuss their relevance to policy makers in an increasingly urbanized continent.

2. Methods

2.1. Literature Search. A systematic search on the impact of urbanisation on malaria transmission in sub-Saharan Africa was carried out in April 2012 by the first named author on the following electronic databases: EMBASE, HMIC, Medline, Maternity and Infant Care, Psycinfo, and Transport through the OVIDSP gateway from 1946 to 2012. The search was performed as follows: [(urban) OR (urbanisation) OR (urbanization)] AND [(malaria) OR (*Plasmodium*) OR (*Anopheles*)].

"Africa" was originally included as a key word, but was subsequently left out as it resulted in several papers being neglected that referred to specific African countries rather than the African continent. Papers referring to Africa were therefore chosen manually.

2.2. Inclusion Criteria. The above key words yielded a total of 1,224 published articles. The authors agreed that the articles included from the search should meet the following criteria: (i) description of malaria burden/transmission/control in urban settings; (ii) study location in sub-Saharan Africa; (iii) English-language abstract. The first named author scanned all articles by title, eliminating those that did not concern sub-Saharan Africa, leaving 326 abstracts to be read. A further 178 abstracts were rejected that did not meet the inclusion criteria. Full texts of the remaining 148 articles were read, unless in a foreign language, in which case the abstracts were read again and studies were excluded if they did not meet the inclusion criteria upon further reading. Where relevance to the inclusion criteria was questioned, the second named author independently evaluated the article and consensus was quickly reached. Finally, 104 English-language articles and nine foreign-language abstracts were identified as relevant to the topic. The literature selection process is summarized in Figure 1.

3. Results of Literature Search

Relevant papers dated back to 1984. Supplementary Table 1 (see Supplementary Material available on line at doi:10.1155/2012/819563) shows the results of the search and the location, publication year, and topics addressed for each study. The authors agreed on which topics to focus on before coding them for each paper. As this paper is largely qualitative, points of interest were noted for each paper and collated for comparative analyses. The number

Table 1: Summary of results by year, location, and number of citations.

Topic	Years published	Locations	No. citations
Urban, peri-urban, rural comparisons	1986–2012	Senegal, Gabon, Kenya, Congo, Mozambique, Ethiopia, Uganda, Cameroon, Tanzania, Burkina Faso, Nigeria, Angola, Ghana, Cote d'Ivoire, Benin, Niger, Djibouti, Dakar, Sudan, DRC, Zambia, and Madagascar	48
Vector factors	1987–2012	Benin, Gabon, Kenya, Tanzania, Senegal, Sudan, Nigeria, Uganda, Ghana, Gambia, and DRC	18
River	1997–2012	Gambia, Mali, Tanzania, Sudan, Cameroon, and Niger	6
Coast	1992–2012	Cote d'Ivoire, Benin	5
Altitude	1993–2012	Tanzania, Uganda, Kenya, and Cameroon	5
Vector breeding sites (artificial & natural)	1986–2012	Mali, Mozambique, Ethiopia, Benin, Cote d'Ivoire, Senegal, Tanzania, Burkina Faso, Kenya, Ghana, Angola, Uganda, Nigeria, Sudan, Cameroon, Gambia, DRC, and Gambia	51
Socio-economic status	1990–2012	Kenya, Tanzania, Ghana, Angola, Nigeria, Malawi, Burkina Faso, and Gambia	12
Household	1993–2011	Ghana, Gabon, Burkina Faso, Tanzania, Cote d'Ivoire, and Gambia	9
Community	2010–2011	Ghana, Gabon	2
Travel	1994–2012	Gabon, Kenya, Guinea, Burkina Faso, Cote d'Ivoire, and Gambia	6
Adaptation/mutualism	2005–2011	Gabon, Kenya, Cameroon, Benin, Burkina Faso, Tanzania, Senegal, Ghana, Nigeria, and Cote d'Ivoire	13
Control	1984–2012	Kenya, Mozambique, Ghana, Tanzania, Angola, Burkina Faso, Uganda, Malawi, Gambia, DRC, and Cote d'Ivoire	22

of papers that contributed information to each topic is summarized in Table 1, along with the location and year of publication. We found that a comparable number of studies addressed the role of the vector breeding sites in urban malaria transmission ($n = 51$) as those addressing the role of geographic disparities ($n = 48$). For papers referring to vector breeding sites, we recorded the number of studies referring to specific sites and summarized these in Table 2. Many papers were synonymous in their findings, so were noted but not necessarily included in the discussion.

4. Discussion

4.1. Urban, Periurban and Rural Transmission. As mentioned in the background, dozens of African cities show a clear trend of increasing malaria transmission from urban to periurban to rural settings [10–12]. For example, in Ouagadougou, Burkina Faso, the *P. falciparum* parasite rate (PfPR) has been estimated at 24.1% in the urban center, 38.6% in its periurban surroundings, and 68.7% in neighboring rural areas [13]. This is largely due to the fact that African cities tend to grow outwards with perimeters consisting of relatively underdeveloped, poorly serviced settlements [14]. Recent migrants from rural areas tend to bring their rural practices with them, creating a multitude of vector breeding sites [15], and poor quality housing provides less protection against mosquito bites [16].

However, it should be noted that this is not a universal trend. In Libreville, Gabon, malaria transmission was found to be the highest in the urban center (EIR of 87.9 infective bites per person per year) and the lowest in the periurban surroundings (EIR of 13.3 per person per year) as a consequence of slum-like conditions in the urban center being surrounded by more affluent periurban suburbs [7]. In Cotonou, Benin, malaria prevalence was highest in an intermediate zone (PfPR among children aged 6–12 of 9.0%) between the urban center (PfPR of 2.6%) and periphery (PfPR of 2.5%). This has been explained by the abundance of urban agriculture in the intermediate zone and a salty lagoon at the periphery making it less conducive to the primary malaria vector *Anopheles gambiae* [17]. This shows that we should not confine our impression of urban malaria simply to urban centres, but we should also base it on an understanding of the underlying geography.

4.2. Malaria Vectors. Malaria in humans results from infection by any of five species of *Plasmodium* transmitted by approximately 50 species of mosquitoes, all belonging to the genus *Anopheles*. In sub-Saharan Africa, the majority of deaths are caused by *P. falciparum* and transmitted by *An. gambiae* s.s. and its close relative *Anopheles arabiensis*. These species are part of a larger species complex, *An. gambiae* s.l., of which *Anopheles melas* is also a member [18]. *An. gambiae* s.s. can further be divided into M and S molecular forms. The M form is better adapted to urban and dry environments and tends to reproduce alongside irrigated fields and permanent or semipermanent swamps. The S form is better adapted to rural and humid forest areas and prefers

Table 2: Urban vector breeding sites by number of citations.

	Vector breeding site	Number of studies
Natural	Swamps	13
	Ponds	8
	Puddles	7
	Marshes	4
	Streams	4
	Seepages	3
	Springs	1
	Lakes	1
	Tree holes	1
Total		42
Artificial	Urban agriculture	36
	Drains/gutters	9
	Ditches	8
	Tyre tracks	8
	Pipes	6
	Domestic containers	5
	Water tanks/reservoirs	5
	Construction	4
	Swimming pools	3
	Canal	3
	Foundations	2
	Septic tanks	2
	Tyres	2
	Bathtubs	1
	Dam	1
Total		95

temporary pools and brick-made ravines [7, 15, 19, 20]. *An. melas* contributes to coastal malaria and is usually found in salt water lagoons [21]. Another vector species, *Anopheles funestus*, also contributes to malaria transmission on the continent and thrives in dry and periurban environments [22]; *Anopheles moucheti,* a rare vector species, breeds in slow-moving rivers [23]. In a recent study in urban Libreville, Gabon [7], *An. gambiae* s.s. S form accounted for 99.5% of all vectors collected, while the M form accounted for 0.2% and *An. melas* accounted for 0.3%. Interestingly, all collected species and sub-species of the *An. gambiae* s.l. complex were positive for malaria sporozoites.

Urban environments are less favourable for vector species, particularly *An. gambiae*, which has a strong preference for unpolluted waters [5]. The lifespan of *An. gambiae* in urban areas was measured to be less than half its lifespan in rural areas (4.1 versus 11 days) in a study in Kinshasa, Democratic Republic of the Congo [24]. Mosquito dispersal is also much more limited in urban areas due to the higher housing density [25], causing urban malaria transmission to be highly focal [18].

4.3. Natural Vector Breeding Sites and Environmental Factors. The heavy burden of malaria in rural Africa is testimony to the ability of natural breeding sites to sustain vector populations. Natural breeding sites, although less common in urban areas, are nevertheless present. Field studies suggest that anopheline larvae are most likely to be found in permanent, shallow, sunlit pools of water of perimeter greater than 10 m [26–28]. Temporary pools are less favoured because they may not provide sufficient time for eggs to develop and emerge as adults. It has also been suggested that they are more likely to be disturbed by human activity [26–28]. A high groundwater table is particularly conducive to breeding sites as the absence of surface runoff allows pools of stagnant water to develop [29]. Of the natural vector breeding sites referred to in the literature search, the most common were ponds ($n = 8$) and swamps ($n = 13$). Also mentioned were seepages, springs, and streams and, in one study, *An. gambiae* were discovered in over 100 trees, suggesting tree holes as a favoured ovipositing site [30].

4.3.1. Coastal Environments. Malaria in coastal African cities has been partially attributed to the colonization of shallow salt waters by *An. Melas*—a less efficient, salt-water-breeding vector species [17, 31]. Clay soils of lagoons have also been noted for collecting stagnant water, providing excellent aquatic conditions for vectors species, with studies in Cote d'Ivoire and Tanzania documenting strong correlations between the presence of clay soil and anopheline mosquitoes [26, 29].

4.3.2. Rivers and Floodplains. Rivers and their floodplains provide great breeding grounds for mosquitoes in riverside urban communities, as demonstrated by the strong association between malaria risk and proximity to a floodplain. Large fields with loamy/clay soils tend to collect stagnant water from rivers and provide optimal conditions for anopheline breeding [32]. In Adama, Ethiopia, for example, households within 250 m of a floodplain have been shown to have a 22 times higher risk of contracting malaria than households further than 950 m away [33]. Sometimes it is the human activity associated with a setting that creates fertile conditions for vector breeding. For example, farms around the confluences of the Blue and White Nile in Khartoum, Sudan, are foci of malaria transmission, as are irrigated rice fields in Dioro, Mali, alongside the Niger River [34].

4.3.3. Altitude. Altitude is commonly thought to play an important role in limiting malaria in the tropical highlands by negatively influencing the development of vector species. In a study of malaria prevalence in south-western Uganda, altitudes higher than 1,500 m were shown to be associated with low malaria risk [35]; however, the presence of vector species at these altitudes cannot be ruled out since a study in the Kenyan highlands revealed high densities of *An. gambiae* mosquitoes in a town 1,650 m above sea level and still more at altitudes higher than 2,000 m [30].

4.4. Artificial Vector Breeding Sites. It is widely regarded that artificial rather than natural vector breeding sites provide the most abundant sources of mosquito larvae in African urban centres [32, 36, 37]. This is reflected in Table 2, which

shows that artificial vector breeding sites were referred to almost three times more than natural sites in this systematic review. Citation numbers are not conclusive evidence for such a comparison; however analysis of the papers from which these numbers were drawn (Supplementary Table 1) does not suggest any obvious bias. Urban agriculture was the most cited breeding site in the literature search ($n = 36$), followed by drains/gutters ($n = 9$), ditches ($n = 8$), tyre tracks ($n = 8$), and water pipes ($n = 6$). Also mentioned were water tanks, construction sites, and swimming pools. Some of these sites, such as tyre tracks and swimming pools, were found to contain all life stages of *An. gambiae*, suggesting that they were particularly productive habitats [26, 38] and were found mainly in poorly-drained, periurban areas [39].

4.4.1. Urban Agriculture. Over the last decade, urban agriculture has become commonplace in sub-Saharan Africa, expanding into the peripheral belts and centres of many towns and cities [15]. Its benefit is that it increases food security while combating malnutrition and poverty; however, it also provides optimal conditions for vector breeding, leading to a higher risk of malaria transmission in its vicinity [36, 40]. Agricultural trenches create ideal breeding sites due to the formation of shallow water between seed beds and, in one study in Abidjan, Cote d'Ivoire, anopheline larvae were present in over half [26]. In another study in Cote d'Ivoire, rice fields were found to have the highest likelihood of anopheline presence throughout both wet and dry seasons [6]. Other breeding sites include irrigation wells, noncemented wells, ditches for furrow systems, and human footprints [29, 41–43]. Larger breeding sites are more productive as they are less likely to be disturbed by irrigation.

Higher mosquito densities naturally lead to elevated levels of malaria transmission for people who either work on or live near urban agricultural fields [15, 40, 44]. For example, in a study in Maputo City, Mozambique, malaria parasitaemia was found to be higher among those who worked in urban agricultural areas throughout the city, irrespective of other factors such as urban or periurban location [45]. Urban agriculture is often associated with socioeconomic advantages, such as piped water, refuse collection, a sewage system, and better education; however, data from Accra, Ghana, suggests that the increase in vector breeding sites is sufficient to counteract these beneficial effects in terms of malaria transmission [8]. There are currently no known initiatives in place for controlling malaria associated with urban agriculture, and control here should be mindful of socioeconomic considerations.

4.4.2. Drains, Ditches, and Gutters. While agriculture provides the most productive urban vector breeding sites, drains and ditches may provide more common habitats. In a study in Dar es Salaam, Tanzania, there were three times more anopheline-positive drains and ditches compared to agricultural breeding sites, and anopheline presence was much more likely in drains that were blocked [32]. Blockages are often due to poor sanitation and lead to reduced water flow and accumulation of stagnant water pools which are ideal for mosquito breeding. Gutters provide a similar breeding site for mosquitoes in both the wet and dry seasons and were specifically noted by a recent study in Abeokuta, Nigeria [46].

4.4.3. Tyre Tracks. Tyre tracks were the second most-cited artificial vector breeding site. In Malindi, Kenya, they accounted for as much as 29% of all water bodies that were positive for mosquitoes [38]. Tyre tracks are more common in areas of high socioeconomic status, which tend to house more vehicle owners while still having roads of sufficiently poor quality to lead to the formation of potholes, tyre tracks, and other artificial breeding sites.

4.4.4. Swimming Pools. In another study in Malindi, unused swimming pools were found to provide a particularly productive habitat for *Anopheles* immature stages [47]. Of the 250 habitats identified in the study, 66 were swimming pools, and these were found to have the highest abundance of *Anopheles* mosquitoes. Hotel workers, tourists, and domestic workers may be at heightened risk of malaria transmission in areas with an abundance of unused pools.

4.4.5. Water Pipes. Water pipes can lead to breeding site formation in a variety of ways, most frequently when they are broken and pools of water collect [5]. Pipes often break as a result of poor installation or quality, clay soil expansion and contraction, construction work, and as an opportunity to procure free water for sale or consumption [48]. Water sources that are further away from pipes are more likely to be anopheline positive because water flow from nearby pipes may disturb the water surface, reducing the breeding site quality [49]. Artificial water storage containers can also serve as breeding sites, and car washing has been found to provide excellent habitats for larval development [39].

4.5. Human Factors

4.5.1. Socio-Economic Status. Higher socioeconomic status is associated with a number of factors that lead to reduced malaria transmission, from piped water and better refuse collection to better education, higher exposure to TV and radio prevention campaigns, and increased ability to afford prevention methods and treatment [50–52]. These factors contribute to a better awareness of vector breeding sites, malaria transmission, and control among people of higher socioeconomic status. The higher socioeconomic status of urban dwellers is a major factor contributing to their reduced risk of contracting malaria [53]; within cities, socioeconomic factors contribute to increased transmission in poorer areas with slum-like conditions, as seen in Libreville, Gabon [7], and in the periurban areas of many cities.

4.5.2. Household Factors. Better-quality housing decreases the risk of malaria as it minimizes entry points for mosquitoes during the night. To illustrate this, a study in Gambia showed that houses with malaria-infected children are more likely to have mud walls, open eaves, and absent

ceilings than those with uninfected children [16]. Floors comprised of earth bricks are also associated with lower malaria risk as inhabitants are more likely to sleep on raised beds to avoid ground moisture, in turn eluding bites from *An. gambiae* mosquitoes which search for blood close to the ground [16]. Interestingly, a study in Burkina Faso found that electricity use was associated with increased malaria risk, as the alternative of biomass fuel burning produces smoke that is thought to deter mosquitoes from entering houses [54]; however, electricity use in better-quality housing would presumably not show this trend.

