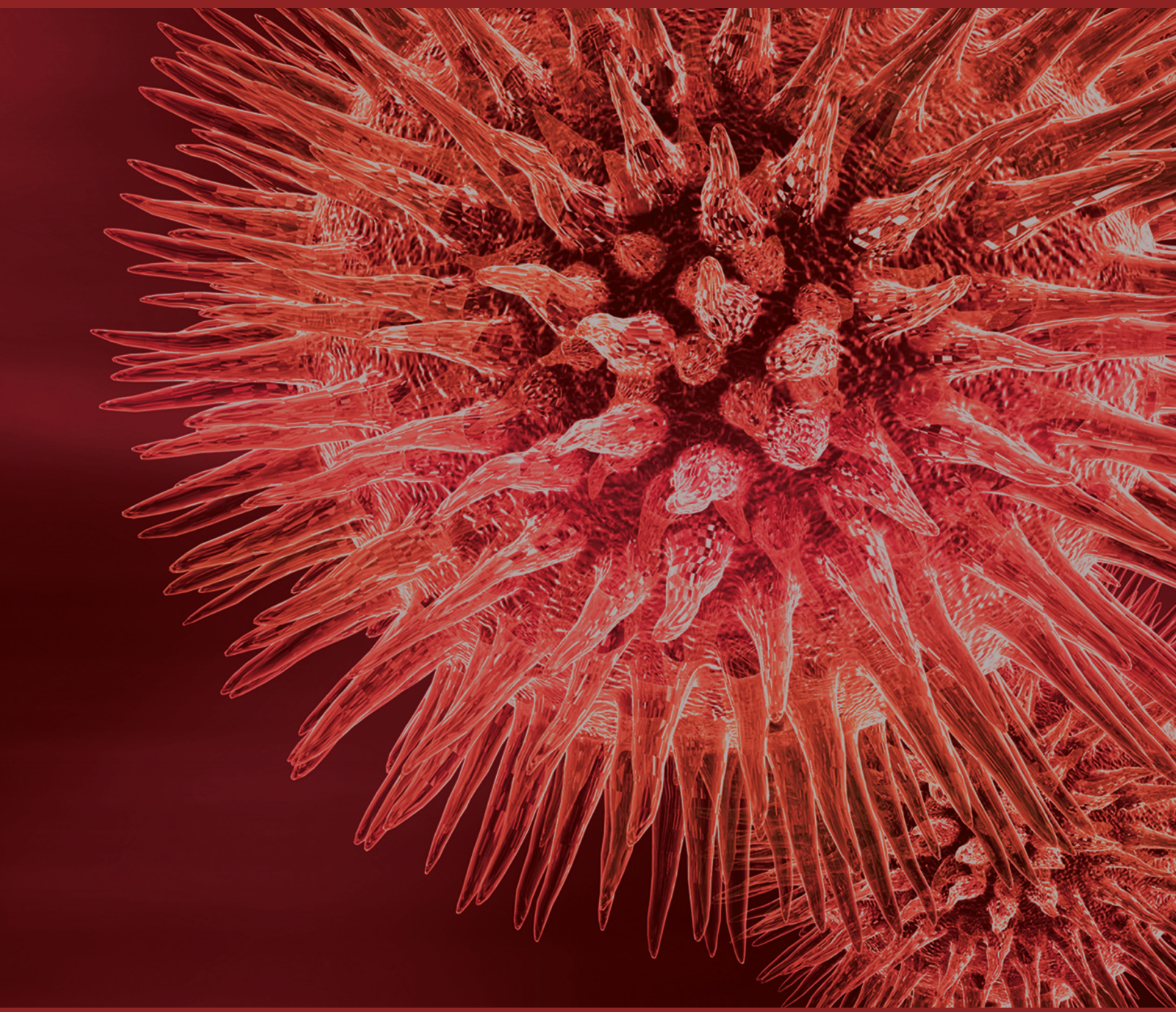
BioMed Research International

Control of Vector-Borne Human Parasitic Diseases

Guest Editors: Fernando A. Genta, Hector M. Diaz-Albiter, Patrícia Salgueiro, and Bruno Gomes



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Editorial

Control of Vector-Borne Human Parasitic Diseases

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Vector-borne diseases (VBD) transmitted by arthropods are responsible for over 1 billion cases and 1 million deaths every year, corresponding to at least 17% of all infectious diseases in human populations [1]. Among them, we can find malaria, leishmaniasis, onchocerciasis, lymphatic filariasis, Chagas disease, and African trypanosomiases, as well as several arboviral diseases (arthropod-borne virus) such as dengue and Zika virus. Some of these have reemerged in new parts of the world and have become a topic of growing importance in public health and in political and scientific agendas [2]. Several factors are contributing towards the reemergence of VBDs. On the one hand, the spread of resistance to drugs in pathogens has become a major obstacle for the effective treatment of some VBDs [3], and the emergence of new strains of arboviruses (*e.g.,* Zika virus in Brazil) has created new challenges for health care systems [4]. On the other hand, an increase in insecticide resistance is threatening the sustainability of vector control programmes in several tropical regions [5]. Additionally, the expansion of different vector populations due to climate change is becoming a growing concern in temperate countries, where vector control programs have been discontinuous for almost 50 years [6, 7]. The scientific community has been trying to overcome these challenges by creating new strategies and tools to improve the diagnosis and treatment of VBDs and by developing new methodologies and targets for vector control campaigns. This special issue of BioMed Research International compiles nine topical articles that explore recent advances in research of an eclectic range of pathogens, vectors, and human diseases affecting several regions of the world.

Malaria remains the human parasitic disease with the highest burden and with risk of reemergence in several areas worldwide. In this special issues there are four papers regarding malaria.

Dahalan et al. [8] focus on a promising target for antimalarial drug, the mitogen-activated protein kinase 2 (*PfMAP2*) and describe for the first time its activity, function, and expression throughout the cycle of the main malaria parasite *Plasmodium falciparum*.

Degarege and Erko [9] summarize the findings of epidemiological studies of *Plasmodium* and helminth coinfection, emphasizing the impact of the coinfection on malaria in a review article.

Mbengue et al. [10] present the results of an IgG binding assay able to discriminate the outcome of cerebral malaria

cases in Senegal, with the prospect of a potential functional-associated assay for symptomatic malaria analysis.

Finally, Ivanescu et al. [11] discuss the association between increasing rates of malaria over a time period where environmental temperature is also increasing. Based on an extrapolation of the climate conditions they predict the risk of malaria re-emergence in Romania.

Aedes aegypti is one of the most important disease vectors in the world. Its control is the main tool available to fight transmission of diseases such as dengue, Zika, or chikungunya. Bellinato et al. [12] bring us an overview of the resistance profile of *A. aegypti* to several insecticides in Brazil, the country most affected currently by dengue and Zika virus.

Presently, lice infestations occur worldwide despite great efforts to maintain high standards of public health. Infectious diseases transmitted by lice remain a public health concern in populations living in crowded and unsanitary conditions, a matter of great concern regarding refugee care. Sangaré et al. [13] detail a state-of-the-art review on the "Management and Treatment of Human Lice," which, like for many other vectors, have been highly affected by insecticide resistance.

Leishmaniasis in the Old and New World is transmitted via the bite of phlebotomine sand flies and is caused by kinetoplastid parasites belonging to the genus *Leishmania*. This collective group of diseases is distributed in 88 countries around the globe with up to 1.6 million estimated cases per year [14–16]. To date, there is no human vaccine and treatment is largely based in 1940's antimony-based drugs which use causes distressing side effects. To date, control of transmission of leishmaniasis has focused mainly on the use of insecticides, a strategy that will eventually result in selection of resistant strains of insects, as seen in other insect vectors, such as *Aedes aegypti* and *Anopheles* spp. The World Health Organization has emphasized the need of developing novel strategies and research on these neglected vector-borne diseases. This special issue includes two papers on leishmaniasis.

Hijjawi et al. [17] assessed the use of molecular tools for the study of human leishmaniasis cases in Jordan, showing that identification of parasite species from dry samples is possible and improves clinical diagnosis. Besides that, they report the occurrence of *Leishmania tropica* in Jordan, which could be a new epidemiological concern related to the Syrian crisis.

Mohamed Mahmoud et al. [18] identified the presence of *Leishmania major* and *L. tropica* in a cutaneous leishmaniasis foci in Errachidia, Morocco, using molecular techniques. The authors also discuss the geographical distribution of *Leishmania* spp. in this area and report, for the first time, the presence of *L. tropica* in the region.

Chagas disease is still a major parasitic disease in the Americas, with up to 7 million cases worldwide. It has gained recent attention due to imported cases in nonendemic regions like USA (>300,000 cases) or Europe (>80,000 cases) [19]. It is caused by the parasite *Trypanosoma cruzi*, which is transmitted by the feces and urine of triatomine kissing bugs. The resurgence of vector populations and some reports of insecticide resistance in triatomines make of special interest the study and development of new strategies for control of these vectors [20]. Henriques et al. [21] studied in detail the action of the Insect Growth Regulator (IGR) Triflumuron (TFM) in *Rhodnius prolixus*, which is a model for triatomine biology. This work shows important effects of TFM on female fertility and gives some new insights in the mechanism of action of this insecticide, as impairments in diuresis and chitin turnover with reflections in insect immunity.

Taken together, the articles in this special issue cover several important aspects of major VBDs, highlighting not only their impact in human health, but also their significance for the development of new concepts and tools for the medical and biological research.

Tribute to Dr. Bruce Alexander. During our time as guest editors for this special number, we lost our friend and colleague John Bruce Alexander, who sadly passed away last March after a quiet battle against cancer. Bruce worked extensively in countries such as Colombia, Ecuador, and Brazil, being an international reference in the field of sampling and control of phlebotomine sandflies. Besides contributing with essential reviews and discussions to the field of leishmaniasis [22–25], he participated in seminal works related to the most diverse topics related to this neglected tropical disease, including transmission by midges in Australia [26], insecticide use and resistance in sandflies [27–30], role of chickens [31, 32], dogs [33, 34], and plants [35, 36] in the maintenance of vectors and parasite life cycle, taxonomic description of new sand fly species [37, 38], sand fly sex pheromones [39, 40], RNAi in sand flies [41], and biological control of sand flies [42]. His work relates to current and successful interventions for disease control, especially in the New World. Because his contribution to this special issue was interrupted in the presubmission stage, we decided to honor him with a very short obituary note here.

Bruce graduated in biology in the University of Edinburgh in 1979 and got his M.S. degree in entomology in the University of London. In his time there, Bruce met Dr. Robert Killick-Kendrick, who introduced him to the study of sand flies. A few years later, Bruce carried out a Ph.D. degree in Florida University on the ecology of sand flies in Northeast Colombia, learning Spanish and Portuguese and working on different aspects of phlebotomine biology. Later he moved to Brazil, where he worked at the Universidade Federal de Minas Gerais and FIOCRUZ. He moved back to the UK and in 2005 started working at the Liverpool School of Tropical Medicine. Later in his professional life, Bruce decided to become an independent researcher and together with his wife Cristina, he successfully started Xeroshield Ltd. in 2005 and Garrapat Ltd. in 2015. The spirit of both companies was to offer "practical solutions to insect control and vector-borne disease prevention to offer safe, sustainable, environmentally friendly alternatives to chemical pesticides." He was an enthusiastic ornithologist, as well. He is survived by his wife, Cristina, and his son, Patrick. We will dearly miss his sense of humor, his wit, and passionate and argumentative discussions.

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Fernando A. Genta
Hector M. Diaz-Albiter
Patrícia Salgueiro
Bruno Gomes

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Research Article

Climate Change Is Increasing the Risk of the Reemergence of Malaria in Romania

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The climatic modifications lead to global warming; favouring the risk of the appearance and development of diseases are considered until now tropical diseases. Another important factor is the workers' immigration, the economic crisis favouring the passive transmission of new species of *culicidae* from different areas. Malaria is the disease with the widest distribution in the globe. Millions of people are infected every year in Africa, India, South-East Asia, Middle East, and Central and South America, with more than 41% of the global population under the risk of infestation with malaria. The increase of the number of local cases reported in 2007–2011 indicates that the conditions can favour the high local transmission in the affected areas. In the situation presented, the establishment of the level of risk concerning the reemergence of malaria in Romania becomes a priority.

1. Introduction

Malaria is a widely spread disease in the tropical and subtropical areas [1].

It is a worldwide spread disease, millions of people being affected by it every year in Africa, India, South-East Asia, the Middle East, and Central and South America, which means that almost 50% of the world's population is at risk of being infected with malaria [2]. Every year, millions of sick people or people carrying the disease travel to malaria-free countries, reintroducing the reemergence risk of this disease. In 2010, Snow and colleagues presented a new cartographic technique that would produce a real image of the spread of malaria worldwide. Only the health reports of 7 out of the 87 endemic countries may be considered rigorous and therefore may be used for the information they provide. There has been a quantification of the anthropic impact on the distribution of malaria in the XXth century, using the geographic information systems and historical maps for the areas with continuous transmission of the disease; this overall estimate shows that malaria prevalence is 50% bigger than that reported by the World Health Organization (WHO) and 200% bigger in the case of the areas outside Africa [3–5]. Climatic factors are directly involved in the geographic distribution and transmission of malaria [6, 7], playing a very important role in the malaria reemergence risk in some parts of the world, where the vectors are present. In 2006 it was proved that an increase of temperatures by 0.5°C in the mountainous areas of Africa would lead to a 100% increase of the mosquito population, which would result in a rapid spread of the disease in this area with sporadic cases as well [8].

In 2011, there have been 40 cases of people infected with *Plasmodium vivax* reported in Greece, in five different districts, in the case of patients with no history of travelling to a malaria-endemic area. Currently, there are preventive measures in place, on a seasonal (spring–autumn) basis, in areas which do not present any risk of spreading malaria in other countries, the affected areas being agricultural and not tourist ones (epidemiological update: malaria in Greece,

20th July 2012, European Centre for Disease Prevention and Control). Some cases of autochthonous malaria have been reported in Germany as well [9]. In the last 10 years, the sporadic malaria transmission has been reported in many countries of Europe: Bulgaria, France, Germany, Greece, Italy, and Spain. Between 21st May and 5th December 2011, in Greece, there have been reported 63 cases of malaria, caused by *Plasmodium vivax*. Based on all available information, it appears that the malaria transmission in Greece would be a result of the annual introduction of the parasite in the country, through the presence of immigrants [10]. Malaria is rarely diagnosed in Europe, but it represents a medical emergency. Malaria was eradicated in Europe, with the exception of Azerbaijan, Georgia, Kyrgyzstan, Tajikistan, and Turkey [11].

Malaria was eradicated in Romania in 1965, being considered in 1967 a malaria-free area by the World Health Organization (WHO) [12]. The presence of the *Anopheles* vector, belonging to the *Anopheles maculipennis* complex, and the existence of the malaria agent in nature with imported malaria cases, as well as the increase in temperatures, are favourable factors for the reemergence of malaria in Romania [13, 14]. All cases of malaria diagnosed in Romania have been "imported," increasing continuously because of the development of tourism and the labour market in malaria-endemic areas [15–17]. The studies carried out by us in Iaşi, Romania, reported the presence of five species of mosquitoes belonging to the *Anopheles maculipennis* complex: *A. atroparvus, A. melanoon, A. maculipennis*, *A. labranchiae, and A. messeae*, species incriminated in the transmission of malaria in Europe. *A. labranchiae* was first reported in Romania, being considered one of the main malaria vectors in Europe, its identification being done using the PCR technique and sequencing, being able to also find the fourth-stage larvae, which indicates that the species adapted to the climate specific to the area [17].

The presence of this species in Romania shows on the one hand the existence of favourable climatic factors, caused by global warming, and on the other hand the increase in people's travels, thus suspecting that the species was "imported" from Italy, Sicily, where many Romanians are working [18, 19].

2. Material and Method

The study carried out had the purpose of establishing the existence of the risk of malaria reemergence in Romania on the basis of the coexistence of the most important factors: the presence of the pathogen causing malaria and the existence of favourable climatic factors for the development of the *Anopheles* vector and its existence in nature.

The work of establishing the risk of malaria reemergence in Romania based on the existence of the malaria agent in nature was done based on the analysis of the data concerning the malaria cases diagnosed in Romania, coming from four diagnosis centres from the country: the Public Health District Authority of Iaşi, Infectious Diseases Hospital of Iaşi, Cantacuzino National Institute of Research, and "Victor Babeş" Infectious and Tropical Diseases Hospital. We must note that in Romania there is not a common, centralised database, where all diagnosed malaria cases are reported; therefore all information presented may not paint 100% the real situation. The cases were distributed and analysed over the course of 7 years, according to the following criteria: age, profession, the strain of *Plasmodium* diagnosed, and the continent where the patients acquired the infection.

Having in mind the purpose of monitoring the influence of the environmental factors on the life cycle of the mosquito vector and on the development of the malaria pathogen inside the vector, we did an analysis of the temperatures recorded between 1961 and 2014, at five weather stations in the country (from Iaşi, Cluj-Napoca, Arad, Bucharest, and Constanţa), all information being granted by the National Weather Administration. There was also a weather forecast made for the year 2030, in order to anticipate the possibility of the malaria reemergence risk in Romania, thus associating with this one the other two essential factors: the mosquito vector and the existence of the malaria pathogen in nature.

The mathematical model we proposed, called ET30, is based on the construction of a nonlinear mathematical function (Lagrange polynomial) which allows us to estimate the average temperature values in the next years, avoiding the weather forecast with fixed point, which is very difficult to do for a short period of time (days) and practically impossible to realise for a longer period of time, such as decades [20]. Using this type of mathematical algorithm allows us to find a function F, through the interpolation of the experimental measurements, which would estimate the average temperature of each month for the year 2030, according to the climate tendency recorded in the last five decades. From a mathematical point of view, the experimental measurements are the solutions to an unknown function f, defined as a table function, represented by the values of air temperature, at the height of 2 m above the ground $(T_{\text{January1961}}, T_{\text{February1962}}, \ldots, T_{\text{December2013}})$, recorded between January 1961 and December 2014, called interpolation points or interpolation nodes. The interpolation function F must have the value of the unknown function f, in the interpolation points T_i, must be a continuous function along the period of time during which the measurements were taken (January 1961–December 2014), and must also be a Lagrange polynomial nonlinear function. Having in mind the purpose of increasing the estimation accuracy of temperatures at ground level, in the next decades, we have used the mathematical model for three sets of temperature values recorded between 1st of January 1961 and December 2014: the first set of values is represented by the average values of the air temperature, during the day, calculated with the arithmetic mean of the values recorded in a day, the second set of values is represented by the average of monthly temperatures, calculated with the arithmetic mean of the values recorded in a month, during the daytime, and the last set of values is represented by the average of temperatures recorded during five consecutive years, for the same month. We have proposed this estimation method with the purpose of reducing the daily fluctuations. In order to see the reliability of the model, it was tested for the years 1991

and 1992, by having built a model, in the same way as was previously presented, for the values recorded between 1st of January 1961 and 31st December 1990 and another model for estimating the values for the years 2011, 2012, and 2013, using the values recorded between 1st of January 1961 and 31st December 2010. The program sequences of model ET30 were calculated using the programming language FORTRAN, the data processing being done using the OringiPro.

3. Results and Discussion

In Romania, the number of malaria cases grew during the war; the strains of *Pl. falciparum* introduced on this occasion increased the gravity of the cases and the mortality rate. The situation was quite drastic in the county of Tulcea, in 1946. In some towns or villages, the malaria infection affected 70% of the population.

Between 1947 and 1948, the fight for the eradication of malaria was at its peak, having used DDT (dichlorodiphenyltrichloroethane), the results however not being exactly in accordance with what had been targeted, namely, complete eradication of the disease. The campaign against malaria continued, starting from 1955 with eradication plans for the disease. The last case was diagnosed locally in 1961. In 1965, the malaria eradication campaign ended, Romania being declared a malaria-free country by the World Health Organization (WHO) in 1967.

Starting from 1967 (the year starting which we had all information regarding the diagnosed cases of malaria) and until 2014, there have been 814 cases diagnosed in Romania, all of them being "imported." Regarding the details about the profession, the age, the strain of *Plasmodium* diagnosed, and the continent where the patients acquired the infection, we had such information only for the last 7 years. In 2008, there were 13 cases diagnosed, in 2009 there were 12 cases diagnosed, in 2010 there were 19 cases diagnosed, in 2011 there were 42 cases diagnosed, in 2012 there were 17 cases diagnosed, in 2013 there were 31 cases diagnosed, and in 2014 there were 39 cases diagnosed, in total being 173 cases, which represent 14% of all diagnosed cases between 1976 and 2014.

Out of the total number of cases diagnosed, 88% of them occurred on the continent of Africa, the continent of Asia following close behind, with 8% of the cases (Figure 1). In 2011, there were 2 cases recorded in Greece (a malaria-free country), these being considered autochthonous cases, which indicates that all three conditions of the vector-borne disease were met: the presence of the *Anopheles* vector, the presence of the *Plasmodium* pathogen, and the existence of favourable climatic factors.

Out of the total malaria cases recorded, 59% were diagnosed in people over 40 years (Figure 2), representing the category of population with the highest labour force level.

Plasmodium falciparum was diagnosed in 75% of the cases, being responsible for the infection with cerebral malaria, all the more dangerous for those living in countries that were not yet affected, meaning that the degree of susceptibility was quite high. *Plasmodium vivax* was responsible for 13% of the cases, being a very dangerous strain, along with

Figure 1: The distribution of malaria cases according to the continent where the infection occurred.

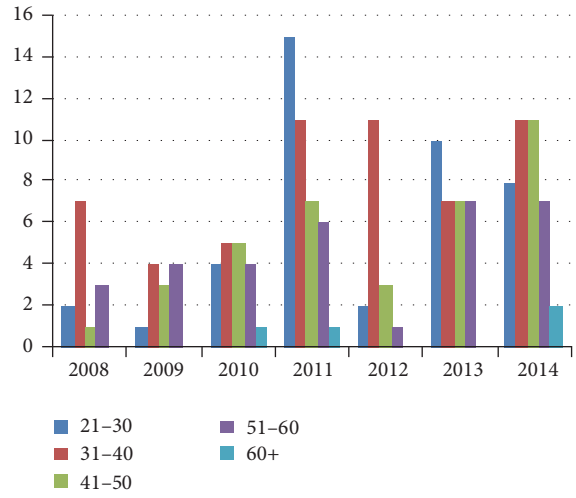

Figure 2: The distribution of malaria cases according to the age factor.

the strains of *Pl. ovale* and *Pl. malariae* (Figure 3), because of the relapse that may occur even 10 years after the infection, the parasite persisting in the patient's liver, in a dormant, hypnozoite stage. The diagnosis was made using multiple techniques: microscopic examination of thick blood smear, rapid diagnostic test, and, in some cases, the PCR test. There is no centralised database in Romania, so many of the patients who were diagnosed with malaria never returned for regular checkups, constituting a real danger in the case of relapse.

In the last 7 years, the labour force was the main reason for travelling, generating 80% of the cases of malaria recorded in Romania, being associated with the onset of the financial crisis of 2007 as well, forcing the population to look for a source of income in places which had not been of any financial interest until that moment (Figure 4). Until 2007, 70% of the total number of diagnosed cases were in the case of people working as sailors. Following close behind, with a percentage of 9%, are students, noting as well an increase

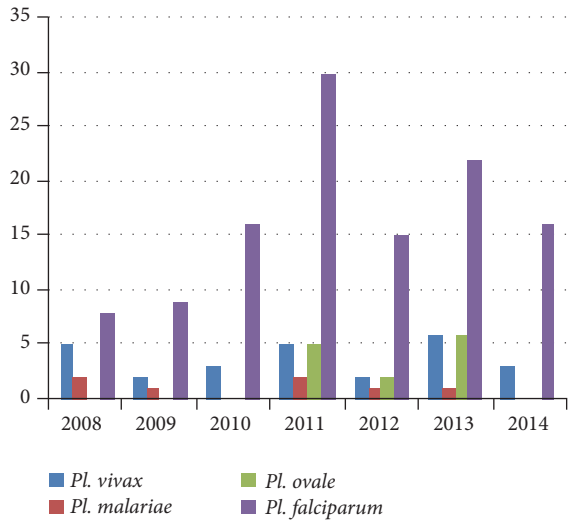

Figure 3: The distribution of malaria cases according to the strain of *Plasmodium* responsible for the infection.

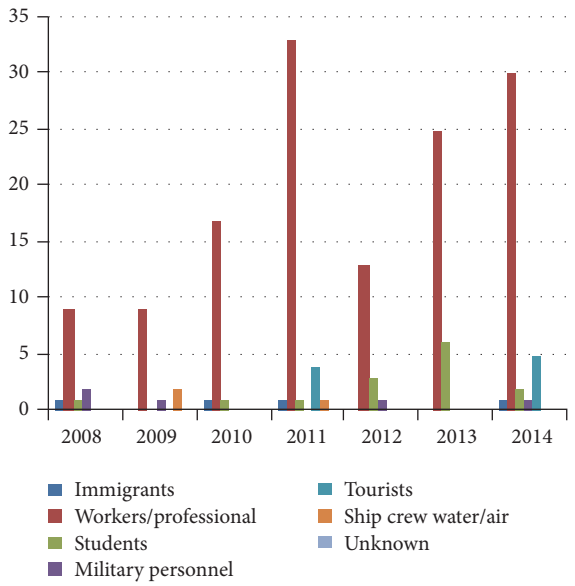

Figure 4: The distribution of malaria cases according to the travel purposes.

of exchange programs and of mobilities between universities on different continents. The majority of people who were diagnosed have not taken any prophylactic medication, have not received any instructions from a doctor before leaving the country, and also did not have any knowledge of the possible dangers existing in tropical areas (the majority of people not having a higher education degree).

The map shown in Figure 5 indicates a higher presence of malaria cases in the North-East and in the South-East parts of the country, areas with a low financial potential and with a high unemployment rate, starting from 2007, after the onset of the financial crisis. Bucharest (the capital city) and Constanţa (maritime city) have been areas of great interest

Figure 5: The distribution of malaria cases recorded in Romania between 1976 and 2014.

Table 1: The increase of current temperatures according to seasons, compared with the malaria eradication period.

Season	(2004–2014) (1961–1970) Iaşi (°C)	(2004–2014) (1961–1970) Romania (°C)
Winter	1.2	1.4
Spring	1.3	0.8
Summer	1.7	0.9
Autumn	0.2	−0.2
Total	1.1	0.725

+ indicates an increase in temperatures.
− indicates a decrease in temperatures.

until 2007, when over 70% of the diagnosed malaria cases were recorded in the case of people working as sailors, after this year the number of cases dropping considerably in these areas, only 13% of malaria cases being recorded.

Using the mathematical model ET30, we realised a temperature curve starting from 1961, until 2014, indicating a constant increase of temperatures by 1.3°C, compared with the malaria eradication period in the county of Iaşi (Table 1).

The temperatures calculated at five weather stations in the country (Iaşi, Bucharest, Arad, Cluj-Napoca, and Constanţa) also show an increase of temperatures, the total being of 0.72°C (Figures 6 and 7).

The second column indicates the increase of temperatures in 2014 (the average for 10 years, 2004–2014), compared with the period in which malaria was eradicated (1961–1970).

The third column indicates the increase of temperatures in 2014, compared with the 60s, for the average calculated from the data provided by the five weather stations in Romania (Iaşi, Bucharest, Arad, Cluj-Napoca, and Constanţa) (Table 1).

Doing an extrapolation of the evolution of temperatures in 2030 (Figure 8), we can see a slight increase of temperatures by an average of 24°C in 2030, which may ensure a favourable climate for the development of Culicidae, the optimal temperatures for development being between 23 and 25°C.

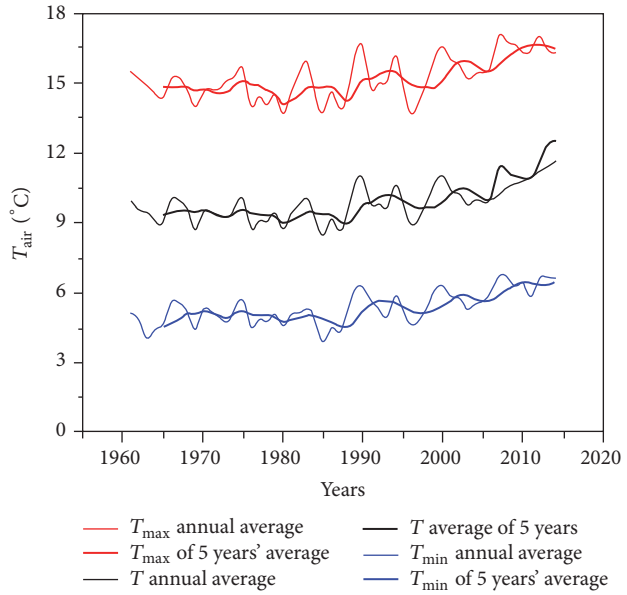

FIGURE 6: The evolution of the annual maximum temperature, the average annual temperature, and the annual minimum temperature between 1st January 1961 and 31st December 2014, also calculated for five consecutive years.

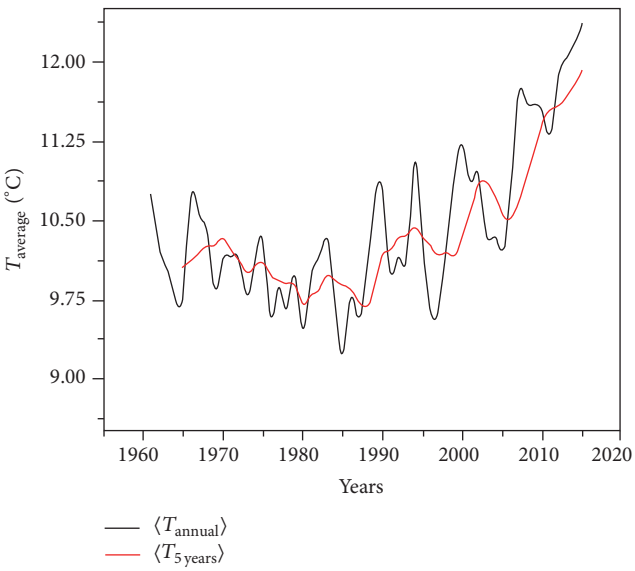

FIGURE 7: The average annual temperature and the average temperature calculated for five consecutive years, both recorded between 1st January 1961 and 31st December 2010, at 5 weather stations in the country.

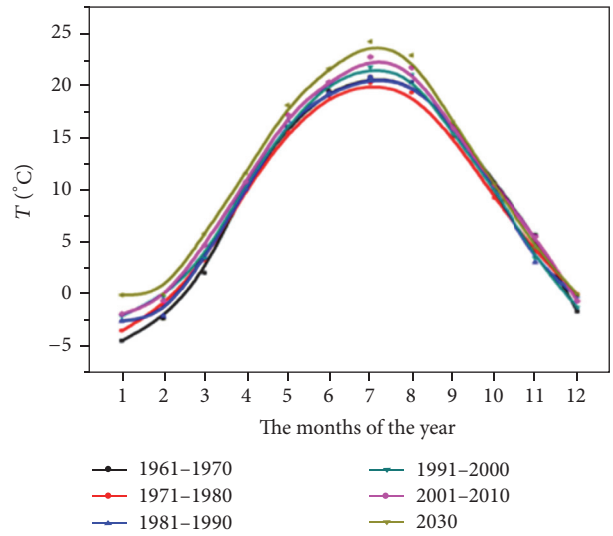

FIGURE 8: The monthly average temperatures calculated according to decades, recorded at the weather station in Iaşi, between 1961 and 2014, and estimated using the mathematical model ET30 for the year 2030, for each of the twelve months.

Anopheles maculipennis is a species widely spread across the country, near forests and on river valleys, the female being zoophilic and occasionally anthropophilic.

Anopheles messeae is spread in all the lowland plains in the country and in places with stagnant waters; it is predominantly zoophilic but may also feed off humans. *Anopheles messeae* may transmit malaria in temperatures even below 4°C, in which case it takes 44 days for the mosquito to become infected. This is how the cases in Scotland and Norway may be explained. In Great Britain, *Anopheles messeae* and *Anopheles atroparvus* are responsible for the cases signaled by Knottnerus [21]. *Anopheles atroparvus* is spread all over the country, in areas with (more or less) salt waters, the female being predominantly anthropophilic. *Anopheles atroparvus* is active in temperatures starting from 15°C, when it may constitute a risk in transmitting malaria.

Anopheles melanoon may be found near fresh waters, marshland areas, stagnant waters covering large areas, the banks of rivers and lakes, ponds, and swimming pools [12].

In Europe and in the Middle East, the transmission of malaria is very low or absent, but there have been some species of the genus *Anopheles* which were considered vectors of malaria: *A. atroparvus*, *A. labranchiae*, *A. messeae*, *A. sacharovi*, *A. sergentii, and A. superpictus* [22].

The illustration of the temperature gradient on the map representing the temperatures in Romania in the month of July was realised using the software ArcGis. For the month of July of the year 2030, we can observe a slight increase in temperatures by 0.9°C (Figure 9).

The estimations obtained by using the model ET30 (slight warming by 0.8°C, Figure 10), which show an increase in temperatures manifesting in the metropolitan area of Iaşi, keep the same tendency like the forecasts regarding the weather at a global level, done by some of the most prestigious research institutes in the world, such as NIES (the National Institute for Environmental Studies in Japan), CCCma (the Canadian Centre for Climate Modelling and Analysis), CSIRO (the Commonwealth Scientific and Industrial Research Organisation in Australia), HCCPR (Hadley Centre for Climate Prediction and Research in the United Kingdom), MPIM (Max-Planck-Institut für Meteorologie), and NCAR (the National Center for Atmospheric Research in the USA). These institutes provide data which show an

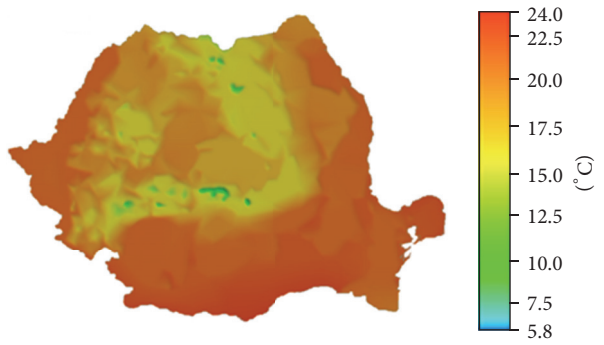

Figure 9: The map of the average temperatures calculated for the month of July in the weather stations of the National Weather Administration network of Romania, for the year 2014.

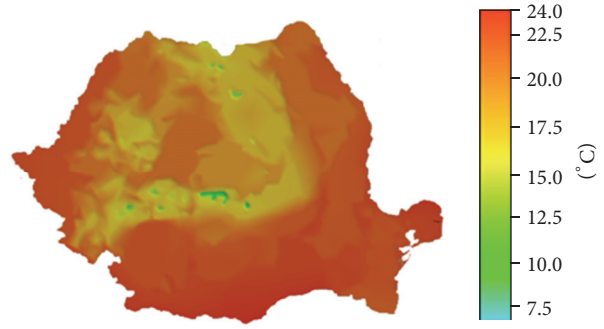

Figure 10: The map of the estimated temperatures for July, for the year 2030, obtained using the model ET30.

increase of temperatures at a global level by 0.8–1.7°C, until the year 2030.

4. Conclusions

Taking into account the coexistence of the two factors that are at the basis of the emergence or reemergence of a vector-borne disease, namely, the presence of the malaria agent in nature, as well as the existence of favourable climatic factors for the development of the *Anopheles* vector and of the parasite inside of it, the risk of malaria reemergence in Romania may become a problem of major interest.

In Romania, there is not a common, centralised database, where all diagnosed malaria cases are reported and also, there is no record being kept; therefore, in many cases there is the risk of having a relapse. Studies regarding the "import" of malaria in Romania have been carried out by researchers such as Neghina et al. in 2008 and 2011, the result being the same: difficulty in diagnosing the disease and in managing the important cases. The annual epidemiological report 2014, emerging and vector-borne diseases, keeps the countries which are part of the European Union (EU) and the European Economic Area (EEA) updated on the situation concerning malaria, which seems to have remained stable, occurring in around one case in every 100.000 inhabitants, registering a slight decrease in 2012, in comparison with 2011 and 2010. 99% of the cases (in the case of which the origin of the disease is specified) are "imported"; these cases occurred in countries belonging to the EU and EEA (European Economic Area), which have strong traditional connections with endemic areas. In Greece, there were some autochthonous cases recorded in 2012 but less than those recorded in 2011. The autochthonous transmission of the disease in the EU remains a possibility and therefore the emphasis is placed on the necessity of having medical supervision for the important cases, training medical staff, educating the population, and improving the access to health care services for migrants. In 2012, there have been 5161 confirmed malaria cases in 25 countries from the EU and in one from Continental Europe. 85% of the cases have been reported by the following five countries: France, Great Britain, Germany, Spain, and Belgium. Most of the confirmed cases have been reported by the United Kingdom, Belgium, Ireland, and Luxembourg.

Twenty-six cases have been identified as being autochthonous, out of which twenty-two were from Greece, three were from Belgium, and one was from France. Therefore, some essential measures need to be put in place in order to fight against malaria and to maintain the status of malaria-free country:

(i) investments in training medical staff and supporting specializations in tropical diseases;

(ii) training medical entomologists, who are very much absent;

(iii) creating a database in order to keep a common record of all the malaria cases recorded at a national level, on Romania's territory;

(iv) following every malaria case diagnosed, through regular medical checkups, especially in the case of cases infected with the strain of *Plasmodium*, which forms hypnozoites in the liver, presenting the risk of relapsing;

(v) providing hospitals with various drugs, specific for malaria treatment, according to the area of infestation;

(vi) keeping an open communication between tourism companies and hospitals, in order to be able to instruct people on the protection measures, in case they travel to tropical areas.

Keeping the status of malaria-free country becomes a growingly serious mission not only for Romania, but also for other countries in Europe.

With every passing year there is an increase in the number of "imported" malaria cases, which proves the lack of information among the population regarding the risk of contracting the disease and the prophylactic measures and treatment needed.

Climatic factors play a very important role and our studies have shown a favourable evolution in this respect, leading to the existence of the emergence or reemergence of some diseases on Romania's territory.

Competing Interests

The authors declare no competing financial interests.

