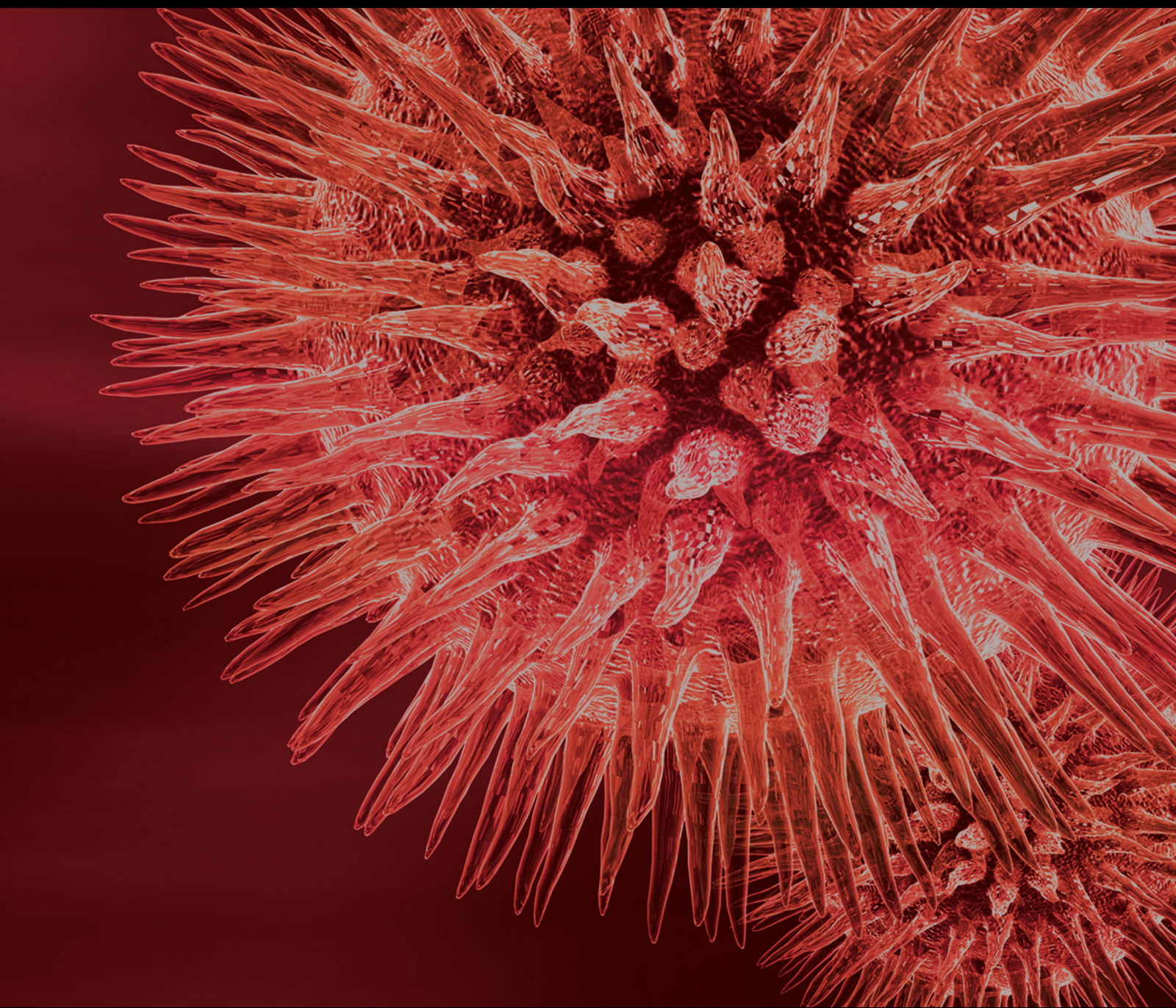
BioMed Research International

Advances in Emerging and Neglected Infectious Diseases 2018

Lead Guest Editor: Charles Spencer
Guest Editors: Jose R. Vasconcelos, Natalie J. Thornburg, and Sara L. Zimmer



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Editorial

Advances in Emerging and Neglected Infectious Diseases 2018

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Emerging and neglected infections are some of the most lethal diseases with mortality rates ranging from 10 to 80%. Despite high mortality for many of these infections, efforts at understanding them comprise an unusually small fraction of the biomedical research community's efforts. However, causative agents of emerging infections are the very ones for which an evolutionary, environmental, or nefarious alteration could allow accelerated spread amongst the human population. Furthermore, these diseases do not respect political boundaries. For example, the emerging tick-transmitted disease foci of the north central and northeastern United States of America have expanded to include parts of Canada, and recent Ebola outbreaks illustrate transmissibility across borders in the age of global economy. Thus, the international research community has a particular responsibility to direct adequate attention to studying these diseases and their etiologic agents.

Emerging and neglected infectious disease continues to be on the radar of the editors of this journal. In 2017, the journal introduced a special issue to illuminate research focused on understanding of and preparing for the emergence of these neglected diseases. The original special issue has been expanded into a continuing series. We present here the 2018 compilation of studies and a review, each addressing a specific topic within this special issue. These articles provide a snapshot of a much broader landscape of the questions and concerns of emerging and neglected infectious disease. Manuscripts published in this special issue address topics in bacterial and parasitic infections ranging from novel treatments and diagnostics to epidemiology and susceptibility. Once again, their authors span the globe, again highlighting the universality of infectious disease.

M. G. R. Muniz et al. from the United States of America demonstrate that obesity results in excessive production of leptin from adipocytes which enhance the cytokine storm following infection with *Francisella tularensis*. This heightened inflammation was associated with increased death of obese mice. This study illuminates a previously unknown risk factor for the development and severity of tularemia.

D. Kosova-Maali et al., in collaboration between France and Mexico, identified variation in two genes of *Nocardia brasiliensis* that together provide a needed method to discriminate between *Nocardia* species. The inclusion of these two genes in phylogenetic analysis can be useful for identification of species type in infected patients and enhancing diagnosis and may even have identified a new species.

V. Tique et al., in collaboration between Colombia and Cuba, analyzed clinical and epidemiological features of undifferentiated tropical febrile illness later identified as leptospirosis with the goal of refining the diagnostic features used in Colombia to identify *Leptospira* infection.

R. Holla et al. from India similarly analyzed clinical and epidemiological features of leptospirosis in coastal southern India. Accumulation of studies of the clinical and epidemiological of leptospirosis in various regions can, collectively, contribute to a better diagnosis for leptospirosis as well as reveal potential unique symptomology due to differences in the predominant strains and their microbiology.

S. C. V. Chaparro et al. from Colombia sought to improve upon the antimicrobial activity of a peptide derived from

lactoferricin B. Using a polyvalent antimicrobial peptide, they demonstrate enhanced antimicrobial activity in gram-negative and gram-positive bacteria, paving the way for development of potential new therapeutics.

Y. Qi et al. from China describe a new method for the diagnosis of *Coxiella burnetti* infection resulting in Q fever. This recombinase polymerase amplification combined with lateral flow was consistent with quantitative PCR-mediated analysis but required less instrumentation. This new visual-based method is rapid and could be very beneficial in resource-limited regions.

P. M. M. Fialho et al. from Brazil present findings after following the seroconversion of *Toxocara* negative children. They identified a strong association between seroconversion and the presence of asthma. This study contributes to understanding the risk factors for seroconversion for *Toxocara* and could expand on the underlying biology of *Toxocara* disease biology.

K. Bi et al. from the United States of America present a literature review of visceral leishmaniosis prevention control strategies. They identified a lack of validation and verification between models and real-work epidemic data. Furthermore, they call for the development of more active control strategies and the advanced simulation models to predict potential pandemics.

In summary, this special issue and the work presented herein highlight the continued demand for increased understanding of infectious diseases that have received arguably less than their needed share of attention. The included studies also underscore that a lot of fundamental qualities of these diseases have yet to be addressed. For a variety of reasons, some infectious organisms and the diseases they cause will always be at the fringes of active research. It would be perilous to ignore them and this special issue is committed to throwing the spotlight on these problems in infectious disease.

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Review Article

Current Visceral Leishmaniasis Research: A Research Review to Inspire Future Study

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Visceral leishmaniasis (VL), one of the deadliest parasitic diseases in the world, causes more than 50,000 human deaths each year and afflicts millions of people throughout South America, East Africa, South Asia, and Mediterranean Region. In 2015 the World Health Organization classified VL as a neglected tropical disease (NTD), prompting concentrated study of the VL epidemic using mathematical and simulation models. This paper reviews literature related to prevalence and prevention control strategies. More than thirty current research works were reviewed and classified based on VL epidemic study methods, including modeling approaches, control strategies, and simulation techniques since 2013. A summarization of these technical methods, major findings, and contributions from existing works revealed that VL epidemic research efforts must improve in the areas of validating and verifying VL mathematical models with real-world epidemic data. In addition, more dynamic disease control strategies must be explored and advanced simulation techniques must be used to predict VL pandemics.

1. Introduction

Visceral leishmaniasis (VL), or kala-azar, is a protozoan disease that, second only to malaria in numbers of fatalities, afflicts millions of people worldwide [1]. VL is primarily distributed in East Africa, South Asia, South America, and Mediterranean Region, with an estimated 50,000 to 90,000 new VL cases each year. Ninety percent of reported VL cases occur in Brazil, Ethiopia, India, Kenya, Somalia, South Sudan, and Sudan [2]. In 2015 the World Health Organization (WHO) classified VL as a neglected tropical disease (NTD) due to relatively minimal granted attention from the public, resulting in high mortality rates (more than 20,000 in 2015), and endemic spreading in poverty-stricken regions around the world [3, 4].

VL is one of the most widespread human diseases, with more than 20 *Leishmania* species identified worldwide [5]. Unlike other common forms of leishmaniasis, such as cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (ML), and post-kala-azar dermal leishmaniasis (PKDL), VL symptoms usually occur internally [1], meaning VL is more difficult to detect and cure than other leishmaniasis. Based on different kinds of susceptible species, VL can be classified as anthroponotic visceral leishmaniasis (AVL) or zoonotic visceral leishmaniasis (ZVL). AVL, which is transmitted between humans via vector carriers, is primarily caused by *L. donovani* throughout East Africa and the Middle East, especially Sudan, Somalia, Yemen, and Saudi Arabia [3]. Since most VL vaccinations for humans are still in research or minimally effective [6], AVL preventions include only early detection and treatment or use of long-term insecticide nets. ZVL, however, is transmitted between humans and other mammals, such as dogs. *L. donovani, L. infantum*, and *L. archibaldi* can result in ZVL, with specific concentrations in East Africa, South America, the Mediterranean basin, and South Asia [6]. Because dogs are the most common mammal carriers of ZVL, ZVL control strategies such as culling dogs, dog vaccinations, and use of insecticide collars are more prevalent than any AVL control strategies [7–10].

The use of mathematical models to describe and predict epidemic transmissions has become a recent trend in disease research area. Mathematical models intuitively exhibit complex VL transmission processes, and they measure variables and system parameters to reveal VL spreading dynamics and

related dominating factors. Rapid advancements in computer technologies have resulted in computer-aided simulation that helps mathematical models directly predict future VL prevalence. Using results from model analysis, parametric estimation, and simulation experiments, researchers can study and anticipate disease transmission dynamics and identify disease control strategies to fight a VL pandemic. Consequently, an increasing number of studies have focused on mathematical modeling and corresponding analysis for VL disease dynamics. Approaches used in these studies can be generally categorized as system dynamic models, including ordinary differential equation (ODE) or partial differential equation (PDE) models, as well as statistic models, or machine learning models. The main contributions and results are concentrated in precise prediction tested by validation, determining the key parameters by sensitive analysis and analyzing the bifurcation point of the disease reproduction number.

A well-defined mathematical model can be used to develop disease control strategies that are ascertained by solving the mathematical model or using numerical experiments. Numerical control strategies are robust and reliable approaches because potential bias from empirical data is not included. Conversely, implementation of real-life control strategies can be cost prohibitive, irreversible, and difficult to apply in a large-scale format, especially for developing countries that lack public health resources. However, computer-aided simulations that compare possible control strategies derived from a mathematical model can be carried out, and they are relatively inexpensive and can be performed repeatedly to examine system sensitivity and determine optimal control parameters. Almost half of corresponding research used mathematical modeling approaches to study potential disease control strategies, including dog culling, use of insecticidal dog collars, vector controls, and insecticide spraying strategies. Using optimal control, parametric analysis, or stochastic control methods, research results provided well-developed guidelines for disease control centers to prevent or mitigate a VL pandemic.

The rest of this paper is organized into comprehensive sections. Section 2 introduces current worldwide VL pandemic situations, and Section 3 presents VL control strategies in existing literature and recent breakthroughs in this field. Section 4 reviews papers on VL mathematical modeling and summarizes new developments and significant contributions since 2013. Section 5 reviews papers on control strategies and the use of numerical simulation, and Section 6 concludes the paper and suggests possible areas for future VL pandemic research. The research tree of this paper is shown in Figure 1.

2. VL Pandemic Recent History

Pigott et al. collected and summarized prevalence data reports of CL and VL from 1960 to 2012 [16]. According to their summarized database, the worldwide VL pandemic has affected at least 55 countries and territories (Figure 2), and another 45 countries have reported unspecified and borderline VL cases. Affected countries are located primarily in Latin America, the Mediterranean basin, Northeastern Africa, and Asia. Moreover, VL outbreaks historically occur most often in developing or agricultural countries because citizens are more likely to be in contact with disease transmission vectors such as dogs and mosquitoes [17]. Worse still, VL traps many families in these developing countries into vicious circle. Families affected by VL have to spend more direct cost (like treatment) and indirect cost (like loss of household income) during the VL epidemic.

According to the WHO neglected tropical diseases (NTD) report in 2007, VL is identified as one of the NTDs [18]. The primary reason WHO classified VL as an NTD is that confirmed VL cases have decreased worldwide from approximately 60,000 to around 20,000 since around 2010 [11], as shown in Figure 3. However, thousands of VL cases may not be covered in the WHO VL estimation report [19] since some countries without public health information systems do not submit their infection report data to WHO (e.g., Chad, the Central African Republic, and the Democratic Republic of the Congo). Even though for the countries with completed public health information system, the reported epidemiological data could be incomplete, and official figures are likely to underestimate grossly [8]. Based on the estimation, there are 500,000 new cases of VL and more than 50,000 deaths from the VL each year [17]. In 2012, another research group from the WHO Leishmaniasis Control Team corrected the number of VL estimation cases into 0.2-0.4 million, and the number of VL deaths into 20,000-40,000 [20]. Therefore, VL continues to be a serious and present threat to public health worldwide.

By observing the VL epidemiological situations for each country in Figure 4 based on the report from WHO [11], the significant drop in reported VL cases can primarily be attributed to a significant decrease in reported VL cases in India and Bangladesh. Between 2006 and 2016 the number of reported VL cases in India decreased from 39,173 to 6,249 and reported cases in Bangladesh declined from 9,379 to 255. The major reason of this decreasing is the wide utilization of insecticide-treated nets [43, 44]. However, VL prevalence did not change significantly for other countries. For example, the number of annually reported VL cases in Brazil were approximately 2,700 throughout the 10-year reporting period.

Conversely, reported VL cases in several African countries have shown significant increase since 2006, as shown in Figure 5. For example, Somalia reported less than 100 cases in 2006 and 781 cases in 2016. Although the population of Somalia (14.32 million) is much less than the population of India (13.24 billion), the proportions of VL cases reported in these two countries were similar in 2016. If no immediate measures are taken in these selected African countries, large-scale VL outbreaks are imminent.

Therefore, VL is still a serious disease which threatens people lives and health especially in the developing countries. To strain the transmission of VL, WHO and health organizations in VL afflicted countries should apply effective prevention and control strategies. In the next section, this paper will review several existing strategies against VL.

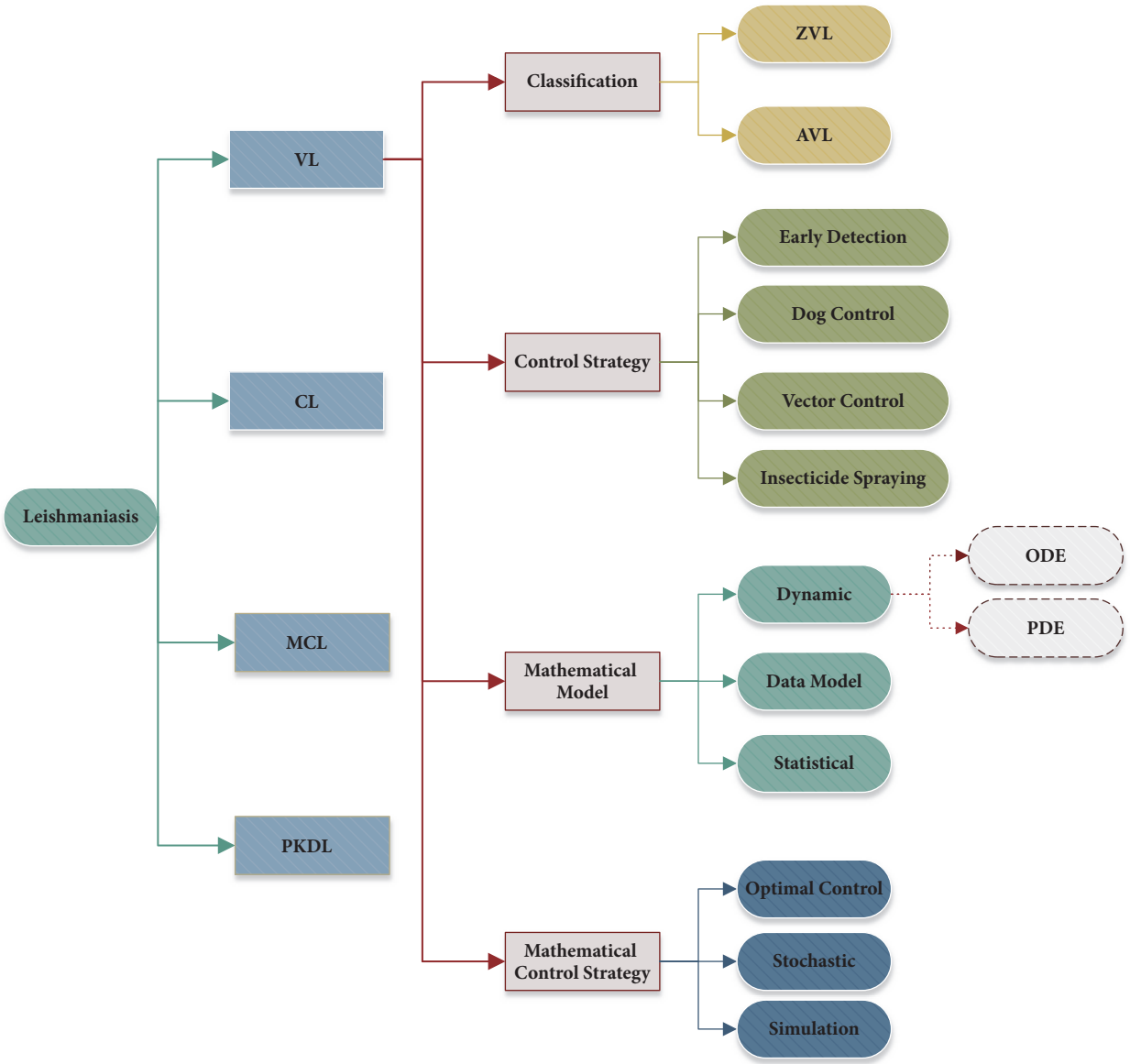

Figure 1: Research tree for this paper.

3. VL Mitigation and Prevention Methods

Since 1995, researchers have focused on ZVL when investigating the intervention and prevention of VL transmission because many ZVL control strategies are related to animals. Tesh categorized former ZVL control strategies into three main classes: early detection of human cases, destruction or treatment of infected dogs, and vector control [10]. Tesh's paper mentioned that treating infected people did not affect the parasite transmission and the drug resistance of *L. infantum* made the treatment even more difficult. For the dog control, many serologically positive dogs are hard-detectible. And the VL infected dogs need expensive and continual treatments. Tesh's paper also proposed that preventing the disease in dogs population is the best control strategies, since it can interrupt the transmission cycle of ZVL. In 2002, Guerin et al. asserted that principal interventions of VL via early diagnosis and treatments, dog controls, and vector controls are effective control strategies [8]. However, Guerin et al. also pointed out that vector controls such as residual-insecticide house spraying are cost prohibitive and rarely used. Especially in India, *Phlebotomus argentipes* (the dominated species of sand fly) is becoming resistant to the insecticide. The authors also mentioned some special challenges in VL endemic areas: lack of financial support (India and Bangladesh), remote places (Brail), and civil war (Sudan). In 2006, Dantas-Torres et al. introduced the Brazilian Leishmaniasis Control Program (BLCP), which includes diagnosis and early treatment of human cases, immunological screening of seropositive dogs, and insecticide spraying [7]. Dantas-Torres et al. indicated that the culling of seropositive dogs has limited effects, as proven by experiments and mathematical models. Authors

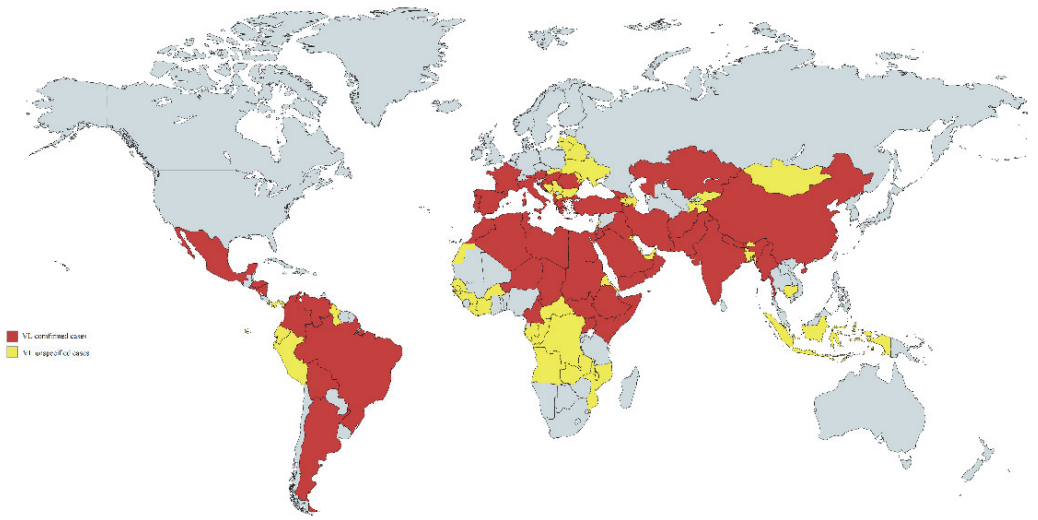

Figure 2: Distributions of confirmed and borderline VL cases from 1960 to 2012.

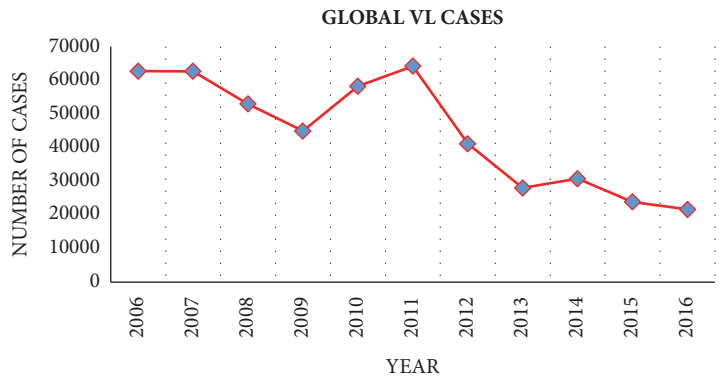

Figure 3: Reported VL cases from 2006 to 2016 [11].

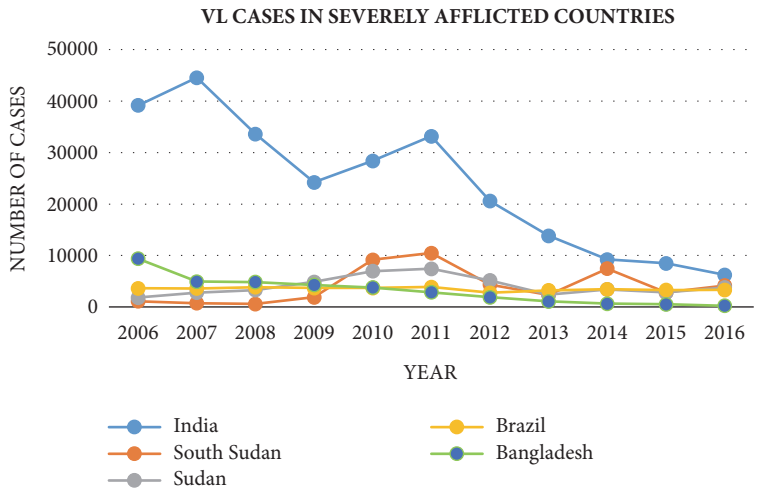

Figure 4: Reported VL cases in severely afflicted countries from 2006 to 2016 [11].

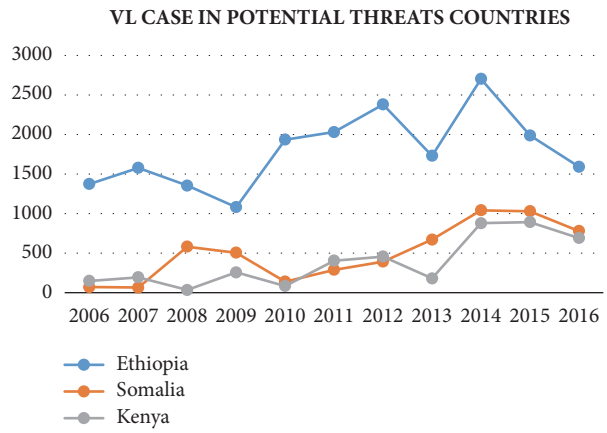

Figure 5: Reported VL cases in vulnerable countries from 2006 to 2016 [11].

summarized several key points of unsuccessful culling dogs: the limitation of the immunological screenings to detect anti-*Leishmania* antibodies, the opposition of dog owners to the culling of asymptomatic dogs, and the lack of evidence that it is an effective intervention strategy. A paper by Quinnell et al. suggested that tropical insecticides-collars and pour-ons can be used to reduce VL incidences for dogs by more than 83% [9]. However, like the insecticide spraying, the cost of this strategy limits the feasibility of the tropical insecticides-collars. The authors offered an alternative method, insecticidal bath, which can protect dogs for at least 3.5 months against *Phlebotomus chinensis*.

In 2014, Werneck considered the effectiveness of control strategies on the basic reproduction number R_0 [22]. The author found that vector controls (e.g., controlling vector density, the ratio of female vector, and the extrinsic incubation period of *L. infantum* in sand flies) and dog controls (e.g., culling infected dogs, dog vaccinations, and insecticide-releasing dog collars) can cause the disease reproduction rate R_0 to decrease to less than 1, meaning that the number of infected individuals will eventually decrease to zero. However, the author did not find the relevant data to support these control strategies. He pointed out some potential implementation difficulties for these strategies, such as costs issues associated with the continual uses of tropical insecticides-collars. Werneck also voiced concern that the effectiveness of using insecticide collars in the large community (like Brazil) may not work so well, since the insecticide collars strategy has the relatively short-term effect and consequent need.

Due the high cost of indoor residual spraying, insecticide-treated nets (ITNs) were introduced as an alternative control strategy for ZVL [21]. Experimental trials in sub-Saharan Africa, Latin American, Thailand, Pakistan, and Iran show that ITNs could reduce infections with malaria by 17%–62%. In 2015, Picado et al. summarized the results of the KALANET project to analyze ITN effectiveness. The KALANET project is a cluster-randomized controlled trial in India and Nepal [23]. The KALANET project was conducted in 26 high-incidence regions (in India and Nepal) with more than 20,000 inhabitants observed over 24 months. Results showed that use of long-lasting insecticidal nets resulted in a 50% reduction of *L. donovani* infections. However, this study also showed that some of those nets were untreated, many were damaged, and most families did not have enough nets to protect all family members. The authors suggested standardizing the color and size of insecticide nets; they also want the government to replace the untreated and damaged nets by new treated nets for free for publics. Although the long-lasting insecticidal nets can prevent VL transmissions while people are sleeping, recent entomological findings in India indicated that vectors are more exophilic than previously thought, meaning that people engaged in outdoor work have more opportunities to become infected [45].

In 2016, Özbel et al. analyzed the geographical distribution, ecological aspects, and species habits of VL vectors (sand flies) in Bangladesh [24]. In general, two genera of sand fly (*Phlebotomus* and *Sergentomyia*) and a total of 13 species were recorded in Bangladesh, among which *Phlebotomus argentipes* is the dominant vector species in VL endemic areas of Bangladesh (>94%). Researchers also found that the *Phlebotomus. argentipes* population peaks around monsoon season and reaches the lowest ebb during winter and summer in Bangladesh. They also determined that eight ecological parameters (soil temperature and moisture, rainfall, air temperature, relative humidity, soil pH, soil organic carbon, and wind speed) can influence the *Phlebotomus. argentipes* population. Future research must enact control strategies based on their ecological aspect. The potential application of this research can provide early warning of the incoming VL epidemic and narrow the range of the insecticide spraying.

As the most effective control strategy for infectious diseases, the successful vaccination on VL is long-awaited for the VL afflicted countries. The experiment on VL vaccination was started in 1990s; researchers tried to utilize the proteins from *L. donovani* to develop vaccines [46]. At the beginning of the 21st century, researchers investigated the possibility of vaccination based on DNA and genetically attenuated parasites [47, 48]. More recently, Duthie et al. studied several different vaccine antigens for VL transmissions using recombinant proteins from *E. coli* [25]. Their research results have shown that several potential vaccine antigen candidates are verified in different platforms. The authors consolidated seven vaccine candidates as recombinant proteins in *E. coli*, and they validated the effectiveness of *E. coli* to *L. donovani* via experiments on mice. However, their research pertained to only nonhuman experiments. Significant research and advancements are still needed to obtain effective vaccination for humans against VL parasites.

For now, even though many contributions have been done for the VL controls and prevention, an effective, feasible, and economical control strategy is still an ongoing effort. The current VL control strategies and corresponding deficiencies are summarized in Table 1. Since the most of VL severely afflicted countries are developing countries, how to balance the effectiveness and costs involved in such VL control plan is delicate tradeoff. This paper will discuss more about this particular issue in Section 5.

Table 1: Current VL control strategies and corresponding deficiencies.

Strategy	Category	Deficiency	Reference
Early detection	Human control	Doesn't affect the parasite transmission	[10]
Culling dogs	Dog control	Opposition of dog owners & Hard to detect infected dogs	[7, 10]
Dog treatments	Dog control	Expensive	[9]
Canine vaccination	Dog control	Expensive & Drug resistant	[9]
Spraying insecticide	Vector control	Expensive & Drug resistant	[8, 21]
Immunological screening of seropositive dogs	Dog control	Expensive & Needs high level technique support	[9, 10]
Insecticide collars	Dog control	Expensive & May not work in large community	[22]
Insecticide-treated nets	Vector control	Damaged and untreated nets have low effectiveness	[23]
Ecological control	Vector control	Needs more time to be applied in the real world	[24]
Vaccination control	Human control	Not available at the current time	[25]

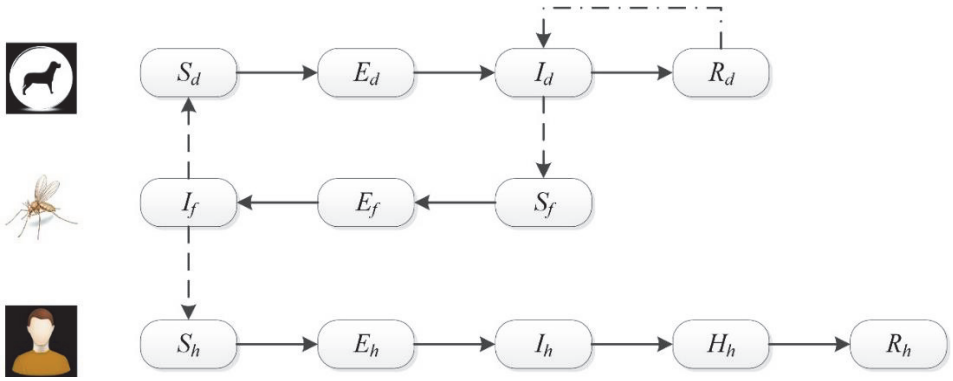

Figure 6: System diagram of ZVL transmission model [12], where d, f, h represent the dog, sandflies, and human species and S, E, I, R, H represent the susceptive, exposed, infected, recovered, and hospitalized population for each species.

4. Mathematical Epidemiology Models for VL

4.1. System Dynamic Model. In 1996 Dye first introduced a 4-equation ODE susceptible-latent-infectious-removed (SLIR) model to describe the VL epidemic [49]. SLIR, respectively, represents the susceptible, latent, infectious, and resistant populations of VL, and the model considered the transitions between these populations. However, the author did not consider the behaviors of dog and vector in his model. Courtenay et al. improved Dye's model by considering the dog population as a key parameter in the SLIR model [50]. Variations of this parameter directly influence the human infection rate. Ribas et al. built an ODE model to describe VL transmissions among humans, dogs, and vectors [14]. The model utilized 11 ODE equations, including the susceptible (dog, sand fly, and human), latent (dog, sand fly, and human), infectious (dog, sand fly, and human), and recovered (dog and human) populations. Although their model was presented without data, simulation, and analysis, it was the first model to describe behaviors for all species involved in VL.

Since WHO's designation of VL as an NTD in 2015 [51], an abundance of research and studies have focused on developing mathematical models of VL. In 2016 Zhao et al. developed a 12-equation ODE model to comprehensively describe a VL epidemic (Figure 6) [12]. They improved the model by including a hospitalized population into the ODE system; this population has a higher probability of survival than infections without hospitalization due to the systematic treatment. The authors utilized a backward bifurcation method to analyze VL equilibrium behavior and the basic reproduction rate R_0. Their research showed that VL equilibrium is highly related to a critical model parameter, R_c, as the epidemic threshold value for R_0. Similarly, Subramanian et al. proposed a compartment-based ODE model of VL transmission to explain disease transmissions in symptomatic VL, asymptomatic VL, and PKDL-infection classes [26]. Sensitivity analysis of model parameters found that the biting and birthing rates of sandflies and the recovery rate of symptomatic humans are dominating factors for VL epidemic control.