4.5.3. Community Factors. Hygiene, sanitation, and waste collection are key determinants of malaria transmission which, while household responsibilities, have a community-level effect on disease transmission. As an example, the more the households dispose of waste properly, the lower the risk of liquid waste collecting in pools of stagnant water and forming vector breeding sites. In Accra, Ghana, being connected to a toilet was found to be even more important than waste removal in reducing community malaria mortality [55]; however, toilets are also potential areas of mosquito activity, and septic tanks within communities are a potential source of vector breeding sites [56].

4.5.4. Travel. The flipside of lower malaria prevalence in urban areas is that immunity is also reduced, making urban dwellers more susceptible to the disease upon exposure. Reduced immunity in urban populations means that, when urban residents travel to rural areas, they are at risk of contracting serious cases of malaria [57]. Due to their reduced immunity, city dwellers are more likely to contract malaria both when they travel to rural areas and when malaria-infected individuals travel to the city. This is supported by studies of urban populations in Burkina Faso, Cote d'Ivoire, and Zambia, all of which reveal a strong association between malaria infection and a recent trip to a rural area [58–60]. Furthermore, in West African cities, heightened EIRs have been observed in October, which is a time when urban dwellers return from their summer vacations in rural areas and rural youths travel to cities in search of work following the rural agricultural season [34].

4.6. Vector Factors (Adaptation and Mutualism). *An. gambiae* is demonstrating a worrying trend of adaptation to polluted waters in urban environments [5]. In recent years, the species has been found breeding in highly polluted water sources in Cote d'Ivoire [6] and Cameroon [19], and in water-filled domestic containers in Accra, Ghana [61]. In Lagos, Nigeria, and Kisumu and Malindi, Kenya, *An. gambiae* s.s. larvae have been found in water sources with high concentrations of heavy metals such as iron, copper, and lead, and other contaminants such as human faeces and petrol [52, 62]. *An. arabiensis*, although tolerant of turbidity, was less tolerant of these pollutants [62], as was *An. funestus* [63], suggesting that these species are less adapted to polluted water sources than *An. gambiae* s.s. These findings suggest that the pollution associated with urbanisation will not necessarily continue to reduce vector densities in African cities, and urban vector control will become increasingly relevant in years to come. Furthermore, the widespread use of ITNs and IRS, combined with insecticide usage in agriculture, is posing a strong selective pressure on vector populations to develop insecticide resistance, suggesting that future IVM programs will need to rely on a wide range of vector control strategies [5].

The mutually beneficial relationship between *Culex quinquefasciatus*—a nonmalaria vector—and *An. gambiae* can lead to elevated malaria vector densities in urban environments [6, 32]. *C. quinquefasciatus* breeds very efficiently in artificial sites like drainage facilities and, once inhabiting these sites, creates an environment in which *An. gambiae* can also breed. How this happens is yet to be explored. In a study in Abeokuta, Nigeria, *An. gambiae* were discovered in gutters blocked by refuse and sewage, but only after they had already been inhabited by *Culex* species [46].

4.7. Implications for Control. The current approach of the WHO to control malaria in sub-Saharan Africa is a combination of vector control, in the form of LLINs and indoor residual spraying with insecticides (IRS), and the distribution of ACT drugs for treatment [64]. Insecticide-treated nets (ITNs) have been shown to be highly efficient at reducing malaria on a community level in urban Ghana [65]; other interventions, such as larviciding and removal of vector breeding sites, are appropriate in both urban and periurban settings. Improved housing, for instance, by using corrugated iron instead of thatched roofing [66], reduces entry points for mosquitoes and is appropriate in less affluent urban settings.

Malaria transmission in urban and periurban areas is highly focused around vector breeding sites, which tend to be more numerous in areas of lower socioeconomic status. Control strategies should therefore adopt an element of spatial targeting rather than targeting a wide urban area uniformly. Vector breeding sites are common in areas with slum-like conditions [7] and in areas where urban agriculture is practiced [36, 40]. Here, emphasis should be placed on both removal of breeding sites and protective measures for the local population. One area where control could be improved is urban agriculture, as there are currently no known initiatives in place that deal with urban agriculture-associated malaria specifically. That said, we must be careful not to hinder the socioeconomic benefits of urban agriculture, such as better education and piped water. Provision of toilets may help to remove some breeding sites [55]. Communities of low socioeconomic status are less likely to be able to afford protective measures such as LLINs and IRS and treatments such as ACTs, so distribution programs and education campaigns should be targeted at these communities [35, 67]. Control strategies should also target urban environments conducive to natural breeding sites, such as coastal lagoons, rivers, and floodplains [26, 29, 32–34].

Sites known to be conducive to vector breeding—such as agricultural fields, tyre tracks, ditches, swimming pools, and construction sites—should be targeted for control.

Larviciding should be prioritised since larvae contained within aquatic sites are easier to control than free-flying adults [6], and its annual cost per individual is less than two-thirds that of ITNs [68]. In Dar es Salaam, Tanzania, larviciding has contributed to reported reductions in malaria transmission of up to 87% [68]. According to the WHO Global Malaria Programme, larviciding should be included as an additional measure to IRS and LLINs, especially in urban areas, where it is cheaper and easier to larvicide the limited urban breeding sites than to distribute nets and apply insecticide to numerous households [69]. Chemical treatment of swimming pools [70] and unclogging and treating stagnant drains will reduce larval densities and decrease larviciding costs even further [32]. Integrated vector management (IVM) provides the WHO's decision-making framework for vector control, and its emphasis on local evidence and participation makes it an ideal framework for effectively utilising a community's resources to control the evolving phenomena of urban malaria [71].

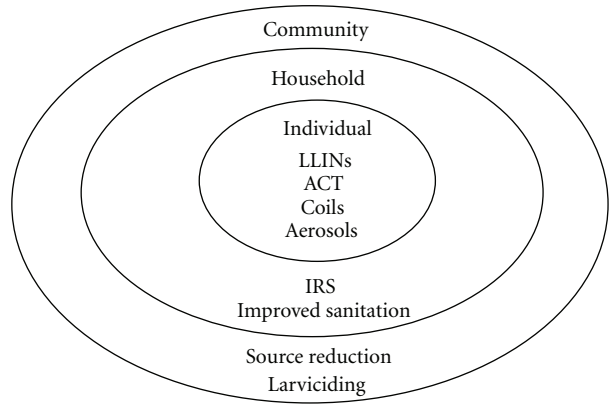

Figure 2: Interventions against urban malaria at the community, household, and individual level.

5. Conclusion

The studies selected for this paper provide a well-rounded picture of the range of factors that contribute to malaria transmission in urban sub-Saharan Africa. Clearly, there is great variation from city to city and from town to town depending on a myriad of environmental, ecological, and socioeconomic factors. However, from a holistic analysis, it is clear that there are patterns of malaria transmission, an understanding of which will help to inform the development of future urban malaria control programs.

In terms of priorities, urban malaria is most efficiently controlled through highly focused, community-level interventions. The emphasis here should be on eliminating vector breeding sites through larviciding and other measures. While LLINs and IRS are the gold standard for vector control in rural areas, there is much greater potential to identify and eliminate breeding sites in urban settings. Attention should be paid to both natural and artificial breeding sites, as summarized here. That said, individual and household-level interventions—for example, LLINs, ACTs, improved sanitation, and IRS—should continue to be strongly encouraged (Figure 2).

The role of monitoring and targeting should be emphasized, as urban malaria is known to be highly focused. These activities should form the basis of an effective IVM program. Predictable areas of high transmission should be closely monitored, including areas of low socioeconomic status, which are often located in periurban areas and are more likely to house vector breeding sites but less likely to have protective measures against vectors and malaria. Another area where close attention should be paid is near urban agricultural fields and environmentally susceptible sites such as coastal lagoons, rivers, and floodplains where human activity can enhance the suitability for breeding sites.

As urbanization continues and malaria vectors continue to adapt to the urban environment, the considerations in this paper will become increasingly relevant. We argue for the continued monitoring of urban malaria, to determine foci of transmission and interventions appropriate to these and other urban areas. We support the continuation of IVM programs, which should be tailored to each individual area, as a growing proportion of sub-Saharan Africa's population represents this demographic.

Acknowledgments

The authors would like to thank Dr. Seydou Doumbia, Dr. Mahamoudou Toure, and Dr. Andre Lin Ouedraogo for helpful discussions on urban malaria transmission in West Africa. J. M. Marshall was supported by a Grant from the Medical Research Council, UK.

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Hindawi Publishing Corporation
Journal of Tropical Medicine
Volume 2012, Article ID 873852, 8 pages
doi:10.1155/2012/873852

Research Article

Transmission Attributes of Periurban Malaria in Lusaka, Zambia, Precedent to the Integrated Vector Management Strategy: An Entomological Input

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Received 30 March 2012; Revised 31 May 2012; Accepted 13 August 2012

Academic Editor: Maria Aparecida Shikanai Yasuda

Globalization and urbanization with their inherent developmental activities and ecological transformations impact on malaria epidemiology. Entomological factors involved in malaria transmission in periurban Lusaka were assessed prior to vector control reintroduction. Data was collected through standard entomological and epidemiological protocols and a pretested structured questionnaire. Larval habitats were characterized as transient (43%), semipermanent (36%), and permanent (21%). *Anopheles arabiensis* and *An. gambiae ss.* were the only vectors identified. A shift in vector population was noted, with the later outnumbering the former. *Plasmodium falciparum* monoinfection rates were 25.6% (95% CI: 20.9–30.7) ($n = 297$). Parasitaemia was 31.8% (95% CI: 23.2–42.2), 25.7% (95% CI: 13.5–41.3), and 23.3% (95% CI: 17.4–29.6) in under 5, 5 to 14, and above 15 age groups, respectively. Low knowledge levels on vector control tools with an average of 7 residents per household were also observed. This study confirmed a local malaria transmission paradigm. The epidemiology necessitated deployment of an integrated vector management strategy with intensified information education and communication.

1. Introduction

Malaria remains a serious global health problem, killing more than one million people per year. The global community has recently had many successes in malaria control. The number of malaria cases has fallen by more than 50% in 43 countries over the past decade [1]. A modeling analysis of malaria prevention activities in 34 African countries suggested that about 730,000 lives were saved between 2000 and 2010, with nearly three quarters of those since 2006 [2]. Funding commitments for malaria have increased nearly 15-fold, from approximately US$ 100 million in 2003 to nearly US$ 1.6 billion in 2010; interest and commitment at global and country levels are very high [3]. However, the problem of malaria parasite transmission remains enormously grave in sub-Saharan Africa where at least 85 to 90% of deaths are attributable to the disease [4–7].

Malaria transmission is driven by a complex interaction of the vector, parasite, human host, and the environment, and is governed by different ecological and social determinants [8, 9]. Globalization and urbanization with their inherent developmental activities and associated ecological transformations have a significant impact on malaria epidemiology [10, 11] and have invariably exacerbated the situation. Malaria transmission depends markedly on local environmental conditions and other compounding factors, that is, presence of drug-resistant parasites and insecticide resistant vectors [12, 13], environmental changes [14], economically driven human population increase and migration [15], poverty levels, climatic changes, natural disasters and political upheavals [16], adaptability of malaria vectors to changing environments [17, 18] and limited investment in research, drug discovery, and optimisation of malaria vector control programmes.

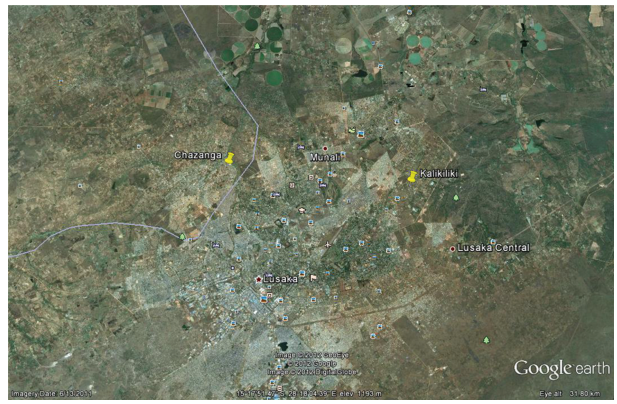

Figure 1: Map of greater Lusaka showing the periurban study site locations.

Transmission patterns and severity of malaria are influenced by the geographic attributes and the socioeconomic environment that vary significantly by city, season, and age group [19]. Accordingly, entomological profiles and clinical patterns are known to vary between urban, periurban, and rural environments [20]. Well-developed urban areas are mostly fringed by underdeveloped and inadequately serviced periurban areas experiencing the highest population growth rates [21] and often lacking infrastructure.

Malaria transmission in peri-urban areas is mostly ascribed to increased vector breeding created by the agricultural and construction activities, lack of drainage of surface water [18, 22, 23], human vector contact due to poor housing and overcrowding [11], and low immunity in children under five and pregnant women, thus increasing the risk of severe disease [19]. In Zambia, between 1950 and early 1980s, vector control reduced malaria cases to a notifiable disease in most urban areas [24]. Ngandu and coworkers reported the resurgence of malaria cases in urban and peri-urban Lusaka [25]. *In vivo* sensitivity tests were also conducted with *Plasmodium falciparum* patients in Lusaka [26], but whether these infections were acquired in urban Lusaka itself or in rural areas was not clear.

Owing to malaria cases resurgence and paucity of entomological data, specific local investigations to appraise and confirm malaria transmission in peri-urban Lusaka were required before approaches to malaria vector control could be considered. We report here on malaria vectors, parasite prevalence rates in febrile patients and knowledge and attitudes of the community pertaining to malaria, precedent to the implementation of the integrated vector management (IVM) strategy.

2. Materials and Methods

2.1. Study Site. Zambia is a landlocked country in southern Africa with an estimated population of 13 million people, 45% are children below 15 years of age [27]. Malaria is endemic across the entire country with transmission peaks coinciding with the rainy season from November to April. This study was conducted in two spatially segregated and randomly selected peri-urban locations of Lusaka district; Chazanga and Kalikiliki (Figure 1) during the cold-dry season from May to July 2003. The two sites have similar ecological characteristics and stretch out in an epidemiological zone characterized by low malaria transmission.

2.2. Mosquito Collections and Laboratory Processing. Mosquito larvae were collected from breeding sites using WHO-standard 250 mL dippers [28], transported to the insectary at the National Malaria Control Centre in Lusaka, and reared to adults while being fed on 1 part yeast and 2 parts dog biscuit. Adults were maintained on 10% sugar solution at 25 ± 2° centigrade temperature and 70–80% relative humidity.

Mosquito breeding sites were characterized into three different categories: transient, semipermanent, and permanent. Transient breeding site refers to temporal water collections, semi-permanent ones are those that would persist for a considerable period of time. Permanent breeding site refers to water bodies available throughout the year.

Adult mosquitoes were collected by the pyrethrum spray catch (PSC) between 06:00 hrs and 08:00 hrs in randomly selected households [28]. *Anopheles* mosquitoes were identified morphologically using standard keys for anophelines of southern Africa [29, 30] and to species by the polymerase chain reaction (PCR) molecular method of Scott et al. [31].

2.3. Parasitemia in Febrile Patients. Plasmodium falciparum infection was determined among febrile patients at health facilities in the study sites. Blood from randomly selected subjects who presented to the health center with febrile symptoms and consenting to participate was screened for parasite species and gametocytes by microscopy using 4% Giemsa thick and thin blood smears for 30 minutes [32]. The age range of subjects was stratified into three age categories: 6 months <5, 5 to <15, and 15 years and over. Participants with positive slide tests were offered free treatment with artemisinin-based combination therapy (ACT) according to Zambia national malaria control programme treatment policy guidelines [33].

Table 1: Entomological survey data.

Breeding sites, larval densities and ratios				
Habitat type	Transient	Semipermanent	Permanent	Total
Abundance	6 (43%)	5 (36%)	3 (21%)	14
Larvae collected	1213 (66%)	508 (28%)	119 (6%)	1840
Anophelenes	212 (17.5%)	161 (31.7%)	30 (25%)	403
Culicines	1001 (82.5%)	347 (68.3%)	89 (75%)	1437
An-Cul ratio	0.21	0.46	0.34	
Larvae/250 mL	70	45	20	
Vector molecular identification				
	Kalikiliki		Chazanga	
An. gambiae ss.	11 (58%)		7 (37%)	
An. arabiensis	0		1 (5%)	

2.4. Knowledge and Attitudes. A pretested structured questionnaire was administered to 150 randomly selected respondents, tested for malaria, to determine community knowledge and attitudes as regards malaria, family demographic data, and possibility of malaria importation from rural areas.

