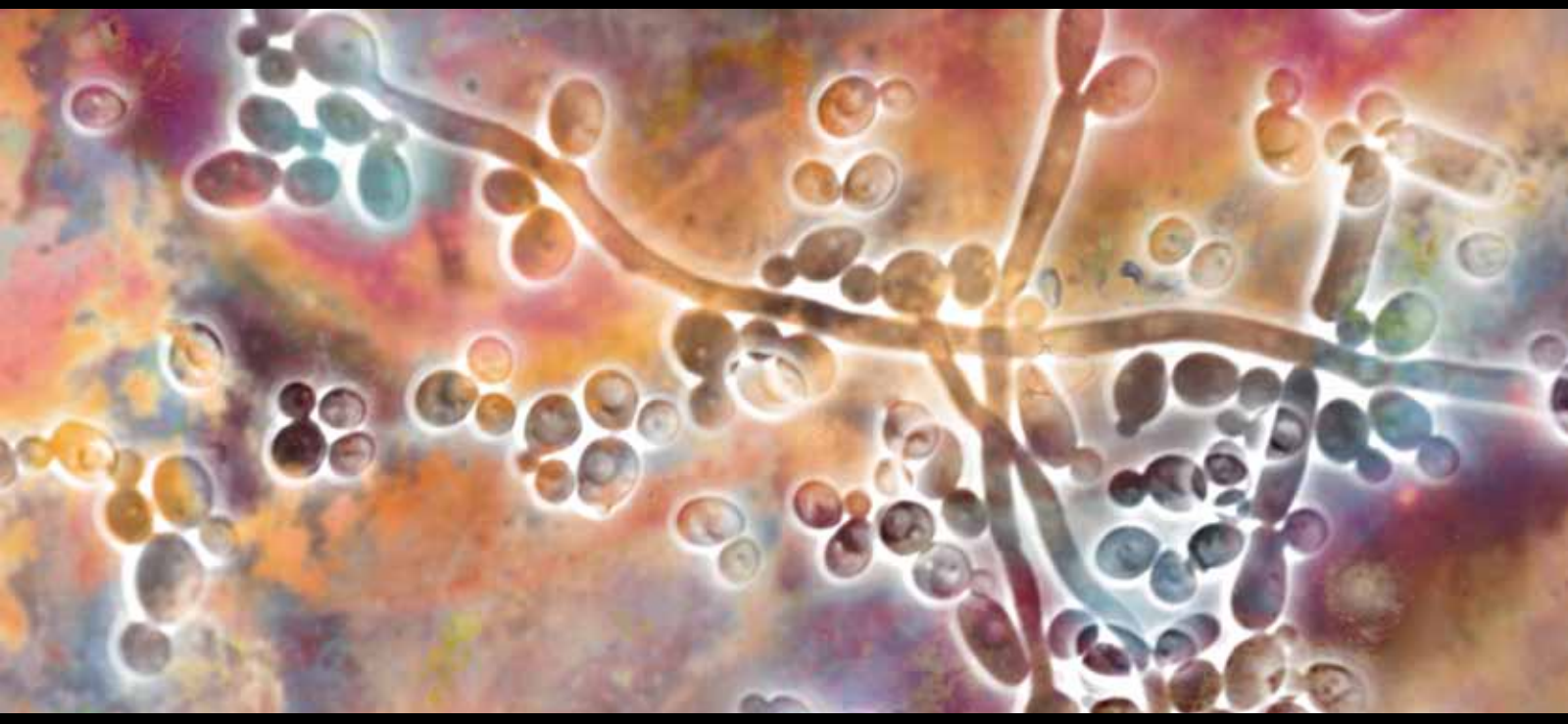
Interdisciplinary Perspectives on Infectious Diseases

Neglected Infectious Diseases: Mechanism of Pathogenesis, Diagnosis, and Immune Response

Guest Editors: Eliete Caló Romero, Fabiana Pimenta, and Décio Diament



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Editorial Board

Contents

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Editorial

Neglected Infectious Diseases: Mechanism of Pathogenesis, Diagnosis, and Immune Response

Eliete Caló Romero,[1] Fabiana Pimenta,[2] and Décio Diament[3]

[1] *Instituto Adolfo Lutz, Av. Dr. Arnaldo 355, 9o andar, 01246-902 São Paulo, SP, Brazil*
[2] *Instituto de Infectologia Emílio RIbas, Av. Dr. Arnaldo, 165 Cerqueira Cesar, 01246-901 São Paulo, SP, Brazil*
[3] *Centers for Disease Control and Prevention, Mail Stop C-02, 1600 Clifton Rd N. E., Atlanta, GA 30333, USA*

Correspondence should be addressed to Eliete Caló Romero, eliete_romero@yahoo.com.br

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Neglected infectious diseases (NIDs) represent a major global health challenge and comprise a group of endemic diseases which occurs in impoverished regions of Asia, Africa, and America. The World Health Organization (WHO) list of 17 diseases includes Buruli ulcer, Chagas disease, cysticercosis, dengue, dracunculiasis, echinococcosis, fascioliasis, human African trypanosomiasis, leishmaniasis, leprosy, filariasis, onchocerciasis, rabies, schistosomiasis, soil-transmitted helminthiasis, trachoma, and yaws. According to the WHO, neglected infectious diseases affect more than one billion human beings, mainly in tropical areas, in 149 underdeveloped or developing countries [1], imposing great economical burden to its overloaded health systems. Preventive measures and treatments are not widely available in developing countries, despite the low cost of some treatments. Due to globalized trade and travel, some diseases may emerge in developed places or reemerge where they once were controlled [2]. The immediate need is to rise comprehensive control programs based on multidisciplinary approaches that include environmental, therapeutic, diagnoses, and other measures. Also, NIDs may spread into the population in developed countries due to demographic change. Recent advances in biology and medicine have introduced new technologies to study and understand the epidemiology, the reinfection and coinfections, and the mechanisms of the development of resistance to treatments in NIDs. Continuous coordinated efforts are needed in order to achieve these goals, and government and nongovernment [3] programs focused on NIDs are on the agenda.

We invited investigators to contribute original research articles as well as review articles that will stimulate the continuing efforts to understand the NIDs, the development of strategies to treat these diseases, and the evaluation of outcomes. We were particularly interested in articles describing the advances in molecular genetics and molecular diagnostics, new insights into reducing morbidity and mortality, and current concepts in the treatment of NIDs.

In this issue, S. D. Hulme et al. described molecular mechanisms of intracellular murine macrophage survival of pathogenic *Salmonella*. Despite its absence on the WHO list of NIDs, salmonellosis remains an important cause of morbidity and mortality in the developing world, as an agent of acute diarrhea and enteric fever [4]. Two papers, one by A. A. Euzébio et al. and another by N. R. B. Zuim et al., evaluated the pathogenicity of different Brazilian strains of *Schistosoma mansoni* using an experimental murine model, regarding granuloma formation. Differences in pathogenicity could explain distinct clinical evolution of disease in humans, leading to better diagnosis and treatment. D. F. Dávila et al. reviewed physiopathogenic aspects of cardiac involvement in Chagas disease, stressing current theories of myocardial dysfunction. Understanding these issues implies diverse treatments. A. Singh and V. K. Kashyap described a PCR method for *Mycobacterium tuberculosis* detection, using triple DNA targets, resulting in better performance in relation to classical methods of smear and culture. More efficient diagnosis could lead to prompt treatment, avoiding complications of tuberculosis.

V. J. Castillo-Morales et al. studied Mexican domestic cat infection with *Toxoplasma gondii* to determine prevalence and risk factors for infection, using molecular and serological methods. A. R. Nimir presented a comprehensive review of ophthalmologic parasitosis, covering protozoan, helmintic, and ectoparasitic etiologies. The eyes are an important source of symptoms and signs and should be always examined in order to promote precocious diagnosis of parasitic diseases. E. Guzman-Marin et al. studied the influence of *Triatoma dimidiata* in modulating the virulence of *Trypanosoma cruzi*. R. M. Bhat and C. Prakash reviewed genetic determinants of host response, clinical aspects, and transmission and immunology of leprosy, which continues to strike poor populations in developing countries, demanding great efforts from the health systems to control it. O. H. Montes et al. analyzed kinetoplast DNA from isolates of *Leishmania mexicana* in order to determine whether a particular minicircle class is exclusive of one strain or if the differences in clinical manifestation are related to any particular minicircles classes. P. Bhargava and R. Singh reviewed tools for leishmaniasis diagnosis, including new molecular and serological methods, and antileishmania drug development, like miltefosine, new amphotericin lipid formulations, new 8-aminoquinolines, and new potential drug targets in parasite metabolic pathways. H. Honarmand made a review of Q fever, a zoonosis that affects several animals and humans.

In closing this introduction to the special issue, we would like to express our gratitude to the contribution of all the authors and reviewers. We sincerely hope that this special issue will stimulate further investigation of neglected infectious diseases.

Eliete Caló Romero
Fabiana Pimenta
Décio Diament

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Research Article

Analysis of Kinetoplast DNA from Mexican Isolates of *Leishmania (L.) mexicana*

Omar Hernández-Montes,[1] Saúl González Guzmán,[1] Federico Martínez Gómez,[2] Douglas C. Barker,[3] and Amalia Monroy-Ostria[1]

[1] *Department of Immunology, National School of Biological Sciences, National Polytechnic Institute, 11340 México City, Mexico*
[2] *Department of Parasitology, National School of Biological Sciences, National Polytechnic Institute, 11340 México City, Mexico*
[3] *Christ's College, Cambridge CB2 3BU, UK*

Correspondence should be addressed to Amalia Monroy-Ostria, amaliahmo@gmail.com

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This study analyzed DNA minicircles of Mexican isolates of *L. (Leishmania) mexicana* to look for genetic differences between strains isolated from patients with diffuse cutaneous (DCL) and localized (LCL) leishmaniasis. The kDNA was analyzed using polymerase chain reaction (PCR), restriction fragment polymorphism analysis of the PCR products (PCR-RFLP) and the PCR products were sequenced. In the PCR with primers specific for the subgenus *Leishmania*, the Mexican isolates gave higher amplification products than the other *L. mexicana* complex strains and with specific primers for the *L. mexicana* complex they were poorly amplified. In the PCR-RFLP analysis with the *Eco RV*, *Hae III*, and *Mbo I* endonucleases, the Mexican isolates displayed similar restriction patterns, but different from the patterns of the other members of the *L. mexicana* complex. In the phylogenetic tree constructed, the kDNA sequences of the Mexican clones formed two groups including sequences of LCD or LCL clones, apart from the other *L. mexicana* complex members. These results suggest that the kDNA minicircles of the Mexican isolates are more polymorphic than the kDNA of other members of the *L. mexicana* complex and have different recognition sites for the restriction enzymes used in this study.

1. Introduction

Cutaneous leishmaniasis (CL) is the most widespread form of leishmaniasis, causing primary localized skin lesions (LCL) from which parasites can disseminate to the nasopharyngeal mucosa and cause the disfiguring lesions typical of mucocutaneous leishmaniasis (MCL) or disseminated to the entire body as nodular lesions (DCL) [1].

American cutaneous leishmaniasis is characterized by a spectrum of clinical presentations. These include LCL caused by *L. (Leishmania) mexicana*, DCL caused by *L. (Leishmania) amazonensis*, *L. (Leishmania) venezuelensis,* and *L. (Leishmania) pifanoi*, all belonging to the *Leishmania mexicana* complex, and MCL caused by members of the *Leishmania braziliensis* complex [2].

Cutaneous leishmaniasis in Mexico is highly endemic with broad geographical distribution of the different clinical manifestations. In endemic areas, LCL or MCL can be caused by members of both *L. mexicana* and *L. braziliensis* complexes [3], making more accurate analysis and identification of the *Leishmania* strains imperative so that opportune and appropriate treatment can be administered.

Polymerase chain reaction (PCR) approaches have been used in parasite DNA analysis. Several PCR molecular targets have been developed, including minicircle kinetoplast DNA (kDNA) [3], the miniexon (spliced leader RNA) gene [4], and the gp63 PCR-RFLP [5], among others.

Kinetoplast DNA contains approximately 10,000 minicircles per cell that are around 800 base pairs (bp) in size, with a roughly 200 bp conserved region and an approximately 600 bp variable region [6, 7]. The sequence classes of minicircles are organized in a uniform scheme and contain one or several highly conserved regions (CR). There are three highly conserved blocks (CSB) within CRs: CSB1

(GGGCGT), CSB2 (CCCCGTTC), and CSB3 (GGGGTT GGTGTA) known as the universal minicircle sequence (UMS) [6, 7]. Minicircles are subjected to frequent insertions and deletions leading to diversity in size; removal and insertion of recognition sites for various restriction enzymes and some size heterogeneity in the overall minicircle size can be observed [8].

In the present study, the analysis of kDNA minicircles of isolates of *L. (L.) mexicana* from skin lesions of patients with DCL or LCL was performed in order to determine whether a particular minicircle class is exclusive of one strain of *Leishmania* or the differences in clinical manifestation of the disease are related to any particular minicircles classes.

2. Materials and Methods

2.1. Leishmania Species and Culture Conditions. This study was reviewed and approved by the Ethics Committee of the Escuela Nacional de Ciencias Biologicas, Instituto Politecnico Nacional, Mexico, in agreement with international ethics guidelines for biomedical research involving human subjects (Ley General de Salud, Mexico).

The study was conducted with seven Mexican isolates of *L. (L.) mexicana* from patients with multiple nonulcerative nodular skin lesions (DCL) or with ulcerative skin lesions developing at the site of the sandfly bite (LCL) from different states in Mexico (Figure 1), included after informed consent was obtained. For parasite isolation, needle aspirates were taken from the edge of cutaneous lesions prior to patients receiving treatment. The Mexican isolates were sent to the London School for Tropical Medicine and Hygiene (UK) for isoenzyme characterization. Reference strains of members of the *L. mexicana* complex were also included (Table 1).

The strains of *Leishmania* were cultured in RPMI medium supplemented with 10% fetal calf serum at 28°C.

2.2. DNA Extraction. DNA was prepared, as described elsewhere [3], by centrifuging 10^9 parasites of an exponential phase of growth culture at 1900 g for 10 min. The pellet was resuspended in 1 mL of NET 100 (100 mMTris-HCl, pH 8.0; 100 mM EDTA; 100 mM NaCl), 1% SDS, and 4 μL of 10 mg/mL proteinase K (Sigma) and incubated at 37°C overnight, followed by two phenol-chloroform extractions and ethanol precipitation. The DNA extracted was washed with 70% ethanol and dissolved in 100 μL distilled water and kept at −70°C until use.

2.3. Polymerase Chain Reaction. The universal primers AJS1 (GGGGTTGGTGTAAAATAG) and DeB8 (CCAGTTTCC CGCCCCG), specific for kDNA minicircles of the *Leishmania* subgenus [9], and the M1 (CCAGTTTCGAGCCCC GGAG) and M2 (GGTGTAAAATAGGGCCGGATGCTC) primers, specific for minicircles of the *L. mexicana* complex [10] were used to amplify kDNA from reference strains and from Mexican isolates. PCR amplifications were done as described elsewhere [9, 10], in a solution of 0.2 mM each of deoxyribonucleotide (Invitrogen Life Technologies, Carlsbad, CA, USA), 50 pmol of each specific primer, 2.5 units of Taq DNA polymerase (Perkin Elmer Cetus), 100 ng of DNA template, 1.5 mM of $MgCl_2$ in a final volume of 100 μL. Samples were denatured at 96°C for 6 min. PCR (35 cycles) consisted of annealing at 60°C for AJS1 and DeB8 primers and 67.5°C for M1 and M2 primers, extension at 72°C for 1 min, and final extension at 72°C for 10 min on a Perkin-Elmer Model 2400 thermal cycler (Perkin Elmer, Roche Molecular Systems Inc., Brancbourg, NJ, USA). PCR products (10 μL) were fractionated by electrophoresis in 1% agarose or 8% acrylamide gels in TBE (90 mMTris-HCl pH 8.3, 90 mM boric acid, and 2 mM EDTA) stained with ethidium bromide (50 g/mL) and were observed under a transilluminator (SIGMA Chemical Co., St. Louis, MO, USA).

Figure 1: Map of Mexico, showing the endemic regions studied in this work, Veracruz, Tabasco, and Campeche states.

2.4. Restriction Fragment Polymorphism Analysis of kDNA. The kDNA amplicons obtained with the AJS1 and DeB8 primers of the *Leishmania* reference strains and the Mexican isolates were digested with the *Bam HI*, *Eco RV*, *Hae III*, *Hind III*, *Kpn I*, *Mbo I*, *Mse I*, *Msp I*, and *Xba I* endonucleases (Gibco BRL). The restrictions were performed following the supplier's instructions, briefly: 10 μg of PCR product, 2.5 μL of the correspondent buffer, and 10 U of the endonuclease in a final volume of 25 μL were incubated at 37°C for 2 h; the *Taq I* was incubated at 67°C. The reactions were analyzed by electrophoresis as mentioned above.

2.5. Sequencing of Minicircles of Mexican Isolates. The kDNA amplicons of the Mexican isolates, MC (from patient with LCL), and GS (from patient with DCL) obtained with the AJS1 and DeB8 primers were gel purified using a QIA quick gel extraction Kit (Qiagen, Germany), ligated into a TA Cloning vector (pCR II Vector) (Invitrogen Life Technologies), and transformed into *Escherichia coli* INV αF′. Plasmid DNA was extracted using the Wizard Plus Minipreps DNA Purification System (Promega Corporation, Madison, WI, USA) and sequenced. Nucleotide sequences were determined using a dideoxynucleotide chain termination sequence kit

Table 1: Mexican isolates and reference strains of *Leishmania mexicana* complex used in this study.