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Research Article

Triflumuron Effects on the Physiology and Reproduction of *Rhodnius prolixus* Adult Females

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We evaluated the efficacy of the growth regulator triflumuron (TFM) in inducing mortality and disrupting both oviposition and egg hatching in *Rhodnius prolixus* adult females. TFM was administered via feeding, topically or by continuous contact with impregnated surfaces. Feeding resulted in mild biological effects compared with topical and impregnated surfaces. One day after treatment, the highest mortality levels were observed with topical surface and 30 days later both topical and impregnated surfaces induced higher mortalities than feeding. Oral treatment inhibited oviposition even at lower doses, and hatching of eggs deposited by treated females was similarly affected by the three delivery modes. Topical treatment of eggs deposited by nontreated females significantly reduced hatching. However, treatment per contact of eggs oviposited by untreated females did not disrupt eclosion. Additionally, oral treatment increased the number of immature oocytes per female, and topical treatment reduced the mean size of oocytes. TFM also affected carcass chitin content, diuresis, and innate immunity of treated insects. These results suggest that TFM acts as a potent growth inhibitor of *R. prolixus* adult females and has the potential to be used in integrated vector control programs against hematophagous triatomine species.

1. Introduction

The insecticide activity of the benzoylphenylurea (BPU) family was discovered in the 1970s by accident during herbicidal screening, after which Diflubenzuron, its first synthesized analogue, started to be commercialized [1]. BPU substances are composed of one benzoyl ring, one aniline ring, and urea bridge, each one playing different roles in activity, varying only in its substituents [2].

There are 15 BPU compounds being commercialized, with over 10000 described [2], which have been used as Insect Growth Regulators (IGRs) against a wide range of crop, pasture, and forest pests [3, 4]. They represent a promising control method for insect populations, able to avoid

the harmful side effects of conventional insecticides on both the environment [5, 6] and human health [7].

BPU substances appear to act by inhibiting the chitin synthesis pathway during insect development [8]. This reduction results in mortality, moulting failure, and/or malformations of the cuticle [9–15]. In addition, adults emerging from BPU-treated larvae display physiological changes, which ultimately lead to decreased physical and reproductive fitness [3]. BPU substances are relatively selective toward arthropods and safe to humans since chitin is absent in vertebrates [16]. Because of that, they are often preferred to broad-spectrum insecticides when control operations are conducted in the field [17, 18].

Chagas disease [19, 20] affects 18 million people in South America [21]. It is transmitted by hematophagous insects of the subfamily Triatominae (order Hemiptera, family Reduviidae) such as *Rhodnius prolixus*. Additionally, *R. prolixus* is a traditional model for physiological and parasite-vector interaction studies [22].

Although several reports describe the effects of BPU substances against disease vectors [23–30], little is known about their implication in the biology and reproductive fitness of Chagas disease vectors [31]. We previously confirmed the effectiveness of triflumuron (TFM) against *R. prolixus* nymphs [32]. The aim of this work is to extend these studies and determine the biological and physiological effects of TFM (Starycide® sc 480 Bayer) on *R. prolixus* adult females. We investigated the effects of different TFM doses administered by three different delivery modes: feeding and topical and continuous contact with impregnated surfaces. Additionally, we investigated some TFM-induced physiological features which might be leading to inhibition of reproduction and insect death. These features include oogenesis, chitin content, diuresis, and prophenoloxidase activation. Altogether, these results might be valuable for evaluation of perspectives for integrated vector control programs against blood sucking triatomine species.

2. Material and Methods

2.1. Insects. Adult females of *Rhodnius prolixus* were reared in laboratory conditions at 28°C and relative humidity of 60–70% [32]. Randomly chosen insects were then allowed to feed upon a membrane apparatus [33] and submitted to biological assays (see Section 2.3).

2.2. Chemicals. We used Starycide sc 480, containing 48 g of TFM per 100 mL, in the experiments. L-3,4-Dihydroxyphenylalanine (L-DOPA) was purchased from Sigma (Cat. no. D9628). Unless mentioned, all solutions were prepared with deionized water (Milli-Q®, Millipore). Other reagents used were of analytical grade.

2.3. Biological Assays. Following ecdysis, we fed fifth-instar female nymphs of *R. prolixus* with defibrinated rabbit blood and separated them until they reached adult stage. After metamorphosis, we submitted primiparous females to three different TFM treatments (see the following). In the control group they were fed with defibrinated rabbit blood only. Oral treatment consisted of adding TFM to the blood meal at concentrations from 0.1 to 10 μL per mL.

Topical treatment consisted of direct application of Starycide to the dorsal surface of each insect abdomen in doses ranging from 0.1 to 10 μL immediately after feeding.

For the impregnated surface treatment, we applied Starycide evenly onto a filter paper placed at the bottom of a Petri dish (⌀ 9 cm) in order to obtain final concentrations ranging from 0.1 to 10 μL/cm^2 after evaporation of solvent.

Immediately after feeding and/or treatment, we placed the adult insects (control and treated groups) in Petri dishes (⌀ 9 cm) by a proportion of one untreated male to 10 treated females and followed them during 30 days. We used only fully engorged insects after only one blood meal throughout the experiments. We replaced the untreated males in case of death. There was no additional surface inside the Petri dish, so the insects were forced to be in contact with the treated paper.

We recorded the biological evaluation of the different treatments by weight of ingested blood, amount of excretion, toxicity (i.e., short and long term mortality, represented by 24 h and 30 days after treatment), malformations of the cuticle, oviposition, and egg hatching.

In another experiment, nonmated adult females were fed with blood containing 1 μL TFM/mL or treated topically with 1 μL TFM after feeding as described above. These insects were weighed daily during 15 days. Controls received no TFM.

Additionally, one-day-old eggs obtained from untreated healthy primiparous females were submitted to topical treatment using 0.1 to 1 μL of TFM applied directly per egg. Eggs deriving from untreated mothers were also submitted to continuous contact with TFM impregnated surfaces (0.1 to 10 μL/cm^2) as described above. The eggs were then observed during 30 days to evaluate hatching. All experiments were repeated at least in triplicate with batches of 10 insects or 10 eggs per group depending on the experiment done.

2.4. Chitin Measurements. We measured chitin according to [34]. After oral (3 μL/mL blood) or topical (3 μL/insect) treatments with TFM, we kept females for 5 days in Petri dishes with an untreated male (mated group) as described above. Another experiment was set without the inclusion of a male in the Petri dish (nonmated group). Insects were ice-anesthetized and then dissected in cold 0.9% NaCl. Gut, ovaries, and fat body were removed and the remaining tissues (named carcass) were then homogenized in liquid nitrogen with the aid of a ceramic mortar and pestle. Powdered tissues from three female insects were then resuspended in 2 mL of water and then 200 μL was centrifuged at 21,000 ×g for 5 minutes at room temperature. We discarded the supernatants, resuspended the pellets in 3% (w/v) sodium dodecyl sulphate, and then incubated them at 100°C for 15 minutes. Samples were cooled and centrifuged as above and supernatants were discarded. Pellets were washed as above with 200 μL water and then resuspended with 150 μL of 2.1 M KOH. Samples were then heated at 130°C for 1 h. After cooling, 400 μL of 75% (v/v) ethanol was added and samples were kept in ice for 15 minutes. Then 60 μL of a Celite 545 suspension

(supernatant of 1 g suspension in 12.5 mL 75% ethanol, resting for 2 minutes) was added. Samples were centrifuged and the pellets were washed with 600 μL of 40% ethanol and then 600 μL of water as above. Those pellets (insoluble chitosan) were frozen at −20°C or kept at 4°C until assay.

Pellets were resuspended in 125 μL water, then 125 μL of $NaNO_2$ 5% (w/v) plus 125 μL of $KHSO_4$ 5% (w/v) was added, and samples were incubated at room temperature for 15 minutes. A control tube consisting of 125 μL water instead of sample pellet suspension was included in this step. After centrifugation (1,500 ×g, 2 minutes, 4°C) two 150 μL aliquots were withdrawn as replicates and each one was combined to 50 μL of ammonium sulfamate 12.5% (w/v) and 50 μL 12.5% (w/v) MBTH (3-methyl-benzo-2-thiazolone hydrazone). MBTH was prepared daily. Samples were then incubated at 100°C for 3 minutes. After cooling, 50 μL of $FeCl_3 \cdot 6H_2O$ 0.83% (w/v) was added and then samples were incubated for 25 minutes at room temperature. Aliquots with 200 μL were transferred to 96-well microplates and absorbance at 650 nm was measured. A standard curve was performed using a 3.3 μg/μL colloidal chitin suspension prepared according to [35] and used to calculate the amount of chitin recovered from each insect.

2.5. Phenoloxidase Assays. Phenoloxidase assays were performed as previously described [36]. Insects were submitted to oral treatment (0–0.5 μL TFM/mL blood) and, 7, 12, or 16 days after feeding, hemolymph was withdrawn using 10 μL glass tubes. Hemolymph samples were diluted 10 times with 10 mM sodium cacodylate buffer pH 7.4 containing 10 mM $CaCl_2$ and then 25 μL was combined with 10 μL of the same buffer above (spontaneous activity) or 10 μL of a 1 mg/mL trypsin solution in the same buffer (total activity). Reaction mixtures were then incubated at 37°C for 20 minutes and phenoloxidase measurements were then started by the addition of 25 μL of a saturated solution of L-DOPA (4 mg/mL). Dopachrome formation was followed by continuous absorbance measurements at 490 nm at 37°C for 60 minutes (Spectra Max, Molecular Devices).

2.6. Oocyte Measurement and Observation of Internal Anatomy. We treated adult females of *R. prolixus* with Starycide orally (3 μL TFM/mL blood) or topically (3 μL/insect) as described above. Control insects received no treatment. Five days after treatment, insects were dissected and oocytes were counted and photographed with a millimeter paper for scale. For measurement of oocyte length, photos were analyzed using the programs Photoshop CS6 Extended or ImageJ. For internal anatomy comparison, photos were taken using a stereomicroscope Leica MZ6 with 10x magnification.

2.7. Data Analysis. The significance of results was analyzed using ANOVA and Tukey's test [37]. Differences between treated and control insects or eggs were considered nonstatistically significant when $p > 0.05$. Probability levels are specified in the text.

3. Results

3.1. Effects of Feeding Treatment with TFM on R. prolixus Females. The biological data of *R. prolixus* adult females after oral treatment with TFM are presented in Table 1. It is important to notice that control insects showed a mortality of 9% one day after feeding, with a mortality of 19% after 30 days. Nevertheless, TFM treatment at all doses (0.1–10 μL/mL blood) induced significantly higher mortality levels than controls, either one day after feeding, with mortalities ranging from 23 to 46% ($p < 0.05$, unpaired t-test), or after 30 days (50–76%, $p < 0.01$, unpaired t-test).

Control females laid an average of 56 ± 1 eggs, with a hatching rate of 70% (Table 1). All TFM doses tested (0.1–10 μL/mL blood) resulted in significantly lower numbers of eggs, ranging from 20 to 40 eggs per female ($p < 0.05$, unpaired t-test).

Egg hatching was also affected by oral treatment with TFM (Table 1). Treatment with 0.1 μL/mL blood did not affect this parameter, but doses equal or above 0.5 μL/mL resulted in significantly lower hatching ratios, equal to or below 16% ($p < 0.001$, unpaired t-test).

3.2. Effects of Topical Treatment with TFM on R. prolixus Females and Eggs. The biological data of *R. prolixus* adult females and eggs after topical treatment with TFM are presented in Table 2. As observed in the previous experiments, controls presented some mortality 1 day after feeding (6±3%), which increased to 23 ± 3% after 30 days. All doses of TFM (0.1–10 μL/insect) resulted in significant higher mortality levels than controls, either 1 day after treatment, ranging from 50 to 90% ($p < 0.01$, unpaired t-test), or after 30 days, above 86% ($p < 0.001$, unpaired t-test).

Control insects laid an average of 60 ± 6 eggs/female, with a hatching ratio of 70% (Table 2). A strong effect was also observed in the number of eggs laid by females, which decreased to 40 eggs/female with 0.5 μL TFM/insect ($p < 0.05$, unpaired t-test) and eventually to 20 eggs/female with 10 μL TFM/insect ($p < 0.001$, unpaired t-test). A similar effect was also observed in egg hatching, with ratios decreasing to 26% (0.5 μL TFM/insect; $p < 0.001$, unpaired t-test) or even 9% (10 μL TFM/insect; $p < 0.001$, unpaired t-test).

The effect of topical treatment with TFM administered directly to eggs freshly laid was also studied (Table 3). Control eggs have shown hatching rate of 93% and, in this case, a significant reduction to 60% was observed with the lowest dose tested (0.1 μL TFM/egg; $p < 0.05$, unpaired t-test). Higher doses resulted in lower hatching ratios, reaching the observed minimum of 10% with 1 μL TFM/egg ($p < 0.01$, unpaired t-test).

3.3. Effects of Impregnated Surface Treatment with TFM on R. prolixus Females and Eggs. The biological data of *R. prolixus* adult females and eggs after continuous treatment with TFM are presented in Table 4. In these experiments, mortalities in the control group were 6 ± 3% and 16 ± 1% after 1 and 30 days of treatment, respectively. Even the lowest TFM dose tested

Table 1: Evaluation of the oral treatment with TFM on *Rhodnius prolixus* adult female mortality and reproduction. Control insects were fully engorged on defibrinated rabbit blood only, whereas experimental groups were fed with defibrinated rabbit blood added with Starycide at concentrations from 0.1 to 10 μL/mL. Superscript letters show differences between treated and control insects: [a] $p < 0.05$; [b] $p < 0.01$; [c] $p < 0.001$ (unpaired t-test). Values are means ± SD of at least three experiments with 10 insects each.

Groups	Mortality		Oviposition, number of eggs per female	Egg hatching (%)
	1 day after treatment (%)	30 days after treatment (%)		
Control	9 ± 3	19 ± 3	56 ± 1	70 ± 20
0.1 μL/mL	23 ± 8[a]	50 ± 10[b]	30 ± 10[a]	66 ± 5
0.5 μL/mL	30 ± 10[a]	63 ± 8[b]	33 ± 9[b]	≤16[c]
1 μL/mL	43 ± 5[b]	70 ± 10[b]	40 ± 30[a]	9 ± 3[c]
10 μL/mL	40 ± 10[b]	76 ± 8[b]	20 ± 10[c]	≤16[c]

Table 2: Evaluation of topical treatment with TFM on *Rhodnius prolixus* adult female mortality and reproduction. Doses from 0.1 to 10 μL of Starycide per insect were applied directly to the dorsal surface of the abdomen immediately after feeding of experimental insects. Control insects received no TFM. Superscript letters show differences between treated and control insects: [a] $p < 0.05$; [b] $p < 0.01$; [c] $p < 0.001$ (unpaired t-test). Values are means ± SD of at least three experiments with 10 insects each.

Groups	Mortality		Oviposition, number of eggs per female	Egg hatching, treated females (%)
	1 day after treatment (%)	30 days after feeding (%)		
Control	6 ± 3	23 ± 3	60 ± 6	70 ± 20
0.1 μL	50 ± 10[b]	86 ± 6[c]	56 ± 7	60 ± 20
0.5 μL	73 ± 3[b]	93 ± 1[c]	40 ± 5[a]	26 ± 9[c]
1 μL	76 ± 5[b]	96 ± 1[c]	23 ± 4[b]	13 ± 3[c]
10 μL	90 ± 3[c]	96 ± 3[c]	20 ± 3[c]	9 ± 2[c]

Table 3: Evaluation of topical treatment with TFM on *Rhodnius prolixus* egg hatching. Doses from 0.1 to 1 μL of Starycide per egg were applied directly to the egg surface. Control insects received no TFM. Superscript letters show differences between treated and control insects: [a] $p < 0.05$; [b] $p < 0.01$ (unpaired t-test). Values are means ± SD of at least three experiments with 10 eggs each.

Groups	Egg hatching (%)
Control	93 ± 3
0.1 μL	60 ± 6[a]
0.5 μL	50 ± 6[a]
1 μL	10 ± 6[b]
10 μL	ND

(0.1 μL/cm^2) resulted in significant effects on mortality, with respective rates of 36% ($p < 0.05$, unpaired t-test) and 86% ($p < 0.001$, unpaired t-test) after 1 and 30 days of treatment. Higher doses resulted in somewhat higher mortalities, with maximum observed values of, respectively, 63% ($p < 0.01$, unpaired t-test) and 96% ($p < 0.001$, unpaired t-test) at the dose of 10 μL/cm^2, after 1 and 30 days of treatment.

Control insects, in these experiments, laid an average of 59 ± 2 eggs/female, with a hatching ratio of 70%. Oviposition was significantly affected by TFM continuous treatment, even at the lowest dose tested (0.1 μL/cm^2), with a decrease in number of eggs to 43 ± 5 eggs/female ($p < 0.05$, unpaired t-test). Higher doses resulted in stronger inhibitions, reaching the observed minimum of 17 ± 1 eggs/female at 10 μL/cm^2 ($p < 0.001$, unpaired t-test). The hatching of eggs laid by treated females was not significantly affected by 0.1 μL/cm^2 ($p > 0.05$, unpaired t-test), but was decreased by doses equal to or higher than 0.5 μL/cm^2, reaching the observed minimum of 3.6 ± 0.3% ($p < 0.001$, unpaired t-test).

The effect of the continuous contact of eggs laid by untreated females with TFM impregnated surfaces was also tested. Interestingly, no significant effect on egg hatching was observed after continuous TFM treatment (Table 5). However, only few of the 1st-instar larvae that emerged were able to survive after eggs eclosion (data not shown).

3.4. Effects of TFM Treatment of R. prolixus Adult Females on Oocyte Development. The effects of oral and topical TFM treatment in *R. prolixus* oocyte development are shown in Table 6. Control insects, 5 days after blood feeding, showed an average of 5 ± 1 oocytes/female, with an average oocyte size of approximately 2 mm. Oral treatment with TFM had no significant effects on average oocyte size ($p > 0.05$, unpaired t-test) but resulted in a significant higher number of oocytes per female (9 ± 2, $p < 0.05$, unpaired t-test). Nevertheless, topical treatment had no significant effect on the number of eggs per female ($p > 0.05$, unpaired t-test) and resulted in significantly smaller oocytes (1.78 ± 0.06 mm, $p < 0.05$, unpaired t-test).

3.5. Effect of TFM Treatment on Chitin Content of R. prolixus Females. The effect of oral and topical treatment with TFM on the chitin content of *R. prolixus* adult females is described in Table 7. We tested the effects of TFM on chitin contents of mated and nonmated females. The amount of chitin was quantitated by the colorimetric technique of [34], which was not sensitive enough for reliable detection of chitin in

Table 4: Evaluation of the effect of *Rhodnius prolixus* continuous contact with TFM impregnated surface on adult female mortality and reproduction. Petri dishes were prepared by applying Starycide to obtain final concentrations of 0.1–10 μL/cm^2 at the bottom of each plate. Controls received no Starycide. Insects were then fully engorged on defibrinated rabbit blood and placed inside the Petri dishes. Superscript letters show differences between treated and control insects: $^a p < 0.05$; $^b p < 0.01$; $^c p < 0.001$ (unpaired t-test). Values are means ± SD of at least three experiments with 10 insects each.

Groups	Mortality in		Oviposition, number of eggs per female	Egg hatching, treated females (%)
	1 day after treatment (%)	30 days after treatment (%)		
Control	6 ± 3	16 ± 1	59 ± 2	70 ± 20
0.1 μL/cm^2	36 ± 8^a	86 ± 8^c	43 ± 5^a	60 ± 10
0.5 μL/cm^2	40 ± 8^a	90 ± 6^c	36 ± 5^b	18 ± 3^c
1 μL/cm^2	40 ± 10^a	93 ± 8^c	20 ± 4^c	9 ± 3^c
10 μL/cm^2	63 ± 6^b	96 ± 3^c	17 ± 1^c	3.6 ± 0.3^c

Table 5: Evaluation of the continuous contact treatment with TFM on *Rhodnius prolixus* egg hatching. Petri dishes were prepared by applying Starycide to obtain final concentrations of 0.1–10 μL/cm^2 at the bottom of each plate. Controls received no Starycide. Freshly deposited eggs were then placed inside the Petri dishes. Values are means ± SD of at least three experiments with 10 eggs each.

Groups	Egg hatching (%)
Control	87 ± 7
0.1 μL/cm^2	ND
0.5 μL/cm^2	100
1 μL/cm^2	93 ± 3
10 μL/cm^2	87 ± 9

Table 6: Effect of oral or topical TFM administration on oocyte development inside treated *Rhodnius prolixus* adult females. Control insects were fully engorged on defibrinated rabbit blood only, whereas experimental insects were fed with blood containing 3 μL TFM/mL blood (oral) or directly received 3 μL TFM on the dorsal surface of the abdomen immediately after feeding (topical). Insects were kept for 5 days and dissected and then oocytes were counted and measured with the aid of a stereomicroscope. Values are means ± SEM of at least three experiments with 10 insects each. Asterisk denotes differences between control and experimental groups (unpaired t-test, $p < 0.05$).

TFM treatment	Oocytes	
	Number	Length
Control	5 ± 1	1.95 ± 0.02
Oral	9 ± 2*	1.87 ± 0.03
Topical	4 ± 2	1.78 ± 0.06*

the internal organs of *R. prolixus*. Therefore, we limited our comparisons to the chitin amounts that could be recovered from insect carcasses, which consisted of the remaining tissues of dissected insects after the removal of gut, ovaries, and fat body.

Mating resulted in a significant increase of carcass chitin contents in control females ($p < 0.01$, unpaired t-test). The amount of carcass chitin in nonmated females was significantly reduced by both TFM treatments ($p < 0.05$, unpaired t-test), with a stronger effect in the topical treatment. However, TFM topical treatment did not significantly change the amount of carcass chitin in mated females ($p > 0.05$, unpaired t-test), which was significantly reduced by the oral treatment only ($p < 0.01$, unpaired t-test).

Table 7: Effect of oral or topical TFM treatment on carcass chitin contents of *Rhodnius prolixus* adult females. Control insects were fully engorged on defibrinated rabbit blood only, whereas experimental insects were fed with blood containing 3 μL TFM/mL blood (oral) or directly received 3 μL TFM on the dorsal surface of the abdomen immediately after feeding (topical). Insects were kept for 5 days and dissected and then carcasses were submitted to chitin quantitation. Values are means ± SEM of at least three experiments with 3 insects each. Asterisk marks denote differences between control and experimental groups (unpaired t-test, $^* p < 0.05$; $^{**} p < 0.01$); hashtag denotes differences between mated and nonmated groups ($^\# p < 0.01$).

TFM treatment	Mated (μg/insect)	Nonmated (μg/insect)
Control	7105 ± 5$^\#$	6760 ± 60
Oral	4700 ± 400**	5100 ± 400*
Topical	6700 ± 500	4300 ± 500**

3.6. Effect of TFM Treatment on Diuresis of R. prolixus Adult Females. To have a better understanding of mechanisms leading to mortality in TFM-treated adult female *R. prolixus*, we followed diuresis after oral and topical treatment. In *R. prolixus*, weight increases several times during a blood meal and slowly decreases during digestion. In the first days after a blood meal, this decrease in weight is mainly caused by the quick excretion of water during haemolysis and concentration of gut contents. For this reason, weight measurements after a blood meal can be used as an indication of effective diuresis.

Treatment with TFM using oral or topical delivery resulted in different effects on blood ingestion (Figure 1, 0 h). Oral treatment resulted in significant less weight gain than that observed in control insects ($p < 0.001$, unpaired t-test). After 24 h, the weight of control insects was indistinguishable from the mean weight of orally treated ones. Nevertheless, insects treated topically with TFM remained significantly heavier than controls up to 48 hours after feeding ($p < 0.01$, unpaired t-test). After 5 days, all groups presented similar average weights ($p > 0.05$, unpaired t-test). These results

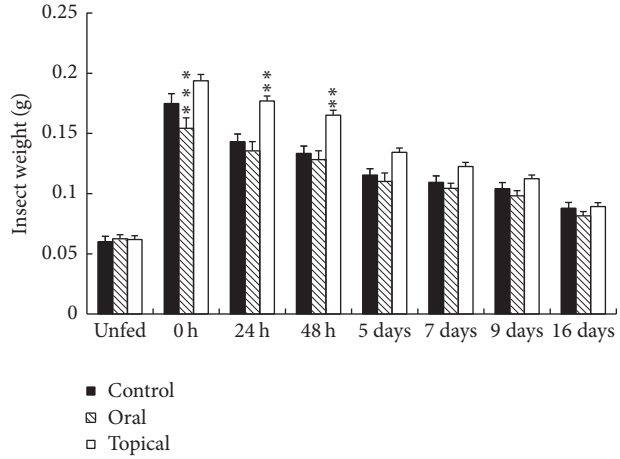

Figure 1: Effect of oral or topical TFM treatment on insect weight of *Rhodnius prolixus* adult females after blood feeding. Control insects were fully engorged on defibrinated rabbit blood only, whereas experimental insects were fed with blood containing 1 μL TFM/mL blood (oral) or directly received 1 μL TFM on the dorsal surface of the abdomen immediately after feeding (topical). Insects were weighted before (unfed) and at different times after blood feeding. Values are means ± SEM of one experiment with 18 insects each. Asterisk marks denote differences between control and experimental groups (unpaired t-test, $^{**}p < 0.01$, $^{***}p < 0.001$).

suggest that insects treated topically with TFM have impaired diuresis in the first two days after blood ingestion. In contrast, protein digestion after 2 days is negligible, and the whole digestive process in *R. prolixus* final stages takes 2-3 weeks [36]. In this respect, TFM effects on blood digestion are less likely because weight loss after 5 days is similar among the three experimental groups.

The suggestion from data of Figure 1 that TFM topical treatment resulted in an impaired diuresis led us to analyze the internal anatomy of treated insects (Figure 2). Control insects and insects that received TFM orally slightly differ in general internal anatomy, with anterior midgut, posterior midgut, and hindgut showing similar placement and size (Figures 2(A) and 2(B)). However, insects topically treated with TFM showed a clear enlargement of the hindgut, with displacement of the posterior midgut (Figure 2(C)).

3.7. Effect of Oral TFM Treatment on Phenoloxidase Activity of R. prolixus Females. Another physiological aspect we followed to better understand the effects of TFM treatment leading to adult female mortality was the prophenoloxidase (PPO) activation system.

This was performed by measuring phenoloxidase (PO) activity in the hemolymph. PO is secreted as a zymogen, prophenoloxidase (PPO), which is activated during infection by pathogens. PPO levels, which we measured as total PO activity (see Section 2 for details), are normally constitutive, being regulated by hormonal and developmental processes. However, PO activity, which we measured as spontaneous activity (see Section 2 for details), is sharply increased after an injury or infection. We hypothesized that insects treated with TFM, with a lower chitin content in the carcass, could be more exposed to infections and present a higher PO activity in the hemolymph.

To test this hypothesis, we orally treated *R. prolixus* adult females with TFM and measured PO and PPO levels 7, 12, or 16 days after treatment (Figure 3). Seven days after TFM treatment, no significant effects on PO levels were observed. However, 12 days after TFM treatment a significant increase in PO activity was observed with higher doses (>0.25 μL/mL blood, $p < 0.05$, unpaired t-test). Sixteen days after feeding with TFM, its effect on PO activity was not detected anymore (Figure 3(a)). This could be related to the high mortality observed with this treatment, since we measured activity only in the survivors that showed no PO response. Another possibility is that insects responded to the treatment and PO activities returned to initial levels. Additionally, no changes in total PO activity were observed independent of time and TFM treatment (Figure 3(b)).

4. Discussion

Triflumuron is BPU and inhibits chitin synthesis in developing insects [4, 28]. TFM is nowadays used against both stored products pests and disease vectors [38–48], strongly affecting insect reproduction by disruption of oviposition and/or eclosion of eggs laid by treated females. Its ovicidal effect is achieved either by direct application onto eggs [39, 49, 50] or by treatment of insects at any life stage [25, 51–53]. BPU-treated immature stages and adults derived from them frequently lay fewer and nonviable eggs when compared to their untreated counterparts [54–59]. This occurs by full or partial disruption of both embryogenesis and gonads development of both sexes [51–53]. Moreover, BPU effects vary according to insect species, developmental stage at time of application, kind of compound, mode, and dose of administration [28, 31, 60, 61].

Our data obtained with adult females of *R. prolixus* corroborate previous observations, showing that TFM is able to induce high levels of mortality after treatment [32]. Regardless of the putative differences in the chitin metabolism between male nymphs [32] and adult females (present work), we registered similar mortalities among these groups. This was at 1 and 30 days after treatment for the three delivery modes tested, with the only exception of long term mortality after oral treatment. Male fifth-instar nymphs showed higher mortalities (86–96%) than adult females (50–76%) 30 days after feeding with similar doses of TFM. This is possibly caused by ingestion of larger blood meals by fifth-instar nymphs when compared to adults [62].

Additionally, TFM significantly inhibits both oviposition and hatching of eggs laid by surviving females. Significant mortality levels after treatment were already observed with the lowest dose for the three delivery modes both 1 and 30 days after treatment. However, there were some differences among the three delivery modes in observed mortality levels. One day after TFM administration, oral delivery showed lower mortalities (23–40%) compared with surface contact (36–63%) or topical (50–90%) treatments. This could be a result of TFM excretion or detoxification. It has been shown

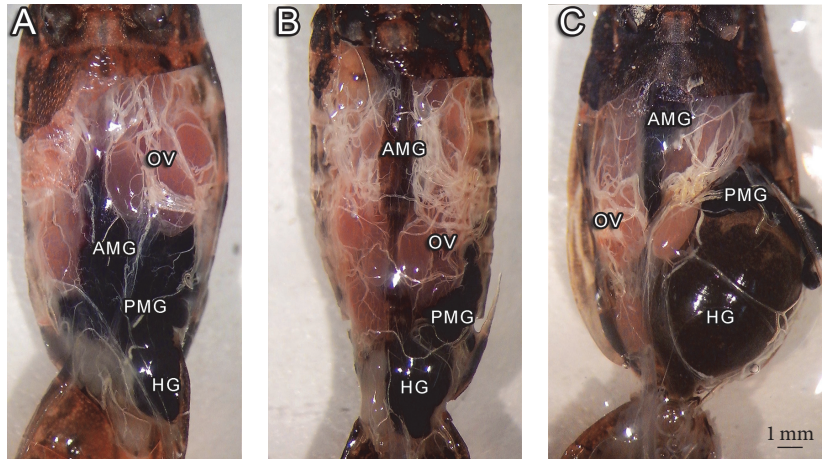

Figure 2: Representative images of internal tissues of *Rhodnius prolixus* adult females after different treatments with TFM. (A) Control insects. (B) Insects fed with defibrinated rabbit blood containing TFM at 1 μL/mL (oral treatment). (C) Insects received 1 μL TFM on the dorsal surface of the abdomen immediately after feeding (topical). OV: ovaries; AMG: anterior midgut; PMG: posterior midgut; HG: hindgut.

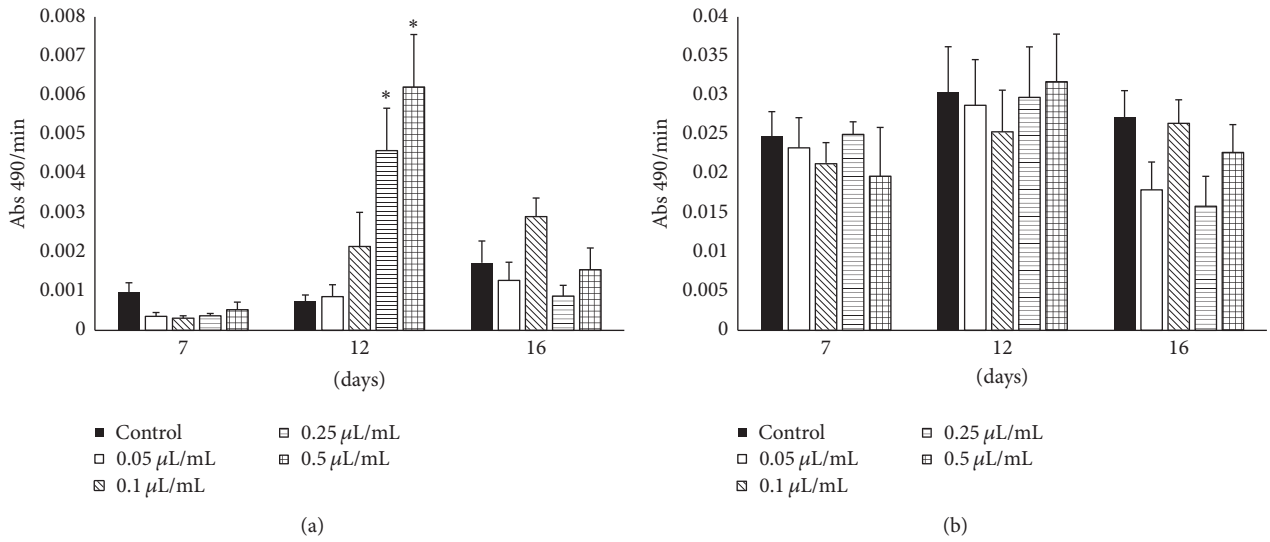

Figure 3: (a) Spontaneous and (b) total phenoloxidase (PO) activities in the hemolymph of *Rhodnius prolixus* adult females orally treated with different doses (0.05–0.5 μL/mL blood) of TFM. Controls were engorged with defibrinated rabbit blood only. Experimental insects were engorged with blood containing TFM and PO activities were measured in the hemolymph 7, 12, or 16 days after feeding. Figures correspond to enzymatic activities measured in 5 samples obtained from one insect each. Asterisk marks denote differences between control and experimental groups (unpaired t-test, $^{*}p < 0.05$).

that gut microbes have an important role in the detoxification of ingested xenobiotics [63], and it has been described that, after a blood meal, there is a huge increase in the bacterial population in *R. prolixus* gut contents [64, 65]. However, our data suggest that oral treatment does not affect diuresis (Figures 1 and 2), so the elimination of TFM in urine after oral treatment should also be considered. This might be an interesting topic for future investigations. Also, the surface contact treatment showed a lower mortality (36–63%) compared with topical one (50–90%), 1 day after treatment. This could be a result of better absorption of TFM through the cuticle in the topical treatment. It is important to consider that the highest dose of TFM used corresponds to 10% of the mass of unfed animals, and this might generate unspecific toxic effects.

When we consider mortality levels 30 days after TFM administration, the oral treatment showed a lower effect (50–76%) than the topical (86–96%) and impregnated surfaces (86–96%) delivery modes. This is possibly due to TFM detoxification at the first days after treatment (see the above). Interestingly, topical and impregnated surfaces treatments showed similar cumulative mortalities after 30 days. This indicates that, in spite of the stronger effect in the first day for the topical treatment, possibly due to better penetration, TFM impregnated surfaces are able to affect the insects during a more prolonged period, suggesting that, under our

experimental conditions, environmental degradation of TFM is negligible [66].

Comparing the effects of different delivery modes on oviposition at the lowest doses used, the oral treatment was more effective (30 eggs/female) than topical (56) or impregnated surface (43). At higher doses, all treatments showed similar effects (17–20 eggs/female). The stronger effect of the oral treatment on oviposition is coherent with the observed accumulation of oocytes in the ovaries of insects treated this way (Table 6) and could be a result of a differential tissue partition of the drug via absorption through the gut. At higher doses, this difference is possibly minimized by the saturation of transport and absorption pathways to the ovaries.

Interestingly, the three delivery strategies had similar effects on the hatching of eggs that were deposited by treated females. This suggests that the amount of TFM, which is absorbed by oocytes during its development in the ovaries, does not depend on the TFM pathway from the insect's surface to the hemolymph. An exception for this rule is the strong reduction in hatching at the higher dose in the impregnated surface treatment (3.6%), which could be explained by a cumulative effect of the drug. Nevertheless, TFM topical treatment resulted in smaller oocytes, when compared to controls or the oral treatment (Table 6). As the oral treatment resulted in an inhibition of oviposition and accumulation of oocytes in the ovaries, these effects could compensate each other since the drug would be absorbed by a higher number of oocytes. It has also been shown that, in *R. prolixus*, hemolymphatic injection of lufenuron, a chitin synthesis inhibitor, decreases the number of eggs laid, modifies eggs shape and color, and reduces the viability of eggs by the disruption of chitin synthesis during oogenesis [31]. In this respect, apparently different BPU substances have similar effects on oogenesis and egg development.

The delivery mode strongly influences the effects of TFM on egg hatching of treated eggs, with strong inhibition after the topical treatment and no effect during the impregnated surface treatment. This could be related to the permeability of the eggshell to TFM, which could change during embryo development. However, it seems more likely that the continuous contact treatment failed due to a smaller egg surface in direct contact with the drug. Additionally, in the topical treatment, TFM was applied to the top surface of the egg, to avoid diffusion of the drug into the paper, and in the continuous contact treatment the bottom surface of the egg was in contact with the paper. In this last condition, absorption of drug is possibly negligible, considering that capillarity and diffusion effects would not occur across the eggshell, which is impermeable. One interesting hypothesis is that in the impregnated surface treatment the drug might be susceptible to environmental breakdown along the experiment, but the observation that first-instar nymphs that were born from the exposed eggs die after few days (data not shown) clearly suggests that the TFM is not significantly degraded. This is coherent with previous studies showing a strong effect of TFM on *R. prolixus* nymph survival [32]. This persistence in the environment, suggested by the effects of TFM in the continuous contact treatment, is an interesting property considering future applications of the drug in control of triatomines in the field. Also, from all the delivery modes tested, the continuous contact treatment was the only one that better reproduced the application of insecticides in control programs.

After characterization of the impact of TFM on the reproductive fitness of *R. prolixus* adult females, we studied some biological and biochemical parameters to have a better understanding of the impact of this drug on the insect's physiology. Initially, we observed that TFM treatment resulted in lower amounts of chitin in adult carcasses. Unfortunately, we were not able to measure chitin content in internal organs as gut or fat body, due to low sensitivity of the colorimetric quantitation technique. In fact, we expected a minimal amount of chitin in internal organs, related to the presence of trachea only. In the gut, the major source of chitin is possibly the cuticle of the foregut (which is vestigial in triatomines) and hindgut, as hemipterans do not have a peritrophic membrane in the midgut. *R. prolixus* midgut shows only the presence of N-acetylglucosamine [67], and only recently the possible presence of chitin in this tissue has been proposed [68].