2.5. Data Management and Statistical Analysis. Randomization was calculated for both study sites. Data was collected and entered in Excel spread sheets (Microsoft Corporation) and statistically analyzed by employing Epi Info version 3.2.2. The Chi-square (χ^2) test was used to determine the differences in parasite prevalence between age categories.

2.6. Ethical Consideration. Ethical approval for the research was granted by the University of Zambia Research Ethics Committee (Assurance number. FWA00000338 IRB00001131 of IOR G0000774). A freely administered informed consent was given to respondents and householders for participation in the study.

3. Results

3.1. Mosquito Collections. Of 1840 larvae collected, 66% (95% CI: 65.7–68.1) were from transient (gardens and abandoned building foundations), 28% (95% CI: 25.6–29.6) semipermanent (abandoned shallow wells and ditches that followed in the wake excavations for building sand or quarrying) and 6% (95% CI: 5.4–7.7) permanent water bodies (perennial streams and dams) (Figure 2). Anophelines accounted for only 21.9% (95% CI: 20.1–23.9). The density of *Anopheles* larvae was comparatively higher in semipermanent (31.7%) followed by the permanent (25%) and transient habitats (17.5%) (Figure 2 and Table 1).

Anophelines constituted 12.83% (95% CI: 8.7–17.9) of the 203 adult mosquitoes collected (Table 1). The mosquito male-to-female ratios and densities per room was 0.59 to 0.26 and 1.7 to 15 for *Anopheles* and *Culex,* respectively. A total of 30 *An. gambiae* ss. were subjected to molecular assays including those reared from larvae. 11 could not amplify a PCR product. All specimens from Kalikiliki ($n = 11$) and Chazanga ($n = 7$), amplified for *An. gambiae* ss. and only 1 from Chazanga amplified for *An. arabiensis* (Figures 3 and 4).

3.2. Parasitemia in Febrile Patients. A total of 297 randomly selected febrile patients were recruited into the study (Table 2). The age of the subjects ranged from 6 months to 60+ years. Seventy-six (25.6%) were positive for malaria parasites with 100% *Plasmodium falciparum* parasite monoinfection. Among the positive slides, 75 (98.7%) exhibited ring form trophozoites and only 1 (1.3%) showed gametocytaemia. The parasitemia in febrile patients per age group was 31.8% (95% CI: 23.2–42.2) for the 0–4 years group, 25.7% (95% CI: 13.5–41.3) for 5 to 15 years, and 23.3% (95% CI: 17.4–29.6) for the 15 years and over ($P > 0.05$).

3.3. Knowledge and Attitudes. Of the 150 respondents 18% (95% CI: 12.4–24.6) were male and 82% (95% CI: 75.4–87.3) were female. The mean age was 29.9 with a range of 18 to 53 years. Forty-eight percent exhibited good knowledge of malaria as a disease. Sixty-three percent were knowledgeable about malaria transmission. Seventy-nine per cent were conversant with causes, signs and symptoms. Sixty-two per cent showed awareness of what to do when they suspected malaria and only forty-six per cent were knowledgeable about vector control interventions. Family demographic data showed an average of seven residents with at least one child under five years per household. Eighty-one per cent of respondents had no history of travel outside Lusaka. There was positive association between knowledge and malaria prevalence in peri-urban Lusaka ($P < 0.05$).

4. Discussion

The malaria vectorial system in Zambia comprises of *An. gambiae* ss., *An. arabiensis,* and *An. funestus* [34, 35], with great divergence in their malaria transmission potential, spatial segregation, and temporal heterogeneity [36, 37]. The pioneering malaria control efforts in the country [38, 39] stimulated unprecedented enthuse in entomological studies [36, 40–44]. Recent studies have demonstrated the presence of *An. nili*, *An. funestus*-like, and *An. rivulorum* although

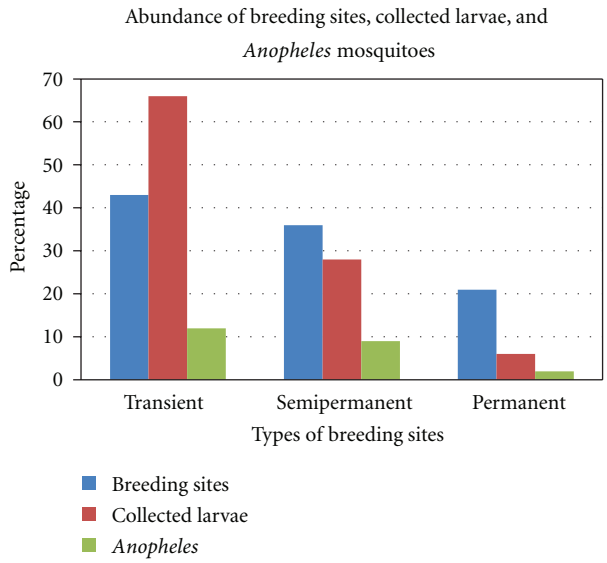

Figure 2: Abundance of breeding sites, collected larvae, and *Anopheles* mosquitoes.

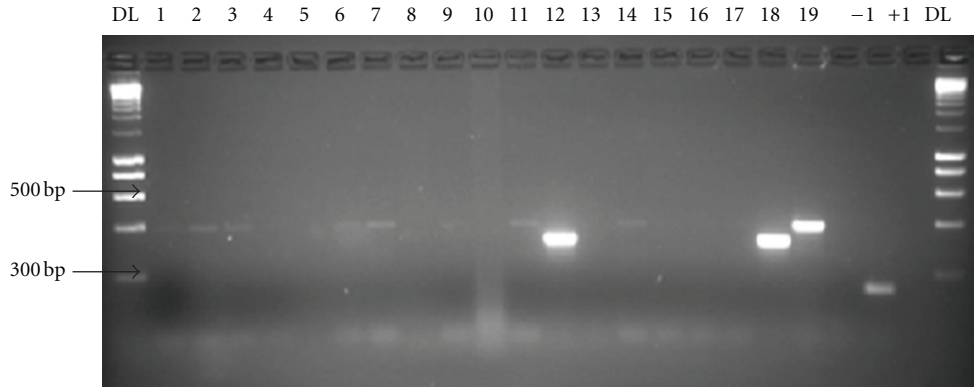

Figure 3: DNA bands produced by ribosomal DNA-polymerase chain reaction (PCR) amplification from the different species in the *Anopheles gambiae* complex from Chazanga. DL: 1-kp DNA ladder size standards, +1: positive control (*A. arabiensis*), −1: negative control. The sample DNA in each of the lanes was as follows: 1, 2, 3, 6, 7, 11, and 14 were amplified for *A. gambiae* ss. (390 bp). 12 was amplified for *A. arabiensis* (315 bp).

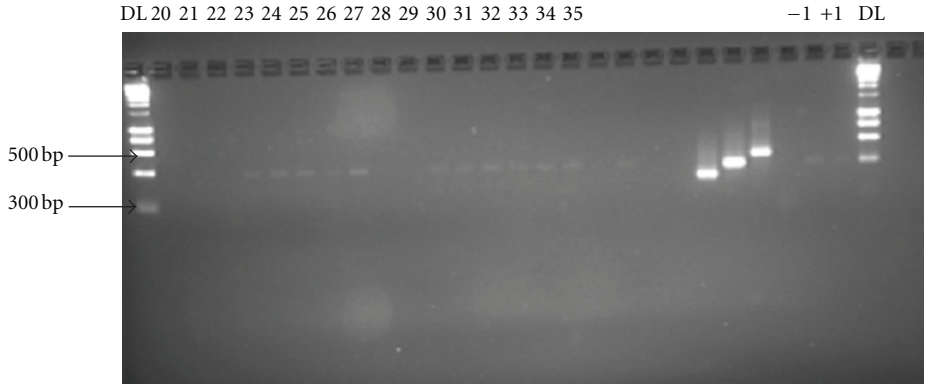

Figure 4: DNA bands produced by ribosomal DNA-polymerase chain reaction (PCR) amplification from the different species in the *Anopheles gambiae* complex from Kalikiliki. DL: 1-kbp DNA ladder size standards, +1: positive control (*A. arabiensis*), and −1: negative control. The sample DNA in each of the lanes was as follows: 23, 24, 25, 26, 27, 30, 31, 32, 33, 34, and 35 were amplified for *A. gambiae* ss. (390 bp).

Table 2: Parasitological survey data.

Parasitemia in febrile patients by age and sex				
Age group	0–4 yrs	5–15 yrs	>15 yrs	Total
Number surveyed	52*	14*	63*	129
	38†	21†	109†	168
Frequency (age)	27 (30%)	9 (25.7%)	40 (23.3%)	76
Frequency (sex)	15*	4*	26*	45
	12†	5†	14†	31
Parasite densities by age				
1–10/100 O.I	25 (62.5%)	3 (33.3%)	15 (55.6%)	43
11–100/100 O.I	9 (19.0%)	2 (22.2%)	4 (17.4%)	15
10/O.I	3 (7.5%)	1 (11.1%)	3 (7.5%)	7
>10/O.I	3 (7.5%)	1 (11.1%)	5 (18.5%)	11

O.I: oil immersion field, †female, and *male.

their role in malaria transmission in Zambia is yet to be established [45].

Urban areas are perceived not to support significant levels of malaria transmission [18]. In this study, three kinds of mosquito breeding habitats: transient, semipermanent, and permanent were characterized with appreciable spatial heterogeneity (Figure 2). *An. gambiae* is known to exploit small open temporal habitats with less predation, increased warmth, and more algae [46]. However, more *Anopheles* larvae were collected from semipermanent habitats than from permanent and transient habitats (Figure 2 and Table 1). This could explain the role of urban development related activities in supporting high malaria transmission levels as observed in peri-urban Lusaka.

While formal urban development typically reduces mosquito densities, informal urbanization has been shown to alter the vector species composition within the *An. gambiae* complex in sub-Saharan Africa, [47]. To illustrate, earlier studies conducted in Zambia indicated 100% *An. arabiensis* [35, 36]. Nevertheless, the profound demographic and extensive environmental changes that have followed in the wake of urbanization have changed the stratification of the vectors. This study demonstrates coexistence of *An. gambiae* ss. and *An. arabiensis* with the former greatly outnumbering the later in complete absence of *An. funestus*. Notably, the predominance of *An. gambiae* ss. validates the premise that informal urban development does transform vector species composition.

The presence of *An. arabiensis*, a species that is typically difficulty to control by IRS and ITNs, and the predominance of the *An. gambiae* ss. which is characteristically amenable to control by IRS and ITNs [48] could have implications for the malaria control programme. The sympatric-existence of these vectors demonstrates the need for an integrated approach for malaria vector control. This study was characterized by low number of mosquito collections due to the unfavorable prevailing environmental conditions during the cold season, lack of data on chromosomal forms of *An. gambiae* ss. and transmission determining parameters, that is, vector infectivity. However, early entomological work in Zambia reported a sporozoite rate of 1.4% in *An. arabiensis* in Lusaka [44]. Notably, there is still a clear paucity of data on malaria vector bionomics in the country.

Malaria had been known to be hyperendemic in hot riverine valleys with perennial transmission, meso-to hypo-endemic on plateaus, and hypo-endemic in urban areas of Zambia [49]. Between 1969 and 2000, parasite rates ranged from 2.0 to 26.4% across the country [39], with parasite species of 86.8% *P. falciparum* and 13.2% *P. malariae* in Ndola rural [50]. By 1999, parasite species was over 97% *P. falciparum* [49]. These findings are corroborated in this study with 25.3% parasitaemia among febrile patients with 100% *P. falciparum* monoinfections. This upsurge of frequency of febrile malaria was further aggravated by the development of chloroquine resistance [51]. Deployment of effective control tools has transformed the epidemiological profile from countrywide high endemicity to three distinct epidemiological strata: very low transmission and parasite prevalence of <1%, low transmission (<10%), and persistent high transmission (>20%).

The prevalence rate of malaria in children under five years is dependent on the intensity of transmission and declines with age as immunity develops and is thus a good indicator of a recent transmission of malaria [52]. The highest prevalence of malaria in Zambia occurs in this age group across the country [49]. In this study, frequency of febrile malaria was highest (31.8%) in the 0–4 years age group and lowest (23.3%) in the 15 years and above group. There was no significant difference in parasitaemia in febrile patients of the three age categories ($P > 0.05$) suggesting a nonimmune population and an area of low transmission. The above 10% parasitaemia observed in children under 5 years of age confirmed that malaria had again become endemic in peri-urban Lusaka [18].

The knowledge and attitudes survey indicated the need for intensified information, education and communication (IEC) on malaria and its prevention. The 46% knowledge level on vector control interventions indicated a weakness in individual efforts to prevent the disease. Population expansion and its health impact has been epitomized by sub-Saharan Africa. In many malaria endemic countries, including Zambia, the population has doubled in the past two

decades, thus greatly increasing the absolute numbers of those at risk [53]. Accordingly, the peri-urban settlements experience the highest population growth rates [11]. This was demonstrated in peri-urban Lusaka where family demographic data showed an average of seven residents with at least one child less than five years per household. Thus, suggesting that congestion in households was probably one of the factors contributing to the increased transmission of malaria in these settings.

It has equally been established that human migration contributes markedly to malaria transmission [54]. In areas of endemicity, encroaching transmission has been demonstrated in areas previously free of transmission and local transmission has been conclusively demonstrated in many African cities [55, 56]. These findings are corroborated in this study which confirmed local transmission in Lusaka as 80% subjects with definitively diagnosed malaria had no history of travel. It was established that there is no significant contribution of migration towards malaria transmission in peri-urban Lusaka ($P > 0.05$). Local transmission of malaria was further strongly inferred by high parasitaemia in children under the age of five and the presence of gametocyte bearers and efficient vectors in the community that perpetuated the transmission cycle. Congestion in households together with the appreciably low levels of knowledge on control and prevention compounded the situation.

The pragmatic data reported on here was an essential prerequisite of evidence-based and effective vector control efforts. The high malaria infection rates in peri-urban Lusaka could be ascribed to the definitively demonstrated local transmission. This necessitated the institution of appropriate control strategies based on the prevailing transmission paradigm. The presence of *An. gambiae* complex species and characterization of their breeding attributes required an integrated vector management (IVM) approach to effectively control transmission. It is noteworthy, that this preintervention study had limitations as the surveys were conducted during the dry season which markedly influenced the malaria vector and parasite populations.

Clearly, the malaria epidemiology in peri-urban Lusaka required an integrated approach involving IRS and ITNs against the adults and larval source management (LSM) against the aquatic stages. Information education and communication (IEC) to increase awareness and knowledge about malaria vector control needed to be intensified. Following this study, IVM was introduced in Lusaka with IRS and ITNs as main thrust interventions and IEC has been strengthened [57]. This has reduced malaria parasite rates to appreciably minimal levels (<1%) [58]. To clear the residual transmission, LSM is being implemented in Lusaka. While monitoring and evaluation of vector control interventions has been strengthened [45], it is imperative that a comprehensive entomological and epidemiological surveillance system is established to detect any increase in the malaria case load.

Conflict of Interests

The authors declare that they have no conflict interests.

Authors' Contribution

E. Chanda: codesigned the study, collected and analysed the data, and drafted the paper. K. S. Baboo: critically reviewed the manuscript. C. J. Shinondo codesigned the study, guided in data analysis and interpretation and contributed to the drafting of the paper and critically evaluated it. All authors read and approved the final paper.

Acknowledgments

The aurhors thank all Ministry of Health staff from the Health Centres and National Malaria Control Centre who participated in the study. They thank Dr. John Govere and the two anonymous reviewers for their useful comments on the paper. They also thank the Lusaka District Health Office for authority to work in Health facilities and the National Institute for Communicable Diseases, South Africa, for analyzing the malaria vector samples. The work was supported by the United States Agency for International Development (USAID).