(a)

Reference strains	International code[1]	Source
L. (*L.*) *mexicana*	MHOM/BZ/62/BEL21	1
L. (*L.*) *mexicana*	MHOM/BZ/62/M379	1
L. (*L.*) *amazonensis*	IFLA/BR/67/PH8	1
L. (*L.*) *amazonensis*	MHOM/BR/73/M2269	1
L. (*L.*) *garnhami*	MHOM/VE/75/HM76	1
L. (*L.*) *garnhami*	MHOM/VE/76/JAP78	1
L. (*L.*) *pifanoi*	MHOM/VE/57/LL1	1

[1]: The WHO *Leishmania* Reference Collection at the London School of Hygiene and Tropical Medicine, London, UK.

(b)

Mexican isolates[2]		Origin
From patients with LCL		
L. (*L.*) *mexicana*	MHOM/MX/88/HRC JS (SOB)	Tabasco
L. (*L.*) *mexicana*	MHOM/MX/88/HRC MC (MC)	Tabasco
L. (*L.*) *mexicana*	MHOM/MX/83/UAYV (YUC)	Campeche
From patients with DCL		
L. (*L.*) *mexicana*	MHOM/MX/84/ISET GS (GS)	Tabasco
L. (*L.*) *mexicana*	MHOM/MX/85/ISET HF (HF)	Veracruz
L. (*L.*) *Mexicana*	MHOM/MX/92/INDRE AM (AM)	Veracruz
L. (*L.*) *mexicana*	MHOM/MX/92/INDRE AG (AG)	Tabasco

[2]: Instituto Nacional de Diagnostico y Referencia Epidemiologicos, Secretaria de Salud, Mexico. Tabasco, Veracruz, and Campeche states are located in the Mexican Southeast.

(ABI PRISM Dye Terminator Cyclers Sequencing Ready Reaction Kit, Perkin Elmer) and an Abi Prism M 310 Genetic Analyzer automated sequencer (Perkin Elmer).

2.6. Sequence Alignments And Phylogenetic Analysis. The sequences were edited with the DNAMAN, Chromas version 2.0, and Seaview software [11]. Multiple sequences were aligned using Clustal-X Ver. 1.83 [12]. An unrooted phylogenetic tree was constructed using the neighbor-joining method [13] with the Clustal-X program. Evolutionary distances were calculated using Kimura's two-parameter method [14], with MEGA (Molecular Evolutionary Genetics Analysis), Version 3.1. [15]. A total of 1000 bootstrapping replicates were used for the neighbor-joining analysis to obtain relative support for internal nodes. The kDNA sequence data were compared with the sequences of the other members of the *L. mexicana* complex previously published in GenBank.

3. Results

PCR specific for the *Leishmania* subgenus performed with the AJS1 and DeB8 primers resulted in the amplification of the kDNA of *L. (L.) mexicana* BEL21 and *L. (L.) mexicana* M379, the reference strains, and the Mexican isolates (lanes 6–12) giving 700 to 870 bp amplification bands; *L. (L.) amazonensis* M2269 and PH8 as well as *L. (L.) garnhami* HM76 and JAP78 gave bands less than 700 bp (Figure 2).

PCR specific for the *L. mexicana* complex, with the primers M1 and M2, resulted in the amplification of the *L. (L.) amazonensis* reference strain, giving 700 bp amplification bands; the other references strains gave 800 bp bands. The Mexican isolates were poorly amplified, giving 680 bp bands (data not shown).

Restriction length polymorphism analysis. In endonuclease digestion of kDNA amplicons with the *Eco RV* endonuclease, the Mexican isolates displayed a six band pattern (lanes 6–12), the reference strain *L. (L.) mexicana* Bel21 showed eight bands (lane 5), the other *Leishmania* strains showed two bands and some were not restricted (Figure 3). With *Hae III* the Mexican isolates all displayed the same pattern, which was several bands in length, the *L. mexicana* complex reference strains also displayed a restriction pattern of several bands, but a different size (data not shown). With *Mbo I*, all the Mexican isolates displayed a similar two-band restriction pattern (lanes 6–12) and the *Leishmania* reference strains showed a pattern of two or four bands (Figure 4). With the *Xba I* only, the Mexican isolates were restricted to displaying patterns several bands long (data not shown); the *Hind III*, *MspI*, and *Spe I* endonucleases restricted only certain strains, but not the Mexican isolates (data not shown). The *Bam HI* and *Kpn I* endonucleases did not digest any *Leishmania* strains (data not shown).

In the cloning and sequencing of kDNA minicircles of the Mexican isolates, seven minicircle clone sequences were obtained, five from the LCL isolated and two from the DCL (data not shown). All seven clones showed high

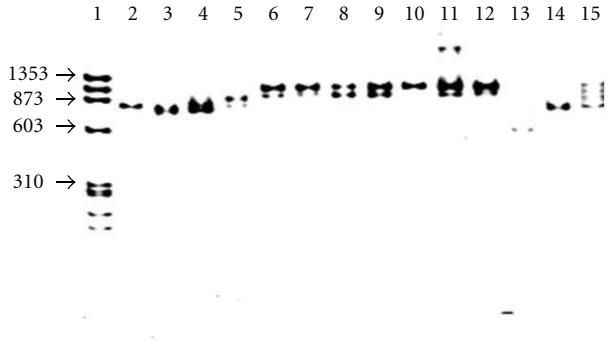

Figure 2: PCR Amplification of kDNA with the primers AJS 1 and DeB 8. Lane 1, MWMX174 Hae III; 2, *L. (L.) granhami* JAP78; 3, *L. (L.) granhami* HM76; 4, *L. (L.) mexicana* M379; 5, *L. (L) mexicana* BEL 21; 6, *L. (L) mexicana* SOB; 7, *L. (L.) mexicana* YUC; 8, *L. (L.) mexicana* MC; 9, *L. (L.) mexicana* GS; 10, *L. (L.) mexicana* HF; 11, *L. (L.) mexicana* AM; 12, *L. (L.) mexicana* AG; 13, *L. (L.) amazonensis* M2269; 14, *L. (L.) amazonensis* PH8; 15, *L. (L.) pifanoi* L11.

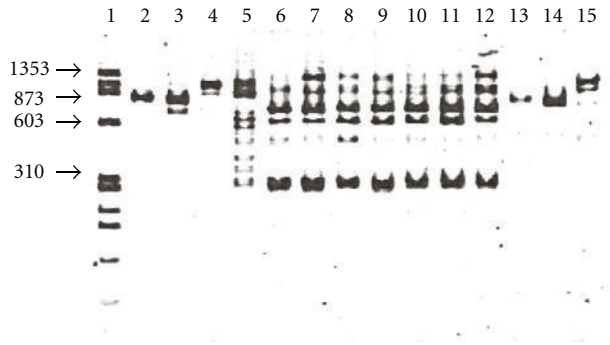

Figure 3: PCR-RFLP analysis with the endonuclease *Eco RV*. Lane 1, MW X174 *Hae* III; 2, *L. (L.) garnhami* JAP78; 3, *L. (L.) garnhami* HM76; 4, *L. (L.) mexicana* M379; 5, *L. (L.) mexicana* BEL 21; 6, *L. (L.) mexicana* SOB; 7, *L. (L.) mexicana* YUC; 8, *L. (L.) mexicana* MC; 9, *L. (L.) mexicana* GS; 10, *L. (L.) mexicana* HF; 11, *L. (L.) mexicana* AM; 12, *L. (L.) mexicana* AG; 13, *L. (L.) amazonensis* M2269; 14, *L. (L.) amazonensis* PH8; 15, *L. (L.) pifanoi* LL1.

homology in their conserved part, presenting the highly conserved block CSB3 (GGGGTTGGTGTA) known as the universal minicircle sequence (UMS) (Shlomai, 2004), but after position 19 two groups of minicircle sequences have been found, one 757–759 bp (LCL6, LCL14, and LCD15), which presents the ACTCCTGGATTT motif, and the 790 to 791 bp group (LCD7, LCL17, LCL5, and LCL4), which presents the TATCCTCTCCT motif. The comparison of the sequences of these two groups revealed differences in deletions and substitutions of bp along the kDNA minicircle (Figure 5).

Based on sequence alignments of kDNA minicircles of Mexican isolates and the other members of the *L. mexicana* complex, phylogenetic trees were constructed. The consensus tree showed nine groupings. The first and second groups were formed with LCL and DCL Mexican clones. A further

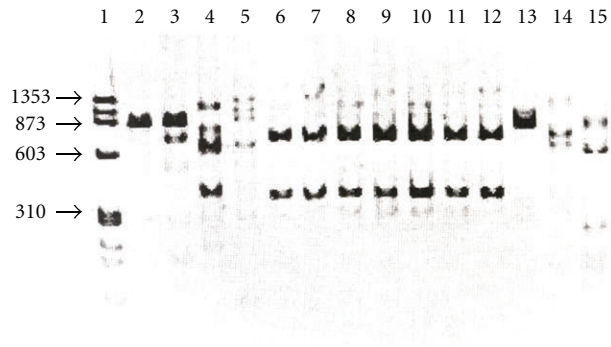

Figure 4: PCR-RFLP analysis with the endonuclease *Mbo I*. Lane 1, MW X174 *Hae* III; 2, *L. (L.) garnhami* JAP78; 3, *L. (L.) garnhami* HM76; 4, *L. (L.) mexicana* M379; 5, *L. (L.) mexicana* BEL 21; 6, *L. (L.) mexicana* SOB; 7, *L. (L.) mexicana* YUC; 8, *L. (L.) mexicana* MC; 9, *L. (L.) mexicana* GS; 10, *L. (L.) mexicana* HF; 11; *L. (L.) mexicana* AM; 12, *L. (L.) mexicana* AG; 13, *L. (L.) amazonensis* M2269; 14, *L. (L.) amazonensis* PH8; 15, *L. (L.) pifanoi* LL1.

two groups were formed with clones of *L. (L.) mexicana* from international strains. Another two groups were formed with *L. (L.) amazonensis* and *L. (L.) mexicana* clones. Another node was formed with two groups including only *L. (L.) amazonensis* clones. The last group was formed with clones of *L. (L.) mexicana* (Figure 6).

4. Discussion

In the amplification of kDNA with the primers specific for the *Leishmania* subgenus, the Mexican isolates showed PCR products that had a slightly higher molecular weight than the *L. (L.) Mexicana* M379, *L. (L.) mexicana Bel21*, *L. (L.) amazonensis*, and *L. (L.) pifanoi* reference strain PCR products (Figure 2). On the other hand, with the specific primers for the *L. mexicana* complex none of the Mexican isolates were properly amplified as the other members of the *L. mexicana* complex had been (data not shown). These results could be due to the fact that the specific primers for *Leishmania* subgenus have the UMS sequence as do the Mexican isolates, and the sequences of the M1 and M2 primers have less homology with the Mexican isolates than with the other members of the *L. mexicana* complex, indicating that the kDNA minicircles of the Mexican isolates are different from the other members of the *L. mexicana* complex.

Interestingly, the Mexican isolates digested with the *Eco RV* (Figure 3), *Hae III* (data not shown), and *Mbo I* endonucleases (Figure 4) displayed similar restriction patterns between them, totally different from the digestion patterns displayed for the other members of the *L. mexicana* complex. Furthermore, with the *Xba I* endonuclease only the Mexican isolates were restricted (data not shown). These results clearly suggest that the kDNA minicircles of the Mexican isolates are more polymorphic than the kDNA of the other members of the *L. mexicana* complex and have different recognition sites for the several restriction enzymes

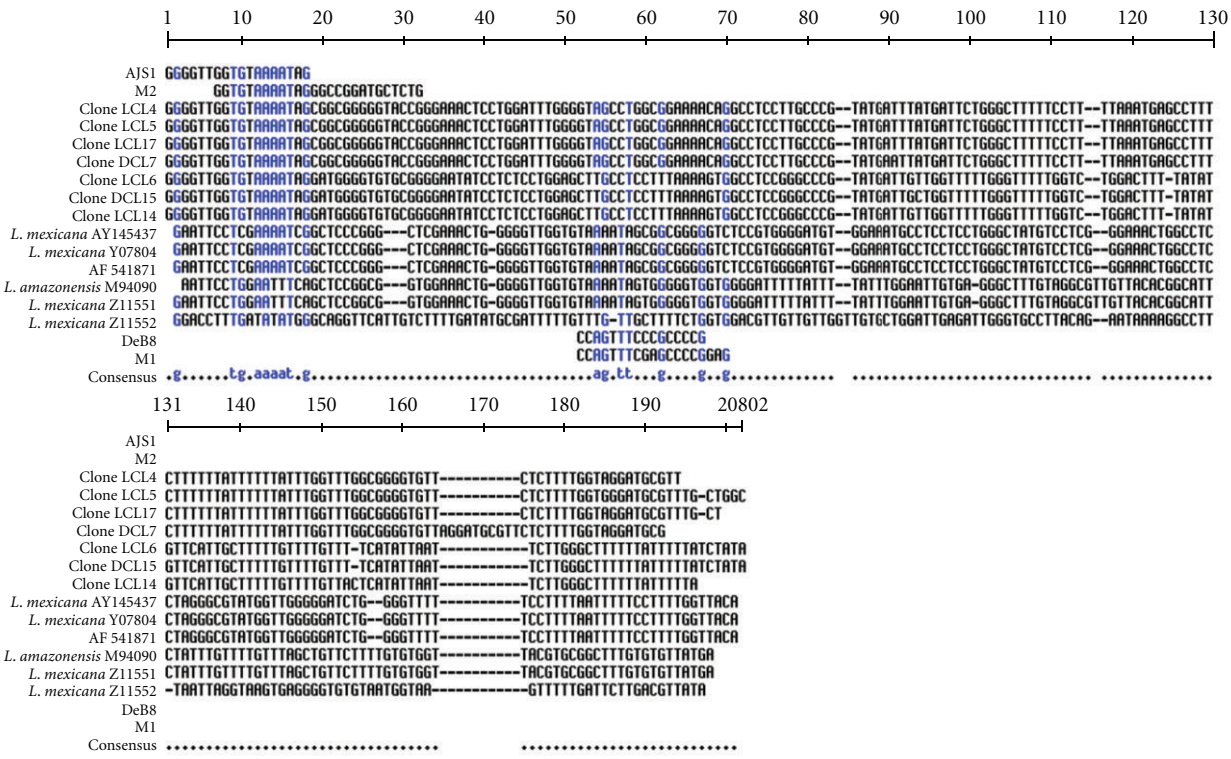

Figure 5: Aligment of the kDNA minicircles sequences classes (from 1 to 200 bp). Clones of the Mexican isolates: LCL 4, DCL 7, LCL 17, DCL 15, LCL 6, and LCL 14. Sequences of other members of the *Leishmania mexicana* complex published in the GeneBank: *L. mexicana*Y07807, AF54187, Y145437; *L. (L.) amazonensis* M 94090; *L. (L.) mexicana* Z11551, Z11552, Z11554, Z11556, and Z11549; *L. (L.) amazonensis* M21325, M 21327; *L. (L.) mexicana* Z11553, Z11555.

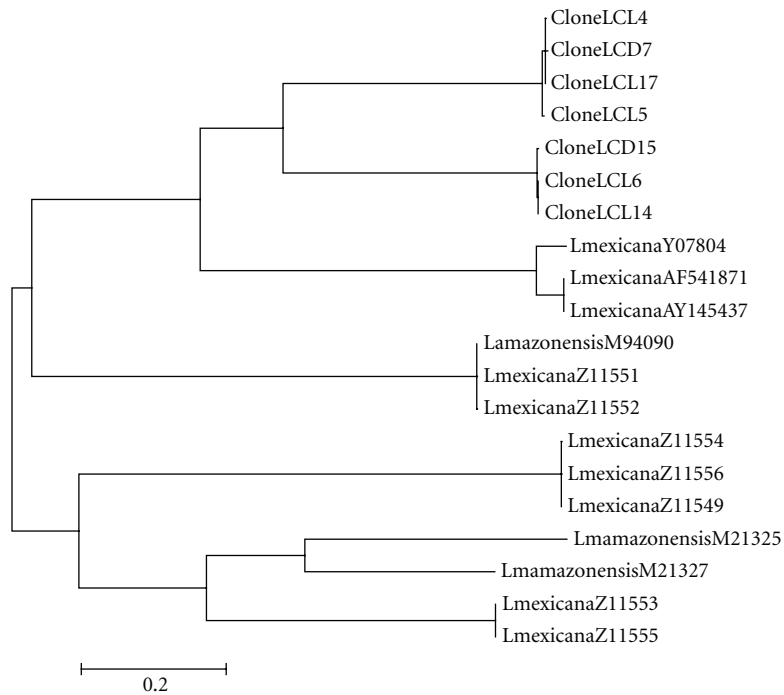

Figure 6: Phylogenetic tree constructed with sequences of kDNA minicircles. *Leishmania mexicana* complex members published in theGenBank: *L. (L.) mexicana* Y07807, AF54187, Y145437; *L. (L.) amazonensis* M 94090; *L. (L.) mexicana* Z11551, Z11552, Z11554, Z11556, and Z11549; *L. (L.) amazonensis* M21325, M 21327; *L. (L.) mexicana* Z11553, Z11555; clones of *L. (L.) mexicana.* Mexican isolates clones: LCL 4, DCL 7, LCL 17, LCL 5, DCL 15, LCL 6, and LCL 14.

used in this study. Furthermore, in studies of PRC-RFLP of kDNA [16] found six basic digestion patterns in Mexican isolates of *L. (L.) mexicana* that were different from the digestion patterns displayed for the other members of the *L. mexicana* complex reference strains.

The Mexican isolate clone sequence classes of minicircles formed two groups including DCL and LCL clones in each group, indicating that a particular minicircle class is not exclusive of one strain of *Leishmania,* furthermore the different clinical manifestation of the disease (LCL or DCL) is not related to any particular minicircle classes (Figure 5). Comparing these sequences with the sequences of other members of the *L. mexicana* complex, it was observed that they did not have extensive sequence homology. These results agree with Barker's studies [17] showing that the minicircles of representative strains of the main complexes of *Leishmania* do not share extensive sequence homology.