Biswas simplified the 12-equation ODE model from Zhao et al. to an 8-equation ODE model by dividing the nonhuman populations (dog and vector) into susceptible and infected population groups [27]. With a simplified ODE model, researchers reduced the complexity of system sensitivity analysis and reduced the numbers of assumed or estimated model parameters. This model also successfully reproduced the 2011 VL epidemic in South Sudan. Shimozako et al. transferred the ODE model in Ribas et al. by considering the dog population as the only source of infection since vectors could not transfer ZVL without dogs [28]. Therefore, their mathematical model contained eight variables corresponding to the susceptible, latent, infectious, and recovery populations for dogs and humans. Le Rutte et al. compared three ODE models and

corresponding simulation results while considering indoor residual spraying [29]. The primary difference between the three models was how relationships between PKDL and the recovery population were modeled. Their research predicted that, using 60%–80% IRS coverage, VL epidemics could be eliminated within three years in Bihar, India. In addition, other researchers have made incremental contributions using various ODE VL epidemic models [30–33].

Although many ODE models describe VL epidemics and transmission, the development of a novel dynamic model is an active area of research in the investigation of complicated transmission behaviors of VL under various situations and the development of improved mitigation and control strategies. Bi et al. introduced a two-dimensional PDE model based on an existing ODE model [13]. The model presented in their research considered human age structures and time as two dimensions since historical data have strongly suggested that VL infection rates are highly correlated to human age groups. For example, children and teenagers (aged from 0 to 20) are more likely to become infected compared to other age groups. This research used computer simulation and mathematical analysis to explain this phenomenon and recover the historical VL endemic data published by WHO.

4.2. Models Based on Real-World Data. VL attracted significant epidemiology research attention; abundant statistical data were collected and reported on the current VL pandemic worldwide by scholars and researchers. Many researchers realized the importance of data utilization in VL model development. Disease data is generally utilized in three ways: use of reported data to build statistical models, use of historical data to predict future prevalence, and use of existing data to calibrate model parameters in mathematical epidemic models.

The primary objective of building a VL statistical model is to statistically identify key parameters in the VL transmission process and determine relationships between the number of parameters and the number of infected population. Werneck et al. used consolidated census tracts to analyze VL disease prevalence data from different regions of Brazil [52]. The authors developed a spherical covariance structure model based on census data from 1992 to 1996 in Teresina, Brazil. By exploring spatial correlation structures of the census data, they found a positive correlation between reported VL incidences and residence in areas of green vegetation, especially in favela. In 2007 Werneck *et al.* extended their previous work by analyzing and comparing the results from 21 statistical models [53]. According to human and canine VL data in Teresina, Brazil, the study found significant correlations between residence in areas with green vegetation and infected dogs and between the human infection rate and urbanization index or socioeconomic status index.

Thompson et al. studied relationships between climate and VL epidemics by establishing the statistical regression model [54]. Their research found that rainfall is the most significant parameter statistically correlated to VL incidences. The influence of geographical features of areas of residence (e.g., cities, plains, or foothills) on VL transmission was also considered. The foothill population statistically revealed a higher risk of VL infection than other populations. De Araújo et al. considered the statistical model with data and found that spatial data is more reliable and accurate for VL epidemic study and analysis [34]. Therefore, they applied spatial statistical modeling and the Bayesian approach to model and estimate risks of VL using historical data from Belo Horizonte, Brail. Their research showed that the relative risk of VL is correlated to income, education, and the ratio of infected dogs to inhabitants in Belo Horizonte. However, as opposed to their previous research findings, residence in areas with green vegetation did not show significant correlations to the risk of VL.

Ecological niche modeling (ENM), stemming from the genetic algorithm [55], has been widely used to predict VL prevalence since 2006. Nieto et al. first used ENM to analyze VL data from northeastern Brazil [56]. Using the geographic information system (GIS), the ENM model can predict prevalence risks in three levels (high, moderate, and low). When being validated with historical data from Bahia, Brazil, the predictive model demonstrated high accuracy (more than 90%) on high-level and moderate-level data. Similar approaches were used to predict VL prevalence in North America and Middle Eastern regions [57, 58]. Several other methods were utilized to predict the trend of VL epidemics with various factors. Elnaiem et al. summarized data from eastern Sudan in GIS and then used that data to build predictive models of VL infections based on rainfall and corresponding distance to a river [59]. Oshaghi et al. built a predictive degree-day model for VL using the single triangulation method [60]. This model predicted temporal and spatial distribution of VL infection density and generations of sandflies. Karagiannis-Voules et al. employed Bayesian geostatistical models to fit the VL incidence data from Brazil, and they identified environmental and socioeconomic predictors using Bayesian variables [15]. Their research results predicted that regions with humid climates and dense vegetation distributions are more vulnerable to VL than other regions.

Parameter estimation is another essential application when validating VL mathematical models using real-world data since the use of assumed system parameters in the model may reduce model reliability. Bi et al. summarized age structures of VL infections in various regions [13]. They revised the constant infection rate into age-dependent distribution of infection rates by studying historical VL prevalence data from existing literature from diverse regions over time (Figure 7). Use of this age-dependent distribution function allowed their PDE epidemiology model to reflect VL human prevalence in both age and time periods. Biswas et al. established the posterior distribution of different parameters and initial parameters based on observation data [27]. Use of parameter distribution allowed the simulation experiments to reflect more than one result with unique possibilities. Even though most mathematical models still use assumptions or estimations with existing literature as their system parameters [12, 26, 28, 29], an increasing number of studies are utilizing real-world data to more accurately estimate system parameters and validate their models.

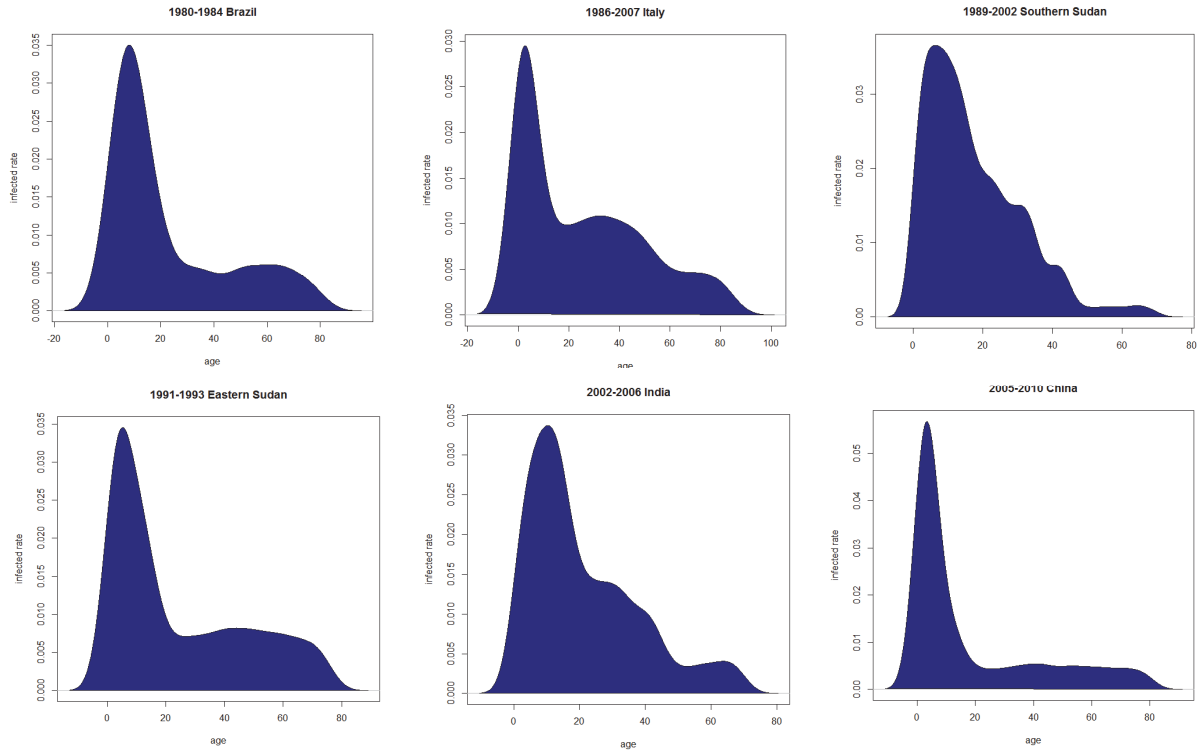

Figure 7: Infection rate distribution based on human age in various countries [13].

5. Optimal Control Strategies Using VL Models

5.1. Parameter Control Strategy. The most generalized control strategy in a VL mathematical model is the parameter control strategy, which assumes that the key parameters in the model are adjustable. When the parameters are adjusted, the model outputs become dependent variables; therefore, the parameter adjustment process can be considered a corresponding real-world control strategy. In 2002 Courtenay et al. introduced the numerical control strategy to the field of VL mathematical modeling [50]. They assumed that the parameter of dog density could be controlled by culling dogs in specific areas, and their research findings showed that dog culling could effectively reduce the proportion of infectious population. Ribas et al. improved the parameter control method by considering additional control parameters [14], including the dog treatment rate, the insecticide collar utilization rate, the dog culling rate (natural mortality rate of dogs), the dog vaccination rate, and the mortality rate of sandflies (vector control). Using the control parameter method, they used simulation experiments to compare the efficiencies of each control strategy, as shown in Figure 9, where vector control proved to be more effective than control strategies such as culling and vaccine. Shimozako et al. incorporated control strategies such as dog treatment, dog vaccination, and use of insecticidal dog collars into an ODE mathematical model of VL [37]. However, they increased the dimension of the model by using control variables to replace the control parameters in their model. A cost-effect analysis and simulation experiments showed that use of insecticidal dog collars should be the most utilized control strategy.

5.2. Optimal Control Strategy. Lev Pontryagin established the optimal control strategy in the 1950s [61, 62]. This strategy provides optimality criterion by maximizing or minimizing a given objective function subject to constraints defined in the differential equation mathematical model. Zhao et al. introduced optimal control into their 12-equation ODE mathematical model of VL [12], including the susceptible, latent, and infectious populations for sandflies, humans, and dogs; recovered human and dog populations; and hospitalized human population. Their study included three control strategies in the model: the control dog prevention level (vaccine protection or dog culling), the control insecticide usage level (insecticide sprayed around buildings), and the control personal protection level (long-lasting insecticide). Consideration of exposed human, infected human, and sandfly populations as well as control cost as the control objective function caused the final control strategy to effectively lower the negative influence of VL by approximately 80% (Figure 9). Agusto et al. considered the use of fabrics and insecticidal animal collars as an additional control [33]. Their research was innovative because they used various combinations of controls instead of utilizing control strategies identical to previous research. Their simulated experimental results showed that use of an ODE mathematical model allowed their control strategies to effectively reduce VL and PKDL infected human populations. Biswas et al. revised a previous

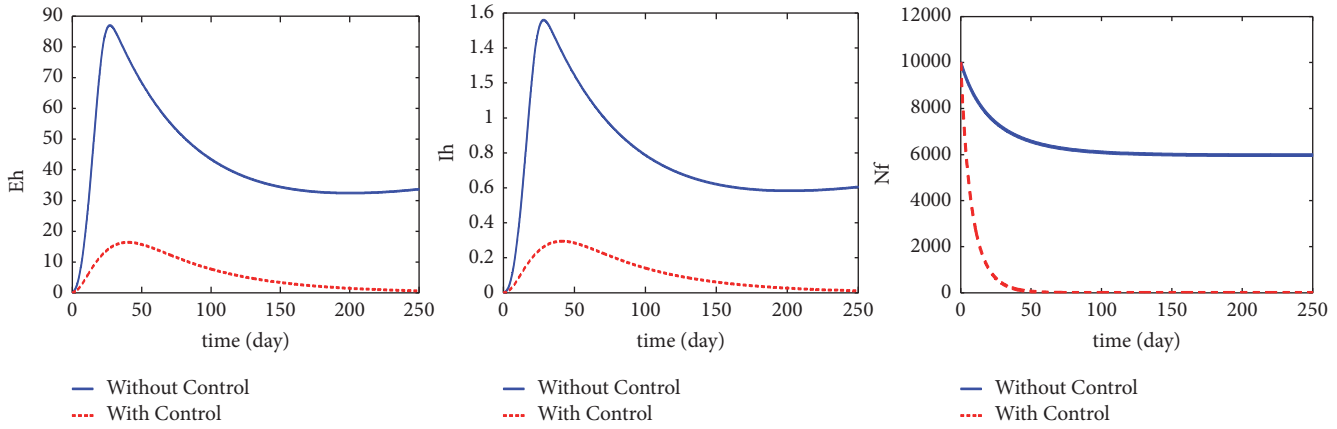

Figure 8: Simulation of dog culling [12].

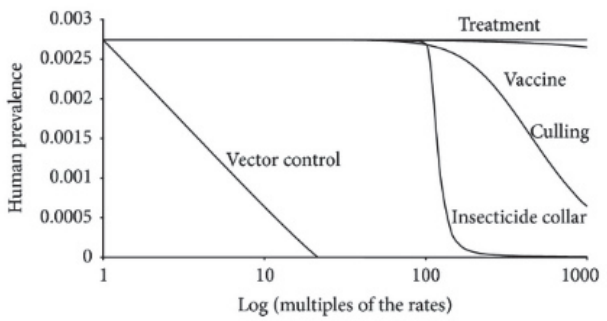

Figure 9: Efficacy comparison of control strategies [14].

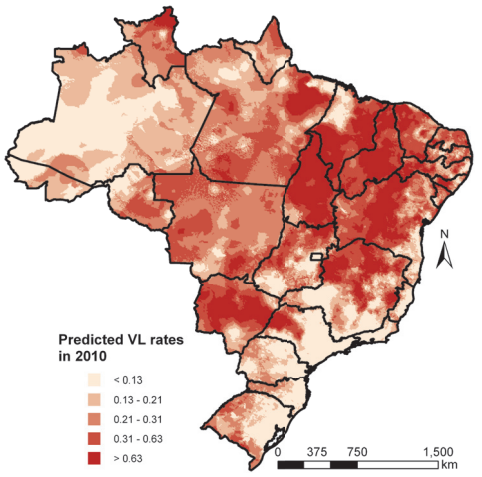

Figure 10: Spatial simulation of predicted VL rates in 2010, Brazil [15].

optimal control by suggesting a strategies mechanism and incorporating various combinations of optimal strategies into the ODE mathematical model with a given objective function [36]. This objective function was a linear combination of VL and PKDL infections with the cost of control strategies. They calculated the corresponding infection averted ratios (IAR) and incremental cost-effectiveness ratios (ICER) for each control strategy, where IAR is the ratio of the number of infections averted to the number of recoveries and ICER is the additional cost per additional health outcome. The researchers then selected the strategy with the highest IAR and lowest ICER as the optimal strategy. An optimal control strategy specifically targeting the human VL vaccination was also analyzed, but no evidence revealed the effectiveness of a human VL vaccination [27].

5.3. Control Strategy Selection Using Simulation. Simulation comparison is the most common method of VL mathematic control modeling in simulation. Figure 8 compares the human infected population with control (dog culling, sandfly control, and human protection) and without control [12]. The solid line in the figure shows epidemic performance (which includes the exposed human E_h, the infected human I_h, and the total population of sand flies N_f) of the control strategy, which reduced disease prevalence to 80%. This simulation proved the effectiveness of the combined control strategies.

Efficacy comparison between control strategies is another type of simulation comparison. Ribas et al. compared the human prevalence influence using vector control, insecticidal collars, dog culling, dog vaccines, and dog treatment [14], as shown in Figure 9. When the parameter was changed to manipulate control levels, the human prevalence by vector control decreased most rapidly, proving that human prevalence is the most sensitive to vector controls. Therefore, vector control is the most effective control strategy.

Spatial simulation is a simulation estimation method that provides spatial information throughout the model behaviors. Using GIS, spatial simulation can exhibit VL prevalence information from various regions, as shown in Figure 10. Karagiannis-Voules et al. utilized historical data (2001–2009) from Brazil to build a statistical model [15]. Their simulation used GIS to predict VL prevalence in Brazil in 2010, thereby directly reflecting high infection density in the eastern region of Brazil.

Table 2: Recent papers on mathematical modeling of VL.

Paper	Published Year	Real Data Involved	Control Strategies	Transmission Models	Simulation
Ribas et al. [14]	2013	No	Yes	Yes	No
Zhao et al. [12]	2016	No	Yes	Yes	Yes
Subramanianet al. [26]	2015	Testing model	No	Yes	Yes
Biswas et al. [27]	2017	Parameter estimated	Yes	Yes	Yes
Shimozako et al. [28]	2017	Testing model	No	Yes	Yes
Le Rutte et al. [29]	2017	Testing model	No	Yes	Yes
Costaet al. [30]	2013	No	No	Yes	No
Sevá et al. [31]	2016	No	No	Yes	No
Zouet al. [32]	2017	No	No	Yes	Yes
Agustoet al. [33]	2017	No	Yes	Yes	Yes
Bi et al. [13]	Not yet	Parameter estimated	No	Yes	Yes
de Araújo et al. [34]	2013	Yes	No	No	No
Karagiannis-Voules et al. [15]	2013	Yes	No	No	Yes
Miller et al. [35]	2014	Yes	Yes	Yes	No
Biswas et al. [36]	2017	Testing model	Yes	Yes	Yes
Shimozako et al. [37]	2017	Testing model	Yes	Yes	Yes
Stauch et al. [38]	2014	Testing model	No	Yes	Yes
Zamir et al. [39]	2017	No	No	Yes	Yes
Boukhalfaet al. [40]	2017	No	No	Yes	Yes
Gorahavaet al. [41]	2015	No	Yes	Yes	No
Rock et al. [42]	2016	No	Yes	Yes	Yes

6. Discussion and Conclusion

Although globally reported, VL-confirmed cases have decreased since 2011; VL prevalence has not improved significantly worldwide except in South Asia (i.e., India and Bangladesh). However, VL outbreaks have increased in Ethiopia, Somalia, and Kenya since around 2008. Moreover, public health agencies in underdeveloped African countries such as Chad and the Central African Republic do not have resources and capabilities to collect and report VL incidences. However, because these countries are near regions severely afflicted with VL, such as Sudan and South Sudan, the number of global VL cases reported from WHO may be underestimated.

This paper reviewed current VL epidemiological research ranging from VL epidemic control strategies to VL mathematical models and related optimal control strategies. The research demonstrated how to use numerical methods such as modeling and sensitivity analysis, as well as equilibrium/stability studies and simulation experiments, to assist mitigation and prevention strategies for a worldwide VL pandemic. Governments and health organizations can utilize the modeling and simulation results to predict or estimate impacts of various control strategies.

Despite significant research efforts using mathematical models for the VL epidemic, research gaps still exist and many areas of study remain unexplored. Table 2 summarizes 21 literature works related to mathematical modeling and VL disease control strategies since 2013. Most of the reviewed research used or proposed system dynamic mathematical models or statistical models; approximately half of these research works considered real-world data and studied possible control strategies. Only one paper included real-world data, control strategies, mathematical models, and numerical simulation experiments.

Future work, thorough VL epidemic research using mathematical or statistical models, ought to consider the four following aspects:

(1) building more sophisticated mathematical models to explain underlying infectious disease transmission dynamics,

(2) including real-world data to aid model validation and verifications,

(3) exploring possible disease control/mitigation strategies to increase understanding of model maneuverability and robustness,

(4) using numerical simulation experiments as a predictive tool to verify the feasibility of model and control strategies.

Moreover, future work in these four aspects of VL mathematical modeling must utilize modern analytical tools. The disadvantage of current modeling is the limited diversity of model types. A majority of existing VL mathematical models are ODE models, which are widely used but produce limited predicted results without details. Therefore, more statistical, machine learning, and PDE models are needed to build sophisticated, comprehensive mathematical models of VL. Statistical and machine learning models can more advantageously utilize real-world data to ensure model prediction accuracy, while use of a PDE model can enrich predicted

results with age, gender, socioeconomic group, ethics, and spatial information. For the second aspect, the inclusion of real-world data, most test data currently used to validate and verify underlying mathematical models are estimated or assumed, consequently limiting the mathematical model to reflect only data from previous VL epidemic episodes. Future research efforts should utilize recent epidemic data with temporal and spatial data during the modeling phase, making the modeling process increasingly dynamic and reflecting real-time data while predicting possible trends of an ongoing epidemic. The current primary disadvantage of the third aspect, exploring possible control strategies, is that the control strategies lack of applicability in the real world. In fact, the most effective control strategies suggested by the mathematical models may not be operable or they may be too cost prohibitive to be implemented. Operable control strategies should be carefully quantized, such as specific consideration of the optimal level of canine culling in a particular time frame or the level of insecticide spraying in each area affected by VL. For the fourth aspect, current studies using numerical simulation experiments frequently provide insufficient information from simulation results. Most simulations of VL models can only predict the trend of VL infections. Future research should focus on spatial simulation and agent-based simulation as well as the study of the interactions between multiple regions or environments.

In conclusion, the use of mathematical models to study, analyze, and predict VL epidemics and to explore effective and implementable control strategies remains an active and study-worthy area of future research. However, research results from more comprehensive studies that use modern analytical tools will help public health organizations understand and prevent the VL disease.

Conflicts of Interest

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

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Research Article

Obesity Exacerbates the Cytokine Storm Elicited by *Francisella tularensis* Infection of Females and Is Associated with Increased Mortality

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Infection with *Francisella tularensis*, the causative agent of the human disease tularemia, results in the overproduction of inflammatory cytokines, termed the cytokine storm. Excess metabolic byproducts of obesity accumulate in obese individuals and activate the same inflammatory signaling pathways as *F. tularensis* infection. In addition, elevated levels of leptin in obese individuals also increase inflammation. Since leptin is produced by adipocytes, we hypothesized that increased fat of obese females may make them more susceptible to *F. tularensis* infection compared with lean individuals. Lean and obese female mice were infected with *F. tularensis* and the immunopathology and susceptibility monitored. Plasma and tissue cytokines were analyzed by multiplex ELISA and real-time RT-PCR, respectively. Obese mice were more sensitive to infection, developing a more intense cytokine storm, which was associated with increased death of obese mice compared with lean mice. This enhanced inflammatory response correlated with *in vitro* bacteria-infected macrophage cultures where addition of leptin led to increased production of inflammatory cytokines. We conclude that increased basal leptin expression in obese individuals causes a persistent low-level inflammatory response making them more susceptible to *F. tularensis* infection and heightening the generation of the immunopathological cytokine storm.

1. Introduction

Infection with the zoonotic pathogen *Francisella tularensis*, the causative agent of human tularemia, elicits a profound overproduction of inflammatory cytokines, culminating in a cytokine storm. Following macrophage uptake of the bacterium, *F. tularensis* escapes into the cytosol where it initiates inflammation [1, 2]. During lethal infection, excessive levels of proinflammatory cytokines, e.g., IFN-γ, TNF-α, and IL-6, are observed in the plasma indicative of a systemic sepsis-like response [3, 4]. This results in excessive immunopathology, including loosening of endothelial tight junctions, edema, hypovolemia, fever, and bradycardia. While low levels of inflammation are necessary to activate the immune response and clear pathogen infection, including *F. tularensis*, excessive inflammation causes this lethal immunopathology.

Excess caloric intake leads to the swelling of adipocytes and the activation of local adipose tissue leukocytes [5–9]. This activation results in increased production of proinflammatory cytokines and adipokines by macrophages and T cells in an attempt to control and remove excess fat and fat-swollen cells [10, 11]. Activation of adipose tissue macrophages results in IL-1β secretion, which in turn triggers production of inflammatory cytokines and adipokines [12–17]. Persistent hypertrophic stress on adipocytes causes continuous production of inflammatory and anti-inflammatory cytokines and adipokines [18–20] and a chronic low-level inflammation in obese individuals [21–24]. Furthermore, extra stores of adipocytes in females are thought to contribute to their increased basal inflammatory response. However, this inflammation is not limited to local adipose tissue as leptin, released into the blood plasma, can activate other immune cells [25]. Leptin receptor is found on T and B lymphocytes, as well as monocytes and macrophages, and stimulates proinflammatory functions of these distant immune cells [12, 13, 26–30].

While obesity increases the risk of several infectious diseases, limited or controversial data exists on its role in an individual's predisposition for bacteremia and sepsis or the severity of the cytokine storm [31, 32]. Since both obesity and *F. tularensis* activate the same inflammatory signaling pathways, we hypothesized an additive activation of inflammation elicited by *F. tularensis* infection of obese individuals. This increased inflammation would then result in an increased cytokine storm and, therefore, increased susceptibility to *F. tularensis*-mediated disease.

2. Materials and Methods

2.1. Ethics Statement. All animal procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee under protocol A-201208-1.

2.2. Animals. Age-matched female C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained on a 12h light/dark cycle with *ad libitum* access to food and water. Diet-induced obesity was caused by providing chow containing 60% kcal from fat for nine weeks (Envigo, Houston, TX). Female mice were selected for this study because of past experience in infectious disease and obesity studies. In a forthcoming publication, we identified a profound difference in the susceptibility of male and female mice to *F. tularensis* infection independent of obesity and obesity-related signaling. In addition, male and female mice deposit excess fat at different rates as well as have profound differences in adipokine production [33]. Furthermore, females have increased severity and mortality from sepsis compared to males [34, 35]. Therefore, in order to focus on the effects of obesity alone, female mice were chosen for this study as the sex more sensitive to sepsis-like disease.

2.3. Bacteria. Francisella tularensis subspp. *holarctica* LVS (Denise Monack, Stanford University) was grown on chocolate agar plates for 24-48 hours. Plates were scraped aseptically and the organisms harvested into sterile PBS with 20% glycerol and stored at -80°C. Concentrations of thawed aliquots were subsequently determined by serial dilution and used for all aliquots.

2.4. Animal Infection. For injection, bacterial stocks were diluted to the indicated concentration in sterile PBS. Mice anesthetized with 3-5% isoflurane inhalation were injected intradermally in the flank above the hind quarters with 50ul of bacteria diluted in PBS. Animals were monitored every 12 hours postinfection for the first 48 hours and every 8 hours thereafter. All animals were weighed before inoculation and every morning thereafter. An animal was considered terminal and humanely euthanized per AVMA standards when it had lost 20% of its baseline weight. In addition, animals were checked for clinical symptoms of disease and considered terminal when lethargic and immobile with prodding.

2.5. Blood Draw. Blood was drawn on days 0, 3, and 5 after infection from the retroorbital capillary sinus using heparinized capillary tubes and at the time of euthanasia (T) by cardiac puncture. As each animal was bled every other day, alternating eyes were used to prevent irritation and ocular damage. Whole blood was fractionated and plasma frozen until completion of the experiment for subsequent analyses.

2.6. Plasma Cytokine Analysis. Concentrations of cytokines and chemokines in the plasma of infected animals were determined by multiplex ELISA (MilliPlex, Millipore Sigma, St. Louis, MO) and analyzed on a Luminex MagPix (Austin, TX) following manufacturer's protocols. Analysis was completed on individual animals at each time point and analyzed by multiparametric t-test.

2.7. Real-Time RT-PCR. At the time of euthanasia, spleen, liver, and lung tissues were harvested, mechanically dissociated, and submerged in RNAlater. Subsequently, RNA was extracted using an RNEasy kit (Qiagen, Germantown, MD) and analyzed by real-time RT-PCR using the CYBRFast 1-step RT-qPCR kit (Tonbo Biosciences, San Diego, CA) and the StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA). ΔCt values were calculated by comparison with GAPDH expression levels and ΔΔCt values by comparison with the average Ct value from uninfected tissue and are reported as fold changes in expression.

2.8. In Vitro Inflammatory Assay. Immortalized C57BL/6 bone-marrow derived macrophages (BEI Resources, NIAID) were seeded at 7.5×10^5 per well in 96-well plates in the presence or absence of 2 ug/ml leptin (Millipore Sigma). Macrophages were chosen due to the tropism of the bacterium for these cells and the ability to culture them *in vitro*. While *F. tularensis* also infects neutrophils *in vivo*, the neutrophil lifespan (12-36 hours) is too short for our *in vitro* culture model which lasts ~60 hrs. In addition, leptin has been shown not to alter the production of inflammatory cytokines from neutrophils following purification [36]. After attachment overnight, macrophages were infected with a multiplicity of infection (MOI)=40 LVS bacteria for 2 hours followed by wash and addition of 20 ug/ml gentamicin containing medium to kill extracellular bacteria and prevent overgrowth of the wells. During all procedures, leptin was continually present in the medium at 2 ug/ml in appropriate wells. 48 hours after bacterial inoculation, the production of cytokines by macrophages in response to infection was determined by MilliPlex.

2.9. Statistical Analysis. Group weights were compared by Mann–Whitney U test, while survival curves of those groups were compared using Mantel-Cox test with 10-15 animals per group. Plasma cytokine levels were analyzed either by ANOVA with Tukey's posttest or multiparametric t-test for repeated sampling measures. Levels of *in vitro* inflammation were compared by Mann–Whitney U test. Significance was determined at the $p \leq 0.05$ level. Statistical analyses and graphs were generated using GraphPad Prism.

3. Results

Following 9 weeks of high fat chow, female mice were, on average, 10g heavier than control mice fed normal fat chow (Figure 1; $p<0.0001$ by Mann–Whitney U test). Little difference was detectable in the blood levels of 20 different cytokines involved in various immunological pathways prior to infection (Figure S1). Serum levels of IL-6, TNF-α, GM-CSF, MIP-3α, and IL-22 were slightly elevated, while IL-15 was strongly reduced (Table S1). However, the only statistically significant differences observed were decreases in the circulating levels of sCD40L and IL-21 in obese animals (Figure S2).

Lean and obese female mice were inoculated with $8x10^5$ cfu *F. tularensis* LVS (LD_{50} for female C57BL/6) and monitored for the following 14 days. While 50% of lean animals survived the infection, none of the obese animals survived (Figure 2(a); $p<0.05$ by Mantel-Cox). Daily weight measurements demonstrated that, as a group, obese mice lost 10% more body weight than lean mice (Figure 2(b); $p<0.02$ by multiple t-test). Decreasing the infectious dose of *F. tularensis* increases survival in wildtype lean mice. Similarly, administration of a $5x10^5$ cfu *F. tularensis* (LD_{25}) resulted in a 75% survival for lean mice, while 35% of obese mice survived (Figure 2(c); $p<0.05$ by Mantel-Cox). Both lean and obese mice became ill at this dose as shown by body weight loss monitoring with obese mice losing 7% more body weight then lean mice (Figure 2(d); $p<0.05$ by multiple t-test). Increased susceptibility of obese mice was independent of bacterial load, as splenic burden showed no differences in bacterial load (Figure 2(e); ns by Mann-Whitne).

F. tularensis infection results in a septic-like response marked by excessive production of inflammatory cytokines. Therefore, temporal analysis of 19 cytokines and chemokines involved in inflammation and the immune response was performed on individual mice. This demonstrated that obese mice infected with *F. tularensis* had significantly higher plasma levels of IL-6, IFN-γ, and TNF-α compared with infected lean animals (Figure 3, Table S1; $p<0.05$ by multiple t-test). This increased proinflammatory response was corroborated by cytokine mRNA transcript expression in infected tissues (Figure 4). Expression of most, but not all, cytokines was upregulated in obese compared with lean mice. In particular, the expression of IL-6, IFN-γ, and IL-21 genes was markedly upregulated, with levels 65-, 15-, and 48-fold higher in obese mice compared with lean tissues, respectively.

Adipocyte hypertrophy triggers the production of the adipokines leptin, resistin, and adiponectin. Indeed, obese mice infected with *F. tularensis* had elevated levels of the inflammatory adipokines leptin ($p<0.005$ by multiple t-test) and a trend for increase production of resistin which became significant at the time of termination (Figure 5, Table S2). In addition, there was a general suppression of the anti-inflammatory adiponectin in infected obese mice compared with lean animals during the peak of the inflammatory response though this did not reach statistical significance (Table S2).

The leptin receptor is expressed by nearly all immune cells and binding of leptin increases production of Th1 and Th17 responses including IL-6, TNF-α, and IFN-γ. Therefore, the direct effects of leptin on macrophage inflammation were determined by *in vitro* culture of *F. tularensis*-infected macrophages. *F. tularensis* triggers inflammation in cultured macrophages observed by the production of IL-6, IL-1β, TNF-α, and IL-23 (Figure 6). Consistent with other studies, addition of leptin to uninfected macrophages at concentrations similar to plasma levels of infected obese mice stimulated a mild increase in the production of these inflammatory cytokines. However, *F. tularensis*-infected macrophages produced significantly higher amounts of IL-6 and IL-β compared with infected macrophages in the absence of leptin (Figure 6; $p<0.03$ by Mann–Whitney t-test). In addition, there was a trend for increased production of TNF-α which did not reach statistical significance ($p=0.1379$ by Mann-Whiteny t-test), while the production of IL-23 was unchanged by the presence of leptin. Together, these data suggest that leptin production from hypertrophic adipocytes in obese mice leads to a heightened inflammation which is exacerbated by the lethal cytokine storm elicited by *F. tularensis* and is associated with increased risk of death following *F. tularensis* infection.

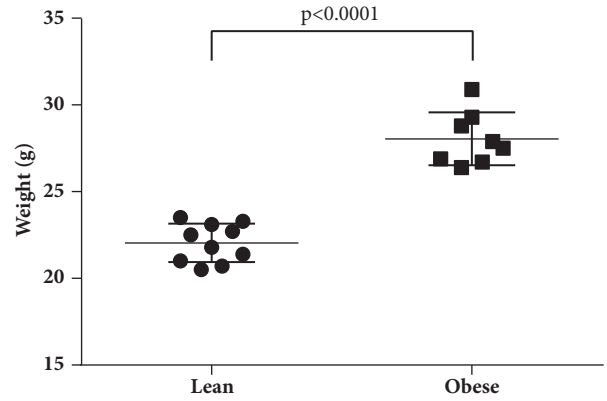

Figure 1: Development of obesity: C57BL/6J mice fed high fat chow for 9 weeks were, on average, 10g heavier ($p<0.0001$, n=15) than their sibling lean counterparts. Data is representative of three replicate experimental groups.

4. Discussion

The immune dysfunction caused by obesity has been linked to an increased susceptibility to a number of infection diseases [37]. However, association studies between obesity and sepsis have had mixed results with either no association, positive association, or negative associations being reporting, reviewed in [38]. *F. tularensis* infection causes disease through overactivation of the inflammatory response, the cytokine storm, resulting in a sepsis-like disease [3, 4]. Disease symptoms are caused by side effects of the cytokine storm resulting in severe immunopathology.