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Hindawi Publishing Corporation
Journal of Tropical Medicine
Volume 2012, Article ID 540858, 6 pages
doi:10.1155/2012/540858

Research Article

Influence of Gender on Cardiac and Encephalic Inflammation in the Elderly with Cysticercosis: A Case Control Study

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Received 25 May 2012; Revised 24 July 2012; Accepted 15 August 2012

Academic Editor: Maria Aparecida Shikanai Yasuda

Background. The present study explores the influence of the host's age and gender upon the inflammatory infiltrate. We aimed to quantify the inflammatory infiltrate caused by cysticercosis, which is related to aging, in the heart and in the encephalon. *Methods*. 75 autopsy protocols with cysticercosis diagnosis from department of pathology at a university hospital from 1970 to 2008 were reviewed. Two groups were formed: elderly with cysticercosis and nonelderly with cysticercosis. We used KS-300 (Kontron-Zeiss) software for morphometric analysis of the inflammation. *Results*. The elderly had an average of 3.1 ± 2.5 cysticerci, whereas the non-elderly had 2.7 ± 3.8 parasites. The non-elderly group with cysticercosis had significantly more inflammation, both cardiac and encephalic, than the elderly group. The elderly females with cysticercosis had more cardiac and encephalic inflammation. *Conclusions*. In this study, we showed that the non-elderly had significantly more cardiac and encephalic inflammation than the elderly, and that such inflammatory infiltrate decreases with age and depends upon the evolutionary stage of the cysticercus. Furthermore, there are differences concerning gender in the intensity of the inflammatory response due to cysticerci in the heart and brain parenchyma during senescence. Even during this period, women continue to have a more intense response to the parasitosis.

1. Introduction

Understanding the changes occurring within an aging immune system is essential if public health authorities are to be equipped to deal with an aging population. Specifically, knowledge of altered immune responses to infectious agents is required if rational clinical interventions are to be tailored to these aging individuals [1].

Aging is a continuous and slow process that compromises the normal functioning of various organs and systems [2]. As the population ages, there is growing interest in understanding host-parasite interaction and eventual prevention of chronic parasitic diseases, including cysticercosis, in elderly individuals.

Cysticercosis is emerging as a serious public health problem in many poor countries in Latin America, Africa and Asia. Although theoretically easy to control, and declared eradicable, cysticercosis remains neglected in most endemic countries [3]. This parasitosis may be asymptomatic or it may cause a variety of clinical manifestations depending on the number, location, and stage of cysticercus lesions. Pleomorphic disease is a result of the presence of the parasite itself (cysticerci), of the inflammatory process that surrounds the larvae, and of residual fibrosis and calcification [4–6]. It is likely that the combination of several factors is responsible for such differences, one of which may be gender-related [7].

The relevance of gender in host susceptibility has been explored in cysticercosis infection. In experimental murine

Taenia crassiceps cysticercosis, female mice were found to be more susceptible than males in different syngeneic and congenic strains of mice [8]. The finding that gonadectomy equalized susceptibility between sexes, by reducing parasite loads in females and increasing it in males, first clearly indicated the relevance of sexual hormones [9].

The cysticercus contains a large number of antigens that can elicit a host immune inflammatory reaction. The inflammatory cellular infiltrate, if present, may be discrete with lymphocytes and eosinophils in the initial stage, or it may be a more intense lymphocyte infiltrate with giant multinucleated foamy macrophages in the necrotic stage [10].

Recent evidence suggests that immunosenescence associated to an immunological alteration caused by cysticercosis leads to a favorable condition for neoplasia development in elderly individuals attacked by the parasitosis. Moreover, it is likely that the patients continue to be infected with cysticercosis as they age [11].

The aim of this study was to quantify the inflammatory infiltrate in the heart and in the encephalon of the elderly with cysticercosis. Our hypothesis is that it is possible to quantify the infiltrated inflammatory among male and female elderly patients, due to the fact that those individuals are undergoing immunosenescence.

2. Material and Methods

2.1. Ethical Aspect. This research paper was approved by Triangulo Mineiro Federal University Research Ethics Committee under protocol no. 486. As this research regards autopsy material, the only risk was the loss of confidentiality. However, as a precautionary measure, the cases were identified by letters and numbers. Also, consent for the autopsy was given in writing by the next of kin after the death of the patient. Then the document was filed in the general hospital and the general pathology discipline records.

A retrospective transversal study of 3639 autopsies of adults collected at the General Hospital of Triangulo Mineiro Federal University, located in Uberaba, MG, Brazil, from 1970 to 2008, was carried out. Diagnosis of cysticercosis was made through histological demonstration or through direct visualization of the cysticercus, meeting the diagnostic criteria proposed by other authors [12], in 75 autopsies, 55 non-elderly, and 20 elderly patients. None of the patients included in the study were diagnosed with cysticercosis before autopsy. Information regarding age, gender, body weight, height, heart weight, brain weight, and the number, location, and evolutionary stage of the cysticercus was registered.

2.2. Material Preparation. In order to analyze the heart and brain inflammatory infiltration, we obtained 33 (13 elderly and 20 non-elderly) samples of heart and brain of patients with cysticercosis the 7 heart samples, amongst which 3 belonged to elderly patients and 4 belonged to non-elderly patients with cardiac cysticercosis, and 26 brains with neurocysticercosis, 10 of which belonged to elderly patients and 16 to non-elderly patients. The other organs were not found at the anatomical specimens' archives from the department of general pathology. The brain and heart samples affected by cysticercosis were fixed in formaldehyde 10% and subjected to routine histological processing so as to obtain 4 μm thick sections, stained with hematoxylin and eosin (HE), for general morphological analysis and for quantification of the inflammatory cell infiltrate.

2.3. Morphometry. A video camera coupled to a standard light microscope and an interactive image analysis system (KS 300 Carl Zeiss) were used. We analyzed ten fields per quadrant; that is, 40 measurements were carried out in each slide. The representative number of measurements was determined through the method of Accumulated Means [13].

2.4. Inflammatory Cell Infiltrate. The HE-stained slides were examined using a standard light microscope with a 20x objective and 800x magnification range. The digital image showed the field where the number of inflammatory cells was counted in absolute value. Quantification was carried out by the observer's identification of such cells and through the staining performed by point-counting method.

2.5. Statistical Analysis. The variables were tested in order to verify the type of distribution using the Kolmogorov-Smirnov test and variance analysis. Student's t-test (t) or Mann-Whitney (T) was used in the comparison of two groups, and ANOVA (F) or Kruskal-Wallis (H) for comparison between three or more groups, followed by Bonferroni or Dunn test when necessary. Correlations between two variables were analyzed by Pearson's or Spearman correlation coefficient (r). Differences in significance levels of less than 5% ($P < 0.05$) were considered statistically significant.

3. Results

Amongst the patients with cysticercosis, the average age of the non-elderly was 47.3 years, ranging from 23 to 58 years old, whereas the elderly had an average age of 66.7 years, ranging from 61 to 75 years old. Male and Caucasian patients predominated in both groups, and analysis of nutritional status showed that the non-elderly had an average body mass index (BMI) of $21.2 \pm 4.4\,\text{kg/m}^2$ and that the elderly with cysticercosis had an average BMI of $20.2 \pm 9.9\,\text{kg/m}^2$.

Heart weight and brain weight of the non-elderly were found to be higher than those of the elderly with cysticercosis, and both elderly and non-elderly male patients had heart weight and brain weight higher than female patients (Table 1).

It was possible to ascertain the evolutionary stage of the parasite in 8 cysticerci of elderly individuals, among whom 4 (50%) were Vesicular Stage, 2 (25%) Colloidal Vesicular Stage, 1 (12.5%) Granular Nodular Stage and (12.5%) Nodular Calcified Stage. Amongst the non-elderly, 4 (21.1%) cysticerci were in the first evolutionary stage, 5 (26.3%) Colloidal Vesicular Stage, 4 (21.1%) Granular Nodular Stage, and 6 (31.5%) Nodular Calcified Stage. The elderly had an average of 3.1 ± 2.5 cysticerci, whereas the non-elderly had 2.7 ± 3.8 parasites.

Table 1: Heart and brain weight according to gender of the elderly and non-elderly patients with cysticercosis.

Groups	Gender	Heart weight (g)	Brain weight (g)
		Mean ± SD	
Elderly		347.2 ± 121.9	1216.1 ± 158.2
Nonelderly		370.1 ± 102.8	1228.3 ± 131.3
		$t, P > 0,05$	$t, P > 0,05$
Elderly	Female	299.8 ± 65.7	1231.0 ± 204.9
	Male	360.0 ± 149.8	1261.1 ± 107.9
Non-elderly	Female	336.6 ± 107.0	1166.7 ± 84.2[1]
	Male	395.2 ± 103.6	1313,6 ± 155.3[2]
		$F, P > 0.05$	$F, P < 0.05$

SD: standard deviation. t: Student's t-test; F: Anova; 1×2 Bonferroni test, $P < 0.05$.

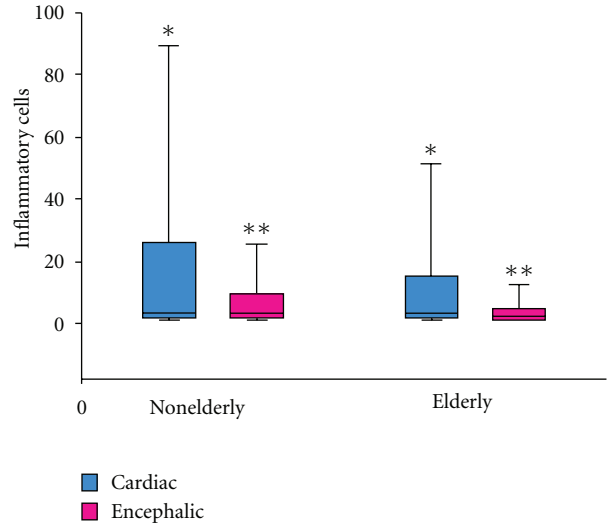


Figure 1: Comparison of the inflammatory infiltrate in heart and brain of non-elderly and elderly patients with cysticercosis.

At all stages was observed some degree of inflammatory reaction around the cysticercus, its intensity decreased with the succession of evolutionary stages. The Colloidal Vesicular Stage showed the highest inflammatory infiltrate, followed by the Granular Nodular Stage in elderly and non-elderly groups (Table 2).

Analysis of the cardiac inflammatory infiltrate indicated that the non-elderly had significantly more inflammation than the elderly patients with cardiac cysticercosis (Figures 1 and 2).

In the non-elderly group, although men had more cardiac inflammation than women, this difference was not significant. Nonetheless, elderly females had significantly more inflammation than the elderly males (Table 3).

Encephalic inflammation was more acute amongst the non-elderly when compared to the elderly with neurocysticercosis (Figure 1). In the elderly group, female patients had significantly more encephalic inflammation than male patients. When contrasting both genders of the non-elderly group, male patients had more inflammation, yet without any significant difference (Table 3).

A positive and not significant correlation between encephalic inflammation and cardiac inflammation was found ($r = 0{,}032$; $P = 0,247$), as well as a negative correlation between age and encephalic inflammation ($r = -0,518$; $P = 0,03$) or cardiac inflammation ($r = -0,385$; $P = 0,186$) in male group. Hence, it was demonstrated that the inflammation decreases with age in men. This relationship was not observed in female group.

4. Discussion

Population aging, which has increased since the last decades of the twentieth century, has changed the demographic and epidemiological profiles of countries such as Brazil. The increase in chronic degenerative diseases, which have replaced infectious and parasitic diseases, has demanded that more emphasis be placed on the prevention and treatment of such diseases, which leads to the need to know about their pathological changes during the aging process.

In the present study, heart and brain weights of non-elderly patients with cysticercosis were found to be higher than those of the elderly group with the parasitosis, without significant difference. Male patients had higher heart weight and brain weight, regardless of age. According to the literature, heart weight ranges from 347 g to 487 g in individuals over 60 years old, and brain weight ranges from 1105 g to 1264 g [14–16]. In an experimental study, not only did adult male rats have higher heart weight, but they also had larger myocardiocytes compared with female rats, which might be related to a higher risk of cardiovascular disease in males [17]. Studies involving patients without encephalopathy showed that the brain weight and the volume and density of the cell undergo a steady decrease with age, whereby male patients have higher brain weight than female patients [18].

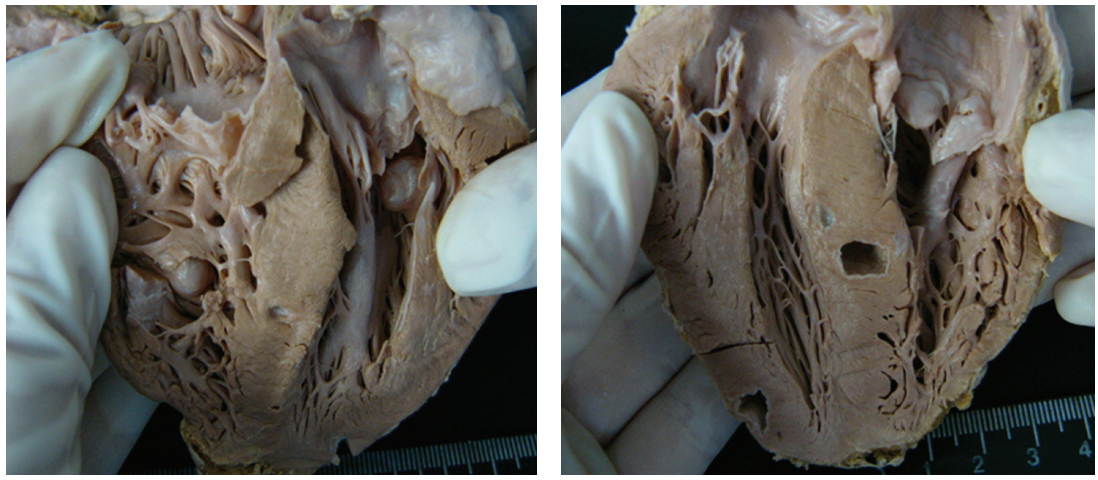
Analysis of the inflammatory infiltrate showed that the non-elderly had significantly more cardiac and encephalic inflammation than the elderly, and that such inflammatory infiltrate decreases with age and depends upon the evolutionary stage of the cysticercus. The inflammatory process caused by cysticerci in the cerebral parenchyma and in the myocardium comprises mononuclear and polymorphonuclear cells, mainly eosinophils, macrophages, and lymphocytes [19, 20]. During the aging process, changes in the expression of functionally important cell receptors, reduction in the population of polymorphonuclear cells, and reduction in the capability of producing antibodies are verified, and these factors may lead to immune dysfunction [21, 22]. Therefore, our data might be related to changes in the immune response, mainly in T cells, which were found in the elderly individuals [23].

The Vesicular Stage was more prevalent among the elderly and Nodular Calcified Stage among non-elderly, and Colloidal Vesicular Stage showed higher inflammatory infiltrate in both groups. Researches show that a more intense inflammation with lymphocyte and macrophage infiltrate can be found around the cysticercus in Colloidal Vesicular Stage [10, 24, 25]. With cysts degenerates, the inflammatory reaction tends to decrease in the Granular Nodular Stage,

Table 2: Comparison of the inflammatory infiltrate in relation the evolutionary stage of the cysticerci in brain of non-elderly and elderly patients with cysticercosis.

Groups	Vesicular stage	Colloidal vesicular stage	Granular nodular stage	Nodular calcified stage
	Median (minimum–maximum)			
Elderly	1.0 (1.0–4.0)	3.0 (1.0–12.0)	2.0 (1.0–6.0)	1.0 (1.0–3.0)
Non-elderly	1.0 (1.0–6.0)	4.0 (1.0–18.0)	2.5 (1.0–8.0)	2.0 (1.0–5.0)
	T, $P > 0.05$	T, $P < 0.05$	T, $P < 0.05$	T, $P > 0.05$

T: Mann-Whitney test.



(a) (b)

Figure 2: Cardiac cysticercosis in elderly individual. (a) Anterior view of the posterior half of organ showing two cysticerci in the endocardium. (b) Posterior view of the anterior half of organ showing cysticercus in the interventricular septum and in myocardium.

denoting continuity in the host reaction against the parasite remnants without, however, having an association with the type or intensity of the inflammatory response [24, 26].

The duration of each of the progressive stages in the natural history of cysticercosis has not been established because there are considerable differences between individuals, particularly in relation to the intensity of the endogenous immune response [27]. Whereas the parasite typically dies few years after infection stimulating a vigorous inflammatory response, probably the acquisition of the parasite occurred most recently in the elderly than in non-elderly patients, or the elderly, due to changes in the immune system with aging, preserve the cysticercus in the initial phase for a long time. However, further research is needed.

Amongst the elderly with cysticercosis, the female patients had more occurrences of cardiac inflammation and encephalic inflammation. There are also some indications that, in human neurocysticercosis caused by *T. solium*, women show a more intense inflammatory profile in the cerebrospinal fluid than men do and, likewise, are more prone to develop a severe and generalized encephalitic process [28]. Women had evidence of cardiac and encephalic inflammation more frequently than men. These observations are in accordance with previous studies in which gender has been associated with the intensity of the inflammatory response against the parasite, possibly promoted by the female sex-steroid levels [7, 9, 28–32]. Therefore, our data showed that even during senescence, when a decrease in the levels of female sex steroids is noticed, women have a more intense immune response towards cysticercosis in comparison with men.