In the phylogenetic tree constructed with these sequences, the other members of the *L. mexicana complex* formed groups with *L. (L.) mexicana* and *L. (L.) amazonensis* clones apart from the groups formed exclusively with the Mexican clones. These may indicate that the Mexican isolates have sequence classes of minicircles that are different from the other members of the *L. mexicana* complex isolated in other countries (Figure 6).

On the other hand, Gutiérrez-Solar et al. [18] found greater homology between minicircles of geographically separated isolates than minicircles of the same stock. Within human species of *Leishmania*, Rogers and Wirth [19] described highly homogeneous sequences only in minicircles of geographically related isolates.

On the other hand, in studies with *L. (L.) amazonensis* resistant to tunicamycin, Lee et al. [20] found that certain minor minicircle sequence were selected to replicate and to replace the dominant type of minicircles and become predominant.

Although *Leishmania* kDNA is very polymorphic, the PCR of kDNA analysis is a very useful and sensitive enough for detecting *L. donovani* genes in skin biopsy specimens from patients and is recommended as a confirmatory diagnostic tool for PKDL [21], and for characterization of cutaneous isolates of *Leishmania* by isoenzyme typing combined with kDNA restriction analysis [22].

In conclusion it seems that the minicircle classes could change in the kDNA network [8]. We cannot say that one or another minicircle sequence class within the kDNA network is permanent or typical of a virulent or less virulent *Leishmania* strain, and the clinical manifestation of the disease (LCL or DCL) is not related to any particular minicircle classes. Furthermore there is no relation with their geographical distribution.

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Research Article

Influence of *Triatoma dimidiata* in Modulating the Virulence of *Trypanosoma cruzi* Mexican Strains

E. Guzman-Marin,[1] M. Jimenez-Coello,[1] M. Puerto-Solis,[1] A. Ortega-Pacheco,[2] and K. Y. Acosta-Viana[1]

[1] *Cuerpo Académico Biomedicina de Enfermedades Infecciosas y Parasitarias, CIR "Dr. Hideyo Noguchi", Universidad Autónoma de Yucatán, Avenida Itzaés No. 490 x Calle 59, 97000 Mérida, YUC, Mexico*
[2] *Departamento de Salud Animal y Medicina Preventiva, Facultad de Medicina Veterinaria y Zootecnia, Campus de Ciencias Biologicas y Agropecuarias, Universidad Autonoma de Yucatan, AP 4-116 Merida, YUC, Mexico*

Correspondence should be addressed to E. Guzman-Marin, gmarin@uady.mx

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The epidemiology of Chagas disease is complex. There are different vectors and reservoirs and different clinical manifestations. In order to assess whether the biological behavior of three strains isolated in southeastern Mexico (H4 isolated from human, Z17 isolated from *Didelphis* sp., and V isolated from *T. dimidiata*) could be modified during passage through the vector *T. dimidiata*, the parasitemia curve, the amount of amastigote nests, and mortality of BALB/c infected with blood trypomastigotes of *T. cruzi* were evaluated. Strains were maintained in continuous passage from mouse to mouse and in animals infected with metacyclic trypomastigotes. The parasitemia curves were significantly different ($P < 0.05$) between mice to mice and triatoma to mice groups in strains H4 and Z17, and was also observed fewer amastigote nests in cardiac tissue ($P < 0.05$ strain H4 with higher number versus all groups and Z17 between mice to mice and triatoma to mice) 45 days after inoculation. It is concluded that *T. dimidiata* influences in modulating the virulence of strains of *T. cruzi* in the region. Further studies of the intestinal tract of the insect in search for some protein molecules involved in regulating may clarify the virulence of the parasite.

1. Introduction

American Trypanosomiasis also known as Chagas' disease remains a public health problem throughout Latin America, where it is estimated that 10 million people are infected with *Trypanosoma cruzi* (*T. cruzi*), and other 25 million are at risk to become infected [1].

The causative agent, *T. cruzi*, has a natural biological pleomorphism with biochemical and molecular differences between isolates, showing great differences in their behavior from both *in vitro* and *in vivo* studies that may explain the large variation in the clinical presentations of the disease [2–4]. These differences have been attributed to many causes such as environmental factors, host immunity, virulence, pathogenicity, and passage through various species of vectors and hosts. Those differences need to be characterized from the clinical-epidemiological and clinical-pathological points of view [4–6].

In Mexico, the heterogeneity among the locally isolated strains has been reported, and differences in the onset and severity of the disease in human hosts have been attributed to the susceptibility to infection, to the parasite, and/or vector [7, 8].

In Yucatan, several studies on the biological behavior of isolated *T. cruzi* strains from human cases and reservoirs have been performed. Entomological, epidemiological aspects, and biological behavior of the vectors have also been conducted [4, 9–11].

Based on data obtained from these studies, it has been determined that *Triatoma dimidiata* (*T. dimidiata*) is the main and effective transmitter of *T. cruzi* in the endemic region of Yucatan, Mexico, and that the presence of different

infected reservoirs including humans contributes to the maintenance of Chagas' disease. However, despite being present the epidemiological conditions for the transmission to humans, only 42 cases were diagnosed with Chagas' cardiomyopathy in acute and chronic stages from 1970 to 1995 [12, 13]. This is a low number of clinical cases compared to the situation in other states of Mexico and other endemic regions of the world. These may be attributed to various reasons such as changes in the virulence of the parasite as it passes through the vectors, the Mayan population resistance to infection, or lack of accurate epidemiological studies.

The objective of this study was to determine the role of *T. dimidiata* modulating the virulence of *T. cruzi* which may explain in part the clinical and epidemiological features of the disease in humans from Yucatan.

2. Material and Methods

2.1. Parasites. Three strains of *T. cruzi* isolated in the State of Yucatan, Mexico, were used: H4 (isolated from human), Z17 (isolated from the reservoir *Didelphis marsupialis*), and V (isolated from the vector *Triatoma dimidiata maculipennis*).

All isolates were maintained by successive passages through mouse to mouse (control group) and *Triatoma* to mouse (experimental group) for their biological characterization.

2.2. Insects. Three groups of five 5th stage nymphs of *T. dimidiata* were used for the triatoma-mouse passes for each strain. This stage was chosen because when insects feed under laboratory conditions, the 4th and 5th stage nymphs feed for longer, and have higher intake capacity, ensuring the capture of the parasite. For the infection of triatomines, mice infected with each of the strains (maintained by successive passages of mouse-mouse) were previously anesthetized with sodium pentothal (40 mg/Kg) intraperitoneally (IP) and kept in a box along with triatomines to feed them for 15 to 30 min. After feeding, triatomines were placed in a jar for 30 to 60 days to become parasitized. Once parasitized, triatomines were compressed in the abdomen to get all the feces and search for parasites; thus, there were obtained inoculums of trypomastigotes to infect each mouse of the experimental groups. Triatomines infected with the different isolates of *T. cruzi* (H4, Z17, and V) were kept for 60 days and then used for the infection of mice from the experimental groups denominated, triatoma to mice.

2.3. Animal Model. A total of 180 BALB/c 35 g, 8 weeks of age male mice were used. BALB/c mice were maintained on a 12 : 12 h light-dark cycle and had access to food and water *adlibitum*.

2.4. Experimental Design. Mice were divided into a total of 18 groups: 9 groups for the experimental condition "mice to mice" and 9 groups to evaluate triatoma to mice groups. Each group included 10 animals. There were 3 strains evaluated, H4, Z17, and V. Each strain was evaluated by triplicated. In the mice to mice groups, *T. cruzi* inoculums were obtained from isolates maintained by passages mouse to mouse. From the triatoma to mice groups, the inoculums were obtained from isolates maintained on passes mouse to triatoma and triatoma to mouse.

Three groups of mice were inoculated with a different isolate H4 (human), Z17 (*Didelphis* sp.), and V (Vector *T. dimidiata*), directly in passage mice to mice, in the same way 3 groups of mice were inoculated with each different isolate. For the infection of mice to mice groups, twenty days after inoculation, mice were bled to obtain an inoculum of 3×10^5 parasites/mL blood trypomastigotes of each of the isolates of *T. cruzi* to study, and for triatoma-mice groups infection, the number of trypomastigotes obtained from infected *T. dimidiata* dejections was adjusted to a final concentration of 3×10^5 parasites/mL with a solution of NaCl (0.85%) and EDTA (0.05%), mixed, and then counted on Neubauer chamber. Each mouse from the 18 groups formed inoculated IP with 200 μL of the mixture [6, 14].

2.5. Biological Characterization. The virulence was evaluated for each experimental and control group in terms of parasitemia curves, tissue invasion, and mortality rate from the infected mice groups. To determine the parasitemia level, the number of blood trypomastigotes was quantified in a Neubauer chamber. A volume of 0.5 mL of peripheral blood complete with 0.85% saline and with 0.05% EDTA was taken from the tail of each mouse. The parasite count was performed every 4 days until the disappearance of parasitemia.

The invasion of tissues was determined by the presence of amastigote nests in different tissue organs evaluated (heart, skeletal muscle, liver, and spleen) and obtained after euthanasia of the mice at day 45 postinfection. Tissue samples were fixed in 10% formalin and processed using histological sections of 5 μm thick each (5 slices per organ); samples were later stained with hematoxylin-eosin (HE) and evaluated in search and quantified of amastigote nests with the 40x objective. Mortality rate was evaluated considering the percentage of spontaneously dead mice during all period of the study.

2.6. Data Analysis. Data obtained from the biological behavior (curves of parasitemia and amastigote nests and mortality) between the experimental and control groups were analyzed with statistical t-Student test and one-way ANOVA with the Tukey posttest. The significance value $P < 0.05$ determines significant differences [15, 16]. The mortality of mouse after inoculation of the strains was analyzed using a life table from the software SPSS v 16. For comparison between groups (mouse-mouse and triatoma-mouse), the logrank test was used.

3. Results

In the H4 strain mouse-mouse, parasitemia started on day 8 postinfection, reaching a peak on day 24 with a parasitemia load of 115×10^5 parasites/mL; the parasitemia level

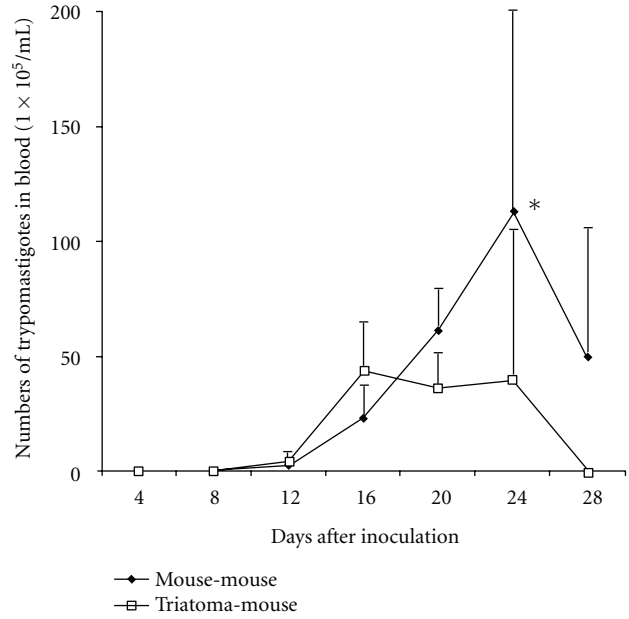

Figure 1: Parasitemia curve of H4 strain (mouse-mouse) versus (triatoma-mouse). (*P value < 0.05 in comparison with all evaluated groups).

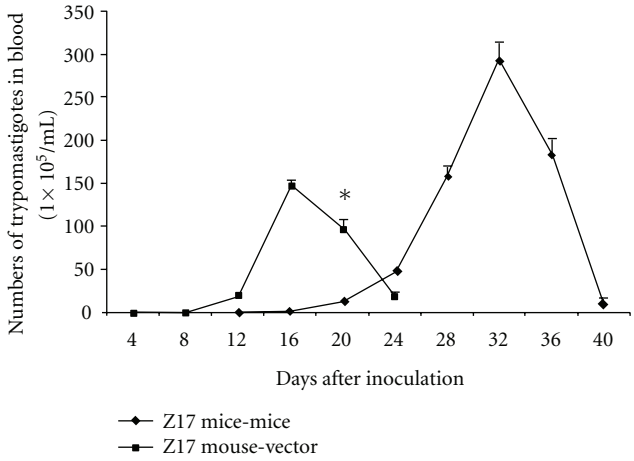

Figure 2: Parasitemia curve of strain Z17 (mouse-mouse versus triatoma-mouse). *P value < 0.05 in comparison between both groups.

showed statistically significant difference in comparison with the other evaluated groups ($P < 0.05$). In the case of the H4 strain triatoma-mouse, the parasitemia began on day 8 with a peak on day 14 when the parasitemia load was 44 parasites/mL (Figure 1).

The infection with Z17 strain, from mouse to mouse parasitemia began on day 12 and peaked at day 32 postinfection with a parasite load of 295×10^5 parasites/mL, for the triatoma-mouse group, the parasitemia began on day 8 reaching a the peak on day 13 postinfection with a parasite load of 149×10^5 parasites/mL (Figure 2) and showed a statistically significant difference when was compared with the Z17 mouse-mouse group at day 20 postinfection ($P < 0.05$), but the duration of the parasitemia of the group Z17 triatoma to mouse was shorter than mouse to mouse.

For the V strain, both mouse-mouse and triatoma-mouse groups, parasitemia starts on day 12 postinfection with the peak on day 28. The parasitemia load postinfection from mouse to mouse was 190×10^5 parasites/mL, and the parasitemia load from triatoma-mouse was 170 parasites/mL (Figure 3).

The virulence of the studied strains (H4, Z17, and V) began between day 8 and 12 with peaks of 115, 295, and 190×10^5 parasites/mL in the mice to mice groups and between on 12 and 32, with peaks of 44, 149, and 170×10^5 parasites/mL in the triatoma to mice groups. Regarding the parasitemia, a significant difference between mice to mice and triatoma to mice groups infected with the strains H4 and Z17. On the other hand, for V strain, the P value was not significant (0.06).

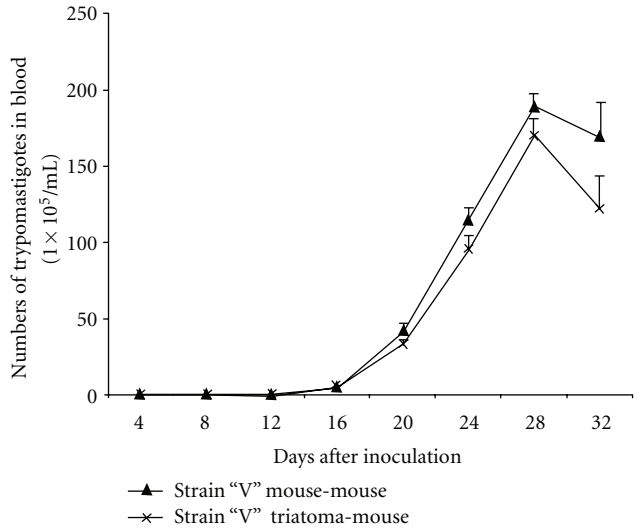

Figure 3: Parasitemia curve of strain "V" (mouse-mouse versus triatoma-mouse).

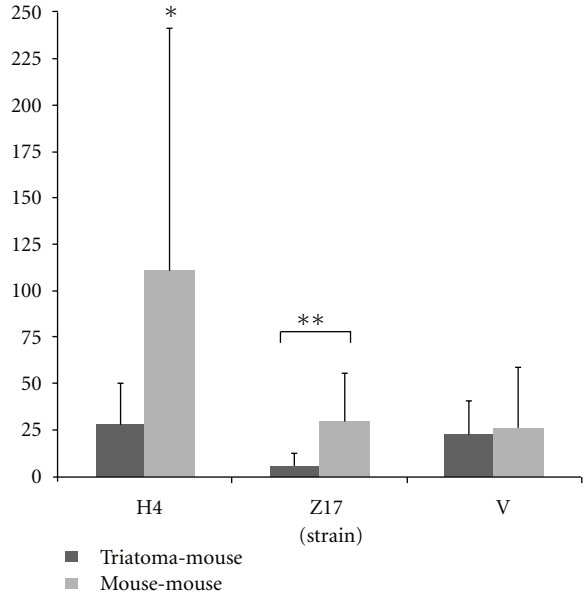

Figure 4: Number of amastigote nests from *T. cruzi* in 100 fields, 40x of cardiac tissue from infected BALB/c mice with the H4, Z17, and V strains (*$P < 0.05$ in comparison with all evaluated groups. **$P < 0.05$ in comparison between both groups).

Amastigote nests were only observed on the cardiac tissue. When comparing the three strains (H4, Z17, and V), the H4 strain mouse-mouse produced the greatest number of nests (<0.05 in comparison with all evaluated groups). In the groups triatoma-mouse, from strains H4 and V, the number of amastigote nests were 28 and 22, respectively, and for the Z17 strain, were counted only 5 nests. This reduction was statistically different (<0.05) when compared between mouse to mouse group versus triatoma to mouse group (Figure 4).

The mortality recorded for the H4 strain was 70% (mouse-mouse) and 100% (triatoma-mouse), for the Z17 strain, 60% (mouse-mouse) and 90% (triatoma-mouse), and for the strain V, 50% (mouse-mouse) and 80% (triatoma-mouse). A significant difference on the survival analysis was found ($P < 0.05$). Mortality occurred faster in the strains H4 and Z17 from the mouse-mouse groups (Figure 5).