In general, treatment with TFM resulted in a decrease in the chitin content of the carcass, which suggests that even in adult stages the amount of chitin depends on a homeostatic balance between synthesis and degradation, which could reflect age-related developmental changes in the cuticle or trachea. However, it is interesting to notice that the effect of TFM on the chitin content of carcass depends on the delivery mode and on the reproductive status of females. For nonmated females, we observed a bigger reduction of carcass chitin contents with the topical treatment, while in mated females the oral treatment resulted in a stronger effect. This could be a result of the activation of metabolism after mating, with higher absorptive and transport rates between the gut, fat body, and ovaries during oogenesis.

As previously discussed, the oral TFM treatment had apparently no significant effect on insect diuresis and a lower toxicity, possibly due to better elimination through gut microbiota, feces, or Malpighian tubules. Nevertheless, topical treatment with TFM resulted in a slower weight loss after blood feeding, especially during the first 48 hours. This may be interpreted as a symptom of impaired diuresis, which was confirmed by inspection of internal anatomy, revealing a great distension of the hindgut in treated insects. The swollen hindgut of treated insects could be interpreted as a result of a detoxification response with higher activity of Malpighian tubules, leading to accumulation of water in this structure. However, it is not clear why the insect did not excrete the urine produced in the first days after blood feeding. This could be a negative feedback response related to optimization of water balance in an insect which detected a continuous xenobiotic challenge.

Interestingly, feeding insects with blood containing TFM resulted in a significant increase of spontaneous PO levels in the hemolymph 7 days after treatment. Considering that in those individuals the amount of chitin in the carcass is reduced, it is possible that these insects are more prone to infection due to a weakened epithelial barrier against

pathogens, resulting in a more activated PPO system. Activation of PPO during bacterial or fungal infection is a conserved and well-documented phenomenon in insects [69], being also already described in *R. prolixus* [36, 70–72]. More studies are necessary to determine if the TFM effect on *R. prolixus* PPO system is a direct effect of the drug on the PPO cascade or if it is an indirect effect involving a more frequent invasion of microorganisms into the hemolymph. It is important to note that the absence of effect on total PO activity registered over time and after TFM treatment is coherent with the constitutive nature of the PPO gene in *R. prolixus* [36] and suggests that expression of this gene is not being affected. Regulation of posttranslational activation of the PPO protein might be responsible for the observed PO activity changes.

In summary, very low concentrations of TFM reduced longevity, oviposition, and hatching of eggs laid by treated *R. prolixus* adult females. In addition, the viability of eggs oviposited by untreated females was affected by TFM topical treatment. Administration of this drug induced major changes in oogenesis, carcass chitin content, diuresis, and PPO activation system, severely affecting the insect physiology and leading to a premature death of treated females.

Chemical insecticides currently constitute a major tool in control programs of vector populations. However, the intense use of several compounds has generated resistance on the target insects [6]. The search of improved technologies involves the development of environmentally safe substances with selective action against target vectors and low risk for nontarget organisms [73]. Growth regulators, especially BPU substances, represent a promising new ecofriendly strategy [3, 7, 27, 53, 64, 74–76]. Our data suggest that TFM and perhaps other chitin synthesis inhibitors should be considered as potential tools for integrated vector control programs against hematophagous triatomine species.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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Research Article

Relationship between Antibody Levels, IgG Binding to *Plasmodium falciparum*-Infected Erythrocytes, and Disease Outcome in Hospitalized Urban Malaria Patients from Dakar, Sénégal

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Background. Management of clinical malaria requires the development of reliable diagnostic methods and efficient biomarkers for follow-up of patients. Protection is partly based on IgG responses to parasite antigens exposed at the surface of infected erythrocytes (iRBCs). These IgG responses appeared low during clinical infection, particularly in severe disease. *Methods*. We analyzed the IgG binding capacity to the surface of live erythrocytes infected by knob positive FCR3 strain. Sera from 69 cerebral malaria (CM) and 72 mild malaria (MM) cases were analyzed by ELISA for IgG responses to five antigens from iRBC and by flow cytometry for IgG binding as expressed in labeling index ratio (LIR). The relationship between IgG levels, LIR, parasitemia, age, and the clinical outcomes was evaluated. *Results*. We found a significant decrease of LIR in adult CM fatal cases compared to surviving patients ($p = 0.019$). In MM, LIRs were correlated to IgG anti-iRBC and anti-*Pf*EMP3/5 levels. In CM, no correlation was found between LIR, IgG levels, and parasitemia. *Conclusion*. The IgG binding assay was able to discriminate outcome of cerebral malaria cases and it deserves further development as a potential functional-associated assay for symptomatic malaria analysis.

1. Introduction

The robust progress in integrated intervention strategies including artemisinin-based combination therapy (ACT), coverage with long-lasting insecticide-impregnated bed nets (LLINs), and systematic diagnosis using rapid tests (RDTs) has considerably reduced the burden of malaria in many countries. However, malaria still remains one of the major infectious diseases in tropical and subtropical regions, causing over 225 million clinical cases and around 600,000 deaths per year [1]. However, efforts are threatened by increasing resistance of parasite and *Anopheles* vectors to drug and insecticides, respectively [2–4]. The control and elimination of malaria require the development of efficient vaccines and of reliable diagnostic and follow-up methods to prevent early individual infection by *Plasmodium falciparum* species which is responsible for most of the deaths in human malaria. It causes severe cases such as cerebral malaria during which the parasites are sequestrated in brain through a complex mechanism that involves interactions between human adhesion molecules and parasitic Var proteins that are highly polymorphic [5]. Parasite sequestration results in brain microvasculature impairment, increased blood volume, and possible occlusion of brain microvessels [6, 7]. Cytokines

and parasite toxins have also been shown to cause direct damage to the blood-brain barrier [8].

P. falciparum virulence mainly relies on immune escape strategies that include the modification of the adhesion properties of infected erythrocytes to the vascular endothelium and the parasite's ability to undergo antigenic variation. The main antigenic ligands responsible for both cytoadherence and antigenic variation are members of the *P. falciparum* Erythrocyte Membrane Protein-1 (*Pf*EMP-1) family [6, 9]. There is evidence that protection against malaria is partly based on antibody responses to diverse parasite antigens [10] including proteins exposed at the surface of infected red blood cells (iRBCs). These antigens are immediate targets for protection-associated antibodies involved in opsonization and phagocytosis of infected erythrocytes [11]. In fact, the key role of antibodies (Abs) in protection against malaria was demonstrated decades ago by passive transfer assays in humans [12, 13]. Additionally, it is known that repeated exposure to *P. falciparum* infection confers a partial immunity to malaria through mechanisms that involve the progressive acquisition of a panel of antibodies that recognize varieties of surface antigens from diverse isolates [14].

There is some evidence that protection against parasite infection is partly based on antibody responses to diverse parasite antigens [10] including those exposed at the surface of infected red blood cells (iRBCs). They are the first line of target antigens accessible for protection-associated antibodies involved in opsonization and immune phagocytosis of infected erythrocytes [11]. The variability of iRBC surface antigens (Ags) upon immune pressure complicates the evaluation of their potential role in controlling parasite density *in vivo*.

Previous studies showed that flow cytometry could measure the ability of Abs to bind to native parasite surface-associated iRBCs Ags [15, 16]. Their potential contribution to immune protection in rural endemic settings [17] and in postvaccinated individuals has been reported [18].

In this study, we further investigated the potential protection-associated role of IgG against iRBCs Ags in urban symptomatic patients hospitalized for mild and cerebral Malaria. We thus evaluated the relationship between IgG binding capacity and Ab responses to *P. falciparum* iRBCs-associated Ags measured by enzyme-linked immunosorbent assay (ELISA) in the context of the bioclinical symptoms from patients hospitalized for confirmed clinical malaria infection. The Ags tested were whole parasite extracts from schizont and IRBC and recombinant IRBC-associated Ags R23, *Pf*EB200, and *Pf*EMP3/Clone5 described below.

2. Materials and Methods

2.1. Study Area and Epidemiologic Context. The study was performed in Dakar (Senegal), an urban area corresponding to a malaria hypoendemic setting with a low level of seasonal transmission. The transmission was estimated to be around 0.5 to 1 infecting bite/person/year and occurred during the rainy season from September to December [19, 20]. The main malaria vector described was *Anopheles arabiensis*, and *P. falciparum* was the most widespread species accounting for 98% of cases [21]. Previous studies in this area revealed that malaria affected all age groups with the highest prevalence occurring in children. A mean incidence of 2.4% of clinical disease has been observed, with no difference between adults and children [21, 22].

2.2. Study Population, Ethical Statements, and Procedures. The study was performed at the Principal Hospital of Dakar. Patients were recruited every year during the rainy season from September to December in three successive years 1999, 2000, and 2001.

An informed consent was obtained from each participant and/or their relatives prior to inclusion, after giving them written or verbal information in their native language. The protocols were approved by the investigators' institutions, the National Ethical Committee and the Ministry of Health of Senegal.

Thin and thick blood smears were prepared from rapid diagnostic test (RDT) positive patients, in order to determine the parasite species and the level of parasitemia. Blood samples used in this study for immunological analysis were collected after determining the parasitological and clinical profiles of the patients. A questionnaire with clinical history and demographic information was recorded. Patients with malaria and any other coinfection were excluded as previously described [23]. Two categories of patients were enrolled: cerebral malaria (CM) and mild malaria (MM) patients.

The CM group consisted of 69 patients hospitalized for unarousable coma (nonpurposeful response or no response to a painful stimulus by Glasgow score < 9) with microscopically diagnosed *P. falciparum* infection and without other clinically evident cause of impaired consciousness such as hypoglycemia, meningitis, and encephalitis according to World Health Organization criteria [24]. Samples were taken at the admission before any treatment. All patients were managed by the same medical staff. The treatment protocol was based on the Senegalese national recommendations which are intramuscular quinine 20 mg/kg followed by 20 mg/kg every 8 h. Patients were examined every 4 h for the first 24 h and every 6 h thereafter. Fatal cases occurred during 1 to 4 days after admission. Surviving patients completely recovered after treatment. A total of 18 CM patients had a fatal outcome (FCM) while 51 subjects recovered with no sequelae (SCM).

Regarding MM, a total of 124 patients who were treated at the outpatient clinic of the hospital were initially enrolled. Of these, 72 patients had fever with *P. falciparum* parasitemia of <25000 parasites/μL of blood, with no evidence of impaired consciousness or seizures at the time of enrolment. Based on previously reported criteria [23, 25], 52 patients were excluded. Blood samples from MM patients were obtained on the day of hospital admission.

2.3. Antigens. Two crude antigenic preparations were used. They consisted of (i) a lysate of *in vitro*-matured schizont-enriched *P. falciparum* from infected erythrocytes (*Pf*Schz) [26] and (ii) erythrocyte membranes from infected red blood cell (iRBCm). Membrane from nonparasitized erythrocyte (nRBCm) was also tested as negative controls. Erythrocyte membranes from iRBCm and nRBCm were prepared

according to the method of Fairbanks et al. [27]. The total protein concentration of the parasite preparations was estimated by the Bio-Rad assay. All Ags were kept frozen at minus 80°C in working aliquots.

Three purified recombinant proteins fused to *Schistosoma japonicum* glutathione S-transferase (GST) in the pGEXA vector were used. R23 contains 11 copies of a 6-amino-acid repeat derived from the central domain of Ag R45, whose consensus sequence is HKSDS N/S/H [28]. *Pf* EB200 contains 13 repeats with characteristic Glu-Glu dimers; it derives from *Pf* 332, a conserved giant protein accessible on the surface of infected erythrocytes [29], in late schizont [30]. *Pf* EMP3/5 (or *Pf* EMP3 clone 5) is a recombinant product expressing the 1,450-bp *Eco*RI fragment recovered from clone 5, isolated from the FUP/SP Palo Alto (alias FCR3) genomic expression library. It expresses the C-proximal region of *Pf* EMP3, a high-molecular weight host membrane-associated protein [31, 32]. The control protein was the GST carrier previously reported [23, 33].

2.4. ELISA Procedure. Antimalarial plasma IgG levels against schizont extracts *Pf* Schz, iRBCm, nRBCm, and purified recombinant proteins were determined using ELISA as described [26, 32]. Crude Ag preparation (15 μg/mL) and recombinant proteins (1 μg/mL) were coated on MaxiSorp® (Nunc®, Roskilde, Denmark) and Immulon-4® (Dynatech®, Springfield, VA, USA) plates respectively, and incubated overnight at 4°C. Coated plates were washed with PBS containing 0.5% Tween 20 and then blocked with PBS-5% bovine serum albumin (BSA) (Sigma® Chemicals St. Louis, MO, USA) for 1 h at 37°C. Plasma samples diluted 1/200 in PBS-1% BSA-0.5% Tween 20 were added and incubated for 2 h at 37°C. Peroxidase-conjugated polyclonal goat anti-human IgG [IgGγ-chain specific] (Cappel®, Organon Teknika, PA, USA) was added to the wells and the plates were incubated for 2 h at 37°C. Bound antibodies were revealed using a citrate buffer (pH 4) containing 160 μg/L orthotoluidine and 10% H_2O_2. The reaction was stopped with 4 N H_2SO_4, and the optical densities (OD) were measured at 450 nm in a plate reader (Tecan® GmbH, Salzburg, Austria). Negative controls consisted of a pool of nonimmune European sera and a pool of naïve African sera. The positive control was a pool of sera from clinically immune adults living in Dielmo. Results were expressed as OD ratios: $OD_{\text{serum sample}}/OD_{\text{negative control}}$. The OD signals of the samples were individually corrected for the GST or nRBCm signal. Serum samples with OD ratios greater than 2 (which is over the OD of naïve control + 3 SD) were considered seropositive [26, 32, 33].

2.5. Flow Cytometry Technique for the Detection of IgG Binding to Live iRBC. To test for binding of plasma IgG to live iRBC, we used a double staining cytofluorimetric technique as previously described [15, 16]. A knob positive (K^+) Uganda Palo Alto FUP strain of *P. falciparum* (alias FCR3) maintained in culture was used as a source of live iRBC. This strain was cultured in complete medium (RPMI 1640, 25 nM HEPES, 2.4 mM L-glutamine, 50 μg/mL gentamicin, and 0.5% w/v Albumax). All these reagents were from Sigma Chemicals (St. Louis, MO, USA). The culture was maintained at 37°C in an atmosphere of 5% CO_2, with daily changes of medium at 5% hematocrit and dilution with red blood cells when the parasitaemia exceeded 5% [34]. K^+ iRBCs were continuously selected after plasmagel flotation at parasitemia about 5% and washed once in phosphate buffered saline (PBS) (Sigma Chemicals, St. Louis, MO, USA).

Membrane-bound IgG were revealed after a first incubation of 30 min at 37°C with plasma diluted 1 : 20, followed by a second incubation of 30 min at 37°C with phycoerythrin-(PE-) conjugated goat anti-human IgG diluted at 1 : 200 (Cappel, Organon Teknica, PA, USA). Live parasites were then labeled for 30 min at 37°C in the dark with thiazole orange (TO) (Retic-Count®; Becton-Dickinson, San Jose, CA, USA). Fluorescence was read within 1 h on a flow cytometer FACS with CellQuest® software (Becton-Dickinson®, San Jose, CA, USA). After gating on the TO-positive iRBC, 5,000 events for the overall iRBC ($FL_1 > 10^1$) were counted. At the end of the first set of analysis, gating of iRBC was changed to select mature forms of the parasites with subsequent nuclear material ($FL_1 > 10^2$), and 2,000 events were counted. In all cases, PE-labeled membrane-bound IgG was measured in the FL_2 channel (Figure 1).

A pool of 30 plasma samples with high levels of IgG to whole schizont extracts, R23, *Pf* EB200, and/or *Pf* EMP3/5 [35], was used as the positive control (SHI), and pools of plasma obtained from non-*P. falciparum* exposed Europeans and Senegalese (Dakar inhabitants) were used as the negative controls. For the quantification of labeled iRBC, a labeling index (LI) ratio (LIR) was defined as $LI_{\text{sample}}/LI_{\text{negative control}}$. The LI was calculated as the percentage of iRBC with bound IgG ($FL_1 > 10^1$; $FL_2 > 10^2$) multiplied by the geometric mean intensity of the peak.

As previously described, this calculation allows interassay comparison [17] resulting in strong correlation between LIR and percentage of iRBC IgG binding. Analyses for flow cytometry were done with Flow Jo X 10.0.6 Software (Tree Star Inc., Ashland, OR, USA).

2.6. Statistical Analysis. As Ab responses are nonnormally distributed, comparisons of Ab levels and/or LIRs in different assays or groups were done using the Wilcoxon signed-rank test and the Spearman rank correlation test for paired data. The Mann-Whitney test was used to compare unpaired data. Comparison of prevalence for positive responders was done using the Chi^2 test. p values of <0.05 were considered significant. Statistical analyses were performed with Statview®, version 5.0, software (SAS Institute, Cary, NJ, USA).

3. Results

3.1. Characteristics and Biological Data of Study Population. A total of 141 malaria patients were selected for the present study. The patients included 69 CM and 72 MM subjects. They suffered of confirmed clinical malaria symptoms within half a day to 14 days before the day of enrolment (mean 6 days).

The age of the patients ranged from 2 to 63 years (mean 17.6 years) without a significant difference between CM and

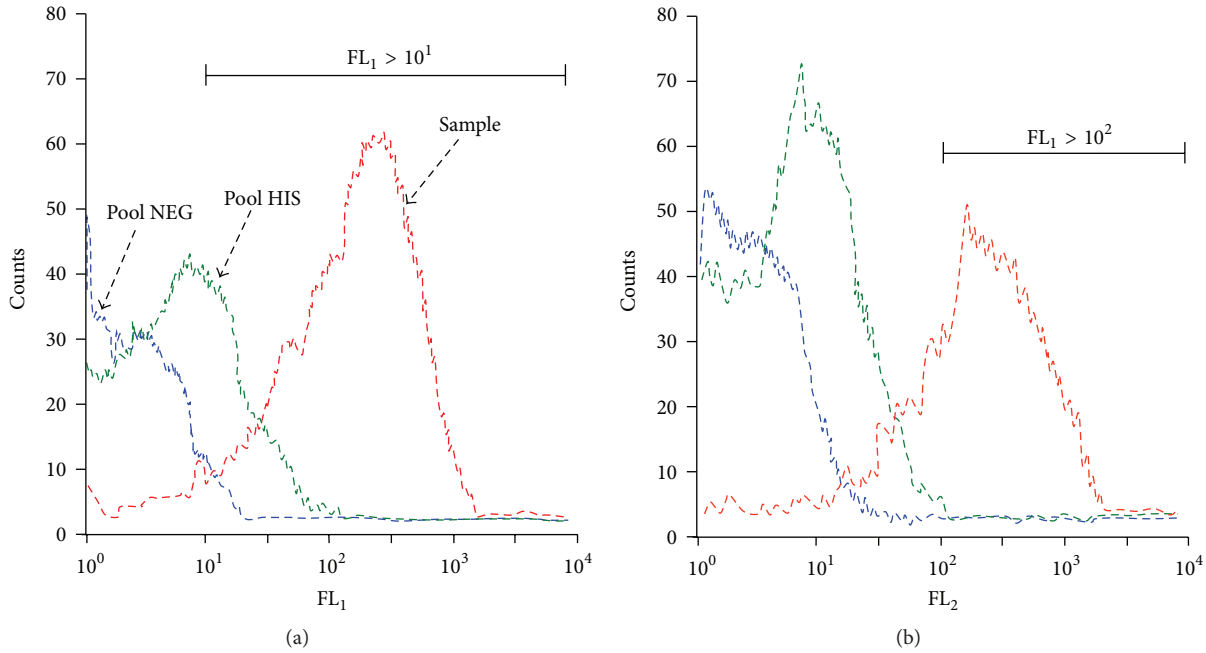

Figure 1: Examples of histograms of flow cytometry data acquisition. The two histograms show results from the acquisition of 5,000 events of a 3% *P. falciparum* culture after gating on $FL_1 > 10^1$ (a) and on $FL_1 > 10^2$ (b). The gating on high FL_1 values ($>10^2$) selected the mature population of the cultured parasites. The binding of human IgG to IRBC was revealed by an anti-IgG conjugated to thiazole orange (TO). The fluorescence of TO-positive iRBCs was measured in the FL_2 channel. The reference negative control (blue curve histogram, Pool NEG), the positive control (green curve, Pool HIS), and a strong responder from Ndiop (red curve, positive sample) are shown. Analysis was done by Flow Jo® Software.

Table 1: Epidemiological and hematoparasitological characteristics of the study population.

Parameters	Cerebral malaria ($n = 69$) Mean ± SE [min–max]	Mild malaria ($n = 72$) Mean ± SE [min–max]	p
Gender (male/female)	39/30	43/29	—
Age (year)	17.9 ± 1.8 [2–63]	17.3 ± 1.2 [5–55]	ns
Age groups (adults/children)	39/30	35/37	—
Parasitemia (Tr/μL)	30150 ± 5510 [175–452100]	28348 ± 3755 [220–141000]	ns
Haemoglobin (g/dL)	8.4 ± 0.33 [3.3–15.1]	11.5 ± 0.51 [8.3–16.1]	*0.031*
Red blood cells count ($\times 10^6/\mu$L)	3.14 ± 0.13 [1.18–4.98]	4.72 ± 0.18 [2.90–7.71]	*0.040*
White blood cells count ($\times 10^3/\mu$L)	12.84 ± 1.15 [4.90–51.10]	8.21 ± 1.01 [4.5–13.3]	*0.012*
Platelet count ($\times 10^3/\mu$L)	143.4 ± 16.2 [11–533]	168.2 ± 11.8 [28–355]	ns
Hematocrit (%)	31.1 ± 0.22 [10.5–46.1]	35.9 ± 0.18 [23.9–49.1]	ns

SE = standard error, Min = minimum, Max = maximum, ns = nonsignificant, and n = number of patients. Data were determined by the hospital's medical laboratory. Adults were patients ≥15 years and children were patients <15 years. p = p value of comparison between CM and MM groups with Mann-Whitney rank test.

MM groups that counted comparable numbers of adults (≥15 years) and young individuals (<15 years) (Table 1).

Regarding biological parameters, hemoglobin level and red blood cells count were significantly lower ($p < 0.05$) while the leucocytes count was significantly increased in CM compared to MM patients ($p = 0.012$). In contrast, there was no significant difference in the platelet count and hematocrit levels between the two groups. Interestingly, the parasitemia at the day of admission was not significantly different between the two groups of patients (Table 1).

There was no significant association between hematology parameters and parasitemia at inclusion in the CM group, contrary to the MM groups where hemoglobin level appeared negatively correlated to parasite densities ($r = -0.53$; $p = 0.017$). Furthermore, hematology data, except for platelet count, correlated together in all clinical groups.

According to age of individuals, that is, adults (≥15 years) versus young individuals (<15 years), no relationship was found between parasitemia and hemoglobin levels, red cells, white cells, or platelets count, in the two groups of patients.

Table 2: Levels and prevalence of anti-IgG responses against tested antigens.

Parameters		Cerebral malaria ($n = 69$)	Mild malaria ($n = 72$)	p
IgG anti-*Pf*Schz	N (%)	35 (50)	50 (69)	*0.021*
	ODrt ± SE*	3.80 ± 0.29	3.06 ± 0.42	*0.063*
IgG anti-iRBCm	N (%)	35 (50)	63 (88)	*<0.01*
	ODrt ± SE	2.75 ± 0.23	3.87 ± 0.39	*0.032*
IgG anti-*Pf*EB200	N (%)	15 (22)	19 (26)	*0.518*
	ODrt ± SE	1.38 ± 0.14	1.21 ± 0.36	*0.235*
IgG anti-R23	N (%)	10 (14)	16 (22)	*0.236*
	ODrt ± SE	2.27 ± 0.33	1.98 ± 0.31	*0.452*
IgG anti-*Pf*EMP3/5	N (%)	37 (54)	30 (42)	*0.155*
	ODrt ± SE	2.59 ± 0.54	3.82 ± 0.38	*0.031*

*IgG responses in OD ratio ± standard error.
N = number of positive responders that is individuals with OD ratio ≥2, (%) = prevalence of positive responses, ODrt = Mean Optical Densities ratio, SE = standard error, p = p value resulting from comparison with Mann-Whitney rank or Chi^2 tests employed, respectively, for ODrt and prevalences of responders.

In CM, no difference of parasitemia was detected between survivors and fatal cases (data not shown). In contrast, hemoglobin, hematocrit, and red blood cells count appeared lower in the fatal CM patients than in recovering individuals ($p < 0.05$). Hyperleucocytosis was significantly found in the group of patients with fatal outcome ($p < 0.03$).

3.2. Levels and Prevalence of IgG Responses in CM and MM Groups. A summary of antibodies levels and prevalence of responders is shown in Table 2. The number of positive responders against parasite extracts (whole schizont, 69% versus 50%; $p = 0.021$; iRBCm, 88% versus 50%; $p < 0.01$) was significantly higher in the MM compared to the CM group. In contrast, the prevalence of responders to the recombinant antigens did not significantly differ between the two groups.

Regarding levels of Ab responses, the mean OD ratios of the MM patients were higher than CMs for IgG to iRBCm (3.87 in MM and 2.75 in CM; $p = 0.032$) and to *Pf*EMP3/5 (3.82 in MM and 2.59 in CM; $p = 0.031$) (Table 2). In addition, IgG responses to iRBCm and to *Pf*EMP3/5 were positively correlated in CM (rho = 0.32; $p = 0.029$) and in MM patients (rho = 0.63; $p < 0.001$). Abs to other Ags were comparable between the two groups.

Regarding age of individuals, no significant difference of IgG Ab levels was found between adults (≥15 years) and children (<15 years) for all Ag tested in MM group (not shown). In CM patients, levels of IgG to iRBCm were positively correlated with the age of individuals (rho = 0.63; $p = 0.001$) and Ab levels were significantly higher in adults with CM than in CM children ($p = 0.01$).

3.3. Variations of IgG Antibodies Levels according to CM Outcome. IgG response against selected Ag was compared between the survivors (SCM) and fatal cases of CM (Figure 2). SCM patients displayed significant higher levels of IgG to the crude extracts (Figure 2(a)) and to the recombinant proteins except for R23 (Figure 2(b)).

In patients with fatal outcomes (FCM), median levels of IgG anti-*Pf*Schz were twice lower: 2.2 versus 4.1, respectively ($p = 0.015$). A similar profile was observed for IgG anti-iRBCm with median values of 2.5 (25% (1st Quartile) = 1.0; 75% (3th Quartile) = 4) in SCM group versus 1.9 (25% (1st Quartile) = 1.2; 75% (3th Quartile) = 2.6) in FCM ($p = 0.041$) (Figure 2(a)).

3.4. Levels of IgG Binding Capacity according to the Severity and Outcome of the Disease. Binding of live iRBCs measured by flow cytometry was analyzed in the context of the clinical status, disease outcome, and age of individuals. As shown in Figure 3(a), sera from MM patients showed a higher but not significant labeling index ratio (LIR) than the CM individuals.

We next compared the IgG binding to live iRBC from SCM versus FCM patients. The median LIR was significantly higher in the SCM compared to the FCM patients (Figure 3(b)). The index of iRBCs IgG binding was highly variable within the two groups, with median LIR significantly lower in fatal cases than in SCM group (6.40 ± 3.01 versus 19.40 ± 2.45; $p = 0.019$, resp.).

This result was partly available after age dichotomization of CM patients, although the difference did not reach significant level in children (Figure 3(c)). In CM surviving children, levels were slightly higher (16.00 ± 3.16) than in those who died (10.10 ± 3.70) ($p = 0.207$).

In adults, a significant ($p = 0.021$) higher level of iRBCs recognition was found in CM surviving (median = 20.70 ± 4.71) compared to fatal (median = 4.90 ± 3.03) cases.

More importantly, index of iRBCs IgG binding from surviving adults and children was not significantly different ($p = 0.178$). Only adults with fatal outcome showed significant lower LIR than children who deceased ($p = 0.034$).

No correlation was found between age of individuals and binding capacity of IgG to iRBCs either as continuous or as stratified variable (young, 2–8 years old; older children, 9–15 years; adults, ≥15 years). Additionally, no relation between parasitemia and LIR was observed in both MM and CM groups.

3.5. Relationship between iRBCs IgG Binding and Antibody Levels. The correlation between levels of IgG anti-*P. falciparum* and magnitude of iRBCs IgG binding was analyzed as

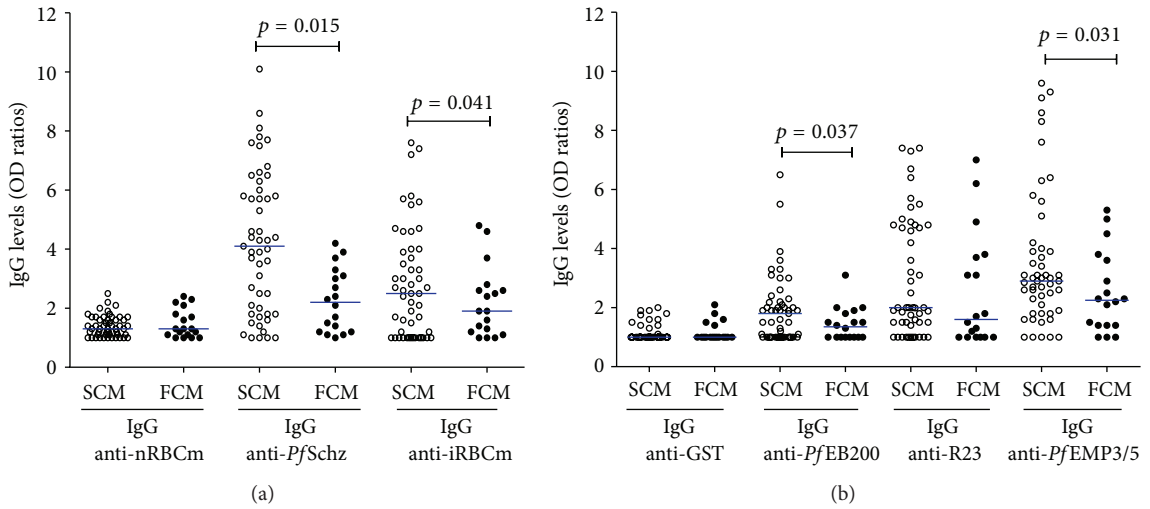

Figure 2: IgG antibodies levels against whole parasite extract antigens (a) and recombinants proteins (b) in survivors and fatal CM patients. Scatter plot of IgG responses to whole parasite extracts antigens (a) and recombinant proteins (b). Comparison between surviving cerebral malaria patients (SCM; $n = 51$; open circles) and fatal cerebral malaria patients (FCM; $n = 18$; black circles) for each antigen. Horizontal blue bar indicates medians of OD ratio value. The Mann-Whitney rank test was employed for between-groups comparison for each antigen. IgG levels were highest in surviving CM group for *P. falciparum* schizont extract, iRBC membrane, *Pf*EB200, and *Pf*EMP3/5. Significant p values are included ($p < 0.05$).

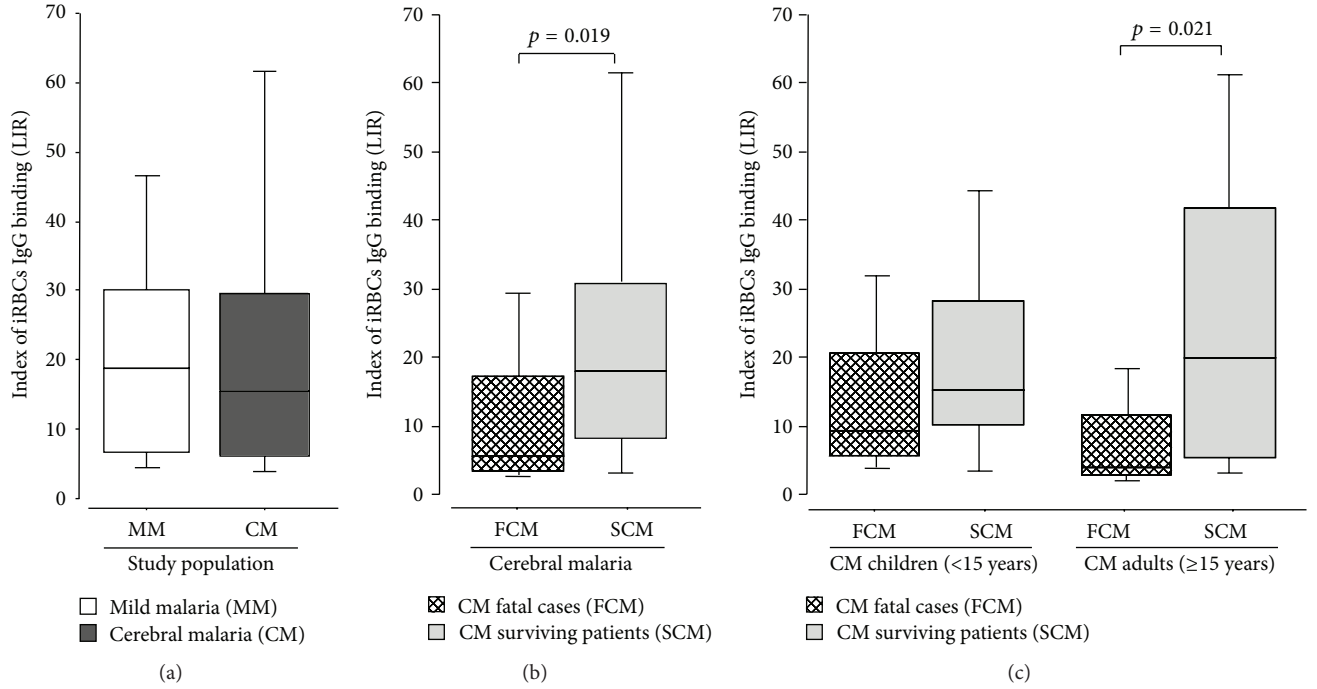

Figure 3: Variations of labeling index ratio according to disease severity (a), CM outcome (b), and age of individuals in CM patients (c). Box plot of labeling index ratio (LIR). The horizontal line within each box represents the median value of LIR. Comparison were done (a) between mild malaria patients (MM, $n = 35$; white boxes) and cerebral malaria group (CM, $n = 69$; dark boxes), (b) between fatal cerebral malaria group (FCM; $n = 18$; hacked boxes) and surviving cerebral malaria patients (SCM; $n = 51$; grey boxes), and (c) in CM patients according to both outcome and age of children (left) and adults (right). The Mann-Whitney rank test was employed for comparison between groups. Sera from survivors showed significant high LIR compared to fatal CM patients ($p < 0.05$) in adults. Significant p values are included.

function of disease severity, age of individuals, and clinical outcome.

In MM group, index of serum IgG binding to iRBCs was positively correlated to IgG levels against infected erythrocyte membrane (iRBCm) (rho = 0.47; $p = 0.029$) (Figure 4(a)), IgG anti-*Pf*Schz (rho = 0.35; $p = 0.037$), and IgG anti-*Pf*EMP3/5 (rho = 0.53; $p = 0.018$). However, LIR had no significant relationship with age of MM patients.

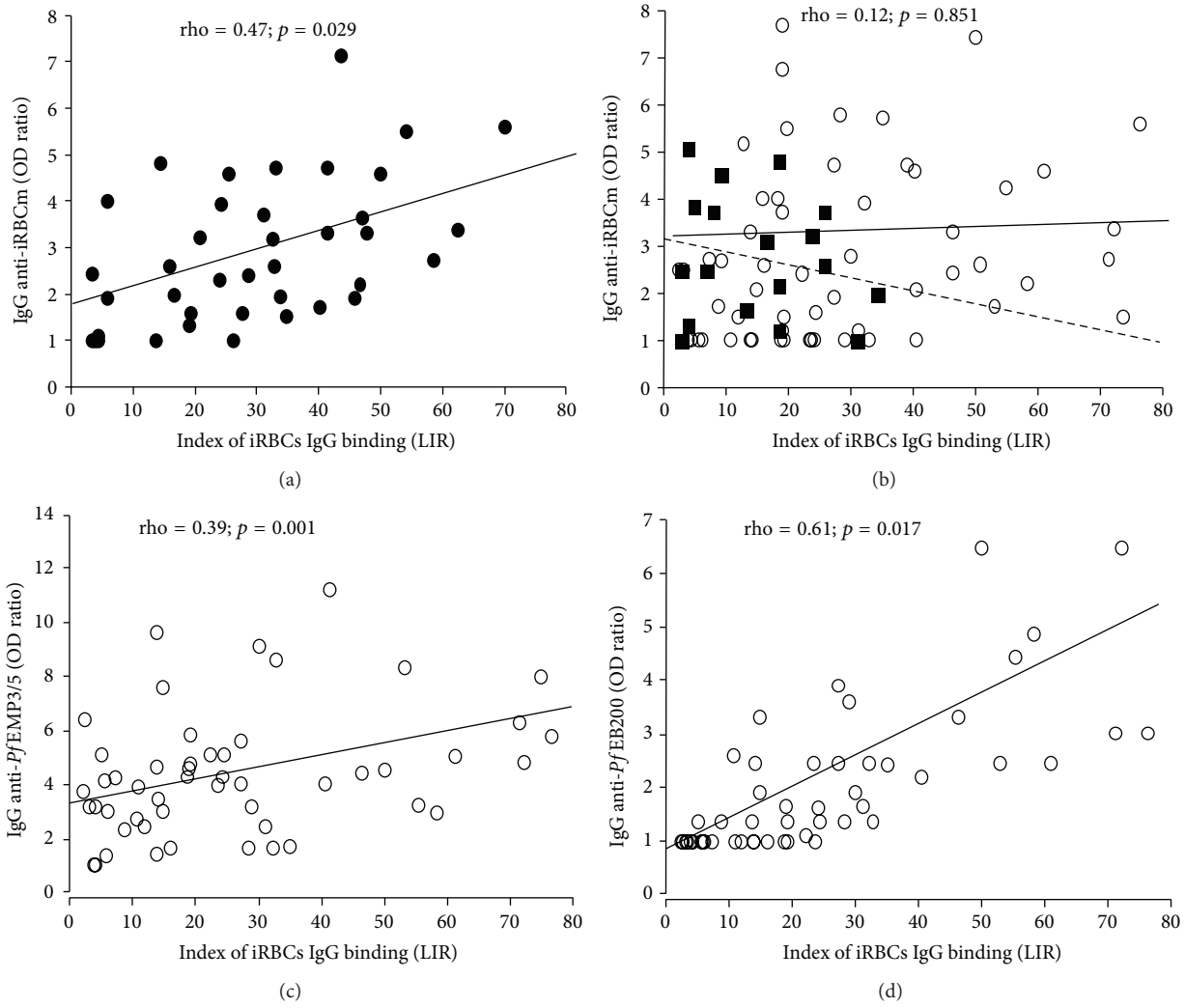

Figure 4: Relationship between labeled index of recognition and IgG responses in MM (a) and CM (b–d) groups. The correlations between labeled index of recognition (LIR) and IgG anti-iRBCm levels (OD ratio) are plotted in MM patients (a) (dark circles) and in CM group (b) with a dichotomization in fatal CM (dark squares) and surviving CM (open circles). Relationships between LIR and the levels of IgG anti-*Pf*EMP3/5 (OD ratio) or IgG anti-*Pf*EB200 levels (OD ratio) are, respectively, plotted in (c) and (d), only in CM surviving patients (open circles). Results from statistical analyses done by nonparametric Spearman rank test are indicated.