Cardiac and encephalic inflammation showed a positive correlation in both groups. Studies have showed that the presence of multiple parasites is more common in older individuals [28]. Encephalic inflammation and cardiac inflammation were more commonly found in the analyzed material, and most of the individuals had cysticercosis in more than one location.

It was found that multiple cysticerci lesions and multiple vesicular cysts were more frequently observed in the elderly without an increase in severity of the clinical symptoms. This observation could indicate that susceptibility to become infected increases with age, whereas susceptibility to follow a pathogenic course of the infection decreases. This suggestion finds additional support in the reduction of leukocyte counts with age. The reverse effect of age upon susceptibility to infection and to resistance against severe disease has been found in other parasite infections such as schistosomiasis [33, 34] and it suggests that susceptibility and pathogenicity involve distinct physiological pathways that are independently regulated [28].

This study presents important findings on the influence of gender on cardiac and encephalic inflammation in the elderly with cysticercosis, although it has some limitations, such as small number of samples for analyses, particularly of the gender influence, loss of many biopsies and retrospective

Table 3: Comparison of the inflammatory infiltrate in heart and brain in relation to the gender of non-elderly and elderly patients with cysticercosis.

Groups	Gender	Cardiac inflammation	Encephalic inflammation
		Med (minimum–maximum)	
Elderly	Female	3.0 (1.0–51.0)[1]	3.0 (1.0–12.0)[3]
	Male	2.0 (1.0–23.0)[2]	2.0 (1.0–6.0)[4,5]
Non-elderly	Female	3.0 (1.0–16.0)	3.0 (1.0–10.0)
	Male	3.0 (1.0–89.0)	3.0 (1.0–25.0)[6]
		H, $P < 0.05$	H, $P < 0.05$

1×2, 3×4, 5×6: Dunn test, $P < 0.05$.

design. Future researches are needed to determine the mechanisms of the differences related to gender and immunosenescence associated to immunological alteration caused by cysticercosis.

5. Conclusions

In this study, we showed that the non-elderly had significantly more cardiac and encephalic inflammation than the elderly, and that such inflammatory infiltrate decreases with age and depends upon the evolutionary stage of the cysticercus. Furthermore, there are differences, concerning gender, in the intensity of the inflammatory response due to cysticerci in the heart and brain parenchyma during senescence. Even during this period, women continue to have a more intense response to the parasitosis.

Conflict of Interests

All authors declare that they had no potential conflict of interests relevant to this paper.

Acknowledgments

This study was conducted at General Pathology Division of Triangulo Mineiro Federal University, Uberaba, MG, Brazil, with grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), and Fundação de Ensino e Pesquisa de Uberaba (FUNEPU).

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Hindawi Publishing Corporation
Journal of Tropical Medicine
Volume 2012, Article ID 281572, 7 pages
doi:10.1155/2012/281572

Clinical Study

American Visceral Leishmaniasis: Factors Associated with Lethality in the State of São Paulo, Brazil

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Received 1 June 2012; Revised 10 August 2012; Accepted 14 August 2012

Academic Editor: Maria Aparecida Shikanai Yasuda

Objectives. To identify factors associated with death in visceral leishmaniasis (VL) cases. *Patients and Methodology*. We evaluated prognostic factors for death from VL in São Paulo state, Brazil, from 1999 to 2005. A prognostic study nested in a clinical cohort was carried out by data analysis of 376 medical files. A comparison between VL fatal cases and survivors was performed for clinical, laboratory, and biological features. Association between variables and death was assessed by univariate analysis, and the multiple logistic regression model was used to determine adjusted odds ratio for death, controlling confounding factors. *Results*. Data analysis identified 53 fatal cases out of 376 patients, between 1999 and 2005 in São Paulo state. Lethality was 14.1% (53/376), being higher in patients older than fifty years. The main causes of death were sepsis, bleeding, liver failure, and cardiotoxicity due to treatment. Variables significantly associated with death were severe anemia, bleeding, heart failure, jaundice, diarrhea, fever for more than sixty days, age older than fifty years, and antibiotic use. *Conclusion*. Educational health measures are needed for the general population and continuing education programs for health professionals working in the affected areas with the purpose of identifying and treating early cases, thus preventing the disease evolution towards death.

1. Introduction

Visceral leishmaniasis (VL) is a disease with broad geographic distribution, being reported mainly in Asia, Europe, Africa, and the Americas, and is one of the six so-called worldwide priorities among endemic diseases. Five countries report 90% of diagnosed cases in the world [1]. In Brazil, VL is present in five regions, in 21 states. Official data from the Ministry of Health reports approximately two to three thousand new cases per year, and the coefficient of incidence has reached 2 : 100.000 inhabitants. The average fatality rate in the period between 1980 and 2005 was 6.1, peaking at 7.5 in 2004 [2–4]. Some factors contributed to expansion of VL and increase in lethality. Factors related to changes in geographic occurrence pattern result from the intense migration of the rural population to the outskirts of medium and large cities [1]. In addition, the network organization process, associated with better assistance for diagnosis and treatment, resulted in increased detection of cases. Increase of lethality due to VL has been associated with the introduction of the disease in new geographic areas and host factors, such as malnutrition; immunosuppression, mainly HIV coinfection and age extremes [5, 6]. Classically, VL is clinically characterized by hepatosplenomegaly, associated

with fever and pancytopenia. The disease is fatal if not treated promptly after the initial symptoms. The most important cause of death in VL patients is, severe anemia, bacterial infections, acute bleeding, sepsis, heart failure, liver failure, and complications arising from the toxicity of antimonials, such as cardiac arrhythmias [6–13].

In the state of São Paulo, until 1998, there were only imported cases of VL from endemic regions. From that year onward, an outbreak of canine visceral leishmaniasis was detected in the western region of the state, and, subsequently, the first human case of VL was diagnosed in 1999. We observed an expansion of VL cases since then: in 2002, the disease was identified in the municipalities located in the region of Bauru and, in 2003, in the region of Marília [14], reaching, until October 2005, 31 municipalities in the regions of Araçatuba, Bauru, Marília, and Presidente Prudente (Figure 1). The VL Control Program from the Brazilian Ministry of Health aims to reduce the rates of morbidity and lethality in humans, by means of diagnosis and early treatment of cases and to decrease the risks of disease transmission through population control of reservoirs and transmitter agents [3, 15]. In order to implement public policies and strategies to improve the clinical management of the disease, as well as epidemiological surveillance programs for the early detection and reduction of lethality, it is necessary to understand the factors associated with the risk of death by AVL. The present study aims to characterize the AVL cases from São Paulo state in the period of 1999 to 2005 and identify factors associated with death.

2. Patients and Methods

2.1. Study Design. A prognostic study nested in a clinical cohort was carried out by data analysis of 376 medical files. All subjects included in this study were natives from São Paulo state in the period between 1999 and 2005. For analysis of the data, the compulsory disease notification system (SINAN database) was used, which defines the autochthonous cases of visceral leishmaniasis in São Paulo state.

After survey of autochthonous cases and their places of notification, the regional directories of health (DIRs) were asked to identify the unit of hospitalization of the patients. Thus, a specific questionnaire was applied to collect the information from the patients' records. The variables included in the questionnaire were related to demographic, clinical, and laboratory data. After authorization, the hospitals were visited in order to get the patient's records and filling out questionnaires.

2.2. Case Definition. A case of human visceral leishmaniasis was defined by clinical and laboratory diagnostic of VL, using direct parasitological examination or culture of specimens collected by venipuncture or biopsy from bone marrow in the routine assessment.

As inclusion criterion, the confirmed cases of AVL had to have São Paulo state as the probable local of infection between 1999 and 2005.

2.3. Variables Analyzed. To identify the probable factors related to lethality, the following variables were used.

(1) Biological and demographic variables: age, sex, ethnicity, housing zone, and municipality of residence.

(2) The probable site of infection: municipality with proof of transmission of VL in São Paulo state, up to the period in which the study was performed.

(3) Care and hospitalization: type of health unit service and date of service.

(4) History of current disease: date of symptom onset, presence of the following signs and symptoms: fever, weight loss, abdominal growth, asthenia, headache, abdominal pain, anorexia, constipation, nausea, vomiting, dry cough, dyspnea, drowsiness, myalgia, hemorrhagic manifestations, skin pallor, jaundice, splenomegaly, hepatomegaly, dehydration, adenomegaly, respiratory alterations, and cardiocirculatory changes.

(5) Early pathological history or associated conditions: diabetes mellitus with or without organ damage, congestive heart failure, chronic obstructive pulmonary disease, peripheral vascular disease, moderate or severe kidney disease, moderate or severe liver disease, malignant neoplasm, leukemia, lymphoma, and solid metastatic tumor, aids, tuberculosis, malnutrition, immunosuppressive medication, and previous splenectomy.

(6) Laboratory tests: levels of hemoglobin, hematocrit, leukocytes, neutrophils, and platelets.

(7) Relapse: symptom recurrence up to 12 months after cure, time of relapse (from the end of previous treatment).

(8) Patient: clinical evolution of cure or death and cause of death.

2.4. Data Collection and Management. An analysis of existing data in the database was conducted based on the variables found in the individual records of epidemiological investigation for human visceral leishmaniasis. A database was developed and analyzed using specific software, Epi-info version 3.2.2, based on questionnaires filled out in consultation together with patient records.

According to the clinical evolution or the type of outcome of confirmed cases of VL, two distinct groups were considered: (1) those who were cured and (2) those who died, in order to identify factors associated with lethality.

2.5. Statistical Analysis. The statistical analysis of the data was performed using the software program Epi-info version 3.2.2. Data tabulation was performed using Microsoft Office Excel 2003. After performing the descriptive analysis and determining the main independent variable frequencies, a bivariate analysis was developed between potential risk factors and death outcome. For the continuous quantitative variables, a mean comparison test was performed among

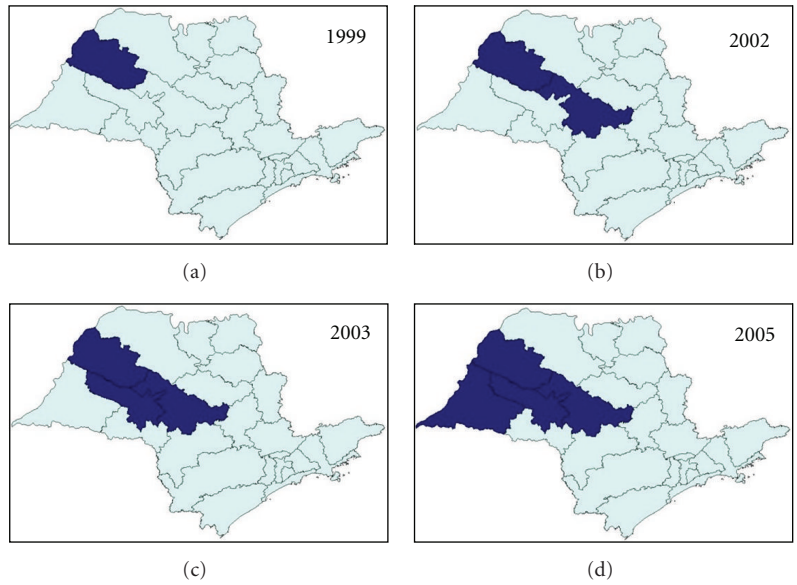

Figure 1: Geographic expansion of confirmed cases of visceral leishmaniasis autochthonous transmission, according to the region of Sao Paulo state, from 1999 to 2005. Data source: SINANW-National Surveillance System for Disease Notification/CVE/SES/SP; TABWIN 3.1.

the factors assessed through evolution to death or cure. Thus, variables with a P value <0.05 were considered for the multivariate logistic regression model by stepwise forward, from the smallest to the largest value of P. The existence of an association between death by VL and the factors of interest was investigated by nonadjusted and adjusted odds ratio, with the respective 95% confidence intervals using logistic regression. The statistical significance of variables in the models was assessed by the likelihood ratio test.

3. Results

3.1. Analysis of Data from SINAN Database, São Paulo State in the Period of 1999 to 2005. During the study period, 945 confirmed cases of VL were reported to the SINAN database, of which 559 were considered autochthonous, with 68 deaths. Lethality reached 14.7% in 2003, with rates remaining at around 11% in the following years.

3.2. Analysis of Data Collected from Patient Records. In order to determine the prognostic factors of VL and identify the risk of lethality 376 patient records of 559 autochthonous cases of VL that had an outcome (67.3%) were assessed. Two separate groups were studied and analyzed according to the patient's clinical evolution: the cases that resulted in death and cases in which the disease was cured. Of 376 cases assessed, 53 resulted in death and 323 in cure. Lethality observed in this group in the study period was 14.1%. The distribution by age groups reflects the predominance of cases among children younger than 10 years (53.7%), while the greater lethality is in the age groups older than 50 years, particularly in those older than 60 years (69%), as demonstrated in Table 1. There was a predominance of males, especially in the age groups older than 10 years. Regarding the signs and symptoms described in patients' records, fever, splenomegaly, hepatomegaly, pallor, asthenia, and weight loss were the most frequent (≥60), and proportional in both study groups (Table 2). Other less frequent findings, such as dry cough, diarrhea, dehydration, hemorrhagic manifestations, edema, and jaundice, were proportionally more frequent in cases that resulted in death. Malnutrition was seldom described in patients' records (1.6%). The age of patients varied from 3 months to 96 years (average = 19 years), with difference between cases that resulted in death and cured patients (44 × 15 years; $P < 0.001$). The mean time between symptom onset and treatment was 34 days (51 × 21 days; $P < 0.001$). Comorbidities were present in 72 patients (19.1), and the most frequent was HIV coinfection ($n = 5$). As for the laboratory data, we observed a mean level of hemoglobin of 8.2 g/dL, 3,390 leukocytes/mm^3, 1,319 neutrophils/mm^3, and 107,000 platelets/mm^3, demonstrating the characteristic thrombocytopenia observed in these patients. The main causes associated with death were sepsis (21/53), bleeding (12/53), liver failure (9/53), and arrhythmia due to antimonial cardiotoxicity (9/53). The bivariate analysis of clinical findings and laboratory data associated with worse prognosis of VL cases is shown in Table 3. The final model analysis using multivariate logistic regression (Table 4) showed to be more strongly associated with VL lethality regardless of the other variables: cardiac abnormalities on admission or during hospital stay (OR = 4.7), presence of diarrhea (OR = 2.7), presence of severe anemia identified by hemoglobin ≤5.0 g/dL (OR = 4.5), increase in total bilirubin ≥2.0 (OR = 7.3), age ≥ 50 years (OR = 29.5), time between fever onset and treatment >60 days (OR = 6.2), and use of antimicrobials during hospitalization (OR = 5.7). Hemorrhagic manifestations remained in the final model only as adjustment variables for the remaining ones, as there was no statistical significance (OR = 2.6).

Table 1: Visceral leishmaniasis-confirmed autochthonous cases, by age group and clinical outcome, state of Sao Paulo, 1999–2005.

Age group (years)	Death		Cured		Total		
	n	%	*n*	%	*n*	%	% Cumulative
<1	3	7.0	40	93.0	43	11.4	11.4
1–4	8	6.5	115	93.5	123	32.7	44.1
5–9	1	2.8	35	97.2	36	9.6	53.7
10–19	2	6.7	28	93.3	30	8.0	61.7
20–29	3	8.6	32	91.4	35	9.3	71.0
30–39	4	13.8	25	86.2	29	7.7	78.7
40–49	4	11.4	31	88.6	35	9.3	88.0
50–59	8	50.0	8	50.0	16	4.3	92.3
≥60	20	69.0	9	31.0	29	7.7	100.0
Total	53	14.1	323	85.9	376	100	

Table 2: Visceral leishmaniasis distribution (*n* = number and %) of the main signs and symptoms, according to the clinical outcome of the confirmed cases, state of Sao Paulo, 1999–2005.