While the effects of obesity on sepsis remain controversial, leptin's detrimental role in sepsis has been documented in several studies. Results from a multinational European survey of sepsis occurrence in acutely ill patients uncovered

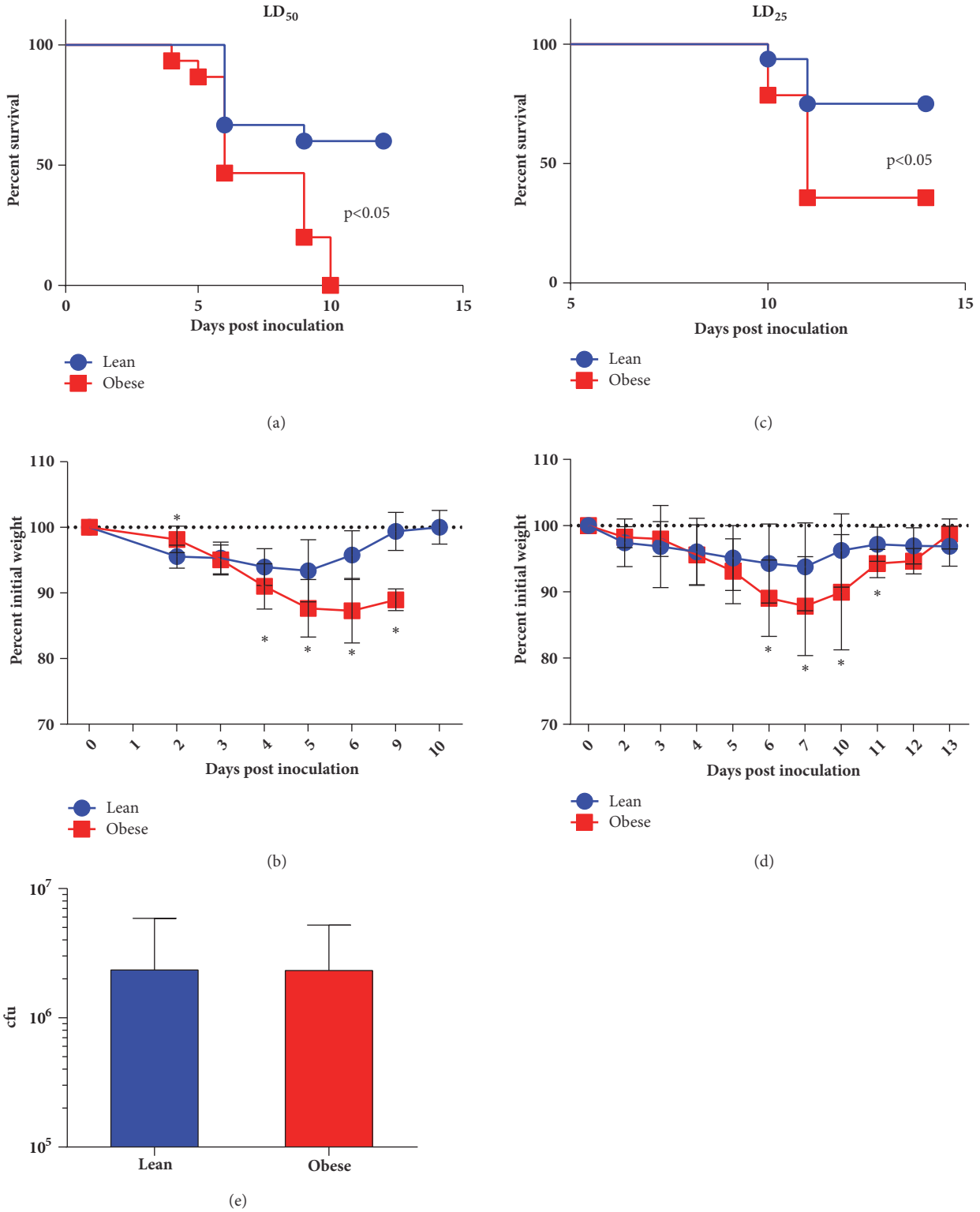

FIGURE 2: Increased susceptibility of obese animals following *F. tularensis* infection. Lean and obese mice were infected with $8x10^5$ (a, b) or $5x10^5$ (c, d) cfu *F. tularensis* LVS (∗ (a, c) $p<0.05$ by Mantel-Cox test; (b, d) $p<0.02$ by multiple t-test). (e) Bacterial load was indistinguishable in target organs between lean and obese animals.

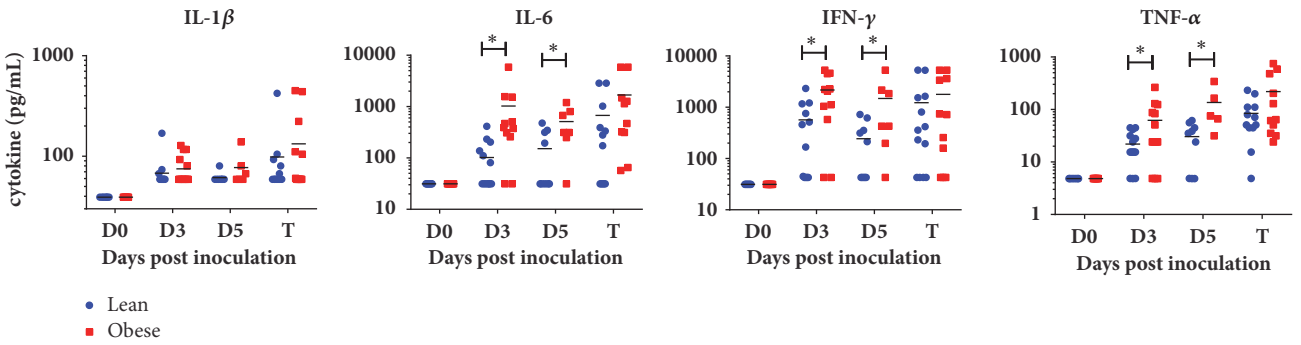

Figure 3: Increased proinflammatory cytokines storm cytokines present in plasma of obese animals. In response to *F. tularensis* initiation, obese mice had significantly higher plasma levels of the inflammatory cytokines compared with lean siblings (∗p<0.05 by multiple t test).

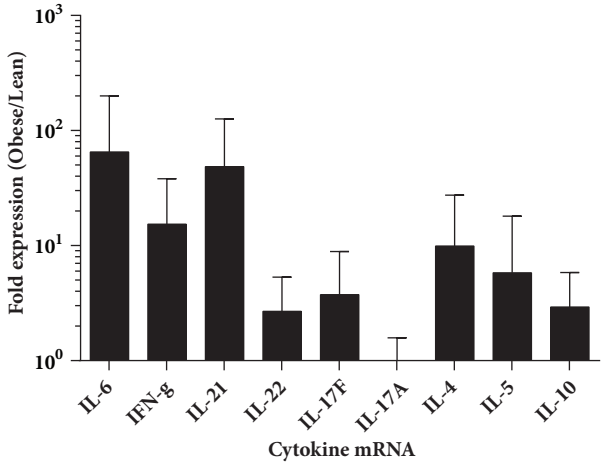

Figure 4: Elevated inflammatory mRNA levels in infected tissues of obese animals. Real-time RT-PCR analysis of mRNA of lean and obese tissue revealed elevated transcript levels of cytokine genes corresponding to cytokine storm cytokines. Data is presented as fold change (2^-ΔΔCt) in expression in obese animals compared with lean animals (N=15-24).

that although men present more frequently with sepsis (63%), females were more likely to develop severe sepsis and have higher mortality (OR =1.4) [34]. Furthermore, females have ~2.5 times higher serum levels of leptin compared to males [39]. Since leptin is an inflammatory adipokine, these associations suggest that leptin could be associated with septic symptoms. Indeed, while associative studies demonstrate no link between occurrence of sepsis and leptin, there was a very strong association in females between leptin and severe sepsis, including death following hospitalization (OR=4.18) [39].

Likewise, hyperleptinemia is associated with increased sensitivity to multiple infectious diseases [40]. In addition, the role of leptin in inflammation and inflammatory conditions has been investigated in multiple models [41–43]. However, it remained unknown whether obesity would affect susceptibility to *F. tularensis* infection. Our data demonstrate an increased susceptibility to this infectious disease through enhanced severity of the cytokine storm. While the exact mechanism of disease is unclear for *F. tularensis* infection, it is clearly a septic response [3, 4]. Our data suggest that the mechanism is independent of IL-6-mediated production of CRP as there was no difference in serum CRP levels between lean and obese infected animals despite differing degrees of disease (data not shown).

As reported here, and elsewhere, the cytokine storm elicited by *F. tularensis* is not solely restricted to proinflammatory cytokines but also includes anti-inflammatory and regulatory cytokines and chemokines. The presence of both proinflammatory and anti-inflammatory/regulatory cytokines is a hallmark of the immune dysregulation seen during the cytokine storm. It is currently unknown whether anti-inflammatory or regulatory cytokines are produced to control in response to the heightened inflammation or whether their production is directly elicited by the infection. In either case, it is clear that the production of anti-inflammatory and regulatory cytokines is insufficient to suppress or modulate the excessive proinflammatory response prior to death [35]. Regardless, obese mice infected with *F. tularensis* displayed a more intense cytokine storm in both plasma and local tissue causing increased *F. tularensis* susceptibility.

It is unsurprising that obese mice have elevated levels of adipokines compared with lean mice as the link between their production and obesity has been widely documented. However, only leptin was associated with the cytokine storm and reduced survival in our study, while resistin was only moderately affected and levels of adiponectin were reduced in infected obese animals, contrary to previous reports [20]. Indeed, in *in vitro* assay utilizing isolated macrophages, addition of leptin increased the production of proinflammatory cytokines following infection with *F. tularensis*. The specific signaling events leading to this increase remain to be determined; however, it is possible that there exists a synergy between the JAK/STAT signaling pathway activated by leptin and the inflammasome signaling pathway activated by *F. tularensis* infection.

Similar to previous reports, obese individuals had slightly higher levels of a number of proinflammatory cytokines though this did not reach statistical significance in our study [44]. However, they were significantly upregulated after a stimulus (infection) induced inflammation. The lack of statistical significance could be explained by a limitation in

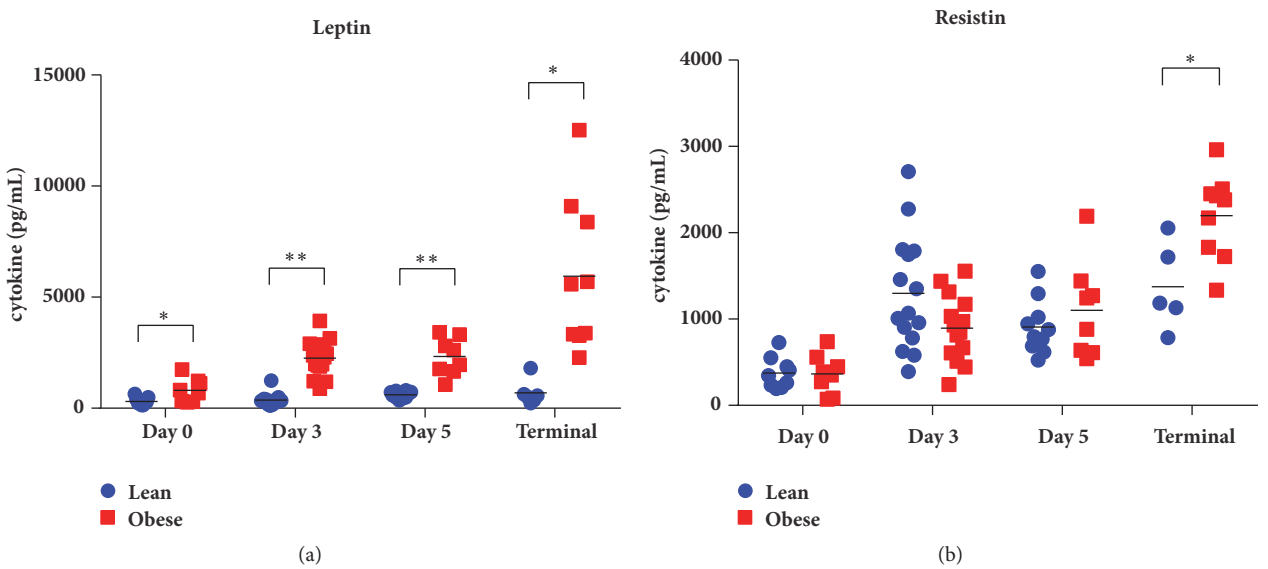

FIGURE 5: Plasma levels of proinflammatory adipokines. (a) Obese mice had significantly higher plasma levels of the inflammatory adipokine leptin compared with lean siblings ($*p<0.05$, $**p<0.001$ by multiple t-test). (b) A trend was observed for higher plasma levels of resistin in obese animals compared with lean animals which became significant at the time of death ($*p<0.05$).

the sensitivity of our assay. Alternatively, despite the lack of a statistical significance, these slight changes may be recognized by a sensitive immune system and be capable of altering the biological response. Regardless, infection of both lean and obese mice activated the inflammatory response, as expected; however, in obese mice, the levels of many of these same inflammatory cytokines were significantly higher than in lean controls.

Interestingly, sCD40L and IL-21 were significantly down-regulated in obese mouse serum prior to infection. sCD40L blocks monocyte activation and activates myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Treg). MDSCs and Treg both function to suppress the immune response [45]. On the other hand, IL-21 is a pleotropic cytokine with both pro- and anti-inflammatory properties. We expect that, in this circumstance, the immunosuppressive functions of IL-21 to inhibit DC activation and maturation and/or induce the production of IL-10 from either T cells or B cells (B10 cells) are dominant [46]. The suppression of sCD40L and IL-21 would therefore serve to enhance inflammation in obese mice.

As stated, only female mice were challenged in this study due to our past experience in infectious disease and obesity studies and prior associative studies with sepsis disease [34, 39]. Our study associated the increased susceptibility to *F. tularensis* disease with the increased inflammation stimulated by leptin production. However, since male and female mice have different amounts of fat as well as adipokines [33–35], the susceptibility of male obese mice may differ from female obese mice. Indeed, we have observed differences in the susceptibility of lean males and females to *F. tularensis* independent of leptin (unpublished observation). Furthermore, since obese humans have an increased inflammatory response compared to lean individuals [34], we anticipate a similar response in obese tularemic patients; however, this remains to be determined.

Herein, we demonstrated that obese female mice are more sensitive to disease following *F. tularensis* infection. Indeed, obese mice had higher serum levels of inflammatory cytokines following *F. tularensis* compared to lean mice. In addition, obese female mice had higher levels of the inflammatory adipokine leptin and reduced levels of the anti-inflammatory adipokine adiponectin. *In vitro* studies demonstrated that exposure of macrophages to leptin resulted in increased inflammation in response to *F. tularensis* infection compared to macrophages infected with *F. tularensis* alone. This increased inflammation was observed by increased production of cytokine mRNA in infected tissues as well as increased serum levels of IL-6, IFN-γ, and TNF-α. These data lead to a model in which the expression of leptin from the activated hypertrophic adipocytes increases the activation of immune cells raising the inflammatory state of female obese mice similar to that reported for human obese females [44]. This higher than normal basal inflammation primes the immune system to generate an even more intense cytokine storm when elicited by *F. tularensis* infection.

Data Availability

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

Disclosure

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

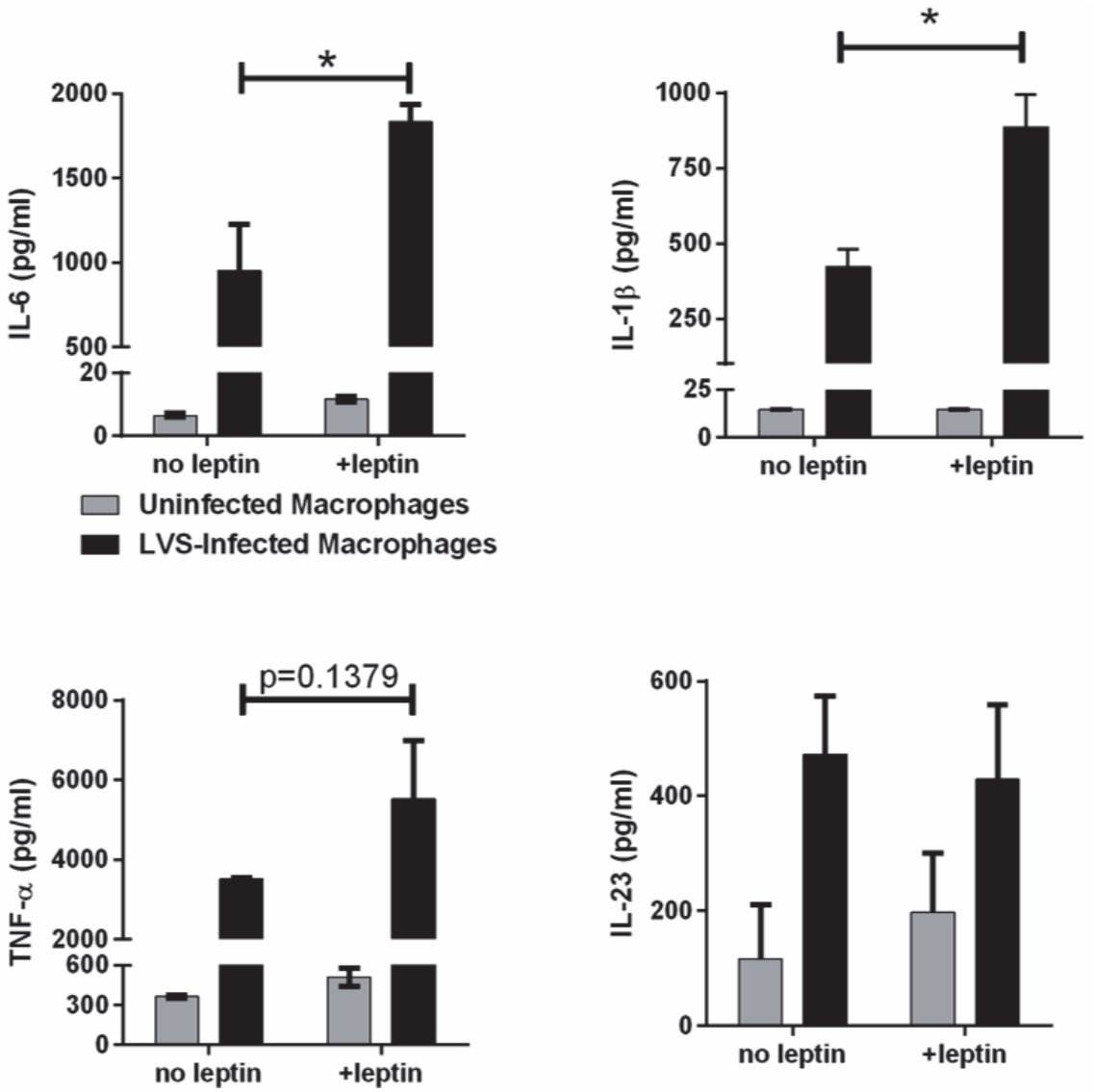

Figure 6: Leptin enhances inflammatory cytokine production by *F. tularensis*-infected macrophages. Macrophages were exposed to leptin prior to, during, and after infection with *F. tularensis* LVS and the production of IL-6 measured as an indicator of inflammation (∗p=0.03 by Mann–Whitney t-test).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Supplementary Materials

Supplemental Figure 1: Obesity-induced changes in plasma levels of inflammatory cytokines preinfection. Plasma levels of 20 cytokines were analyzed by multiplex ELISA and analyzed by ANOVA with Bonferroni posttest. Data is displayed as the mean and confidence intervals for the differences between the lean and obese animal groups (∗p<0.05 by two-way ANOVA). Supplemental Figure 2: IL-21 and sCD40L were reduced in the plasma of obese animals compared with lean mice. Prior to infection, only IL-21 and sCD40L were significantly altered in obese mice blood plasma compared with lean mice (∗p<0.05 by Mann–Whitney t-test). Supplemental Table: Detailed levels of plasma cytokines in lean and obese animals prior to (D0) and after infection with *F. tularensis*. *(Supplementary Materials)*

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Research Article

Effect of Polyvalence on the Antibacterial Activity of a Synthetic Peptide Derived from Bovine Lactoferricin against Healthcare-Associated Infectious Pathogens

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Antimicrobial peptides (AMPs) are gaining interest as potential therapeutic agents. Peptides derived from bovine lactoferricin B (LfcinB) have been reported to exhibit antimicrobial activity, and the LfcinB RRWQWR sequence is the smallest known motif that exhibits antibacterial and cytotoxic activity. Our goal was to examine the effect of multicopy arrangements of the RRWQWR motif, on its antibacterial activity against healthcare-associated infections (HCAIs). Linear and branched peptides containing the RRWQWR motif were generated using solid phase peptide synthesis-Fmoc/tBu methodology, purified, and characterized using reverse phase-high performance liquid chromatography and matrix-assisted laser desorption/ionization time of flight mass spectrometry. For each peptide, the antibacterial activity against *Staphylococcus aureus* (ATCC 25923 and 33591 strains) and *Klebsiella pneumoniae* (ATCC 13883 and 700603 strains) was assessed by measuring the minimum inhibitory and the minimum bactericidal concentrations, in the exponential phase. Cells were observed by scanning electron microscopy, and the hemolytic activity of the peptides was assessed. The overall results demonstrate that, compared to linear analogues, polyvalent presentation of the RRWQWR motif enhances its antibacterial activity against both Gram-negative and Gram-positive bacteria even on resistant strain.

1. Introduction

Until very recently, "nosocomial" was the term used to refer to any disease acquired by a patient under medical care [1], particularly with reference to infections acquired by hospitalized patients. Recently, a new expression, "healthcare-associated infections" (HCAIs), has been established to refer to infections due to hospitalization. HCAIs are a major risk factor for serious health problems and can lead to death [2].

Several microbes, such as protozoans, fungi, viruses, and mycobacteria, can cause HCAIs, but bacteria are responsible for approximately 90% of the infections [3]. The incidence of various bacteria in HCAI infection changes over time and depends on the region [1]. Currently, pathogens that commonly caused HCAIs include *Staphylococcus aureus*, *Klebsiella pneumoniae, Pseudomonas aeruginosa, Escherichia coli*, and *Enterococcus faecalis* [4].

Furthermore, the excessive and improper use of traditional broad-spectrum antibiotics, especially in healthcare venues, increases the prevalence of bacteria resistant to conventional antibiotics and has resulted in an increase in HCAI infections caused by drug-resistant pathogens [1]. The design of new antimicrobial molecules effective against resistant bacteria is crucial to overcome and control the antibiotic resistance.

One promising group of potential lead structures for antibiotics is antimicrobial peptides (AMPs), as they are naturally derived compounds with antimicrobial activity. The rational design of new AMPs offers hope for enhanced biological activity and cheaper, more-efficient production. Rational

design methodologies include in silico methodologies. Large-scale, high-quality recombinant production can be done using tobacco mosaic virus and gene-editing techniques such as CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) recombinant peptide biosynthesis [5]

In this AMP group, peptides derived from LfcinB protein have been reported to exhibit antimicrobial activity. Peptides derived from bovine lactoferricin B (LfcinB) have been reported to exhibit antimicrobial activity [6]. The RRWQWR sequence of LfcinB is the smallest motif that exhibits antibacterial activity [7, 8], as well as cytotoxic effect [9]. Previous studies of our group have shown that this sequence motif has antibacterial activity although it is minimal [6, 8, 9]. Our goal was to examine the effects of various arrangements of multiple copies of the RRWQWR motif on its antibacterial activity against HCAI bacteria.

2. Materials and Methods

2.1. Microorganisms. All microorganisms were purchased from the American Type Culture Collection (ATCC). To determine the antibacterial activity of synthetic peptides against HCAI bacteria, the *Staphylococcus aureus* strains ATCC 25923 and ATCC 33591 were employed as sensitive and resistant strains, respectively, of a Gram-positive microorganisms species, and *Klebsiella pneumoniae* ATCC 13883 and ATCC 700603 as sensitive and resistant strains of a Gram-negative species.

2.2. Antibacterial Peptides. To improve the antibacterial activity of the RRWQWR motif against *S. aureus* and *K. pneumoniae*, our approach was based on presenting motif repetitions as linear or branched derived peptides [10]. The peptides were synthesized using solid phase peptide synthesis- (SPPS-) Fmoc/tBu methodology, as previously reported by our group [8, 9]. Bearing in mind that the amidated peptides have greater biological activity, the peptides synthetized and evaluated in this work were carried out using Rink-amide as a solid support, in order to obtain peptides with amide function at their carboxyl end. The purity of the peptides was >90%, as determined via reverse phase-high performance liquid chromatography (RP-HPLC) analysis. All peptides had the expected molecular weight, as verified by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MS MALDI-TOF). Synthetized peptides were stored as lyophilized products. To obtain the dimeric peptide, di-FMOC-protected lysine was used, which enabled simultaneous synthesis of the two peptide chains (one from the α-amino group and the other, from the ε-amino group of this amino acid) [6]. The tetrameric peptide was obtained through disulfide bond formation (Figure 1). Briefly, the purified peptide $(RRWQWR)_2$-K-Ahx-C was oxidized using 10% dimethyl sulfoxide in a buffer PBS at pH 7.5 at room temperature, in accordance with Leon-Calvijo et al. [6].

The peptides molecules were synthesized and characterized by the "Síntesis y Aplicación de Moléculas Peptídicas (SAMP)" research group of the Faculty of Science of the Universidad Nacional de Colombia.

2.3. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). The antibacterial activity assay for peptides and controls was performed according to the National Committee for Clinical Laboratory Standards method M7-A7 [11]. MIC and MBC values were determined using a broth microdilution and growth inhibition method [12] with some modification. Briefly, MIC assays were performed by liquid inhibition growth assay in an untreated sterile 96-well flat-bottom tissue culture plate. Bacteria were cultured overnight on Mueller-Hinton agar (MHA). Three colonies were transferred to 8 mL of Mueller-Hinton broth (MHB) and incubated at 37°C until the mid-exponential phase of growth. The turbidity of the cultures was measured and adjusted spectrophotometrically to McFarland standard 0.5, then diluted to a final concentration of 5×10^7 colony forming units (CFU)/well. Synthetic antibacterial peptide candidate stock solutions were diluted to final concentrations per well of 200, 100, 50, 25, 12.5, and 6.25 μM. Each concentration was evaluated in duplicate in three repetitions of the assay.

Wells containing MHB with bacterial inoculum only served as bacterial growth controls. Additional controls included MHB alone and MHB with ciprofloxacin (CIP) (2 μg/mL) and bacteria as a positive control. The microplate was incubated for 24 h at 37°C, and growth inhibition was examined by monitoring the optical density at 620 nm (OD_{620}). MIC was defined as the lowest peptide concentration that inhibited bacteria growth >90%.

To determine the MBC, an aliquot from each well of the MIC assay was spread onto MHA. After 18 h at 37°C, the concentration that inhibited bacterial growth was determined. Each of these tests was performed four times. MBC was defined as the lowest concentration of peptide that reduced the number of bacteria by 99.9% *in vitro* [12].

2.4. Electron Microscopy. Scanning electron microscopy (SEM) was used to observe bacterial morphology. *S. aureus* and *K. pneumoniae* strains were grown to mid-logarithmic phase and adjusted spectrophotometrically to the McFarland 0.5 standard, corresponding to $\sim 1 \times 10^8$ CFU/mL. Subsequently, 1 mL of bacterial suspension was distributed into 3 tubes, and peptides (dimeric and tetrameric molecules) were added to two tubes at 3x the MIC. The third culture, without peptide, was used as a control. Samples were incubated aerobically at 37°C for 2 h, and the bacterial suspension was centrifuged at 1459 ×g for 3 min and washed twice with Millonig's Phosphate Buffer (0.10 M, pH 7.4). For SEM, each sample was fixed with 1 mL of 2.5% glutaraldehyde at 4°C for 2 h. The fixed samples were dehydrated in an ethanol gradient (50, 70, 80, 90, and 100%) for 20 min and centrifuged at 1459 ×g for 10 min. The bacterial pellet was suspended in 100% ethyl alcohol and air-dried. Finally, the slides were taped onto stubs, coated with gold using a Quorum Q150R sputter coater, and observed with an FEI Quanta 200-r SEM.

2.5. Haemolytic Activity. Human erythrocytes collected from the blood samples of healthy humans were harvested by centrifugation for 7 min at 162 ×g and washed three times in phosphate-buffered saline (PBS). The erythrocytes (2%

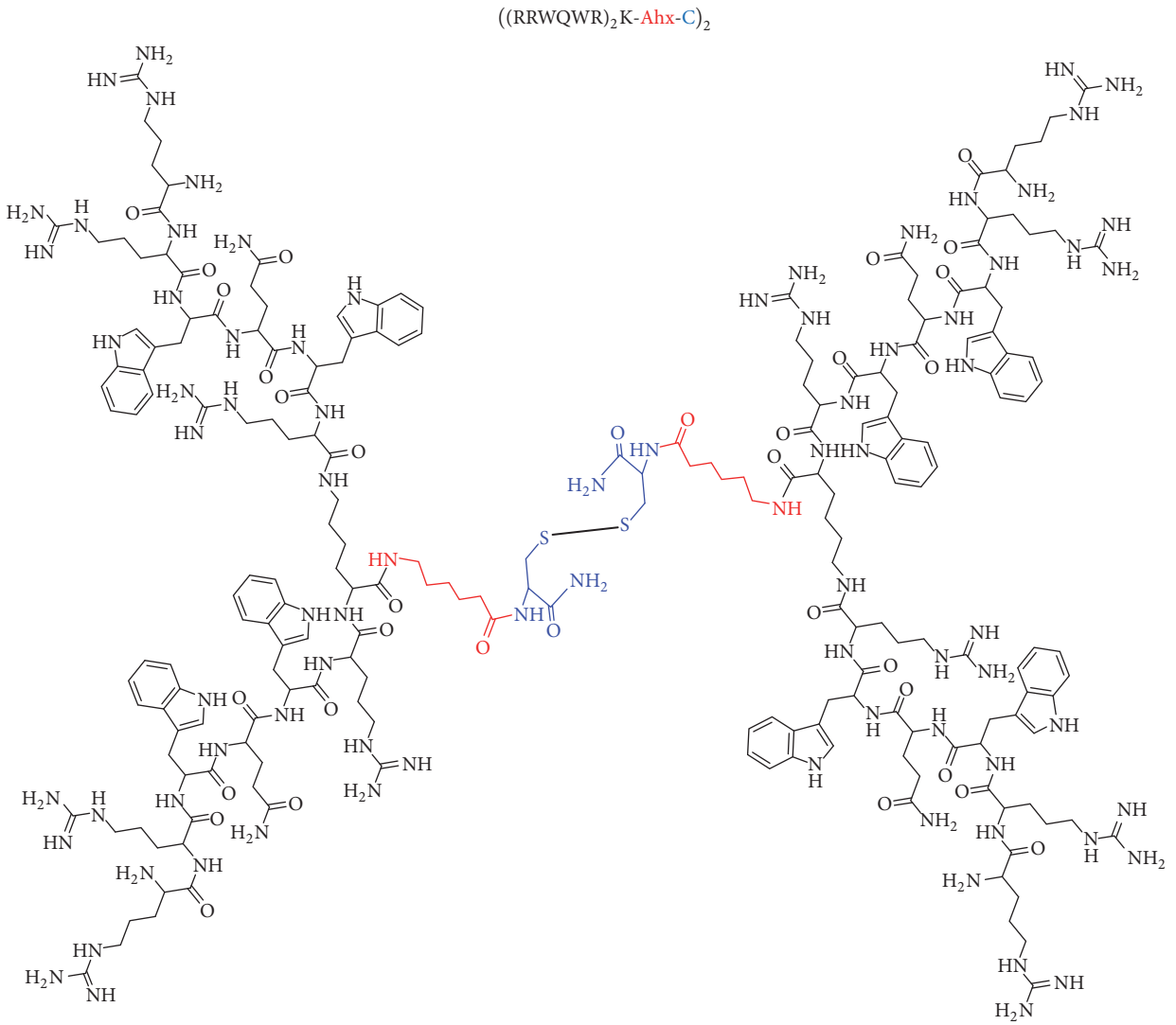

Figure 1: Representation of the tetrameric RRWQWR motif peptide molecule.

haematocrit in PBS) were incubated with peptide molecules at several concentrations (6.25, 12.5, 25, 50, and 100 μM), for 2 h at 37°C. PBS was used as a negative control, and sterile distilled water was used as a positive control for haemolysis (100% of haemolysis). The plate was subsequently centrifuged at 1459 ×g for 10 min at 4°C. Aliquots of the supernatants of each well (75 μL) were carefully transferred to a new sterile 96-well plate, and haemolytic activity was evaluated by measuring the OD_{492} using an Asys Expert Plus Microplate reader. The experiments were performed in duplicate, and peptide haemolysis activity was calculated.

3. Statistical Analysis

Data were analyzed using SPSS 11.0 software and are presented as the mean ± standard deviation.

4. Results

4.1. Antibacterial Peptides. For this research, five peptides (Table 1) containing the RRWQWR motif were synthesized through SPPS, using the manual Fmoc/tBu strategy. Specifically, we generated a linear LfcinB motif (20–25), a palindromic sequence (PLS), and LfcinB (17–31), which was considered an antibacterial peptide reference, according to results previously reported by Leon-Calvijo et al. [6]. Palindromic sequence is not really palindromic as it is but it shifted sequence in order to increase the size of the motif peptide in a linear designed while maintaining the same net charge. Additionally a dimeric peptide was synthesized and a tetrameric peptide as branched derived. Peptide solutions were prepared in sterile water for injection, sterilized by 0.22 μm filtration, and stored at −20°C.

Table 1: Antibacterial peptides derived from LfcinB in this study.