Globally, in CM groups no correlation was found between LIR and levels of IgG anti-iRBCm (rho = 0.12; $p = 0.851$) or IgG anti-*Pf*Schz (rho = 0.06; $p = 0.587$). According to CM outcome, a trend for negative correlation was observed between levels of IgG anti-iRBCm and LIR in the group of fatal cases (rho = −0.16; $p = 0.057$) (Figure 4(b)). Furthermore, considering age and disease outcome, the previous trend was found in fatal CM adults but not in CM children.

For recombinant proteins, levels of IgG anti-*Pf*EMP3/5 (rho = 0.39; $p = 0.001$) (Figure 4(c)) and IgG anti-*Pf*EB200 (rho = 0.61; $p = 0.017$) (Figure 4(d)) were positively correlated to LIR in SCM group. When CM adults and children were analyzed separately with stratification on the outcome, the positive correlation were stronger in surviving CM adults (rho = 0.44; $p < 0.001$ for IgG anti-*Pf*EMP3/5 and rho = 0.72; $p = 0.011$ IgG anti-*Pf*EB200) than in CM children who recovered (rho = 0.29; $p < 0.01$ for IgG anti-*Pf*EMP3/5 and rho = 0.54; $p = 0.031$ IgG anti-*Pf*EB200) (Figure 4(b)).

4. Discussion

In this study, we analyzed Ab responses against *P. falciparum* total and recombinant Ags in individuals living in an urban area of low endemicity who suffered of acute symptoms of malaria requiring hospitalization. Two groups of patients (MM and CM), considered as nonimmune or partially immune and residing in the same locations, were selected and categorized on the basis of the disease severity. These individuals, regardless of age, of previous history of infection, and of individual conditions of exposition to infective bites, can be considered as being equally at risk for infection resulting in confirmed clinical outcomes with or without severe cerebral symptoms.

Antibodies are known to reduce morbidity and parasite densities in patients with malaria as demonstrated by passive transfer experiments [13, 36]. Intraerythrocytic circulating parasites synthesize a family of proteins displayed at the surface of the red blood cell. The proteins at the host-parasite interface play an important role in malaria pathogenesis: these antigens are under constant immune pressure and diversify to avoid immune detection while maintaining conserved features required for their function in host-parasite interactions. Antibody response against iRBCs surface proteins is a crucial step in initiating the adaptive immune response against the malaria infection as they can promote destruction and/or phagocytosis of iRBCs and subsequent parasite clearance. Analyzing how antibodies interact with iRBCs could be important in understanding the transition from innate to adaptive immunity and develop iRBCs-derived vaccine candidates and a reliable functional-associated monitoring technique of the IgG binding to iRBCs surface.

In this study, we aimed to evaluate the capacity of acquired IgG responses to bind live iRBCs with regard to potential relationship with defined iRBCs antigens and potential prognostic significance in symptomatic urban malaria. We used two whole parasite extracts and three recombinant proteins associated with the surface of iRBCs and shown to protect monkeys by eliciting strong opsonizing Ab responses [37].

The flow cytometry technique has been already used as a relevant surrogate marker in IgG binding to iRBC in different contexts: before and after vaccination trials [18], as function of age and hemoglobin type in Malian children [38] and as a differential marker in Senegalese individuals living in rural settings with different levels of endemicity [17]. In urban setting, previous studies focused on relationship between antibody responses to defined antigens and clinicoepidemiology such as age of individuals and severity of outcome [23, 33]. However, as reported in rural symptomatic malaria, the amount of sequestered parasites increased with disease severity [7], and the binding of *P. falciparum*-infected RBCs to microvascular endothelial cells induces pathological sequelae in microvessels that are associated with the symptoms and severe manifestations such as CM [39].

In this study, the iRBCs binding assay did not show any significant difference of LIR between CM and MM groups but, importantly, there was a significant lower IgG binding capacity to iRBCs in CM patients with fatal outcome compared to surviving group. These results are in agreement with the protection-associated role of IgG to iRBCs expressed antigens [40]. However, such significant difference between surviving versus fatal cases was observed here in adults (≥15 years); this may be attributed to the high anti-*P. falciparum* immune responses described in adults compared to children [8, 23]. To assess the potential predictive role of IgG binding capacity to iRBCs in severe malaria, more studies investigating this phenomenon are warranted, including IgG subclasses that can differentially promote several mechanisms [41] including immune phagocytosis [42].

When regarding technical aspects, the use of live parasite and flow cytometry supports substantial standardization [43] providing comparable interassays results in different studies [15, 17, 18]. Furthermore, the use of FCR3 strain of *P. falciparum* adapted to *in vitro* culture has been investigated; a number of changes in plasmatic membrane including a strong expression of knob-like structures involved in vascular adhesion and sequestration were described [44]. Indeed, the FCR3 strain used here has a limited relationship with natural parasites infecting population in urban Dakar; it may probably underestimate the actual individual IgG binding capacity. To circumvent the limited Ag repertoire expressed *in vitro* by FCR3 strain of *P. falciparum*, further analysis using local strains of parasites should be conducted. Implication of some other factors such as host genetic [38] and hemoglobin types [17] should be also investigated in urban setting.

For the determination of antibody responses, the use of whole parasite extract antigens and recombinants proteins showed that IgG responses to iRBCm and *Pf*EMP3/5 were positively correlated and their levels were significantly higher in MM compared to CM patients. These results are in agreement with previous findings of association with protection reported in *Saimiri* models [37] and in human [31]. This antigen, derived from the parasite-encoded protein *Pf*EMP3, is expressed in knobs structures and described as inducing a strong propensity for iRBCs to adhere in the vasculature [31]. However, protective role of Abs to *Pf*EMP3/5 and other iRBC expressed proteins such as *Pf*EB200 is difficult to establish for several reasons. Firstly, a large array of antigens expressed on the surface of infected erythrocytes [45] have been identified as potential targets of protective immunity and promising vaccine candidates [10]. Secondly, Bull et al. demonstrated that severe malaria resulted from a substantial limitation of the antibodies repertoire to variant antigens (VSAs) [11] and the main antigenic ligands responsible for both cytoadherence and antigenic variation are members of the *P. falciparum* Erythrocyte Membrane Protein-1 (*Pf*EMP1) family [44–46].

These iRBC surface proteins are highly diverse and undergo clonal antigenic variation under selective pressure by immune responses [47, 48]. They are dominantly recognized by antibodies from individuals with uncomplicated malaria [49], and the surface antigens of those parasites seem to be geographically conserved [50], supporting the hypothesis that the surface molecules expressed by parasites causing severe malaria, especially cerebral malaria, maintain relatively conserved epitopes. Indeed, the age-related acquisition of protection in endemic areas requires both strain-specific and strain-transcending IgG responses to iRBCs as reported by measuring anti-VSA binding assay in different settings of Tanzania [51]. In addition the strain-specific cumulative repertoire increases but was shown not to be infinite with a relative cross-geographical conservation [50]. It is clear that optimal anti-IRBCs Ab response would require additional anti-VSA measure, but VSA measure requires heavy simultaneous culture procedure of multiple strains which is more difficult than a potential use of one strain and/or defined Ag targets in the case of hospitalized patients with severe symptoms.

Thus, recombinant proteins tested in our study such *Pf*EMP3/5 and *Pf*EB200 are predominantly expressed in *P.*

falciparum knobs structures [29, 30] whereas the presence of knobs may not necessarily result in sequestration. For example, *P. malariae* has knob structures but does not sequester, while *P. chabaudi* sequesters without knobs [52].

Taken together, our results underline the difficulty to establish a clear correlation between antibodies analyzed by ELISA and protection against disease outcome [40, 42, 53]. The use of ELISA alone cannot provide information about the function of antibodies [48]. The live iRBCs binding assay introduces a potential functional-associated assay for symptomatic malaria analysis. Here the absence of correlation of LIR with parasitaemia agrees with previous studies reporting measuring anti-erythrocyte surface antibodies [40] or iRBC binding capacity by agglutination [53] with protection from clinical malaria in endemic area. It is likely that these antibody responses are short-lived [54] and the presence of antibodies without parasites may simply reflect a recently treated acute infection, possibly a marker of increased susceptibility [40].

5. Conclusion

ELISA provides distinct measure of the anti-*P. falciparum* IRBC responses compared to iRBCs binding assay. Our results showed a significant difference of LIR between surviving and fatal outcome in patients with confirmed cerebral malaria. This IgG binding assay has potency for analysis of symptomatic malaria; it deserves further investigation to determine to what extent it could provide a relevant indicator highlighting a risk of fatality in cerebral malaria.

Competing Interests

There are no existing competing interests.

Authors' Contributions

Ronald Perraut and Alioune Dieye designed the study. Babacar Mbengue, Mouhamadou Mansour Fall, and Birahim Niang were in charge of recruitment of patients. Babacar Mbengue, Antoine Marie Diatta, and Ronald Perraut performed the tests and were in charge of database management and the plasma database. Babacar Mbengue and Ronald Perraut conducted data analyses with input from Maguette Sylla Niang, Rokhaya Ndiaye Diallo, Marie Louise Varela, Kantome Ndiaye, Moustapha Mbow, and Alioune Dieye. Babacar Mbengue and Ronald Perraut drafted the paper with input from Alioune Dieye. All authors read and approved the final paper.

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Review Article

Management and Treatment of Human Lice

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Of the three lice (head, body, and pubic louse) that infest humans, the body louse is the species involved in epidemics of louse-borne typhus, trench fever, and relapsing fever, but all the three cause pediculosis. Their infestations occur today in many countries despite great efforts to maintain high standards of public health. In this review, literature searches were performed through PubMed, Medline, Google Scholar, and EBSCOhost, with key search words of "*Pediculus humanus*", "*lice infestation*", "*pediculosis*", and "*treatment*"; and controlled clinical trials were viewed with great interest. Removing lice by hand or with a lice comb, heating infested clothing, and shaving the scalp were some of the oldest methods of controlling human lice. Despite the introduction of other resources including cresol, naphthalene, sulfur, mercury, vinegar, petroleum, and insecticides, the numbers of lice infestation cases and resistance have increased. To date, viable alternative treatments to replace insecticides have been developed experimentally *in vitro*. Today, the development of new treatment strategies such as symbiotic treatment and synergistic treatment (antibiotics + ivermectin) *in vitro* has proved effective and is promising. Here, we present an overview on managing and treating human lice and highlight new strategies to more effectively fight pediculosis and prevent resistance.

1. Lice and Their Public Health Impact

1.1. Overview. Lice have been parasites of humans for thousands of years and differ according to their habitat on the host [1]. The three sucking lice that infest humans are the body louse (*Pediculus humanus humanus*), the head louse (*Pediculus humanus capitis*), and the pubic or "crab" louse (*Pthirus pubis*) [2]. Body lice and head lice harbor the same endosymbiotic microorganism that seems to be essential for the production of nutritional components such as the B vitamins lacking in host feedings [3, 4]. In 2006, Sasaki-Fukatsu et al. were the first to describe the phylogenetic placement of the primary endosymbiont of human body lice; they identified this endosymbiont as a γ-proteobacterium and named it *Candidatus* Riesia pediculicola [5].

Each year, louse infestations still affect hundreds of millions of people worldwide [6], 6 to 12 million children in the United States annually [7]. Analysis of data on the global incidence of pediculosis has shown that this remains a major health problem in many countries [8]. Thus, the head louse is prevalent in all countries, and outbreaks have been described at all levels in society [9]. However, children of primary school age constitute the largest group of people affected [10]. Approximately 6–12 million cases of infestation occur each year in the United States among children 3–12 years old [11]. Infestation may be increased in school children with more siblings and of lower socioeconomic group [12]. The pubic louse is usually a sexually transmitted organism, although atypical locations such as eyebrows and eyelashes have been reported [9, 13]. The body louse lives in clothes and multiplies when cold, promiscuity, lack of hygiene, and war are present. Its prevalence also reflects the socioeconomic level of society [14]. During the civil wars in Burundi, Rwanda, and Zaire in the 1990s, the prevalence of lice infestations reached 90–100% [15]. The body louse is the species known to be involved in epidemics of louse-borne infectious diseases [16], but all the

three cause pediculosis which is highly contagious and easily transmitted by close body-to-body contact or contact with infested linen, brushes, or clothes, according to the species of louse. A louse-infested person can be infested by thousands of lice, each of which bites on average five times per day for body lice [17]. In literature, several methods were used to get rid of lice infestations. Thus, this review summarizes the management methods and various strategies used in treating these hematophagous parasites.

1.2. Physiology and Exponential Multiplication of Lice. Humidity is a critical factor for lice; the optimal humidity for survival is in the range of 70–90% [9]; they cannot survive when this value falls below 40%. Temperature is also highly influential on the louse's physiology. According to Maunder, laboratory lice prefer a temperature between 29 and 32°C [9]. At 50°C, body lice die, and this temperature is critical when washing clothes, as water and soap alone will not kill lice [43]. Although eggs can survive at lower temperatures, their viability is limited to 16 days [43].

A louse typically feeds five times a day and each female can have several successive partners. At maturity, lice can mate every day and each female lays 8–10 eggs per day, with a female able to lay up to 300 eggs during her lifetime. During the prolonged mating process, both the male and the female will continue to feed [9]. Eggs are laid in the folds of clothing for body lice and in the hair for head lice; they are held in place by an adhesive produced by the mother's accessory gland [13]. Hatching occurs 7–10 days after laying. There are three nymphal stages (L1, L2, and L3), moulted the third, fifth, and tenth days after hatching and which are differentiated by their size.

1.3. Remarks on the Genetics of the Louse (Body Lice versus Head Lice) and Its Symbiont. Genetic tools were used to separate human lice into head lice and body lice. The first study was based on the 18S rRNA gene [44], and subsequent studies focused on mitochondrial genes [45–47] and intergenic spacers [48, 49]. These studies revealed that there are three clades of head lice, one of which may also be body lice (clade A) [46, 47]. Subsequently, a fourth clade including both head and body lice was detected in Congo [50]. A transcriptome study of human head and body lice revealed that there is only one gene present in body lice but not in head lice. Otherwise, the main differences identified between head lice and body lice concern gene expression levels [51]. Indeed, 14 putative differentially expressed genes were identified by comparing head louse and body louse data. However, head lice and body lice have almost the same genomic content but are phenotypically different (different ecotypes) as a result of differential gene expression [52]. Thus, a rapid multiplex real-time PCR assay was established that differentiates between head and body lice with 100% specificity and sensitivity [53]. Based on these studies, we can suggest that the clade A head louse has a deleted genome and originated from the body louse. The opposite hypothesis was considered evident for years [9, 45, 54].

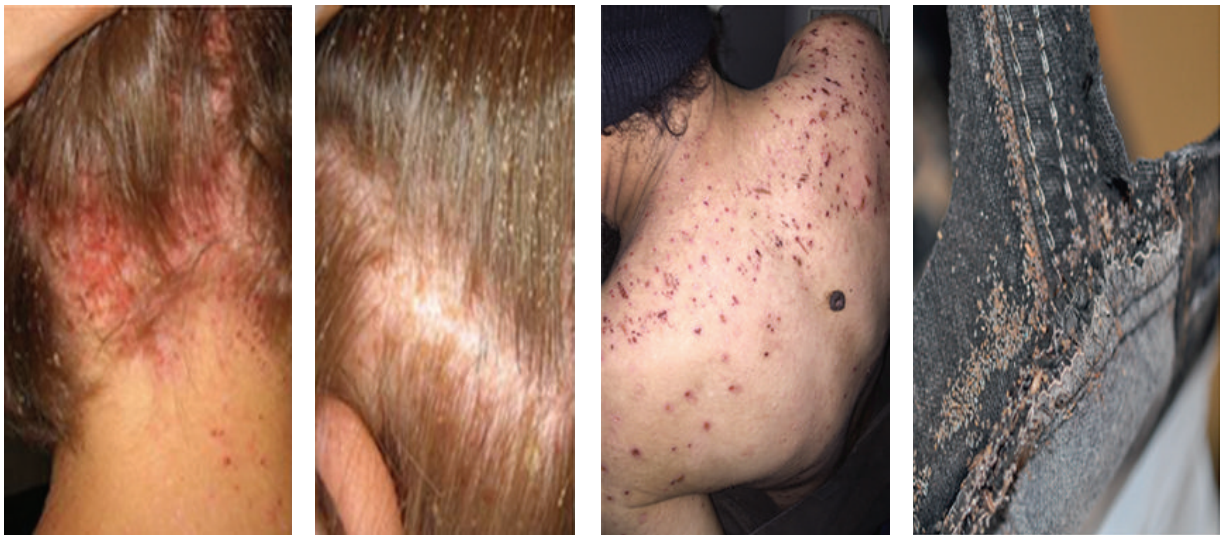
The primary endosymbiont of human body lice is a bacterium belonging to the family Enterobacteriaceae in the γ-Proteobacteria class [5]. Organs called mycetome host the primary endosymbiont, except during passage to the ovaries for transovarial transmission [55]. A recent study of the genome of *Candidatus* Riesia pediculicola revealed a small genome, 574 kB, similar to what is found in other insect primary endosymbionts [56]. The reduction in genome size and the high AT-bias suggest an ancient association between the louse and its primary endosymbiont [56]. Thus, *Candidatus* Riesia pediculicola is an insect primary endosymbiont (P-endosymbiont) that has been associated with the louse for 13–25 million years [52].

1.4. Louse-Borne Infectious Diseases. Louse-borne diseases are associated with a high prevalence of body louse infestation and have recently reemerged in jails and refugee camps in central and eastern Africa [15], in rural communities in the Peruvian Andes [57], in rural populations in Russia [58], and in homeless populations living in poor hygiene conditions in developed countries [59–63]. Given the phagocytic activity of their immune system, it is more likely that body lice are vectors of pathogens than head lice [64]. The physiological difference between head and body lice is that head lice do not transmit human diseases, whereas body lice are vectors of bacterial diseases transmitted to humans, including trench fever caused by *Bartonella quintana*, relapsing fever caused by *Borrelia recurrentis*, and epidemic typhus caused by *Rickettsia prowazekii* [1, 57, 65]. *Acinetobacter baumannii* was found in 21% of the 622 body lice collected worldwide [66], but the transmission of the infection *A. baumannii* by body louse has not yet been demonstrated. In Morocco in the 1940s, the causative agent of plague, *Yersinia pestis*, was recovered from a body louse collected from a septicemic patient [67, 68]. In the Democratic Republic of the Congo, DNA from *Yersinia pestis* was observed in one head louse and two body lice [50, 69]. In addition, the experimental model to evaluate the human body louse as a vector of plague has been demonstrated in our laboratory [70].

Head lice can carry pathogens although their role as a vector has not yet been clarified. DNA from *B. quintana* was found in head lice from Nepalese children in 2006 [71], from homeless individuals in the USA in 2009 [65], and from the local population in Congo, Madagascar, and Senegal [72]. It was also found in head louse nits from a homeless person in Marseille, France [73], and in head lice from Ethiopian [74] and Senegalese [75] patients. DNA from *A. baumannii* was detected in 33% and 3% of the head lice collected from schoolchildren in Paris, France [76], and Diankabou, Mali, respectively [77].

2. Diagnosis of Lice Infestations

Lice infestation is a common problem and diagnosis is generally based on the presence of nits or lice. The characteristic itching or pruritus that accompanies infestation may in some cases be complicated by bacterial infections (Figures 1(a) and 1(b)) that occur when the skin becomes excoriated [78, 79]. Lice may be seen on the scalp, in the hair, or on clothing of the infected person. However, the techniques used for



(a) (b)

Figure 1: Nuisance related to lice: (a) scalp infection caused by head lice; (b) scraping lesions related to body lice infestation.

diagnosing a louse infestation (*Pediculus humanus*) are a source of controversy. Most epidemiological studies have used direct visual examination (visual inspection). Direct visual examination (Figure 2(a)) with a magnifying glass and combing with louse comb (Figures 2(b) and 2(c)) are two frequently used methods; but the first is not a reliable method for diagnosing living lice on hair [80]. It underestimates active infestation and is only useful with heavily infested patients. Elsewhere, Balcioglu et al. have demonstrated in their study that plastic detection comb is better than visual screening for diagnosis of head louse infestation [81].

3. Treatment Strategies

The fight against pediculosis is certainly a very ancient concern and various methods have been used to get rid of it.

3.1. Historical Methods. Removing lice by hand (Figure 2(a)) or with a lice comb (Figures 2(b) and 2(c)) and shaving the scalp were some of the oldest methods of controlling human lice [82, 83].

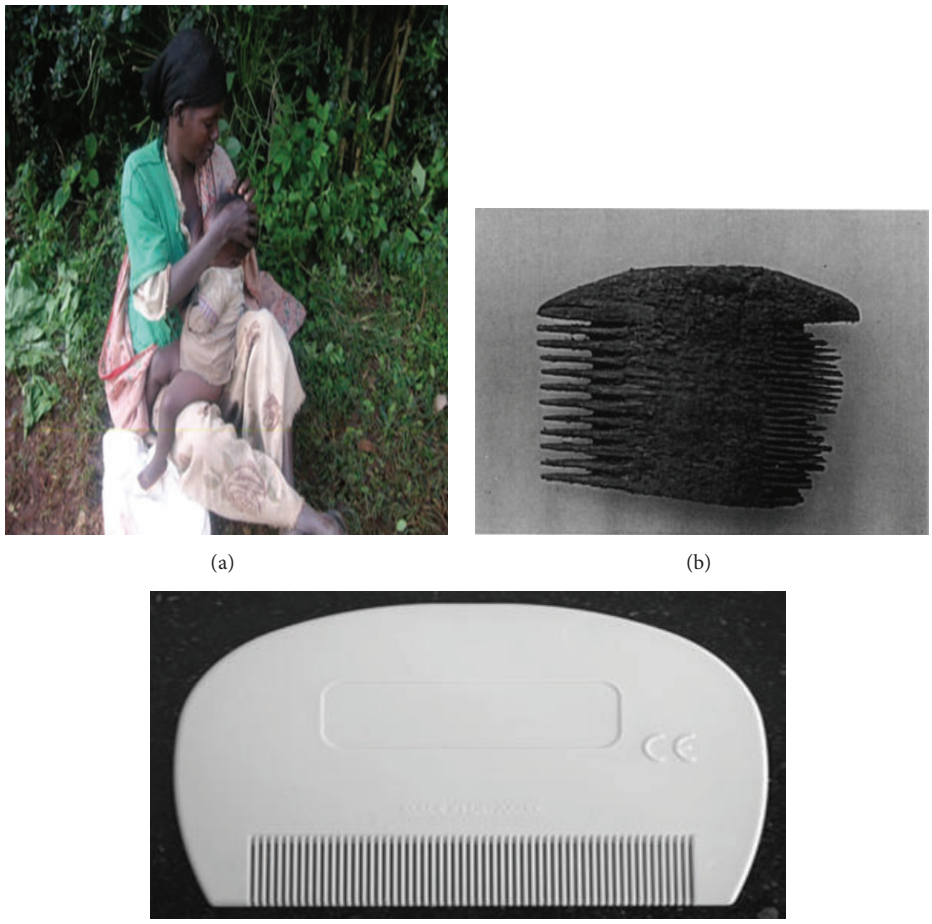
3.1.1. Using Hands. This was the first means used before the comb was invented. Crushing lice with your fingers should be strongly discouraged because it can lead to bacterial penetration through the cutaneous route. Manual removal of nits (especially the ones within 1 cm of the scalp) can only be recommended after treatment with a product [84]. One study showed that manual removal is less effective than pediculicides and does not improve results even when used in addition to a pediculicide treatment [35].

3.1.2. Using Combs. Combs for the removal of adult lice and nits have been used since ancient times [82]. Today, many different types of combs are sold to control lice. Combing can be undertaken every 1–3 days. This method not only is for treatment but can be for prevention, to remove mature lice which might otherwise lay eggs and perpetuate the life cycle. However, it was also demonstrated that the diagnosis of louse infestation using a louse comb is four times more effective (25% versus 6%) than direct visual examination and twice (57 seconds versus 116 seconds) as fast [80]. This study was proven by Balcioglu and colleagues in 2008 [81]. Compared to phenothrin in clinical trials, the bug busting method is effective for managing head lice infestation [41]. Thus, we can conclude that using a louse comb to screen patients for lice infestation and treating with a pediculicide are effective.

3.1.3. Shaving. Head shaving can be a simple method to remove the lice and eggs. It was noted that short hair does not prevent head lice infestations [78]. However, head shaving should be avoided whenever possible because it is humiliating, especially for girls. Complete shaving of the head generally does eliminate lice and prevents reinfestation but is rarely an appropriate measure to take in response to infestation [85].

3.1.4. Using Heat. A further step is heating infested clothing and bedding with hot water to destroy all stages of lice. The heat necessary for the destruction of both lice and nits is 52°C for 30 minutes [86]. This method can be easily applied to infested clothes using hot water but cannot be used on infested hair. Lice may, however, make themselves heat resistant by a hormonal process. Heat resistance is achieved by lice excreting heat resistant, protective secretion through their outer skeleton. This is part of the natural defense mechanism of lice. When the lice have become heat resistant, they can tolerate very high temperatures (above 100°C) [87].

3.1.5. Chemical Products. Cresol, naphthalene, sulfur, mercury, petroleum, naphthalene, and petrolatum were



(a) (b) (c)

Figure 2: A few historical methods to get rid of lice. (a) Detecting lice or nits by direct visual examination; (b) wooden comb found at Ein Rachel (Negev Desert) (100 BC–200 AD) containing 10 head lice and 5 nits; (c) modern plastic comb.

employed alone or, for some of these products, in combination with oil or vinegar [83, 88]. Due to major adverse effects and/or resistance, all of these methods have now been discontinued.

3.2. Local Products

3.2.1. Study Methods: Sensitivity and Resistance. Measurement of the susceptibility or resistance per se may be performed only by using doses of insecticide applied via an inert carrier, and this is the basis of the WHO tests and their variants [89–93]. The majority of studies have used WHO-recommended protocols [94] or modified versions of those techniques. Studies have sought to measure the effectiveness of pediculicides in controlled laboratory test systems. Measurement of sensitivity to insecticide in formulations cannot be generic and must be measured either *in vivo* by means of a clinical trial or *in vitro/ex vivo* by using the whole formulation [95]. The methodology of *ex vivo* tests conducted by Meinking and colleagues [96, 97], in which the lice were allowed to continuously bubble on wet sponge with pediculicidal and without subsequent washing, may have given unrepresentative results in some cases. Two studies evaluated resistance to permethrin using techniques on the basis of a WHO method employing single concentration applications of insecticide to filter papers and measuring time to death of the test insects, in order to record LT50 and LT95 [94]. However, in one of these studies the levels of insecticide used to measure the lethal dose were considerably lower than those recommended by WHO [98], so in some cases the measure may have been more of vigor tolerance (reduced sensitivity of an insect population due to elimination of the least robust insects by weak selection) than resistance [99]. Tests using laboratory-reared body lice may be less discriminatory than tests employing wild-caught head lice, and they do not identify variations of effectiveness likely to arise as a result of selection pressure [96]. Observations of lice that survived adequate clinical applications of insecticide products containing more than 13% of monoterpenes suggest they have already become resistant to these chemicals [95].

3.2.2. Pediculicides. Since World War II, many insecticides have been used against lice. Among those for treatment of head and body lice were organochlorines (DDT, lindane), organophosphates (malathion), carbamates (carbaryl), pyrethrins (pyrethrum), and pyrethroids (permethrin,

Table 1: Main products used in clinical trials in humans: efficacy and safety.

Comparison of treatments	Efficacy	Safety	References
1,2-Octanediol versus *malathion*	More effective	Adverse effects reported	[18]
1,2-Octanediol versus *placebo*	Effective	No serious adverse events	[19]
Cocamide DEA versus *permethrin*	May be as effective	Adverse effects reported	[20]
Phenothrin versus *wet-combing*	May be as effective	No evidence of harms from combing	[21]
Tocopheryl acetate versus *permethrin*	More effective	No adverse effects reported	[22]
Dimeticone versus *permethrin*	More effective	No serious adverse events	[23, 24]
Dimeticone versus *malathion*	More effective	No adverse effects reported	[25]
Dimeticone versus *dimeticone plus nerolidol*	As effective	No adverse effects reported	[26]
Dimeticone 4% lotion versus *phenothrin*	Equally effective	Few adverse effects reported	[27]
Ivermectin versus *malathion*	As effective	No major adverse effects observed	[28, 29]
Ivermectin versus *placebo (vehicle)*	More effective	No adverse effects reported	[30]
Malathion lotion versus *phenothrin*	More effective	No adverse effects reported	[31]
Malathion versus *permethrin*	More effective	No adverse effects reported	[32]
Lindane versus *permethrin*	As effective	Adverse effects reported	[33]
Permethrin versus *lindane*	More effective	No adverse effects reported	[34]
Permethrin versus *combing*	More effective	No adverse effects reported	[35]
Permethrin versus *placebo*	More effective	No adverse effects reported	[36]
TMP-SMX plus permethrin versus *permethrin alone*	More effective	No major adverse effects reported	[37]
Combined insecticides versus *herbal oils*	As effective	No clinically detectable adverse effects	[38]
Soya oil-based shampoo versus *permethrin*	More effective	No serious adverse events	[39]
Coconut and anise in spray versus *permethrin*	More effective	Adverse effects reported	[40]
Combing versus *phenothrin*	More effective	No evidence of harms from combing	[41]
Bug Buster kit versus *malathion or permethrin*	More effective	No information on adverse effects	[42]

phenothrin, and bioallethrin) [82]. Most of these pediculicides were tested in clinical trials to assess their effectiveness and safety (Table 1). However, their pediculicidal and ovicidal efficacy may vary by product components (Table 2).

(1) Dichlorodiphenyltrichloroethane (DDT) and Lindane. Organochlorines (DDT and lindane) were the first of the synthetic organic insecticides used. The development of DDT during the 1940s had enormous impact. It was immediately used to dust prisoners of war to control body lice and won wide acceptance, not only for use on humans but also for animals [100]. Lindane has been available since 1951. However, its effectiveness has been compared to other products [33, 34]. Physiological resistance among both head and body louse populations to lindane is widespread [101]. These two organochlorides are neurotoxic for parasites. Due to developed resistance [91] and safety concerns, the use of these products should be discussed [6].

(2) Malathion. An organophosphorous insecticide formulated in concentrations of 1.0% and 0.5%, malathion, has been widely used in the USA and Europe. It worked rapidly against adult lice and was usually effective ovicide [102]. Its effectiveness has been tested in clinical trials [31, 32, 42]. However, resistance of body lice to malathion has been reported in Burundi [103] and Ethiopia [104]. In France, head lice resistance to malathion is reported to be based on clinical failure to control infestations [105]. In addition, a randomized study in 22 volunteers found no evidence that malathion was dangerous in the treatment of head lice when the products were applied in accordance with the instructions for use [106]. However, its use in children under 6 months should be avoided [6].

(3) Carbaryl. Used since 1976, more recent reviews have reported carbaryl to be less effective than previously thought [107]. Potentially carcinogenic in rodents, its prescription was restricted in UK [6].

(4) Natural Pyrethrins or Synthetic Pyrethroids (Phenothrin). These pyrethroids are closely related to permethrin and are combined with a synergist (piperonyl butoxide) or

Table 2: Main formulations of physical acting and insecticide-based products available in France.

Physical acting products	Principal component(s)	Insecticide-based products	Principal component(s)	Activity
Pouxit® XF Extra Fort	Dimeticone-1.6, dodecatrien-3-ol 3,7,11-trimetyl PEG/PPG dimeticone co-polymersilica silylate	Prioderm®	Malathion	100% pediculicidal and ovicidal activity
Duo LP Pro®	Triglycerides, lipid esters (Oxyphthirine)			
Itax®	Oily silicone based complex			
Altopou®	Cyclomethicone 5, dimeticone	Marie-Rose	Pyrethrin	
Pouxit	Cyclomethicone 5, dimeticone	Para® Special Poux	Alethrin (prallethrin)	100% pediculicidal activity and insufficient ovicidal activity
Paranix® mousse	Dimeticone, paraffin oil	Para Plus	Malathion, permethrin	
Paranix new formule action double	Dimeticone, mineral oil			
Marie-Rose® une seule application	Cocamidopropyl betaine cocamide DEA			
Parasidose® lotion traitante	Ricinus, paraffin, cocamide DEA, cocos	Pyreflor® lotion	Permethrin 25/75	Insufficient pediculicidal activity and insufficient ovicidal activity
Parasidose nouvelle formule Biococidine®	Biococidine	Sklice® lotion	Ivermectin	
Yapapou®	*Cocos nucifera*, cocamide DEA, citric acid, cocamidopropyl			
Poux Apaisyl®	Coconut oil derivatives			

nonsynergist insecticide (permethrin) [6]. Like malathion, these products can be a fire danger, and burns have been reported [108]. As with permethrin, resistance to this compound has already appeared in France [109], UK [110], and the Czech Republic [111]. In clinical trials, phenothrin has been demonstrated to also be more effective than wet-combing [21]. It seems likely that resistance to pyrethroids will develop much more rapidly than was the case with older compounds [112]. However, many studies worldwide have already described resistance to pyrethrins and pyrethroids [91, 99, 113].

(5) Permethrin. Synthetic pyrethroid, introduced for the first time in the 1986s, 1% permethrin, was approved and was available over the counter for use in 1990 [114]. It is one of the most frequently used treatments against human ectoparasites (head lice and scabies) among lindane, malathion, and carbaryl [34, 115]. However, resistance to permethrin has been reported in many studies throughout the world [116–120]. In clinical trial, permethrin compared to soya oil-based shampoo, coconut, and anise in spray has been less effective [39, 40].

(6) Dimethicone. Some studies found dimethicone to be a safe product and more effective than permethrin [121]. In their randomized controlled trial, Burgess et al. confirm efficacy of a single application of 4% dimeticone liquid gel in comparison with two applications of 1% permethrin [23]. Used in many clinical trials, its effectiveness may depend on product [24–27, 122]. However, resistance to dimethicone is unlikely due to its physical mode of action [114].

(7) Oxyphthirine®. The Oxyphthirine lotion in a single application in treatment of human lice infestations was revealed to be the safest as it was shown to be nonflammable. The product showed a high efficiency (100%) and certain ability to remove attached nits [123]. The lotion, a patented meta-emulsion suitable for treatment of human head lice (*Pediculus capitis*), has a mechanical action that asphyxiates lice and nits [123].

(8) Benzyl Benzoate/Benzyl Alcohol. In concentrations of 10% to 30%, this substance has been widely used for treatment of pediculosis and scabies, although it is not always effective ovicide. This product may cause allergic reactions and skin irritations. It is no longer registered for lice control in the USA, and in Canada it is only available on prescription [112]. Benzyl alcohol 5% is nonneurotoxic and kills head lice by asphyxiation. Its side effects are pruritus, erythema, pyoderma, and ocular irritation [114].

(9) Spinosad. Spinosad 0.9% topical suspension is a new ovicidal and pediculicidal treatment against head lice created by fermenting *Saccharopolyspora spinosa*, a bacterium found in the soil [124]. However, the most common adverse reactions observed include erythema, ocular irritation, and irritation at the application site [125].

(10) Other Products. 1,2-Octanediol, cocamide diethanolamine lotion (cocamide DEA), and tocopheryl acetate 20% were tested in clinical trials against head lice to assess their efficiency and safety (Table 1) [18–20, 22]. However, the adverse effects were reported with 1,2-octanediol and

cocamide diethanolamine except tocopheryl acetate. Thus, continued study is recommended to establish long-term safety of new and alternative agents [126].

3.3. Per Os Treatment. As all other external treatments, per os treatment may have more secondary effects.

3.3.1. Ivermectin. Macrocyclic lactone, ivermectin, is widely used throughout central and western Africa as a microfilaricide to control the transmission of human onchocerciasis and Bancroft filariasis and has been effective against ectoparasites and nematodes in veterinary medicine. It is similar to macrolide antibiotic agents but without antibacterial activity. Clinicians have explored using ivermectin for human ectoparasitics, specifically for head lice (and scabies). In clinical trials, its effectiveness has been compared to other products [29, 30]. Ivermectin appears to provide encouraging results in the treatment of head lice [127] with a need for further doses [128], despite a potential resistance to this molecule being demonstrated in laboratory conditions [129]. However, in a cohort of homeless subjects in Marseilles, France, a prevalence of 85% for body lice was observed [130]. This infestation was reduced temporarily to 19% with three doses of oral ivermectin administered at seven-day intervals [130]. Currently, resistance to ivermectin has been widely demonstrated in many arthropods [131, 132] and is an increasing problem for ectoparasite and nematode control. In clinical trials, oral ivermectin, given twice at a 7-day interval, may be more effective [28] or as efficacious [29] as topical 0.5% malathion lotion.

3.3.2. Cotrimoxazole. Although this product (trimethoprim/sulfamethoxazole, TMP/SMX) has also been reported as a treatment in head lice [133], these compounds are not currently recommended for controlling body lice. Moreover, using this molecule as a pediculicide was stopped due to the multiple adverse effects (nausea, vomiting, rash, transient pruritus, and allergic-type reactions) recorded in participants in clinical trials [37].

4. New Approaches

The posttreatment reemergence of lice is common and still remains a real challenge. Treatment success depends on improving our knowledge of the fundamental biology and physiology of the louse.

It is important to notice that per os treatment may have more secondary effects as all other oral medicines than external products.