4. Discussion

Chagas disease is an important endemic protozoa infection in Mexico. One of the main pathologic characteristic, as seen in other countries, is the cardiac damage. However, despite that in Yucatan the vector is widely distributed and the parasite is present in several reservoir mammals, there is no

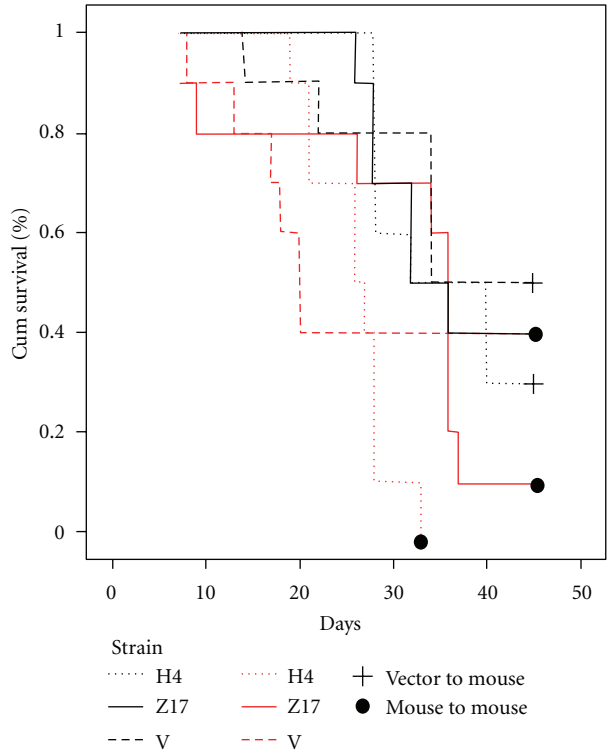

Figure 5: Survival rate of *T. cruzi* strains H4, Z17, and V in BALB/c mice infected "mice to mice" and "triatoma to mice" during 45 days.

important number of clinical reports from cardiomyopathies or fatal cases in the region even when there are no official strategies for the control of the vector.

The clones H4, Z17, and V isolated from the Yucatan area showed a remarkable difference in the parasitemia level when the parasites from the inoculums were passed through the vector *T. dimidiata*. It should be noted that the biological, biochemical, and molecular strains of *T. cruzi* have been characterized by several authors so that each strain sharing these aspects could be representative of a single group. Due to the heterogeneity of the strains, *T. cruzi* has a natural pleomorphism responsible of the severity of the disease when present in the human host and other mammals [5, 17].

Current research has shown that biological and biochemical characteristics of three strains (19 SF, 21 SF, and 22 SF) were unchanged after its passage through three different species of triatomines. The maintenance of their biochemical characteristics indicates that virulence may be a variable parameter, but the genetic patterns are stable phenotype [18].

The presence of different strains of the parasite found from vectors, reservoirs, and regions from the countries may be responsible of the levels of parasitemia and invasion of certain organs. This variation on their behavior may be due to several reasons such as environmental factors, immunity, virulence, pathogenicity, and deferential step transmitters and host species [3, 19].

In the present study the parasitemia and its duration were reduced in the triatoma-mouse group similarly as described by Vallejo et al. [20], which states that the interactions of subpopulations of the trypanosomes with different species and populations of Triatominae determine the epidemiology of the human-infecting trypanosomes in Latin America.

The mortality rates from the studied strains in mice experimentally infected with metacyclic parasites (obtained from a vector) from the southeast of Mexico are higher than others previously reported in the occident of Mexico [8]. The lower velocity of mortality observed in the triatoma-mouse groups may indicate a lower virulence when compared with mouse-mouse H4 and Z17 strains of *T. cruzi*.

A Bolivia reference strain (strain Bolivia) of *Trypanosoma cruzi* (*T. cruzi*) demonstrates clear muscular tissue tropism, however different clones of the same may have affinity for different organs may migrate to produce skeletal muscle or cardiac disorders [21]. Similarly Mexican strains have shown tropism for skeletal and cardiac muscle [8] in coincidence with the ones studied in this work, however these characterizations were made in direct passes and should be noted that the behavior changes (mainly in the intensity of virulence) when the parasite passes through the vector as demonstrated in this study. It would be important to know the behavior of different clones of these strains to evaluate their behavior as they pass through the vector [22].

A study comparing *T. cruzi* strains from Mexico with other Mexican strains show significant differences in several biological parameters by Gómez-Hernández et al., [8], which suggests the intraspecies variations observed in the other

Mexican strains might be related to differences in biological behavior *in vivo*. Also was cited that clonal genotypes of *T. cruzi* differ significantly in terms of infectivity, demonstrating an association between genotype diversity, tropism, and pathogenicity [20].

However, in the present study, when parasitemia level was compared, a significant difference ($P < 0.05$) between strains H4 and Z17 was found between mouse-mouse and triatoma-mouse groups, and one strain undergoes changes that are not significant (strain V isolated from a vector *T. dimidiate* from the studied area), indicating that the biological behavior of the studied strains was different, and that maybe it could be modified when passing through the *T. dimidiata* vector and their virulence being reduced, explaining partially why the number of human cases of the disease in the Yucatan, Mexico, area is low. However, because of the chronic-degenerative characteristic of the disease, there may be an important amount of cases that are not properly diagnosed or are undiagnosed (persons infected but in the indeterminate phase of the disease, without any evident clinical sign). It is important and interesting to point that perhaps the behavior of certain strains is influenced by the predominant vector in each endemic area, which in this case *T. dimidiata* is the only recognized vector of *T. cruzi* present in the region. This confirms a great adaptation of strains to the only existing vector in the region. In cases of *Leishmania infantum* infection, a virulence deficit occurs by successive *in vitro* passages as a result from an inadequate capacity of the protozoa to differentiate into amastigote forms [22]. This low virulence is a reversible phenomenon, since serial passages on susceptible mice allow the parasite to recover a virulence phenotype. In the case of *T. cruzi*, when the parasite is in the midgut of the vector, it has been described than the interactions between the parasite with digestive enzymes, hemolysins, agglutinins, and antimicrobial compounds involve a mechanism of defense reactions that maybe responsible of regulating the development of *T. cruzi* in the triatomine vectors gut, and the capability of the parasite to establish an infection could be affected depending the lineage involved [23]; even these evidences have been found in *Rhodnius prolixus*. These interactions may also reduce the virulence of the strains as they pass from vectors and may partially explain the results from the present study.

5. Conclusion

This paper demonstrates that the vector *T. dimidiata* may influence in modulating to some degree of the virulence of *T. cruzi* strains isolated from mammals in the southeast of Mexico (Yucatan area), which leads to the need for further studies of the intestinal tract of the vector in search of some protein molecules that interact with the parasite by modulating their virulence. Further studies evaluating and characterizing virulence of *T. cruzi* strains should include real-time PCR from hearth tissue and correlate it with the parasitemia in peripheral blood and heart tissue.

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Review Article

Q Fever: An Old but Still a Poorly Understood Disease

Hamidreza Honarmand

Cellular and Molecular Research Center, Guilan University of Medical Sciences, Rasht, Iran

Correspondence should be addressed to Hamidreza Honarmand, honarmand_36@yahoo.com

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Q fever is a bacterial infection affecting mainly the lungs, liver, and heart. It is found around the world and is caused by the bacteria *Coxiella burnetii.* The bacteria affects sheep, goats, cattle, dogs, cats, birds, rodents, and ticks. Infected animals shed this bacteria in birth products, feces, milk, and urine. Humans usually get Q fever by breathing in contaminated droplets released by infected animals and drinking raw milk. People at highest risk for this infection are farmers, laboratory workers, sheep and dairy workers, and veterinarians. Chronic Q fever develops in people who have been infected for more than 6 months. It usually takes about 20 days after exposure to the bacteria for symptoms to occur. Most cases are mild, yet some severe cases have been reported. Symptoms of acute Q fever may include: chest pain with breathing, cough, fever, headache, jaundice, muscle pains, and shortness of breath. Symptoms of chronic Q fever may include chills, fatigue, night sweats, prolonged fever, and shortness of breath. Q fever is diagnosed with a blood antibody test. The main treatment for the disease is with antibiotics. For acute Q fever, doxycycline is recommended. For chronic Q fever, a combination of doxycycline and hydroxychloroquine is often used long term. Complications are cirrhosis, hepatitis, encephalitis, endocarditis, pericarditis, myocarditis, interstitial pulmonary fibrosis, meningitis, and pneumonia. People at risk should always: carefully dispose of animal products that may be infected, disinfect any contaminated areas, and thoroughly wash their hands. Pasteurizing milk can also help prevent Q fever.

1. Introduction

Q fever is a zoonosis caused by *Coxiella burnetii*, an obligate gram-negative intracellular bacterium. Most commonly reported in southern France and Australia, Q fever occurs worldwide. *C. burnetii* infects various hosts, including humans, ruminants (cattle, sheep, goats), and pets—and, in rare cases, reptiles, birds, and ticks. This bacterium is excreted in urine, milk, feces, and birth products. These products, especially the latter, contain large numbers of bacteria that become aerosolized after drying. *C. burnetii* is highly infectious, and only a few organisms can cause disease.

Because of its sporelike-life cycle, *C. burnetii* can remain viable and virulent for months. Infection can be acquired via inhalation or skin contact, and direct exposure to a ruminant is not necessary for infection. Rare human-to-human transmissions involving exposure to the placenta of an infected woman and blood transfusions have been reported [1]. Sexual transmission is also possible [2].

C. burnetii infection in livestock often goes unnoticed. In humans, acute *C. burnetii* infection is often asymptomatic or mistaken for an influenza-like illness or atypical pneumonia. In rare cases (<5%), *C. burnetii* infection becomes chronic [3], with devastating results, especially in patients with preexisting valvular heart disease. Because of its highly infectious nature and having an inhalational route of transmission, *C. burnetii* is recognized as a potential agent of bioterrorism. The Centers for Disease Control and Prevention (CDC) classify Q fever as a Category B agent.

The pathogenic agent is to be found everywhere except New Zealand [1]. The bacterium is extremely sustainable and virulent: a single organism is able to cause an infection. The common way of infection is inhalation of contaminated dust, contact with contaminated milk, meat, wool, and particularly birthing products. Ticks can transfer the pathogenic agent to other animals.

Some studies have shown more men to be affected than women [2, 3], which may be attributed to different

employment rates in typical professions. "At risk" occupations include, but are not limited to: veterinary personnel, stockyard workers, farmers, shearers, animal transporters, laboratory workers handling potentially infected veterinary samples or visiting abattoirs, and people who cull and process kangaroos.

2. History

It was first described by Edward Holbrook Derrick [4] in abattoir workers in Brisbane, Queensland, Australia. The "Q" stands for "query" and was applied at a time when the causative agent was unknown; it was chosen over suggestions of "abattoir fever" and "Queensland rickettsial fever," to avoid directing negative connotations at either the cattle industry or the state of Queensland [5].

The pathogen of Q fever was discovered in 1937, when Frank Macfarlane Burnet and Mavis Freeman isolated the bacterium from one of Derrick's patients [6]. It was originally identified as a species of *Rickettsia*. H.R. Cox and Gordon Davis isolated it from ticks in Montana, USA in 1938 [7]. *Coxiella burnetii* is no longer regarded as closely related to Rickettsiae, but as similar to *Legionella* and *Francisella*, and is a proteobacterium.

3. Bacteriology

C. burnetii is an obligate intracellular, small gram-negative bacterium (0.2 to 0.4 μm wide, 0.4 to 1 μm long). Although possessing a membrane similar to that of a gram-negative bacterium, it is usually not stainable by the Gram technique. The Gimenez method is usually used to stain *C. burnetii* in clinical specimens or laboratory cultures. Since *C. burnetii* cannot be grown in axenic medium and has long been recovered from ticks, it has been classified in the *Rickettsiales* order, the *Rickettsiaceae* family, and the *Rickettsiae* tribe together with the genera *Rickettsia* and *Rochalimaea* [8]. However, phylogenetic investigations, based mainly on 16S rRNA sequence analysis, have shown that the Coxiella genus belongs to the gamma subdivision of *Proteobacteria* [9], with the genera *Legionella*, *Francisella*, and *Rickettsiella* as its closest relatives.

C. burnetii expresses a low degree of genetic heterogeneity among strains by DNA-DNA hybridization. the genome size is highly variable among different *C. burnetii* strains, ranging from 1.5 to 2.4 Mb [10]. The inability to localize origin function by standard methods could well be related to the fact that *C. burnetii* probably has a linear rather than a circular chromosome and thus may not have conventional bidirectional replication [10]. *C. burnetii* gene sequences partially or completely available in the GenBank or EMBL databases include 23 chromosomal sequences and 17 plasmid sequences. The *C. burnetii* genome comprises facultatively a 36- to 42-kb plasmid, whose function remains undetermined.

C. burnetii displays antigenic variations similar to the smooth-rough variation in the family *Enterobacteriaceae*. Phase variation is related mainly to mutational variation in the lipopolysaccharide (LPS) [11]. Phase I is the natural phase found in infected animals, arthropods, or humans. It is highly infectious and corresponds to smooth LPS. In contrast, phase II is not very infectious and is obtained only in laboratories after serial passages in cell cultures or embryonated egg cultures. It corresponds to rough LPS. Compared to phase I, phase II displays a truncated LPS and lacks some protein cell surface determinants [12].

Genetic variability among different *C. burnetii* strains, as demonstrated by different RFLP-based genomic groups, specific plasmid regions, and LPS variations, were tentatively related to virulence. Genomic groups I, II, and III were associated with animal, tick, or acute Q fever human isolates, referred to as acute strains, whereas groups IV and V were associated with human Q fever endocarditis isolates, referred to as chronic strains. Group VI isolates, obtained from feral rodents in Dugway (Utah), were of unknown pathogenicity. Comparison of the various isolates for LPS variations, using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting, resulted in isolates being placed into groups similar to the genomic groups [13]. Some investigations suggest that predisposing host factors are more important than genomic strain variation in the explanation of the occurrence of acute or chronic Q fever diseases in humans [14, 15]. Moreover, recent data shows that genetic variation has an apparently closer connection with the geographical source of the isolate than with the clinical presentation.

4. Clinical Manifestation

Clinical signs of Q fever are often subclinical or extremely mild. In acute infection. The incubation period has been estimated to be approximately 20 days (range 14 to 39 days). There is no typical form of acute Q fever. The clinical signs vary greatly from patient to patient. The most important diagnostic clue is the epidemiological circumstance. Typically, three major presentations are described. These are as follows.

(i) Self-limited flu-like syndrome. A self-limited flu-like syndrome is the most common manifestation of Q fever. In Spain, this form of Q fever has been demonstrated as the cause of 21% of episodes of fever lasting for more than 1 week and less than 3 weeks. The most frequent symptoms, usually following a sudden onset, are high-grade fever (104°F or 40°C), fatigue, headache, and myalgias. The duration of fever increases with increasing age.

(ii) Pneumonia. Atypical pneumonia is one of the most commonly recognized forms of acute Q fever. Most cases are clinically asymptomatic or mild, characterized by a nonproductive cough, fever, and minimal auscultatory abnormalities, but some patients present with acute respiratory distress. Pleural effusion can also be present. Findings on the chest radiograph are nonspecific. Marrie et al. [16] demonstrated that 3.7% of all patients with community-acquired pneumonia admitted to a tertiary-care teaching hospital in Nova Scotia over a 5-year period were due to

C. burnetii, which is similar to the findings of Lieberman et al. in Israel (5.8%) [17].

(iii) Hepatitis (inflammation of the liver) is the predominant form of acute Q fever and it manifests mostly as a granulomatous hepatitis. The duration of symptoms varies from 10 to 90 days. The mortality rate ranges from 0.5 to 1.5%, depending upon the series [18].

(iv) Hepatitis. Three major forms of hepatitis may be encountered: an infectious hepatitis-like form of hepatitis with hepatomegaly but seldom with jaundice, clinically asymptomatic hepatitis, and prolonged fever of unknown origin with characteristic granulomas on liver biopsy [19].

(v) Other manifestations. Many other clinical manifestations of acute Q fever are possible: maculopapular or purpuric exanthema in 10% of patients [18], pericarditis and/or myocarditis (which is frequently fatal), and severe headache. Aseptic meningitis and/or encephalitis, which occur in 0.2 to 1.3% of patients with Q fever [20], are rarely accompanied by seizures and coma. Polyradiculoneuritis, optic neuritis, hemophagocytosis, hemolytic anemia, transient hypoplastic anemia, thyroiditis, gastroenteritis, pancreatitis, lymphadenopathy mimicking lymphoma, erythema nodosum, bone marrow necrosis, inappropriate secretion of antidiuretic hormone, mesangioproliferative glomerulonephritis related to antiphospholipid antibodies, and splenic rupture are uncommon manifestations of acute Q fever [21].

Chronic infection: chronic Q fever was initially described as lasting for more than 6 months after the onset. It occurs in approximately 5% of patients infected with *C. burnetii* and may develop insidiously months to years after the acute disease. In the chronic form of Q fever, *C. burnetii* multiplies in macrophages, and a permanent rickettsemia results in very high levels of persistent antibodies. Typically, the heart is the most commonly involved organ, followed by arteries, bones, and liver [22]. Endocarditis usually occurs in patients with previous valvular damage or those who are immunocompromised [23]. Chronic Q fever represents 3% of all cases of endocarditis in England and Lyon, France [24], and 15% in Marseille, France [25], and its annual incidence is 0.75 cases per 1 million population in Israel [26]. Clinically, the disease usually presents as a subacute or acute blood culture-negative endocarditis [27]. Symptoms are not specific. Arterial embolism occurs in about 20% of patients [28]. Vegetations are only rarely seen by transthoracic cardiac ultrasonography. They are usually smooth and nodular [27]. Because of the lack of specificity of symptoms, the diagnosis is often delayed 12 to 24 months, resulting in an increased mortality rate. Other manifestations of chronic Q fever include infections of aneurysms or vascular grafts [25], isolated hepatitis possibly complicated by hepatic fibrosis and cirrhosis [22], and osteoarthritis and osteomyelitis [29]. Rare cases of pericardial effusion [30], pulmonary interstitial fibrosis, pseudotumor of the lung, lymphoma-like presentation, amyloidosis, and mixed cryoglobulinemia have been reported in the literature.