Peptide	Sequence															m/z $[M+H]^+$	Net charge[a]	GRAVY[a]	pI[b]
Motif				^{20}**R**	**R**	**W**	**Q**	**W**	**R**^{25}							986.7	+3	−3.133	12.30
Palindromic					**R**	**W**	**Q**	**W**	**R**	W	Q	W	R			1488.6	+3	−2.678	12.30
LfcinB reference	^{17}F	K	C	**R**	**R**	**W**	**Q**	**W**	**R**	M	K	K	L	G	A^{31}	1994.7	+6	−1.207	11.74
Dimeric				**(R**	**R**	**W**	**Q**	**W**	**R)$_2$**	K	Ahx					2198.5	+6	- -	- -
Tetrameric				**(R**	**R**	**W**	**Q**	**W**	**R)$_4$**	K_2	Ahx_2	C_2				4594.6	+12	- -	- -

[a]Net charge and grand average of hydropathy (GRAVY) were calculated using the Antimicrobial Peptide Calculator and Predictor (http://aps.unmc.edu/AP/prediction/prediction_main.php). [b]Theoretical pI values were calculated using the Compute pI/Mw Tool (https://web.expasy.org/compute_pi/).

Table 2: Antibacterial activity of synthetic peptides derived from the LfcinB RRWQWR motif against HCAI pathogens.

Peptide	*Staphylococcus aureus*				*Klebsiella pneumonia*			
	ATCC 25923 Sensitive		ATCC 33591 Resistant		ATCC 13883 Sensitive		ATCC 700603 Resistant	
	[a]MIC	[b]MBC	MIC	MBC	MIC	MBC	MIC	MBC
Motif	>197 (200)	>197 (200)	>197 (200)	>197 (200)	>197 (200)	>197 (200)	>197 (200)	>197 (200)
Palindromic	74 (50)	148 (100)	9 (6.25)	74 (50)	74 (50)	148 (100)	74 (50)	148 (100)
LfcinB reference	>398 (200)	>398 (200)	100 (50)	199 (100)	>398 (200)	>398 (200)	>398 (200)	>398 (200)
Dimeric	14 (6.25)	110 (50)	55 (25)	110 (50)	27 (12.5)	55 (25)	27 (12.5)	220 (100)
Tetrameric	29 (6.25)	57 (12.5)	57 (12.5)	57 (12.5)	29 (6.25)	115 (25)	57 (12.5)	115 (25)

[a]The minimum inhibitory concentration expressed in μg/mL and (μM). [b]The minimum bactericidal concentration expressed in μg/mL and (μM). Data are the averages of four independent experiments, each performed in duplicate.

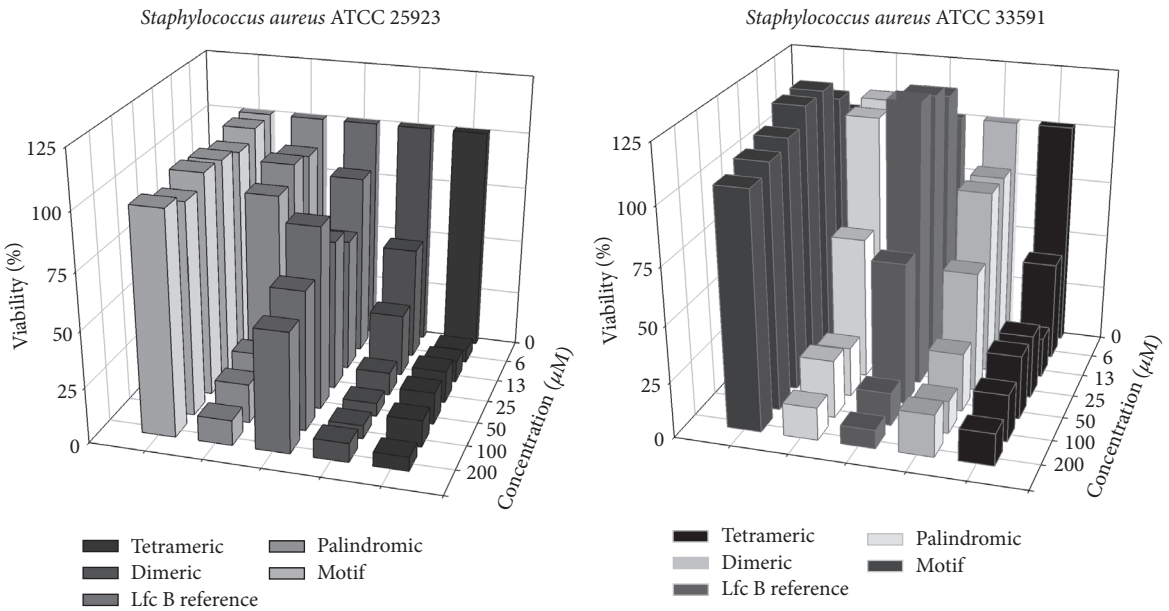

Figure 2: Dose-dependent effects of the peptides on two *Staphylococcus aureus* strains.

4.2. Antibacterial Activity of the Designed Peptide Molecules. We examined the antibacterial activity of the peptides at several concentrations. Specific dose-response profiles were observed for each peptide molecule, and the profiles differed according to the strain evaluated and its sensitivity to antibiotics.

4.3. Determination of MIC and MBC. Figure 2 shows the specific antibacterial activity profiles for each peptide against representative sensitive and resistant strain of the Gram-positive microorganism *S. aureus*. The peptide motif had no significant effect on either strain. However, the palindromic peptide in general displayed antibacterial activity at concentration ≥74 to 148 μg/mL (≥50 to 100 μM) on both the sensitive and resistant strains. Similarly, the LfcinB reference peptide, which was previously reported to exhibit antibacterial activity on other bacterial pathogens, displayed antibacterial activity, but only affected the resistant strain, at concentration ≥ 100 μg/mL (≥50 μM). Lower, concentrations of this molecule did not exhibit antibacterial activity, and only concentrations ≥ 199 μg/mL (≥100 μM) exhibited significant antibacterial effects. The dimeric and tetrameric peptide molecules exhibited a strong effect on bacterial growth, with dose-dependent effects on both the sensitive and resistant *S. aureus,* and the strongest effect was observed on the sensitive strain, at concentration ≥ 14 μg/mL (≥6.25 μM) and ≥29 μg/mL (≥12.5 μM), respectively.

Concentration expressed in μg/mL for each peptide is reported in Table 2.

Conversely, results obtained with Gram-negative *K. pneumonia* were similar for both strains tested (sensitive and resistant) with all analyzed peptide molecules. Figure 3 shows the specific antibacterial activity profile of each peptide against *K. pneumoniae*. The peptide motif had no substantial

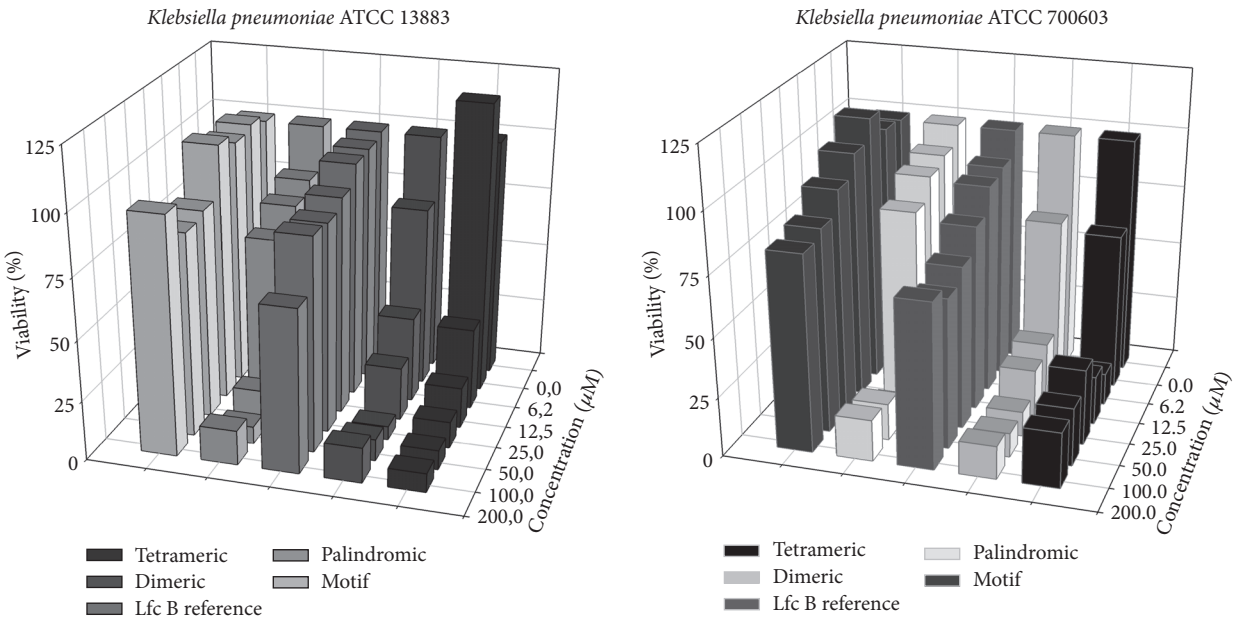

Figure 3: Dose-dependent effects of the peptides on two *Klebsiella pneumoniae* strains.

effect on either strain, at any concentration. The palindromic peptide exhibited similar antibacterial activity against the sensitive strain at concentration ≥74 μg/mL (≥50 μM); however the resistant strain required a higher concentration to induce similar antibacterial activity ≥148 μg/mL (≥100 μM). The LfcinB reference peptide exhibited similar antibacterial activity to the motif peptide on both strains, while the branched dimeric and tetrameric peptides both exhibited strong antibacterial activity at concentrations ≤27 μg/mL (≤12,5 μM) and ≤57 μg/mL (≤12,5 μM), respectively, against both sensitive and resistant *K. pneumoniae*.

Concentration expressed in μg/mL for each peptide is reported in Table 2.

According to these results, molecules containing repetitions of the RRWQWR motif exhibited antibacterial activity against both sensitive and resistant strains of Gram-positive and Gram-negative bacteria, in the following order:

$$\text{Tetrameric} > \text{Dimeric} > \text{Palindromic} \quad (1)$$

The MIC for each peptide against sensitive and resistant strains of *S. aureus* and *K. pneumoniae* were measured using a broth microdilution assay. The MIC value for the five peptide molecules studied against the infecting organisms is shown in Table 2.

The MIC values for the RRWQWR motif showed no antibacterial activity against *S. aureus* and *K. pneumoniae* (>197 μg/mL–200 μM) (Figures 2 and 3; Table 2). Similar results were observed for the LfcinB reference peptide (>398 μg/mL–200 μM), except for the *S. aureus*-resistant strain, against which significant antibacterial activity was demonstrated (≥100 μg/mL–≥50 μM, Table 2). The palindromic peptide had a higher MIC for the sensitive strain (≥74 μg/mL–≥50 μM, Table 2), than the resistant strain (≥9 μg/mL–≥6,25 μM, Table 2), and was more effective against the resistance strain. Remarkably, the dimeric and tetrameric peptides exhibited the strongest inhibitory activities against both *S. aureus* and *K. pneumoniae*.

For *S. aureus*, the dimeric (≥14 μg/mL–≥6.25) and tetrameric (≥29 μg/mL–≥6.25 μM) peptides exhibited greater antibacterial activity than the palindromic on the sensitive strain (Table 2). By contrast, the palindromic (MIC ≥ 9 μg/mL–≥6.25 μM) peptide showed increased activity compared to the tetrameric (MIC ≥ 57 μg/mL–≥12.5 μM) and the dimeric peptide (MIC ≥ 55 μg/mL–≥25 μM), against the resistant strain. Interestingly, the palindromic peptide displayed a difference in the MBC obtained with the sensitive (≥148 μg/mL–≥100 μM) and resistant (≥74 μg/mL–≥50 μM) strains, requiring higher dose to induce the same effect, whereas the branched molecules dimeric (≥110 μg/mL–≥50 μM) and tetrameric (≥57 μg/mL–≥12.5 μM) induced stronger antibacterial effects on both sensitive and resistant strains (Table 2).

For *K. pneumoniae*, the palindromic (≥74 μg/mL–≥50 μM) and dimeric (≥27 μg/mL–≥12,5 μM) peptides did not exhibit any differences in MIC on the sensitive and resistant strains, while tetrameric peptide induced higher antibacterial effects on the sensitive (≥29 μg/mL–≥6,25 μM) that resistance strain (≥57 μg/mL–≥12,5 μM).

The MBC values for each peptide were described as a function of the MBC of the tetrameric peptide, because this molecule exhibited the greatest antibacterial activity on the strains tested in this study.

For *S. aureus*, the tetrameric MBC was ≥57 μg/mL (≥12.5 μM). Therefore, the efficacy profile expressed in concentration unit (μg/mL) for this organism was tetramer (1x MBC) > dimer (1.9x MBC) ≥ palindromic (2.6x MBC and

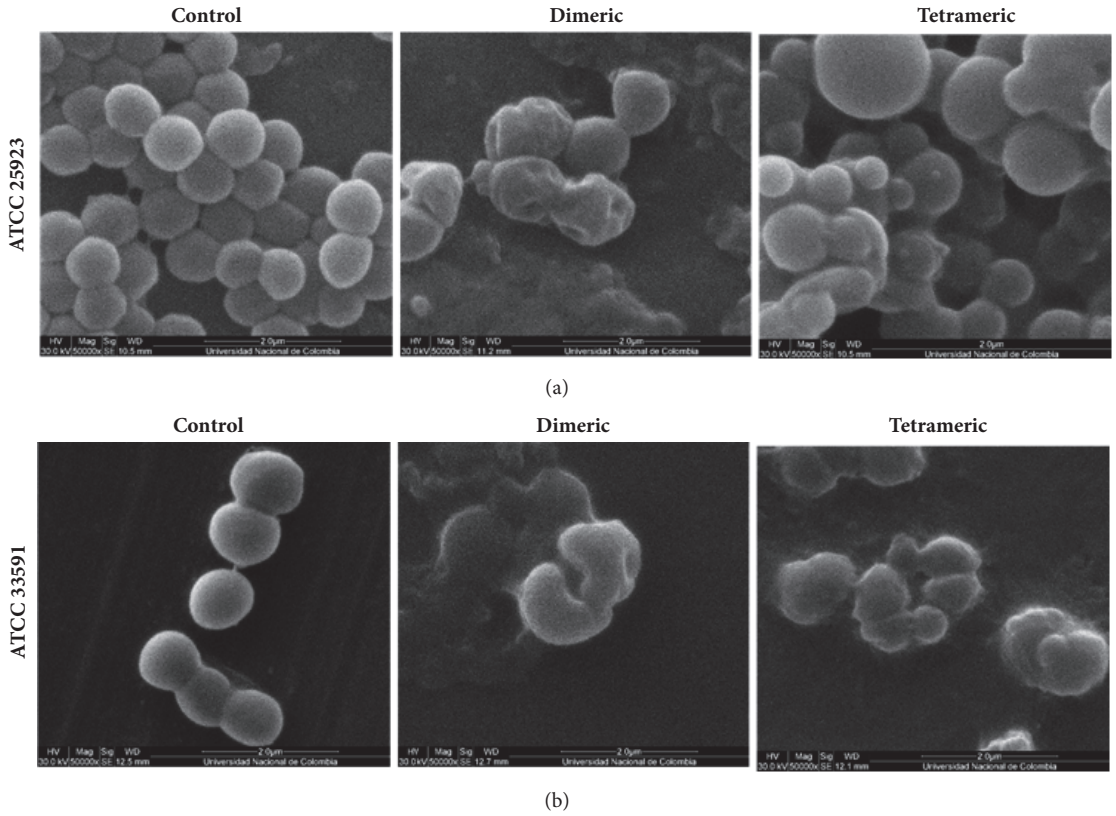

Figure 4: Scanning electron microscopy (SEM) images of *Staphylococcus aureus* after treatment with dimeric and tetrameric peptides.

1.3x MBC for the sensitive and resistant strain, respectively) with similar effects on the sensitive and resistant strains, with the exception of the palindromic molecule.

For *K. pneumonia,* the tetrameric MBC was ≥115 μg/mL (≥25 μM). Therefore, the efficacy profile expressed in concentration unit (μg/mL) for this organism was tetramer (1x MBC) ≥ dimer (0.5x MBC and 1.9x MBC for the sensitive and resistant strain, respectively) > palindromic (1.3x MBC), with similar effects on the sensitive and resistant strains, with the exception of the dimeric molecule.

In the case of *K. pneumoniae*, the palindromic, dimeric, and tetrameric peptides did not exhibit dramatic differences in the MBCs obtained with the sensitive and resistant strains, and the branched molecules (dimeric and tetrameric) induced stronger antibacterial effects.

4.4. SEM. To examine how the dimeric and tetrameric peptides affect *S. aureus* and *K. pneumoniae*, the morphologies of the microorganisms were observed using SEM analysis. As shown in Figure 4, untreated (control) *S. aureus* was spherical, with a smooth surface and minimal mucus and aggregated in grape-like clusters in both the sensitive and resistant strains. Sensitive *S. aureus* (Figure 4(a)), after treatment with dimeric peptide for 2 h, organized into short chains of cells with morphological alterations such as pitted and wrinkled surfaces. Tetrameric peptide treatment of the same strain induced a reduced number of cells, heterogeneous appearance, and alterations of the bacterial surface. The tetramer induced aggregates of several spherical sizes, and protrusions formed on the bacterial surface. The resistant *S. aureus* strain (Figure 4(b)) exhibited a reduction in the bacterial cell population with both treatments. Dimeric peptide treatment induced cell membrane alterations on the surface and abnormal cell shapes. The tetrameric peptide resulted in abnormal cell shapes and aggregations, along with surface changes. With both treatments, the leakage of cellular contents may have contributed to the creation of the observed aggregates.

The untreated (control) sensitive strain of *K. pneumoniae* resembled typical coccobacilli, with an encapsulated, smooth surface, and a population of heterogeneous size and shape (Figure 5). After 2 h treatment with dimeric peptide, sensitive *K. pneumonia* (Figure 5(a)) exhibited bacilli with less-defined capsules and morphological alterations such as loss of shape and ruptured cell membranes, generating bacilli with irregular edges. Treatment with the tetrameric peptide induced a reduction in cell number, a very heterogeneous population, and formation of protusions on the bacterial surfaces. The tetramer induced total loss of typical bacilli shape and the formation of aggregates of several shapes and sizes. The untreated resistant *K. pneumoniae* strain (Figure 5(b)) exhibited a bright capsule, mucus, and a smooth surface with no visible damage, whereas dimeric peptide treatment induced cell membrane alterations on the surface such as shrivelling

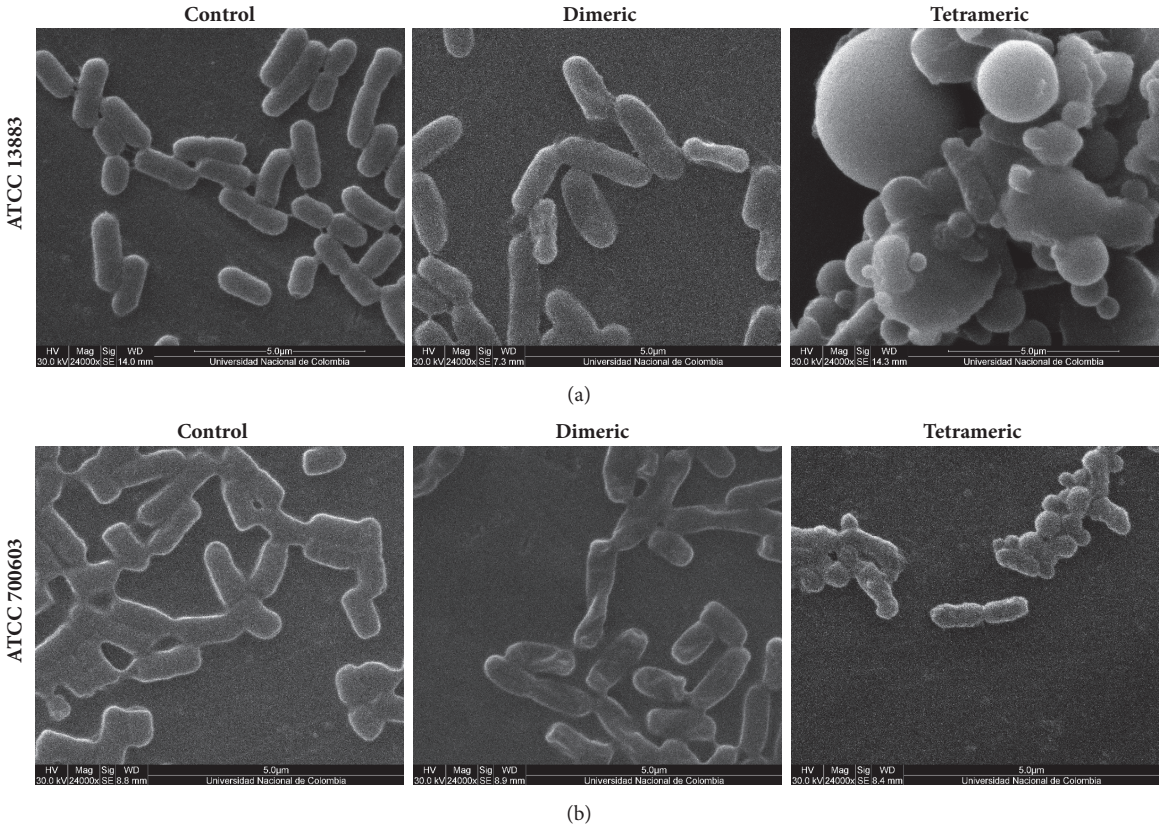

Figure 5: Scanning electron microscopy (SEM) images of *Klebsiella pneumoniae* after treatment with dimeric and tetrameric peptides.

and reduced brightness, as well as decreased size and bacterial population. The effects of tetrameric peptide were even more dramatic, with surface changes and the aggregation of cells of different shapes. In both treatments, the leakage of cellular contents may have contributed to the creation of the observed aggregates.

4.5. Haemolytic Activity. Lastly, to evaluate the effects of the peptides on normal human erythrocytes, their haemolysis activity was investigated (Figure 6). The percentage of haemolytic activity on human red blood cells after 2 hours was determined via the standard microtitre dilution method. The peptide concentration is reported as μM.

Figure 6 demonstrates that none of the peptides studied reached HC_{50} (the concentration that induces the lysis of 50% of human erythrocytes). The palindromic and the tetrameric peptides exhibited greater haemolysis activity (24.8% and 49.1%, respectively) at the highest peptide concentration tested (100 μM) by inducing permeabilization of the human erythrocytes. Looking at the results in detail (Figure 6), the RRWQWR motif showed no haemolysis activity, and the maximum value (7.1%) of haemolysis activity was obtained with 25 μM peptide. Similarly, the LfcinB reference peptide exhibited the greatest haemolysis value (7,9%) at 12.5 μM. Interestingly, the dimeric peptide exhibited the lowest haemolytic profile at all concentrations tested. In summary,

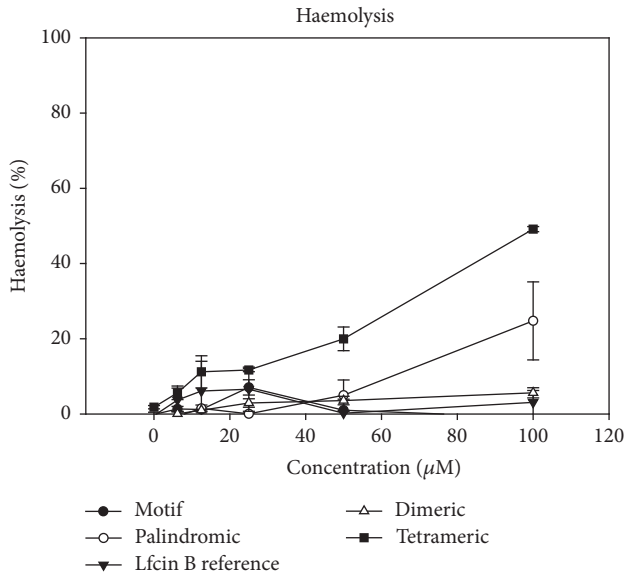

Figure 6: *Haemolytic activity of antibacterial synthetic peptides derived from LfcinB*. Symbols indicate mean and range ($n = 2$).

linear and tetrameric motif increased the haemolytic activity; however, the dimeric motif exhibited low haemolysis activity (<5%). Thus the haemolytic activity profiles for the peptides

tested here were tetrameric > palindromic > dimeric > LfcinB reference > motif.

5. Discussion

AMPs are fascinating prospect for novel antibiotics because of their broad-spectrum activity, including against drug-resistant bacteria LfcinB-derived peptides which are an excellent example. Previous work has shown that how structural changes to the RRWQWR motif can influence the antimicrobial activity of the resulting peptides [13], as well as several studies demonstrated that branched short peptides are more active than lineal one [14–16]. In this study, we investigated how polyvalent presentation of the LfcinB-derived RRWQWR motif affects its antibacterial activity against representative Gram-positive and Gram-negative HCAI bacteria.

RRWQWR motif-derived peptides (linear and branched repetitions) were designed with the aim of developing shorter peptides, to increase antibacterial activity, reduce toxicity, and decrease the coupling reaction steps required and therefore the cost of synthesis. The motif contains positively charged and hydrophobic aromatic amino acids (arginine and tryptophan, respectively), which confer key amphipathic properties at physiological pH.

Cationic and hydrophobic physicochemical properties have been utilized as structural features to improve the function of antibacterial peptides [17]. The crucial physicochemical parameters of the peptides were calculated by using on-line tools. As shown in Table 1, the net charge of the peptides varied from +3 to +12, and this was directly proportional to the antibacterial activity demonstrated. Previous studies have shown that the cationic segments of AMPs facilitate the initial electrostatic attraction and enable interactions with negatively charged components on the bacterial membrane surface [18–20]. However, more recent studies have demonstrated that the relationship between charge and antibacterial activity is nonlinear and that, above a certain threshold (usually +6), increasing the positive charge does not improve antibacterial activity [21, 22]. Even if the results here demonstrate that increasing the number of motif repetitions in the designed peptide induced a higher net charge and that this was related to higher antibacterial activity, it is necessary to consider the relationship between higher antibacterial activity and specificity/selectivity. The hydrophobicity of motif-derived peptides ranged from −3.133 (motif) to −1.207 (LfcinB reference peptide). Therefore, increasing the hydrophobicity and reducing the isoelectric point did not improve antibacterial activity in this study.

Antibacterial activity is related to the nature of bacterial cell membranes, which contain negatively charged lipids in greater abundance than mammalian cell membranes. For this reason, cationic and amphipathic peptides preferentially bind to bacteria by electrostatic attraction, resulting in the specific targeting of bacteria over human cells [23]. Although LfcinB exhibits strong antimicrobial activity, against Gram-positive bacteria [24], Gram-negative bacteria [25], and fungi [26, 27], including multidrug-resistant pathogens, our goal was to establish the effect induced by the polyvalent motif presentation on well-known HCAI pathogens, meaning resistant bacteria. We did not consider cyclic forms, as it has previously been reported that, in spite of the naturally cyclic structure of LfcinB, cyclization is not required for antibacterial activity [9, 28, 29] or *in vitro* cytotoxic effects in some cancer cell lines [9, 30–32]. Our results showed that three of the five molecules studied exhibited antibacterial activity on both microorganisms. The motif and LfcinB reference peptides did not exhibit significant antibacterial activity against these microorganisms, although it was possible to improve their biological activity with linear or branched motif repetitions. Interestingly, the three active molecules (palindromic, dimeric, and tetrameric) exhibited a broad spectrum of activity on both the Gram-positive and Gram-negative species tested, including resistant microorganisms requiring always higher MIC and MBC values for resistance reference strains that for, respectively, sensitive strain (Figure 2; Table 2).

The results demonstrate that palindromic, dimeric, and tetrameric molecules exhibited antibacterial activity, and there was stronger antibacterial activity (defined as molecules with lower MIC and MBC values) with branched motif repetitions for both Gram-positive and Gram-negative microorganisms, as well as for both sensitive and resistant strains (Figures 3 and 4; Table 2).

Interestingly, dimeric and tetrameric molecules have a lower MIC for sensitive *S. aureus* than for sensitive *K. pneumoniae*. Paradoxically, these same molecules on the resistant S. *aureus* strain have a higher MIC than *K. pneumoniae*. These results demonstrate that these cationic molecules (dimeric and tetrameric) exhibit different specificity profiles for Gram-positive and Gram-negative molecules, as well as for sensitive and resistant strains (Table 2). Here we have demonstrated that dimeric and tetrameric molecules exhibited a stronger antibacterial effect on *S. aureus*.

The profile differences may be explained by differences in bacterial membrane components and lipid composition between Gram-positive and Gram-negative species [33] and different responses to environment changes [34, 35] and antibiotic exposure [36–38]. It is well known that different bacterial strains can have unique membrane compositions [38–44].

S. aureus is Gram-positive and *K. pneumoniae* is Gram-negative, and both are relevant HCAI pathogens. Gram-positive bacteria have only one membrane (the cytoplasmic membrane that surrounds the cell), while Gram-negative bacteria have two: the cytoplasmic membrane and an outer membrane. This may explain why *K. pneumoniae* required a higher dose of peptide, antibacterial effect similar to that observed with *S. aureus*.

While both Gram-positive and Gram-negative bacteria have a peptidoglycan layer on the outer side of the cytoplasmic membrane, the peptidoglycan layer is much thicker in Gram-positive bacteria. Perhaps this is the reason that the *S. aureus* strains exhibited lower MIC and MBC values (Table 2) due to the fact that thicker peptidoglycan layer makes it easier for peptide to reach the surface membrane and induce formation of pores. SEM microscopy (Figure 5) clearly showed that, after 2 h of treatment with 3x MIC, the

K. pneumoniae surface membrane exhibited fewer pores and less damage in comparison with *S. aureus* (Figure 4), which displayed dramatic changes under similar conditions. The bacteria surface modifications showed by SEM photography are similar for the changes induced with other antimicrobial cationic peptides on *S. aureus* [45, 46] and *K. pneumoniae* [47] as reference strains. Then, according to the results, our hypothesis is that action mechanism of this branched Lfcin B derived peptides could attach to the bacterial surface and induce the pore formation that disturb the functions of the bacterial membrane as for another cationic peptides was previously described. We are focused on action mechanism studies to elucidate it.

Peptide-lipid interactions are another critical factor. Cationic peptides are facilitated with the negatively charged phospholipids of the microbial membrane. However, it is important to note that the membrane lipid content is diverse. Whereas Gram-positive bacteria contain lipoteichoic acid (LTA) or teichuronic acid (TA), in Gram-negative bacteria it is lipopolysaccharide (LPS) that forms the major lipid component of the outer leaflet of the outer membrane. Consequently, our results could indicate that dimeric and tetrameric molecules have a preference for molecules like LTA and/or TA, expressed on the Gram-positive cytoplasmic membranes, establishing an electrostatic interaction that induces strong antibacterial activity against *S. aureus* strains.

It is possible that branched polyvalent molecules have a higher probability of reaching the bacterial microorganism and establishing electrostatic interactions with anionic molecules and that this depends on the lipid composition of the strain and the Gram classification of the bacteria. Once the dimer and tetramer molecules reach the microorganism, the electrostatic interaction is established, which then leads to microbial lysis and death, perhaps followed by subsequent membrane permeabilization.

The safety of the newly designed molecules is an important aspect to consider for future clinical application. Although the tetrameric and dimeric presentations both demonstrated high antibacterial activity, broad-spectrum activity, and low production cost, their haemolytic activity results indicated that the most innocuous and specific antibacterial molecule was the dimer, at concentrations $\leq 100\,\mu$M, whereas the tetramer has limited therapeutic use at lower concentrations of 12,5 μM. As none of the peptides reached HC_{50}, they exhibit (tetrameric 49.3% with 100 μM) and display interesting antimicrobial activity. Our future work will involve detailed study of the mechanisms of antibacterial action used by these dimeric and tetrameric peptides and its specificity profile in terms of security.

6. Conclusion

RRWQWR motif repetitions in linear and branched conformations resulted in favorable effects on antibacterial activity against Gram-positive and Gram-negative ATCC strains evaluated in this study. Molecules with branched structures were the most promising even on resistance reference strains.

Conflicts of Interest

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

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Research Article

Clinical and Epidemiological Status of Leptospirosis in a Tropical Caribbean Area of Colombia

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Objective. To describe and analyze the clinical and epidemiological status in 28 confirmed cases of human leptospirosis at the main public hospital of Cordoba. *Methods.* Between 2012 and 2013, we conducted an active surveillance at the main hospital of Cordoba to establish the etiologic diagnosis of the undifferentiated tropical febrile illness (UTFI) cases. UTFI is defined as a fever without an infection focus in the initial physical examination or in basic laboratory tests. Patients in acute phase were accompanied by prodromal symptoms, including myalgia, arthralgia, headache, asthenia, chills, icterus, dyspnea, abdominal pain, rash, and nausea. Samples were collected on admission and at discharge. Clinical and epidemiological data were collected for each patient. Microscopic agglutination test (MAT) was performed. *Results.* The 28 leptospirosis cases presented the following gender distribution: male (n=24) and female (n=4). The duration of hospitalization was 10.39 days. The main symptoms and clinical manifestations were fever, headache and nausea, vomiting, and abdominal pain, all of which occurred in up to 60% of patients. Of the 28 cases studied, 4 were fatal. The most frequent infecting serogroups were Ballum and Canicola. *Conclusion.* Leptospirosis is a common cause of undifferentiated tropical febrile illness in Colombia; it is important to establish ongoing and accurate surveillance for acute febrile illness to facilitate the detection of cases of leptospirosis.

1. Introduction

Human leptospirosis is caused by bacteria that belong to the genus *Leptospira.* This genus comprises at least 22 species grouped into three categories containing pathogenic, intermediate, and saprophytic species. Currently, there are more than 250 named, potentially pathogenic serovars [1].

The disease results in high morbidity and considerable mortality in areas of high prevalence. It is estimated that around 10,000 cases of severe leptospirosis are hospitalized annually worldwide. The disease is usually endemic in areas with rainy season, humidity, close human contact with livestock, poor sanitation, and workplace exposure to the organism [2]. In recent years, a new trend in human leptospirosis outbreaks has been observed related to recreational activities among wildlife (a form of tourism that is becoming increasingly popular) and army expeditions, either for training or for combat-related purposes in similar environments [3]. A systematic literature review of Leptospirosis Burden Epidemiology Reference Group (LERG) reports an estimated global annual incidence of endemic and epidemic human leptospirosis ranging from 5 to 14 cases per 100,000. Endemic human leptospirosis rates have varied by region from 0.5/100,000 in Europe to 95/100,000 in Africa. Based on global data collected by International Leptospirosis Society surveys, the incidence was estimated to be 350,000–500,000 severe leptospirosis cases annually [4].