4.1. Symbiotic Treatment. Only a few P-endosymbionts have been described among 14,000 species of hematophagous insects [134, 135]. The endosymbiont *Candidatus* Riesia pediculicola (Figure 3(a)) is a microorganism hosted by body (*Pediculus humanus corporis*) and head lice (*Pediculus humanus capitis*) that appears to be essential for the production of nutritional components such as the B vitamins lacking in host feeding [3, 4]. In our laboratory, we developed an experimental *in vitro* feeding model using an artificial membrane to demonstrate that doxycycline (an antibiotic belonging to the family of tetracyclines) given at different doses (10, 20, and 50 μg/mL) daily for up to 10 days affects the endosymbiont of lice (Figure 3(b)) and also decreases egg production [136]. It was demonstrated that the symbiont *Candidatus* Riesia pediculicola is a possible target for the development of louse-control strategies [56], because loss of these bacteria would mean the death of their hosts. However, symbiotic treatment remains promising and it would be interesting to evaluate the effectiveness of other drugs alone or in combination on lice by targeting their endosymbiont bacterium.

4.2. Synergistic Treatment (Antibiotics + Ivermectin): Figures 3(c) and 3(d). Several compounds, including antibiotics, have been shown to increase intracellular concentrations of macrocyclic lactones [137]. Indeed, antibiotics such as doxycycline, erythromycin, or azithromycin were recommended to treat some infections linked to lice [138]. In addition, it was shown that drug combinations including ivermectin provide antifilarial activity with ancillary benefits on intestinal helminths and ectoparasites, such as chiggers and lice [139]. However, in experiments with adult worms, when doxycycline was combined with macrocyclic lactones in ivermectin the effectiveness was approximately 80% versus 9% for treatment with doxycycline alone [140, 141]. The effectiveness of this combination has also been confirmed in naturally infected dogs with *Dirofilaria immitis* [142]. Recently, in our laboratory we have demonstrated the effectiveness of drug combinations (especially doxycycline + ivermectin, erythromycin + ivermectin, rifampicin + ivermectin, and azithromycin + ivermectin) on body lice reared on rabbits in our laboratory [143]. Thus, we conclude that the synergistic effect is one of the most effective means of lice treatment and also prevents reemergence and resistance.

5. Future Efforts

Lice have been intimately associated with humans for centuries. Infestations are increasing worldwide due to insecticide resistance. To date, viable alternative treatments to replace insecticides have been developed experimentally *in vitro*. However, it will be interesting to develop these methods *in vivo* in other studies in order to achieve the complete eradication of lice and avoid the selection of a resistant population of lice. Thus, future efforts should be directed toward the development of pediculicides based on new chemicals such as avermectins and antibiotics.

Competing Interests

The authors declared that there is no conflict of interests.

Authors' Contributions

Abdoul Karim Sangaré, Ogobara K. Doumbo, and Didier Raoult conceived and wrote the final version of the paper.

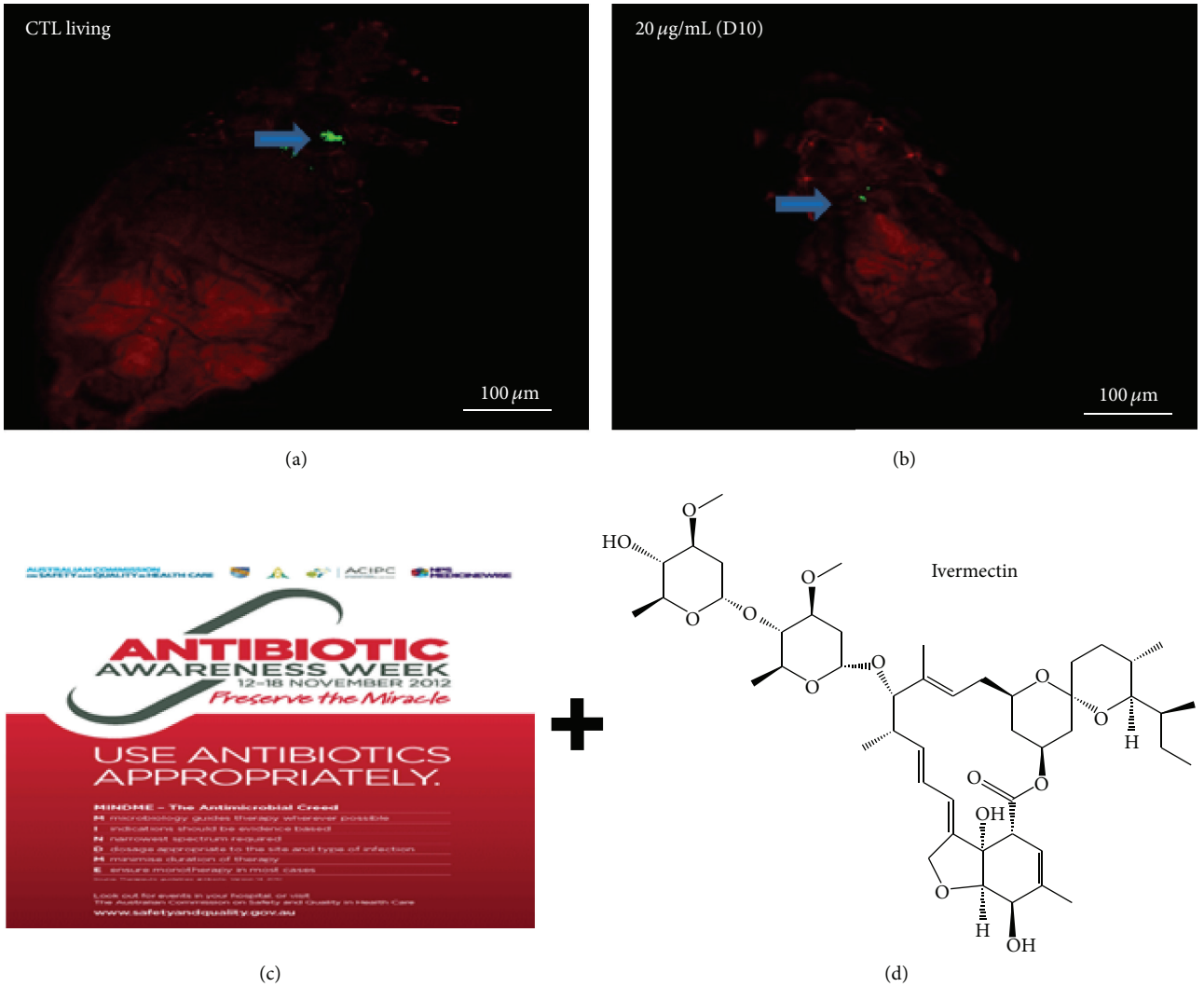


FIGURE 3: New approaches to get rid of lice: *symbiotic treatment* ((a) living control showing higher bacterial fluorescence; (b) louse treated with doxycycline 20 μg/mL taken at day 10 showing lower bacterial fluorescence) and *synergistic treatment* ((c) antibiotics; (d) ivermectin: chemical structure).

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Research Article

Phosphorylated and Nonphosphorylated PfMAP2 Are Localized in the Nucleus, Dependent on the Stage of *Plasmodium falciparum* Asexual Maturation

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Plasmodium falciparum mitogen-activated protein (MAP) kinases, a family of enzymes central to signal transduction processes including inflammatory responses, are a promising target for antimalarial drug development. Our study shows for the first time that the *P. falciparum* specific MAP kinase 2 (PfMAP2) is colocalized in the nucleus of all of the asexual erythrocytic stages of *P. falciparum* and is particularly elevated in its phosphorylated form. It was also discovered that PfMAP2 is expressed in its highest quantity during the early trophozoite (ring form) stage and significantly reduced in the mature trophozoite and schizont stages. Although the phosphorylated form of the kinase is always more prevalent, its ratio relative to the nonphosphorylated form remained constant irrespective of the parasites' developmental stage. We have also shown that the TSH motif specifically renders PfMAP2 genetically divergent from the other plasmodial MAP kinase activation sites using Neighbour Joining analysis. Furthermore, TSH motif-specific designed antibody is crucial in determining the location of the expression of the PfMAP2 protein. However, by using immunoelectron microscopy, PPfMAP2 were detected ubiquitously in the parasitized erythrocytes. In summary, PfMAP2 may play a far more important role than previously thought and is a worthy candidate for research as an antimalarial.

1. Introduction

The spread of antimalarial resistance, in *Plasmodium falciparum* populations, is an important factor in our inability to effectively control this deadly cause of malaria. Consequently, there is an urgent need to identify novel targets for a new generation of antimalarials. MAP kinases have a potential role in the regulation of cell cycle machinery of the malaria parasite upstream of the signal transduction pathways involved in the control of *Plasmodium falciparum* proliferation. The *Plasmodium* parasite is unable to survive in the human red blood cell without protein kinases [1]. *Plasmodium* kinases first reported in 1997 [2, 3] are a promising target for new antimalarials due to divergence in the properties and phylogenetic differences between *Plasmodium* and the host which leaves a space to exploit and inhibit specific enzymes that are crucial to *Plasmodium* [4–6].

In *P. falciparum*, two types of MAP kinases are known: PfMAP1 and PfMAP2 [7]. Initially, only PfMAP1 was thought to be expressed in both sexual and asexual stages of the malaria parasite. Further studies confirmed that PfMAP2 is also expressed in both stages, sexual and asexual. Both MAP kinases differ from a normal 3-cascade MAPK usually found in eukaryotes (MAPKKK-MAPKK-MAPK). Reverse genetic

analysis showed that PfMAP2 plays an important role in *P. falciparum* survivability in the intraerythrocytic life cycle [7]. PfMAP2 is also known to play a compensatory role to PfMAP1 [7]. Although PfMAP1 does not play an essential role in schizogony and Gametocytogenesis its knockout harbours an increase in PfMAP2 protein suggesting PfMAP1 is necessary for asexual cycle survivability [3, 7]. PfMAP2 possesses an atypical activation site which is the TSH motif instead of the TXY motif conserved in MAPKs in other eukaryotes [3]. PfMAP2 is unique in that it cannot be confined to a specific MAPK family. Although both enzymes were originally grouped within the extracellular signal-regulated kinase 1, ERK1/ERK2 family of MAP kinases, a comprehensive phylogenetic analysis [4] of the entire complement of human protein kinase sequences indicates that PfMAP1 is clearly closer to the recently described ERK7/8 [8, 9]. The uniqueness of PfMAP2 is further strengthened by the finding that no members of the MAPKK family are found in the *Plasmodium* genome [8, 9].

Thus, PfMAP2 is seen as a potential drug target for further investigation in hemotherapeutics. However, very little is known about this protein and the function of PfMAP2 is poorly understood. This study was carried out to comprehend and enhance the understanding of PfMAP2 protein in order to convey it as a potential drug target.

2. Material and Methods

(i) In Vitro Culture of P. falciparum. *P. falciparum* 3D7 strain was cultured with some alterations [10, 11]. Percentage of *P. falciparum* used ranged between 8% and 10% parasitemia at 5% hematocrit.

(ii) Synchronization of P. falciparum Culture. *P. falciparum* culture was synchronized using 5% D-Sorbitol as described by Radfar et al. [11]. Briefly, the culture was pooled and centrifuged at 2000 ×g for 5 minutes at room temperature. Supernatant was discarded and 5% D-Sorbitol was added at a ratio of 1 : 1 (v/v) for 15 minutes followed by centrifugation (2000 ×g, 5 minutes, at room temperature). Pellets were washed with washing medium (RPMI1640, gentamycin, HEPES, and hypoxanthine) twice before being added into a new culture flask containing complete medium and freshly processed O+ type blood at 2% hematocrit.

(iii) Extraction of P. falciparum Protein. Synchronized cultures of *P. falciparum* were collected in a 15 mL tube. Subsequently, proteins from the parasites were extracted. Parasites were subsequently harvested by suspending the red blood cell (RBC) for 10 minutes at room temperature in a saponin lysis buffer. Samples were centrifuged at 1300 ×g for 10 minutes and RBC was continuously lysed with saponin twice. Parasites were then washed with phosphate buffered saline (PBS) and the pelleted parasites were subsequently frozen at −80°C.

Lysis buffer containing 1% Triton-X, 50 mM Tris HCl pH 8, protease inhibitor cocktail (Sigma USA), and phosphatase inhibitor cocktail were added to the parasite pellet at a concentration of 1 to 5×10^8 parasites per millilitre. Samples were left on ice for 30 minutes and centrifuged at 13,000 ×g for 10 minutes. A total of 20 μL supernatant containing the parasites were aliquoted for protein analysis using the Bradford assay [12]. The remaining supernatant was mixed equally with 2x-sample buffer (0.5 M Tris HCl pH 6.8, glycerol, 10% SDS, 0.05% 2-mercaptoethanol, and 0.5% bromophenol blue) boiled for 5 minutes and centrifuged at 250 ×g, 5 minutes.

(iv) Western Blot. Samples were subjected to SDS-PAGE [13] and blotted on a nitrocellulose membrane [14]. Immunoblotting was carried out using a rabbit-anti-PfMAP2 antibody and a rabbit anti-phosphorylated PfMAP2 antibody (PPfMAP2) at a dilution of 1 : 3000 overnight in +4°C. Both primary antibodies were prepared by Biogenes GmbH, Germany. Briefly, two New Zealand White rabbits were immunized with a synthetic peptide derived from PfMAP2 amino acid sequence retrieved from PlasmoDB database raised towards sequence CLKKQLTSHVVTR (residue 285–296). Terminal cysteine residue was added on the N-terminus in order to enable direct conjugation to the protein carrier. The purification step allows the antibody to differentiate between a phosphorylated and nonphosphorylated peptide. Rabbit-anti-phosphorylated-PfMAP2 serum is the phosphorylated form of the antibody which is the active form of the protein. The activation motif of PfMAP2 protein is reported to be TSH which is different from the common MAPK which is TXY [3]. The purified phosphorylated-specific antibody recognizes the phosphorylation site (-pT – pS). Membranes were washed using TBST solution three times followed by incubation for an hour on an orbital shaker (50 rpm) with anti-rabbit secondary antibody HRP-conjugated (1 : 15,000). Membranes were washed, incubated with Enhanced Chemiluminescent Western Blotting solution (ECL, Pierce) for 5 minutes prior to film exposure. Densitometry analyses of PfMAP2 and PPfMAP2 bands were performed using Bio-1D software [15].

(v) Indirect Immunofluorescence Assay (IFA). The method used is based on Ramasamy et al. [16] and Dorin et al. [3] with some modifications. Blood smears of *P. falciparum*-infected red blood cells were prepared on slides at different stages of the parasite life cycle. Slides were then fixed with a mixture of methanol and acetone (1 : 1 v/v) for one minute. Control slides were prepared by incubating slides with 200 U of Lambda phosphatase (Calbiochem USA) for 30 minutes at 4°C. Next, 10% Fetal Bovine Serum (FBS) diluted in 1x PBS was used to block the slides at 37°C for 2 hours. Slides were then washed with 1x PBS for 5 minutes, 3 times followed by primary antibody incubation (PfMAP2 or phosphorylated PfMAP2 antibody at 1 : 100 dilutions) for 1 hour at 37°C. Subsequently, slides were washed at intervals of 5 minutes for 3 times. Next, slides were incubated in secondary antibody (conjugated FITC at 1 : 200 dilution) for 1 hour at 37°C and then were washed with PBS. Slides were then stained with 4′, 6-diamidino-2-phenylindole (DAPI) (2 μg/mL) for 30 minutes at 37°C. After incubation, slides were washed with 0.05% phosphate buffered saline-Tween (PBST) for 2 times and once with 1x PBS. Slides were mounted with mounting medium and cover-slipped. Slides were observed

using Olympus fluorescence microscope and images were analyzed using Image Analyzer.

(vi) Statistical Analysis. The effect of parasite stage of development and phosphorylation status on the relative intensities (expression) of PfMAP2 were analyzed using a repeated measures 2-way ANOVA, with a Bonferroni post test (Graph Pad Prism).

(vii) Phylogenetic Analysis. *Plasmodium* and human MAP kinase sequences were retrieved from PlasmoDB and GenBank, respectively. Similarity searches were done using the Basic Local Alignment Search Tool (BLAST). A total of 30 amino acids which contain the activation motives from each MAP kinases were used. The activation motif of *Plasmodium* MAP1 is TDY [3], TSH for *Plasmodium* MAP2 [3, 17], TEY for ERK [18], TGY for p38 [19], and TPY for JNK [20]. MEGA 4 software was used to construct the Neighbour Joining (NJ) phylogenetic tree with 1000 bootstrap replicates. NJ analyses were performed with distances calculated with the p-distance parameter [21–23].

(viii) Bioinformatic Analysis of Localization of PfMAP2. All computational studies were performed on a few bioinformatics tools able to predict the subcellular localization of PfMAP2 protein. PfMAP2 sequence was retrieved from PlasmoDB (http://www.plasmodb.org/). Subcellular localization of PfMAP2 protein was carried out using program CELLO [24, 25], BaCelLo [26], Loctree [27], PProwler [28], Euk-mPLoc 2.0 [29–32], and PredictProtein [33]. Nuclear localization signals, PredictNLS [33], and NetNES 1.1 [34] in PfMAP2 and analysis of PfMAP2 protein were done using Signal Peptide [35] and Secretome v 2.0 [36].

(ix) Immunoelectron Microscopy (IEM) of Infected Erythrocyte. Immunoelectron microscopies were performed as described by Bannister and Kent [37]. *P. falciparum*-infected blood cultures were collected in a 15 mL tube and subsequently centrifuged (2000 ×g, 15 minutes). The pellet was suspended and fixed in McDowell Trump fixative prepared in 0.1 M phosphate buffer (pH 7.2) [37, 38]. Fixed specimens were washed for a few times, dehydrated, and embedded in London White Resin [39]. Ultrathin sections about 70 nm in thickness were collected by using 200–300-mesh nickel grids. The grids were incubated in blocking reagents for 30 minutes at room temperature and then incubated in primary antibody (rabbit-anti-PPfMAP2 antibody) for two hours at room temperature (dilutions of 1 : 10 were used). After repeated washing in TBS-Tween 20 buffer for 5 times, 5 minutes each, the grids were incubated in goat anti-rabbit IgG conjugated to 5 or 10 nm gold particles with dilution of 1 : 50. After washing step with the same buffer, the grids were stained with uranyl acetate for 2 minutes and examined with a transmission electron microscope H-9500.

3. Results and Discussion

Our studies showed that not only is PfMAP2 expressed in all stages of asexual *Plasmodium* development (Figure 1), it is also expressed specifically in the nucleus irrespective of MAPK activation suggesting a more profound role being played by PfMAP2 in contrast to PfMAP1.

We obtained a single band of 59 kDa in our Western blot using the PfMAP2 and PPfMAP2 antibodies (Figure 1(a)). Figure 1(b) showed that the intensity of PfMAP2 protein is highest (2.3×10^5) at the ring stage compared to the trophozoite (1.9×10^5) or schizont (1.6×10^5). The percentage of activation, phosphorylated protein, is about 15.73% more compared to the nonactive form of the protein during ring stage. In the trophozoite stage, the percentage of activation is slightly lower with total of 14.17%. During the schizont stage, although the amount of PfMAP2 protein is lowest compared to the rest, its phosphorylated intensity value is highest at 16.69% more than the nonphosphorylated PfMAP2. Our studies also showed a greater degree of PfMAP2 expression in the ring stage as opposed to the trophozoite and schizont stages suggesting that PfMAP2 is required for *Plasmodium* maturation since it is widely accepted that the ring stage undergoes the most prolific cell differentiation compared to the other asexual stages [4]. This is supported by microarray results [4] which showed higher Pfmap2 mRNA in ring stage as compared to later stages. A functional *map2* gene is required for *P. falciparum* asexual growth [7]. The results showed that expression of PfMAP2 protein is not gametocyte-specific. The presence of both PfMAP2 and phosphorylated PfMAP2 in all stages supports the idea that PfMAP2 plays a crucial role [40] in the *P. falciparum* asexual stage development of the asexual cycle especially during the phase of parasite growth and maturation.

We have also provided evidence showing that the blocking peptide binds completely to the PfMAP2 and PPfMAP2 antibodies as shown by the lack of signal on the Western blots (data not shown). This confirms that both the phosphorylated and nonphosphorylated form of antibodies generated using the TSH motif is highly specific. Therefore, the signals generated in the Western blots and IFA are likely indicative of the expression and localization of the PfMAP2 protein in the parasite. This is the first report on the localization of PfMAP2 in the nucleus of the parasite as contrary to the previous report of PfMAP2 being present in the cytoplasm of the parasite [7]. We explain this as our antibody centres around the TSH motif whereas the previously published expression of PfMAP2 was based on the construction of a knock-in which lacked the complete TSH motif [7]. Moreover, although the expression of PfMAP2 in our study is concentrated in the nucleus, there is a certain degree of expression in the cytoplasm in the areas closest to the nucleus and this is somewhat in agreement to what was observed in the previous report [7] that there exists an overlap in expression in the cytoplasm as well as the nucleus.

Figure 2(a) shows the Indirect Immunofluorescent Analysis (IFA) using PfMAP2 antibody without phosphatase and Figure 2(b) shows PfMAP2 antibody with phosphatase treatment. While Figure 2(c) shows the IFA result with PPfMAP2 antibody without phosphatase treatment Figure 2(d) shows PPfMAP2 antibody with phosphatase treatment. All results barring the control experiment shown in Figure 2(d) showed that PfMAP2 and PPfMAP2 are localized in the parasite

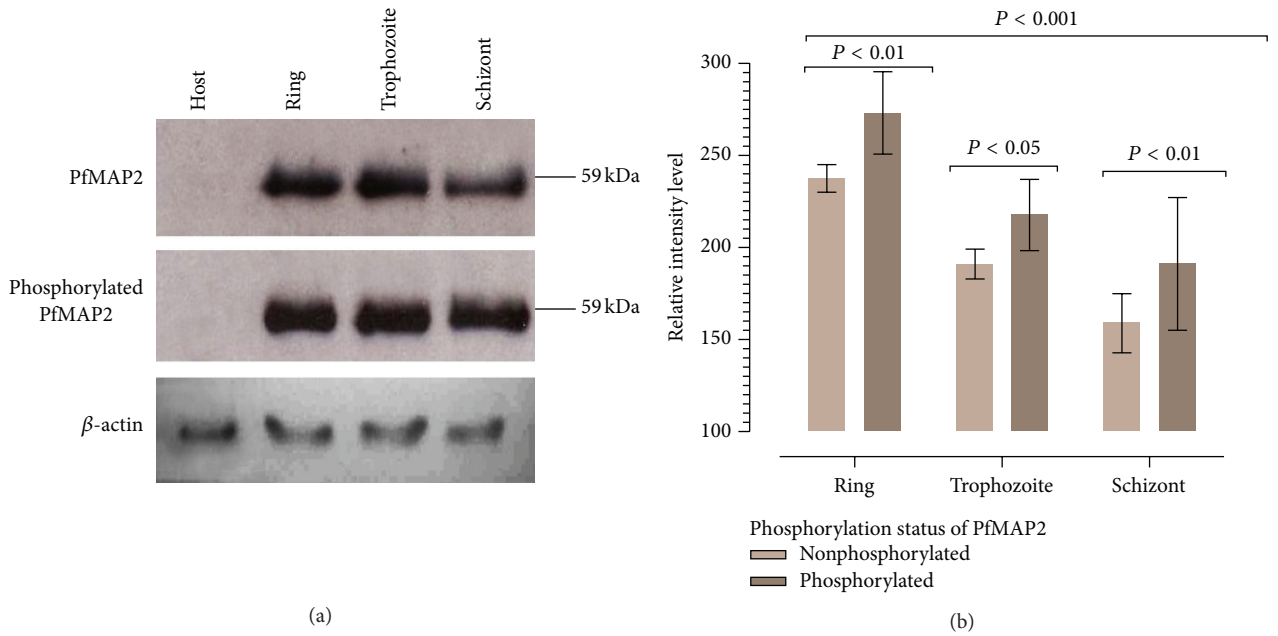

Figure 1: Expression levels of PfMAP2 at different stages of *P. falciparum*. (a) Expression pattern of PfMAP2 and phosphorylated PfMAP2 (PPfMAP2) within the developmental stages of *P. falciparum*. Stage-specific Western blot analyses were performed. H, host; R, ring stage; T, trophozoite stage; S, schizont stage. β-actin was used as a standard to allow semiquantitative comparison between samples over time. (b) Result shown is the average value of triplicate result ($n = 3$) +SE. A repeated measures 2-way ANOVA with Bonferroni post hoc analysis showed that the overall RI of (PfMAP and PPfMAP2) significantly decreased as the parasite developed from ring to schizont ($F = 43.8$, DF = 2,12 $P < 0.0001$), and that the RI of PPfMAP2 was always significantly higher than PfMAP2. However the ratio of PfMAP2 and PPfMAP2 did not significantly differ between stages of development; that is, there was no interaction effect ($F = 0.28$, DF = 2,12 $P = 0.759$ (not shown in the figure)). Immunoblotting was carried out using PfMAP2 peptide and PPfMAP2 peptide (1 : 3000).

nucleus in all intraerythrocytic stages (ring, trophozoite, and schizont). We explain this in terms of the importance of subcellular localization playing a vital role in physiological functions, for example, in metabolic pathways, signal transduction cascades, and structure associated functions [41]. For a protein to function properly, translocation to the right compartments (intra/extracellular) occurs when the protein assumes a soluble form or is attached to a membrane [41]. MAP kinases activated in the nucleus will activate the mitogenic response and hence aid in parasite survivability. Several other proteins are reported to be translocated into the nucleus to play important roles in parasite survivability. Similar to PfMAP2, *Pf60* gene is located within the nucleus in all mature blood stages [42] and its possible function is in regulating homo- or heteromeric interactions in the parasite nucleus. Apart from *Pf60* gene, an unusual peroxiredoxin protein of *P. falciparum*, PfnPrx (earlier known as MCP1) is also localized to the nucleus of *P. falciparum*. PfnPrx is associated with the parasite chromatin suggesting its potentially essential role in the protection of nuclear DNA against oxidative stress which is similar to *Pf60* gene. Pfmrk is similar to mammalian CDK7 and is activated by PfMAT1. It is interesting to note that even though the nuclear localization signal (NLS) sequence is absent in the amino acid sequence of the parasite [43], both Pfmrk and PfMAT1 proteins are localized in the nucleus. Pfmrk activates downstream substrates; PfRFC-5 and PfMCM6, hence suggesting that Pfmrk is important in regulating DNA synthesis [43].

Table 1: PfMAP2 subcellular localization prediction through 6 available servers for eukaryotic proteins.

Bioinformatics tool	Subcellular localization
CELLO	Nucleus
BaCELLO	Cytoplasm-nucleus-cytoplasm
LocTree	Cytoplasm
PProwler	Other than mitochondria peroxisome, secretory pathway
Euk-mPLoc 2.0	Cytoplasm, nucleus
PredictProtein	Nuclear

The PfMAP2 sequence was analyzed using different bioinformatics prediction software as described in the methodology section and shown in Table 1. Four out of six bioinformatics software programs predicted PfMAP2 would be in the nucleus of the parasite. The NES sequence (nuclear export signal) is found to be present in the PfMAP2 sequence; hence the likelihood of it aiding nuclear import-export mechanism in the parasite is high. Interestingly, NES is a lysine residue which potentially interacts with the receptors that export out protein from the cell's nucleus [44] (Figure 3). Upon further investigation, PfMAP2 does not contain a nuclear localization signal (NLS). NLS is a signal or sequence in a protein which transports protein into the cell nucleus by nuclear transport. The function of NLS is known to be in opposition to NES [42, 44, 45].

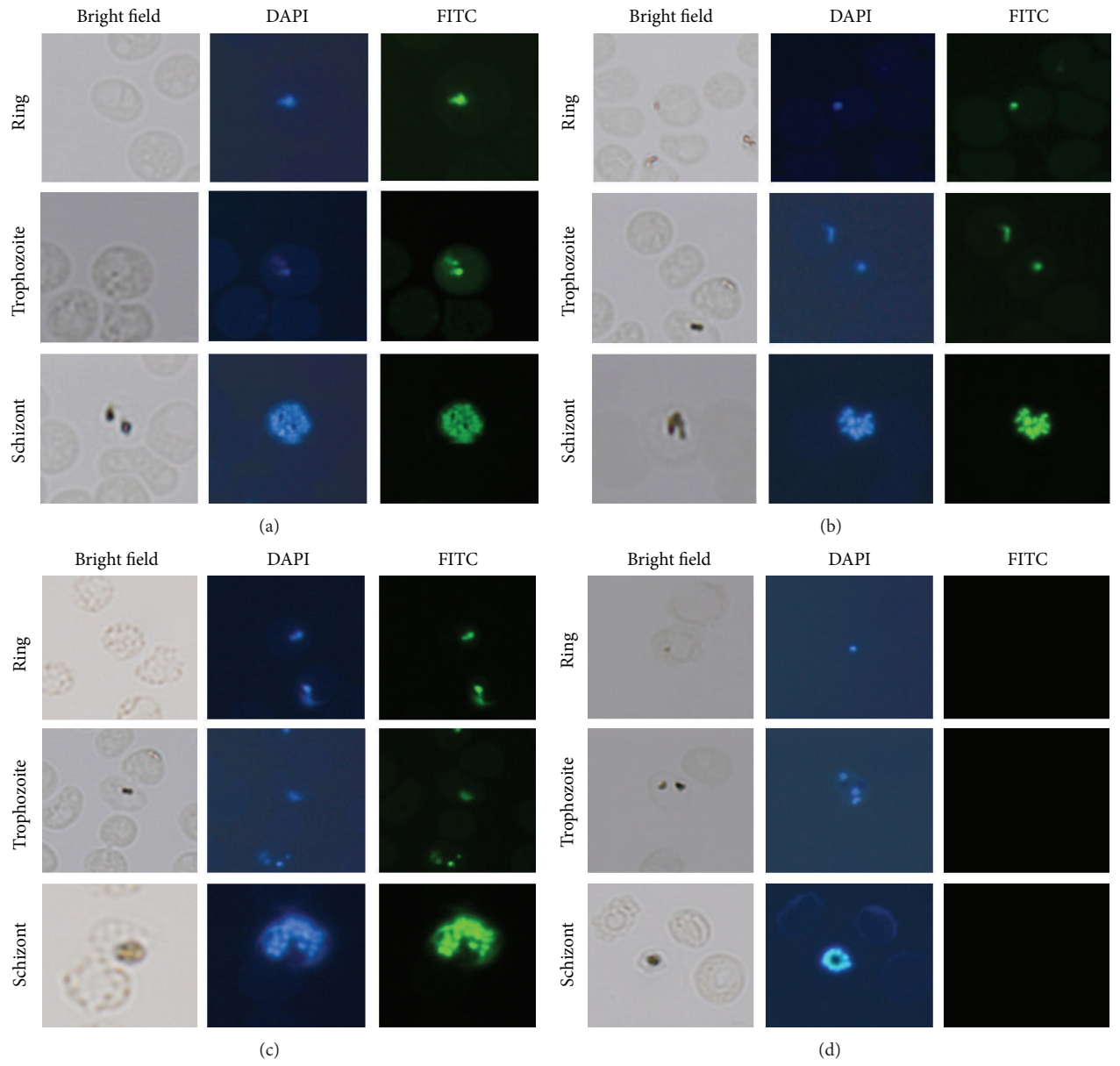

Figure 2: Subcellular distribution of PfMAP2 in *P. falciparum*. Immunofluorescence analysis of (a) PfMAP2 without phosphatase treatment localization in *P. falciparum*-infected red blood cells, (b) PfMAP2 with phosphatase treatment localization in *P. falciparum*-infected red blood cells, (c) PPfMAP2 without phosphatase treatment localization in *P. falciparum*-infected red blood cells, and (d) PPfMAP2 with phosphatase treatment localization in *P. falciparum*-infected red blood cells. *P. falciparum*-infected red blood cells were treated with DAPI (blue) and incubated with primary antibody PfMAP2 (1 : 100) and secondary antibody conjugated-FITC (green) (1 : 200).

Furthermore, removal of the phosphorylation activity of the parasite by the phosphatase treatment further supports the specificity of the PPfMAP2 antibody which we designed focussing on the TSH motif due to the complete lack of signal as shown in Figure 2(d). MAPK is activated when it is phosphorylated on both threonine and tyrosine [46]. However, in *P. falciparum*, PfMAP2 is shown to have atypical property of MAPK as its activation motif is TSH as compared to typical MAPK which is TXY [2, 3]. This divergence of the plasmodial MAPK from the human protein kinase makes them attractive as a potential and promising drug target [5, 47, 48]. The human phosphorylated mitogen-activated extracellular signal regulated protein kinase (MAPK/ERK-P), phosphorylated protein kinase of 38 kDa (p38-P), and phosphorylated stress-activated protein kinase (SAPK/JNK-P) expression have been examined in Alzheimer's disease (AD), Pick's disease (PiD), progressive supranuclear palsy (PSP), and corticobasal degeneration (CBD) [49]. Inhibition of ERK activation reduced *P. falciparum* oocyst load and infection prevalence in *Anopheles stephensi* as well as enhancing TGF-b1-mediated control of *P. falciparum* development [50]. PfMAP2 is reported to be related to SNF-1 family [4].

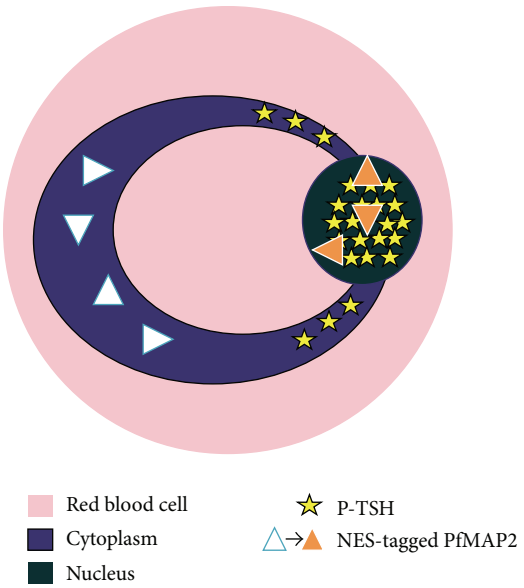

Figure 3: Schematic representation of the localization of phosphorylated PfMAP2. PPfMAP2 was shown to be in the nucleus. Expression of PPfMAP2 also extends into the cytosol in the areas surrounding the nucleus (P-TSH). NES-tagged PfMAP2 are thought to be transported from the cytosol to the nucleus. Upon phosphorylation, it will localize to the nucleus and activate downstream substrates.

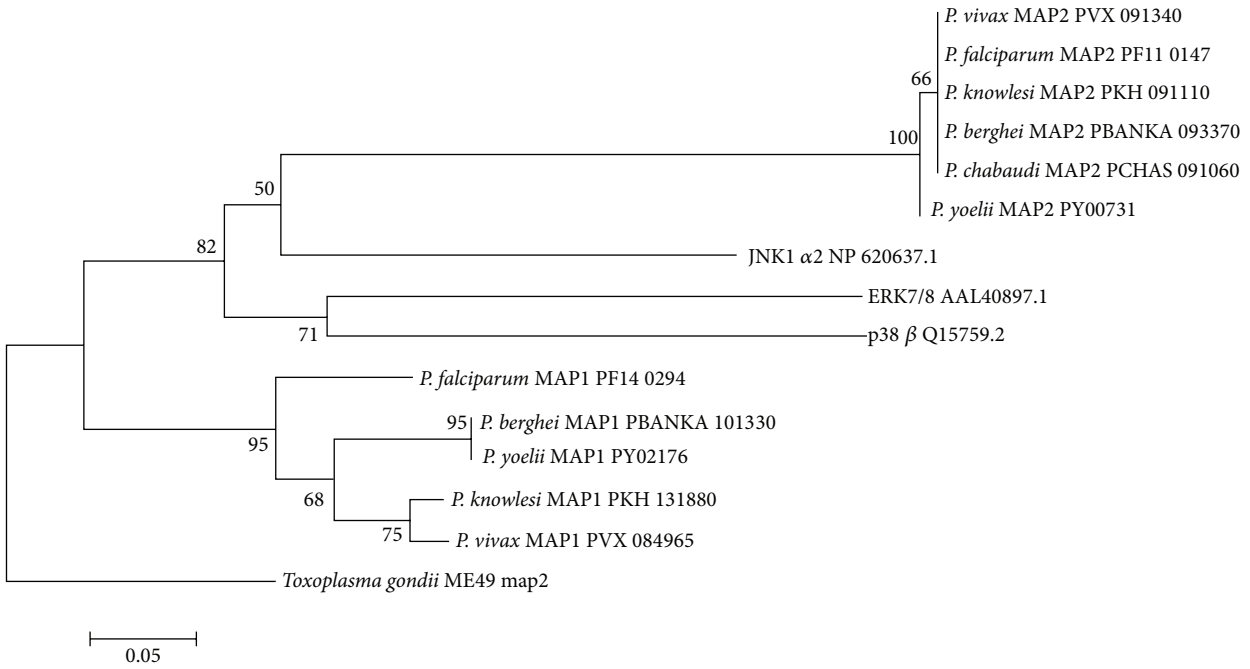

Figure 4: Phylogenetic analysis of PfMAP2 protein. Phylogenetic analysis using Neighbour joining (NJ) tree displaying the genetic relationships among MAP kinases in *Plasmodium* species. Bootstrap support of more than 65% and distance is indicated. *Plasmodium* MAP kinases sequences were retrieved from PlasmoDB and *Homo sapiens* MAPK sequences representing ERK 7/8, JNK, and p38 were retrieved from NCBI.

SNF-1 is a subfamily of protein kinase group which plays a crucial role in regulating lipid metabolism and cell stress response [51]. Activation of PfMAP2 protein in all the stages supports the parasite metabolism by activating downstream substrates which might be diverted compared to mammalian MAP kinases.