Signs and symptoms	Death ($n = 53$)		Cured ($n = 323$)		Total ($n = 376$)	
	n	%	*n*	%	*n*	%
Fever	51	96.2	320	99.1	371	98.7
Splenomegaly	50	94.3	319	98.8	369	98.1
Hepatomegaly	44	83.0	244	75.5	288	76.6
Pallor	39	73.6	199	61.6	238	63.3
Asthenia	44	83.0	191	59.1	235	62.5
Weight loss	35	66.0	189	58.5	224	59.6
Dry cough	29	54.7	127	39.3	156	41.5
Diarrhea	20	37.7	52	16.1	72	19.1
Hemorrhagic manifestations	18	34.0	22	6.8	40	10.6
Edema	10	18.9	11	3.4	21	5.6
Dehydration	12	22.6	8	2.5	20	5.3
Cardiac abnormality	9	17.0	10	3.1	19	5.1
Jaundice	10	18.9	6	1.9	16	4.3
Malnutrition	2	3.8	4	1.2	6	1.6

4. Discussion

Visceral leishmaniasis is a disregarded emerging tropical disease, which shows modifications in its epidemiological behavior, occurring in new areas [5, 10]. The increase in human VL cases in endemic regions, or even the emergence of the disease in regions where it did not previously occur, can be explained by the change in geographical occurrence patterns, with the reporting of cases in urban centers [16–19]. As reported by other authors, we clearly observe that the introduction of VL in São Paulo state caused an initial increase in lethality, likely by the misdiagnosis of the disease by health care professionals, and, subsequently, the maintenance of fatality levels came as a result of the occurrence of the disease in vulnerable populations, such as those infected with HIV. VL lethality in São Paulo state during the study period was 14.1%, whereas it was 6.7% in the rest of Brazil. The data analysis showed that the quality of the data and mainly the lack of update were limiting factors. On the other hand, it was observed that the vast majority of patients examined came from urban areas and had access to health services and, even so, there was a delay in the diagnosis. The high incidence of the disease in the age groups younger than 10 years observed in data analysis of patients' records corresponds to that found in the literature and the official data of the Ministry of Health. The prevalence of males observed in the most predominant age groups is also a constant characteristic found in several studies [16–19]. Regarding age, the study found high levels of lethality in those aged 50 years and older, with the mean age in the group of patients who died of 44 years, while the mean age in the group of patients that were cured was 15 years. The greatest strength of association was found between the evolution to death and age 50 years and older (OR = 20.16; $P < 0.0001$). The association between death and age with a cutoff of 30 years was also statistically significant, but with less strength (OR = 7.25; $P < 0.001$). Symptoms and clinical findings pointed to severity, and, therefore, factors associated with death, such as evolution to hemorrhagic manifestations, edema, and jaundice, were highlighted in the study, as shown by the largest percentage in the group of deaths [7–13].

Table 3: Statistical analysis of factors associated with VL lethality, state of Sao Paulo, 1999–2005 (bivariate analysis).

Features	Outcome ($n = 376$)			OR (95% CI)	P value
	Death $n = 53$	Cured $n = 323$	Total $n = 376$		
Signs and symptoms					
Asthenia	44	191	235	3.38 (1.59–7.16)	0.0008
Cardiac abnormality	9	10	19	6.53 (2.28–18.65)	<0.0001
Dehydration	12	8	20	12.11 (4.66–31.47)	<0.0001
Diarrhea	20	52	72	3.16 (1.68–5.93)	0.0002
Dyspnea	12	22	34	4.00 (1.84–8.69)	0.0001
Edema	10	11	21	6.75 (2.7–16.86)	<0.0001
Hemorrhagic manifestation	18	22	40	7.24 (3.54–14.83)	<0.0001
Jaundice	10	6	16	12.88 (4.45–37.31)	<0.0001
Pallor	39	199	238	2.02 (1.02–4.01)	0.040
Dry Cough	29	127	156	1.86 (1.04–3.35)	0.035
Vomiting	13	35	48	2.67 (1.30–5.48)	0.006
Drowsiness	9	11	20	5.8 (2.27–14.79)	<0.0001
Laboratory analysis					
Total bilirubin ≥ 2.0	13	6	19	17.17 (6.18–47.7)	<0.0001
Hypoalbuminemia ≤ 3.0	15	37	52	3.05 (1.53–6.07)	0.0009
Thrombocytopenia ≤ 100.000	31	108	139	2.8 (1.55–5.08)	0.0004
Aspartate aminotransferase > 40	20	62	82	2.55 (1.37–4.74)	0.002
Comorbidities					
Liver disease	3	2	5	9.79 (1.59–60.11)	0.020
Diabetes	5	4	9	8.46 (2.19–32.62)	0.003
Peripheral vascular disease	7	5	12	9.86 (3.0–32.4)	<0.0001
Splenectomy	2	0	2	undefined	0.019
Congestive heart failure	4	2	6	13.33 (2.38–74.78)	0.004
Use of immunosuppressive drugs	3	0	3	undefined	0.002
Tuberculosis	3	1	4	19.65 (2.0–192.74)	0.009
Fever					
≥60 days	13	26	39	3.71 (1.76–7.80)	0.0002
≥30 days	26	79	105	2.97 (1.64–5.39)	0.0002
Age					
≥30 years	36	73	109	7.25 (3.85–13.66)	<0.0001
≥50 years	28	17	45	20.16 (9.74–41.73)	<0.0001
Complications	53	64	117	undefined	<0.0001
Opportunistic infections	21	43	64	4.27 (2.26–8.08)	<0.0001
Pneumonia	13	29	42	0.40 (0.18–0.89)	0.023
Bleeding	24	2	26	26.1 (5.77–117.78)	<0.0001
Sepsis	28	0	28	undefined	<0.0001
Antimicrobial use	34	59	93	8.71 (4.56–16.64)	<0.0001
Blood derivatives	36	95	131	5.61 (2.93–10.72)	<0.0001

The classic symptoms, such as fever, hepatosplenomegaly, and pallor, showed no statistical difference between the two groups, as they are not criteria of diagnostic suspicion that influence disease prognosis, and therefore should be equally present in the two groups [9, 11–13]. Other signs and symptoms associated with death, such as cardiac alterations at admission or during hospitalization, dehydration, diarrhea, vomiting, abdominal pain, dyspnea, dry cough, and drowsiness, are also already described in study of risk factors for death. Concomitant diseases and conditions associated with the diagnosis of VL that contributed to the death outcome were moderate liver disease and severe cardiovascular diseases, tuberculosis, and the use of immunosuppressive drugs; these conditions are already known to be risk factors, involving mainly immunity impairment [9–13]. HIV coinfection increased the risk of death but did not show statistical significance, probably by the small number of cases and fatalities evaluated. It is known that

Table 4: Final model of factors associated with VL lethality, state of Sao Paulo, 1999–2005 (multivariate analysis).

Variable	Clinical outcome			OR (95% CI)*	*P* value**
	Death	Cured	Total		
	$n = 53$	$n = 323$	$n = 376$		
High total bilirubin					
Total Bilirubin ≥ 2.0 g/dL	13	6	19	7.36 (1.65–32.76)	<0.0001
Severe anemia					
Hemoglobin ≤ 5.0 g/dL	7	13	20	4.56 (1.17–17.48)	<0.0001
Antimicrobial agents	34	59	93	5.76 (2.27–14.64)	<0.0001
Age ≥ 50 years	28	17	45	29.54 (10.6–82.6)	
Length of illness (days)					
Fever >60 days	13	26	39	6.23 (2.05–18.92)	<0.0001
Hemorrhagic manifestations	18	22	40	2.62 (0.93–7.4)	0.0001
Cardiac abnormality	9	10	19	4.73 (1.3–17.23)	<0.0001
Diarrhea	20	52	72	2.76 (1.03–7.43)	<0.0001

* Odds ratio (95% confidence interval). ** Likelihood ratio.

this coinfection is emerging and is considered to be of high severity [10, 13, 20].

The major complications of the disease found in the study, associated with worse prognosis, corroborate those already described in the literature: bacterial infections, such as pneumonia, skin infections and ear infections, bleeding, and sepsis [8–13, 21]. The use of antimicrobials and the need for blood transfusions and blood products were also associated with greater lethality alone, reflecting the severity of cases and, consequently, the development of complications. Febrile neutropenia and adverse reactions to antimonials did not show statistical significance for the death outcome, although it has been discussed in other studies as factors of poor prognosis. Still, one of the leading causes of death was found to be cardiac arrhythmias due to antimonial use toxicity. Sepsis, bleeding, and liver failure were also causes of death, compatible with the data from the literature [9, 10]. In addition to these, medullary aplasia and aids appeared as causes of death among the evaluated patients.

Laboratory alterations that indicated disease severity, compatible with the literature, were also associated with deaths analyzed in the study. Thrombocytopenia ($\leq$100,000 mm^3), severe anemia, hypoalbuminemia, hyperbilirubinemia, in addition to the increase in aspartate aminotransferase (AST) levels were associated with statistical significance for the outcome of death. Leukopenia and neutropenia showed unexpected statistically significant differences, requiring further analysis when relating them to other events, where opportunistic infections constitute a complication and not lethality cause [10, 12, 13].

Factors associated with the risk of death in the multivariate analysis were the presence of diarrhea and hemorrhagic phenomena, cardiac abnormalities on admission or during hospitalization and treatment, hemoglobin levels $\leq$5.0 g/dL, and total bilirubin $\geq$2.0 g/dL, constituting serious anemia and jaundice, respectively. All of these factors have already been addressed in studies as associated with worse prognosis of the disease. Still, the need for the use of antimicrobials as predictor of death, notably in the multivariate analysis, reflects the concomitant infections and results from the severity of the disease; these variables did not remain in the final model, as they represent the same nature of the outcome in question, that is, death. In other words, if antibiotics are used, the reason is the presence of infection, or pneumonia, or otitis, or sepsis, and, conversely, if there is infection, then the use of antimicrobials is required.

Long-term symptoms were found to be associated with greater lethality, a factor that reflects the delay in the detection of cases and areas of recent VL diagnosis in addition to the lack of knowledge on the part of the population that inhabits these periurban and urban areas, as they have universal access to health services through the public health system. The conclusions to be drawn from this study are aimed at the implementation of intervention measures, prevention, and control in certain regions of the state, for certain sectors of the population, with specific clinical findings of paramount importance for the reduction of lethality due to visceral leishmaniasis in the state of São Paulo in Brazil.

5. Conclusions

The appearance of visceral leishmaniasis in the state of São Paulo is recent, dating from the year 1999, being considered an emerging disease in our state.

High incidence of cases was observed in children under 10 years and high lethality in those older than 50 years.

Average lethality for the state of São Paulo in the period 1999 to 2005 was 14.1, which is considered high in comparison with the epidemiological data from other states and even in Brazil as a whole, which shows up to 7.0 of lethality.

Deaths must be investigated to improve the clinical management of cases, through the knowledge of their determining factors and population groups at greatest risk, in addition to increase the epidemiological surveillance system.

The prognostic factors that were more strongly associated with death, regardless of all other variables were

major anemia, hemoglobin ≤5.0 g/dL, hemorrhagic manifestations, cardiac abnormalities at admission or during hospitalization and treatment, total bilirubin level <2.0 g/dL, diarrhea, age >50 years or older, time period between symptom onset, indicated by the onset of fever, and treatment longer than 60 days and the need of antimicrobials.

Investment in health education of the population and continuing education programs for health professionals working in the affected areas is of paramount importance for the early detection of cases, thus preventing the evolution towards death.

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Hindawi Publishing Corporation
Journal of Tropical Medicine
Volume 2012, Article ID 429586, 8 pages
doi:10.1155/2012/429586

Research Article

Public Knowledge about and Detection of Canine Visceral Leishmaniasis in Urban Divinópolis, Brazil

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Received 29 May 2012; Revised 11 July 2012; Accepted 27 July 2012

Academic Editor: Maria Aparecida Shikanai Yasuda

Background. Leishmaniases are diseases with a wide spectrum of clinical manifestations including cutaneous (CL) and visceral (VL) forms. Many factors may affect their occurrence and expansion including environmental, geographic, and social conditions. In the past two decades, Divinópolis, Minas Gerais State, Brazil, has exhibited the potential for a disease outbreak, with the appearance of CL, and VL cases (human and canine). Hence, this study was initiated to monitor public knowledge of the disease. Questionnaires were administered in four neighborhoods (Jardim Belvedere, Esplanada, Danilo Passos I and II) where most of the human and canine cases have been reported. The analyses demonstrated that public knowledge of the disease is sparse and fragmented. A strong perception of the dog as the main reservoir was observed. Five veterinary clinics were evaluated for the presence of canine VL using serological (RIFI and ELISA) and molecular (PCR-RFLP) techniques. This is the first study demonstrating the occurrence of *Leishmania infantum* in Divinópolis, suggesting a possible urbanization of VL.

1. Introduction

Leishmaniases are a group of diseases caused by the protozoan *Leishmania* (Kinetoplastida: Trypanosomatidae) affecting 12 million people in 88 countries. The disease exhibits a wide spectrum of clinical manifestations ranging from benign cutaneous lesions (CL) to the fatal visceral form (VL) [1]. In Latin America, especially Brazil, both forms are widely distributed and are transmitted by the bite of phlebotomine sand flies (Diptera: Psychodidae) [2]. Wild and domestic reservoirs including foxes, marsupials, rodents, dogs, and cats are the main sources of sand fly infection [3–8].

Many factors may have contributed to VL and CL expansion and urbanization [9], including deforestation [10], human migration [11], vector adaptation [12, 13], drug resistance [14], poverty [15], and social conflicts [16]. As a result of anthropic modifications, VL has been increasingly reported in urban areas of major Brazilian cities including Natal, Teresina, Sobral, and Belo Horizonte [5, 17–20].

The city of Divinópolis, Minas Gerais State, Brazil, has a population of approximately 210,000. It has grown dramatically, with 90.5% of its territory completely urbanized in 2000 [21]. During the 1990s, 135 CL cases were detected

Table 1: Number of leishmaniasis cases reported (2004–2010) in Divinópolis according to regions.

Region	2004			2005			2006			2007			2008			2009			2010		
	CL	VL	CVL	CL	VL	CVL	CL	VL	CVL	CL	VL	CVL	CL	VL	CVL	CL	VL	CVL	CL	VL	CVL
Central[a]	—	—	0	—	—	2	—	—	2	—	—	1	—	—	13	—	—	32	2	1	39
Northeast[b]	1	—	1	—	—	0	—	—	1	—	—	1	1	—	0	—	1	13	5	2	63
Far Northeast	1	—	0	—	—	0	1	—	0	—	—	0	—	—	0	—	—	4	—	—	4
Northwest	—	—	0	—	—	3	—	—	0	1	—	0	—	—	2	—	—	15	2	—	32
Far Northwest	—	—	0	—	—	0	—	—	0	—	—	0	—	—	1	—	—	0	—	1	5
West	—	—	0	—	—	0	—	—	0	—	—	0	—	—	2	—	—	3	—	—	3
Southeast	—	—	0	—	—	0	—	—	0	—	—	1	—	—	1	1	—	19	2	1	47
Far Southeast	—	—	0	1	—	0	—	—	0	—	—	0	—	—	0	—	—	0	—	—	2
Southwest[d]	—	—	0	—	—	0	—	—	0	—	—	1	—	—	1	—	—	14	3	—	14
Far Southwest	—	—	0	—	—	0	—	—	0	1	—	0	—	—	0	—	—	0	2	—	6
Total	2	0	1	1	0	5	1	0	3	2	0	4	1	0	20	1	1	100	16	5	215

[a]Region of Esplanada (see Figure 2 for details); [b]region of Danilo Passos I and II (see Figure 2 for details); [c]region of Jardim Belvedere (see Figure 2 for details); CL: human cutaneous leishmaniasis; VL: human visceral leishmaniasis and CVL: canine visceral leishmaniasis. Data obtained from CREVISA [24].

by health authorities. Most of those cases were reported in the neighborhoods of Jardim Belvedere, Esplanada, São José, Catalão, and Candelária, all in the vicinity of Mata do Noé forest, where a large area was deforested. More recently, another forest remnant, Gafanhoto Park, was reported to be a potential CL focus, where known vectors and reservoirs were detected [22]. Between 2004 and 2008, 33 canine VL cases were detected, and this number increased up to 215 in 2010. This was also followed by an increase in the number of human VL and CL cases (5 and 16, resp.) for the same period (Table 1) [23, 24]. Those data were primarily based on notification by health professionals rather than due to a detailed epidemiological and serological survey in the city. Based on these observations, as a part of a wider study on leishmaniasis in Divinópolis, this work aimed to confirm the presence of *Leishmania infantum* in the city after 2009 and to assess the level of public awareness of the disease and aspects of its transmission.

2. Materials and Methods

2.1. Study Area and Data Collection. Divinópolis (20°8′21″S, 44°53′17″W) is located in west central Minas Gerais State, (Figure 1). Data on canine and human leishmaniasis (2004–2010) were obtained from the Reference Center of Epidemiological Surveillance (CREVISA) and the Epidemiology Department of City Hall (DEDCH), respectively [23, 24]. The project was approved by the Ethical Committee from FUNEDI/UEMG (protocol 63/2007) and FIOCRUZ (protocol P-0119-02).