Q fever during pregnancy. Both acute and chronic Q fever have been described during pregnancy. In mammals, *C. burnetii* undergoes reactivation during pregnancy and thus is responsible for higher rates of abortion, prematurity, and low birth weight [31]. In humans, it has been isolated from the placenta of a woman who became pregnant 2 years after an episode of acute Q fever [32], but few cases have been reported. Clinically, although most cases seem to be asymptomatic [33], complications may complicate the course of the disease, such as in utero fetal death [34], placentitis, or thrombocytopenia [35]. Although intrauterine transmission of *C. burnetii* has been documented, the consequences of congenital Q fever remain to be determined.

5. Epidemiology

Q fever is a worldwide zoonosis. The reservoirs are extensive but only partially known and include mammals, birds, and arthropods, mainly ticks. While an important reservoir seems to be small wild rodents, the most commonly identified sources of human infection are farm animals such as cattle, goats, and sheep. Pets, including cats [36], rabbits, and dogs, have also been demonstrated to be potential sources of urban outbreaks. Cats are suspected as an important reservoir of *C. burnetii* in urban areas and may be the source of urban outbreaks [37–39]. In Canada, 6 to 20% of cats have anti-*C. burnetii* antibodies [36]. Wild rats have been suspected as an important reservoir in Great Britain [40]. All these mammals, when infected, shed the desiccation-resistant organisms in urine, feces, milk, and, especially, birth products [41]. Reactivation of infection occurs in female mammals during pregnancy. Q fever causes abortions in goats and, less frequently, sheep and causes reproductive problems in cattle [42]. High concentrations of *C. burnetii* (up to 10^9 bacteria per g of tissue) are found in the placentas of infected animals. Due to its resistance to physical agents, probably related to its sporulation process [43], *C. burnetii* survives for long periods in the environment.

In humans, infection results from inhalation of contaminated aerosols from amniotic fluid or placenta or contaminated wool. Therefore, Q fever is an occupational hazard. At greatest risk are persons in contact with farm animals, but also at risk are laboratory personnel who work with infected animals. When looking for the source of *C. burnetii* exposure, the investigator should search for contact with a parturient or newborn animal. Mammals also shed *C. burnetii* in milk, and thus, consumption of raw milk could be a source of infection [44]. Sexual transmission of Q fever has been demonstrated in the mouse [45] and has been suspected in humans [46]. Sporadic cases of human-to-human transmission following contact with an infected parturient woman have been reported and have been suspected to occur by direct aerosol transmission. It has also been proven to occur via transplacental transmission, resulting in congenital infections [34], via intradermal inoculation, and via blood transfusion [47]. Ticks transmit *C. burnetii* to domestic mammals but not to humans [41]. *C. burnetii* may persist asymptomatically

Table 1: Cutoff proposal for Q fever diagnosis by using microimmunofluorescence and interpretation of serological results obtained with a single serum sample.

Phase II antibody titer		Phase I antibody titer (IgG)	Interpretation
IgG	IgM		
≤100			Active Q fever improbable
≥200	≥50		Acute Q fever (100% predictive)
		≥1 : 800	Chronic Q fever (98% predictive)
		≥1 : 1,600	Chronic Q fever (100% predictive)

in humans throughout life. However, pregnancy, a cardiac valvular abnormality, a vascular aneurysm or prosthesis, hemodialysis [48], and immunodeficiency, including AIDS [49], may promote reactivation of dormant *C. burnetii*.

In Europe, acute Q fever cases are more frequently reported in spring and early summer. They may occur at all ages, but they are more frequent in men than in women. Q fever is usually benign, but mortality occurs in 1 to 11% of patients with chronic Q fever [50]. *C. burnetii* is endemic in every part of the world except New Zealand [51]. Since the clinical presentation is very pleomorphic and nonspecific, the incidence of Q fever among humans is probably underestimated, and diagnosis particularly relies upon the physician's awareness of the symptoms of Q fever and the presence of a reliable diagnostic laboratory. In southern France, 5 to 8% of cases of endocarditis are due to *C. burnetii*, and the prevalence of acute Q fever is 50 cases per 100,000 inhabitants [18]. Seroepidemiological surveys have shown that 18.3% of blood donors in Morocco, 26% in Tunisia [52], 37% in Zimbabwe [53], 44% in Nigeria [54], 10 to 37% in northeast Africa, and 14.6 to 36.6% in different areas of Canada [22] had anti-*C. burnetii* antibodies. Large outbreaks of Q fever have also been reported in the Basque country in Spain [55], in Switzerland [56], in Great Britain [57], and in Berlin, Germany [58].

In addition, a large number of Q fever cases have been reported in The Netherlands since 2007, with over 3700 human cases reported through March 2010. Infected dairy goat farms are believed to be the source of the outbreak, and most human cases have been reported in the southern region of the country [59].

6. Laboratory Diagnosis

Blood cultures are typically negative (Note that, although possible, attempting to isolate the organism from blood is a dangerous practice; cases of Q fever have developed in laboratory technicians).

C. burnetii can be seen on smears or frozen tissue prepared with a routine Giemsa stain. Histopathologic changes consistent with doughnut granulomata the liver and bone marrow may be observed, but these are not specific for *C. burnetii*. They can also occur in Hodgkin lymphoma, typhoid fever, cytomegalovirus infection, infectious mononucleosis, and allopurinol hypersensitivity.

Most cases of Q fever are diagnosed based on detection of phase I and II antibodies (between acute and convalescent paired sera); a 4-fold rise in complement-fixing antibody titer against phase II antigen occurs and yields the highest specificity. This requires a baseline sample and another sample in 3-4 weeks. Thus, serologic tests are not helpful acutely but may later confirm the diagnosis: seroconversion generally occurs between days 7 and 15 and is almost always present by 21 days.

The 3 serologic techniques used for diagnosis include indirect immunofluorescence (IIF) (method of choice), complement fixation, and enzyme-linked immunosorbent assay (ELISA) (comparable to IIF). As noted above, significant titers may take 2–4 weeks to appear. Laboratory values vary considerably, so clinicians must interpret results according to their local standards [60].

Raoult et al. recommended serologic testing 2 years following treatment in patients with valvulopathy after acute infection [61], whereas Healy et al. recommended serial testing every 4 months for 2 years, with additional investigation in those with elevated phase 1 immunoglobulin G (IgG) titers greater than 800 [62]. Maurin and Raoult recommendations are shown in Table 1 [63].

Serologic followup to detect a rise in phase I IgG titers of 1 : 800 or more can be performed twice every 3 months. If detected, transesophageal echocardiography and serum real-time polymerase chain reaction (PCR) techniques can be performed in an attempt to diagnose endocarditis [61, 63]. Sensitivities may be as low as 18% in early disease.

Interpretation of Q fever serology is challenging in regard to discordance of the serologic results from different reference laboratories [64]. None of these results should be used in isolation, and their interpretation should always be applied in the appropriate clinical context. False-positive serologic results may occur in legionellosis and leptospirosis.

IIF findings in acute Q fever include the following.

(i) A rise in IgG and IgM against phase II antigen [65].

(ii) Phase II IgM of 1 : 50 or more; usually undetectable after 4 months but can last 12 months or more.

(iii) Phase II IgG of 1 : 200 or more.

(iv) Phase II titers of 1 : 100 or less make the diagnosis of acute Q fever unlikely.

(v) In a reference French laboratory, these values showed 100% specificity.

IIF findings in chronic Q fever include the following.

(i) A rise in IgG and IgA against phase I antigen [65, 66].

(ii) Phase I IgG of 1 : 800 or more is considered diagnostic of endocarditis (one of major modified Duke criteria).

(iii) Phase II IgM titers are lower or absent.

(iv) Phase II IgG titers are usually greater than 1 : 1600; they can last up to 12 years after an outbreak.

(v) The main predictive criterion of clinical cure is detection of phase I IgG titer of less than 1 : 200.

Complement fixation is less sensitive and specific than IIF, and the time to positivity may take longer than IIF. Different cutoff values are also used. IgG levels usually fall within 3 years.

In acute Q fever, the anti-IgG titer is at least 200, and the anti-IgM titer level is at least 50. In chronic Q fever, the anti-IgA titer for phase I is greater than 50, and the anti-IgG titer for phase I is greater than 800.

In certain reference laboratories, polymerase chain reaction (PCR) techniques can be used with tissue specimens, such as resected cardiac valves, with greater sensitivity than serum assays, but these are not generally available commercially. *C. burnetii* organisms can persist in tissues even after prolonged antimicrobial treatment [66]. Although still controversial, serum PCR may be used to diagnose acute Q fever in the first 2 weeks of the disease. It should also be reserved for seronegative patients in the subsequent 2 weeks and not used later than 4 weeks following onset [62].

7. Prevention

In the United States, Q fever outbreaks have resulted mainly from occupational exposure involving veterinarians, meat processing plant workers, sheep and dairy workers, livestock farmers, and researchers at facilities housing sheep. Prevention and control efforts should be directed primarily toward these groups and environments.

The following measures should be used in the prevention and control of Q fever.

(i) Educate the public on sources of infection.

(ii) Appropriately dispose of placenta, birth products, fetal membranes, and aborted fetuses at facilities housing sheep and goats.

(iii) Restrict access to barns and laboratories used in housing potentially infected animals.

(iv) Use only pasteurized milk and milk products.

(v) Use appropriate procedures for bagging, autoclaving, and washing of laboratory clothing.

(vi) Vaccinate (where possible) individuals engaged in research with pregnant sheep or live *C. burnetii*.

(vii) Quarantine imported animals.

(viii) Ensure that holding facilities for sheep should be located away from populated areas. Animals should be routinely tested for antibodies to *C. burnetii*, and measures should be implemented to prevent airflow to other occupied areas.

(ix) Counsel persons at highest risk for developing chronic Q fever, especially persons with preexisting cardiac valvular disease or individuals with vascular grafts.

A vaccine for Q fever has been developed and has successfully protected humans in occupational settings in Australia. However, this vaccine is not commercially available in the United States. Persons wishing to be vaccinated should first have a skin test to determine a history of previous exposure. Individuals who have previously been exposed to *C. burnetii* should not receive the vaccine because severe reactions, localized to the area of the injected vaccine, may occur. A vaccine for use in animals has also been developed, but it is not available in the United States.

8. Treatment

Doxycycline is the first line treatment for all adults, and for children with severe illness. Treatment should be initiated immediately whenever Q fever is suspected. Use of antibiotics other than doxycycline or other tetracyclines is associated with a higher risk of severe illness. Doxycycline is most effective at preventing severe complications from developing if it is started early in the course of disease. Therefore, treatment must be based on clinical suspicion alone and should always begin before laboratory results return. If the patient is treated within the first 3 days of the disease, fever generally subsides within 72 hours. In fact, failure to respond to doxycycline suggests that the patient's condition might not be due to Q fever. Severely ill patients may require longer periods before their fever resolves. Resistance to doxcycline has not been documented [3].

There is no role for prophylactic antimicrobial agents in preventing Q fever after a known exposure and prior to symptom onset; attempts at prophylaxis will likely extend the incubation period by several days but will not prevent infection from occurring. Patients should be treated for at least 3 days after the fever subsides and until there is evidence of clinical improvement. Standard duration of treatment is 2-3 weeks [3].

The use of doxycycline is recommended to treat Q fever in children of all ages who are hospitalized or are severely ill. Unlike older generations of tetracyclines, doxycycline has not been shown to cause staining of permanent teeth, and most experts consider the benefit of doxycycline in treating Q fever in children younger than 8 years of age with severe illness or who are hospitalized greater than the potential risk of dental staining. Children with mild illness who are less than 8 years of age may be treated with co-trimoxazole, but therapy should be switched to doxycycline if their course of illness worsens.

In cases of life-threatening allergies to doxycycline and in pregnant patients, physicians may need to consider alternate antibiotics. Treatment of pregnant women diagnosed with acute Q fever with once daily co-trimoxazole throughout pregnancy has been shown to significantly decrease the risk of adverse consequences for the fetus [3, 67].

Recommended treatment for Chronic Q fever in Adults is: Doxycycline 100 mg every 12 hours and hydroxychloroquine 200 mg every 8 hours. Standard duration of treatment is 18 months [3].

9. Conclusion

Although described 60 years ago, Q fever is still a poorly understood disease. Its reservoirs seem to be related to any mammal, but ticks may also be reservoirs. The clinical presentation is very pleomorphic and includes severe forms with a poor prognosis. Most often, acute cases present as asymptomatic infections, as a flu-like syndrome, as a pneumonia, or as hepatitis. Host factors probably play an important role in the development of chronic disease, which may present as a blood-culture-negative endocarditis or as an infected aneurysm. Although its exact prevalence is unknown, it is likely that the number of cases of Q fever is underestimated. Therefore, the diagnosis must be considered in the case of an unexplained fever, especially if the fever recurred following contact with possibly contaminated mammals. The best tests for diagnosis are those which permit the direct detection of bacteria. They include shell vial cell culture, PCR amplification, and immunodetection with tissue biopsy specimens. All these techniques require a level 3 biosafety laboratory and trained personnel due to the extreme infectivity of *C. burnetii*. In chronic cases, the techniques that allow the direct detection of *C. burnetii* in blood or tissues should be used before the beginning of therapy. As for indirect specific diagnosis, the technique to be used should be very sensitive and should detect antibodies early in the course of the disease. Although many techniques have been described, immunofluorescence assay is the reference method. It is both very specific and sensitive. In case of acute Q fever, diagnosis would be confirmed by an immunofluorescence assay titer greater than or equal to the cutoff value (which must be determined for each geographical area) or by a fourfold increase in the antibody titer detected by immunofluorescence assay, complement fixation, ELISA, or microagglutination. The presence of cross-reacting antibodies should be investigated by cross-adsorption followed by Western immunoblotting.

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Research Article

Experimental Evaluation of the Pathogenicity of Different Strains of *Schistosoma mansoni*

Antônio Aurélio Euzébio Jr.,[1] Nádia Regina Borim Zuim,[2] Arício Xavier Linhares,[2] Luiz Augusto Magalhães,[2] and Eliana Maria Zanotti-Magalhães[2]

[1] *Department of Pediatrics, School of Medical Sciences, State University of Campinas (Unicamp), Rua Tessália Vieira de Camargo, 126 Cidade Universitária Zeferino Vaz, 13083-887 Campinas, SP, Brazil*
[2] *Department of Animal Biology, Institute of Biology, State University of Campinas (Unicamp), Rua Monteiro Lobato, 255 Cidade Universitária Zeferino Vaz, 13083-862 Campinas, SP, Brazil*

Correspondence should be addressed to Eliana Maria Zanotti-Magalhães, emzm@unicamp.br

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The pathogenesis of three different *Schistosoma mansoni* strains from the Brazilian states of Minas Gerais (BH strain) and São Paulo (SJ and SD strains) was evaluated in experimentally infected mice. Observations of the most severe clinical cases among local patients treated (SD strain) in the city of Campinas (São Paulo, Brazil) formed the basis of this study. Mice were used as definitive hosts and were infected with cercariae from *Biomphalaria tenagophila* (SJ and SD strains) and *Biomphalaria glabrata* (BH strains). The parameters analyzed were as follows: number of *S. mansoni* eggs in mice feces; number of granulomas per tissue area in liver, spleen, lungs, pancreas, and ascending colon; measurements of hepatic and intestinal granulomas; number of adult worms; and measurements of trematode eggs. The comparison among the three strains indicated that the SD strain, isolated in Campinas, presented a higher worm recovery relative to the number of penetrating cercariae. In addition, when compared to the SJ and BH strains, the SD strain demonstrated similar pathogenicity to the BH strain, with a greater quantity of granulomas in the viscera, as well as larger granulomas and eggs. Furthermore, a greater quantity of trematode eggs was also shed in the feces.

1. Introduction

After malaria, schistosomiasis is the most important global parasitic disease and is a serious public health problem mainly in tropical and subtropical regions. In Brazil, the spread of schistosomiasis is caused by the single species occurring in the country, *Schistosoma mansoni*. It has been shown that the spread of *S. mansoni* is directly related to the economic history of the country via human migratory events from hyperendemic regions and the presence of three species of snails, which are the natural intermediate hosts of the parasite. Schistosomiasis, initially described as a typically rural disease, now has an urban character and is prevalent mainly on the outskirts of large cities in São Paulo (Brazil), the most populous state in Brazil. Another feature of the disease in São Paulo state is the absence of severe cases [1], which is favorable in one sense; however, due to the absence of clinical manifestations that bring patients to the public health clinics, schistosomiasis often remains undiagnosed, and the infected individuals may continue to contaminate fresh water sources where the intermediate hosts breed.

In 1963 [2], the BH and SJ strains of *Schistosoma mansoni* were initially described based on the observation that *Biomphalaria glabrata* from Belo Horizonte (in the state of Minas Gerais, Brazil) could be infected with a local strain (BH) of *S. mansoni* but was resistant to infection by *S. mansoni* isolated from the Vale do Rio Paraiba do Sul (the South Paraiba River Valley) (SJ strain) from São Paulo, Brazil. Moreover, *Biomphalaria tenagophila* from the Vale do Rio Paraíba do Sul was susceptible to the local *S. mansoni* (SJ strain) but refractory to infection by *S. mansoni* from Belo Horizonte (BH strain).

Studies in mice [3, 4] revealed morphological differences between the worms of these two strains as well as differences

in their pathogenicity [5–7]. The authors [7] concluded that a much smaller number of BH-strain parasites lead to equivalent levels of damage caused by greater numbers of SJ-strain worms.

In BH-strain endemic areas, individuals with decompensated hepatosplenomegaly are commonly observed. In areas where patients are infected with the SJ strain, the commonly described clinical manifestations include the intestinal form with a slightly enlarged spleen [8].

In Campinas, a city located in the state of São Paulo (Brazil) with a population of approximately one million, the first native case of schistosomiasis from *S. mansoni* was observed in 1959, according to the records of the Health Secretariat of São Paulo, and originated from a focus at the Piçarrão stream [9]. Since then, new cases and new infection foci have been recorded in Campinas and its surrounding regions. However, most of the cases have been asymptomatic.