We have also shown that the TSH motif of PfMAP2 sets it apart from PfMAP1 as our Neighbour Joining analysis with 1000 bootstrap replicates suggests that it is less related to both the human MAP kinases and the TXY motif of plasmodial MAP1 as shown in Figure 4. This is in agreement with the extensive divergence of the kinome of *P. falciparum* which

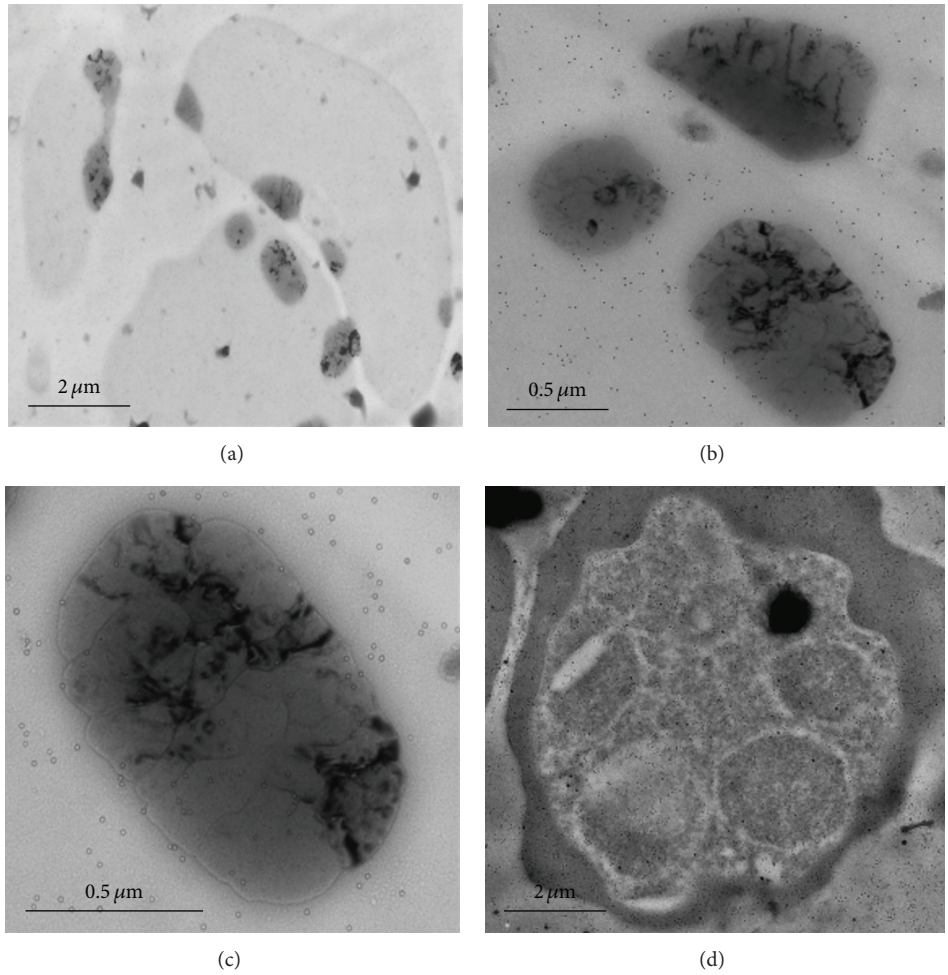

Figure 5: Localization of PfMAP2 by immunoelectron microscopy. Ultrathin sections of *P. falciparum* parasites at intraerythrocyte stage were labelled with rabbit anti-PPfMAP2 antibody followed with anti-rabbit secondary antibody conjugated with 15 nm colloidal gold particle. Ultrathin sections of infected erythrocyte were observed under 7 000x (a), 25 000x (b), and 40 000x (c) magnification. Similarly PPfMAP2 staining in the schizont was observed under 8 000x magnification (d).

showed that PfMAP2 is located in a basal position relative to other MAP kinases whereas PfMAP1 is clearly associated with the human MAP kinase, ERK8 [4]. The divergence of PfMAP2 from PfMAP1 as shown in Figure 4 using only the activation sites of these proteins suggests that the TSH motif is not only highly unique but it is also evolutionarily divergent from the other MAP kinases inclusive of plasmodial MAP1 and human MAP kinases.

The data shown do not allow us to draw any conclusion regarding detailed function of PfMAP2 during the intraerythrocytic cycle. However, the additional information on PfMAP2 protein could possibly aid in finding downstream substrates and hence predict PfMAP2 pathway that is involved in its protective mechanism similar to JNK/p38 and also assist in parasite proliferation which is similar to ERK function [52–54].

Immunogold labelling together with the use of transmission electron microscopy would aid in a more precise localization of the activated protein. Therefore, to further confirm the localization of PfMAP2, ultrathin sections of parasitized red blood cell were probed with anti-PPfMAP2 antibodies. At lower magnification, PPfMAP2 staining was detected ubiquitously. When the parasitized erythrocytes were observed at higher magnification, the immunogold was detected in various vesicular structures including nucleus (Figure 5). Since we do not have a specific PPfMAP2 antibody which is nucleus-specific, we have come to understand that the expression of PfMAP2 is regionalised in various organelles of the *Plasmodium* parasite. Here, we suggest that since PfMAP2 have a functional role during the pathological development of the parasite [4], our data renders that further detailed investigation should be done on this protein. Further studies on the placement of PfMAP2 in the nuclear architecture will aid in understanding on the gene regulation involved in its function and the role of nuclear structure in MAP kinase antigen variation.

4. Conclusion

The finding that the PfMAP2 protein is highly localized in the parasite's nucleus at all intraerythrocytic stages and its expression is significantly dependent on the stage of *P. falciparum* asexual maturation further supports future potential

manipulation of PfMAP2 as a protein kinase inhibitor to halt the spread of malaria.

Competing Interests

The authors declare that the grant mentioned in Acknowledgments does not lead to any conflict of interests. Additionally, the authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contributions

Hasidah Mohd Sidek, Mohammed Noor Embi, and Noraishah Mydin Abdul-Aziz conceived and designed the experiments. Farah Aida Dahalan and Mogana Das Murtey performed the experiments. Farah Aida Dahalan, Hasidah Mohd Sidek, Mohammed Noor Embi, Jamaiah Ibrahim, Lim Fei Tieng, and Noraishah Mydin Abdul-Aziz analyzed the data. Farah Aida Dahalan, Nurul Aiezzah Zakaria, Hasidah Mohd Sidek, and Noraishah Mydin Abdul-Aziz wrote the paper.

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Research Article

Geographical Distribution and New Situation of *Leishmania* Species after the Control of Cutaneous Leishmaniasis Foci in Errachidia Province, Morocco, in 2014

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In Errachidia province, the incidence of cutaneous leishmaniasis (CL) has increased over the past decade and it was higher in 2010 (860.34 per 100,000 inhabitants), with 3445 cases. The number of cases declined sharply and decreased from 3445 cases in 2010 to 8 cases in 2014 following the control action plan interventions. The total of patients was diagnosed only on clinical basis and the lesions were considered caused by *L. major*. The epidemiological study was conducted between 2001 and 2014 and the molecular detection of CL was studied to identify the circulating parasite species in this province by using the ITS1-PCR-RFLP methods. In 2010, the molecular identification of 11 samples revealed the presence of *L. major* in the most affected circles: Goulmima, Er-Rissani, and Errachidia. In 2014 the molecular characterization of 7 among 8 cases reported in this year showed the presence of *L. tropica* in Errachidia circle. This is the first molecular identification of *L. tropica* in Errachidia province. The detection of this species after the intensified control measures strategies suggests that it was probably dissipated through the dominance of *L. major*.

1. Introduction

In Morocco, cutaneous leishmaniasis (CL) is caused by three species of *Leishmania*: *L. major*, *L. tropica,* and *L. infantum*. CL due to *L. major* is considered as a major public health threat. The clinical characteristic is a single localized cutaneous lesion, which is often severely inflamed as well as ulcerated and healing within 2–8 months, but a polymorphism can be observed [1, 2]. This disease is characterized by its wide geographical distribution in arid zones in the palm groves of the southern foothills of the Anti-Atlas and High Atlas, especially in Jerada, Figuig, Errachidia, Ouarzazate, Zagora, and Tata provinces (Figure 1) [1]. The distribution of *L. major* is conditioned by the presence of suitable reservoir hosts mainly the commensal rodent *Meriones shawi* (*M. shawi*) and sand fly vector mainly *Phlebotomus papatasi* [3]. The parasite-reservoir host combination was subsequently observed by formal identification of the parasite [4–6]. This reservoir (*M. shawi* rodent) is the most ubiquitous species in Morocco and its distribution largely exceeds *L. major* repartition [7], proving its role in transmission of disease. In terms of vectors, the bioclimate affects the vectors distribution and density and hence disease prevalence [8]; *P. papatasi* is the main vector of *L. major,* found common to all environments but with a varied predominance, especially in saharan environment where it was the most prevalent species [9].

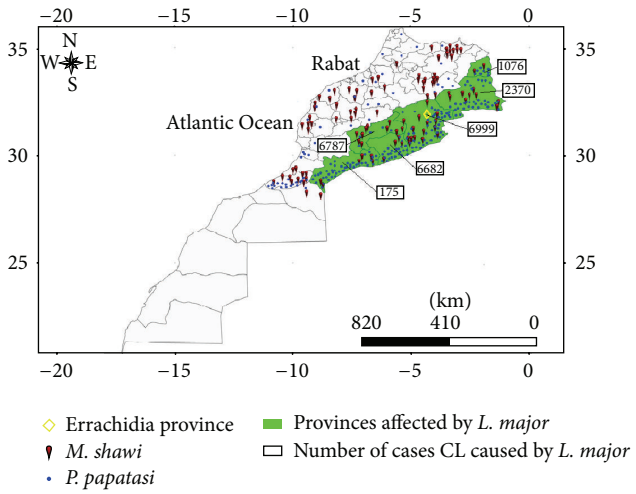

FIGURE 1: Repartition of *M. shawi* rodent and densities of *P. papatasi* and CL caused by *L. major* distributions in Morocco from 2001 to 2014 [1, 7, 10].

The CL caused by *L. major* in Morocco is located in the east of the country [1], which is consistent also with the high densities of vectors and rodents in the south and southeast of country according to Laqraa et al. [10] and Echchakery et al. [7], respectively. The *M. shawi-P. papatasi-L. major* association seems to constitute quite stable zoonotic systems as shown in Figure 1. Isoenzymatic characterization of *L. major* indicated the presence of a unique zymodeme MON-25 [8]. In 2010, the number of *L. major* cases saw a great increase with 6444 cases; the highest rate was recorded in Errachidia province including over than 3000 cases recorded. In 2011, the number of cases saw a large decrease following the control action plan interventions. In the present study, after the new epidemiological situation and a better control of leishmaniasis adopted, an epidemiological study and ITS1-PCR method were used for update and identification of *Leishmania* parasite species responsible for the recent CL cases in Errachidia province.

2. Materials and Methods

2.1. Study Area. Errachidia is a province located in the south east of Morocco (31°45′0″N/4°30′0″W), which was separated administratively since 2009. This province is constituted by four circles currently having 29 communes with 7 urban and 22 rural communes. This province covers an area of 59,585 km^2; it is bordered in the north by Midelt province, in the northeast by Figuig province, in the south and southeast by Algeria, and in the west by Tinghir and Zagoura provinces. The climate is semidesert; maximum and minimum mean temperatures in Errachidia amount to 5°C in January and 31.5°C in July. With low rainfall and spread of irregular way in time and space, most of the territory sits down within 100 mm of rain per year. Due to its typical climate, it is characterized by low density of vegetation cover. The total population amounted to 418,451 in 2014 [11]. The rural population makes out around 53.61% with an overall density of 7.02 inhabitants per square kilometer [11].

2.2. Sampling and Diagnosis. Samples of DNA were isolated from the positive skin smears in 2010, for 11 CL lesions performed in Errachidia, Goulmima, and Er-Rissani circles which are the most affected. In 2014 we collected 7 skin smears of CL from 8 cases only recorded in circle of Errachidia. These slides were examined by the provincial laboratory and sent to the national laboratory of leishmaniasis in National Institute of Hygiene for control and confirmation. Smears (Giemsa-stained slides) were prepared from skin lesions and examined microscopically for the detection of *Leishmania* amastigotes.

2.3. Extraction of DNA from Stained Smears and PCR Analysis. DNA was extracted and purified from positive smears using a kit "high pure template PCR" according to the instructions of the manufacturer. The ribosomal internal transcribed spacer (ITS1) region was analyzed using the primer pair L5.8S and LITSR by PCR-restriction fragment length polymorphism (RFLP) approach for identification of the *Leishmania* parasites. The product was loaded and analyzed on 1.5% agarose gels by electrophoresis and visualized by UV lights [13]. Positive controls contain DNA of *L. infantum* (MHOM/MA/1998/LVTA), *L. tropica* (MHOM/MA/2010/LCTIOK-4), and *L. major* (MHOM/MA/2009/LCER19-09). Negative controls (distilled water) were included during PCR to ensure reliability and validity and to check for possible contaminations of the amplification reactions. PCR product was followed with the RFLP analysis using Hae III enzyme.

2.4. Data Analysis. Epidemiological data were exploited by using Microsoft Office Excel 2010 and analyzed by R Software version 3.2.2. We used chi-square tests to compare proportions. Quantum Geographic Information System (GIS) was used to design and develop CL distribution maps.

3. Results

During the period 2001–2014, there were a total of 7653 CL cases notified in Errachidia province. The epidemiologic situation remained stable until the year 2004 with an average of 2 cases per year; over 2007–2014 the number of cases had an exponential increase with fluctuations. Period from 2007 to 2011 has known a great epidemic. The number of cases has decreased slightly in the following years, with a new recrudescence until 8 cases in 2014. The maximum incidence peak was reached in 2010 with 860.34/100,000 inhabitants (3445 cases) (Figure 3). The spatial as well as temporal distribution of CL cases in the four circles of Errachidia province is illustrated in Figure 2. The first recorded CL cases appeared in 2001 towards the end of 2006 (Figure 2(a)). In the following years (2007–2012), the outbreak spread to the north and west of the province towards Sahara in the south and extending to the Algerian border. We observed higher rates of CL (over 1000 cases) in the four circles, while the highest rate for CL was found in northern of the province (Figure 2(b)). Considering its recent control, CL

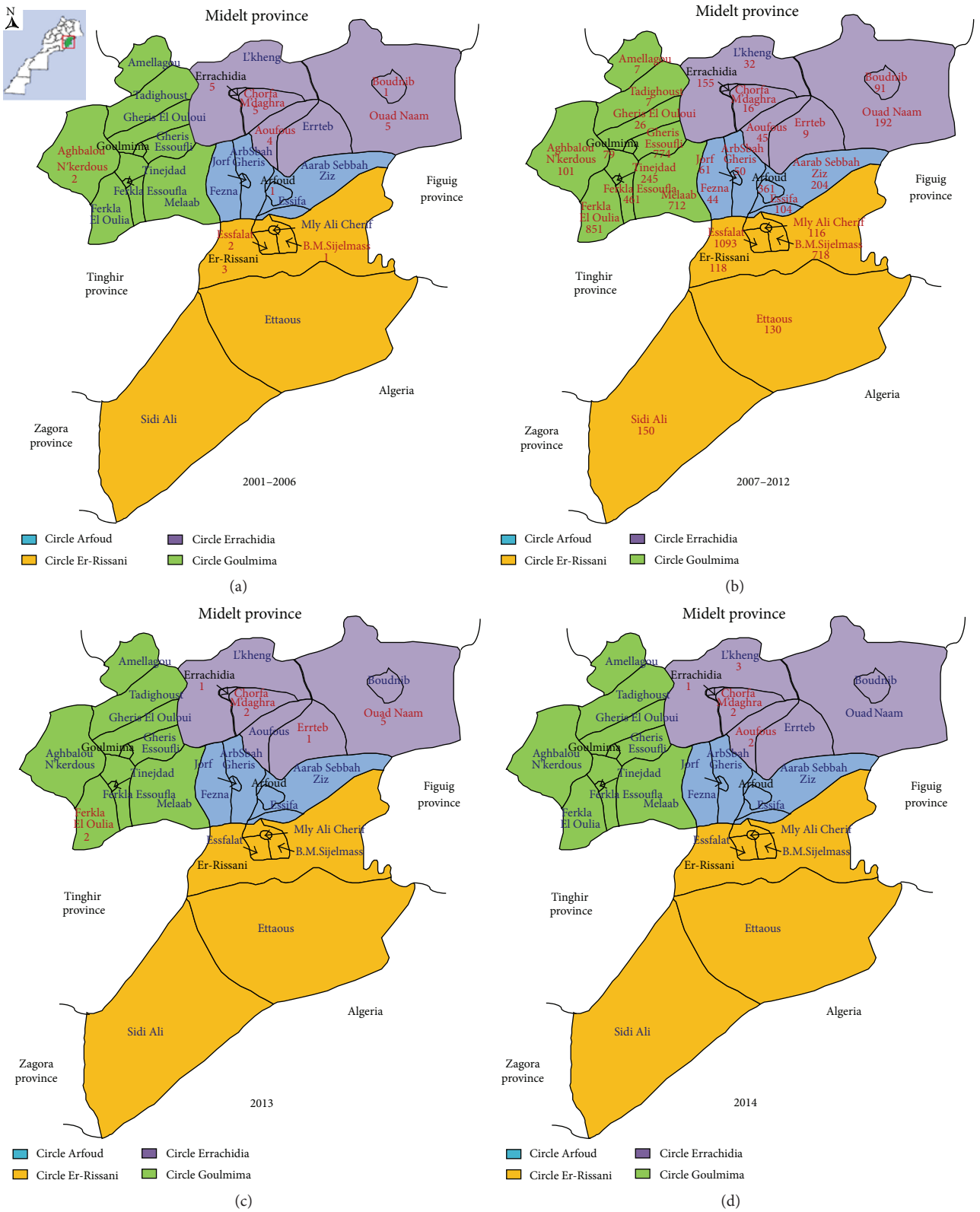

FIGURE 2: Map of Morocco showing Errachidia province. The study area and geographical distribution of CL from 2001 to 2014. The four circles of Errachidia province following the administrative map are colored by green, purple, blue, and orange.

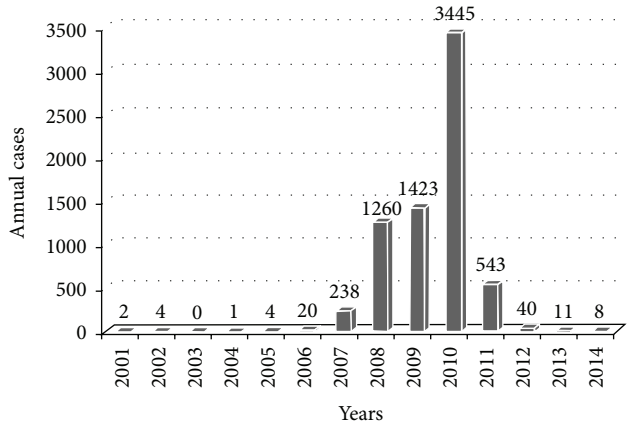

Figure 3: Evolution of CL cases in Errachidia province showing cumulative number per year for the period 2001 to 2014, with major peak in 2010 [12].

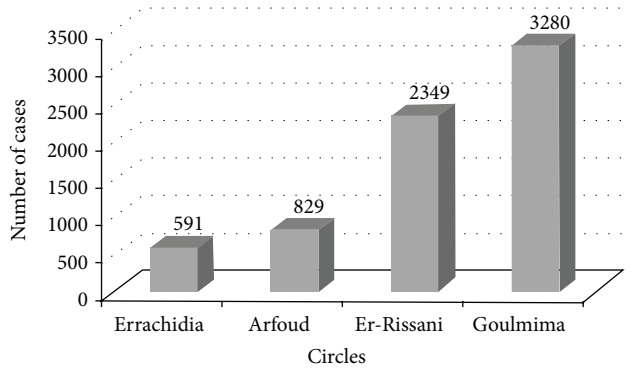

Figure 4: Evolution of CL cases in the four circles of Errachidia province from 2001 to 2014.

cases dropped to 11 cases for the rest of 2013 (Figure 2(c)). CL case numbers subsequently decreased during 2014; the few cases recorded are located in the north of the province at the Errachidia circle (Figure 2(d)). Among four circles in this province, the temporal and spatial distribution of CL recorded from 2001 to 2014 showed that 79.86% of cases were recorded in Goulmima and Er-Rissani circles (3 urban and 13 rural districts among 29 total districts) and 11.76% and 8.38% were declared, respectively, in Arfoud and Errachidia circles (Figure 4).

3.1. Distribution of CL by Age and Sex. Viewing the important CL cases recorded in Errachidia, we were able to make an exploitation of the patient files and represent the distribution of cases by age and sex. Usually, all ages are affected by the disease, with a high prevalence for age of 10–19 years (32.03%) followed by the age of 0–9 years (21.39%). Other age groups (from 20–39 to over 50 years) were weakly affected with, respectively, 12.93%, 11.74%, and 12.98%. However, age group of 40–49% is less affected with 8.91% (Figure 5).

The difference of CL cases from 10 to 19 year old was statistically significant in regard to the other age groups (χ^2 = 1523.3, p value < $2.2e-16$).

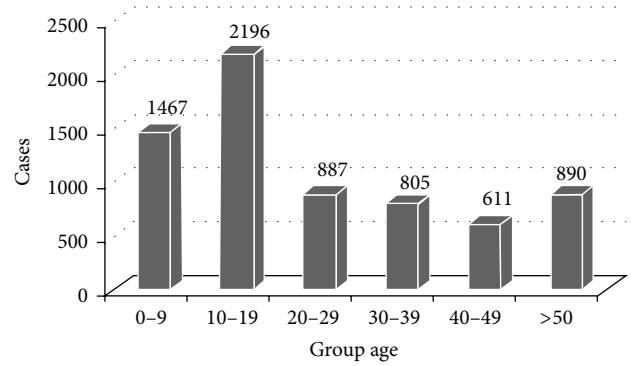

Figure 5: Distribution of CL according to age from 2001 to 2014 in Errachidia province.

Both sexes are affected by CL, and females were more affected with a sex ratio of F/M = 1.44. The difference between males and females was statistically significant (χ^2 = 229.55, p value < $2.2e-16$).

3.2. Results of PCR. The results of the molecular study of 11 samples collected in 2010 showed that CL was due to *L. major* in Errachidia, Er-Rissani, and Goulmima circles. In 2014, the molecular identification of 7 among the 8 cases reported in the Errachidia circles showed the presence of only *L. tropica* (Table 1, Figures 2 and 7).

4. Discussion

Since the appearance of CL in 1914 in Morocco, the numbers of cases have showed some fluctuation. In recent years the diagnosis of patients was performed only on clinical basis; at the national level, the highest numbers of CL cases due to *L. major* were noted in 2010 (6444 cases), among them nearly 50% (3445 cases) were recorded in Errachidia province [12]. The Ministry of Health has decided to implement the response strategy in the fight against leishmaniasis based mainly on diagnosis and treatment of patients, vector, and reservoir control measures for reducing the incidence of disease. Besides the literature, recent study shows that the best way to control *L. major* is to combine reservoir and vector control [14]. In Errachidia, the rodents control was targeted for control policy of CL; the control of vectors and reservoir hosts is based on improving hygiene conditions and chemical measures for controlling rodent populations by using the rodenticides giving beneficial results in the active burrows in all infested areas. However, the systematic application of poisoned bait in the CL-affected areas in Errachidia province has probably contributed to the decrease in CL incidence, as it led to a 95% reduction in the number of active rodent burrows in treated areas, which was sustained for each of the 3 years of the intervention [15]. In addition, all these measures of controlling actions are supported by education and raising public awareness with collaboration of local collectives and associations.

The data show that Goulmima and Er-Rissani circles were the most affected by CL. This could possibly be explained by

Table 1: Results of molecular study of slides of the most affected circle in Errachidia province.

Province	Year	Circle	Commune	Results of ITS1-PCR	Results of RFLP by HaeIII
Errachidia	2014	*Errachidia*	L'kheng	Positive	*L. tropica*
	2014		L'kheng	Positive	*L. tropica*
	2014		M'daghra	Positive	*L. tropica*
	2014		Aoufous	Positive	*L. tropica*
	2014		Errachidia	Positive	*L. tropica*
	2014		Errachidia	Positive	*L. tropica*
	2014		M'daghra	Positive	*L. tropica*
Errachidia	2010	*Er-Rissani*	Er-Rissani	Positive	*L. major*
	2010		Er-Rissani	Positive	*L. major*
	2010		Er-Rissani	Positive	*L. major*
	2010		Er-Rissani	Positive	*L. major*
	2010		Er-Rissani	Positive	*L. major*
Errachidia	2010	*Goulmima*	Goulmima	Positive	*L. major*
	2010		Goulmima	Positive	*L. major*
	2010		Goulmima	Positive	*L. major*
Errachidia	2010	*Errachidia*	Errachidia	Positive	*L. major*
	2010		Errachidia	Positive	*L. major*
	2010		Errachidia	Positive	*L. major*

the dominance of rural area of high densities of populations near to *L. major* reservoir host biotopes. These areas host the largest oases of Errachidia, namely, Ferkla, Tinjdad, Goulmima, and Er-Rissani oasis, which are adjacent to the great watersheds Ziz and Rhéris of Errachidia. Therefore in arid areas, the huge mobilization of water resources and the creation of new irrigated zones probably favored the proliferation of both sandflies and rodents, including gerbils and jirds living in burrows with *P. papatasi*, the vector of *L. major* [16]. This rodent (the main reservoir host of *L. major*) is considered as an agricultural pest. *M. shawi* is the main infected animal in south Morocco [17]. Most recent studies show in the rural area poorer farmers might live close to their animals, which promotes humidity and the synthesis of organic material for breeding of sandfly [2].

During this study designed to evaluate the overall situation of CL and molecular identification of circulating species in the Errachidia province, we noted clearly that the number of cases was significantly declined during the last 4 years; this decline is due to the outbreak control measures focusing on rodents and sandflies control. According to age, the majority of the cases occurred in an age group less than 19 years; this could be explained by their weak immunity system. In general, CL tends to affect a younger age group because they are consistently more exposed to phlebotomine sandfly bite by their habit to play near breeding sites [18]. However, the majority of cases infected were diagnosed by passive detection, which could possibly explain the higher rate of infection in women (sex ratio F/M = 1.44) over men, by the fact that women consult more than men because of the unsightly lesions [19, 20].

Frequently in all provinces known by CL due to *L. major*, the *Leishmania* species are identified based on their geographical distribution and on the clinical manifestations. The diagnosis of the infecting species based on clinical symptoms is not crucial, because symptoms can vary and may be confused with other etiologic agents [21]. In Morocco and particularly in Errachidia province, the diagnosis is done only by the clinical methods. The "wet" type of *L. major* is a classical lesion aspect, associated with infiltrated nodule causing the localized cutaneous lesions (LCL) form. These various clinical manifestations of *L. major* are dug which can evolve into an ulceration covered by crust. It is also often severely inflamed, ulcerated, and healing within two to eight months. In addition, a remarkable polymorphism of lesions caused by *L. major* was also observed with 11 different forms (vegetative, impetiginoid, eryseloid, necrotic, warty, erythematosquamous, lupoid, sporotrichoid, papulous, eczematoid and recidivans) [1, 18, 22–27].

Conversely, the lesions caused by *L. tropica* are dry, lupoid, small (2 cm in diameter), unique, self-healing, and mainly located on the face and can last up to a year [28, 29]. A clinical polymorphism of this cutaneous form was also described with eight different forms (impetiginized, ulcerocrusted, noduloulcerative, severe, vegetant inflammatory, large, multiple and limbs infections) [1, 30]. According to these data concerning this clinical manifestation the confirmation of the circulation of *Leishmania* species cannot be differentiated and detected without molecular biology analysis.

On the other hand, molecular identification revealed for the first time the presence of *L. tropica* (Figures 6 and 7). We think that the presence of this species could be related to semiarid climate in part of High Atlas in this region where the conditions are favorable, which is consistent with the weak distribution of its vector *P. sergenti* [10]. Therefore, we can say that *L. tropica* was present but at low density compared to *L. major*.

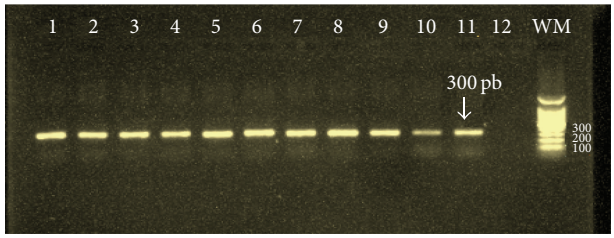

Figure 6: Gel electrophoresis of *Leishmania* (ITS1-PCR product) from Giemsa-stained lesion smears isolates obtained from patients. Lanes 1–10 correspond to clinical materials from patients. Sample from slides, lane 11: positive control, lane 12: negative control, and WM: weight marker, 100 pb.

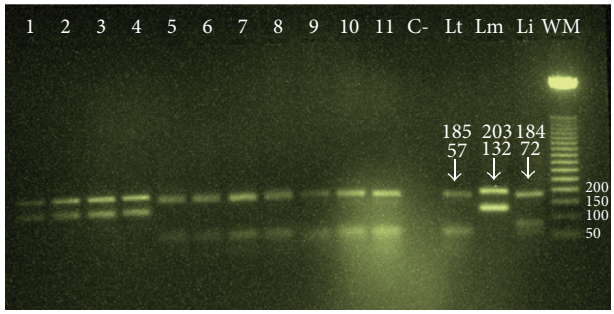

Figure 7: Agarose gel electrophoresis analysis of ITS1-PCR-RFLP amplified products from different slides collected, lanes 1–4 (*L. major* detected in the samples 2010), and lanes 5–11 (*L. tropica* detected in the samples 2014), C: negative control, Lt: *L. tropica,* Lm: *L. major,* Li: *L. infantum,* and WM: weight marker (50 pb).

Our study has some limitations. Case is based on physicians' expertise and the availability of laboratory facilities with trained staff. Unfortunately, it was not possible to conduct the most of skin lesion smears during the epidemic period especially in 2010.

5. Conclusion

These results show the success of the implementation of the response strategy which reduced dramatically *L. major* CL. However, molecular analysis of persistent cases showed the existence of *L. tropica* which was probably dissipated through the dominance of *L. major*.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

El Alem Mohamed Mahmoud designed the study, carried out all the technical experiments, and drafted the paper; Sebti Faiza conceived and directed the study and revised the paper based on all authors' amendments; Med Lemine participated in the technical experiments; Chichaoui Smaine collected the statistics data; El Bachir Adlaoui, Habbari Khalid, Sadak Abderrahim, and Fellah Hajiba revised and approved the paper. All authors read and approved the final version of the paper.

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Research Article

Resistance Status to the Insecticides Temephos, Deltamethrin, and Diflubenzuron in Brazilian *Aedes aegypti* Populations

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Insecticides are still largely applied in public health to control disease vectors. In Brazil, organophosphates (OP) and pyrethroids (PY) are used against *Aedes aegypti* for years. Since 2009 Insect Growth Regulators (IGR) are also employed in the control of larvae. We quantified resistance to temephos (OP), deltamethrin (PY), and diflubenzuron (IGR) of *A. aegypti* samples from 12 municipalities distributed throughout the country, collected between 2010 and 2012. High levels of resistance to neurotoxic insecticides were detected in almost all populations: RR_{95} to temephos varied between 4.0 and 27.1; the lowest RR_{95} to deltamethrin was 13.1, and values higher than 70.0 were found. In contrast, all samples were susceptible to diflubenzuron ($RR_{95} < 2.3$). Biochemical tests performed with larvae and adults discarded the participation of acetylcholinesterase, the OP target, and confirmed involvement of the detoxifying enzymes esterases, mixed function oxidases, and glutathione-*S*-transferases. The results obtained were discussed taking into account the public chemical control component and the increase in the domestic use of insecticides during dengue epidemic seasons in the evaluated municipalities.

1. Introduction

Currently dengue is spreading worldwide, placing at risk around 40% of the global population [1]. To date, no specific drugs are available and dengue treatment is restricted to supportive care. Although several candidate vaccines, directed against the four dengue serotypes, are presently submitted to human clinical trials, or even licensed for commercialization, none of them attains high protection levels [2]. The major dengue vector is *Aedes aegypti* (Diptera: Linnaeus, 1762), a highly anthropophilic and synanthropic mosquito, distributed throughout tropical and subtropical areas of the world [3–5], mainly between latitudes 35°N and 35°S [6, 7]. In addition, the recent chikungunya and Zika virus dispersion throughout the globe is also primarily attributed to *A. aegypti* [8].

Actions against dengue are mostly focused on the reduction of mosquito densities, and vector control can be accomplished through mechanical, biological, and chemical approaches. Mechanical control is based on the elimination or on the adequate protection of potential breeding sites; biological control makes use of larvae predators, such as small fishes, or formulations with entomopathogenic bacteria, like *Bacillus thuringiensis var. israelensis* (*Bti*); chemical control consists in the use of insecticides against larvae or adults of the vector mosquito [6, 7, 9].

Insecticides, still largely utilized by a number of vector control programs, belong to four main classes; all of them are neurotoxic compounds: carbamates (CA), organochlorates (OC), organophosphates (OP), and pyrethroids (PY) [10]. Nowadays, PY and OP are the most used. Recently two

additional classes became available, the spinosyns, modulators of acetylcholine receptors [11], and the Insect Growth Regulators (IGR), a group that includes the chitin synthesis inhibitors (CSI) [12]. It should be noted that the Brazilian Dengue Control Program (PNCD) only employs insecticides that are recommended by the World Health Organization Pesticide Evaluation Scheme (WHOPES) for use in potable water or properly approved for space spraying applications [13, 14].

The intensive and prolonged use of insecticides can select resistant specimens in the natural vector populations, decreasing the frequency of susceptible individuals and reducing variability of field populations [5]. Insecticide resistance can derive from different mechanisms, the main ones being modifications in the target sites and higher ability to detoxify xenobiotic compounds; the former mechanism is known as target site resistance and the other as metabolic resistance [5, 6].

The voltage gated sodium channel (Na_V) is the target site of pyrethroids; these insecticides keep Na_V in its opened conformation, resulting in repetitive pulses. Na_V substitutions that affect its susceptibility to PY are known as knockdown resistant ones (kdr) [15]. Such mutations have been reported in *A. aegypti* populations from several countries worldwide [16–18]. In Brazil two major kdr Na_V alleles related to PY resistance are spreading and increasing in frequency. A clear regional distribution pattern is observed with $Na_V{}^{R1}$ (mutant at position 1534 of the channel protein) present throughout the country while $Na_V{}^{R2}$ (mutant at both 1534 and 1016 positions) is more frequent in central and southeastern municipalities [19, 20].

The target site of OP insecticides is acetylcholinesterase (AChE), an enzyme that hydrolyzes acetylcholine molecules; as a consequence, this neurotransmitter persists in the synaptic cleft, resulting in the exacerbation of nerve impulse transmission [21, 22]. To our knowledge there are no confirmed evidences of AChE alterations related to OP resistance in field *A. aegypti* populations.

The main detoxifying enzyme classes participating in the xenobiotic metabolizing processes are the Phase I mixed function oxidases (MFO) and esterases (EST) that trigger chemical modifications in the substrates and the Phase II conjugating enzymes, glutathione-*S*-transferases (GST) [21]. Each of these enzyme families is composed of several molecular entities, bearing distinct levels of specificity [5]. In general, evaluations of *A. aegypti* detoxifying mechanisms worldwide associate ESTs and OP resistance as well as GST and MFO alterations with PY resistance [23–26]. However such relations are not always straightforward due to the variability of enzymes participating in the insecticides detoxification and to the resistance multifactorial character [5, 10].

In Brazil, during more than three decades, only temephos was employed in the control of *A. aegypti* larvae. Resistance to this OP was originally detected at the end of the years 1990, and registers of the dissemination of this phenomenon persist up to the present [23, 24, 27–30]. Since 2009 temephos is being substituted by IGR in the country and a strategy of larvicide rotation, each 3-4 years, is attempted. Development of resistance was also verified for PY shortly after its use has been implemented for the control of adults, since 2000 [23, 31, 32].

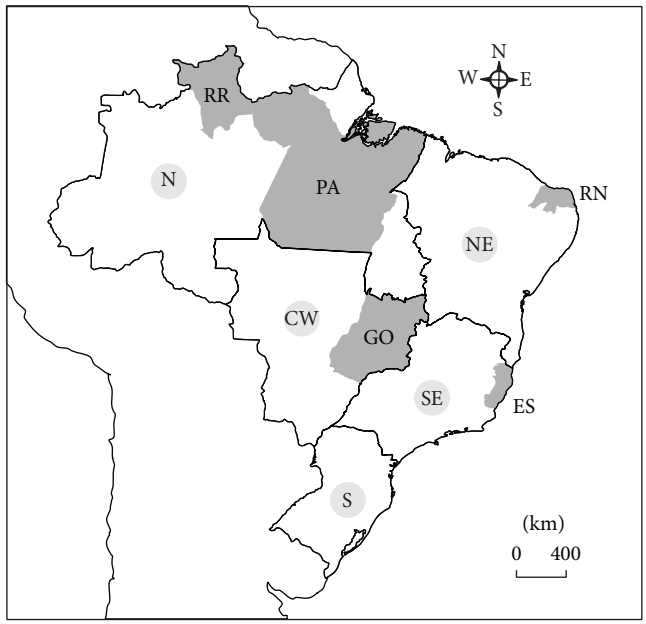

Figure 1: Brazilian map with the states used in the study in grey. RR: Roraima; PA: Pará; RN: Rio Grande do Norte; ES: Espírito Santo; and GO: Goiás. The continuous lines indicate the different regions of the country. N: north; NE: northeast; SE: southeast; S: south; and CW: central-west.

The insecticide susceptibility profiles of several Brazilian field *A. aegypti* populations are presented (Figure 1). Resistance to compounds employed by the PNCD was investigated, in order to collaborate with the elucidation of both the resistance dynamics and the potential related mechanisms. Chemical insecticides are still a relevant control tool employed by public managers against dengue vectors. In addition, in general, dengue epidemic periods are related to a significant increase in the domestic use of insecticides, mainly adulticides. This collective behavior has the potential to contribute to a rapid increase in resistance levels, and it has already been detected in Brazil against pyrethroid compounds [33]. Taking these aspects into account, the results obtained were evaluated in the scope of several parameters, such as the Ministry of Health (MoH) supply of insecticides to the Brazilian States, the local historic of dengue outbreaks, and the frequency of kdr mutations, majorly responsible for PY resistance in the country.

2. Materials and Methods

2.1. Data on Insecticide Distribution and Dengue Cases. The Brazilian MoH coordinates the distribution of insecticides used in public health to all states and to all disease vector control programs. Insecticides are stored in a warehouse of Rio de Janeiro State Health Secretariat, in charge of stock control and supply of the products to the different States. We got MoH authorization to access these data, sorted by year,

since 2003. Figure 2 illustrates the insecticides employed by PNCD from 2003 until 2012, the latter corresponding to the year of collection of the last samples in the field.