However, data emerging from prospective surveillance studies suggest that most human leptospiral infections in endemic areas may be mild or asymptomatic. Development of more severe outcomes likely depends on three factors: epidemiological conditions, host susceptibility, and pathogen virulence [5]. Fatality rates reported worldwide vary from 5% to 30%. This epidemiological picture is not reliable because

in many areas the occurrence of the disease is not well documented. In addition, mild cases may not be diagnosed as leptospirosis [6]. Case fatality for pulmonary hemorrhagic syndrome and Weil's disease is more than 10% and 70%, respectively [5].

The Caribbean and Latin America, the Indian subcontinent, Southeast Asia, Oceania, and to a lesser extent Eastern Europe are the most significant foci of the disease, including areas that are popular travel destinations. According to Pappas et al., 2007, the annual incidence of leptospirosis cases per 100.000 in Latin America shows the following distribution: Costa Rica 67,2, Uruguay 25, Cuba 24,7, Brazil 12,8, Ecuador 11,6, Argentina 9, Venezuela 3,8, Chile 1,6, Colombia 1,6, and Panama 1,3. In Seychelles there were 432,1, Trinidad and Tobago 120,4, Barbados 100,3, Jamaica 78, Sri Lanka 54, Thailand 48,9, El Salvador 35,8, New Zealand 26, and Nicaragua 23,3 [3].

In Colombia, leptospirosis has been considered an event of mandatory notification to the National Surveillance System (SIVIGILA) since 2007 and has gained interest from health authorities, especially due to the increase in cases related to the rainy season and floods that have occurred in recent years. Knowledge about the characteristics of the disease is essential for improved surveillance and control of such events. Despite the increase in the notification of cases of leptospirosis per year in the country [7], no data are available regarding the current situation of the disease; most recent publications in Colombia have been focused on the characterization of outbreaks or have involved seroprevalence studies. In recent and previous study, we documented that leptospirosis was the most common cause of undifferentiated tropical fevers in this region [8].

The aim of the present study was to describe and analyze the clinical and epidemiological status in 28 confirmed cases of human leptospirosis at the main public hospital of Cordoba.

2. Material and Methods

2.1. Study Area. Cordoba is a region in the Caribbean Sea on the northern coast of Colombia. Monteria has an altitude range of 20-100 m above sea level, is covered by tropical dry forest vegetation, has an average temperature of 24°C, and receives between 1000 and 2000 mm^3 of rain per year. Cordoba is a state devoted to agriculture and livestock production. Geographically the department of Córdoba can be divided into two regions based on ecosystems, separated by the mountains (Serranía) of San Jerónimo and influenced by its two main rivers: the Sinú and San Jorge. These two rivers, their valleys, and the mountain range that separates them create habitat conditions that could influence the ecology and distribution of *Leptospira* serogroups in the department of Cordoba.

2.2. Patients and Data Collection. Between 2012 and 2013, we managed an effective surveillance at the main hospital of Cordoba to determine the etiologic diagnosis of the undifferentiated tropical febrile illness (UTFI) cases. UTFI is defined as a fever without an infection focus in the initial physical examination or in basic laboratory tests [9]. In Colombia, UTFI are frequent infections; some of these strike during the year and in rainy or even during dry season (The Indian Society of Critical Care Medicine Tropical fever G). Throughout our analysis, there was no El Niño phenomenon nor floods and the occurrence of rain was normal. The examined area is not endemic for yellow fever or West Nile virus disease

Patients with acute phase were admitted to the emergency ward with febrile illnesses escorted by prodromal symptoms typical of UTFI infection, including myalgia, arthralgia, headache, asthenia, chills, icterus, dyspnea, abdominal pain, rash, and nausea. Patients were registered in a clinical trial for UTFI at the University of Cordoba. Serum samples were taken on admission and at discharge. Clinical and epidemiological data were collected for each patient during their hospital stay including age, sex, municipal origin, occupation, history of illness (date of onset of disease and date of admission), symptoms, physical findings, laboratory findings, and medical care. Case definition of leptospirosis was defined as specified by the National Health Institute of Colombia [7, 10].

2.3. Sampling. From each patient, we collected one acute phase and one convalescent phase (15-20 days after illness) peripheral venous blood sample. Seroconversion was described as negative serology which became positive in a convalescent serum sample; therefore the escalation of detectable antibodies (usually a fourfold titer increase) between the first and second sample was used as a definition in the present study.

2.4. Leptospirosis Diagnostics. Microscopic agglutination test (MAT) was performed according to the standardized protocol in the Reference Laboratory of Spirochetes, Institute of Tropical Medicine "Pedro Kourí", Cuba. In the test were used 16 different serovars: *L. interrogans* Icterohaemorrhagiae Copenhageni strain M20, *L. interrogans* Icterohaemorrhagiae Icterohaemorrhagiae strain FGA; *L. interrogans* Canicola Canicola strain Hond Utrecht IV; *L. interrogans* Pomona Pomona strain Pomona; *L. borgpetersenii* Ballum Castellonis strain Castellon 3; *L. borgpetersenii* Sejroe Sejroe strain M 84; *L. interrogans* Sejroe Hardjo strain Hardjoprajitno; *L. interrogans* Sejroe Wolffi strain 3705; *L. interrogans* Pyrogenes Pyrogenes strain Salinem; *L. interrogans* Hebdomadis Hebdomadis strain Hebdomadis; *L. borgpetersenii* Tarassovi Tarassovi strain Perepelitsin; *L. interrogans* Australis Australis strain Ballico; *L. interrogans* Autumnalis Aautumnalis strain Akiyami A; *L. interrogans* Bataviae Bataviae strain Swart; *L. noguchii* Panama Panama strain CZ 214K; *L. kirschneri* Cynopteri Cynopteri strain 3522 C*; L. borgpetersenii J*avanica Javanica strain V Batavia 46; *L. kirschneri* Grippothyphosa Grippothyphosa strain Moskva V; *L. biflexa* Semaranga Patoc strain patoc I. Titers ≥1:160 were considered positive.

In addition, the Panbio® *Leptospira* IgM ELISA (Catalog E-LEP01M/E LEP01M05, Queensland, Australia) was used for serological screening. The test has demonstrated a diagnostic sensitivity of 96.5% and specificity of 98.5%.

2.5. Conventional PCR Assay. PCR was performed for 27 of the samples from blood anticoagulated with EDTA or sodium citrate. The PCR amplified a fragment of 146 bp of the lipoprotein gene *lipL32*, which is found only in pathogenic strains of *Leptospira* spp. The primers used were pfLp32-1 5′-TAGAATCAAGATCCCAAATCCTCC-3′ and pfLp32-2 5′-CCAACAGATGCAACGAAAGATCC-3′ for forward and reverse primers, respectively [11].

The PCR was performed in a 50 μL volumes containing 0.125 U of *Taq* polymerase (QIAGEN, Germany), 1ul of each primer, PCR buffer 1X (dNTP 0.02 mM, $MgCl_2$ 0.25 mM, KCl 0.025 M, Tris HCl 0.025 M, mg/mL), and 5 μL of template DNA in a final volume of 50 μL. PCR cycling conditions were carried out in automated MJ Research PTC-100TM thermal cyclers as follows: 1 cycle at 95°C for 3 min, followed by 40 cycles of 20 sec at 95°C, 30 sec at 60°C, and 30 sec at 70°C. PCR products were analyzed on a 2% agarose gel and visualized with SYBR safe in transilluminator (Biorad).

2.6. Differential Diagnosis. To establish other tropical prevalent pathologies in the studied area, serodiagnosis tests for malaria, hantavirus, *Rickettsia, Brucella*, Hepatitis A, and Hepatitis B were carried out [8].

2.7. Ethical Aspects. The investigation committee of the Institute of Tropical Biological Research of the University of Cordoba and Hospital San Geronimo of Monteria permitted the ethics protocol, and knowledgeable permission was achieved from all enrolled patients. Patients were anonymized using a numeric code. The study incorporated procedures, management and conservation of samples, and technical-administrative procedures for health research required by resolution 8430 of the Ministry of Health of Colombia, in 1993 [12], and declaration of Helsinki for ethical and medical research in human subjects [13].

3. Results

3.1. Sociodemographic and Geographic Characteristics. The 28 leptospirosis cases presented the following gender distribution: male (n=24) and female (n=4). The mean age was 26.7 years. Six people were linked to farming, while the other 22 were engaged in other activities. Of the 28 cases, 14 belonged to each of the two geographic regions, with 11 men and 3 women; 4 people died, two in each region. The age of those affected was statistically the same in the two regions, 27.7 years in San Jorge region and 25.8 years in Sinú river region. Figure 1 shows the serogroups in the two studied regions. The prevalence of serogroups was different; in the San Jorge region were identified Pomona (n=3), Ballum (n=3), Cynopteri (n=3), Icterohaemorrhagiae (n=2), Sejroe (n=1), Tarrassovi (n=1), and 1 negative. Conversely, in the Sinú region the distribution was Canicola (n=4), Grippothyphosa (n=2), Australis (n=1), Patoc (n=1), Hebdomadis (n=1), Pyrogenes (n=1), Panama (n=1), Ballum (n=2), and 1 negative. Ballum was the only serogroup present in the two regions, in San Jorge region (n=3) and in Sinú region (n=2), and the Ballum was most prevalent in the study.

Figure 1 shows the distribution of cases of leptospirosis serogroups and annual incidence in the different affected municipalities. The analysis of the distribution of annual incidence (cases per 100,000 inhabitants) identified two clusters with epidemic activity, one in the San Jorge region and another in the Sinú region. The San Jorge conglomerate was formed by the municipalities of Ayapel (I=14.2), La Apartada (I=6.8), Buenavista (I=4.7), and Montelibano (I=3.9). The Sinú conglomerate was made up of the municipalities of Arboletes (I=5.2), Los Córdobas (I=4.5), Puerto Escondido (I=3.6), and most likely Valencia (I=4.9). In the conglomerate of the regions of San Jorge and sinu the serogroups are present.

3.2. Clinical Presentation of the Disease. The average duration of illness was 6.7 days and the duration of hospitalization was 10.4 days. The main symptoms and clinical manifestations are shown in Table 1. The main clinical symptoms were fever, headache, and nausea. Vomiting and abdominal pain presented in up to 60% of patients. Other important symptoms were myalgia, arthralgia, chills, jaundice, hepatomegaly, coluria, mucocutaneous pallor, and dyspnea. The main clinical manifestations were thrombocytopenia, lymphocytosis, neutrophilia, and leukocytosis. The clinical picture was statistically the same in patients from the two geographical regions; minor clinical manifestations (not shown) were also statistically the same between the two geographical regions. Hemorrhagic manifestations were infrequent; only 5 patients presented with gingivorrhagia. Two patients presented with malaria coinfection and another with dengue and hantavirus.

The major antibiotics administered to the patients were as follows: 13 (46.4%) were given ceftriaxone, 4 (14.3%) clindamycin, 4 (14.3%) vancomycin, and 3 (10.7%) penicillin. Acetaminophen was administered to 18 (64.3%) of the patients.

Three categories were established taking into account the compatibility of the leptospiral diagnostic leading to the following final diagnoses: (i) high compatibility with leptospirosis or hemorrhagic fevers in 12 cases; (ii) incompatibility in 6 cases; (iii) slight compatibility in 10 cases. Of 28 cases studied only 4 were classified with a diagnosis of leptospirosis at discharge; one of the patients who presented with IgM ELISA positive and MAT negative died.

3.3. Lethal Cases of Leptospirosis in Córdoba. Of the 28 cases studied, 4 were fatal (Table 2). One of these cases was negative by MAT and positive by IgM but he presented signs and symptoms consistent with the disease. Four cases were considered febrile syndrome or icteric hemorrhagic syndrome and only one of them was diagnosed as leptospirosis.

3.4. Conventional PCR Results. We attempted to amplify a fragment of the lipL32 gene; however, all blood samples were negative.

4. Discussion

The present study shows a cohort of 100 patients who were enrolled in a tropical febrile disease trial; 28 patients were

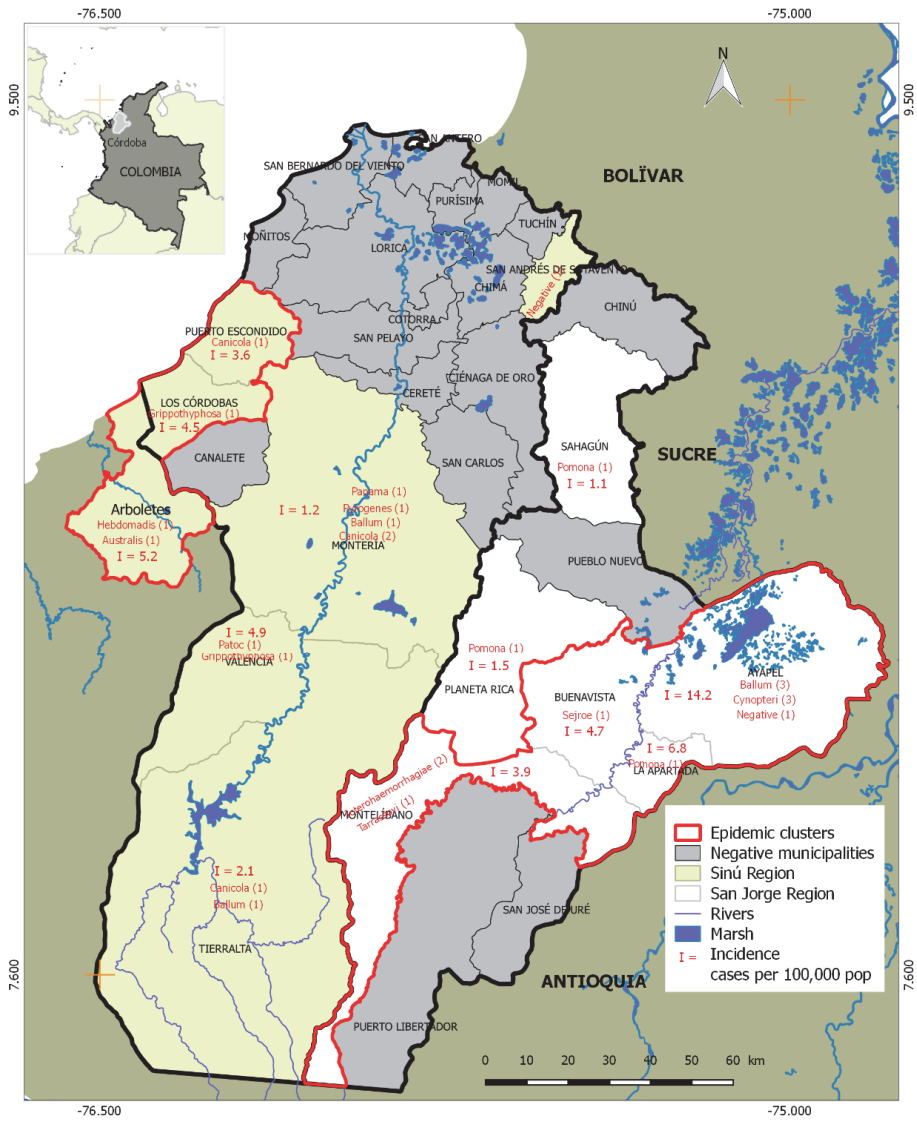

Figure 1: Distribution of leptospiral serogroups in the department of Córdoba.

diagnosed with leptospirosis by laboratory tests of MAT and ELISA IgM. Leptospirosis is a major concern because of its high prevalence of 28% in this region of the country [8].

In Colombia since 2009, 16.989 cases have been notified to SIVIGILA but only 7.481 (44%) have been confirmed. A national incidence of 1.15/100.000 has been established. In Córdoba state in 2016 were reported 55 cases; only one of them was confirmed as fatal [7]. These data demonstrate the importance of studying this disease, particularly in endemic and tropical areas. (Table 3).

The highest incidence of leptospirosis was in the area of Ayapel (San Jorge region), where there is a large lagoon with an important agricultural activity and artisanal fishing. It is likely that being a flood prone area resulted in increased incidence of leptospirosis. In addition, it is an area lacking basic public services, which contributes to the proliferation of tropical diseases. The area is also affected by contamination with heavy metals from illegal mining and spraying of pesticides. The next highest frequency of leptospirosis was Monteria, an important urban area with semirural areas and nearby rivers. The socioeconomic conditions of patients affected in Monteria also make them prone to leptospirosis, since there are no public services such as sanitary sewage and potable water.

Some studies have reported the circulation of *Leptospira* spp. in Colombia, from the late 1960s, with seroprevalence ranges from 3.9% to 35.8%. However, an accurate situation of the disease is unknown in most regions of Colombia [14–16]. In a study carried out in Villavicencio state of Meta (Colombia) in the south east and near the Venezuelan border, the seroprevalence of *Leptospira* spp. in an apparently healthy population and in groups at risk was determined. In the low risk group the seroprevalence was 5.2% and 19% for groups at risk. Three factors were found to be associated with higher seroprevalence: rural social level, having a pet dog, and contact with rodents in the workplace [17]. Although our

Table 1: Main symptoms, clinical manifestations, and laboratory findings of patients with leptospirosis.

Main symptoms and clinical manifestations	Frequency	%	Frequency by area		p-value
			San Jorge	Sinú	
Fever	28	100,0	14	14	1.000
Headache	20	71.4	11	9	0.403
Nausea	19	67.9	9	10	0.686
Vomiting	18	64.3	9	9	1.000
Abdominal pain	17	60.7	11	6	0.053
Myalgia	16	57.1	9	7	0.445
Arthralgia	16	57.1	9	7	0.445
Chills	15	53.6	9	6	0.256
Jaundice	13	46.4	6	7	0.705
Hepatomegaly	13	46.4	5	8	0.256
Coluria	11	39.3	5	6	0.699
Mucocutaneous pallor	10	35.7	5	5	1.000
Dyspnea	9	32.1	5	4	0.686
Thrombocytopenia	20	71.4	12	8	0.094
Lymphocytosis	11	39.3	6	5	0.699
Neutrophilia	11	39.3	6	5	0.699
Leukocytosis	8	28.6	4	4	1.000

seroprevalence is higher (28%), the same factors associated with the increase of seroprevalence were found in the present study in patients from rural areas.

In a study carried out during 2007 and 2008, sera were collected from 220 nonmalarial acute febrile and convalescent patients from the rural and urban zones of Necocli, Turbo, and Apartado, areas close to our study area [18]. These authors found a frequency of infection for leptospirosis of 14.1%, as well as 12 coinfection cases of leptospirosis-dengue and one of leptospirosis-rickettsiosis-dengue. Although the frequency of infection for leptospirosis in our study was double (28%), we found two coinfections, namely, leptospirosis-malaria and leptospirosis-dengue-hantavirus. These coinfections could be common in this region of the country due to the endemicity of tropical diseases as dengue, rickettsiosis, and hantavirus.

Seroprevalence studies may represent a good indicator of the circulation of the pathogen. In that sense, in Uraba, Antioquia near the Panama border, a seroprevalence of 12.5% was detected [14]. No differences were observed according to race, gender, occupation, age, living conditions, or time living in the area. Our results are higher than others in a similar area near the present study. Other studies in the same region have found that the practice of barefoot walking in domestic environments represented a 4.27-fold higher risk for leptospirosis [19]. Although the present study did not evaluate these aspects, barefoot walking is a common practice in the study area; it is necessary to carry out follow-up studies in these municipalities to evaluate this factor.

In a study carried out in Tunja (Colombia), a prevalence of 21.7% in humans was found [20]. In the same study, in the canine population a seroprevalence of 67.2% was found; in another study, in three municipalities of Tolima (Colombia) a seroprevalence of 21.4% was found [21]. These findings suggested that dogs are potential reservoirs of *Leptospira* in these areas of the country.

MAT is usually positive 10 to 12 days after the onset of the first symptoms and clinical signs, but seroconversion may occur as early as 5-7 days after the onset of the disease. The antibody response can be delayed if antibiotic therapy is started before the test is performed. The antibody titer should be interpreted in light of the date of collection of the sample in relation to the first clinical signs; the evolution of antibody titers between the two or three successive samples; the causal serogroup; the treatment given [6]. In the present study all patients were positive for ELISA (IgM) and two were negative for MAT. 39% (11/28) of the patients presented a seroconversion with an increase in titers up to four times. Caution is necessary in the interpretation of serological data. Several factors must be taken into consideration, including the technique used, the serogroup involved, the chronological order of the samples taken during the disease, and the treatment with antibiotics if any. The specific gender test tends to be positive earlier in the course of the disease than the MAT.

The presence of cases of Leptospira of the present study is corroborated with the study in Córdoba, where a high seroprevalence of 75.8% in humans is established [22]. They also detected seven L. interrogans sensu lato strains isolated from different sources (pigs, dogs, and water). High seroprevalence in humans, concomitant to isolation of strains, demonstrates that, in Cordoba, transmission exists among animals, the environment, and humans [22].

Regarding clinical symptoms, signs, hematological alterations, and hemorrhagic presentations, there were no remarkable findings to allow us to differentiate among the frequent tropical acute infectious diseases found in that area. It is difficult to differentiate between leptospirosis, dengue,

Table 2: Lethal cases of leptospirosis in Córdoba.

Id/Gender Years Procedence	Devol	Dhos	Clinical manifestations	Blood count	IgM (Value)	MAT 1 (title)	MAT 2 (title)	MAT Results (serogroups)	Antibiotic therapy	Diagnosis at admission	Discharge diagnosis
1/F 14 Puerto Escondido.	7	13	Fever, headache, nausea, jaundice, vomiting, hyporexia, asthenia, coluria, cough abdominal pain, diarrhea, abdominal examination, mucocutaneous pallor, dyspnea, pleural effusion, ascites, hepatomegaly, tachypnea.	Leukocytosis, mild anemia, grade I thrombocytopenia	30.1 Positive	160	640	canicola	DA, CN, AMP, SAM	Viral hepatitis, abdominal pain	Hepatic failure, hepatitis under study
2/M 14 San Andrés Sotavento.	4	2	Fever, headache, nausea, vomiting, chills, abdominal pain, diarrhea, abdominal examination, convulsions, mucocutaneous pallor, dyspnoea, pleural effusion, ascites, hepatomegaly, splenomegaly, tachycardia, tachypnea, algidity, hypotension, bilateral infiltration, hypoventilation.	Neutrophilia, mild anemia, severe leukopenia, grade III thrombocytopenia.	31.6 Positive	0	0	Negative	DA,CRO,ACET	Dengue	Leptospirosis
3*/M 47 Montelibano	10	6	Fever, headache, nausea, vomiting, chills, retroocular pain, abdominal pain, diarrhea, abdominal examination, seizures dyspnea, pleural effusion, tachypnea. Crepitos.	Lymphocytosis, mild anemia, mild leukopenia, grade II thrombocytopenia.	14.7 Positive	320	640	icterohaemorrhagiae	DA, Antimalarico.	Malaria	Malaria
4/M 45 Ayapel	5	38	Fever, headache, nausea, vomiting, chills, asthenia coluria, hyperconjunctival injection, abdominal pain, abdominal examination, mucocutaneous pallor, dyspnea, pleural effusion, asitis, hepatomegaly, tachycardia, tachypnea, hypoventilation.	Leukocytosis, neutrophilia, grade II thrombocytopenia.	27.8 Positive	40	160	Ballum	CN,SAM,MEM.	Hemorrhagic icteric syndrome	Unspecified jaundice, unspecified renal failure

F: female, M: male, Devol: days of evolution at admission, Dhosp: days of hospitalization, ICU: intensive care unit, DA: clindamycin, CN: gentamicin, AMP: ampicillin, SAM: ampicillin & sulbactam; CRO: ceftriaxone, MEM: meropenem, ACET: acetaminophen. *Patient with underlying arterial hypertension, required ICU admission, coinfection with malaria.

Table 3: Total cases of leptospirosis reported by national public health surveillance system, Colombia, 2009-2017.

Years	Number of reported cases	Suspected	Confirmed	(%)
2009	1815	827	988	54.44
2010	2261	1026	1235	54.62
2011	2478	1237	1191	48.06
2012	1986	943	1043	52.52
2013	1940	1073	867	44.69
2014	2305	1368	846	36.70
2015	2007	1225	782	38.96
2016	2197	1635	529	24.07
2017*	113	-	-	-
Total**	16989	9334	7481	44.03

Source: SIVIGILA, National Institute of Health, Colombia, 2009-2017. ∗ Leptospira cases to epidemiological week 12 of 2017. ∗∗Not including 2017.

and malaria due to the overlap in clinical symptoms, signs, hemorrhagic presentations, and hematological alterations (Table 1). For example, thrombocytopenia was found in 80% of patients with dengue and 60% of those with leptospirosis. Epistaxis, petechiae, gingival hemorrhage, leukopenia, neutrophilia, and anemia were seen without a remarkable predominance in dengue and leptospirosis. We studied only 28 patients and it may be possible that some pathologies were unrepresented, making it difficult to establish a definitive conclusion for this disease.

Leptospirosis is among the leading zoonotic causes of morbidity worldwide and accounts for numbers of deaths, which approach or exceed those for other causes of hemorrhagic fever. Highest morbidity and mortality occur in resource-poor countries, which include regions where the burden of leptospirosis has been underappreciated [5]. The lethality in our study was 14%, but of the 4 patients who died, only 1 was diagnosed with leptospirosis, demonstrating the complexity of diagnosis due to the similarity of the clinical presentation of the cases that can be confused with dengue, malaria, hantavirus, arenaviruses, rickettsiosis, and salmonellosis. Regarding the antibiotics administered to the patients, only 2 (57%) received ceftriaxone and penicillin, both with activity against *Leptospira*, and the remaining 2 were treated erroneously.

Despite some limitations such as the small number of patients that do not allow definitive conclusions, the study allowed us to define some clinical and epidemiological features of patients with leptospirosis in the state of Córdoba. It also highlighted some failures in the clinical diagnosis and management of cases of leptospirosis.

In conclusion, it is important to establish ongoing and accurate surveillance for acute febrile illness to facilitate the detection of cases of leptospirosis. Early recognition and treatment of patients have been shown to reduce the duration and severity of illness. Surveillance is also useful for identifying outbreaks early where mass prophylaxis could be considered, especially in areas with high numbers of cases and limited access to healthcare [23]. These results will guide interventions in health and environmental control in the area of this disease forgotten or confused with other endemic febrile syndromes such as dengue or malaria.

Data Availability

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

Conflicts of Interest

The authors do not have any conflicts of interest.

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Research Article

High Intraspecific Genetic Diversity of *Nocardia brasiliensis*, a Pathogen Responsible for Cutaneous Nocardiosis Found in France: Phylogenetic Relationships by Using *sod* and *hsp65* Genes

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This study aims at genetic characterization and phylogenetic relationships of *Nocardia brasiliensis* focusing by using housekeeping *rrs*, *hsp65,* and *sodA* genes. *N. brasiliensis* is the species responsible for 80% of cases of actinomycetoma, one form of cutaneous nocardiosis which occurs mainly in tropical regions reaching immunocompetent patients in which the disease can lead to amputation. We analyze 36 indigenous cases of *N. brasiliensis* that happened in France. Phylogenetic analysis targeting *rrs* gene showed no robustness at phylogenetic nodes level. However, the use of a concatenation of *hsp65* and *sodA* genes showed that the tested strains surprisingly ranked in 3 well-defined genotypes. Genotypes 2 and 3 were phylogenetically closer to each other and both diverged from genotype 1 sustained by a high bootstrap of 81%. This last genotype hosts all the cases of pulmonary forms (3), the sole cerebral form, and almost all the cases of immunocompromised patients (3 out of 4). Moreover, excepting one of them, all the strains belonging to this group present a susceptibility to imipenem which is not the case in the other genotypes that rarely count among them strains being susceptible to this drug. The haplotype diversity (Hd) of *hsp65* (0.927) and *sodA* (0.885) genes was higher than that of *rrs* (0.824). For this gene, we obtained 16 polymorphic sites whereas, for *hsp65* and *sodA* genes, up to 27 and 29 were identified, respectively. This study reveals that these two genes have an important genetic discriminatory power for the evaluation of the intraspecies genetic variability of *N. brasiliensis* and they may be useful for identification purposes at species level. This study also reveals the possible existence of a new species harbored by genotype 1.

1. Introduction

Nocardia is a genus belonging to the aerobic actinomycetes group of bacteria which are Gram-positive bacilli and showing branching filamentous forms [1]. They are saprophytic ubiquitous bacteria which can be found in several environments such as fresh water and saltwater, soil, dust, decaying vegetation, and decaying fecal deposits from animals [1]. Nevertheless, these environmental bacteria can be opportunistic pathogens and lead to human

infectious diseases called "nocardiosis" [2]. Nocardiosis can be discriminated into two groups: invasive infection, mainly caused by *N. asteroides,* presenting commonly as pneumonia in patients who are immunocompromised, have underlying chronic lung disease, and are with a possible dissemination to other organs [3], and cutaneous infection via a cut or abraded skin, which can be manifest clinically as (i) abscess and cellulitis, (ii) lymphangitis, (iii) skin infection secondary to dissemination, and (iv) actinomycetoma. This latter group is the most amazing infection due to their severity characterized by the presence of tumefaction, subcutaneous nodules, destructive granulomata, fistulas, and pus [2, 4].

N. brasiliensis is the species isolated from the majority (approximately 80%) of cases of cutaneous nocardiosis, especially in actinomycetoma [2]. This species is more commonly isolated in areas with tropical or subtropical climates such as South America, Asia, and Africa. Due to false diagnosis, rural lifestyles, and poor access to care in these countries, *N. brasiliensis* nocardiosis constitutes a real public health problem that can lead, in the absence of treatment, to amputations and death in young populations. On the basis of epidemiological surveys conducted in France, the number of cases of nocardiosis between 2000 and 2007 according to the French Nocardiosis Observatory (OFN) was 607 with *N. farcinica* and *N. nova* being the most frequent species [5]. However, no data currently exists on the phylogenetic relationships between the indigenous *N. brasiliensis* strains of tropical origin and native strains isolated in France. Routine genus/species identification of *Nocardia* was based on macroscopic, microscopic, and biochemical characteristics. The methods described by Boiron et al. [6] were used to determine the decomposition of adenine, casein, hypoxanthine, tyrosine, and xanthine. In addition to the phenotype-based methods, species-level identification is mainly genetically based, nowadays. Classically, 16S rRNA *(rrs)* gene sequencing is generally used for the species-level identification [7, 8], but it fails to discriminate among some species of *Nocardia* because it does not have enough polymorphism to differentiate them at the species level. Multilocus sequence analysis (MLSA) using concatenated sequences of several housekeeping genes such as superoxide dismutase A *(sodA)* and heat shock protein 65 *(hsp65)* has been increasingly used to provide higher accuracy and discriminatory power in the molecular identification of *Nocardia* spp. [9, 10]. Indeed, a recent study seeking to identify new molecular targets shows that the polymorphism observed in the *sodA* gene sequence contains variable regions that allow the discrimination of closely related *Nocardia* species [9].

The aim of the present study was to perform a genetic characterization and assess the phylogenetic relationships of *Nocardia brasiliensis* focusing on using housekeeping *rrs*, *hsp65,* and *sodA* genes, for 36 autochthonous *N. brasiliensis* strains isolated in France and analyzed by the OFN between 2002 and 2012. Phenotypic characterization was also conducted by assessing antimicrobial resistance profiles, metabolic profiles, and culture condition.

2. Materials and Methods

2.1. Bacterial Strains and Culture Media. A collection of 36 human clinical strains of *N. brasiliensis* was studied (Table 1). All strains were identified as such, at species level by the French Nocardiosis Observatory (OFN) by genetic approach. Moreover, six *Nocardia* reference strains belonging to *N. brasiliensis* clade [9] were also used: *N. brasiliensis* ATCC 19296^T (unknown), *N. altamirensis* DSM 44997^T (karstic cave), *N. boironii* DSM 101696^T (pus sample), *N. iowensis* DSM 45197^T (garden soil), *N. tenerifensis* DSM 44704^T (rhizosphere), and *N. vulneris* DSM 45737^T (human leg wound). Prior to the assays, strains were cultured 72 hours in Bennett medium (made in the laboratory) aerobically at 37°C.

2.2. Growth Test on Culture Media. From 0.5 McF bacterial suspension, bacterial growth was evaluated on three culture media: (i) bromocresol purple (BCP) (Biomérieux, Marcy l'étoile), (ii) Bennett (made in the laboratory), and (iii) Middlebrook (Biomérieux, Marcy l'étoile). One hundred microliters from bacterial suspension standardized was inoculated on the different plate of culture media. The plates were incubated at 37°C and the observations were performed at 48, 72, and 96 hours.

2.3. Antimicrobial Susceptibility. The susceptibility of the isolates to different antimicrobials was determined by disk diffusion method with a panel of 31 antibiotics (Biorad, Marnes-la-Coquette France) on Muller Hinton E medium (Biomérieux, Marcy l'étoile, France). Susceptibility testing was done with amikacin 30 μg, gentamycin 15 μg, tobramycin 10 μg, ciprofloxacin 5 μg, levofloxacin 5 μg, moxifloxacin 5 μg, minocycline 30 μg, doxycycline 30 μg, tigecycline 15 μg, cefotaxime 30 μg, ceftriaxone 30 μg, cefepime 30 μg, cefuroxime 30 μg, amoxicillin 25 μg, amoxicillin + clavulanic acid 20/10 μg, ampicillin 10 μg, ertapenem 10 μg, meropenem 10 μg, imipenem 10 μg, vancomycin 30 μg, pristinamycin 15 μg, erythromycin 15 μg, trimethoprim + sulfamethoxazole 1.25/23.75 μg, rifampicin 30 μg, and linezolid 30 μg.

From visible colonies, bacterial suspension was done in sterile water, using a cotton swab to obtain a concentration of 0.5 McFarland according to the Clinical and Laboratory Standards Institute standard M24-A2 [11]. Seeding was done according to the swab method. In this latter, the bacterial inoculum was spread on the agar using a sterile cotton swab in three different directions. The disks were dispensed with a dispenser and the plates were incubated at 37°C for 72 hours and read manually according to the thresholds defined in the recommendations of the SFM 2013 [12].

2.4. Substrate Degradation. The methods of Boiron et al. [6], Goodfellow et al. [13, 14], and Goodfellow and Lechevalier [15] were used to determine the decomposition of adenine, casein, and uric acid [9]. Clinical strains of *N. brasiliensis* and the strains of species belonging to the *N. brasiliensis* clade (*N. brasiliensis, N. altamirensis, N. iowensis, N. tenerifensis*, *N. boironii,* and *N. vulneris*) were tested [9]. Strains *N. boironii*

Table 1: Table of clinical strains including the type of tropism observed in host, their corresponding *sodA/hsp65* genotypes, and drug phenotypes.