Autochthonous cases of schistosomiasis from *S. mansoni* have been reported in the Jardim São Domingos (São Domingos Garden) neighborhood of Campinas since 1998. More recently, a strain of the trematode was isolated in a laboratory from *B. tenagophila* captured at Lagoa Boa União (Boa União Lake), which is located in the Jardim São Domingos neighborhood. Freitas and Oliveira [10] and Freitas et al. [11] found signs of portal hypertension and, in some cases, myelopathy in patients with autochthonous schistosomiasis treated at the Jardim São Domingos Medical Center. These facts led us to conduct comparative studies of the pathogenicity of different *S. mansoni* strains isolated from Jardim São Domingos (Campinas, São Paulo, Brazil), BH strains from Belo Horizonte (Minas Gerais, Brazil), and SJ strains from the Vale do Rio Paraíba do Sul (São Paulo, Brazil).

2. Materials and Methods

The experiments were approved by the Animal Experimentation Ethics Committee from our institution and were certified under protocol number 642-1.

Swiss specific pathogen free (SPF) female mice were exposed to infection by *S. mansoni* cercariae of BH strains (originally from Belo Horizonte, Minas Gerais, Brazil), SJ strains (originally from the Vale do Rio Paraíba, São Paulo, Brazil), and SD strains (originally from Jardim São Domingos, Campinas, São Paulo, Brazil). The mice were distributed into three groups: Group 1, mice infected with *S. mansoni* cercariae of the BH strain; Group 2, mice infected with *S. mansoni* cercariae of the SJ strain; and Group 3, mice infected with *S. mansoni* cercariae of the SD strain.

Starting at the third week of infection, mice fecal matter was collected weekly to verify the elimination of *S. mansoni* eggs. At the eighth week of infection, all surviving mice were sacrificed for worm recovery.

The evaluated parameters included the following: the number of penetrating cercariae; the number of eggs shed in feces; the number of worms in the mesenteric-portal system on the day the mice were sacrificed; the number of granulomas in the liver, spleen, intestine (ascending colon), pancreas, and lung; tissue area of granulomas found in the liver and intestine; and *S. mansoni* egg size.

2.1. Infection of Mice. Mice were infected with *S. mansoni* cercariae from sympatric snails bred in the laboratory from *B. tenagophila* captured at Lagoa Boa União, Jardim São Domingos (23°02′21.31″S 47°06′17.01″W) in Campinas. These snails were exposed to miracidia of the SD strain. The *B. glabrata* from Belo Horizonte was exposed to miracidia of the BH strain, and the *B. tenagophila* from the Vale do Rio Paraiba do Sul was exposed to miracidia of the SJ strain.

Mice were individually exposed for two hours to cercariae from the snails infected with different strains. Infection was performed percutaneously by exposure of the tail to 70 cercariae, according to the method described by Magalhães [12].

2.2. Checking the Number of S. mansoni Eggs Shed in the Feces. The number of eggs eliminated in the feces of infected mice was determined using the method described by Kato-Katz starting from the third week of infection [13]. Glass slides were prepared for each group of five animals, and the final report results represent the arithmetic means of the readings from all glass slides.

2.3. Collection and Counting of Worms. Animals were sacrificed by cervical dislocation at the eighth week of infection. Worms were collected through the perfusion of the hepatic portal system according to the method described by Yolles et al. [14]. The number of male and female worms was recorded, whether they were isolates or pairs.

2.4. Collection, Counting, and Measurement of Schistosomal Granulomas of the Viscera. After perfusion, fragments of the liver, spleen, colon, lung, and pancreas of the sacrificed animals were extracted for granuloma counting and measurement. The fragments were fixed in aqueous Bouin's solution, and 5 μm thick histological sections were stained with Masson's trichrome stain. The sections were analyzed with an optical microscope to calculate the number of granulomas per tissue area (1.2469 mm^2) based on the method described by Magalhães et al. [6]. The sizes of the granulomatous reactions were determined based on their area. Only granulomas containing an *S. mansoni* egg in their center were measured, as the egg verified the proximity of the sectioning to larger granulomatous reactions. The measurements were performed using the Image Pro Lite software, version 4.0 for Windows 95/NT/98.

2.5. Collection and Measurement of S. mansoni Eggs. Eggs of the BH, SJ, and SD strains of *S. mansoni* were collected from the feces of infected mice. The length, width, and length of the egg spicules were measured using Image Pro Lite, version 4.0 for Windows 95/NT/98. Statistical analysis of the data included examination of the length, width, spicule size, length to width ratio, length to spicule ratio, and width to spicule ratio. The PROC GLM (General Linear Procedure)

Table 1: Mean numbers of penetrating cercariae and mean numbers of male and female worms in mice infected with BH, SJ, and SD strains of *S. mansoni* and sacrificed at the eighth week of infection.

S. mansoni strain	Number of infected mice	Penetrating cercariae	Male worms	Female worms	Duncan's Test*	
SJ	10	66.90			A	
SD	10	64.50			A	B
BH	15	62.67				B
BH	15		1.73		A	
SD	10		1.30		A	
SJ	10		0.90		A	
SJ	10			0.30	A	
BH	15			0.13	A	
SD	10			0.10	A	

* Means followed by same letter do not differ significantly from each other ($\alpha = 0.05$).

Table 2: Mean numbers of mating worm pairs, total number of worms, and mean percentages of worm recovery relative to the number of penetrating cercariae in mice infected with BH, SJ, and SD strains of *S. mansoni* and sacrificed at the eighth week of infection.

S. mansoni strain	Number of infected mice	Mating worm pairs	Total number of worms	Worm recovery (%)	Duncan's test*	
SD	10	14.80			A	
SJ	10	11.60			A	B
BH	15	7.87				B
SD	10		31.00		A	
SJ	10		24.40		A	B
BH	15		17.60			B
SD	10			48.62	A	
SJ	10			36.67	A	B
BH	15			28.47		B

* Means followed by same letter do not differ significantly from each other ($\alpha = 0.05$).

function in the SAS software package (2006) was used for the analysis [15].

3. Results

All animals infected with all three strains of *S. mansoni* survived for the eight weeks of the experiment.

Tables 1 and 2 list the mean numbers of penetrating cercariae, male worms, female worms, mating worm pairs, total number of worms, and percentage of worms recovered relative to the number of penetrating cercariae in mice infected with the BH, SJ, and SD strains of *S. mansoni*.

Statistical analysis indicated that the penetration capacity of the cercariae in mice was significantly different among the strains ($P = 0.0241$). The SJ cercariae presented greater penetration than the BH cercariae (Table 1).

The number of mating worm pairs and the total number of worms were significantly different among the strains ($P = 0.0020$ and $P = 0.0036$, resp.). The mice infected with the SD strain had a significantly higher number of worms (mating pairs and total) than the mice infected with the BH strain (Table 2).

The percentage of recovered worms relative to the number of penetrating cercariae was significantly different by strain ($P = 0.0065$). As listed in Table 2, the worm recovery percentage was higher from mice infected with the SD strain. Statistical analysis (Table 1) also revealed that the numbers of male worms and female worms recovered from mice did not differ significantly among all three strains ($P = 0.7389$ and $P = 0.4560$, resp.).

Table 3 lists the number of granulomatous reactions around the *S. mansoni* eggs per tissue area (1.2469 mm^2) found in the viscera of mice infected with the BH, SJ, and SD strains of *S. mansoni*.

The mean number of granulomatous reactions found in the liver was significantly different ($P = 0.0032$) by strain. Mice infected with the BH and SD strains exhibited a greater number of granulomatous reactions compared to those infected with the SJ strain (Table 3).

There was a significant difference in the mean number of splenic granulomas in mice infected with different strains ($P = 0.0390$), and the SD strain produced a higher number of granulomas compared to the BH and SJ strains. The difference between the values for the BH and SJ strains was not significant (Table 3).

Histological analysis of the pancreas sections revealed a greater mean number of schistosomal granulomas for the SD strain (Table 3) compared to the other strains ($P = 0.0189$).

Statistical analysis of the number of granulomas in the intestine (ascending colon) and lung did not reveal any significant difference among all three strains ($P = 0.2134$ and $P = 0.2473$, resp.). However, the SD strain produced

Table 3: Mean numbers of granulomas per tissue area (tissue area of 1.2469 mm^2) in mice infected with the BH, SJ, and SD strains of *S. mansoni* and sacrificed at the eighth week of infection.

S. mansoni strain	Number of mice	Number of granulomas (mean)/1.2469 mm^2					Duncan's test*	
		Liver	Spleen	Pancreas	Intestines	Lungs		
BH	15	15.27					A	
SD	10	13.80					A	
SJ	10	10.60						B
SD	10		1.00				A	
BH	15		0.13					B
SJ	10		0.10					B
SD	10			3.40			A	
BH	15			2.00			A	B
SJ	10			0.10				B
SD	10				4.50		A	
BH	15				3.87			
SJ	10				2.50		A	
BH	15					1.07	A	
SD	10					0.40	A	
SJ	10					0.00	A	

* Means followed by same letter do not differ significantly from each other ($\alpha = 0.05$).

Table 4: Mean areas of the hepatic and intestinal granulomas at the eighth week of infection in mice infected with BH, SJ, and SD strains of *S. mansoni*.

S.mansoni strain	Number of granulomas	Granuloma area (mean) μm^2		Duncan's test*
		Hepatic	Intestinal	
SD	206	132194		A
BH	403	111586		B
SJ	137	109472		B
SD	32		78313	A
BH	41		72340	A
SJ	16		38667	B

* Means followed by same letter do not differ significantly from each other ($\alpha = 0.05$).

a greater mean number of granulomas in the intestines. In addition, the SD strain did not produce a greater number of granulomas in the lung than the BH strain (Table 3).

In general, the histological analysis of sections from mice infected with all three strains indicated that the liver had the greatest number of granulomatous reactions around the *S. mansoni* eggs, followed by the intestines. The third-greatest number of granulomas for the SD strain was found in the pancreas, followed by the spleen and lungs. For the BH strain, the pancreas also had the third-greatest number of visceral granulomas; however, unlike the SD strain, the pancreas was followed by the lungs and then the spleen. For the SJ strain, similar numbers of granulomas were found in the pancreas and spleen. No granulomas were observed in the lungs of animals infected with the SJ strain.

The values obtained for the average size of granulomatous reactions and the number of eggs per gram of feces are presented for each strain in Tables 4 and 5.

The data in Table 4 indicate that the largest granulomatous area in the liver was found for the SD strain. The hepatic granulomas in mice infected with the BH and SJ strains were smaller and did not differ significantly from each other.

The intestinal granulomas were larger in animals infected with the SD and BH strains, followed by those infected with the SJ strain (Table 4).

By the seventh and eighth weeks of infection, it was evident that mice infected with the SD strain tended to eliminate similar numbers of eggs to the BH strain (Table 5), despite having *B. tenagophila* as an intermediate host. However, it is important to note that the total worm number and mating worm pair recovery in the mesenteric portal system of mice infected with the SD strain was greater on average than in mice infected with the BH strain (Table 2).

Table 6 lists egg measurement data. The length and width of the SD-strain eggs (Table 6) were significantly longer than the other eggs from other strains ($P < 0.0001$ and $P < 0.0001$,

Table 5: Mean numbers (log) of *S. mansoni* eggs from mice infected with the BH, SJ, and SD strains per gram of feces.

S. mansoni strain	Number of infected mice	Number of eggs (log-mean)	Duncan's test*	Infection Time (week)
SJ	10	1.417	A	
BH	15	0.944	A	3rd
SD	10	0.000	A	
SJ	10	3.186	A	
BH	15	0.944	A	4th
SD	10	0.000	A	
SJ	10	4.342	A	
SD	10	3.967	A	5th
BH	15	2.409	B	
SD	10	4.309	A	
SJ	10	3.804	A	6th
BH	15	3.275	A	
SD	10	6.787	A	
BH	15	6.579	A	7th
SJ	10	4.524	B	
SD	10	7.248	A	
BH	15	6.746	A	8th
SJ	10	5.563	B	

* Means followed by same letter do not differ significantly from each other ($\alpha = 0.05$).

Table 6: *S. mansoni* egg measurements from the BH, SJ, and SD strains.

S. mansoni strain	Number of eggs	Egg (mean) μm			Duncan's test*
		Length	Width	Spicule	
SD	23	155.60			A
SJ	31	141.11			B
BH	31	139.34			B
SD	23		64.63		A
SJ	31		59.37		B
BH	31		56.89		C
BH	31			21.22	A
SJ	31			21.09	A
SD	23			20.85	A

* Means followed by same letter do not differ significantly from each other ($\alpha = 0.05$).

resp.), and the eggs of the BH and SJ strains did not differ in length. Eggs from the BH strain had the shortest width.

The size of the spicule (Table 6) was not significantly different among the strains ($P = 0.9383$); however, some eggs from the SD strain had spicules with a very curved tip, as shown in Figure 1. The measurement ratio data in Table 7 indicate that the strains differed only in the width to spicule length ratio ($P = 0.0855$), which was highest for the SD strain.

4. Discussion

Geographically isolated strains of *S. mansoni* present significant differences in pathogenicity, which is attributed to greater impairment of organs due to the greater extent and wider distribution of *S. mansoni* eggs [16], the number of eggs produced by the parasite [7, 17], and the degree of susceptibility of the snail vector [1, 16, 18, 19]. According to Chieffi and Waldman [1], the rarity of hepatosplenic forms in autochthonous cases in São Paulo State (Brazil) may be due to the low rates of snail infection by *S. mansoni*, which reflects the lower susceptibility of infection of *B. tenagophila* compared to *B. glabrata* and *B. straminea*.

In a previous study, Yoshioka et al. [20] demonstrated that the Santa Rosa (SR) strain of *S. mansoni* isolated in Campinas, São Paulo State (Brazil), was less pathogenic than the BH and SJ strains. Mice infected with the SR strain exhibited the fewest worms, eggs shed in the feces, and granulomas. The parasite eggs also had the smallest diameter of granulomatous reaction around them.

Bina and Prata [17] have shown an association between the development of the hepatosplenic disease form and the quantity of *S. mansoni* eggs eliminated in patient stools. The lesions caused by *S. mansoni* are rarely restricted to one organ, whether it is the liver or intestine. *S. mansoni* eggs are usually found in almost all organs and tissues, where they

Table 7: *S. mansoni* egg ratio measurements for the BH, SJ, and SD strains.

S.mansoni strain	Number of eggs	Egg (mean)			Duncan's test*	
		Length/width	Length/Spicule	Width/spicule		
SD	23	2.45			A	
BH	31	2.41			A	
SJ	31	2.38			A	
SD	23		7.63		A	
BH	31		6.93		A	
SJ	31		6.87		A	
SD	23			3.16	A	
SJ	31			2.89	A	B
BH	31			2.80		B

* Means followed by same letter do not differ significantly from each other ($\alpha = 0.05$).

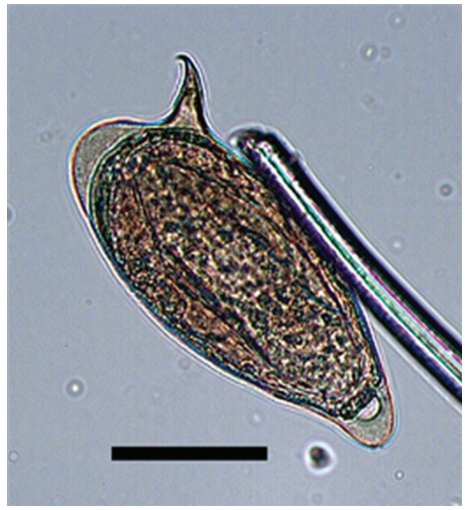

Figure 1: Egg from the SD strain of *S. mansoni* (bar = 50 μm) observed in the feces of mice. Note the curved spicule tip.

produce some similar reactions. Certain differences arise from the specific characteristics of the affected tissues.

In the studied strains, schistosomal granulomas were found in all studied organs, except for the lungs of mice infected with the SJ strain (Table 3).

The results presented here indicate high pathogenicity for the São Domingos strain (SD), which was initially isolated from the São Domingos neighborhood in Campinas (São Paulo state). This strain had similar levels of pathogenicity to the BH strain, when considering the number and size of granulomatous reactions in the viscera. The greater involvement of tissues and organs can be attributed to the greater number of eggs shed by the worms and to the larger egg size of the SD strain. In addition to being more numerous, the eggs of the SD strain were larger (Table 6) and had a distinct morphological feature, a curved spicule tip (Figure 1).

From an epidemiological point of view, these results are relevant due to the greater worm recovery relative to the number of penetrating cercariae and higher number of eggs shed in the feces of animals infected with the SD strain.

The data obtained for the SD strain of *S. mansoni* also indicate that this strain is the most pathogenic of all strains currently described that have *B. tenagophila* as an intermediate host. The greater pathogenicity of the SD strain observed experimentally in laboratory animals confirms the observations obtained from clinical examinations of patients who were infected while bathing in areas contaminated with *B. tenagophila* [10, 11]. Freitas and Oliveira [10] have observed myelopathy in some of these patients. Myelopathy is considered one of the most serious problems caused by *Schistosoma mansoni* infection, and all evidence suggests that parasite eggs are responsible for its clinical manifestation [21].

The results of the present study seem to indicate that the more severe clinical forms of *S. mansoni*-related schistosomiasis in immunologically competent individuals may be caused by higher parasite load, higher fecundity of the worms, and greater numbers of eggs. All of these lead to a more severe compromise of tissues due to a greater number of schistosomal granulomas. The severity of the disease may also be attributed to the larger size of the trematode eggs, which generate more antigens in the host organism, thereby raising the host's sensitivity and promoting inflammation, which, in turn, produces a greater extent of tissue damage.

Based on the data presented in this study, an accurate evaluation of *S. mansoni* pathogenicity has to be based on several biological characteristics of the parasite: the immunoregulatory role involved in the granulomatous response, the trematode egg antigens responsible for inducing the granuloma, the genetic peculiarities of the host [22], and the parasite load in the clinical manifestation of schistosomiasis in mice and humans.