Dengue incidence rates were based on the historical series of cases available at the MoH website for each municipality [34] and on the 2010 population census data conducted by the Brazilian Institute of Geography and Statistics [35].

2.2. Mosquitoes. Natural *A. aegypti* populations were collected between 2010 and 2012, in 12 municipalities belonging to a total of five States (Figures 1 and 2): Roraima (RR) and Pará (PA) at the north, Rio Grande do Norte (RN) at the northeast, Espírito Santo (ES), at the southeast, and Goiás (GO) at the central-west region. In all cases, sampling of vector eggs was performed with ovitraps according to MoReNAa (the Brazilian *A. aegypti* insecticide resistance monitoring network) guidelines, as described elsewhere [28, 36]. Depending on the number of buildings in each municipality, 150 to 300 ovitraps were installed during 5–7 days, representing the whole area.

Rockefeller mosquitoes (Rock), a reference strain of insecticide susceptibility [37], were employed as control in all bioassays and also in the biochemical and molecular analysis.

2.3. Mosquitoes Rearing. Eggs derived from field populations were allowed to hatch for two days in plastic cups containing 2.5 L of dechlorinated water and a small amount of cat food (Friskies®, Purina, São Paulo, SP). Pools of 1,000 larvae were then transferred to transparent plastic trays (33 × 24 × 8 cm) filled with 1.0 L of water and fed with 0.5 g of cat food every three days. The resulting pupae were transferred to cartoon cages (18 × 17 cm) and the *A. aegypti* emerging female and male adults were separated from other mosquito species, scored and reared in cages in order to proceed to blood feeding and egg laying. Adult females were fed on xylazine and ketamine-anaesthetized guinea pigs [38] for 30 minutes; oviposition cups were placed inside the cages three days later. Achievement of F1 and F2 generations in the laboratory was performed essentially as described elsewhere [28]. The whole procedure took place at 26 ± 1°C and 80 ± 10% relative humidity.

2.4. Larval Bioassays. In order to maximize synchronous development, egg hatching was induced during one hour in dechlorinated water. Afterwards, groups of 1,000 larvae were reared in plastic trays, as described above, until the third instar (L3).

Dose response bioassays with temephos (Pestanal®, Sigma-Aldrich) were performed with 10 different concentrations of the OP, designed to kill between 10 and 95% of each population. Four 100 mL replicas were employed per concentration and 20 L3 larvae per replica. Mortality was registered after 24 hours of exposure [29, 39, 40].

For the CSI diflubenzuron (Sigma-Aldrich), each dose response bioassay employed eight insecticide concentrations, also designed to be effective between 10 and 95%. Four 150 mL replicas per concentration and 10 L3 larvae per replica were employed. Both the bioassay methodology and the evaluation criteria were adapted from previous work [41, 42]. In this case, records were made each other day. Replicas were covered with a nylon mesh in order to avoid escaping of adults. The assay was considered terminated when all the specimens from the control group, nonexposed to the CSI, emerged as adults.

Two internal controls were placed at every bioassay: (a) Rockefeller larvae exposed to two different insecticide concentrations, the ED_{99} (effective dose) and half of it, and (b) field specimens kept with the solvent, in the same amount used for the experimental samples.

2.5. Adult Bioassays. Female adults were submitted to dose response bioassays to quantify resistance to the pyrethroid deltamethrin (Sigma-Aldrich) following the World Health Organization [43] methodology of impregnated papers, with some modifications [40, 44]. Assays employed 10 deltamethrin concentrations, ideally killing between 10 and 95% of each mosquito population. In all cases three replicas with 15–20 non-blood-fed females, 1–5 day-old, were used. After exposure to the pyrethroid during one hour, mosquitoes were recovered for 24 hours in insecticide-free compartments, when mortality was recorded. Adult bioassay controls followed the same rational employed for larvae ones: Rockefeller specimens exposed to two different deltamethrin concentrations and field derived adults exposed to the solvent.

2.6. Biochemical Assays. The potential mechanisms involved with resistance were evaluated through biochemical assays that quantified the activity of several classes of enzymes according to WHO and CDC procedures [23, 45, 46]. Two Phase I enzyme classes were evaluated, MFO and EST. While MFO was indirectly measured, three substrates were employed for EST: α- and β-naphtyl and ρ-nitrophenyl acetates, accounting, respectively, for activities named α-EST, β-EST, and ρNPA-EST. The Phase II GST and the OP target site AChE were also evaluated. For AChE, both total activity and activity inhibited by the carbamate propoxur were assayed. According to WHO criterion [47], AChE inhibition higher than 70% points to an activity compatible with insecticide susceptibility. Dosage of total proteins was done with the Bio-Rad protein assay/dye reagent concentrate (500-0006), and the results were used to calculate enzymatic specific activities.

Tests with each population employed approximately 90 individual non-blood-fed young females (up to 24 hours after adult emergence) and 90 late L3-early L4 larvae. All specimens were stored at −80°C until use.

Enzyme activities were classified essentially according to what was established previously [23]: after calculating the Rockefeller 99 percentile, the rate of specimens above this value was estimated for each enzyme and population. Activities were classified as unaltered, altered, or highly altered if this rate was, respectively, below 15, between 15 and 50, or above 50%.

2.7. Molecular Assays. The results of kdr genotyping were previously published [20] and herein explored in parallel with the bioassays. Briefly, the genotyping was conducted with a customized real-time PCR TaqMan SNP Genotyping Assay (Thermo Fisher), for Val1016Ile (AHS1DL6) and Phe1534Cys

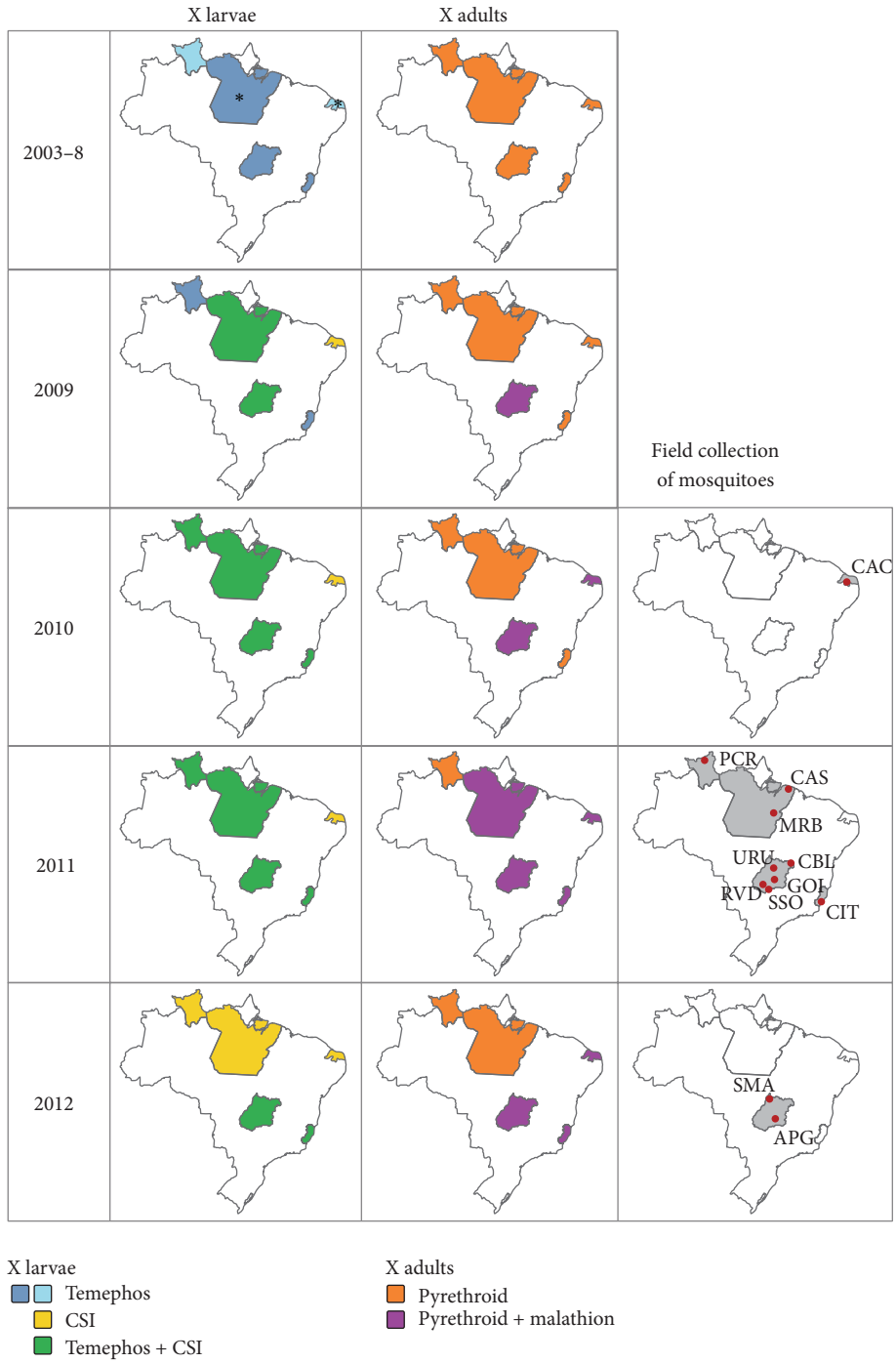

Figure 2: Historic of insecticide distribution, between 2003 and 2012, by the Brazilian MoH to the States where mosquitoes samples were collected, in the indicated years. The municipalities evaluated are highlighted with red dots in the panels at right. During 2003–2008, two states, shown in lighter blue in the upper map at left, did not receive temephos continuously: at Roraima (RR) temephos supply started at 2005; at Rio Grande do Norte (RN) it ended at 2004. (∗) States that also received CSI (diflubenzuron or novaluron) during 2003–2008 (RN at 2004 and PA at 2008). Compounds against adults: all pyrethroids distributed by the MoH were considered, and not only deltamethrin (see text for details). Municipalities—APG: Aparecida de Goiânia; CAC: Caicó; CAS: Castanhal; CBL: Campos Belos; CIT: Cachoeiro de Itapemirim; GOI: Goiânia; MRB: Marabá; PCR: Pacaraima; RVD: Rio Verde; SMA: São Miguel do Araguaia; SSO: São Simão; and URU: Uruaçu.

(AHUADFA). In general 30 individual adult males preferentially from the parental generation were used in two independent reactions, one for each Na_V kdr SNP (1016 and 1534). The allelic and genotypic frequencies of each population were calculated based on variations at both positions, assuming that they are under linkage disequilibrium, which resulted in the alleles Na_V^S (1016 Val^+ + 1534 Phe^+), Na_V^{R1} (1016 Val^+ + 1534 Cys^{kdr}), and Na_V^{R2} (1016 Ile^{kdr} + 1534 Cys^{kdr}) [20].

2.8. Interpretation of Results. Results of bioassays for each population and every active compound derived from three or four tests performed in different days. The lethal concentrations (LC) in the case of neurotoxic insecticides or the concentrations inhibiting adult emergence (EI), when the IGR was considered, were calculated using probit analyses [48] (Polo-PC, LeOra Software, Berkeley, CA). Resistance ratios (RR_{50}, RR_{95}) were acquired dividing the results obtained for each population by the equivalent Rockefeller's values. For all insecticides, the resistance status of mosquito populations was classified according to the criterion utilized in the country to temephos evaluation. This criterion, recommended by PNCD, considers that populations with RR_{95} above 3.0 are resistant [23, 49] (see Section 4).

3. Results

3.1. Insecticides Employed against Aedes aegypti in the Field. Figure 2 exhibits the recent history of insecticides distributed by the Brazilian MoH to the States where field collection of *A. aegypti* populations took place. All larvicides evaluated in the present work are depicted. Beyond these products, Bti was also employed in the field (not shown), during most of the period between 2003 and 2009, except for the central-west State of Goiás. For adulticides, besides the organophosphate malathion, deltamethrin was the pyrethroid elected against *A. aegypti*. However, several PY compounds were also used in the scope of the control of other vectors. Therefore, Figure 2 depicts all pyrethroids distributed for this purpose by the MoH, since these products can interfere with *A. aegypti* populations' susceptibility status. It should also be taken into account that the uncontrolled domestic use of pyrethroids plays an important role in the dissemination of insecticide resistance [32]. However, information regarding domestic use is very difficult to obtain.

Up to 2011, the larvicide temephos was continuously distributed to the states evaluated, with two exceptions: temephos supply to RR started only in 2005 and to RN it lasted until 2004 (Figure 2, light blue states in the 2003–8 line). In this latter state, control of larvae employed Bti between 2005 and 2008. Since 2009-2010 the CSI diflubenzuron was introduced in the *A. aegypti* larvae control, in addition to the organophosphate temephos in all states. The exception was RN where, as mentioned above, temephos had been previously discontinued; in this case, the CSI remained the sole larvicide adopted in *A. aegypti* control from 2009 on.

Control of adult mosquitoes was performed exclusively with pyrethroids between 2003 and 2008. Since 2009, the organophosphate malathion was gradually introduced. At 2011, all the states received both compounds, except RR that employed exclusively PY. At 2012, malathion was not distributed to the state of Pará.

3.2. Dengue Incidence in the Evaluated Municipalities. Figure 3 shows the incidence of dengue reported cases for all municipalities evaluated here. The aim in this case was to investigate if there were local outbreaks that could be related to a domestic intensification of insecticide use and, potentially, to an increase in *A. aegypti* resistance to these compounds. The period chosen ranged from 2008, two years before the collection of the first *A. aegypti* samples here evaluated, up to 2012. Incidence values for municipalities and the corresponding states are also presented in Table S1 of the Supplementary Material available online at http://dx.doi.org/10.1155/2016/8603263.

According to the Brazilian MoH, dengue incidence rates higher than 300 cases/100,000 inhabitants are already indicative of an epidemic situation [50]. In almost all municipalities, at least once, the dengue incidence of notified cases was compatible with this scenario. When the whole 2008–2012 period was considered, only Marabá, PA, was the exception. However, the localities of Castanhal, PA, and Caicó, RN, only presented high dengue incidence at 2012, after collection of vector samples was made (compare Figures 2 and 3). In some situations this "epidemic status" persisted throughout the whole evaluated period; this was the case of Pacaraima, RR, and of the GO adjacent municipalities Goiânia and Aparecida de Goiânia. The number of registers well above the threshold value of 300 cases per 100,000 inhabitants also attracted attention. For instance, reported incidence equivalent to at least 1% (1,000 cases/100,000 inhabitants) was found during 2009 and 2010 in half of the evaluated municipalities. Notably, the dengue incidence of Aparecida de Goiânia remained above 1% during the whole evaluated period. In the adjacent locality, Goiânia, those high dengue rates were registered during three years between 2008 and 2012. One should be aware that in general dengue epidemic periods are related to a significant increase in the domestic use of insecticides against adult mosquitoes (see Section 4).

3.3. Bioassays with Larvae. Table 1 summarizes the results of quantitative bioassays performed with the two main larvicides recently employed against *A. aegypti* in Brazil, the OP temephos and the CSI diflubenzuron. Table S2 shows details of these assays, such as effective concentrations and confidence intervals. Data are organized by year and then by geographic region.

All the populations evaluated were considered resistant to temephos. The higher resistance values were obtained in 2012. Nevertheless, since there were no municipalities examined in consecutive years, it is not possible to claim that temephos resistance is increasing in the country, based on the data presented here. In general, temephos resistance was higher at the central-west region: six out of seven municipalities presented RR_{95} above 10. São Miguel do Araguaia, GO, exhibited the higher temephos RR_{95} value, above 27. In contrast, Pacaraima, the municipality with the lower resistance level to the OP, is located at RR, a state where temephos

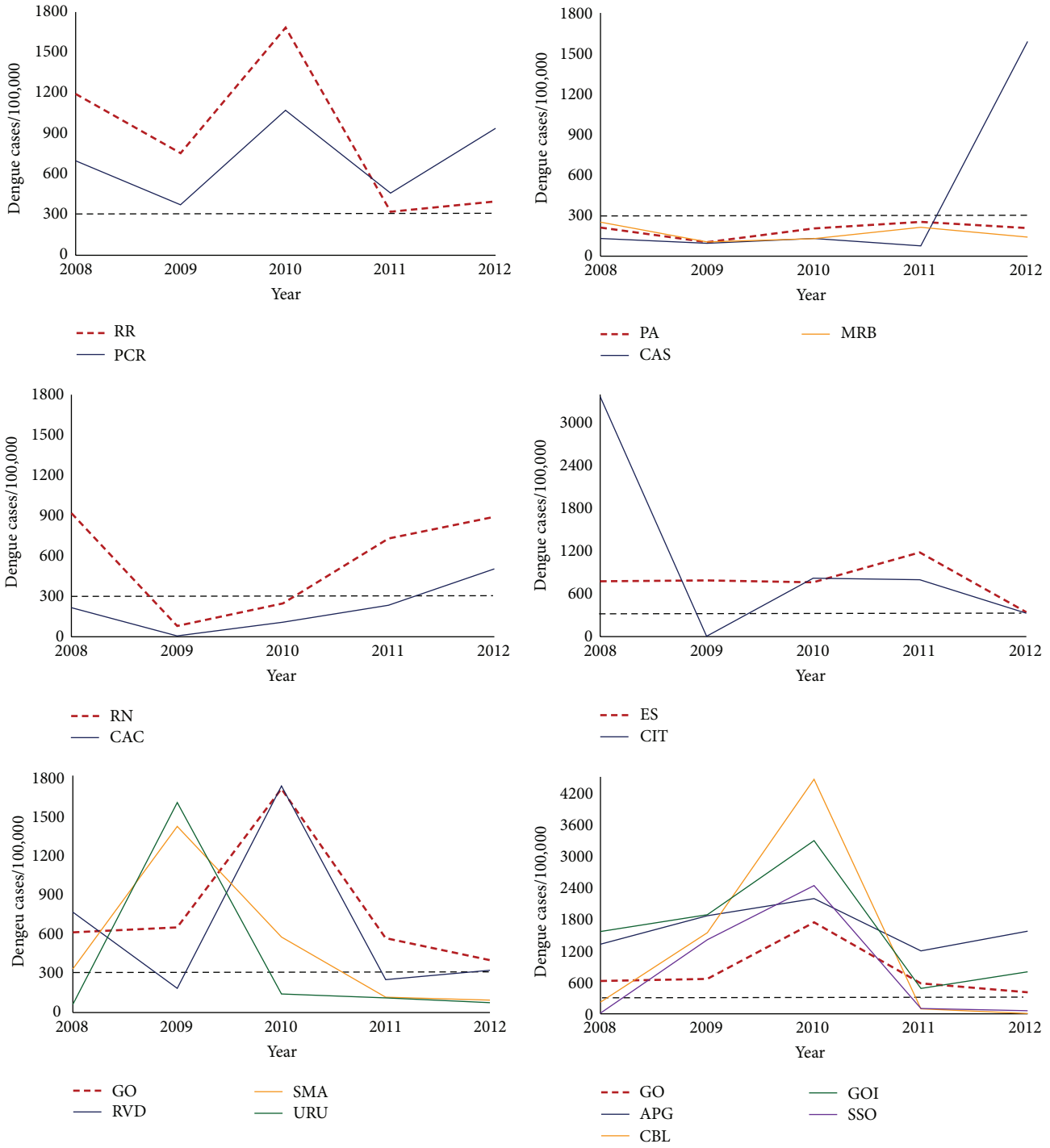

Figure 3: Incidence of reported dengue cases in the municipalities evaluated from 2008 to 2012 (values refer to the rate of notified cases per 100,000 inhabitants). In each panel, the thick red line refers to the dengue incidence in the corresponding State. The dashed line indicates the point above which dengue incidence is considered high. Note that in some panels it was necessary to change the y-scale in order to include all values.

supply started later than in the other states (Figure 2). In comparison with Rockefeller, a general higher heterogeneity of field populations was detected, as judged by their low slope values.

In contrast to the results obtained for temephos, all populations analyzed were susceptible to diflubenzuron ($RR_{95} < 3.0$). This was true even for mosquito populations bearing high temephos resistance rates, suggesting absence of cross resistance between these compounds in the localities examined. In contrast to the results obtained with temephos, field populations seemed more homogeneous than Rockefeller strain regarding diflubenzuron resistance profiles.

Table 1: Resistance status of several Brazilian municipalities to the larvicides temephos (OP) and diflubenzuron (CSI).

Year	Region	State	Municipality/strain	Generation	Temephos			Generation	Diflubenzuron		
					RR_{50}	RR_{95}	Slope		RR_{50}	RR_{95}	Slope
2010	NE	RN	Rockefeller		1.0	1.0	6.20		1.0	1.0*	4.85
			Caicó	F2	8.4	**9.6**	5.07	F2	2.2	*1.7**	6.56
2011	N	RR	Rockefeller		1.0	1.0	5.03		1.0	1.0	4.16
			Pacaraima	F2	4.3	**4.0**	5.70	F2	1.7	*1.5*	5.05
		PA	Castanhal	F2	8.2	**11.2**	3.53	F2	1.4	*1.2*	4.75
			Marabá	F2	8.0	**10.3**	3.76	F3	1.8	*1.6*	4.91
	SE	ES	Cachoeiro de Itapemirim	F1	18.4	**17.1**	5.57	F2	1.9	*1.6*	5.03
			Campos Belos	F2	9.1	**12.0**	3.68	F2	1.7	*1.6*	4.38
	CW	GO	Goiânia	F2	7.9	**8.6**	4.56	F2	1.6	*1.8*	3.63
			Rio Verde	F2	11.5	**14.8**	3.77	F2	2.0	*1.6*	5.32
			São Simão	F2	12.1	**14.8**	3.98	F2	2.0	*2.3*	3.57
			Uruaçu	F2	10.5	**12.5**	4.08	F2	1.9	*1.5*	5.78
2012	CW	GO	Aparecida de Goiânia	F1	17.9	**16.6**	5.59	F2	1.1	*2.1*	2.43
			São Miguel do Araguaia	F1	21.1	**27.1**	3.77	F1	1.6	*1.7*	3.82

RR_{50} and RR_{95}: resistance ratios; profiles corresponding to RR_{95} below or above 3.0 (italic font or bold font numbers) were classified as susceptible or resistant, respectively.
*RR_{80} is informed. See Table S2 for additional details.

Table 2: Resistance status of several Brazilian municipalities to the pyrethroid deltamethrin and allelic frequencies of the major kdr mutations found in the country.

Year	Region	State	Municipality/strain	Generation	RR_{50}	RR_{95}	Slope	Na_V allelic frequencies		
								S	R1	R2
2010	NE	RN	Rockefeller		1.0	1.0	2.96			
			Caicó	F2	6.0	**13.1**	2.51	0.917	0.067	0.017
2011	N	RR	Rockefeller		1.0	1.0	4.55			
			Pacaraima	F2	33.2	**60.3**	2.65	0.000	0.600	0.400
		PA	Castanhal	F2	9.9	**14.9**	3.05	0.667	0.300	0.033
			Marabá	F2	47.4	**70.7**	3.07	0.690	0.310	0.000
	SE	ES	Cachoeiro de Itapemirim	F1	49.0	**78.6***	2.16	0.103	0.224	0.672
			Campos Belos	F2	25.3	**52.3**	2.43	0.310	0.086	0.603
	CW	GO	Goiânia	F2	47.6	**46.5**	4.68			
			Rio Verde	F2	32.5	**56.2**	2.74	0.241	0.207	0.552
			São Simão	F2	30.4	**51.6**	2.78	0.083	0.383	0.533
			Uruaçu	F2	38.6	**51.6**	3.37	0.450	0.117	0.433
2012	CW	GO	Aparecida de Goiânia	F1	33.0	**57.2**	2.74	0.207	0.362	0.463
			São Miguel do Araguaia	F1	39.6	**49.4**	3.59	0.414	0.293	0.293

RR_{50} and RR_{95}: resistance ratios; profiles corresponding to RR_{95} above 3.0 (bold font numbers) were classified as resistant.
*RR_{80} is informed. See Table S3 for additional details.
Kdr allelic frequencies S, R1, and R2 refer to the positions 1016 and 1534 of the gene coding for the voltage gated sodium channel (Na_V) as follows: S (susceptible) = 1016 Val^+/1534 Phe^+; R1 (single mutant) = 1016 Val^+/1534 Cys^{kdr}; and R2 (double mutant) = 1016 Ile^{kdr}/1534 Cys^{kdr}. Kdr data have been originally published by Linss et al. 2014 [20].

3.4. Bioassays and Molecular Assays with Adults. Resistance rates resulting from bioassays with the adulticide PY deltamethrin are depicted in Table 2, together with the Na_V allelic frequencies, where R1 and R2 are the kdr alleles related to PY target site resistance. Additional details of the bioassays are presented in Table S3 that also includes the kdr allelic frequencies for each position (1016 and 1534) separately [20].

Very high deltamethrin resistance levels were found for all populations; RR_{95} was always above 10.0. Caicó, RN, at the northeast region, and Castanhal, PA, in the north region, exhibited the lowest RR_{95}, respectively, 13.1 and 14.9. Accordingly, these municipalities presented the lower frequencies of R1 and R2 kdr alleles. In all other municipalities values remained above 45.0. In two localities, Marabá, PA, and Cachoeiro de Itapemirim, ES, RR_{95} was higher than 70.0. In this latter locality, due to the high resistance level detected, there was lack of enough specimens to reach LC_{95} (note that in Tables 2 and S3 the higher value shown for

Table 3: Quantification of the enzymatic activity of *A. aegypti* larvae from different Brazilian municipalities. Numbers refer to the rate of specimens with activity higher than the 99 percentile of Rockefeller (% >p99). Municipalities are arranged in descending order of temephos resistance (RR_{95} OP).

Year	Region	State	Municipality/strain	RR_{95} OP	ACE	MFO	GST	A-EST	B-EST	PNPA-EST
2012	CO	GO	São Miguel do Araguaia	**27.1**	0	0	*20*	*28*	*21*	***59***
2012	CO	GO	Aparecida de Goiânia	**16.6**	5	*31*	***85***	8	***54***	***63***
2011	CO	GO	Rio Verde	**14.8**	1	0	*40*	*19*	*38*	8
2011	CO	GO	São Simão	**14.8**	1	4	*40*	*35*	*33*	*32*
2011	CO	GO	Campos Belos	**12.0**	1	15	9	1	5	0
2011	N	PA	Castanhal	**11.2**	0	*48*	*34*	3	*29*	***75***
2010	NE	RN	Caicó	**9.6**	0	4	*19*	0	0	0
2011	CO	GO	Goiânia	**8.6**	3	6	*49*	5	*34*	*36*
2011	N	RR	Pacaraima	**4.0**	6	1	*21*	0	14	3

Activities were classified as normal (regular font), altered (italic and underlined font) or highly altered (italic and bold) if these values ranged respectively below 15%, between 15 and 50% or above 50%.

Table 4: Quantification of the enzymatic activity of *A. aegypti* adults from different Brazilian municipalities. Numbers refer to the rate of specimens with activity higher than the 99 percentile of Rockefeller (% >p99). Municipalities are arranged in descending order of deltamethrin resistance (RR_{95} PI).

Year	Region	State	Municipality/strain	RR_{95} PI	ACE	MFO	GST	A-EST	B-EST	PNPA-EST
2011	SE	ES	Cachoeiro de Itapemirim	**78.6***	0	***80***	***98***	***67***	3	***70***
2011	N	RR	Pacaraima	**60.3**	0	*17*	*40*	*48*	13	2
2012	CO	GO	Aparecida de Goiânia	**57.2**	1	***57***	***94***	***70***	10	***73***
2011	CO	GO	Rio Verde	**56.2**	13	***74***	4	***81***	8	13
2011	CO	GO	Campos Belos	**52.3**	0	8	***65***	14	1	6
2011	CO	GO	São Simão	**51.6**	*21*	9	***59***	***58***	14	15
2012	CO	GO	São Miguel do Araguaia	**49.4**	4	***97***	8	*46*	0	8
2011	CO	GO	Goiânia	**46.5**	0	***99***	***78***	***55***	5	*38*
2010	NE	RN	Caicó	**13.1**	3	0	11	***63***	*22*	6

Activities were classified as normal (regular font), altered (italic and underlined font) or highly altered (italic and bold) if these values ranged respectively below 15%, between 15 and 50% or above 50%. *RR_{80} is informed.

Cachoeiro de Itapemirim corresponds to LC_{80}). The supply of pyrethroids for the bulk of the states evaluated here was continuous since at least 2003 (Figure 2). Comparison of the slope values obtained for deltamethrin assays shows that, in general, field populations evaluated demonstrate higher heterogeneity than the Rockefeller strain.

3.5. Biochemical Assays. Tables 3 and 4 present the results of biochemical assays for, respectively, larval and adult stages. In both tables, data are organized by decreasing RR_{95} order for the neurotoxic insecticides temephos (larvae) and deltamethrin (adults). Additional details of these assays are shown in Tables S4 and S5.

According to the WHO criterion, measurements of inhibition of AChE activity, the OP target site, point to unaltered activity for all populations and development stages (data not shown). This was verified because the carbamate propoxur induced more than 70% of AChE activity inhibition in all cases [47]. In addition, quantification of total AChE activity [45, 46] revealed values compatible with susceptibility, in all cases, with exception of adults from one population (São Simão, GO, Table 4).

For all the remaining enzymes, changes were detected for both stages. In the larval stage, major changes were noted in the activities of GST, β-EST, and ρNPA-EST, while moderate increases were noted for MFO and α-EST. The intensity of enzymatic alterations appeared to be higher at the adult stage, mainly when MFO, GST, and α-EST were considered. In general, populations with a higher RR also tended to exhibit a higher increase in detoxifying enzymes activity, taking into account both the number of altered classes and the intensity of activity increment.

4. Discussion

The use of insecticides in the control of the dengue vector in Brazil has been broad and continuous, a procedure that favored the selection of resistant specimens over the years. In order to assist in the rational use of pesticides, in 2006, the Brazilian Dengue Control Program adopted a functional criterion for the evaluation of the temephos status of *A. aegypti* populations. This criterion, also employed here to classify both deltamethrin and diflubenzuron resistance status, considers that populations with RR_{95} above 3.0 are resistant. This is the cutoff to conduct insecticide substitution in the

field, and the adoption of this parameter took into account Brazilian operational aspects of insecticide management, like the period of time necessary for the effective implementation of the insecticide substitution in all affected localities. This strategy aimed to preserve the insecticides in the field [23].

Functional validation of this criterion has been previously obtained through simulated field assays with temephos and, more recently, with pyrethroids [23, 51]. Resistance to diflubenzuron was not established in the country and therefore a functional criterion has not yet been defined for this IGR by PNCD. However, Fontoura et al. [42], using simulated assays, did not find impairment of the efficacy of another CSI, novaluron, in *A. aegypti* populations bearing $RR_{90} < 2.0$.

The use of OP pesticides in Brazil for *A. aegypti* control dates back to the 1960s, and it was intensified since 1986, when the DENV-1 virus was introduced in the country [52, 53]. As a result, resistance to temephos has been reported in Brazilian populations of *A. aegypti* collected from 1998 on [23, 27, 28]. Resistance to temephos spread around the country so intensely that, since 2009, PNCD does not recommend the use of this OP as the larvicide of choice [54]. Accordingly, all populations here evaluated between 2010 and 2012 were resistant to temephos.

Investigation of putative resistance mechanisms present in *A. aegypti* larvae excluded the participation of acetylcholinesterase, the OP target site. Regarding metabolic resistance, MFO enzymes are strongly associated with insecticide resistance in several *A. aegypti* populations around the world [25, 26, 55]. We found only discrete alterations in this class of enzymes in larvae from Brazilian *A. aegypti* populations, while adult specimens exhibited levels of MFO alteration equivalent to EST and GST enzymes. In 2007, *A. aegypti* adult EST activities were associated with resistance to both OP and PY in Brazil [23]. Connections between OP resistance and significant alterations of EST as well as association between PY resistance and both MFO and GST elevated activity rates were also reported in other countries [25, 26, 55].

In general, higher RR levels against OP and PY neurotoxic insecticides correlated to increased metabolic alterations in terms of both number of enzymes affected and intensity of activity enhancement [30, 55, 56]. Usually detoxifying enzymes that trigger metabolic resistance participate in the general insect metabolism. These are somewhat generic molecules, with a variable affinity for a number of insecticides or other xenobiotics. Although resistance to the IGR diflubenzuron has not yet been detected in the country, the development of metabolic resistance against these compounds is a potential mechanism that should be monitored.

For some localities here depicted, previous evaluations of the temephos susceptibility status are available. In these cases, the resistance dynamics profiles were compared to the insecticide distribution performed by the MoH to each state (Figure 2). Increase in the temephos resistance status was noted whenever the application of this OP persisted. Examples are the central-west municipalities of Goiânia (GOI) and Aparecida de Goiânia (APG), at GO state: between 2003 and 2011, GOI RR_{95} increased more than twice, from 3.3 to 8.6 (Table 1, [23]). In APG the temephos RR_{95} also increased significantly between 2006 (11.2) and 2012 (16.6) (Table 1, [57]). In contrast, only a low decrease in the temephos resistance status was noted following its interruption. This was the case of Caicó (CAC), at RN state, where no temephos was provided since 2004 (Figure 2). At that year, CAC temephos RR_{95} was 12.5; six years later this value dropped only slightly, to 9.6 (Table 1, [23]).

One major consequence of the high and disseminated Brazilian *A. aegypti* temephos resistance status was the inclusion of the CSI diflubenzuron in the chemical control of larvae since 2009. Bioassays of mosquito samples obtained between 2010 and 2012 confirmed the susceptible status of all evaluated populations to this product. Diflubenzuron resistance ratios below 3.0 were also found for field *A. aegypti* populations from Cabo Verde, Malaysia, and Martinique [58–60]. Together, these data point that the use of this class of insecticides in the control of larvae of the dengue vector is still viable.

The bulk of results obtained by the *A. aegypti* insecticide resistance monitoring Brazilian network guided the option to rotate products against larvae in the country. Insect Growth Regulators were adopted. Due to operational issues, the maximum period of four years was fixed for alternation of products [61]. The aim of this resistance management strategy was to preserve the few available larvicides. In this regard, it is ought to mention that Brazil only employs larvicides recommended by WHOPES for use in drinking water [14]. Currently, the IGR used against *A. aegypti* larvae is the juvenile hormone analogue (JHA) pyriproxyfen [62].

Some decades were necessary until the spread of resistance to the OP temephos in Brazil could compromise its use in the control of *A. aegypti*. In contrast, in the case of pyrethroids, introduced in the whole country in 2000 by PNCD, only a few years were enough to the achievement of extremely high resistance levels [23, 31, 32]. If, on the one hand, chemical control of *A. aegypti* adults is recommended by the MoH only to block outbreaks or on the imminence of a dengue epidemic [9], on the other hand, unlike temephos, PY insecticides are available in the retail market. The domestic use of pyrethroids is intensified at every dengue epidemic period and certainly contributes significantly to the rapid dissemination of resistance [33]. Accordingly, *A. aegypti* deltamethrin resistance levels doubled between 2009 and 2011 at Cachoeiro de Itapemirim and Goiânia; in the same period, an eightfold increase was observed for this parameter at Marabá (Table 2; Bellinato D, personal communication). Cachoeiro de Itapemirim and Goiânia faced dengue outbreaks in this interval, corroborating the hypothesis that the intensification of the domestic use of PY collaborated in the resistance increase. However no dengue outbreak was noted at Marabá in this period. Marabá is located in the Amazon region, where almost all Brazilian malaria cases are reported. Control of *Anopheles* malaria vectors also employs PY and this could explain the increased resistance observed. In contrast, the lowest PY resistance levels were found for Castanhal at 2010 and Caicó at 2011, two municipalities that had not experienced dengue epidemics since 2008.

To date, the PY resistance ratios found here are among the highest ones reported in the country. In spite of that,

heterogeneity levels exhibited by those vector populations suggest that the insecticide resistance character is still not irreversibly fixed. In relation to the PY target site resistance, the voltage gated sodium channel, this heterogeneity confirms previous observations, reporting the presence of the Na_V susceptible allele (S) with allelic frequencies between 0.0 and 0.92 (Table 2). Regarding metabolic resistance of adult mosquitoes, as for temephos resistance and larvae biochemical profile (mentioned above), a general positive correlation between the resistance level and the magnitude of altered enzymes was also found. It should be noted that, in this case, except for Caicó, all populations exhibited PY resistance levels above 45.0. Hence, while Caicó detoxifying enzymes effects were restricted to esterases, in the remaining populations, GST or MFO activities (and both enzymes in some cases) were significantly enhanced. In addition, GST, MFO, and EST have already been correlated with PY resistance [5, 23, 44, 63].

Due to the spread of high pyrethroid resistance levels throughout the country, since 2009 PY is being gradually replaced by the OP malathion for the control of *A. aegypti* adults, employed in residual applications and UBV [16, 54, 64].

Currently, chemical control is still largely applied in public health, favoring the insecticide resistance dissemination of vector populations. Accordingly, we detected high resistance levels against the OP temephos and the PY deltamethrin, insecticides long used in the country. In some municipalities, comparison with the incidence of dengue outbreaks suggested significant participation of the domestic use of PY insecticides in the rapid resistance increase. The introduction of IGRs is recent in Brazil, and all *A. aegypti* populations here evaluated were susceptible to the CSI diflubenzuron. Together our results indicate the potential of this IGR against the vector but also point to the need for rational use of chemical tools. In this sense, the adoption of rotation of compounds with different mechanisms of action is a positive step. It still remains, however, to invest in awareness campaigns, directed to both managers and the general society, regarding the importance of the mechanical control of vectors as a priority. Spreading the concept that chemical control is a complementary antivector strategy is the best way to preserve insecticides.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Ademir J. Martins, Diogo Fernandes Bellinato, Denise Valle, José Bento Pereira Lima, and Simone Costa Araújo conceived and designed the experiments. Diogo Fernandes Bellinato and Priscila Fernandes Viana-Medeiros performed the experiments. Diogo Fernandes Bellinato, Denise Valle, Priscila Fernandes Viana-Medeiros, and Simone Costa Araújo analyzed the data. Denise Valle and José Bento Pereira Lima contributed reagents/materials/analysis tools. Diogo Fernandes Bellinato and Denise Valle wrote the paper. All authors read and approved the final version of the paper.