2.2. Elaboration of Questionnaires and Distribution. Four neighborhoods were evaluated (Figure 1): Esplanada and Jardim Belvedere, where the majority of human cases have occurred (1989–1991) (34) (52.5 and 32.5%, resp.), and Danilo Passos I and II (6) (12.5 and 2.5%, resp.). One hundred questionnaires (25 per neighborhood) were administered and sample size was calculated as described elsewhere [25]. The interviewed areas had similar characteristics. For example, only houses were present in the streets and buildings were absent. The questionnaires were based on previous VL studies (but also applicable to CL) and included general questions on sociodemographics, transmission, prevention,

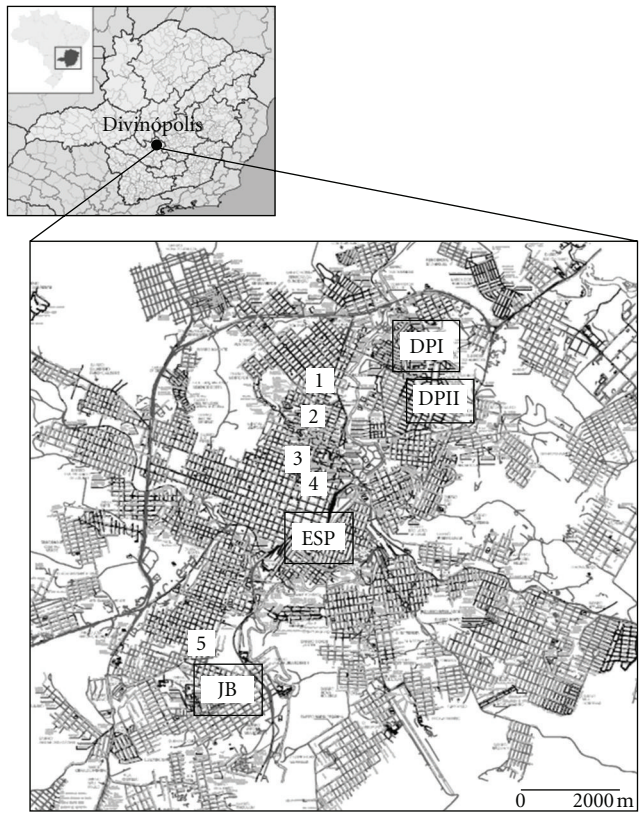

Figure 1: Divinópolis urban area. Rectangles indicate the four neighborhoods where questionnaires were applied, and numbers (1–5) indicate the five veterinary clinics surveyed. DPI, Danilo Passos I; DPII, Danilo Passos II; JB, Jardim Belvedere; ESP, Esplanada.

treatment, and environmental conditions [26]. All questionnaires were administered by the same person (M.E.O.), and informed consent was obtained. Selection of interview subjects was as follows: all streets in each neighborhood were numbered, and five were randomly selected. One home per block was randomly selected, totaling five houses per street and 25 homes per neighborhood. After selection of a given house, the next house would be in the subsequent block. The houses were always in the center of the block and never on a corner or at the end of the street.

2.3. Analyses. The percents of each response were calculated using Statistical Analysis System (SAS) software. Data were analyzed using the Kruskal-Wallis test. $P < 0.05$ was considered statistically significant.

2.4. Immunofluorescence and ELISA. Sixty-nine dogs suspected of being infected with *Leishmania* from five veterinary clinics were subjected to serological tests (ELISA and RIFI) to detect *Leishmania* infection. Canine IFI-leishmaniasis and canine leishmaniasis EIA kits (Bio-Manguinhos/FIOCRUZ) were used according to the manufacturer's instructions for immunofluorescence and ELISA assays, respectively. Canine serum samples obtained from five veterinary clinics (Figure 1) were diluted from 1 : 40 (the cut-off value) to 1 : 640 in phosphate-buffered saline (PBS), and the cut-off value of absorbance was considered >0.05 OD. Data are a representation of two experiments in triplicate.

2.5. Restriction Fragment Length Polymorphism (RFLP-PCR). Blood samples were subjected to DNA extraction for *Leishmania* detection [20, 22]. PCR reactions and thermal profile followed the procedure previously published [27, 28]. Amplified PCR products were digested with *HaeIII* (1 U, 3 h, 37°C) and visualized in polyacrylamide gels (8%). Data are a representation of two experiments.

3. Results

3.1. Social and Demographic Indicators. Females represented 71% percent of respondents in all neighborhoods, an indication that the majority of residents found at home were homemakers. In all studied areas, 69% of the population had completed high school and 63% of family incomes ranged from $300 to $900 (2008-2009). Education and income levels were similar among the four neighborhoods ($P > 0.05$).

3.2. Awareness of Leishmaniasis Transmission and Prevention. There was no difference among the four areas with respect to knowledge about leishmaniasis ($P > 0.05$). Approximately half of the respondents were unaware of the disease and its transmission routes and mechanisms (Table 2). Twenty-nine percent were aware that transmission occurred through the bite of the sand fly. In all interviewed individuals, dogs were identified as the main reservoir (49%), followed by rats (17%) and cats (4%) in all four areas ($P = 0.0021$). Thirty percent of the respondents did not know about reservoirs. In all neighborhoods, no difference was observed regarding prevention measures. Cleaning of yards and vacant lots was the most cited (38%), followed by dog euthanasia (17%). Thirty-three percent were not aware of any method of prevention. Most interviewees did not know about treatment (41%) and would take a suspected patient to a hospital (77%) or health agent (19%) (Table 2). Among the eight interviewees reporting previous leishmaniasis infection, four cases occurred in Esplanada (50%), two (25%) each in Jardim Belvedere and Danilo Passos II, and none in Danilo Passos I (data not shown), conforming to our prior information on incidence in the area [23, 24].

3.3. Environmental Conditions. In all surveyed neighborhoods, 60% of homes included pets, with a predominance of dogs (88.3%), followed by cats (3.3%) and other animals (8.3%) ($P = 0.0346$) (Table 3). No difference was observed in the number of dogs while comparing Danilo Passos I/II and Esplanada/Jardim ($P > 0.05$). The perception of the presence of hematophagous insects and rodents in the homes was reported in all neighborhoods (above 60%), with no observed difference among them ($P > 0.05$) (Table 3).

The majority of the surveyed homes in Jardim Belvedere (88%) and Danilo Passos II (72%) were near vacant lots. No difference among the four studied areas was observed with respect to proximity to water, green areas, presence of yard, and yard cleaning (Table 3). All homes had their trash regularly collected (data not shown).

3.4. Dog Survey. Twenty-seven dogs (39.1%) tested positive using serological tests, with infection rates among the clinics varying from 6.25% to 50% (Table 4). Seventeen dogs (24.6%) were positive with both tests, and ten dogs (14.5%) were positive only with ELISA. For this reason, a more sensitive technique (PCR-RFLP) was conducted in those animals to confirm infection. A 120 bp fragment confirmed *Leishmania* sp. DNA in 100% of the blood samples. In the gel, PCR from nine blood samples is represented (lanes 2–10, Figure 2(a)). After digestion with *HaeIII*, *L. infantum* was confirmed as the species causing canine VL (lanes 1–6, Figure 2(b)).

4. Discussion

4.1. Urbanization as a Current Problem in Leishmaniases. Despite control programs, reports of leishmaniasis have been increasing. Many factors are involved, but it is clear that the lack of a vaccine, the adaptation of vectors and reservoirs to human environments, lack of effective drugs, and therapeutic failures contribute [29]. In Brazil, VL urbanization has been observed in places including São Luís, Natal, Teresina, Aracaju, Sobral, Boa Vista, Santarém, Cuiabá, Campo Grande, and Araçatuba [30–33]. In Minas Gerais state, VL urbanization has occurred in Montes Claros and Belo Horizonte [13, 30, 34]. It is not known if this phenomenon is occurring in the city of Divinópolis. Although human and canine cases have been reported, only serological and clinical diagnoses were made, with no parasitological investigation. Few studies have assessed the public perceptions of and attitudes toward the disease in this city.

Table 2: Frequency of responses regarding leishmaniasis knowledge in four neighborhoods of Divinópolis, MG, Brazil.

Question	Number (%) Neighborhood			
	DPI	DPII	ESP	BEL
Do you know what leishmaniasis is?*				
Yes	13 (52)	10 (40)	13 (52)	13 (52)
No	12 (48)	15 (60)	12 (48)	12 (48)
Do you know how it is transmitted?*				
Do not know	13 (52)	13 (52)	13 (52)	13 (52)
Sand fly	7 (28)	8 (32)	7 (28)	7 (28)
Dog	4 (16)	4 (16)	4 (16)	5 (20)
Other	1 (4)	0 (0)	1 (4)	0 (0)
Do you know the reservoir?*				
Dog	10 (40)	11 (44)	12 (48)	16 (64)
Rat	6 (24)	3 (12)	4 (16)	4 (16)
Cat	1 (4)	2 (8)	0 (0)	1 (4)
Do not know	8 (32)	9 (36)	9 (36)	4 (16)
Do you know how to prevent?*				
Do not know	8 (32)	10 (40)	10 (40)	5 (20)
Yard cleaning	9 (36)	11 (44)	7 (28)	11 (44)
Dog euthanasia	4 (16)	2 (8)	6 (24)	5 (20)
Water accumulation	1 (4)	2 (8)	0 (0)	3 (12)
Insecticides	2 (8)	0 (0)	1 (4)	1 (4)
Other	1 (4)	0 (0)	1 (4)	0 (0)
What would you do to help a suspected victim?				
Take to hospital	19 (76)	21 (84)	18 (72)	19 (76)
Take to a health agent	6 (24)	3 (12)	4 (16)	6 (24)
Do not know	0 (0)	1 (4)	1 (4)	0 (0)
How would you treat leishmaniasis?**				
No treatment	11 (44)	8 (32)	10 (40)	4 (16)
Glucantime	2 (8)	0 (0)	1 (4)	1 (4)
Antibiotics	0 (0)	2 (8)	1 (4)	1 (4)
Vaccine	1 (4)	1 (4)	0 (0)	4 (16)
Do not know	8 (32)	12 (48)	11 (44)	10 (40)
Other	3 (12)	2 (8)	2 (8)	5 (20)

DPI: Danilo Passos I; DPII: Danilo Passos II; ESP: Esplanada; BEL: Belvedere. *No statistical difference was observed among the four neighborhoods (Kruskall-Wallis, $P > 0.05$). **No treatment in this case means that they are not aware that leishmaniasis has a treatment for humans.

4.2. Leishmaniasis Transmission, Prevention, and Environmental Conditions. Analysis of the questionnaire administered in this study indicated a lack of knowledge of the disease. In all surveyed neighborhoods half of the interviewed subjects were unaware of the disease and aspects of its transmission. Similar situations have been reported in Belo Horizonte [35], Maringá [36], São Luís [26], and Tancredo Neves [37]. Similar results were also found in an area endemic for LTA in Venezuela, where 68% of the population had an insufficient level of information about transmission and prevention [38]. In our study, there was no difference in the level of knowledge about the risk factors and transmission of the disease. Forty-nine percent were able to identify the dog as a possible domestic reservoir. In a similar survey in São Luís, 87.2% of the respondents implicated the dog in leishmaniasis transmission [26].

The association of the vector with a domestic vertebrate host was not clear. Although 71% of the respondents reported the presence of hematophagous insects indoors, this does not indicate that they were sand flies. Sand flies are extremely small and difficult to identify compared to mosquitoes [39]. Only 29% of the house-holders knew that phlebotomine sand flies were responsible for leishmaniasis transmission. Similar results were observed in a transmission area in India, where VL is a major health problem, with 61% of the respondents believing mosquitoes to be the vectors of the disease. Currently there is no available information on sand fly species in the urban area of Divinópolis. Margonari et al. (2010) observed a high diversity of sand flies, including CL vectors, in Gafanhoto Park, a forest remnant in the city [22]. Consistent with those data, our questionnaires found some individuals that reported having been infected with CL

Table 3: Frequency of responses regarding transmission risk of leishmaniasis in four neighborhoods of Divinópolis, MG, Brazil.

Question	Number (%) Neighborhood			
	DPI	DPII	ESP	BEL
Do you have pets?				
Dog	14 (93)	14 (93)	15 (79)	10 (90)
Cat	0 (0)	1 (7)	1 (5)	0 (0)
Other	1 (7)	0 (0)	3 (16)	1 (10)
Did you notice blood-sucking insects in the house?*				
Yes	18 (72)	20 (80)	18 (72)	15 (60)
No	7 (28)	5 (20)	7 (28)	10 (40)
Did you notice rodents around the home area?*				
Yes	14 (56)	16 (64)	5 (20)	10 (40)
No	11 (44)	9 (36)	20 (80)	15 (60)*
Are there any vacant lots in the surroundings?				
Yes	3 (12)	18 (72)	2 (8)	22 (88)
No	22 (88)	7 (28)	23 (92)	3 (12)
Is there any water collection/river close to the house?				
Yes	24 (96)	19 (76)	16 (64)	20 (80)
No	1 (4)	6 (24)	9 (36)	5 (20)
Is there any green area close to the house?				
Yes	18 (72)	25 (100)	8 (32)	24 (96)
No	7 (28)	0 (0)	17 (68)	1 (4)
Is there any backyard at home?				
Yes	14 (56)	16 (54)	18 (72)	15 (60)
No	11 (44)	9 (36)	7 (28)	10 (40)
Do you clean your backyard regularly?				
Yes	24 (96)	23 (92)	21 (84)	24 (96)
No	1 (4)	2 (8)	3 (12)	1 (4)

DPI: Danilo Passos I; DPII: Danilo Passos II; ESP: Esplanada; BEL: Belvedere. *Based on population perception and not sampling.

Table 4: Proportion of dogs from five veterinary clinics in Divinópolis, MG, Brazil, positive for leishmaniasis with serology tests (ELISA/RIFI) and PCR-RFLP.

Clinic	Samples *n*	Serology *n* (%)
1	10	5 (50)
2	16	1 (6.25)
3	24	12 (50)
4	19	8 (42)
5	2	1 (50)

in the 1990s, especially in the neighborhoods of Esplanada and Jardim Belvedere, strong evidence that the disease is occurring in town. The respondents were unclear as to the difference between the cutaneous and visceral forms of the disease.

Many reports have suggested that prevention measures face difficulties during implementation due to the lack of a public informed on basic concepts of the disease [26, 35, 37]. The large majority of the interviewed subjects were unaware of prevention measures and treatment, with the primary response being to take a victim to a hospital. In our survey, cleaning of yards and vacant lots was indicated as possible preventative measures. This probably reflects a common habit of the population rather than an action specific to prevention of leishmaniasis. Cleaning of yards is important to control vector proliferation [40] and synanthropic rodent occurrence. However, 45% of respondents reported the perception of the presence of those animals near their homes. In other urban and rural areas, some studies have incriminated them as *Leishmania* reservoirs, especially in the state of Pernambuco, Brazil [41, 42]. In a survey in the state of Minas Gerais, the presence of *L. mexicana*, *L. braziliensis*, and *L. donovani* complex species was detected in wild and synanthropic (*Rattus rattus*) rodents [43]. In Divinópolis, although rodents have been observed in wild areas (Gafanhoto Park), there was no parasite isolation/detection from those reservoirs. The presence of *L. braziliensis* and *L. infantum* was confirmed in this area after examination of sand flies [22]. More studies should be conducted to identify the role of wild and urban rodents as potential reservoirs for leishmaniasis in Divinópolis. There was no difference in the environmental aspects of the survey neighborhoods regarding proximity to forests or water bodies or collection

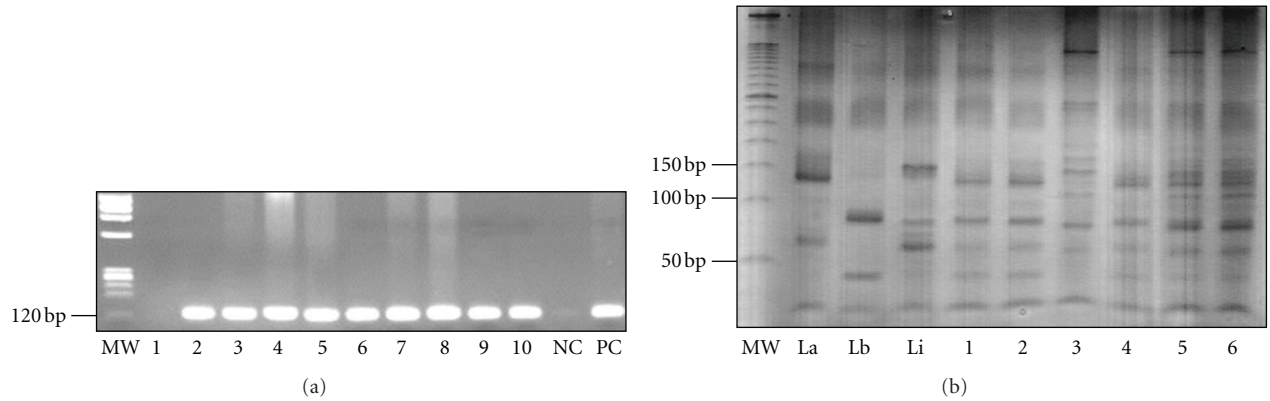

Figure 2: Molecular detection of *Leishmania infantum* from canine samples obtained in five veterinary clinics in Divinópolis, Brazil. (a) Detection of *Leishmania* sp. Legend: MW, molecular weight; lane 1, negative dog; lanes 2–10, positive dogs; NC, negative control; PC, positive control. (b) MW, molecular weight 50 bp ladder (Invitrogen, Carlsbad, CA, USA), lanes 1–3, positive controls represented by *Leishmania amazonensis*, *L. braziliensis* and *L. infantum*; Lanes 1–6, positive dogs for *L. infantum*.

areas. During our statistical analyses, we could not correlate their answers to any sociodemographic parameter. For this reason, a more detailed epidemiological analysis crossing those variables was not performed.