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Research Article

A Study of the Granulomatous Responses Induced by Different Strains of *Schistosoma mansoni*

Nádia Regina Borim Zuim, Silmara Marques Allegretti, Arício Xavier Linhares, Luiz Augusto Magalhães, and Eliana Maria Zanotti-Magalhães

Department of Animal Biology, Institute of Biology, State University of Campinas (UNICAMP), Cidade Universitária Zeferino Vaz, Rua Monteiro Lobato 255, 13083-862 Campinas, SP, Brazil

Correspondence should be addressed to Eliana Maria Zanotti-Magalhães, emzm@unicamp.br

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The increased pathogenesis of the *Schistosoma mansoni* BH strain compared with the SJ strain has been attributed to the number of granulomas formed in experimental infections, which increase the mortality in definitive hosts. The aim of the present study was to investigate the development of granulomas around the eggs of the *S. mansoni* BH and SJ strains and to determine whether this host reaction was strain specific. Four experimental groups were analyzed. Two groups contained mice inoculated in the caudal vein with eggs from the *S. mansoni* BH or SJ strains and the other two contained mice that were infected with cercariae of the BH strain prior to being inoculated with eggs. The number of granulomas per tissue area in the lungs and liver, as well as the size of the granulomas, was analyzed to characterize the response to schistosome infection. The largest granulomatous responses were observed around eggs of the BH strain. Granulomas covered a larger area in the lungs of mice that were previously infected with cercariae and subsequently inoculated with eggs of the BH strain. These results indicated that specific granulomatous responses occurred following an infection with the BH and SJ strains of *S. mansoni*.

1. Introduction

Schistosomiasis is considered to be the most notable parasitic disease after malaria due to its wide geographic distribution, the large number of people affected by the disease, its severity, and its association with poor sanitary conditions [1, 2].

The primary pathogenic mediators in schistosomiasis are the trematode eggs. A schistosomal granuloma, the characteristic lesion, forms around mature eggs that have been deposited in the tissues of the definitive host. The distribution of eggs in host tissues, the extent of the granulomatous response, and the degree of infectivity of the parasite strain are key factors in the pathogenesis of schistosomiasis [3].

The diverse behavior of different strains of *Schistosoma mansoni* [4–11] may account for the regional variation observed in clinical schistosomiasis [12]. The degree of morbidity in human schistosomiasis varies regionally, possibly due to variations in parasite infectivity and fecundity [13].

Previous studies have demonstrated a difference in the development of the BH and SJ strains of *S. mansoni* in the definitive host [4, 6, 7, 14–16]. More hepatic granulomas were observed in mice infected with the *S. mansoni* BH strain, and a significantly lower percentage of mice survived in this group than in the group of mice that were infected with the SJ strain. In mice, fewer parasites of the BH strain were necessary to obtain equivalent pathogenesis than those with the SJ strain [6]. These experiments demonstrated an increased pathogenicity of the BH strain [6, 10, 17, 18]. In areas that are considered endemic for the BH strain, there are frequent cases of decompensated hepatosplenomegaly, which does not occur in regions where the SJ strain is present. Instead, almost all cases of infection with the SJ strain are asymptomatic, with the exception of rare cases of compensated hepatosplenomegaly [19, 20]. In addition, adult worms and eggs of the BH strain are typically larger than those of the SJ strain [10, 14, 21–27].

A number of factors seem to be fundamentally important for the development of severe forms of schistosomiasis: the number of eggs and the antigens they released; the reinfections; the genetic influence of the host, the hosts immune response to the formation of granulomas, the development of periportal fibrosis and factor modulators, and the associations with aggravating factors such as alcoholism, malnutrition, and hepatitis (particularly hepatitis B and C) which compromise the liver [28]. Mice were experimentally infected and submitted to a low-protein diet using the BH strain of *S. mansoni*. In spite of the reduced number of hepatic granulomas, as well as a reduction in the size of the granuloma, mortality rates among the animals were high [29]. Recent studies [30] used different strains of mice and established an association between malnutrition and the development of hepatic fibrosis. In the state of Minas Gerais (Brazil), the hepatosplenic form of schistosomiasis in children was strongly associated with bathing in streams [31]. In parts of the state of São Paulo (Brazil), characterized by low endemicity, where the main risk factor for *S. mansoni* infection is from leisure in water, there is a significant correlation between the intensity of the infection and the prevalence. The infection rate of the intermediate host *B. tenagophila* was 0.4% in this case [32]. All of these associated factors indicated that the evolution of mansoni schistosomiasis should be seen as a multidisciplinary phenomenon and individual analysis of each case should be performed to gain a better understanding of the infection.

Considering the importance of *S. mansoni* eggs in the pathogenicity of schistosomiasis and the fact that the induced granulomatous immune response is stage specific [33], the aim of the present study was to investigate the inflammatory response in the lung tissue of mice inoculated in the caudal vein with eggs of the *S. mansoni* BH and SJ strains to determine whether differences existed between the two strains in terms of the inflammatory response around eggs.

2. Materials and Methods

The present study received approval from the Animal Experimentation Ethical Committee of the UNICAMP (Comissão de Ética na Experimentação Animal-CEEA-IB-UNICAMP) under protocol number 870-1.

2.1. S. mansoni Strains and Egg Collection. Two *S. mansoni* strains were used in the present study: the SJ strain from São José dos Campos (SP, Brazil), which was maintained in populations of sympatric *B. tenagophila* and the BH strain, originally from Belo Horizonte (MG, Brazil), which was maintained in populations of sympatric *B. glabrata*. Cercariae obtained from the snails were used to infect Swiss SPF (specific pathogen free) mice [34]. The mice were exposed to 100 cercariae for two hours. After this time period had elapsed, the cercariae that remained in the test tubes, where the tails of the mice were immersed, were counted.

The eggs from both strains (BH and SJ) of *S. mansoni* were obtained from the intestinal wall of infected mice. Mice were inoculated in the caudal vein with approximately 1000 mature eggs in 0.3 mL of saline solution [35].

2.2. Experimental Groups. Four experimental groups were established in the present study. Group I contained 12 mice inoculated with eggs from the *S. mansoni* BH strain. Group II contained 12 mice inoculated with eggs from the *S. mansoni* SJ strain. Group III contained 12 mice infected percutaneously with 100 cercariae of the *S. mansoni* BH strain 8 weeks prior to being inoculated in the caudal vein with eggs from the *S. mansoni* BH strain. Group IV contained 12 mice infected percutaneously with 100 cercariae of the *S. mansoni* BH strain 8 weeks prior to being inoculated in the caudal vein with eggs from the *S. mansoni* SJ strain.

Two to three animals were used to obtain the mature eggs of the BH strain to be inoculated in the caudal vein of mice. Five to six previously infected animals were used to obtain mature eggs of the SJ strain. The mice in each group were euthanized by cervical dislocation 1, 8, 15, or 34 days after inoculation with the eggs. On each of these preestablished days, 3 mice from each group were euthanized. The worms were then recovered from the mice in groups III and IV via perfusion of the hepatic portal system [36]. Finally, the recovered worms were separated by gender.

2.3. The Number and Size of the Granulomas in the Liver and the Lungs. In order to count and measure the schistosome granulomas in the liver (Groups III and IV) and in the lungs (Groups I, II, III, and IV) of the euthanized mice, histological slices (5 μm thick) were fixed in Bouin's solution, stained with Masson's trichrome and examined using an optical microscope. The number of granulomas per tissue area (0.984704 mm^2) and the extent of the granulomatous response were determined using the techniques described by Magalhães et al. [17]. Only granulomas that contained an *S. mansoni* egg at the center were measured. Measurements were performed using Image-Pro Lite software, (version 4.0) for Windows 95/NT/98.

2.4. Statistical Analysis. The data were analyzed using SAS software for Windows 8.01, 2000 [37].

3. Results

Table 1 displays the number of cercariae that effectively penetrated and recovered trematodes from Groups III and IV. There was no significant difference between the number of penetrating cercariae in the two groups ($P = 0.4667$). The number of trematodes was significantly higher in Group III than in Group IV ($P = 0.0012$, $P = 0.0036$, $P = 0.0019$; male, female, and total number of trematodes, resp.).

3.1. Granuloma Area in the Lung (Table 2, Figures 1, 2, 3, and 4). Table 2 displays the number of pulmonary granulomas found in each of the experimental groups. Figure 1 displays the beginning of the granulomatous reaction one day after inoculation of the BH eggs (Group I) and the SJ eggs (Group II).

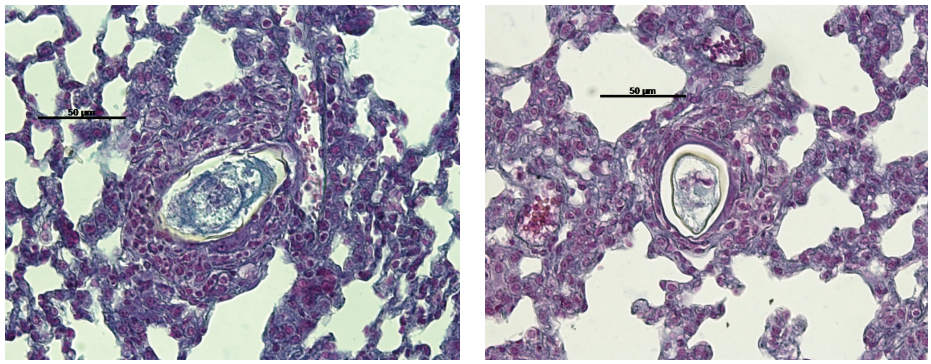
Table 1: The table presents the mean number of cercariae and male and female trematodes that were recovered from mice exposed to 100 cercariae of the *S. mansoni* BH strain and inoculated 8 weeks later with eggs from the *S. mansoni* BH (Group III) or SJ (Group IV) strains. The mice were euthanized 1, 8, 15, and 34 days after being inoculated with eggs in the caudal vein.

Group	Infecting cercariae	Female	Male	Total trematodes	Duncan's test
III	94.90				A
IV	95.00				A
III		17.75			A
IV		9.10			B
III			18.87		A
IV			8.50		B
III				36.62	A
IV				17.60	B

There is no significant difference between means with the same letter ($\alpha = 0.05$).

Table 2: Number of pulmonary granulomas recorded, with the mean and standard deviation values from the different groups of mice, which were euthanized after 1, 8, 15, and 34 days.

Groups	Days	Number of observations	Mean area (μm^2)	Standard deviation
I	1	8	13908.4392	10297.1036
	8	35	14455.5974	8873.95994
	15	12	30432.241	11432.0316
	34	36	17290.6786	8834.70254
II	1	8	13558.7594	10318.3571
	8	17	15336.7856	5982.09941
	15	17	9571.12058	4399.63715
	34	27	14944.4057	5278.07681
III	1	13	20343.3065	6016.07255
	8	35	38047.4264	16323.5628
	15	14	45384.2903	21215.4849
	34	53	28413.4525	9918.21986
IV	1	31	22506.9705	9944.37182
	8	15	25709.9754	15222.4076
	15	37	29895.0112	10174.3078
	34	22	30799.1796	15791.2374



(a) (b)

Figure 1: Formation of a halo cell around mature (a) *S. mansoni* BH (Group I) and (b) SJ (Group II) eggs in the pulmonary parenchyma one day after inoculation of the eggs in the caudal vein of mice.

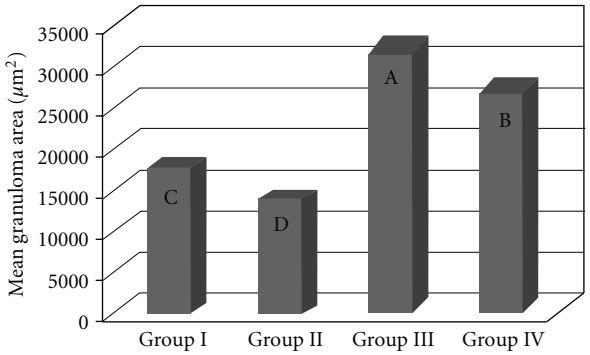


Figure 2: The granuloma area in the lungs of mice that were inoculated with eggs from the *S. mansoni* BH (Group I) or SJ (Group II) strains and of those that were previously infected with the *S. mansoni* BH strain and then inoculated 8 weeks later with eggs from the *S. mansoni* BH (Group III) or SJ (Group IV) strains. The means with different letters exhibited a significant difference ($\alpha = 0.05$).

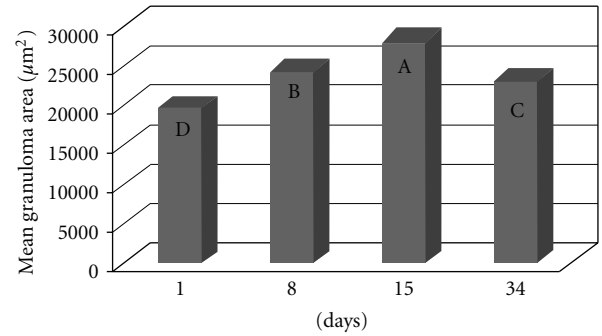


Figure 3: The area of the granulomatous response around the eggs of *S. mansoni* in the lungs of the mice in all 4 experimental groups (I, II, III, and IV) is depicted based on the time since the inoculation of the eggs in the caudal vein. The means with different letters exhibited a significant difference ($\alpha = 0.05$).

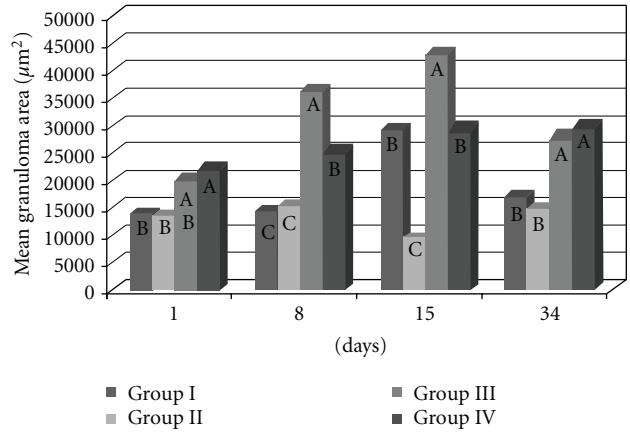


Figure 4: The mean granuloma area in the lungs of the mice that were inoculated with eggs of the *S. mansoni* BH (Group I) or SJ (Group II) strains and of those that were previously infected with the *S. mansoni* BH strain and then inoculated 8 weeks later with eggs from the *S. mansoni* BH (Group III) or SJ (Group IV) strains. The mice were euthanized 1, 8, 15, and 34 days after inoculation with the eggs. At each time point, the means depicted with the same letter do not differ significantly ($\alpha = 0.05$).

The area covered by granulomas differed significantly between the groups, independently of the time since inoculation ($P < 0.0001$). The largest granulomas occurred in Groups III and IV (previously infected with the *S. mansoni* BH strain) and the smallest granulomas were found in Groups I and II (inoculated with eggs from the BH and SJ strains, resp.) (Figure 2). The granulomatous area around the eggs from the BH (Group I) strain was significantly larger than that of the SJ (Group II) strain (Figure 2). The area of the granulomas altered throughout the postinoculation period ($P < 0.0001$), with the smallest granulomas recorded 1 day after inoculation and the largest granulomas recorded after 15 days (Figure 2). Fifteen days after inoculation, the BH eggs had induced a significantly larger granulomatous response than the SJ eggs (Figure 4). The mice previously infected with the *S. mansoni* BH strain (Groups III and IV) exhibited larger granulomas in the lung than the mice in Groups I and II (Figure 2). There was also a significant difference in granuloma size 8 days ($P < 0.0001$) and 34 days ($P < 0.0001$) after inoculation with the eggs (Figure 4). The comparison of mice infected with the *S. mansoni* BH strain and subsequently inoculated with the BH (Group III) or SJ (Group IV) eggs revealed that the granuloma area surrounding BH eggs was significantly larger 8 and 15 days after inoculation (Figure 4).

3.2. The Number of Lung Granulomas (Figures 5 and 6). There were significantly ($P < 0.0001$) more granulomas in the lungs of the mice infected and inoculated with eggs from the BH strain (Group III) (Figure 5). There were no significant differences in the number of granulomas among the other groups (I, II, and IV). Most granulomas were observed 8 and 34 days after inoculation, and the fewest granulomas were found on the first day after inoculation with the eggs. Fifteen days after inoculation, there were no significant differences in the number of granulomas between the four experimental groups. Significantly more granulomas formed on the first day after inoculation in the lungs of previously infected animals (Groups III and IV) than in that of naive mice (Groups I and II). Eight days after inoculation, there were significantly fewer granulomas in the mice from Group IV than in the other groups, whereas no significant difference existed between Groups II and III. Thirty-four days after inoculation, there were significantly more granulomas in Group III than in the other groups (Figure 6).

3.3. The Granulomatous Area in the Liver (Figures 7 to 9). The granulomatous area was assessed in the livers of mice that were previously infected with the *S. mansoni* BH strain (Groups III and IV). No significant difference was found between the two groups in terms of the area covered by hepatic granulomas ($P = 0.7412$) (Figure 7), even when the time after infection was considered (Figure 8). In both

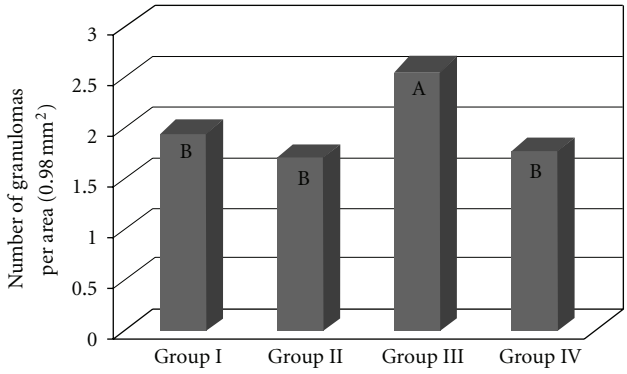


Figure 5: The number of lung granulomas of the mice that were inoculated with eggs of the *S. mansoni* BH (Group I) or SJ (Group II) strains and of those that were previously infected with the *S. mansoni* BH strain and inoculated 8 weeks later with eggs from the *S. mansoni* BH (Group III) or SJ (Group IV) strains. The means with different letters exhibited a significant difference ($\alpha = 0.05$).