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Research Article

Molecular Diagnosis and Identification of *Leishmania* Species in Jordan from Saved Dry Samples

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Diagnosis of the endemic cutaneous leishmaniasis (CL) in Jordan relies on patient clinical presentation and microscopic identification. Studies toward improved identification of the causative *Leishmania* species, especially in regions where multiple species exist, and the introduction of these techniques into medical diagnosis is paramount. This study looked at the current epidemiology of CL in Jordan. Clinically diagnosed 41 patients with CL were tested for the presence of *Leishmania* parasite using both Giemsa staining from skin scraps on glass slides and ITS1-PCR from samples blotted onto storage cards (NucleoCards®). Microscopically, 28 out of the 41 (68.3%) collected samples were positive for amastigotes, whereas the molecular ITS1-PCR amplification successfully identified 30 of the 41 samples (73.2%). Furthermore, PCR-RFLP analysis allowed species identification which is impossible microscopically. Of the 30 PCR positive samples, 28 were *Leishmania major* positive and the other two samples were *Leishmania tropica*. This indicates that *L. major* is the most prevalent species in Jordan and the two *L. tropica* cases originated from Syria indicating possible future *L. tropica* outbreaks. Diagnosis of CL based on clinical presentation only may falsely increase its prevalence. Although PCR is more sensitive, it is still not available in our medical laboratories in Jordan.

1. Introduction

Leishmaniasis threatens about 350 million people in 88 countries around the world [1]. It is believed that about 12 million people are currently infected with leishmaniasis, with about 1-2 million estimated new cases occurring every year [1]. The global incidence ranges of visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL) are 0.2 to 0.4 cases and 0.7 to 1.2 million cases per year, respectively. More than 90% of the global VL cases occur in six countries, excluding Jordan. CL cases are more widely distributed and one-third of the cases occur in three epidemiological regions: the Americas, the Mediterranean basin, and western Asia from the Middle East to central Asia including Jordan [2].

Cutaneous leishmaniasis is endemic in many Middle Eastern countries such as Syria, Iraq, Saudi Arabia, and Jordan and is still regarded as a major health problem which requires international awareness [3]. Syria reported very high incidence of CL and although several Middle Eastern countries have well-established national control programs for controlling the spread of the vector (sand fly) and the treatment of the infected individuals, still the disease continues to spread especially in the past few years due to the human migration within this area which occurred due to the political instability in the region [3].

Cutaneous leishmaniasis due to *L. major* is an endemic disease in Jordan which is known as "Jericho boil." Since 1985, outbreaks have appeared in areas where CL was previously unknown [4–7]. The occasional outbreaks of leishmaniasis in Jordan have occurred in endemic and nonendemic foci such as Aqaba, North Agwar, and South Shuneh. Based on

an annual report released in 2009 by the Jordanian Ministry of Health, Alvar et al. [2] reported 227 CL cases per year between 2004 and 2008. However, this number is probably skewed due to a spike in 2007 where 354 cases were reported. The most recent Jordanian annual report identified a total of 2,560 CL cases throughout Jordan between 1994 and 2014 [8]. Severe underreporting of CL is suspected in Jordan, which impacts its eradication [9]. Many factors can lead to this underestimation, some of which include the self-healing nature of the disease, the lack of awareness of the physicians of the importance of disease notification, and the occurrence of the majority of the CL cases in endemic rural areas which have limited resources for treatment due to the scarcity of clinics.

In Jordanian rural areas, during outbreaks of CL, the diagnosis and treatment are usually made directly in the field based on epidemiological data and clinical presentation (i.e., lesion morphology). However, the definitive diagnosis of CL can be sometimes challenging even for expert clinicians, since the symptoms can vary and may be confused with other etiological agents. Thus, correct diagnosis is important for the selection of appropriate treatment and for the reduction of its complications [10]. The classical diagnosis of specimens that are taken from CL patients relies on the visualization of the parasite stage (amastigotes inside and outside macrophages) in Giemsa-stained smears. Microscopic examination of stained smear is rapid, cheap, and easy to perform but sometimes lacks sensitivity due to the generally low number of parasites in tissue samples [11], in addition to the need for experienced microscopist. Parasite culturing is more sensitive than microscopy but is time consuming, requires sophisticated laboratory setups, harbors the risk of contamination, and cannot identify species, as different species are morphologically indistinguishable [12].

Molecular tools using a number of molecular markers and polymerase chain reaction (PCR) protocols have been developed for the detection and identification of *Leishmania* [13–18]. For epidemiological investigation and clinical case management especially during outbreaks of leishmaniasis, the determination of the causative *Leishmania* species is extremely important and many of those molecular tools have already been used in different parts of the world including Jordan to differentiate species of *Leishmania* [19–21]. In the absence of molecular tools, geographical distribution, clinical presentation, and the species of sand fly vectors and animal reservoirs are commonly used to attempt to identify species, which is clearly inadequate, particularly where multiple species coexist [5, 17].

The present study is aimed at describing the epidemiology of human CL in Jordan using ITS1-PCR and RFLP in addition to Giemsa-stained smears microscopy. During the study period, we collected skin scrapping from 41 patients with suspected CL lesions. NucleoCards were used in the field to dry-preserve the genetic material for later molecular testing. Then NucleoCards ability to retain the genetic material and aid in CL diagnosis was compared to routine Giemsa-stain smear microscopy identification. The NucleoCards ability to hold parasites was not investigated in this study.

2. Materials and Methods

2.1. Study Population. The study participants were suspected patients with CL who were presented to the Ministry of Health Clinics in different parts of Jordan for the evaluation of skin lesions from early 2009 until late 2011. All patients gave their informed consent to participate in the study, which was reviewed by the institutional review board and approved by the Research Ethics Committee at Hashemite University and the Ministry of Health. A total of 41 patients thought to be infected with CL based on clinical examination only (i.e., size, number, location, and type of lesion) were recruited. These patients were interviewed to fill a demographic questionnaire, and then skin scrapings from the lesions were taken for the study.

2.2. Sample Collection for DNA Extraction and PCR. Samples from suspected *Leishmania* lesions were obtained from 41 patients. After removing any overlying scab or crust with moistened gauze, a DNA storage card, NucleoCards (740403.100, Macherey-Nagel, Germany), was gently pressed onto the moist ulcer base to allow the tissue fluid and scrapings to diffuse into the NucleoCards. At least two different samples/spots were collected on two different collection circles on the same card. The card was then allowed to air-dry for 30–60 min and stored at room temperature for subsequent DNA extraction. All cards were labeled with the patients' information and date of collection.

2.3. Sample Collection for Giemsa Staining. The same 41 patients were also screened by microscopy. After cleaning with topical antiseptic, material from the lesion (tissue scrapings) was collected from the ulcer base and border using a sterile lancet and spread on a glass slide. Slides were stained using a routine Giemsa staining procedure. All slides were viewed under oil immersion with 100x objective lens for confirmation of amastigotes stages inside and outside macrophages.

2.4. Extraction of DNA from NucleoCards. The DNA was extracted from the dry clinical samples (skin scrapings) collected on the NucleoCards using phenol-chloroform extraction manual procedure [22]. Briefly, two discs (5 mm) were punched out from each clinical sample blotted onto NucleoCards using a sterile blade and transferred to a sterile DNAase free tube containing 250 μL cell lysis buffer (50 mM NaCl, 50 mM Tris, and 10 mM EDTA; pH 7.4), 2.5 μL Triton X-100 (9002-93-1, Bio Basic Inc., Canada), and 1.26 μL Proteinase K (20 mg/mL) (EO0491, Fermentas GmbH, Germany). The samples were incubated overnight at 60°C. The DNA was pelleted and dried at room temperature for 30 min and redissolved in 100 μL of TE buffer (pH 8) (V6231, Promega, USA). The samples were kept at −80°C until assayed.

2.5. PCR Amplification. Amplification of the ribosomal internal transcribed spacer 1 (ITS-1) gene was carried out using the LITSR (forward) (5′CTGGATCATTTTCCGATG-3′) and L. 5.8S (reverse) (5′-TGATACCACTTATCGCACTT-3′)

Table 1: Major characteristics of the study participants ($n = 41$).

Characteristic (N)				
Gender	M (27) 66%	F (14) 34%		
Age (years)	0.5–73	Average 18.3	Median 14	Mode 14
Education	None (21)	<grade 10 (13)	>grade 10 (7)	
Nationality	Jordanian (34)	Syrian (6)	Egyptian (1)	
Infected body parts	Facial (13)	Upper limbs (23)	Lower limbs (16)	Trunk (2)
Stage of infection	Cured (7)	Papule (2)	Ulcerated (31)	Ulcerated + papule (1)
Number of lesions	1–8 (average 1.99)	Average M 1.99	Average F 1.97	
Microscopic results	Negative (13)	Positive (28)		
PCR results (*Leishmania* species)	*L. major* (28)	*L. tropica* (2)	Negative (11)	
Treatment	Yes (37)	No (4)		
Residency in current address (years)	<5 (13)	5–10 (3)	>10 (25)	
Outdoor activities	Yes (30)	No (11)		
Travelled outside Jordan in the past year	Yes (3)	No (38)		
Sand fly knowledge	Yes (9)	No (32)		
House type	Cement (33)	Hair tent (8)		
Protections from sand flies	None (25)	Some (16)		
Living nearby valley, stream, or farm	Yes (38)	No (3)		

N: number; M: male; F: female; some protection: window sieve, insecticides, and/or insect repellent.

primers [22]. Both primers and positive controls of *Leishmania* strains were kindly provided by Dr. Abdedelmajeed Nasereddin (Al-Quds University, Palestine). Amplification reactions were performed in a 25 μL volume containing 1.5 μL extracted DNA or control samples, 12.5 μL of 2x PCR master mix (M7502, Promega, USA), 1.5 μL of each primer (10 pmol/μL), and 8 μL nuclease-free water. Amplification was performed in a thermocycler (I Cycler, Bio-Rad, USA) according to El Tai et al. [22] with modification; thermal profile involved initial denaturation at 95°C for 2 min followed by 32 cycles consisting of denaturation at 95°C for 20 sec, annealing at 53°C for 30 sec, and extension at 72°C for 1 min. This was followed by a final extension at 72°C for 6 min. 5 μL of the amplification products was assayed using 2% agarose gel electrophoresis (V3125, Promega, USA) at 120V for 45 min in 1x TBE buffer (0.045 M Tris-borate (H5131, Promega, USA) and 1 mM EDTA (H5031, Promega, USA)) after staining with of ethidium bromide (0.5 mg/mL) (H5041, Promega, USA) for 15 min. DNA bands of 300–350 bp that confirmed the presence of *Leishmania* DNA were visualized by ultraviolet transparent gel tray (UVI, EEC) and photographed using the IP-010-SD photo documentation system program (Vilber Lourmat, EEC). Each PCR run was routinely assessed against negative and positive controls. The two negative controls are clean NucleoCards without template DNA and NucleoCards spotted with healthy blood. Positive controls for *Leishmania* DNA included *L. tropica*, *L. major*, *L. infantum*, and *L. donovani*. After comparing to controls, an image was taken for a group of positive samples (Figure 1(a)).

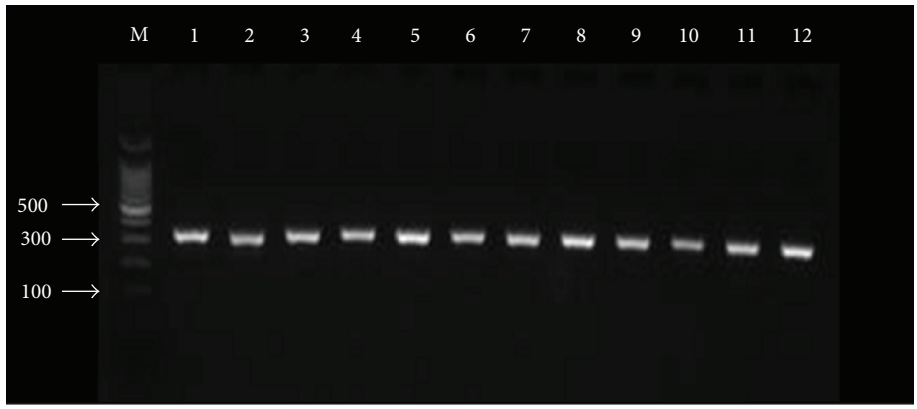
2.6. Restriction Fragment Length Polymorphism (RFLP) Analysis of Amplified ITSI Products. To digest the PCR product, 15 μL of the PCR products was mixed with 2 μL of restriction enzyme buffer (10x), 0.2 μL of BSA acetylated, 1.8 μL of nuclease-free water, and 1 μL of 10 U/μL of the restriction enzyme *Hae* III as recommended by the supplier (R6175, Promega, USA). The mixture was incubated at 37°C for 2 hrs and then the restriction fragments were separated using 2% agarose gel electrophoresis as described above. An image was taken for a group of positive samples in addition to positive and negative controls after restriction (Figure 1(b)).

3. Results

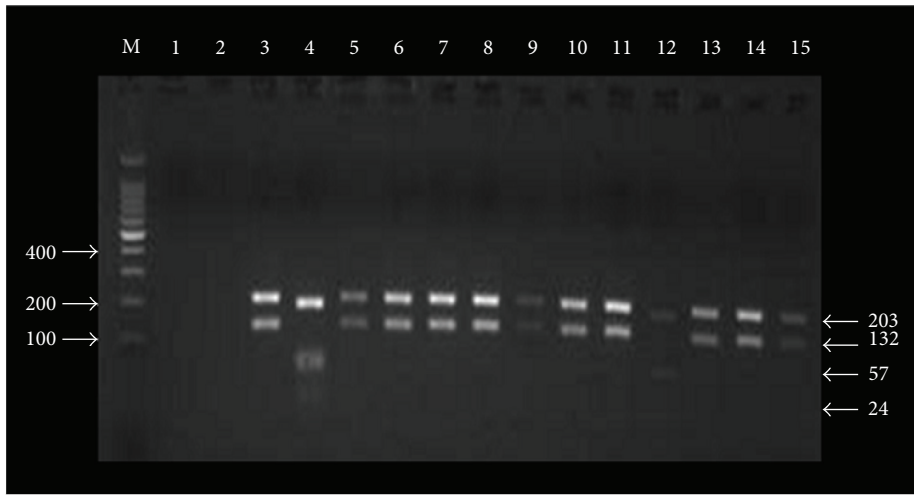
3.1. Demographics. Briefly, samples were collected from patients diagnosed with CL based on clinical presentation attending various clinics in different parts of Jordan. The majority of the samples collected for this study were from Jordanians (83%), with six Syrians (15%) and one Egyptian. Detailed demographic data is shown in Table 1.

3.2. Comparison of Cutaneous Leishmaniasis Diagnosis Based on Clinical Presentation, Microscopy, and PCR Analysis. Based on clinical presentation, all 41 cases were suspected as CL patients. By microscopy, *Leishmania* amastigote stage was detected in 28 of these 41 samples (68.3%, 60.4–82.5%). The PCR procedure was used for the diagnosis and identification of *Leishmania* species for clinical samples saved on NucleoSave cards. Of the 41 samples, PCR amplicon could only be successfully extracted from 30 clinical samples and all of these were positive by PCR (72.3%, 59.6–87.5%). Figure 1(a) shows the PCR products (300–350 bp) from 12 randomly selected positive samples.

3.3. Species Identification by Restriction Analysis of the Amplified ITSI Region. RFLP analysis of the 30 PCR-positive clinical specimens with the restriction enzyme Hae III identified 28 samples infected with *L. major* and only two were infected with *L. tropica* (Figure 1(b)).



(a)



(b)

Figure 1: Representative pictures showing agarose gel electrophoresis (2%) of random PCR products (300–350 bp) which were extracted from the 30 positive *Leishmania* samples. Lane M: 100 bp DNA ladder. Lanes 1–12: PCR products randomly selected from 30 clinical samples (a) and (b) showing the digestion of amplified ITS1 regions for different *Leishmania* species with the restriction endonuclease Hae IIII. Lane M: 100 bp DNA ladder. Lane 1: negative control. Lane 2: healthy individual control. Lane 3: *L. major* positive control showing two bands (203 bp and 132 bp). Lane 4: *L. tropica* positive control showing three bands (185 bp, 57 bp, and 24 bp). Lanes 5, 6, 7, 8, 9, 10, 11, 13, 14, and 15: random samples for *L. major* detected in clinical samples. Lane 12: *L. tropica* detected in clinical samples.

4. Discussion

The aim of this study is to use the ITS1-PCR and RFLP to describe the epidemiology of human CL and species identification of leishmaniasis in Jordan from dry-preserved samples on NucleoCards. The sensitivity of the PCR-RFLP technique was also compared with the clinical diagnosis and routine Giemsa-stain microscopy method. According to the study results, samples collected from CL suspected patient were positive for either *L. major* (28 samples) or *L. tropica* (2 samples) using the PCR-RFLP method. The median age of the study sample was 14 years and almost two-thirds of them were males (66%). The sample population was mainly Jordanians; however, there were some Syrians and one Egyptian. Based on the clinical diagnosis, most CL infections were in the usually exposed parts of the body such as upper and lower limbs and the face in patients who had resided in the same address for more than 10 years and nearby valleys. Despite living in endemic leishmaniasis area for many years, the majority of those patients showed little or no knowledge of the vector (sand flies) and, therefore, did not know how to protect themselves from CL infection (Table 1).

The PCR-RFLP method was found to be highly sensitive and specific in detecting samples that have *Leishmania* when compared to the Giemsa-stain microscopy method, which is congruent with previous reports [16, 23, 24]. Of the 41 samples, PCR amplicons could only be successfully extracted from 30 samples and all of these were positive by PCR. The other 11 samples did not have enough DNA concentration on the cards (<5 ng/μL), hence the negative results, which indicate the need for more sample quantity during collection. A previous study reported the use of molecular detection of CL from lesions stored on NucleoCards filter paper (modified cards which are different from the normal filter paper) [14]. However, this study compared lesion scrapings for microscopic identification with blots on NucleoSave cards from lesions for its ability to diagnose CL and discriminate the infecting species based on dried DNA.

Obtaining clinical samples (lesions aspirates and scrapings) for CL diagnosis by microscopy and *in vitro* culturing in Jordan is the routine procedure. Nevertheless, it frequently causes pain and discomfort to patients and requires technical expertise [25, 26]. For existing and emerging foci of CL in Jordan, accurate diagnostic tools are required to detect parasites directly in clinical samples and distinguish all relevant species of *Leishmania*. The ITS1 sequence (300–350 bp depending on the species) was chosen in the present study as the target for our PCR assay for several reasons. Many recent studies have shown that ITS1-PCR followed by restriction fragment length polymorphism analysis is a suitable tool for diagnosing and identifying *Leishmania* species [17, 27]. The major advantage of ITS1-PCR is that species identification can be achieved by digesting the PCR product by just one restriction enzyme (Hae III) and this is sufficient to distinguish almost all medically important *Leishmania* species [17].

All cases of leishmaniasis reported from Jordanian patients are cutaneous and caused by the two species, *L. major* and *L. tropica* [9, 10]. The different *Leishmania* species are morphologically indistinguishable, but they can be differentiated by isoenzyme analysis, molecular methods, or monoclonal antibodies. Molecular tools have been developed for the differentiation of the pathogenic species of *Leishmania* in different parts of the world [19–21]; however, recently, just one study evaluated using such a PCR technique in Jordan [28] but still molecular based assay is not a readily available diagnostic technique in our medical laboratories despite the fact that species discrimination is important for epidemiological and clinical reasons.

Until recently, relatively little information has been available on the epidemiology of CL and the causative species in different parts of Jordan. Leishmaniasis due to *L. major* species is considered hyperendemic in the Jordan Valley and Wadi Araba regions, with some areas exhibiting 80% positivity using *Leishmania* skin testing among exposed populations in hyperendemic areas and an infection rate of 5.5% and 23% in the vector and reservoir hosts, respectively [29]. There appears to be an evolutionary divergence between the *L. major* zymodemes isolated from the Jordan Valley and the Jordanian plateau [30]. These regions are separated by a mountainous region where no cases of *L. major* have been reported. In the North where the biotope is more Mediterranean and the land is rocky, only *L. tropica* infections have been reported [31, 32]. This area has a semi-Mediterranean climate and is about 600 meters above sea level. No evidence for the presence of rodent hosts of *L. major* (*P. obesus*) was found [33].

In the North of Jordan, *L. tropica* has been reported as a causative agent for CL, whereas *L. major* has been reported in all other areas of the country. Those findings are based on geographical distributions, data collected by the Health Department and local studies, clinical presentations, vectors, and animal reservoirs. The Jordan Valley is home to endemic areas with very high infection rates. In the hyperendemic area of Swaimeh, 100% of individuals over 5 years old were found positive in a *Leishmania* skin test survey in 1992, which is similar to our results where the average age of patients was 18.3 years [34]. In the same survey, higher infection rates (72.4%) are recorded in males than females (27.6%) in all age groups which is also congruent with our findings where 66% and 34% of the suspected CL patients were males and females, respectively. It was also reported that CL is more prevalent in children under 5 years (24%) than in those older than 50 (8%), as we also noted in this study which may be explained by the natural vulnerability of this population [34].

The available epidemiological evidence indicated that all forms of CL in Jordan are mainly zoonotic diseases [35], where the most prevalent *Leishmania* species in Jordan is *L. major* which is responsible for 75% of the cases [35, 36]. Large areas of the country, particularly the Southern and Eastern regions, correspond to desert biotopes in which *L. major*, a zoonotic infection, is maintained by the reservoir host *Psammomys obesus, Meriones libycus*, and the sand fly vector *Phlebotomus papatasi*. Cases from *L. tropica* are rare and reported from the northern regions of Jordan. The suspected vector for *L. tropica* is the sand fly *Phlebotomus sergenti* and the reservoir host is still unknown but evidence has suggested that canines or hyraxes, which are present in all *L. tropica* foci in Jordan, are both suspected to be the reservoir hosts [1].

In regions such as the Middle East including Jordan, patients suffering from leishmaniasis often live in remote and isolated low socioeconomic areas with limited access to clinical care and education [10]. When they become infected with *Leishmania* they are often undiagnosed patients and when they present to their local medical center, there are insufficient diagnostic tools and expertise. We believe that using sample storage cards (NucleoCards) is very effective and helpful in such regions. The material can be collected on the NucleoCards which can be then mailed via normal local mail to the central diagnostic facility where molecular tools and skilled specialist are available to properly diagnose the material in a relatively short period of time, allowing better patient management and treatment.

Cutaneous leishmaniasis is well known to be an endemic disease not only in Jordan but also in neighboring countries such as Syria [37]. In the year 2012 an official figure reported 52,982 confirmed cases of CL in Syria [38]. With the political instability in Syria, millions of Syrian refugees entered the neighboring countries of Lebanon, Turkey, and Jordan [38]. In Jordan, only 20% of the refugees reside in the Al Zaatari camp while the remaining 80% reside within the Jordanian population in major cities. The extensive displacement of the Syrian population also increased the incidence of the vector-borne disease within Syria and its spread into neighboring countries [38]. In this study we found that the majority of CL is caused by *L. major* (28 cases) in a mixture of nationalities of Jordanians (22), Syrians (5), and one Egyptian. There were only two cases of CL that were caused by *L. tropica*. One of them is a Syrian and the other is a Jordanian who indicated a previous travel to Syria. As a result, it is obvious that future outbreaks of CL caused by *L. tropica* more than *L. major* might be unavoidable in Jordan especially in areas that have a high number of refugee residents. This requires higher alert level by the Ministry of Health in Jordan and proactive role in preventing outbreaks and the spread of CL. Early detection, proper diagnosis, and treatment are the gold

standard recommendations. Using ITS1-PCR to identify the infecting *Leishmania* species in clinical samples could be the future routine test that needs to be adopted in Jordan.

Competing Interests

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

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Review Article

Epidemiology of *Plasmodium* and Helminth Coinfection and Possible Reasons for Heterogeneity

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Understanding the impact of helminth infections on clinical malaria is useful for designing effective malaria control strategies. Plenty of epidemiological studies have been conducted to unravel the nature of interactions between *Plasmodium* and helminth infection. Careful broad summarization of the existing literature suggests that *Schistosoma mansoni* and hookworm infections may increase the risk of clinical malaria and associated morbidities, but *Trichuris trichiura* infection is not associated with the occurrence of clinical malaria and related outcomes. However, findings about effect of *Ascaris lumbricoides* and *Schistosoma haematobium* infection on clinical malaria are contradictory. Furthermore, the nature of relationship of helminth infection with severe malaria has also not been determined with certainty. This review summarizes the findings of epidemiological studies of *Plasmodium* and helminth coinfection, placing greater emphasis on the impact of the coinfection on malaria. Possible reasons for the heterogeneity of the findings on malaria and helminth coinfections are also discussed.

1. Introduction

Although the nature of interaction remains uncertain, studies showed that an apparently true biological association exists between *Plasmodium* and helminths when they coexist in a host [1, 2]. Hence, the presence of helminth can affect the risk of malaria and severity of the disease; or the occurrence of *Plasmodium* infection may in turn impact the upcoming helminth infections and related morbidities [1, 2]. As a result, disease due to one of these parasites could be exacerbated or ameliorated due to the cooccurrence of the other species resulting in synergistic or antagonistic impacts on the infected host.

Despite such a bidirectional nature of interactions between the two groups of parasites [1], studies usually evaluate the impact of helminth coinfection on malaria. This could be due to the perceived larger public health impact of malaria compared to helminth infections. In addition to this, helminths are known for their strong immune-modulatory impact on other coinfecting parasites compared to *Plasmodium* [3]. Helminths also reduce the amount of red blood cells necessary for *Plasmodium* to reproduce. Moreover, anaemia caused by helminth infection can lead to cardiovascular compensation, hyperventilation of CO_2, and increased lactates which can make the host more attractive to mosquitoes [4].

This paper provides a brief review of epidemiological studies on helminths and malaria interactions with emphasis on the impacts of the coinfection on malaria. Possible reasons for the inconsistency of the findings on malaria and helminth coinfections are discussed. Scholar search in PubMed, Embase, and Google was performed without restricting to language, publication date, study design, and nature of the study participants (e.g., age, sex, pregnant, and health status). Combinations of key words such as "helminths and malaria or *Plasmodium*", "*Ascaris* and malaria or *Plasmodium*", "*Schistosoma* and malaria or *Plasmodium*", "hookworm and malaria or *Plasmodium*", "*Trichuris* and malaria or *Plasmodium*", and "*Strongyloides* and malaria or *Plasmodium*" were used to search relevant references. Studies on *Plasmodium* and helminth coinfections in animals were excluded. In addition, human studies on the immunological evidences of *Plasmodium* and helminth, *Ascaris*, *Schistosoma*, hookworm, or *Trichuris* interactions were excluded. More than 7560 published articles were identified following the

literature search. After exclusion of duplicates and screening of the titles and abstracts, a total of 107 articles were found that fulfill the inclusion criteria. About 50 articles were further excluded after reading the full-text. The characteristics of the studies on epidemiology of *Plasmodium* and helminth coinfection are provided in Table 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/3083568.

2. *Schistosoma* and Malaria

Many studies have been conducted to evaluate the nature of the interactions between *Schistosoma* and *Plasmodium*. More than 20 of these studies were conducted under natural conditions in humans. Of these studies, five looked at immunology, thus excluded from the current epidemiology focused review.

Most of the epidemiological studies were conducted among children infected with *P. falciparum*. However, findings about malaria prevalence or incidence, density of the parasite, and associated morbidities during *S. haematobium* infection were heterogeneous. While some studies reported decreased malaria prevalence [5], incidence [6], *Plasmodium* density [5], disease severity [5], and associated splenomegaly [7], others reported increased prevalence or risk of *Plasmodium* infection and density of the parasite [7–10] or low haemoglobin level [11] and enlarged spleen [12] among individuals infected with *S. haematobium*. Still, other studies reported similar level of prevalence of *Plasmodium* infection [13, 14] or density of the parasite [6, 15, 16] and haemoglobin level [6] among children infected and uninfected with *S. haematobium*.

On the other hand, prevalence [17, 18] or incidence [19, 20] and density of asexual [21] or gametocyte [22] stages of *P. falciparum* infection and related anaemia [20] or hepatosplenomegaly [23] increased in individuals infected with *S. mansoni* alone [17, 19, 21, 23] or both *S. mansoni* and *S. haematobium* [20, 22]. However, two studies reported lack of association between prevalence of falciparum malaria and *S. mansoni* infection [13, 24]. The nature of association between *Schistosoma* and malaria seems to vary with the age of the individuals [6] and intensity of *Schistosoma* [19, 25] infection.

3. *Ascaris lumbricoides* and Malaria

Randomized controlled trials in Madagascar and Comoros Islands reported increased *P. falciparum* incidence and density of the parasite after treatment of children for *A. lumbricoides* infection [26–28]. Similarly, a cohort study in Brazil documented a lower drop in haemoglobin level among children coinfected with *P. vivax* and *A. lumbricoides* compared to children infected with only *P. vivax* [29]. In addition, cross-sectional studies in pregnant women, children, and adults reported association of *A. lumbricoides* infection with a low prevalence [30] or incidence [31] and density of *Plasmodium* infection [32]. *A. lumbricoides* infection was also negatively correlated with the occurrence of cerebral malaria and body temperature among patients in Thailand [33, 34].

On the other hand, in a longitudinal study among pregnant women in Gabon and a case control study in Thailand, patients reported association of *A. lumbricoides* infection with increased incidence of malaria [35]. In addition, positive association between *A. lumbricoides* infection and prevalence of malaria was observed among patients in Ethiopia and pregnant women in Ghana [17, 36]. Severe malaria was also found to be more common among children infected than uninfected with *A. lumbricoides* [37]. On the other hand, other studies documented lack of association between *A. lumbricoides* infection and prevalence [13, 38–41] or incidence [42] of malaria and *Plasmodium* density [29]. Overall, findings about the effect of *A. lumbricoides* infection on clinical or severe malaria are inconsistent and it is difficult to make a clear conclusion about the nature of relation between the occurrence of *A. lumbricoides* infection and the risk of malaria based on the existing evidences.

4. Hookworm and Malaria

Hookworm is widely distributed in most tropical regions where malaria is endemic [43]. As a result, malaria and hookworm coinfection is common in many parts of the world especially in tropics and subtropics [44]. Moreover, hookworm is a known cause of anaemia and could strongly predict *Plasmodium* infection and associated morbidities [4].

Cross-sectional studies among pregnant women in Thailand, Ghana, and Uganda reported increased malaria prevalence during hookworm coinfection [30, 36, 45]. Hookworm infection was also associated with increased malaria prevalence [13, 41, 46, 47] and *Plasmodium* density [22, 32] among children in Zimbabwe, Ethiopia, Uganda, Kenya, Côte d'Ivoire, and Colombia. On the other hand, some studies showed lack of association between hookworm infection and prevalence [24, 31, 48] or incidence [42] of malaria and *Plasmodium* density [29]. Yet, the existing epidemiological evidences tend to suggest positive association between hookworm infection and occurrence of clinical malaria and associated morbidities.

5. *Trichuris trichiura* and Malaria

Unlike other helminth species, there are few epidemiological studies examining the relationship between *T. trichiura* infection and malaria. Perhaps this could be due to the restricted distribution of *T. trichiura* infections in the equatorial regions of Africa which may have resulted in a decreased risk of coinfection with *Plasmodium* [44]. Majority of the studies on *T. trichiura* and *Plasmodium* coinfection showed lack of association between the two groups of parasites. Whilst two studies observed association of *T. trichiura* infection with high prevalence of *P. falciparum* infection among patients in Thailand and Ethiopia [17, 49], three studies among pregnant women in Ghana, Kenya, and Uganda [36, 39, 45], one study among patients in Columbia [41], and another study among primary school children in Zimbabwe [13] did not show relationship between *T. trichiura* infection and the occurrence of malaria or related outcomes. Other studies also reported lack of association between *T. trichiura* infection and

malaria incidence [42] or *Plasmodium* density [29, 32] among school-age children and adults. This suggests that *T. trichiura* infection may not affect the occurrence of clinical malaria or associated morbidities. The two studies which showed positive association between *T. trichiura* infection and malaria were conducted among individuals visiting the outpatient clinics. Patients may have other infections or health problems that might have confounded/distorted the direction of relationship between these two groups of parasites.

6. Pooled Intestinal Helminth and Malaria Data

Some studies considered pooled data of different helminth species as one variable while evaluating the nature of interactions between *Plasmodium* and helminths rather than considering intestinal helminth species independently. Although variation may exist in their pathogenicity, different helminth species have similar immunopathological impact on hosts. Most gastrointestinal helminths and *Plasmodium* affect host nutrition in a similar manner. Hence, it seems plausible to consider different gastrointestinal helminth species together while assessing the impact of helminth coinfection on malaria.

While some studies reported increased prevalence [17, 36, 50–52] or incidence [53–55] of malaria and gametocyte carriage [55], some studies reported lower occurrence of clinical and severe malaria [32, 56–58] in helminth-infected individuals compared to the uninfected ones. Two studies also reported decreased reticulocyte count and haemoglobin concentration among individuals infected with helminths and *Plasmodium* [59, 60]. Still, other studies reported lack of association between intestinal helminth infection and prevalence [30, 38, 61] or incidence [29, 42, 62] and density of *Plasmodium* infection [15].

7. Factors Contributing to the Heterogeneity of the Findings about Malaria and Helminths Coinfection

Factors related to methodology, environment, host, and parasite may explain the lack of uniformity of the findings about the nature of relationship between helminth and malaria.

7.1. Methodological Factors. Most previous studies on malaria and helminth coinfection were not uniform in terms of design and methods used for assessing *Plasmodium* and helminth infection, sample size, and the degree to which they control the effect of confounders. They were either cross-sectional, case control, or retrospective analysis of data collected previously for other purposes. Hence, it is difficult to confirm whether helminth infection occurred before the occurrence of malaria. Additionally, sample sizes in some previous studies were small. This might have reduced the power of the studies to detect differences in the occurrence of malaria and associated morbidities between helminth-infected and uninfected individuals. Variation also existed among previous studies on how the study subjects were selected and diagnosed for parasitic infection. Single Kato-Katz technique is less sensitive for examining light intensity helminth infection [63]. In addition, the performance of microscopy in the diagnosis of *Plasmodium* infections relies on blood film quality and experience of the examiner [64]. Thus, intensity of infection could be underestimated or light infections could be missed and *Plasmodium* species may not be correctly identified when the examiner is less experienced.

While intensity and species of helminths are central determinants of infection-related impact on clinical and immune functions of malaria [1, 2], studies usually fail to consider these factors while testing the relationship between the two groups of parasites. Furthermore, some studies did not control for the effect of socioeconomic conditions, place of residence, housing condition, education status, and nutrition status while examining the nature of relationship between the two groups of parasites. Thus, results obtained could have been distorted.

The nature of relationship between helminth and malaria could vary based on the type of helminth species [1, 2]. Hence, analyzing data after combining different helminth species into a single group may yield a different result. If two or more helminth species which can associate with malaria positively and negatively coexist, one may cancel or suppress the effect of the other. For example, in a study by Boel et al. [30], stratified analysis based on the type of helminth species showed that incidence of malaria is positively associated with hookworm infection but negatively associated with *A. lumbricoides* infection. However, this relationship was not maintained when data was analyzed after pooling the different helminth species in one group. Another study also showed negative correlation between *S. haematobium* and *Plasmodium* density, but this relationship was not maintained when analysis was done after pooling different helminth species together [15].

7.2. Environmental Factors. Contradictions in findings about the nature of relationship between malaria and helminth could also be due to variation among different geographic locations in their degree of endemicity for malaria and helminth. In areas where there is frequent exposure to malaria, strong immunity will be developed that can be affected during helminth coinfection. In contrast, malaria immunity will be less developed; therefore, the effect of helminth coinfection will be minimal when transmission intensity is low. Indeed, a study in Uganda, where malaria transmission is low, failed to show any association between helminth and malaria even when data were analyzed after stratifying by the type of helminth species and intensity of infection [42].

7.3. Host Factors. In addition to methodological and environmental variations, previous studies vary in the nature of the study population. While most studies were conducted in children, some studies were conducted in adults. Some studies also involved pregnant women or immunocompromised individuals who were malnourished or had other viral, bacterial, parasitic, or chronic infections. Moreover, the

genetic makeup of individuals, which may affect susceptibility of individuals to malaria, was likely different among the study population in previous studies. These factors may confound the nature of relationship between these two groups of parasites.

7.4. Parasite Factors. Intensity and species of helminths and *Plasmodium* were not similar among studies examining malaria and helminth interactions. Although different helminth species affect the immune system in similar manner, variation may exist in their degree of potency [3]. For example, *S. mansoni* is known for its strong influence on the immune system [3]. In addition, some helminth species such as hookworm destroy RBCs to a level where *Plasmodium* cannot replicate [44]. Intensity of helminth infection is also important in the nature of association [1].

Similarly, the types of *Plasmodium* species could also be indispensable in evaluating the nature of relationships between helminths and malaria. *P. falciparum* and *P. vivax* vary in their pre-erythrocyte immunity profile [65], suggesting differences in their degree of association with helminths. Indeed, a study was able to confirm association between different intestinal helminth species and *P. falciparum* malaria but failed to confirm the association among individuals infected with *P. vivax* [17].

8. Conclusions and Recommendations

Studies have shown that helminth infection may affect the epidemiology of malaria. Careful broad summarization of the existing evidence suggests that *S. mansoni* and hookworm infections may increase the risk of clinical malaria and associated morbidities, but *T. trichiura* infection is neither associated with the occurrence of clinical malaria nor associated with the morbidities. However, findings about association of *A. lumbricoides* and *S. haematobium* infection with clinical malaria are contradictory. Findings about the nature of relationship of helminth infection with severe malaria are also heterogeneous. It is indicated that most previous studies had limitations in methodology and design and there is a possibility that different socioeconomic and environmental conditions could confound the nature of interaction between helminth and malaria. Thus, well designed randomized controlled clinical trials involving periodic treatment with anthelmintic treatment from well-characterized populations are indispensable to make firm conclusion about the effect of helminth infection upon clinical or severe malaria and related morbidities. This will help to design effective disease management program. To make the conclusion more robust, studies should also focus on immunological analysis of the interaction considering different helminth species independently. Additionally, future studies should evaluate the effect of age, transmission intensity, and nutrition status on the nature of interaction between the two parasites.

Competing Interests

The authors declare that they have no competing interests.

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