Sample date	Nature of sampling	Patient record	Immunosuppressed	Genotype *sodA/hsp65*	Tropism	Pristinamycin	Imipenem	Amikacin	Trimethoprim + sulfamethoxazole
04/2002	Intraoperative tissue	02.56	No	G3	Cutaneous	R	R	S	S
12/2003	Pus from cutaneous thigh abscess	04.21	Yes	G1	Cutaneous	R	S	S	S
2004	Pus from cutaneous abscess	04.101	No	G3	Cutaneous	R	R	S	S
07/2005	Expectoration then LBA	05.64	Yes	G1	Lung	R	S	S	S
01/2005	Skin biopsy	05.12	No	G1	Cutaneous	R	S	S	S
07/2005	Phalanx biopsy	05.63	No	G3	Cutaneous	R	R	S	S
2005	CSF	05.77	No	G1	Brain	R	R	S	S
11/2005	-	3247	No	G3	Unknown	R	S	S	S
2007	Cutaneous abscess	07.168	No	G3	Cutaneous	R	R	S	S
10/2008	Wound of forehead	08.178	No	G3	Cutaneous	R	S	S	S
11/2008	Subcutaneous abscess	08.188	No	G3	Subcutaneous	R	R	S	S
10/2008	Elbow abscess	2985	No	G3	Cutaneous	R	R	S	S
03/2008	-	9044	No	G2	Unknown	R	R	S	S
03/2009	Pus of leg abscess	09.71	No	G2	Cutaneous	R	S	S	S
04/2009	Bronchial aspiration	09.106	No	G1	Lung	R	S	S	S
10/2009	Bronchial aspiration	09.244	Yes	G1	Lung	R	S	S	S
12/2009	Finger skin	09.280	No	G3	Cutaneous	R	R	S	S
10/2009	Pus from the lip	10.16	No	G3	Cutaneous	R	R	S	S
02/2010	Finger abscess	10.35	No	G2	Cutaneous	R	R	S	S
05/2010	Hand abscess	10.82	No	G3	Cutaneous	R	R	S	S
05/2010	Toe abscess	10.93	Yes	G3	Cutaneous	R	R	S	S
09/2010	Leg wound	10.146	No	G3	Cutaneous	R	R	S	S
11/2010	Pus finger	10.180	No	G3	Cutaneous	R	R	S	S
11/2010	Leg wound	12786	No	G2	Cutaneous	R	R	S	S
04/2010	-	14229	No	G2	Unknown	R	S	S	S
07/2010	Sepsis hand	45762	No	G3	Cutaneous	R	R	S	S
2011	Pus	11.44	No	G2	Cutaneous	R	R	S	S
05/2011	Cutaneous abscess	11.73	No	G1	Cutaneous	R	S	S	S
05/2011	Hand wound	11.80	No	G1	Cutaneous	R	S	S	S
08/2011	Cutaneous	11.116	No	G2	Cutaneous	R	R	S	S
2011	Thigh abscess	11.140	No	G2	Cutaneous	R	R	S	S
09/2011	Knee wound	11.151	No	G3	Cutaneous	R	R	S	S
2011	Hand wound	11.172	No	G3	Cutaneous	R	R	S	S
12/2011	Cutaneous abscess	11.189	No	G3	Cutaneous	R	R	S	S
2012	-	12.08	No	G1	Unknown	R	S	S	S
02/2012	Cutaneous abscess	12.28	No	G3	Cutaneous	R	R	S	S

DSM 101696^T, *N. brasiliensis* ATCC 19296^T, and *N. vulneris* DSM 45737^T were incubated at 37°C, and *N. altamirensis* DSM 44997^T, *N. tenerifensis* DSM 44704^T, and *N. iowensis* DSM 45197^T were incubated at 28°C [9]. The readings were performed at 3, 7, 10, 14, 17, and 21 days.

2.5. Methods of DNA Extraction. DNA extraction from *Nocardia* strains was performed with achromopeptidase according to the method reported by Rodríguez-Nava et al. [10]. Colonies were picked off with a loop, and one loopful was suspended in 200 μL of sterile water containing a dozen glass beads and vortexed for 5 minutes. The mixture was then incubated for 15 minutes at 70°C. The suspension supplemented with 3.4 μL of achromopeptidase (Sigma, Steinheim, Germany) at 10 U/mL was incubated at 55°C for 15 minutes. The suspensions were then centrifuged for 5 minutes at 13,000 rpm. The supernatants were stored at −20°C until use.

2.6. Amplification and Sequencing

Gene rrs. A 606-bp fragment of the rrs gene was amplified with primers Noc1, 5′-GCTTAACACATGCAAGTCG-3′, and Noc2, 5′-GAATTCCAGTCTCCCCTG-3′, and PCR program and reaction mixture were carried out according the recommendations of Rodríguez-Nava et al. [10].

Gene hsp65. A 441-bp fragment of the *hsp65* gene encoding the 65-kDa heat shock protein was amplified with primers described by Telenti et al. (TB11: 5′-ACCAACGATGGTGTGTCCAT-3′ and TB12: 5′-CTTGTCGAACCGCATACCCT-3′) [16]. PCR program and reaction mixture were carried out according to the recommendations of Sánchez-Herrera et al. [17].

Gene sodA. A 440-bp fragment of the *sodA* gene was amplified and sequenced with primers SodV1 (5′-CAC CAY WSC AAG CAC CA-3′) and SodV2 (5′-CCT TAG CGT TCT GGT ACT G-3′) where Y = C or T, W = A or T, and S = C or G. The amplification was also done according to the recommendations of Sánchez-Herrera et al. [17].

All resulting PCR products were sequenced and verified (Biofidal, Lyon, France).

The breakpoints for identification based in *sodA* and *hsp65* genes are 99% for each one [17, 18]. For the *rrs* gene, a higher breakpoint of 99.6% is used, according to CLSI [19].

2.7. Phylogenetic Analysis. The *rrs* gene sequences which we obtained for the 36 clinical isolates of *N. brasiliensis* and the reference strains were aligned manually for the comparative phylogenetic analysis using the Seaview program.

MLSA was performed using *hsp65* and *sodA* sequences of the strains collection. The trimmed aligned sequences were concatenated in the order *sodA-hsp65* to generate an 846 bp sequence using the Seaview program. The Seaview program was also used to infer the evolutionary trees according to the neighbour-joining method [20] and Kimura's two-parameter model [21]. The robustness of the tree was performed with a bootstrap of 1000 replicates.

Taking into account the breakpoints for identification at species level of *sodA* and *hsp65* genes individually, the breakpoint for concatenated sequence has been also fixed at 99%.

2.8. DNA Polymorphism of rrs, hsp65, and sodA Genes. The number of haplotypes, the haplotype diversity (Hd), the number of polymorphic sites, and other variables were obtained with DnaSP software [22].

3. Results

3.1. Growth on Culture Medium. The three culture media allowed the growth of clinical strains of *N. brasiliensis*. The Bennett medium showed abundant and rapid growth (48 hours). Middlebrook medium showed strong growth but also it was slightly slower (72 hours). The BCP medium presented interesting results with good rapid growth at 48 hours. *N. brasiliensis* clade tested type strains showed similar patterns to the clinical strains, except that *N. boironii* had a difficult growth on BCP and no growth on Middlebrook; this seems a peculiarity of this species.

3.2. Antimicrobial Susceptibilities. Eight out of 31 antibiotic molecules tested were active on all the strains' collection: linezolid, tigecycline, trimethoprim + sulfamethoxazole, moxifloxacin, amikacin, amoxicillin + clavulanic acid, tobramycin, and gentamycin. Regarding the imipenem and pristinamycin molecules, resistance was observed on the majority of clinical isolates of *N. brasiliensis* (Table 1).

3.3. Degradation of Substrate. The assimilation test of adenine and uric acid proved negative for all the strains tested of the *N. brasiliensis* clade including clinical and reference ones. The casein degradation test showed that all clinical strains are able to metabolize casein except the clinical strain 12.28. In addition our result showed that some types of strains such as *N. vulneris*, *N. tenerifensis*, *N. boironii,* and *N. iowensis* are also able to degrade casein in the same way as *N. brasiliensis* except *N. altamirensis*. Casein is ultimately a marker that can be used for the phenotypic identification of the *N. brasiliensis* clade and not the *N. brasiliensis* species as it has been believed for many years.

3.4. Phylogeny. Primers Noc1 and Noc2 amplified the expected 606-bp fragment of the *rrs* gene for all the collection strains. Phylogenetic trees (Figure 1) based upon *rrs* showed homogeneity within clinical strains of *N. brasiliensis*. For this, the *rrs* gene is not relevant to show intraspecies diversity.

In addition, based upon the concatenation of *sodA* and *hsp65* housekeeping genes, the phylogenetic tree generated (Figure 2) had several distinct genotypes: (i) genotype 1 containing clinical strains, (ii) genotype 2 harboring some clinical strains, and (iii) genotype 3 harboring some clinical strains and *N. brasiliensis* ATCC 19296^T. For the tropical *N. brasiliensis* HUJEG01 strain, it is observed that it does not belong to any of the 3 genotypes and is positioned alone

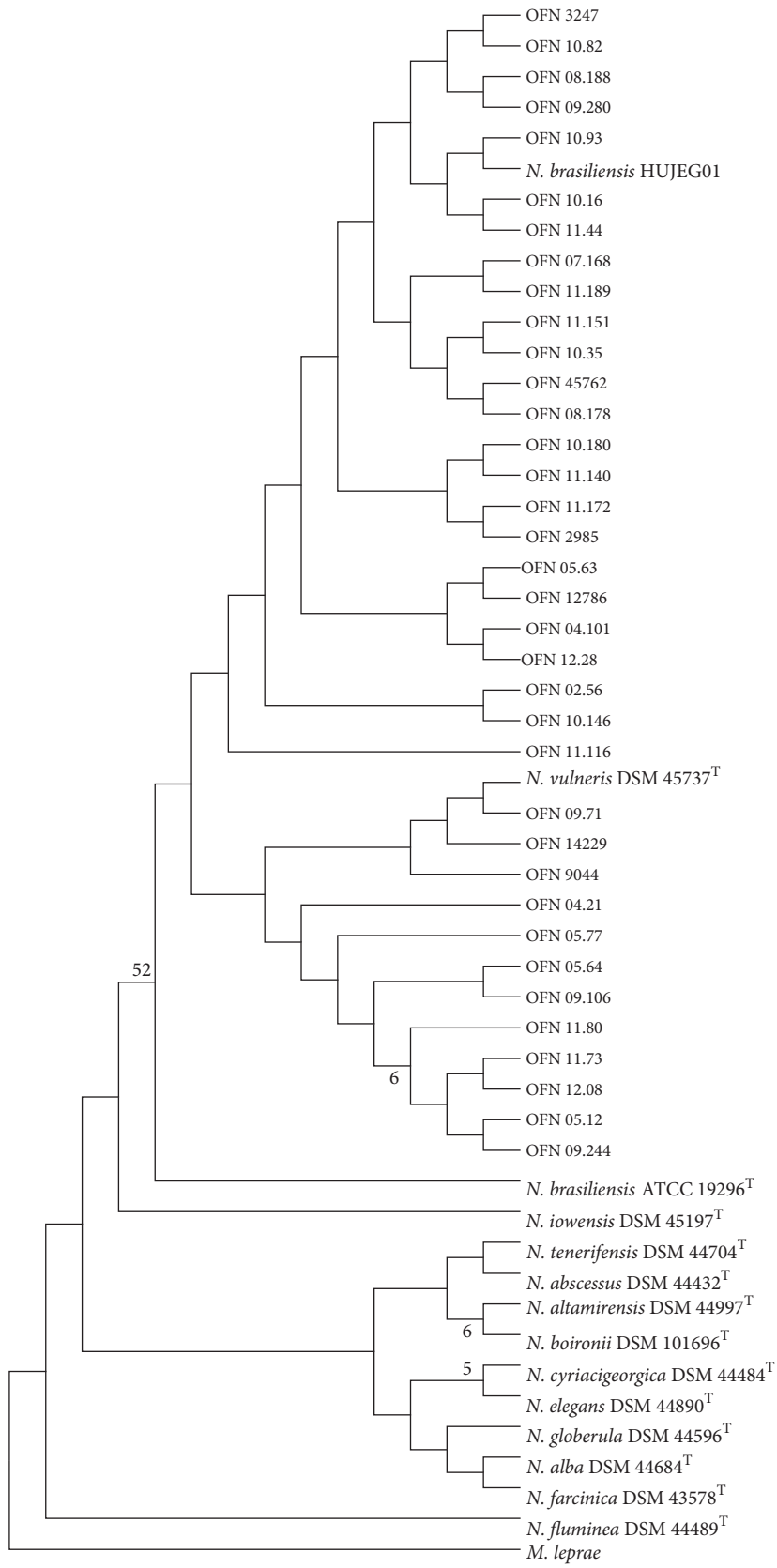

Figure 1: Phylogenetic distribution of *rrs* gene of 36 *N. brasiliensis* clinical strains analyzed in this study using neighbour-joining method, Kimura's two-parameter model, and bootstrap of 1000. Only values of bootstrap significance greater than 50% (Seaview) were reported.

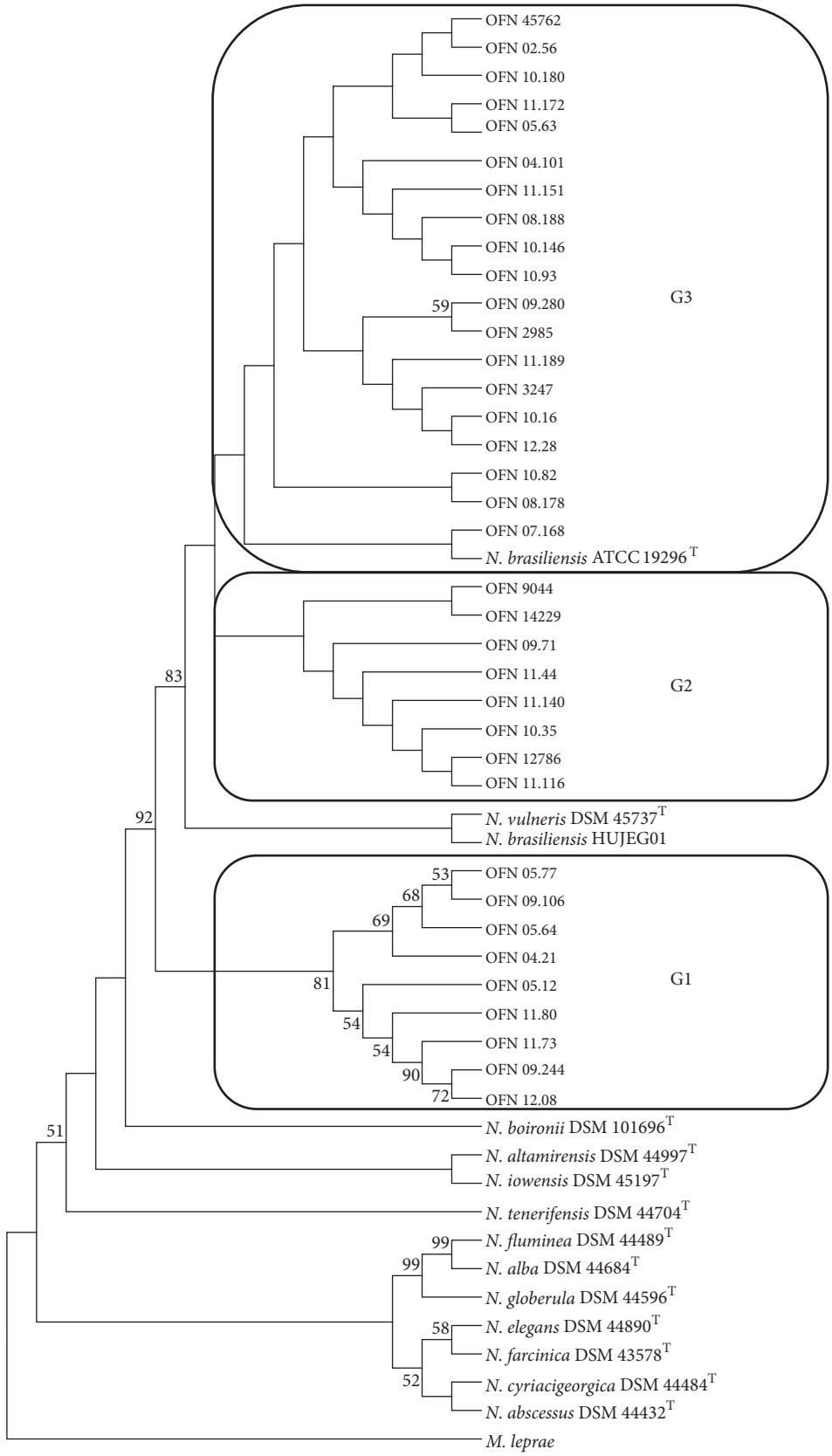

Figure 2: Phylogenetic distribution of concatenation *sodA-hsp65* genes of 36 *N. brasiliensis* clinical strains analyzed in this study using neighbour-joining method, Kimura's two-parameter model, and bootstrap of 1000. Only values of bootstrap significance greater than 50% were reported.

in the tree between genotypes 1 and 2. This distribution of clinical strains of *N. brasiliensis* in 3 different genotypes shows an intraspecies diversity rather important. To better understand the polymorphism showed by phylogenetic trees, we studied the percentages of the similarities between the sequences. The average percentages of similarities based on the *rrs* gene (Table 2) range from 99.39% to 99.57% between the clinical strains and the 2 reference strains of *N. brasiliensis* (type and tropical strains). According to the CLSI, the similarity percentage needed for identification at species level must be greater than or equal to a threshold of 99.6% [19]. The clinical strains that showed a similarity percentage lower than this threshold for both reference strains of *N. brasiliensis* were anyway considered as belonging to this species because no higher similarity percentage was obtained for any other species. In the same way, the *N. vulneris* type strain was also revealed to be close to clinical strains according to the average of percentage of similarity (98.77%). Between the 2 reference strains of *N. brasiliensis* the percentage of similarity is higher, up to 99.82%. The percentages of similarities based on the concatenation of the *sodA-hsp65* genes (Table 2) decrease and range now from 97.99% to 99.19% between the clinical strains and the 2 reference strains of *N. brasiliensis*. Between the type and reference strains of *N. brasiliensis* the percentage of similarity does not reach 99% this time. The comparison of the 3 genotypes between them (based on the representation of each genotype by 3 clinical strains) by using *sodA-hsp65* genes shows that genotypes 2 and 3 are closer to each other (98.97% of similarity). The average of the percentages of similarity between genotypes 1 and 2 were 97.97%. and 98.28% between genotypes 1 and 3. Finally this value goes up to 98.97% between genotypes 2 and 3. This means that the more distant genotypes between them are 1 and 2 and the closer ones are 2 and 3.

In parallel, an epidemiological study based on the clinical files was carried out, and the data were presented in Table 1. In order to know the link between the genetic diversity and the tropism of the clinical strains, a superposition of data was made between the phylogenetic tree obtained by the concatenation of *sodA* and *hsp65* and the tropism of the clinical strains (Figure 2). Thus, we can see that in genotypes 2 and 3 we have almost all the clinical strains that have a cutaneous tropism except the 08.188 strain which has a subcutaneous tropism. Regarding genotype 1 it is more heterogeneous with various tropism: (i) pulmonary, (ii) cerebral, and (iii) cutaneous. Regarding the immunocompetence of patients, we have only 4 patients who have immunodepression factors, whose strains are in genotype 1 except the 10.93 strain which is in genotype 3.

3.5. Analysis of rrs, hsp65, and sodA Genes Polymorphism. The 36 clinical strains and 2 reference strains of *N. brasiliensis* studied showed (i) for *rrs* gene 16 polymorphic sites sharing 16 haplotypes and showing a Hd of 0.824; (ii) for *hsp65* gene, 27 polymorphic sites and up to 22 different haplotypes with a Hd of 0.927; and, (iii) for *sodA* gene, up to 29 polymorphic sites sharing 14 haplotypes having a Hd of 0.885 (Table 3).

4. Discussion

Nocardia spp. are common soil-inhabiting bacteria that frequently infect humans through traumatic injuries or inhalation routes and cause infections, such as actinomycetoma and nocardiosis, respectively. *N. brasiliensis* is the main aetiological agent of actinomycetoma in various countries [23]. The input data used in this study highlight the existence of indigenous cases of cutaneous and subcutaneous (such as actinomycetoma) nocardiosis caused by *N. brasiliensis* in France. Moreover, we can observe that *N. brasiliensis* is also responsible for severe cases of disseminated nocardiosis in immunocompromised patients (pulmonary and cerebral cases).

To determine whether there is an association between clinical tropism of strains and their genetic profile we performed genetic characterization of 36 indigenous cases of *N. brasiliensis* that happened in France.

The three culture media allow the growth of clinical strains of *N. brasiliensis*. However, on Bennett's medium more abundant and fast growth (48 hours) was observed. But the downside of this medium is its inaccessibility in the hospital because it is not marketable. Middlebrook medium shows strong growth but also it was slightly slower (72 hours). This medium is very expensive and not accessible to all budgets. However, it is an interesting alternative in isolating *Nocardia* from a complex sample. It is a selective medium of Mycobacteria, which promotes the growth of some *Nocardia* to the detriment of other external bacteria or commensal flora that may be in the biological sample analyzed. The BCP medium, used routinely in hospitals for Gram-negative bacteria, has interesting results with good fast growth (48 hours). It would therefore be advisable to use it as isolation medium for urgent cases, by the speed of growth.

Antibiograms results show resistance of most of the clinical strains to imipenem. This can pose therapeutic problems since it is part of molecules proposed during a phase of a general treatment for nocardiosis [24]. However, all clinical strains of *N. brasiliensis* were sensible to SXT and would be an effective molecule during treatments. The sensibility of *N. brasiliensis* type strain to this antibiotic has already been observed by Gilquin et al. [9].

Our study confirms that all clinical strains of *N. brasiliensis* are capable of degrading casein except 12.28 clinical strain. As shown by Seol et al. the *N. brasiliensis* type strain is able to degrade casein as well [25]. However, the test on the reference strains reveals that *N. vulneris*, *N. tenerifensis*, *N. boironii,* and *N. iowensis* are also capable of degrading casein in the same way as *N. brasiliensis,* as also shown by Gilquin et al. [9]. This type of test is used in some countries without the necessary molecular biology tools to identify *N. brasiliensis*. But, now, they must be aware that with this test we target several species of clade *N. brasiliensis*. So, it is no longer a criterion of identification proper to *N. brasiliensis*.

Phylogenetic tree based on the *rrs* gene sequence of our collection showed a low genetic diversity resulting in low polymorphism sequence. In addition, we can note that *N. vulneris* DSM 45737^T, identified as a new species by Lasker et al., present a genetic sequence very close to *N. brasiliensis*

Table 2: Percentage of similarity expressed in interval and mean for *sodA-hsp65* and *rrs* genes.

		N. brasiliensis HUJEG01		*N. brasiliensis* ATCC 19296^T		*N. vulneris* DSM 45737^T	
		sodA-hsp65	*rrs*	*sodA-hsp65*	*rrs*	*sodA-hsp65*	*rrs*
N. brasiliensis ATCC 19296^T	Similarity average (%)	98.51	99.82	-	-	-	-
N. vulneris DSM 45737^T		98.38	98.73	98.51	98.55	-	-
N. altamirensis DSM 44997^T		95.91	98.24	95.41	98.07	95.91	97.47
N. boironii DSM 101696^T		96.53	98.07	96.28	97.89	96.15	97.29
N. iowensis DSM 45197^T		94.91	97.89	95.29	97.71	94.54	97.64
N. tenerifensis DSM 44704^T		96.28	96.67	96.03	96.49	95.91	96.03
Genotype 1	Similarity range (%)	(97.77–98.38)	-	(97.77–98.26)	-	(97.52–98.14)	-
	Similarity average (%)	98.01	-	97.99	-	97.67	-
Genotype 2	Similarity range (%)	(98.39–98.88)	-	(98.88–99.38)	-	(98.63–98.88)	-
	Similarity average (%)	98.73	-	99.19	-	98.80	-
Genotype 3	Similarity range (%)	(98.14–98.88)	-	(98.26–99.00)	-	(98.01–98.76)	-
	Similarity average (%)	98.63	-	98.76	-	98.50	-
All clinical strains	Similarity range (%)	-	(98.57–100)	-	(98.38–99.82)	-	(98.01–99.09)
	Similarity average (%)	-	99.57	-	99.39	-	98.77

Table 3: DNA polymorphism of *rrs*, *hsp65,* and *sodA* genes from clinical *N. brasiliensis* strains isolated in France.

Nocardia species	Genes (bp)[a]	Number of haplotypes (Hd, S^2, SD)[b]	Number of of polymorphic sites
N. brasiliensis (N = 38: 36 clinical strains and 2 reference strains)	*rrs* (569)	16 (0.824, 0.00300, 0.055)	16
	hsp65 (401)	22 (0.927, 0.00087, 0.029)	27
	sodA (406)	14 (0.885, 0.00080, 0.028)	29

[a]Resulting fragment size without the primers sequences; [b]Hd: haplotype (gene) diversity, S^2: variance of haplotype diversity, and SD: standard deviation of haplotype diversity.

strains [26] with percentages of similarities on average greater than 98%.

Analysis of the phylogenetic tree (Figure 2) based on the MLSA by the concatenation of *sodA* and *hsp65* housekeeping genes showed that the isolates are surprisingly classified according to 3 genotypes. These groups were formed upon similarity percentages and existing phylogenetic distances between the sequences of the strains studied. Genotype 1 concerns a well-defined cluster containing 9 clinical strains only which is sustained by a bootstrap of 81%. This genotype hosts all the cases of pulmonary forms (3), the sole cerebral form, and almost all the cases of immunocompromised patients (3 out of 4). Moreover, eight out of twelve strains susceptible to imipenem can be found in this group. There is just one remaining strain in this group not presenting this kind of susceptibility. The reason may be an acquired resistance to this drug due to a previous treatment. This well-defined genotype evokes the possible existence of another species or a strong variability in this case. This may have been caused by environmental pressures in the ecosystem of these isolates which may have resulted in the selection of strains that may have acquired, by mutations or genetic transfer with other microorganisms, new virulence characters different from that of the strain type *N. brasiliensis*. Regarding genotypes 2 and 3, they include clinical strains and *N. brasiliensis* ATCC 19296^T type strain. However, the discrimination between these 2 genotypes is less clear than that with genotype 1 because of being in weak bootstrap that is less than 50. The genetic differences do not allow distinguishing them properly and their phenotypic behavior remains similar. Then, it would be interesting to study on another gene capable of generating more divergences, for example, *gyrB* and *rpoB* genes, which have already successfully been used for studying the polymorphism of some other *Nocardia* species [27, 28].

Concerning the percentage of similarity between the type and tropical strain of *N. brasiliensis*, it is 99.82% according to the *rrs* and goes down to 98.51% with the concatenation of *sodA* and *hsp65*. The fact of highlighting a greater dissimilarity with the concatenation between the type and tropical *N. brasiliensis* strain shows the advantage of the use of 2 markers like *sodA* and *hsp65* vis-à-vis the *rrs*. The discriminatory power of these two genes may be explained by the presence of more polymorphic sites (*hsp65*: 27; *sodA*: 29) than in the case of *rrs* gene (16) and also by having Hd values higher than that of *rrs* gene (*hsp65*: 0.927; *sodA*: 0.885; *rrs*: 0.824).

It would be interesting to identify the genes involved in the virulence of different genotypes, including those of actinomycetoma. Interesting leads can be considered: (i) as identification of virulence genes expressed using the RNAseq method or (ii) to identify noncoding RNAs [23]. In addition, to genomically distinguish *N. brasiliensis* and *N. vulneris* a specific PCR to *N. vulneris*, using a specific gene of the species, should be developed.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Leptospirosis in Coastal South India: A Facility Based Study

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Background. Leptospirosis is a major neglected public health problem and is highly underreported in India. The spectrum of clinical features ranges from subclinical infection to multiorgan failure. The burden of leptospirosis is more in developing countries. *Objective.* The present study was designed to assess the sociodemographic characteristics, clinical feature, and outcome of leptospirosis patients. *Methods.* This record based retrospective study was conducted in hospitals affiliated to Kasturba Medical College, Mangalore. The registries of the leptospirosis patients during the period of four years between January 2011 and December 2014 were reviewed and the information on demographic and clinical profile of the leptospirosis patients was recorded in the data capture sheet. The information obtained was analyzed using SPSS version 11.5. *Results.* The study included 202 leptospirosis victims. The mean age of the study population was 40.48 (±14.8) years. Majority of the patients presented with fever (92.1%) at the time of admission. Acute renal failure was found to be the most common complication (79.2%). Case fatality rate was found to be 3.5%. *Conclusion.* High proportion of cases indirectly reflects the endemic nature of the disease in the study setting. The clinical presentation of the leptospirosis is highly protean and may vary from a mild illness to life-threatening complications as evident from the current study.

1. Introduction

Leptospirosis is essentially a zoonotic disease that is caused by spirochetes of the genus *Leptospira* and is prevalent worldwide [1]. It is an infectious disease caused by pathogenic strains of *Leptospira* species, of which almost 20 serogroups and 200 serovars are currently known. The problem of the disease has not been completely addressed even though it has been recognized for decades, the primary reason being the presence of the major burden of the disease in poor, developing countries [2]. Leptospirosis has been recognized as major public health problem and multiple epidemics have been reported, owing to the occurrence of natural disasters and the prevalence of poor sanitary conditions [3, 4]. Very little is currently known regarding the true incidence of leptospirosis. However, it is estimated that 10 or more per 100,000 people are affected with this disease each year in tropical climates. If there is an epidemic, the incidence can soar to 100 or more per 100,000 people [3]. While humans are accidental hosts, the primary reservoir hosts include infected animals such as rodents, dogs, cattle and pigs, and infection is acquired by humans through direct or indirect contact with water or soil contaminated by the urine of infected animals [5].

Important epidemiological risk factors in the occurrence of the disease include contaminated environment and rainfall [6]. Children acquire the infection from dogs more commonly than do adults. Occupational exposure is a major cause of infection and the risk groups include agricultural and livestock farmers, workers in underground sewers, meat and animal handlers, and veterinarians [4].

The spectrum of the disease ranges from subclinical infection to a severe syndrome characterized by multiorgan dysfunction. Clinical features include headache, fever, myalgia, jaundice, conjunctival suffusion, bleeding tendencies, oliguria, and pulmonary manifestations like cough, breathlessness, and hemoptysis [7]. The mild, anicteric form of the disease is more common and presents with nonspecific

symptoms while the icteric form of the disease is potentially fatal and presents with jaundice, renal dysfunction, and bleeding diathesis [8]. In many cases, leptospirosis can present without any classical features [5]. Laboratory investigations are essential for the confirmation of disease as vague clinical symptoms make the diagnosis difficult [7]. Common complications of the disease include renal failure, respiratory failure, neuroleptospirosis, and Disseminated Intravascular Coagulation [8]. Leptospirosis is highly underreported in India, most likely due to lack of diagnostic modalities and lack of awareness among clinicians [9]. It is important to diagnose the disease timely as early initiation of antibiotic therapy is highly beneficial in interrupting the course of the disease [10].

The burden of leptospirosis is more in developing countries and there is paucity of literature available on the burden and varied clinical manifestations of this disease in India. The present study is being undertaken in one of the coastal districts of Southern India where agriculture-related activities form a major occupation of the population and are at risk of acquiring leptospirosis. The present study was designed to assess the sociodemographic characteristics, clinical feature, and outcome of leptospirosis patients.

2. Materials and Methods

The present registry-based retrospective study was carried out among all patients admitted with a diagnosis of leptospirosis at the hospitals affiliated to Kasturba Medical College, Mangalore. It acts as a referral center for coastal part of Karnataka and northern parts of Kerala. Informed consent was not obtained as it was a retrospective case record study; however, approval was taken from the Institutional Ethics Committee (IEC) of Kasturba Medical College, Mangalore (Manipal Academy of Higher Education), before commencement of the study. Permission was then obtained from the department of General Medicine and Medical Superintendent of the hospital to access the registries of leptospirosis patients from the medical records department. The registries of the leptospirosis patients during the period of four years between January 2011 and December 2014 were reviewed and the information on demographic and clinical profile of the leptospirosis patients seeking healthcare (only serologically confirmed cases through Lepto IgM ELISA) was recorded in the data capture sheet. The information obtained was analyzed using SPSS (Statistical Package for Social Sciences) version 11.5 for descriptive statistics, and the results were expressed in proportions, mean, and standard deviation.

3. Results

The baseline characteristics of leptospirosis patients are displayed in Table 1. The mean age of the study population is 40.48 (±14.8) years and the age ranged from 11 to 90 years. Majority of the leptospirosis victims were in the age group of 20–40 years (n = 80, 39.6%) and 41–60 years (n = 82 40.6%), thus affecting the working population. It was observed that majority of the victims were males (n = 142, 70.3%). When the duration of the hospital stay was analyzed, it was seen that most of the victims were discharged from the hospital within 5 days (n = 94, 46.5%). However the median duration of stay in the hospital was 6.0 days [IQR: 4–9 days], ranging from 1 to 23 days.

Table 1: Baseline characteristics of leptospirosis patients (N = 202).

Baseline characteristics	Number	Percentage
Age group (years)		
<20	022	10.9
20–40	080	39.6
41–60	082	40.6
>60	018	08.9
Sex		
Male	142	70.3
Female	060	29.7
Religion		
Hindu	191	94.5
Muslim	007	03.5
Christian	004	02.0
Hospital		
Government	121	59.9
Private	081	40.1
Duration of stay (days)		
<5	094	46.5
6–10	073	36.1
>10	035	17.4

Table 2: Distribution pattern of the chief presenting complaints (N = 202).

Clinical presentation	Number*	Percentage
Fever	186	92.1
Myalgia and generalized weakness	073	36.1
Vomiting	066	32.7
Fever with chills and rigors	054	26.7
Oliguria	052	25.7
Jaundice	045	22.3
Abdominal pain	038	18.8
Headache	037	18.3
Cough	021	10.4
Conjunctival suffusion	009	04.5

*Multiple responses.

Table 2 shows the distribution of leptospirosis patients according to clinical presentation at the time of admission. Majority of the patients presented with fever (n = 186, 92.1%) at the time of admission followed by myalgia and generalized weakness (n = 73, 36.1%) and vomiting (n = 66, 32.7%).