4.3. Dog Survey and Detection of Leishmania Infantum. The domestic dog (*Canis familiaris*) is the main reservoir for VL and responsible for the endemic foci of leishmaniasis in urban and rural areas [3, 44]. In many transmission areas, a high incidence of human cases overlaps with high prevalence in canines [5, 45]. A recent study in Montes Claros, Minas Gerais, confirmed this using geo-referenced data identifying the main transmission areas in the city [46]. Since the dog was identified as the most common domestic reservoir by questionnaire respondents, our next step was to investigate the occurrence of canine VL in the city of Divinópolis. For this purpose, five strategically located veterinary clinics (Figure 1) selected animals suspected of being infected with *Leishmania* for the survey. The veterinaries knowledge was not assessed in this survey. After serological diagnosis, the presence of *Leishmania* sp. was detected. However, ELISA and RIFI did not identify the species involved. A more sensitive PCR-RFLP technique was performed that confirmed the presence of the parasite and identified *L. infantum* as the etiological agent of VL in Divinópolis. These data confirmed the parasite in the urban area and the dog as an important reservoir in the city. However, a more detailed epidemiological study is still warranted to describe incidence and prevalence.

5. Conclusions

This is the first study assessing public knowledge of several aspects of leishmaniasis in Divinópolis, Brazil, where many human cutaneous and visceral cases have been reported in the past two decades. Our data indicated that public knowledge is sparse and fragmented, suggesting the urgent need for leishmaniasis education and development of preventive methods. The study also demonstrated for the first time the occurrence of *L. infantum* in the canine population of the surveyed region, reflecting a possible disease urbanization process in recent years.

Acknowledgments

This work was supported by the Fundação de Amparo à Pesquisa de Minas Gerais (FAPEMIG, Processo APQ-016957-11). R. P. Soares and M. N. Melo are research fellows of Conselho Nacional de Pesquisa e Desenvolvimento (CNPq, 305042/2010-6). M. N. Rocha is supported by CNPq (142361/2009-7).

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Hindawi Publishing Corporation
Journal of Tropical Medicine
Volume 2012, Article ID 534649, 4 pages
doi:10.1155/2012/534649

Research Article

Mortality Related to Chagas Disease and HIV/AIDS Coinfection in Brazil

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Received 30 May 2012; Accepted 27 July 2012

Academic Editor: Maria Aparecida Shikanai Yasuda

Chagas disease in patients with HIV infection represents a potentially serious event with high case fatality rates. This study describes epidemiological and clinical aspects of deaths related to Chagas disease and HIV/AIDS coinfection in Brazil, 1999–2007. We performed a descriptive study based on mortality data from the nationwide Mortality Information System. Of a total of about 9 million deaths, Chagas disease and HIV/AIDS were mentioned in the same death certificate in 74 cases. AIDS was an underlying cause in 77.0% (57) and Chagas disease in 17.6% (13). Males (51.4%), white skin color (50%), age group 40–49 years (29.7%), and residents in the Southeast region (75.7%) were most common. Mean age at death was significantly lower in the coinfected (47.1 years [SD ± 14.6]), as compared to Chagas disease deaths (64.1 years [SD ± 14.7], $P < 0.001$). Considering the lack of data on morbidity related to Chagas disease and AIDS coinfection, the use of mortality data may be an appropriate sentinel approach to monitor the occurrence of this association. Due to the epidemiological transition in Brazil, chronic Chagas disease and HIV/AIDS coinfection will be further complicated and require the development of evidence-based preventive control measures.

1. Introduction

Chagas disease, caused by the protozoan parasite *Trypanosoma cruzi*, is a well-known opportunistic infection in people living with HIV/AIDS [1–6]. Reactivation of chronic indeterminate Chagas disease in patients with HIV infection represents a serious event with high case fatality rates [1, 3, 4]. New aspects of the immunopathology of Chagas disease have been described recently in patients infected with HIV, and unusual clinical manifestations such as skin lesions, involvement of the central nervous system (meningoencephalitis), and/or serious heart damage (myocarditis) related to the reactivation of the disease have been reported [2–4].

The first case of HIV/*T. cruzi* coinfection was reported in the 1980s, but data on several issues are still scanty, such as the frequency of its occurrence, clinical and laboratorial profile of subjects with coinfection, survival rates, and mortality [1, 7, 8].

Chagas disease is endemic in 21 Latin American countries. Due to migration of Latin Americans, an increasing public health impact has been observed in nonendemic countries, such as in Australia, Canada, Japan, Spain, and the United States [5–7, 9]. Thus, the overlap of HIV infection and *T. cruzi* may occur not only in endemic areas, but also in wealthier regions that receive an increasing number of potentially infected migrants [1, 6].

Despite the relevance, the clinical importance of this coinfection and its epidemiology is unknown in Brazil and other endemic countries [1]. Here, we present an analysis of deaths related to Chagas disease and HIV/AIDS coinfection in Brazil, based on multiple causes of death.

2. Materials and Methods

2.1. Study Design and Population. We performed a descriptive study on population-based nationwide mortality data, obtained from the Brazilian Mortality Information System (*SIM—Sistema de Informação sobre Mortalidade*). *SIM* data sets are based on the death certificates (*Declaração de óbito*), consisting of standardized forms to be filled out by the physicians in charge. Death certificates contain demographic data (age, gender, education, race, marital status, municipality of residence, and municipality of occurrence of death) and clinical information (underlying and associated causes of death).

SIM data are public domain and freely available at the website of the Informatics Department of Unified Health System (*DATASUS*, http://tabnet.datasus.gov.br/cgi/deftohtm.exe?sim/cnv/obt10uf.def).

We included deaths that occurred in Brazil between 1999 and 2007, in which Chagas disease and AIDS were mentioned in the same Death Certificate, both as underlying or associated cause of death (so-called multiple causes of death).

2.2. Data Processing and Analysis. Downloading of data sets and data processing has been described in detail previously [10]. Briefly, a total of 243 mortality data sets with about 9 million entries were downloaded. We obtained the study population of coinfected individuals by selecting data sets where Chagas disease and HIV/AIDS were mentioned in any field of death certificates. Available demographic and clinical data were used to characterize the study population. We described frequencies and proportions by gender, age, race, region of residence, residence and occurrence in state capital, and year of death.

Chagas disease as a cause of death corresponded to the category B57 (Chagas disease), including all clinical forms of the Tenth Revision of the International Statistical Classification of Diseases and Related Health Problems (ICD-10) [11]. HIV/AIDS as a cause of death was identified in ICD-10 by the group B20-B24 (disease by human immunodeficiency virus—HIV) [11].

In addition, we present clinical forms of Chagas disease and HIV/AIDS that were associated with coinfection. The description was performed by disease or disorders coded according to ICD-10. Individual death certificates may have more than one clinical form of Chagas disease or AIDS.

Data were stored and analysed by STATA version 11 (Stata Corporation, College Station, TX, USA).

2.3. Ethics. This study was solely based on publicly available secondary anonymous data, with no possibility of identification of individuals. Thus, approval by an ethical review board was not necessary.

3. Results

Between 1999 and 2007, a total of 8,942,217 deaths occurred in Brazil, with 53,930 (0.6%) deaths related to Chagas disease, and 103,075 (1.1%) related to HIV/AIDS. We identified 74 deaths in which Chagas disease and HIV/AIDS were mentioned on the same Death Certificate, either as underlying or associated causes of death.

Of these coinfected cases, AIDS was an underlying cause of death in 57 (77.0%), while Chagas disease was listed in 13 (17.6%). Chagas disease and HIV/AIDS were presented as associated causes in four deaths with other underlying causes: liver cancer (2), acute myocardial infarction (1), and skin abscess (1).

Table 1 depicts epidemiological characteristics of deaths related to Chagas disease and HIV/AIDS coinfection. Males (51.4%), whites (50%), 40–49 year olds (29.7%), and residents of the Southeast region (75.7%) were most common. The mean age at death was significantly lower in the coinfected (47.1 years [SD ± 14.6]), as compared to the total number of Chagas disease deaths (as published previously [10]: 64.1 years [SD ± 14.7], $P < 0.001$).

Table 1: Sociodemographic characteristics of deaths related to Chagas disease and HIV/AIDS co-infection in Brazil, from 1999 to 2007 ($n = 74$).

Characteristics	*N*	%
Sex		
Male	38	51.4
Female	36	48.6
Age group (years)		
<15	2	2.7
15–29	4	5.4
30–39	18	24.3
40–49	14	18.9
50–59	22	29.7
60–69	11	14.9
≥70	3	4.1
Age at death		
<50	38	51.4
≥50	36	48.6
Race/color		
Caucasian	37	50.0
Brown	18	24.3
Black	11	14.9
Ignored	8	10.8
Region of residence in Brazil		
Southeast	56	75.7
Central West	10	13.5
Northeast	4	5.4
South	4	5.4
Residence in state capital		
No	51	68.9
Yes	23	31.1
Death in state capital		
Yes	39	52.7
No	35	47.3

Table 2: Distribution of deaths related to Chagas disease and HIV/AIDS, according to clinical presentation, Brazil, from 1999 to 2007 ($n = 74$).

Clinical form (ICD-10)	*N*	%
HIV/AIDS*		
HIV disease resulting in other infectious and parasitic diseases (B20.8)	28	37.8
Unspecified human immunodeficiency virus [HIV] disease (B24)	22	29.7
HIV disease resulting in multiple infections (B20.7)	18	24.3
HIV disease resulting in other bacterial infections (B20.1)	3	4.1
HIV disease resulting in multiple diseases classified elsewhere (B22.7)	2	2.7
HIV disease resulting in mycobacterial infection (B20.0)	1	1.4
HIV disease resulting in other specified conditions (B23.8)	1	1.4
Chagas disease**		
Chagas disease (chronic) with heart involvement (B57.2)	57	77.0
Acute Chagas disease with heart involvement (B57.0)	6	8.1
Acute Chagas disease without heart involvement (B57.1)	5	6.8
Chagas disease (chronic) with nervous system involvement (B57.4)	5	6.8
Chagas disease (chronic) with digestive system involvement (B57.3)	2	2.7
Chagas disease (chronic) with other organ involvement (B57.5)	1	1.4

* In one case two clinical forms were presented.
** In two cases two clinical forms were presented.

Clinical manifestations of HIV/AIDS included other infectious and parasitic diseases (ICD-10: B20.8) (37.8%) and unspecified HIV disease (B24) (29.7%; Table 2). In Chagas disease, the chronic cardiac forms (B57.2) were predominant (77%; Table 2). Acute Chagas disease with cardiac involvement (B57.0) (8.1%) and chronic Chagas disease affecting the nervous system (B57.4) (6.8%; Table 2) were more common among the HIV-infected as compared to all deaths by Chagas disease (2.5% and 0.3%, resp.).

4. Discussion

This is the first national population-based analysis of Brazilian mortality data related to Chagas disease and HIV/AIDS coinfection. In fact, *T. cruzi*/HIV coinfection has not been systematically evaluated in the majority of endemic countries for Chagas disease [7]. The data show an association of coinfection with early mortality, compared to deaths from Chagas disease only, as described in a previous study [10]. The magnitude of both AIDS and Chagas disease in Brazil as chronic conditions will probably increase the likelihood of occurrence of coinfection in the future [12].

The epidemiology of Chagas disease has changed in recent decades, with a shift to older age groups, as a consequence of the control of its main vector (the kissing bug *Triatoma infestans*) and the control of transmission by blood transfusion [1, 10]. The control of these main means of transmission of Chagas disease may have caused this observed higher frequency of deaths in the chronic phase [10, 13]. Our study shows that in contrast to this trend deaths from coinfection were found predominantly in young adults. Our observation is consistent with the epidemiological profile of coinfected subjects described in previous studies: adult males from endemic regions, with serological diagnosis in the indeterminate form of the chronic phase and reactivation of Chagas disease [7].

The lower survival rate of subjects with coinfection is related to reactivation of Chagas disease and complications of both diseases [7]. Myocarditis and meningoencephalitis played also an important role in coinfection deaths as compared to Chagas disease. This indicates that Chagas reactivation in the central nervous system and myocardium is usually severe, often with fatal results [7]. Reactivation is suspected when the coinfected subject presents clinically acute Chagas disease or clinical decompensation of the chronic phase, organic impairment uncommon in Chagas disease, or pseudotumoral brain lesions [5, 7, 12]. In the case of absent or controlled reactivation, survival is directly related to the complications of Chagas disease and of HIV/AIDS infection. In the case of central nervous system involvement, delay in diagnosis of Chagas neurological damage and late introduction of specific therapy against *T. cruzi* increases case fatality [3, 8]. However, predictive factors for reactivation of Chagas disease are not yet fully understood [7, 14].

Reactivation of Chagas disease has been recognized as an opportunistic disease and was included as an AIDS-defining event in Brazil in 2003 [15]. Brazil has developed since 2006 a National Network of Attention and Studies in *T. cruzi*/HIV coinfection that currently involves cooperation with other countries, like Argentina and Spain [1].

Overlapping of HIV and *T. cruzi* infections also occurs in nonendemic areas of North America and Europe. The implementation of screening programs for migrant populations is necessary for early diagnosis of Chagas disease [6, 9].

Due to the lack of systematic data on morbidity related to Chagas disease and AIDS coinfection, the use of mortality data may be an appropriate sentinel approach to monitor the occurrence of this association. Mortality data can be considered as valid in Brazil, as they are well recorded in DATASUS database and undergo quality control [16, 17]. Limitations of the study may include problems arising from

disease notification and data entry [16], and secondary data may have shown inconsistencies in the quantity and quality of information [17]. Deaths may be underreported, despite the progress made during the observation period in terms of *SIM* coverage and quality of information on causes of deaths. The coverage (ratio of deaths reported/estimated) also presents variations between regions in the country, with lower coverage mainly in the North and Northeast Regions [17]. The results of this study show internal consistency and coherence with existing knowledge about Chagas disease and HIV/AIDS.

We consider data as highly representative, since all death certificates during the period 1999 to 2007 were included, in a country of continental dimensions.

5. Conclusions

The use of multiple causes of death allowed to describe the magnitude and epidemiological characteristics of mortality related to Chagas disease and HIV/AIDS coinfection in Brazil. Due to the ongoing epidemiological transition from the predominance of infectious diseases to more chronic and lifestyle-related ones in Brazil, chronic Chagas disease and HIV/AIDS coinfection require comprehensive and reliable information that supports the development of preventive control measures. There is a clear demand for comprehensive care from primary service providers to reference centers and to structure a network of comprehensive care to deal with this situation, with the mobilization that goes from primary care to highest level of technological complexity.

Acknowledgments

JH is a research fellow at the *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq/Brazil). The authors would like to thank the *Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES/Brazil) for granting a Ph.D. scholarship to CHA and *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq/Brazil) for granting a Master scholarship to FRM. The authors would like also to thank Duane Hinders for critically reviewing the paper.

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