The laboratory profile of most of the patients was consistent with elevated serum bilirubin levels (n = 118, 76.6%) followed by elevated AST levels (n = 116, 73.4%) and thrombocytopenia (n = 113, 71.5%). The mean Hb value was 11.6 (±2.5) gm% and ranged from 4.7 to 18.5 gm%. The median total leukocyte count was 11,300 [IQR: 7350–17825]. The total

Table 3: Laboratory profile of leptospirosis patients.

Lab parameter	*N*	*n* (%)*
Anemia (Hb < 11 gm%)	159	059 (37.1)
Leukocytosis (TC > 11000)	164	083 (50.6)
Neutrophilia (*N* > 70%)	143	095 (66.4)
Lymphopenia (*L* < 20%)	140	096 (68.6)
Thrombocytopenia (<1.5 lakhs)	158	113 (71.5)
Serum hyperbilirubinemia (>1.2 mg/dl)	162	109 (67.7)
Serum direct bilirubin > 0.2 mg/dl	154	118 (76.6)
Serum urea > 45 gm/dl	156	099 (63.9)
Serum creatinine > 1.4 mg/dl	168	088 (53.0)
Serum hypoproteinemia (TP < 6.0 gm/dl)	139	074 (53.2)
Serum hypoalbuminemia (Alb < 3.2 gm/dl)	143	087 (60.8)
Elevated liver enzymes		
AST (>40 units)	158	116 (73.4)
ALT (>40 units)	160	098 (61.2)
ALP (>129 units)	147	074 (50.7)

*Multiple responses.

Table 4: Clinical outcome of leptospirosis patients (*N* = 202).

Outcome	Number	Percentage
Recovered without complications	151	74.7
Recovered with complications	044	21.8
Death	007	03.5

leukocyte count ranged from 800 to 77000. Among the leptospirosis victims who were investigated for renal parameters, it was seen that more than half of them had elevated urea (63.9%) and creatinine levels (53%) as depicted in Table 3.

When the clinical outcome of the leptospirosis patients was analyzed, it was observed that most of the patients recovered without any complications (n = 151, 74.7%) and nearly one-fifth of them recovered with complications (n = 44, 21.8%). When the type of complications was further analyzed it was noted that acute renal failure was the most commonly seen (n = 35, 79.2%). The case fatality was found to be 3.5% as observed in Table 4.

4. Discussion

Leptospirosis is prevalent worldwide but is most commonly seen in tropical and subtropical regions due to excessive rain and flooding. It is often transmitted through water and food contaminated by urine of infected rats whereas human to human transmission is rare [2]. A hospital based case record study was carried out in leptospirosis patients to determine the sociodemographic profile, symptomatology, and the outcome of the disease.

Mean age of the leptospirosis patients was found to be 40.4 years in the present study, while in a study done in Maharashtra it was found to be 42 years [10]. This is in congruence with the findings recorded in studies conducted at different parts of Northern India [7–9, 11]. A study done at southern part of India to analyze the changing pattern of leptospirosis patients revealed that though the people of all age group were affected, maximum number was observed in adulthood because of their work pattern [6]. In line with our study findings, people of productive age group were affected in other studies conducted in different parts of South India [12–14] and central India [15], thus imparting economic misery to the affected families.

Predomination of males over females were observed among leptospirosis cases in studies conducted at Ludhiana [7], Thirupathi [12], Mangaluru [13], and Chennai [14] which is similar to the present study wherein three-fourths of the victims were males. In contrast to a study conducted at Maharashtra where the mean duration of hospital stay was sixteen days, the duration in the present study was observed to be eleven days [10].

Leptospirosis is febrile systematic disease and symptoms manifested depend on the organs involved. It mainly involves central nervous system, reproductive system, liver, lung, eyes, kidney, and reproductive system [16]. Fever was the most common clinical presentation in the present study which was seen among more than four-fifths of the leptospirosis patients. This is consistent with the findings observed in the studies conducted at different parts of northern [8, 10] and Southern India [12–14] and sub-Himalayan region [11]. A study conducted at western Maharashtra has revealed jaundice as the most common symptom which was noted among three-fourths of the leptospirosis patients [10], whereas one-fifth of the patients were presented with jaundice in the current study. Myalgia was seen in almost 40% of the patients at the time of admission which was similar to the observation made in a study conducted in Chandigarh [8]. Other common symptoms at the time of admission include headache, vomiting, and oliguria. This is similar to results of various studies which are carried out in North India, Kolkata, and Punjab [7, 9, 17]. Respiratory symptoms were seen in 10% of the patients in the current study which is comparable to that reported by a study done at North India [8].

Laboratory findings are essential to confirm a case of leptospirosis as clinical manifestation of leptospirosis is nonspecific [16]. The case definitions of leptospirosis, that is, suspect, probable, and confirmed, have been followed for managing the case in our hospitals. Serologically the cases were confirmed by doing Lepto IgM ELISA. On recording the laboratory parameters, it was observed that more than half of the patients had total leukocyte count of more than 11000. This is similar to the results observed in a study conducted in Chandigarh [8]. Hemoglobin values of less than 11 gm% were recorded in one-third of the patients whereas in a study conducted in Ludhiana only one-tenth of the patients had anemia [7]. Thrombocytopenia was seen in almost three-fourths of the study population. This is in concordance with a study which was carried out in Kolkata [17]. Most of the patients were found to have elevated liver enzymes and increased bilirubin levels which is consistent with various other studies conducted on leptospirosis patients [7, 9, 10]. Serum creatinine values of more than 1.4 mg/dl were found in more than half of the patients in the present study while a study done at North India observed increased creatinine levels in less than 10% of the patients [7].

Complications of leptospirosis include pulmonary hemorrhage, renal failure, icterus, myocarditis, and uveitis [16]. It was evident from our study that acute renal failure was the most common complication seen in leptospirosis patients. Studies which were conducted in western Maharashtra [5], Northern India [10], and southern part of India [12, 14] have also stated the same. The rarer complications encountered in our study included ARDS (Acute Respiratory Distress Syndrome), dilated cardiomyopathy, sepsis, and multiorgan dysfunction syndrome. In a study done at Chandigarh, hemorrhagic pneumonia, neuroleptospirosis, and Disseminated Intravascular Coagulation were seen as rarer complications [8]. In the same context, a study done at western Maharashtra showed meningoencephalitis, Deep Vein Thrombosis, and hepatic encephalopathy as few of the rare complications seen in leptospirosis patients [10].

Case fatality rate for leptospirosis can be as high as 30% [18]. Present study showed that most of the patients recovered without any complications while the case fatality rate was only 3.5% which is comparable to the study done at Punjab (5.9%) [9]. However higher case fatality rate was observed in a study conducted at coastal part of South India [13]. The lesser case fatality rate in the present study can be attributed to dialysis for renal failure patients and good nursing care at the study setting. On the other hand, study done at sub-Himalayan region of North India did not encounter any deaths in their study population [11].

5. Conclusion

Leptospirosis remains a significant public health problem mainly affecting the population of productive age group. High proportion of cases indirectly reflects the endemic nature of the disease in the study setting. The clinical presentation of the leptospirosis is highly protean and may vary from a mild illness to life-threatening complications as evident from the current study. As the disease is endemic and can have a fatal outcome, it should raise a high index of suspicion among the clinical fraternity when they come across a patient with fever and jaundice. A well planned multicentric study done at different geographical locations could bring out better insight to the epidemiology of leptospirosis.

Data Availability

The data used to support the findings of the present study is available and will be made available by the corresponding author upon request.

Disclosure

Findings of the current study were presented at "45th National IAPSM & 19th Maharashtra State Joint Conference of IAPSM & IPHA-2018" as oral presentation.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Asthma and Seroconversion from *Toxocara* spp. Infection: Which Comes First?

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The aim of this study was to estimate the incidence of seroconversion of *Toxocara* spp. infection and related variables. We conducted a cohort study of 77 children aged 2–12 years who had negative serology in a previous cross-sectional study. Univariate and bivariate analyses were performed to describe the cohort, using socioeconomic, behavioral, and health conditions as variables. Logistic regression analysis was performed using seroconversion as the dependent variable, and the remaining variables are treated as independent variables. Asthma was the only independent variable that showed an association with seroconversion, with an odds ratio = 3.57 (1.01–12.6). The incidence of seroconversion from *Toxocara* spp. infection in the children followed was 10.4 per 100 per year. Previous studies reporting an association of asthma with toxocariasis have only been carried out using cross-sectional studies. Therefore, this study is one of only a few describing the incidence of seroconversion from *Toxocara* spp. infection, which is relevant for understanding the burden of this parasite.

1. Introduction

Toxocariasis is a cosmopolitan disease that occurs in various regions worldwide, more frequently in developing countries [1–4]. Owing to an increasing human population in large cities, with consequential increases in canine and feline populations, the feces of these animals are increasingly contaminating the urban environment. Therefore, toxocariasis is currently a common anthropozoonosis in developed countries, with a reported prevalence of 13.9% in the United States [5] and a range from 4.2% to 65.4% in Brazil [6].

Among the most commonly reported factors associated with toxocariasis is contact between humans and dogs [7, 8]. Asthma is described in various studies as a toxocariasis-associated factor [5, 8, 9]; however, the direction of this association is unclear. The definitive factors in this association must be clarified, and appropriate methodology is needed in order to further elucidate the relationship.

Various studies describe the prevalence of toxocariasis in humans [10–13]. However, its incidence is rarely studied unless a population sample is being described. Therefore, the current study aimed to estimate the incidence of seroconversion from *Toxocara* spp. infection as it related to a cohort of randomly selected children living in the city neighborhood of Campinas, São Paulo State, Brazil.

This study determined the incidence of seroconversion resulting from *Toxocara* spp. infection and tested several variables for association with this seroconversion. Asthma was the only variable that showed an association.

2. Materials and Methods

A closed prospective cohort study was carried out using a sample of children who had negative serology for *Toxocara* spp., and whose place of residence was in a neighborhood of São Marcos, in the Northern District of the city of Campinas, São Paulo, Brazil. The study was approved by the Research Ethics Committee of the Faculty of Medical Sciences, State University of Campinas, and Tropical Medicine Institute of the University of São Paulo.

Table 1: Questions asked during each follow-up visit of the cohort regarding the presence of rashes and any respiratory complaints. Campinas, SP, Brazil, 2015.

(1) "Has your son or daughter experienced a cough, shortness of breath, or wheezing during the last month?"
(2) "Has your son or daughter visited any health-service provider owing to respiratory problems during the last month?"
(3) "Has any doctor told you that your son or daughter has or had asthma?"

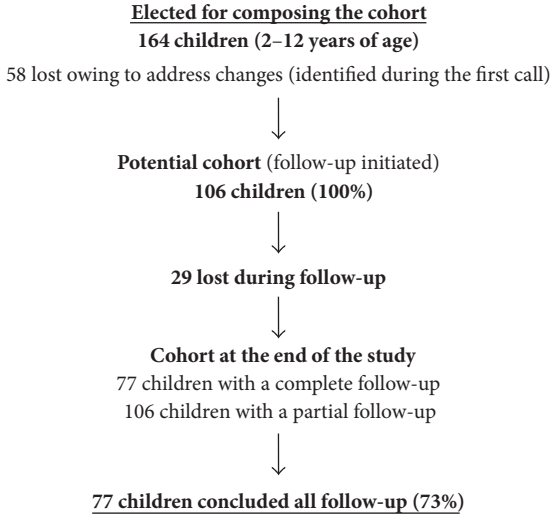

Figure 1: Cohort dynamics. Campinas, SP, Brazil, 2015.

The first stage was performed between 2012 and 2014 as part of a population-based study [6] comprising 200 children between the ages of 2 and 12 years. The study population was selected through a random sampling of houses registered in the Basic Attention Information and Management System (SIGAB, Sistema de Informação e Gerenciamento de Atenção Básica) of the Campinas Health Department. Initially, 192 children were evaluated for the presence of anti-*Toxocara* spp. antibodies, of which 28 presented with positive serology and were treated. The 164 who demonstrated negative serology against *Toxocara* spp. comprised the cohort of the current study. Because the cohort was open, 87 total losses were incurred (Figure 1). These losses were mainly caused by address changes (92%). The results of the current study are with reference to the 77 children that had completed all follow-up.

The cohort was followed over a 12-month period. Each child was visited four times, and all were asked about the presence of a rash and whether they had any respiratory complaints. Questions were formulated following a pretest performed with a limited number of individuals. The questions were designed to correspond with the International Study of Asthma and Allergies in Childhood (ISAAC) program. The questions asked during the evaluations are listed in Table 1.

2.1. Blood Samples and ELISA. One year after their last serological test, the children of the cohort underwent a new serological test for the diagnosis of IgG anti-*Toxocara* spp. antibodies. Blood collection from each child was performed using digital puncture and filter paper (Whatman™ Grade number 3) [14]. The collected samples were stored in notebooks, previously prepared using 1 cm wide filter paper strips separated by cellophane strips and filed according to the sequence of the sample collection. The child's ring finger was disinfected with 70% alcohol and the fingertip pierced using a disposable lancet. Blood was collected by sliding the ribbon filter paper on the finger until it was completely absorbed. The strips were dried at room temperature and stored at −20°C [6]. For processing, the blood samples were sent to the laboratory, eluted, and subjected to analysis using enzyme-linked immunosorbent assays (ELISAs) to detect IgG class antibodies using *T. canis* excretory-secretory (TES) antigen as a capture antigen. This technique has been previously described by de Savigny [15] and subsequently modified by Bach-Rizzatti [16].

2.1.1. Preparation of the Eluate. Pieces of Whatman #3 filter paper were cut to size (1.0 cm^2) from strips containing the blood collected from the fingertips of the study participants. The filter paper and 330 μL of PBS-0.01, pH 7.2, were added to Eppendorf microtubes and incubated at 4°C for 16 hours.

2.1.2. ELISA Plate Preparation. The reaction was performed in flat bottom 96-well polystyrene microplates (Costar 3590, high binding). The wells were coated with 100 μL TES antigen solution diluted in PBS (10 μg/mL). The plates were covered and incubated at 37°C for 2 hours, followed by 16 hours at 4°C. Prior to use, the nonspecific sites were blocked with 200 μL of PBS-Tween-Gelatin (0.05%) for one hour at 37°C.

2.1.3. Adsorption of Eluates. The eluates and control sera were adsorbed with total *Ascaris suum* antigenic extract to avoid cross-reactions with common *Ascaris* antigens. *Ascaris suum* antigen was diluted 1 : 200 in PBS-Tween to prepare an adsorption solution. The positive and negative control sera were diluted 1 : 320 in the adsorption solution (PBS-Tween-*Ascaris*) and incubated as with the eluates.

After blocking, the wells of the ELISA plates were washed three times with PBS-Tween (5 min each). Subsequently, 100 μL of the preadsorbed eluates were added to the wells, in duplicate, and 200 μL of known positive-patient sera were preabsorbed. In the subsequent rows, 100 μL of PBS-T was put into each well, and 100 μL from the first set of wells was passed using a multichannel pipette to the following rows, resulting in twofold serial dilutions. The last 100 μL was discarded. Subsequently, 100 μL of standard negative sera, absorbed with PBS-T-*Ascaris*, as well as two blanks with PBS-T, were placed into the first six wells. The plate was incubated for 40 minutes at 37°C. Further, the plate was washed three times with PBS-T.

The enzyme conjugate anti-IgG labeled with peroxidase was diluted 1 : 5000 in PBS-TG and applied to all the wells and incubated at 37°C for 40 minutes. Another wash cycle was performed as previously described. Visualization was carried out by adding 100 μL of an orthophenylenediamine solution in citrate phosphate buffer, with 5 μL of 30% hydrogen peroxide (H_2O_2) and incubated in the dark at room temperature for 20 minutes. The reaction was stopped by adding 50 μL of 4 N H_2SO_4 solution. The plates were read using a Multiskan™ ELISA reader with 492 nm filter. The cut-off density-point in the serological assays varied from 0.330 to 0.390 and was determined for each test using the mean optical density of 30 sera from the negative control group plus two standard deviations [6].

2.2. Statistical Analyses. Data analysis initially included the cohort description according to each variable studied, calculating absolute and relative frequencies, and their respective 95% confidence intervals (CI). A bivariate analysis was then performed for socioeconomic, behavioral, and health conditions as variables as a function of seroconversion from *Toxocara* spp. infection. Fisher's exact test was performed as indicated. When the association had $p > 0.20$, it was included in the regression model.

Logistic regression was performed, considering the presence of *Toxocara* spp. infection as a dependent variable, and the remaining variables as independent. The Forward Euler method was used, where one variable at a time was added in sequence, starting with the variables presenting a higher correlation value with the response variable. The logistic regression coefficient was raised to the power of "e" (Euler's number) in order to obtain the odds ratio (OR) of the association. The critical limit adopted for the test was 10% [17]. All statistical analyses were performed using SAS 9.4v and Epi Info 7 software.

3. Results

The results of the present study are based on 77 children who had negative serology in a previous cross-sectional study. The incidence of seroconversion from *Toxocara* spp. infection in this cohort was 10.4% (8/77; 10.4 cases per 100 children) per year. Table 2 summarizes the descriptors for the children in the cohort who had completed the entire follow-up ($n = 77$). The mean age of the children was 7.7 years (range 2–12) with 63.6% of the children being male.

Results of the bivariate study of the cohort according to whether toxocariasis serology was positive are presented in Table 3. In the current study, we were unable to identify any association between the seroconversion from *Toxocara* spp. infection and socioeconomic, behavioral, or health-condition variables. Logistic regression analysis showed that asthma was a risk factor for seroconversion from *Toxocara* spp. infection with an OR of 3.57 (CI: 1.01–12.6). The remaining variables such as the type of property border, the number of residents in the home, and contact with dogs or cats did not present as statistical significance risk factors associated for seroconversion (Table 4).

Table 2: Description at the start of the study ($T0$[a]) of the children ($n = 77$[b]) in the cohort that completed follow-up, relative to the characteristics considered in the study. Campinas, SP, Brazil, 2015.

Variable	n	%	95% CI[c]
Toxocariasis (seropositive)			
Yes	0	0	
No	77	100	95.32–100.00
Property borders			
Walled	67	92	82.96–96.92
Fenced	2	3	0.33–9.55
None	4	5	1.51–13.44
Number of residents in the home			
<6	66	10	81.24–96.06
>6	7	90	3.94–18.76
Contact with dog(s)			
Yes	59	81	69.92–89.10
No	14	19	10.90–30.08
Contact with cat(s)			
Yes	31	42	30.97–54.59
No	42	58	45.41–69.03
Asthma			
Yes	18	24	14.89–32.25
No	57	76	64.75–85.11

[a]$T0$ = time 0; [b]n = number of participants. [c]Confidence interval.

4. Discussion

The data from our study indicate that the incidence of seroconversion from *Toxocara* spp. infection was 8/77 per year for the children that were followed-up or 10.4 per 100 children per year. This incidence was not significantly different from the one found by Correa and Bismarck [18] in the same territory. Subsequent to the study by Correa and Bismark, there has been an increase in the number of houses with sanitation, and also additional asphalt paving. This reinforces the cosmopolitan character of this parasitosis. Although this parasitic infection depends on the presence of geohelminth eggs in the environment, canine or feline contact was not an independent variable associated with this infection, possible owing to the small sample size. Therefore, other factors must be considered as they relate to the way in which a child may come into contact with these geohelminth eggs.

Among the evaluated independent variables, only the presence of asthma was found to be a risk factor for a positive serologic result for infection by *Toxocara*. The association between asthma and toxocariasis has been previously reported in various cross-sectional studies [8, 9, 19], which present an association between these two variables. However, in these types of studies it is not possible to conclude which condition is chronologically preceded by the other. In the current cohort study, we found that the presence of asthma preceded seroconversion from *Toxocara* spp. infection; thus,

Table 3: Bivariate analysis of the 77 children in the cohort regarding seroconversion from *Toxocara* spp. infection and independent variables. Campinas, SP, Brazil, 2015.

Variable	Seroconversion of *Toxocara* spp.				p^*
	Yes		No		
	n	%	*n*	%	
Property borders					
Walled	8	11.0	59	80.8	0.48
Fenced	0	0.0	2	2.7	
None	0	0.0	4	5.5	
Number of residents in the home					
<6	6	8.2	38	52.1	0.21
>6	2	2.7	27	37.0	
Contact with dog(s)					
Yes	7	9.6	1	1.4	0.35
No	52	71.2	13	17.8	
Contact with cat(s)					
Yes	5	6.8	3	4.1	0.14
No	26	35.6	39	53.4	
Asthma					
Yes	4	5.6	4	5.6	0.08
No	14	19.4	50	69.4	

*Fisher's exact test.

Table 4: Logistic regression model[a] for the 77 children of the cohort with seroconversion from *Toxocara* spp. infection. Campinas, SP, Brazil, 2015.

Variable	Odds ratio	90% CI[b]
Asthma	3.57	1.01–12.6

[a]Forward Euler method of selection. [b]Confidence level.

asthma preceded the serologic shift. One explanation is that there is a period of time between infection and the serologic shift, with the respiratory symptoms initiating prior to the serologic shift. Thus, asthma would be identified, not only as a disease, but also as an "unfavorable conditions *proxy*" that facilitates infection by the parasite, which is related to the seroconversion.

Several articles have previously identified the association of asthma with the presence of antibodies against *Toxocara* spp. The current study, based on the adopted model, allowed us to identify asthmatic children who presented with anti-*Toxocara* antibodies. This result suggests a factor, probably related to inflammation, that would be present in asthmatic children and that could facilitate them to seroconvert. Several published reports suggest that allergic manifestations, such as asthma, may be a consequence of parasitic infections [5, 8, 20, 21]. For more than two decades the hygienist theory has suggested that the increase in allergic diseases is a consequence of the decrease in parasitic infections [22, 23].

In the current work, using a cohort study, the results were contradictory. We observed that asthmatic children developed anti-*Toxocara* antibodies. These data, however, may be explained by an observation made by Maizels [23] in which the inflammatory response triggered by a parasite varies depending whether the host is the definitive or paratenic host of the parasite. In the case of *Toxocara*, the human is a paratenic host, and thus the inflammatory response is expected to be different from that triggered by another parasite of which the human being is the definitive host, which is the case for *Schistosoma mansoni* [24].

However, it is not possible to avoid the association of the data from this study with those obtained by Poorisrisak et al. [25]. While studying the association of asthma with infection by respiratory syncytial virus (RSV) in monozygotic twin infants, they found that asthma susceptibility influences infection by RSV. Data found in this study suggest that the presence of asthma may be a marker for whom would be infected by *Toxocara canis* or, at least, present antibodies for this parasite. Other children may be infected without producing antibodies. However, these conclusions should be considered with caution, because at this point, the data only suggest hypotheses to be tested in subsequent studies using a larger number of children involved.

The average age of our cohort was 7.7 ± 3.9 years, which is consistent with the age of participants in other studies in which higher toxocariasis prevalence was found [26]. Anaruma Filho et al. [13] found for the same region that the type of property border was a factor that contributed to the prevention of infection by *Toxocara*. This protection may be associated with the fact that obstacles such as walls or fences may limit access of dogs to the peridomicile. However, this association was not found in our study, possibly because of the fact that almost every house of both infected and noninfected children presented property borders such as gates, walls, and fences. Therefore, we could not discriminate between the hosts infected and not infected by the parasite. There are no epidemiological differences that reinforce the

claim that the parasite is cosmopolitan and adapts to the social conditions of the territory.

Fan et al. [27] observed that a high prevalence of toxocariasis occurred similarly in individuals who had contact with dogs and those who had not and thus suggested that both groups present the same risk for infection by *Toxocara canis*. It is important to highlight that various parents/guardians of the children reported that they did not have dogs or cats at the home, but that the child did have daily contact with these animals at the homes of their relatives.

A mother's level of education was another variable that was not statistically significant, which contrasts the findings of Ferreira et al. [28] who attributed a significant decrease in the prevalence of enteroparasitosis in children living in the city of São Paulo, Brazil, to the mother's level of education and to the income improvement observed over the last decades. However, it is worth noting that this was a study conducted in a neighborhood of Campinas, Brazil, that has a low human development index (HDI), and where 25% of pregnant women are younger than 20 years of age and on average have a lower level of education [29].

An important factor in the current study is the significant number of children who abandoned the cohort. This loss occurred exclusively because of families moving from the study region, probably because of social factors that forced their relocation. Such a significant loss was unexpected; however, these losses did not compromise the findings or the impact of the work.

It is also important to note that access to families and the territory where they lived was assessed with the knowledge and consent of those responsible for the region's Primary Care Unit and was facilitated by the community health agent responsible for that location. The cohort was assessed during the same time period as the largest dengue outbreak in Campinas' history, which brought an important outcome to this study.

The diagnosis of toxocariasis is performed through antibody detection. Various techniques have been developed, with the most commonly used being ELISA in which larval excretory-secretory products are used [30]. The current ELISA being used has a 78% sensitivity and a 92% specificity [31]. This makes it possible to identify truly positive or truly negative individuals using these data, since a sensitive test rarely fails to detect individuals inflicted with the disease, while a highly specific test will rarely incorrectly categorize individuals who do not have the disease.

It is important to emphasize in this discussion that the presence of detectable anti-*Toxocara* antibodies does not necessarily indicate an active infection. False-positive reactions may occur in individuals suffering from ascariasis, schistosomiasis, or filariasis. It was not feasible to perform a geohelminth survey during our study because of the low adherence by the cohort in providing fecal samples for parasitology tests.

The majority of children infected with *Toxocara* are asymptomatic. However, as a result of the overall number of infected children, the development of more severe clinical manifestations is possible, which may compromise their quality of life. In general, the awareness by health professionals, government workers, and educators with respect to toxocariasis as a public health problem is both necessary and urgently needed. It is also important that preventive and educational measures are employed in order to control this parasitosis.

Disclosure

Current address of Paula Mayara Matos Fialho is as follows: Departamento de Saúde Coletiva, Faculdade de Ciências Médicas, Universidade Estadual de Campinas, Brasil.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Rapid and Visual Detection of *Coxiella burnetii* Using Recombinase Polymerase Amplification Combined with Lateral Flow Strips

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Coxiella burnetii, a global-distributed biological warfare agent, is the causative agent of Q fever. Correct diagnosis of Q fever is challenging and developing a fast, simple, and reliable detection method is necessary. In this study, recombinase polymerase amplification (RPA) assay combined with lateral flow (LF) test was developed targeting 23S rRNA gene of *C. burnetii* Xinqiao strain. Primers and probe were designed and synthesized, with one set with high amplification efficiency screened for establishment of the method. Reaction conditions were optimized. Sensitivity, specificity, and accuracy were evaluated. The established RPA-LF reaction could be completed in 30 minutes by combining RPA at 37°C with LF at room temperature, with visually judged results. The method showed good specificity without recognizing other bacteria evaluated. It detected positive plasmid and genomic DNA at levels of 10 copies/reaction and 7 copies/reaction, respectively, levels comparable to that of real-time quantitative PCR (RT-qPCR) targeting 23S rRNA gene established previously. Both RPA-LF and RT-qPCR were used to detect *C. burnetii*-infected mouse samples and the results were fully consistent. The method showed superior detection performance and will provide technical support against *C. burnetii* in resources-limited areas.

1. Introduction

C. burnetii is the causative agent of Q fever, which is globally distributed and listed as a biological warfare agent [1]. Its broad host range includes domestic and wild animals as well as humans [2, 3]. Acute Q fever is usually transmitted to humans by inhalation of aerosols generated by infected animals and may progress to chronic disease complicated by endocarditis, chronic hepatitis, and/or osteomyelitis [4, 5], which are sometimes incurable [6, 7].

The largest outbreak of Q fever ever reported in the literature occurred in the Netherlands from 2007 to 2010, and *C. burnetii*-infected dairy sheep or goats were the source [8, 9]. However, accurately diagnosing Q fever is challenging, because its clinical signs, such as flu-like signs or pneumonia, are not pathognomonic [10, 11], leading to misdiagnosis, delayed treatment, serious pneumonia, and even fatal disease [12]. To reducing such major impacts of Q fever on public health, the development of accurate diagnostic techniques is firstly required [13].

Specialized laboratories detect Q fever by diagnostic techniques employing antibodies or nucleic acid. The most commonly used and reliable antibody detection methods include complement fixation [14], indirect immunofluorescence [15], and ELISA [16]. These methods rely on antibodies generating usually in 1 to 2 weeks after infection, which may delay proper treatment. Also preparing the diagnostic *C. burnetii* whole cell antigens is hazardous and laborious [3, 13, 17]. The culture of the antigen usually needs a biological safety protection third-level laboratory, where lots of human

Table 1: Primers and probe for PCR, RPA, and qPCR.

Usages	Primer or probe	Nucleotide sequences (5′-3′)
PCR	CbF1	AAGGATCCAATTAACCGTTGTAGTT
	CbR1042	CGGAATTCTCACTCTTTCCTATGTT
RPA	CbF310	TCCTTGTCGGGTAAAAAATTGCCCCGCTAA
	CbF340	ACTGTAAAGTTTAGTGATAAAGTCAGCTCA
	CbF370	TATCGGGGGAACCCTCCTGCTTTTTAGCAA
	CbF399	AAGGGCAATCCCGAGGGAAGTCTTAAATGA
	CbR484	Biotin-AGTCAGCGTATTGCACACAAATGCGTGCCT
	CbR507	Biotin-TGAGTATAAACCCAAGGGCAAGAAGTCAGC
	CbR533	Biotin-TAAATTCTCCATAGTCACTTACTTCTTGAG
	CbR564	Biotin-CATACCATGGCTCTAAATGTAAATACATAA
	Cbprobe408	FAM-CCCGAGGGAAGTCTTAAATGACCCCGTAAC-[THF]-ACTGATCCGAAAGGT-PO_4
RT-qPCR [22]	CbF	CGGCTGAATTTAAGCGATTTATTTTT
	CbR	CGTAACCACACACGCATCTCA
	TaqMan-MGB probe	TGCAATGGGTTCGG

and material resources are needed, or there is a risk of leakage to threat public health. As a nucleic acid detection method, real-time quantitative PCR (RT-qPCR) is the most frequently used for direct detection of *C. burnetii* in whole blood or buffy coat aliquots collected at onset of symptoms and before antibiotic treatment [18, 19]. This method relies on highly equipped centralized laboratories as the fluorescent quantitative machine is too expensive, especially in resource-limited areas where these diseases are endemic.

It appeared, thus, necessary to develop a fast, simple, and reliable detection method for *C. burnetii*, which is suitable for diagnosis in the field, in simple and crude removable laboratory, or in some basic medical unit such as county or township hospital. Recombinase polymerase amplification (RPA) is an isothermal DNA amplification method in which amplification reaction can be completed in 10 to 20 min at 24°C to 45°C [20]. The successful application of RPA is evident as shown in many publications [21]. Furthermore, the amplicons can be visually detected using a lateral flow (LF) test at room temperature. A viable point-of-care nucleic acid detection method combining RPA with LF is promising for utilization in resources-limited areas. In this study, such a detection method for *C. burnetii* was established and evaluated.

2. Materials and Methods

2.1. Ethics Statement. The animal experiments were approved by the Administrative Committee for Laboratory Animals of Huadong Research Institute for Medicine and Biotechniques and the animal care met the standard of the committee. Mice were well cared for during their stay in the facility and all efforts were made to minimize suffering. The use of human blood samples was approved by the Ethics Committee of Huadong Research Institute for Medicine and Biotechniques and consent form was signed.

2.2. Preparations of DNA. DNA from spleens of *C. burnetii* (Xinqiao strain)-infected mice was kindly given by Professor Bohai Wen from State Key Laboratory of Pathogens and Biosafety of China. Briefly, 9 female, 5-week old C57BL/6 mice were infected with *C. burnetii* through intraperitoneal injection, and 9 control mice were injected with phosphate buffered saline (PBS). One week after infection, mice were sacrificed and spleens were collected. DNA was extracted from 10 mg of the spleens using a QIAamp Blood and Tissue Mini DNA kit (Qiagen, CA, USA) as per the manufacturer's instruction.

Genomic DNAs of *C. burnetii* (Xinqiao strain), *Rickettsia rickettsii* (Sheila Smith), *Rickettsia heilongjiangensis* (054 strain), and *Rickettsia sibirica* (246 strain) were also kindly given by Professor Wen. Bacteria of *Orientia tsutsugamushi* (Karp-like strain), *Staphylococcus aureus*, and *Streptococcus suis* were kindly given by Research Institute for Medicine of Nanjing Command [23–25]. Genomic DNAs of these bacteria were extracted from the corresponding bacteria using a QIAamp Blood and Tissue Mini DNA kit (Qiagen).

For human blood DNA, 5 mL of blood samples was collected from cubital veins of health volunteers and DNA was extracted using a QIAamp Blood and Tissue Mini DNA kit (Qiagen).

For quality control, the presence of DNAs of *C. burnetii*, *R. rickettsii*, *R. heilongjiangensis*, *R. sibirica*, *O. tsutsugamushi*, *S. aureus*, and *S. suis* was detected using RT-qPCR as described previously [22, 26–31] and that of human blood was detected using PCR targeting GAPDH gene [32].

2.3. Design of Primers and Probes. The sequence of 23S rRNA gene of *C. burnetii* (Genbank: AE016828) was analyzed using DNAman version 5.2.2 software and Nucleotide BLAST online (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Partial sequence of the 23S rRNA gene, from 169923 to 170964 of the genome sequence, was selected as target gene and its specificity was evaluated by Nucleotide BLAST online. Polymerase chain reaction (PCR) primers were designed using software Primer premier 5.0 to include restriction enzyme cutting sites of *Bam*H I or *Eco*R I as shown in Table 1.

The primers and probe for RPA were designed manually as indicated in Table 1, including 4 forward primers, 4 reverse primers, and 1 probe. The 5′ end of the reverse primer was labeled with biotin. The 5′ end of the probe was labeled with Carboxyfluorescein (FAM), the 3′ end was blocked with a phosphate group, and a base analog tetrahydrofuran (THF) was inserted between the 30th and 31st base. All primers and probes were synthesized by Genscript company of Nanjing.

2.4. Construction of Recombinant Plasmid. The partial gene of 23S rRNA was amplified by PCR using primers indicated above. Briefly, 12.5 μL of 2x PCR premix solution (Premix Taq™ Version 2.0, TaKaRa, Dalian, China), 1.5 μL of each primer (10 nM), 2 μL of genomic DNA (1×10^8 copies/μL), and 7.5 μL of dH2O were mixed together to initiate the reaction. The reaction was conducted as described by the manufacturer's instruction. Plasmid pUC19 was purified from *E. coli* cells using a TaKaRa MiniBEST Plasmid Purification Kit. Both the amplified gene and plasmid were digested using *BamH* I and *Eco*R I restriction enzymes and linked using a Takara DNA Ligation Kit. Competent *E. coli* cells were transformed with ligation product, scraped to solid LB medium with ampicillin, and incubated overnight at 37°C. Recombinant plasmid 23SrRNA-pUC19 from positive bacterial colony was purified and digested using both *BamH* I and *Eco*R I, and the product was analyzed by agarose gel electrophoresis to confirm the target gene was linked. The concentration of the recombinant plasmid was measured using Nanodrop 2000, and the copies were calculated according to Avogadro constant.

2.5. Establishment and Optimization of RPA Assay. For establishment of the RPA method, each of the 4 RPA forward primers was combined with each of the 4 RPA reverse primers to make 16 groups of primers. An initial RPA reaction system recommended by the manufacturer's instruction of TwistAmp® RPA nfo kit (TwistDx™ Limited, Cambridge, UK) was used to screen the best primer group. Briefly, 29.5 μL of rehydration buffer, 2.1 μL of forward or reverse primer (10 μM), 0.6 μL of probe (10 μM), 1 μL of 23SrRNA-pUC19 or pUC19 (1×10^4 copies/μL), and 12.2 μL of dH_2O were mixed together, vortexed, and spun briefly. The mixture was then added to the 0.2 mL tube which contained the freeze-dried reaction pellet to reconstitute it. The reaction pellet consisted of the recombinase, polymerase, and single-stranded binding protein. 2.5 μL of magnesium acetate (280 mM) was pipetted into the tube lids. To initiate the reaction, the lids were closed and the magnesium acetate was spun down using a microcentrifuge mySPIN 6 (Thermo Scientific) for 5 sec to the mixture. The tube was incubated at 37°C for 4 min, followed by another brief vortex and incubation for 16 min at 37°C.

For analysis of the amplified product, Millenia Genline Hybridetect-1 (MGH) strips (Millenia Biotec GmbH, Gieben, Germany) were used. Two μL of the amplified products were mixed with Tris-buffered saline to a total of 100 μL in a well of a 96-well plate. The sample pad of each MGH strip was immersed into the dilution of amplified product in each well. After 3 to 5 min of incubation at room temperature, the results were determined visually by naked eyes from the test line (T line) and the control line (C line) on the strips. Briefly, as a positive result, both the T line and the C line were developed, indicating that the DNA labeled with both FAM and biotin existed in the amplicons; only the C line being developed indicates a negative result; only the T line being developed indicates that the strip should be replaced.

To optimize the RPA-LF system, various concentrations of reverse primer (10 μM, 5 μM, and 2.5 μM) and probe (5 μM and 2.5 μM) were evaluated in the reaction mixture, a series of amplification times were explored, and various volumes of amplified products (1 μL, 2 μL, and 5 μL) were used to develop the MGH strips. In the optimization experiment, 1×10^4 copies of 23SrRNA-pUC19 (experimental group) or pUC19 plasmid (negative control) was used as template. All the reactions were conducted in duplication.

2.6. Evaluation of Sensitivity and Specificity. Both recombinant plasmid 23SrRNA-pUC19 and genomic DNA of *C. burnetii* were used to evaluate the sensitivity of the established RPA-LF detection method.

The recombinant plasmid was diluted into a series of concentrations from 1×10^4 copies/μL to 1 copy/μL with Elution buffer (TaKaRa MiniBEST Plasmid Purification Kit Ver.4.0) and added to the optimized RPA reaction system as templates to evaluate the detection limit of the method for detection of positive plasmid. The genomic DNA of *C. burnetii* was diluted into a series of dilutions with human blood DNA solution and the DNA copies were evaluated using RT-qPCR as described previously [5, 22]. Primers and probe sequences used in RT-qPCR were indicated in Table 1. Then these dilutions were used as templates to evaluate the detection limit of the method for detection of genomic DNA.

For evaluation of specificity, the purified genomic DNAs of *C. burnetii*, *R. rickettsii*, *R. heilongjiangensis*, *R. sibirica*, *O. tsutsugamushi*, *S. aureus*, and *S. suis* were used as experimental or control templates to conduct the RPA detection method.

All the reactions were conducted in duplication.

2.7. Detection of Infected Mouse Samples. DNA from infected or uninfected mouse spleens was prepared as described above. *C. burnetii* DNA in the samples was detected using RT-qPCR targeting 23S rRNA gene as described previously [5, 22] which was considered as the golden standard in this research. The primers and probe used in RT-qPCR were indicated in Table 1. Then the samples were used as templates to evaluate the established RPA detection method. The results of RPA were compared with those of RT-qPCR to check their accuracy.

3. Results

3.1. Specific Sequence and Recombinant Plasmid. Partial sequence of 23S rRNA gene of *C. burnetii* was selected as a specific sequence. Its specificity was evaluated using Nucleotide BLAST online with *C. burnetii* (taxid: 777) excluded in the interface of the software. As shown in Figure 1(a), the partial sequence selected was very specific

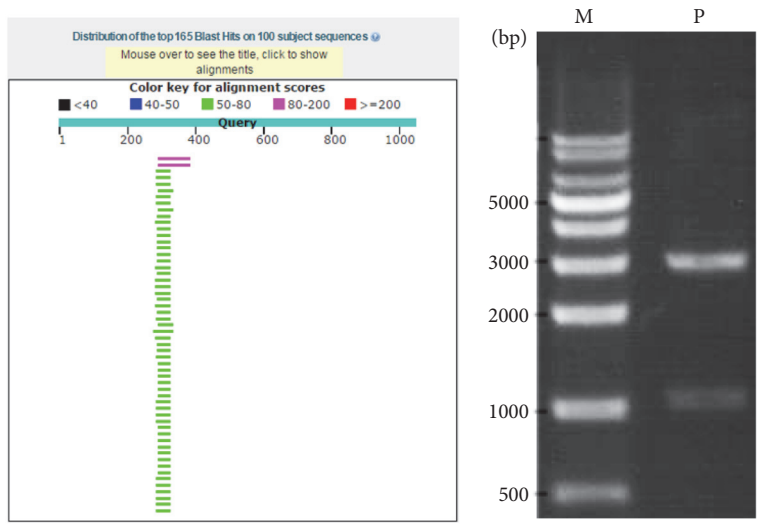

Figure 1: Specificity analysis of the partial sequence of 23S rRNA gene and verification of the recombinant plasmid. (a) The partial sequence was analyzed using Nucleotide BLAST with genome of *C. burnetii* excluded and the BLAST hits were shown; (b) the recombinant plasmid was digested with *Bam*H I and *Eco*R I, and the products were analyzed using agarose gel electrophoresis; M, DNA marker; P, digested products.

and the alignment scores were no higher than 200 aligned with DNA sequence of any other species. This ensured the "in silico" specificity of the RPA assay.

The DNA sequence was linked with pUC19 plasmid to make a recombinant plasmid, which was verified by digestion with *Bam*H I and *Eco*R I and analyzed with agarose gel electrophoresis as shown in Figure 1(b). The band on the gel corresponded to the actual size of the DNA sequence of 1042 bp, indicating the successful construction of recombinant plasmid.

3.2. RPA Assay Establishment and Optimization. Four forward primers, 4 reverse primers, and 1 probe were designed, synthesized, and combined to make 16 primer groups. The best primer group was screened using the initial RPA reaction system. A best primer group should not only lead to a high amplification efficacy, but also lead to a good specificity. So except for the recombinant 23SrRNA-pUC19 as the experimental template, an empty plasmid pUC19 was used as the control template. After the amplification, the products were developed with lateral flow detection strips and visually determined the results [21].

As shown in Figure 2 and Table 2, T lines of the experimental strips in groups 10, 11, 14, and 16 developed band with deeper color while these of the control strips developed no band. These four groups of primers performed best amplification efficacy and specificity. In the other groups, T lines of the experimental strips developed lighter color band, or T lines of both experimental and control strips developed band, indicating a low amplification efficacy or poor specificity. The primer group 16 was selected for the experiment as shown in Table 2.

The RPA-LF detection system was optimized using primer group 16. Different concentrations of reverse primer and probe were combined and introduced to the RPA assay. As shown in Figure 3(a) and Table 3, T lines of the experimental strip in groups 1 (10 μM of CbR564 and 5 μM of Cbprobe408) and 2 (5 μM of CbR564 and 5 μM of Cbprobe408) developed band with deeper color while those of the control strip developed no band. Considering the material cost, a lower concentration of reverse primer was selected with 5 μM of CbR564 and 5 μM of Cbprobe408. Various loading volumes of the amplified products were used to develop the MGH strips. As shown in Figure 3(b), the

Table 2: Results of screening of primer combinations.

Group number	Primer pairs	Results
1	CbF310 + CbR484	−
2	CbF310 + CbR507	−
3	CbF310 + CbR533	−
4	CbF310 + CbR564	−
5	CbF340 + CbR484	−
6	CbF340 + CbR507	−
7	CbF340 + CbR533	−
8	CbF340 + CbR564	+
9	CbF370 + CbR484	−
10	CbF370 + CbR507	+
11	CbF370 + CbR533	+++
12	CbF370 + CbR564	−
13	CbF399 + CbR484	−
14	CbF399 + CbR507	+++
15	CbF399 + CbR533	−
16	CbF399 + CbR564	+++

$^{-}$Bad; $^{+}$good; $^{+++}$excellent.

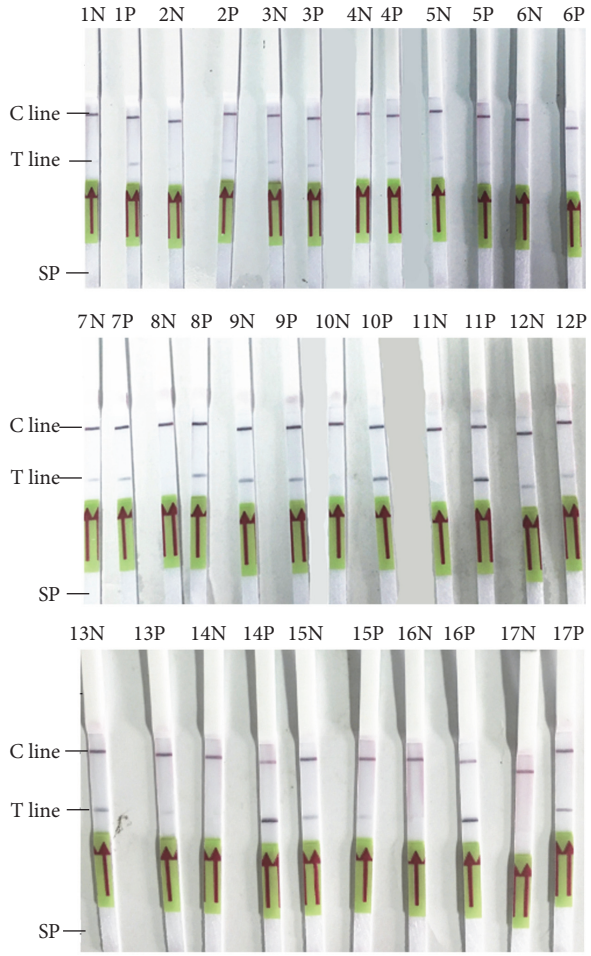

Figure 2: Screening of primer combinations. Sixteen groups of primers (indicated in the table) were used to conduct the RPA-LF reaction with recombinant plasmid 23SrRNA-pUC19 as positive template (P) and plasmid pUC19 as negative template (N). Group 17 using primers, probe, and template from the TwistAmp RPA nfo kit was conducted as a control. SP, sample pad of the strip.

Table 3: Results of RPA-LF using reverse primer and probe with various concentrations.

Group number	Concentrations (CbR564 & Cbprobe408)	Results
1	10 μM & 5 μM	+++
2	5 μM & 5 μM	+++
3	2.5 μM & 5 μM	+
4	10 μM & 2.5 μM	−
5	5 μM & 2.5 μM	−
6	2.5 μM & 2.5 μM	−

$^{-}$Bad; $^{+}$good; $^{+++}$excellent.

color intensities of the bands were proportional to the loading volumes and 5 μL of the amplified products developed the deepest band on the strip. Various amplification times were set for RPA assay to determine the most proper time. As shown in Figure 3(c), an amplification time of 15 min could lead to a modest development on the strip and 20 min to the deepest band. Considering a better sensitivity, a loading volume of 5 μL of amplified product and an amplification time of 20 min were chosen in the optimized RPA-LF detection method.

3.3. Sensitivity and Specificity. The sensitivity of the RPA-LF detection method in detecting recombinant plasmid 23SrRNA-pUC19 or genomic DNA of *C. burnetii* was evaluated. As shown in Figure 4(a), 10 to 10,000 copies of recombinant 23SrRNA-pUC19 could be detected in the reaction while fewer copies could not. The detection limit of the method is 10 copies/reaction in detecting recombinant plasmid. For genomic DNA, 5 dilutions were tested with both the established RPA-LF method and RT-qPCR. After the RPA reaction, Genomic DNA of *C. burnetii* in dilutions 2 to 5 could be detected while that in dilution 1 was not detected (Figure 4(b)). In the RT-qPCR, genomic DNA copies in dilutions 2 to 5 were detected as 7 copies/μL, 51 copies/μL, 1002 copies/μL, and 14526 copies/μL, respectively, while those in dilution 1 were undetected (Figure 4(c)). Compared with RT-qPCR, the detection limit of RPA-LF method in detecting genomic DNA could be as low as 7 copies/reaction, which is similar to that of RT-qPCR.

Except for genomic DNA of *C. burnetii*, genomic DNAs of other pathogens, including *R. rickettsii*, *R. heilongjiangensis*, *R. sibirica*, *O. tsutsugamushi*, *S. aureus*, and *S. suis* were used to evaluate the specificity of the RPA-LF method. The concentrations of these genomic DNAs were determined to be 1×10^5 to 1×10^8 copies/μL by RT-qPCR. As a result shown in Figure 5, the established method showed no cross-reaction with the genomic DNAs of these 6 kinds of pathogens and performed a good specificity.

3.4. Infected Mouse Samples Detection. The effectiveness of the established RPA-LF method was evaluated with DNA of spleens from *C. burnetii*-infected mice. The existence of *C. burnetii* DNA was verified by RT-qPCR. As shown on Figure 6, *C. burnetii* DNA in the 9 samples was all detected while that in the 9 control samples was not. The results showed a good coincidence between RPA-LF method and RT-qPCR. The established method is effective in detection of infected mouse samples with both sensitivity and specificity of 100%.

4. Discussion

RPA is a new developed technology and is considered as a replacement of PCR in the future [33]. It mainly relies on three proteins, a recombinase, a polymerase, and a single strand binding protein. The optimum reaction temperature of these proteins is 37°C. In the reaction process, constant change of temperature like PCR or RT-qPCR was not necessary, resulting in a highly efficient amplification and consuming less than 20 min. However, until now, no reliable method of primer or probe design for RPA exists and optimal sets of primers and probes need to be screened. In this research, 4 of the 16 primer groups were screened to lead to a high amplification efficacy and a modest specificity. In

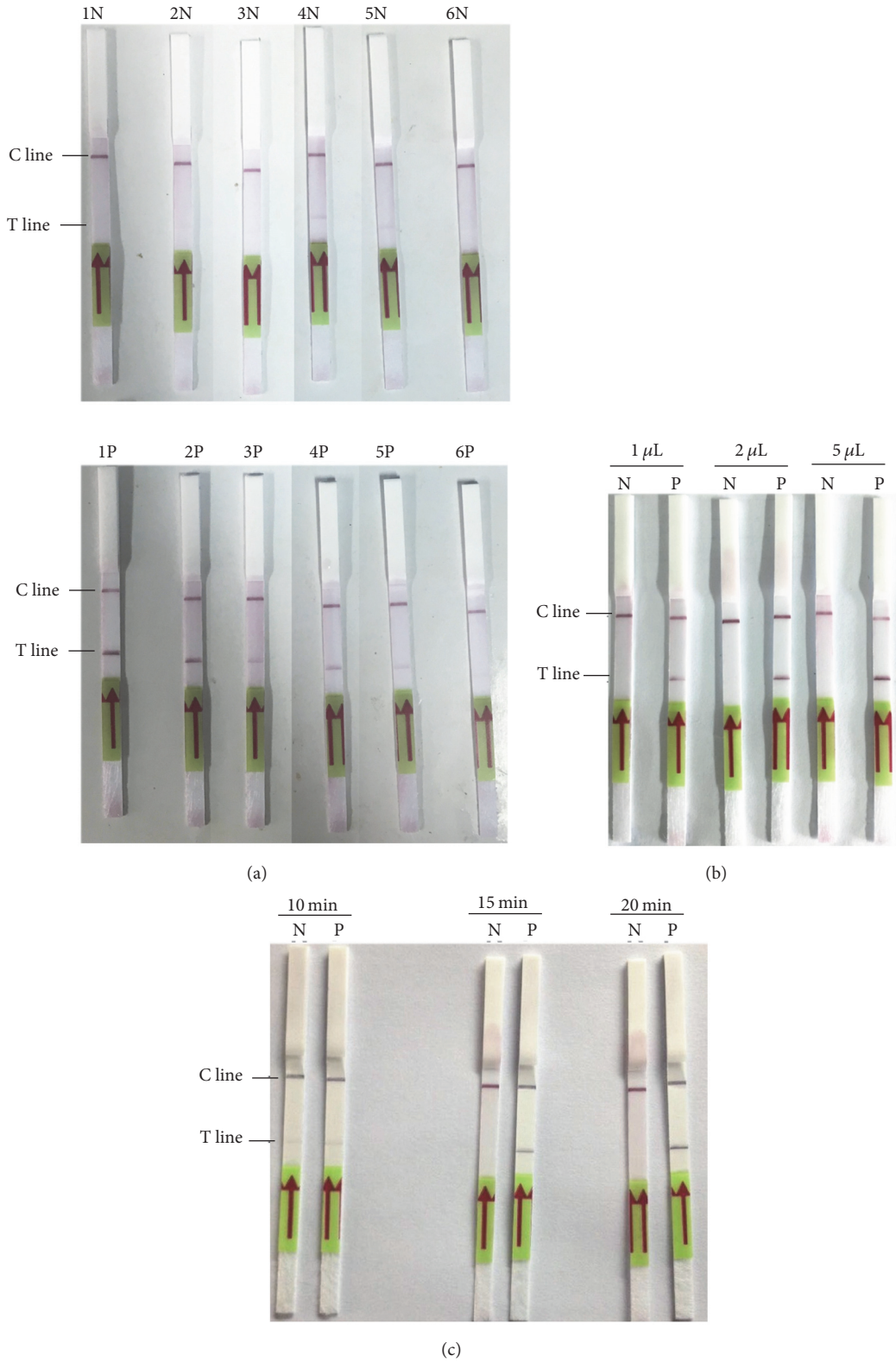

Figure 3: Optimization of the RPA-LF detection system. (a) Various concentrations of reverse primer and probe were combined to make 6 groups as indicated in Table 3 and evaluated using RPA-LF; (b) various volumes (1 μL, 2 μL, or 5 μL) of the amplified products were used to develop the MGH strips; (c) various of amplification times (10, 15, or 20 min) were used in RPA-LF and developed with MGH strips. Each group used both recombinant plasmid 23SrRNA-pUC19 as positive template (P) and plasmid pUC19 as negative template (N).

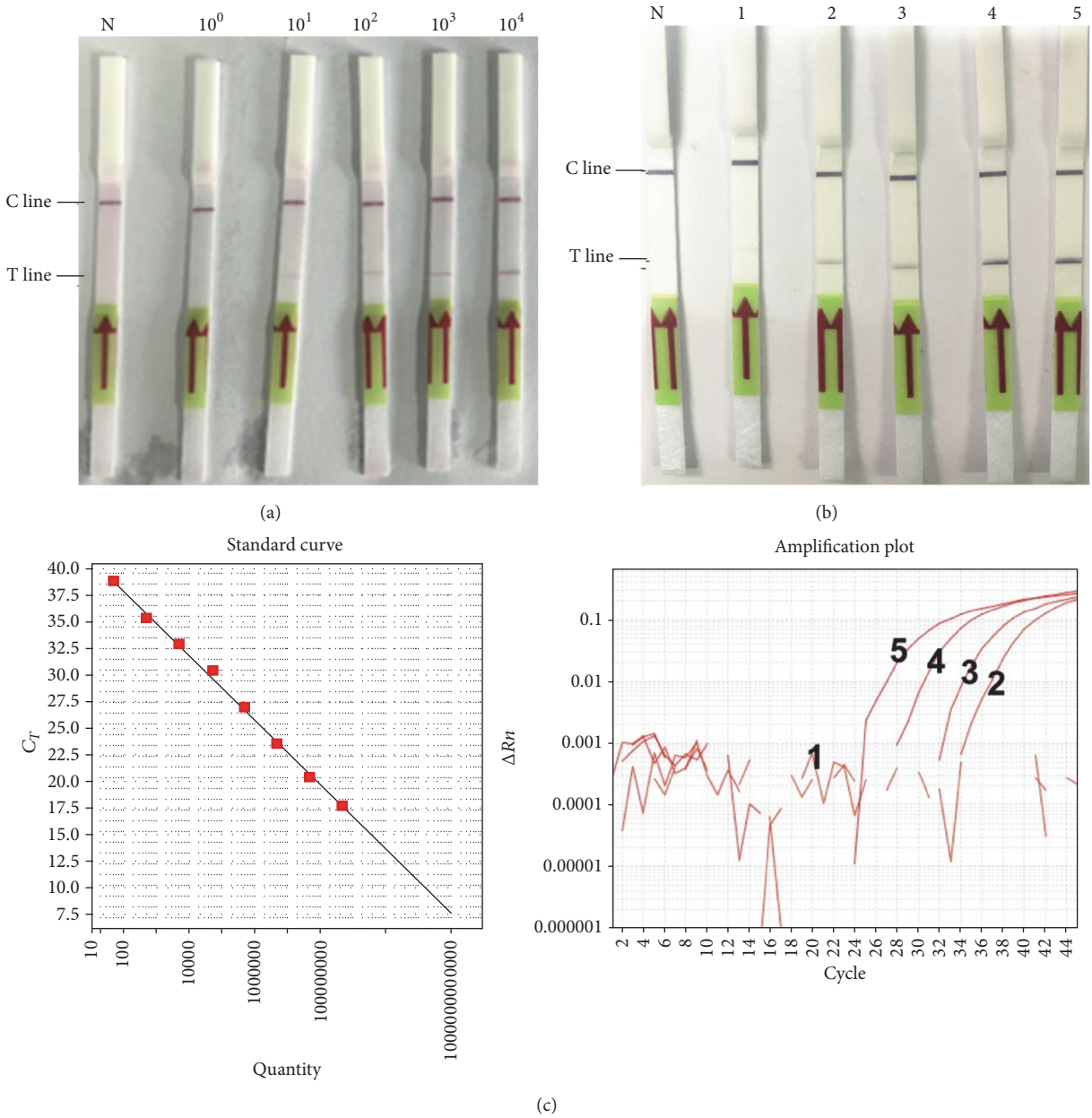

Figure 4: Sensitivity analysis of RPA-LF detection method. Sensitivity of RPA-LF detection method was evaluated with various copies of 23SrRNA-pUC19 (a) or various of dilutions of genomic DNA of *C. burnetii* (b). Copies of genomic DNA of dilutions 1 to 5 (as shown on (b)) were evaluated as undetected, 7 copies/μL, 51 copies/μL, 1002 copies/μL, and 14526 copies/μL, respectively, by RT-qPCR (c). N, negative control using template of pUC19 plasmid (a) or human blood DNA (b).

the optimization, though we chose an amplification time of 20 min for a better sensitivity, the 15 min of amplification did lead to a deep enough band on the strip. In the actual clinical detection application, the amplification time can be set from 15 to 20 min.

In this study, partial sequence of 23S rRNA gene of reference strain RSA 493 was chosen and finally a sequence of 166 bp between primers CbF399 and CbR564 was selected as target sequence. To ensure that the established method could detect all the other strains of *C. burnetii*, the target sequence was aligned with the corresponding sequence of almost all the strains in Genbank database using Nucleotide BLAST online (https://blast.ncbi.nlm.nih.gov/Blast.cgi), including strains Schperling (Genbank: CP014563.1), Heizberg (Genbank: CP014561.1), Henzerling (Genbank: CP014559.1), 18430 (Genbank: CP014557.1), 2574 (Genbank: CP014555.1), 701CbB1 (Genbank: CP014553.1), 14160-001 (Genbank: CP014551.1), 14160-002 (Genbank: CP014836.1), CbCVIC1 (Genbank: CP014549.1), 42785537 (Genbank: CP014548.1), Scurry_Q217 (Genbank: CP014565.1), RSA 439 (Genbank:

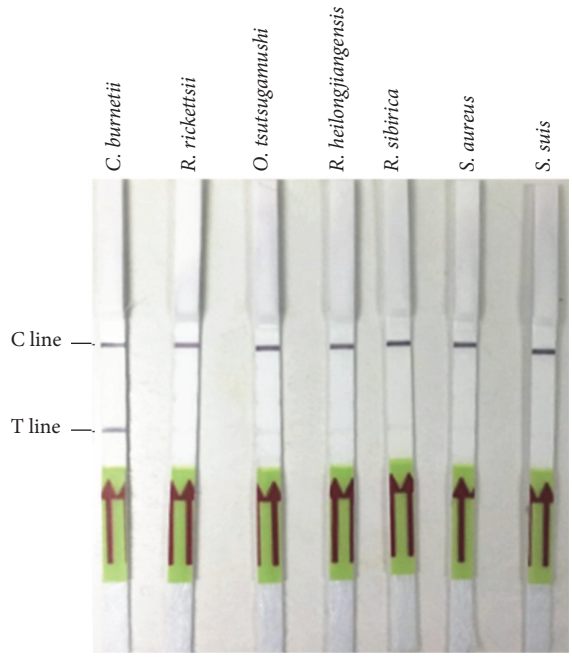

Figure 5: Specificity analysis of RPA-LF detection method. Genomic DNA from various organisms including *C. burnetii*, *R. rickettsii*, *R. heilongjiangensis*, *R. sibirica*, *O. tsutsugamushi*, *S. aureus*, and *S. suis* was used as experimental or control templates to conduct the RPA-LF detection method.

CP018005.1 and CP020616.1), MSU Goat Q177 (Genbank: CP018150.1), RSA 493 (Genbank: AE016828.3), 3345937 (Genbank: CP014354.1), 3262 (Genbank: CP013667.1), Cb175_Guyana (Genbank: HG825990.3), Namibia (Genbank: CP007555.1), Z3055 (Genbank: LK937696.1), CbuK_Q154 (Genbank: CP001020.1), CbuG_Q212 (Genbank: CP001019.1), RSA 331 (Genbank: CP000890.1), and Dugway 5J108-111 (Genbank: CP000733.1). All the sequences from various strains are of 100% identity, indicating that the established method could detect almost all *C. burnetii* strains theoretically.

The amplicons of RPA assay can be detected using agarose gel electrophoresis, lateral flow test, or real-time fluorescent detection. Both the agarose gel electrophoresis and real-time fluorescent detection need expensive devices or are not convenient enough for basic medical unit like some county or township hospitals. So we chose lateral flow test for the product detection in which results can be visually determined. To do this, a nfo (a kind of endonuclease) enzyme, a biotin-labeled reverse primer, and a FAM-labeled, phosphate-blocked, and THF-inserted probe were introduced. After a normal RPA process, the amplified products will be labeled with biotin. Then the probe will be annealed with the amplicons, and the blocked 3′ end of the probe will be cut off by nfo enzyme at the THF site. The left probe sequence labeled with FAM will initiate another amplification as a forward primer with the biotin-labeled reverse primer, resulting in a double labeled product, which will be recognized by the MGH strips. The design by the company is artful; however, the role that the forward primer plays is limited and we suspect that even if the forward primer is removed, the whole reaction still works. This will be tested and discussed in the future. During the research, it is found that the amplified products were easy to spread when pipetted to develop the strips, leading to a potentially important contamination and false positive in the future experiment. So maybe using a XCP cassette to replace the MGH strips is a good option as suggested by Chao et al. [21].

The RPA assay has been shown to detect product amplified from a single molecule [20, 21]. This research shows that RPA is a method that could be used to detect plasmid DNA, DNA extracted from *C. burnetii*-infected mice, or pure organisms with a detection limit of 10 copies or less, within 20 minutes, and without cross-reaction with other organisms. This is generally comparable to that of RT-qPCR considering they have a similar detection limit (Figure 4). Actually, RPA is more attractive. A cheap heating block is the only needed device instead of an expensive fluorescent quantitative machine in RT-qPCR, which liberates some detection limits for many different laboratories with limited instruments and infrastructure.

In the specificity analysis, 4 kinds of bacteria (*R. rickettsii*, *R. heilongjiangensis*, *R. sibirica*, and *O. tsutsugamushi*) from order of Rickettsiales were chosen as relative control for *C. burnetii* shares similar biological characteristics and was classified into the order of Rickettsiales in the past. Also two irrelevant kinds of bacteria (*S. aureus* and *S. sui*s) were used to exclude potential influence or interference from other disease. The established method did not recognize any of these bacteria, indicating a good specificity of this method.

In this research, it is a pity that we did not collect any clinical samples from patients. So samples from *C. burnetii*-infected mice were used to evaluate the established method. The results totally agree with those of RT-qPCR. For acute human cases, whole blood or buffy coat aliquots are most useful for diagnosis [19]. So in the sensitivity evaluation experiment, the genomic DNA was diluted with human blood DNA solution, which partly simulated the patient samples and excluded the interference of human DNA in the method. All the results demonstrated the potential clinical application of the assay.

In conclusion, based on RPA assay and LF strips, we successfully establish a prompt, accurate, sensitive, specific method for detection of *C. burnetii*, with a visible result judged by naked eyes. The method had a detection limit similar to that of RT-qPCR and a modest specificity without recognizing other organisms like *R. rickettsii*, *R. heilongjiangensis*, *R. sibirica*, *O. tsutsugamushi*, *S. aureus*, or *S. suis*. and samples from mouse or human. The results evaluated with infected mouse samples were in complete agreement with those of RT-qPCR. The isothermal amplification, at a constant temperature of 37°C, obtained by RPA-LF makes the method promising for a wide use in the field, though more clinical patient samples are needed to evaluate the method in the future.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

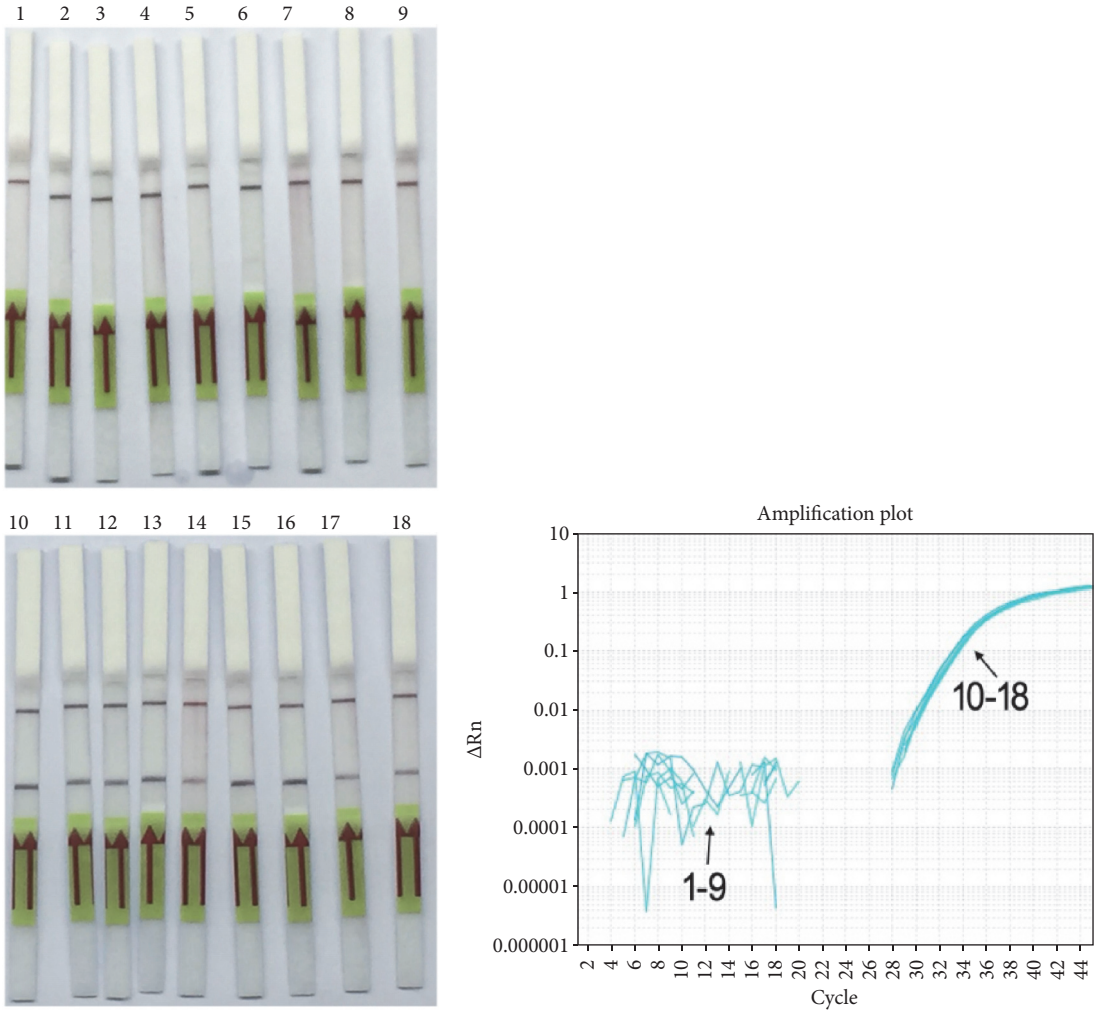

FIGURE 6: Evaluation of the established RPA-LF method with *C. burnetii*-infected animal samples. DNAs of uninfected (1 to 9) or *C. burnetii*-infected (10 to 18) mouse spleens were detected with RPA-LF method (a) or RT-qPCR (b).

Authors' Contributions

Yong Qi and Qiong Yin contributed equally to this research.

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