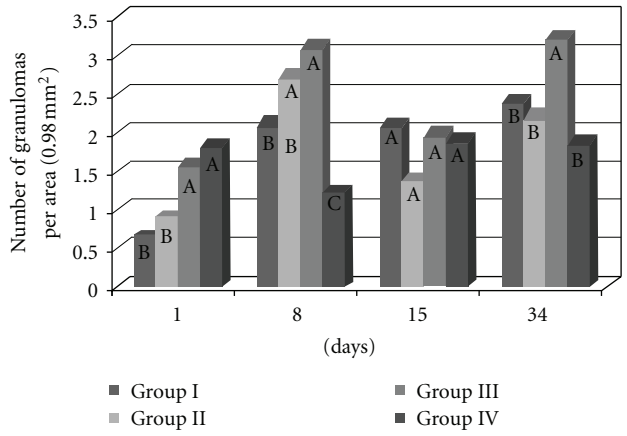


Figure 6: The number of lung granulomas of the mice that were inoculated with eggs of the *S. mansoni* BH (Group I) or SJ (Group II) strains and of those that were previously infected with the *S. mansoni* BH strain and then inoculated 8 weeks later with eggs from the *S. mansoni* BH (Group III) or SJ (Group IV) strains. The mice were euthanized 1, 8, 15 or 34 days after inoculation with the eggs. At each time point, the means depicted with the same letter do not differ significantly ($\alpha = 0.05$).

groups, the hepatic granulomatous area increased 8 and 15 days after infection ($P = 0.0010$) and then decreased 34 days after inoculation (Figure 9).

3.4. The Number of Granulomas in the Liver (Figures 10 and 11). Significantly more granulomas were observed in the livers of mice from Group III than in the livers of mice from Group IV ($P < 0.0001$) (Figure 10). In addition, more granulomas were found 1 and 8 days after inoculation ($P < 0.0001$). At these time points, there were significantly more granulomas in mice from Group III than in mice from Group IV (Figure 11).

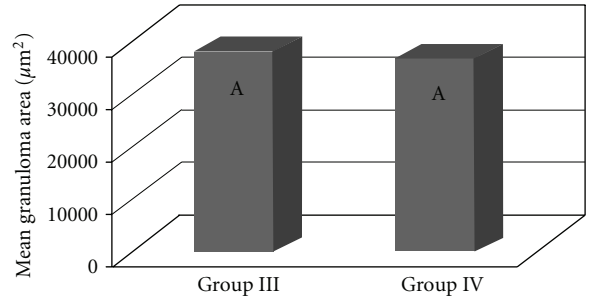


Figure 7: The total granulomatous area in the liver of the mice that were previously infected with the *S. mansoni* BH strain and then inoculated 8 weeks later with eggs from the *S. mansoni* BH (Group III) or SJ (Group IV) strains. The means with same letter do not differ significantly ($\alpha = 0.05$).

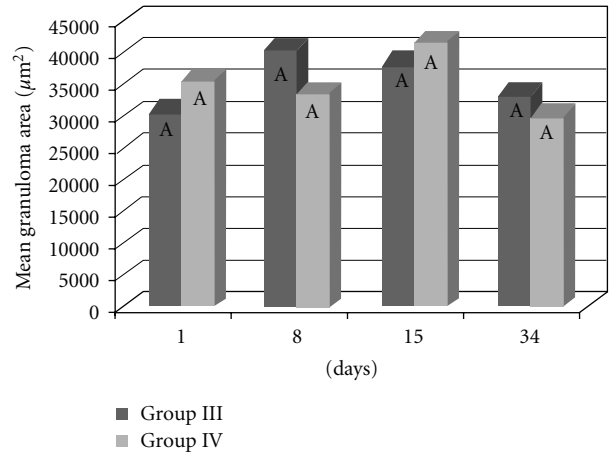


Figure 8: The granulomatous area in the liver of the mice that were previously infected with the *S. mansoni* BH strain and then inoculated 8 weeks later with eggs from the *S. mansoni* BH (Group III) or SJ (Group IV) strains. The mice were euthanized 1, 8, 15, or 34 days after inoculation with the eggs. At each time point, the means depicted with the same letter do not differ significantly ($\alpha = 0.05$).

4. Discussion

The granulomatous response induced by the eggs of *S. mansoni* is a protective mechanism initiated by the host organism, although its appearance is also responsible for the disease pathology. The degree of the response by the host organism depends on the stimulating capacity of the parasite and the integrity of the host immune system. According to Lichtenberg [38, 39], the length and size of the granuloma are proportional to the persistence of the egg in the lesion and the ability of the host cells to destroy antigens. The granulomatous reaction plays an important role in protecting host tissues to sequester antigens released by the eggs, while at the same time causing the pathogenesis [39]. The granulomatous response that was induced in the lungs by the BH eggs (Group I and III) was greater than that induced by the SJ eggs (Groups II and IV) (Figure 2). The increased granulomatous response in the lungs was

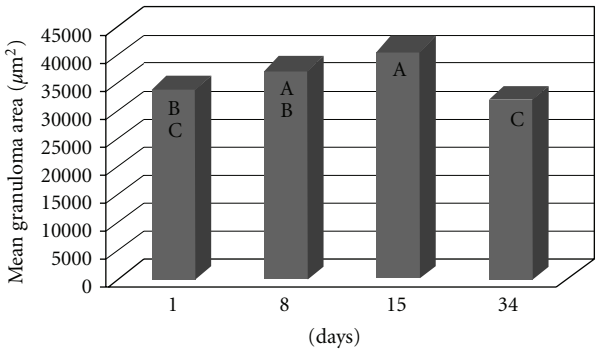


Figure 9: The granulomatous area in the liver of the mice that were previously infected with the *S. mansoni* BH strain and then inoculated 8 weeks later with eggs from the *S. mansoni* BH (Group III) or SJ (Group IV) strains. The mice were euthanized 1, 8, 15, or 34 days following inoculation with the eggs. The means depicted with the same letter do not differ significantly ($\alpha = 0.05$).

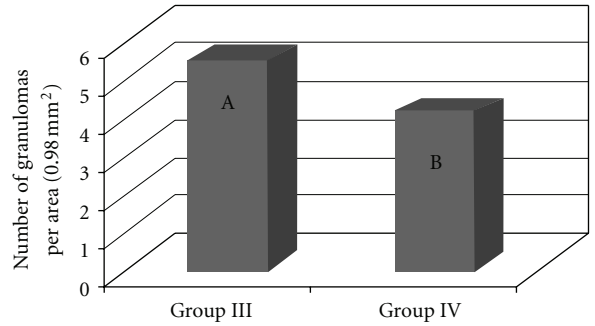


Figure 10: The number of granulomas in the liver of the mice that were previously infected with the *S. mansoni* BH strain and then inoculated 8 weeks later with eggs from the *S. mansoni* BH (Group III) or SJ (Group IV) strains. The means depicted with different letters differ significantly ($\alpha = 0.05$).

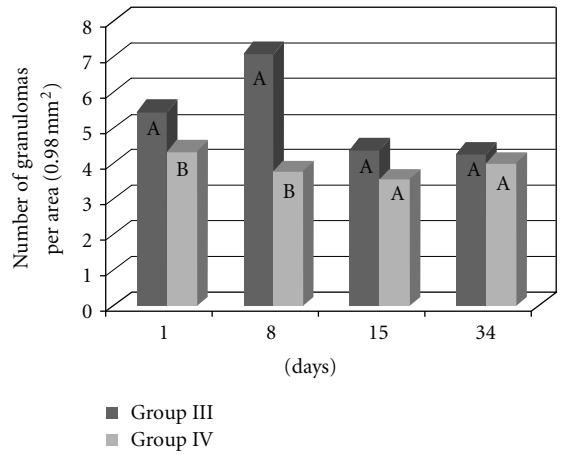


Figure 11: The number of granulomas in the liver of the mice that were previously infected with the *S. mansoni* BH strain and then inoculated 8 weeks later with eggs from the *S. mansoni* BH (Group III) or SJ (Group IV) strains. The mice were euthanized 1, 8, 15, or 34 days after the inoculation with the eggs. For each time point, the means depicted with the same letter do not differ significantly ($\alpha = 0.05$).

observed 15 days after inoculation with the eggs (Figure 3). Among all of the groups of the present study, the largest granulomas were observed around the BH eggs in mice that were previously infected with the *S. mansoni* BH strain 8 and 15 days after inoculation in the caudal vein (Figure 4). The response around the SJ eggs, after 15 days in mice without prior infection of the *S. mansoni* BH (Group II) and after 8 and 15 days in mice that were previously infected with the *S. mansoni* BH strain (Group IV), was lower than the responses around eggs of the BH strain. As the number of eggs inoculated in the caudal vein was similar for all groups and there was a similar number of granulomas around both BH and SJ eggs (Groups I and II, Figure 5), the larger size of the BH eggs, which has been reported by several authors [26, 27], appears to influence the extent of the granulomatous response in the lung. Infecting mice with cercariae from the BH strain before they were inoculated with eggs (BH or SJ strain, Groups III and IV) induced a more robust granulomatous response around the eggs in the lungs of infected animals. However, the reaction was significantly lower around SJ eggs, which indicated that the antigenic identity of the eggs from each strain had unique aspects (Figure 2). The granulomatous response in the lungs around the BH eggs (Group III) was significantly greater than the reaction around the SJ eggs (Group IV) 8 and 15 days after inoculation (Figure 4), suggesting a specificity of the host response against the two strains. Although demonstrating host immunological integrity, the large granulomatous responses around the *S. mansoni* eggs compromized the host tissues and blood circulation. The granuloma-inducing antigens, which were secreted and/or excreted by the eggs, were located below the mature eggshell that surrounded the miracidium [40, 41]. Therefore, larger eggs, such as those of the BH strain, could produce an increased amount of antigen. Schramm et al. [41] reported that the stimulation of the immune response by egg antigens was dose dependent. The greater size of the BH eggs (in relation to the SJ eggs) would constitute a greater antigenic volume to be processed by the defense mechanisms of the host and could result in a greater retention of antigenic stimulus, as well as the actual mechanical effect represented by its greater volume. Morphometric and biological differences were also found in the Japanese and Formosan strains of *S. japonicum*. The resistance to infection was greater when the animals were initially exposed to the Formosan strain as opposed to the Japanese strain [42]. A study of the protein composition of mature and non-mature *S. mansoni* eggs of the Porto Rico strain identified a different composition depending on the stage of development, the miracidium, and the hatching fluid [43]. Differences in antigenicity were found between eggs that were inoculated and eggs that were produced by worms in active infections [44]. Even though mature eggs were used in the present study, it is possible that antigens could have been lost during attainment and handling of the eggs and tissues and during the inoculation of the caudal vein. The vast behavioral diversity of *S. mansoni*, with its various developmental stages in both vertebrate and intermediate hosts, is a reflection of the large number of genes and their different expression patterns during each stage of the

life cycle [45]. One could hypothesize that the *S. mansoni* SJ strain is different because *B. tenagophila* is the natural intermediate host. Geographical strains of *S. mansoni* have significant pathogenic differences stemming from the degree of organ impairment, which is dictated by the distribution of *S. mansoni* eggs [9], the number of eggs produced by the parasite [6, 46], and the degree of susceptibility of the snail vector [9, 10, 18].

The increased number of granulomas in the lungs and the liver observed in the animals from Group III (infection with *S. mansoni* and inoculation with BH eggs) can be attributed to the greater number of eggs resulting from the greater number of worms recovered (Table 1). Granulomatous pulmonary reactions around the eggs were found in mice infected with the BH strain [47]. The granulomas observed in the livers of the mice in Groups III and IV were probably caused by eggs laid by the BH trematodes, since an examination of the livers of the mice in Groups I and II (inoculated with the BH and SJ eggs only) did not reveal the presence of granulomas. The smaller hepatic granulomas found 90 days after infection (34 days after inoculation, Figure 9) resulted from the immunomodulation of the inflammatory response of the eggs during chronic schistosomiasis [48]. The results of the present study showed that granulomas covered a larger area in the lungs of mice that were previously infected with cercariae and subsequently inoculated with eggs from the BH strain. These results indicated that specific granulomatous responses occurred following an infection with the BH and SJ strains of *S. mansoni*.

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Review Article

Developments in Diagnosis and Antileishmanial Drugs

Prachi Bhargava[1] and Rajni Singh[2]

[1] *Amity Institute of Biotechnology, Amity University Uttar Pradesh, Sector 125, Noida 201303, India*
[2] *Amity Institute of Microbial Biotechnology, Amity University Uttar Pradesh, Sector 125, Noida 201303, India*

Correspondence should be addressed to Rajni Singh, rsingh3@amity.edu

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Leishmaniasis ranks the third in disease burden in disability-adjusted life years caused by neglected tropical diseases and is the second cause of parasite-related deaths after malaria; but for a variety of reasons, it is not receiving the attention that would be justified seeing its importance. Leishmaniasis is a diverse group of clinical syndromes caused by protozoan parasites of the genus *Leishmania*. It is estimated that 350 million people are at risk in 88 countries, with a global incidence of 1–1.5 million cases of cutaneous and 500,000 cases of visceral leishmaniasis. Improvements in diagnostic methods for early case detection and latest combitorial chemotherapeutic methods have given a new hope for combating this deadly disease. The cell biology of *Leishmania* and mammalian cells differs considerably and this distinctness extends to the biochemical level. This provides the promise that many of the parasite's proteins should be sufficiently different from hosts and can be successfully exploited as drug targets. This paper gives a brief overview of recent developments in the diagnosis and approaches in antileishmanial drug discovery and development.

1. Introduction

Protozoan parasitic diseases remain a major concerned public health problem, especially in tropical regions. The major death toll is due to malaria, African and American trypanosomiasis, and leishmaniasis, whose high mortality rates in underdeveloped developing countries are associated to poor hygienic conditions and lack of efficient prophylactic measures [1].

For many years, the public health impacts of the parasitic diseases have been grossly underestimated, mainly due to lack of awareness of its serious impact on health. Protozoan parasites of the genus *Leishmania* cause severe diseases that threaten human beings, both for the high death rates involved and the economic loss resulting from morbidity, primarily in the tropical and subtropical areas [2]. It ranks the second only to malaria, and the control of leishmaniasis remains a serious problem with ever increasing cases worldwide. It has become a major focus of concern and a serious third world problem affecting the poorer sections of the society [3].

Leishmaniasis is included in the list of neglected tropical diseases (NTDs) [4] and has a strong link to poverty [5]. The disease has been reported in 88 countries in five continents—Africa, Asia, Europe, North America, and South America out of the seven continents (22 in the new world and 66 in the old world) [6], out of which 16 are developed countries, 72 are developing, and 13 of them are among the least developed [7]. Approximately, 350 million individuals are at risk of leishmaniasis and 20 million people are infected worldwide, and an estimated 2.0 million new cases occur each year [8] with an incidence of 1.5 million cases per annum of the disfiguring cutaneous leishmaniasis (CL) and 0.5 million cases per annum of the potentially fatal visceral leishmaniasis (VL) [9, 10]. However, with increasing travel to and from endemic regions, there is an increase in the number of patients suffering from leishmaniasis [10–12]. The importance of this parasitic disease is further stressed out by the rise of *Leishmania*/HIV coinfection in many parts of the world including European countries such as Spain, Italy, France, and Portugal where up to 9% of the AIDS patients suffer from fatal visceral leishmaniasis

[13]. However, due to underreporting and misdiagnosis, the number of actual cases is expected to be higher.

No effective vaccines are available against *Leishmania* infections as yet and the treatment relies solely on chemotherapy, with pentavalent antimonials as first-line drugs and amphotericin B and pentamidine as second-line agents [10, 11]. Miltefosine is the first recognized oral treatment for leishmaniasis, but resistance to miltefosine may emerge easily during treatment due to single point mutation [14]. There is a pressing need for the identification of novel drug targets, virulence factors, and development of vaccines to expand our understanding of the prevention and treatment of leishmaniasis.

1.1. Epidemiology: Impact and Geographical Distribution. Leishmaniases cause considerable morbidity and mortality and are a typical example of an anthropozoonosis. The majority of infections are originally zoonotic, although some cases of transmission of *L. donovani* from human to human are also known. The different epidemiological cycles are (i) a primitive or sylvatic cycle (human infection is accidental, transmission occurring in wild foci), for example, *L. braziliensis*; (ii) a secondary or peridomestic cycle (the reservoir is a peridomestic or domestic animal, the parasite being transmitted to humans by anthropophilic sand flies), for example, *L. infantum*; (iii) a tertiary, strictly anthroponotic cycle (in which the animal reservoir has disappeared or has not yet been identified, and the sand fly vectors are totally anthroponotic), for example, *L. donovani*. Nevertheless, many unknown factors remain. For example, the main animal reservoir of *L. braziliensis* is still unknown [15]. *L. tropica* was considered to be a strict anthroponosis, but several cases of canine infection have been described [16–18].

Leishmaniasis is a complex disease caused by haemoflagellate obligate intracellular protozoa belonging to the genus *Leishmania*, family Trypanosomatidae of the order Kinetoplastida. Visceral leishmaniasis (VL), cutaneous leishmaniasis (CL), and rarer manifestations such as mucosal leishmaniasis and post-kala-azar dermal leishmaniasis (PKDL) are the major forms of this disease. Parasites are transmitted by female sandflies via anthroponotic or zoonotic cycles [19]. Theparasites have a dimorphic lifecycle; flagellated promastigotes develop in the gut of female Phlebotomine sandflies to infectious forms that are transmitted to mammalian hosts [20, 21]. Inside the host, the parasites survive and multiply as amastigotes within parasitophorous vacuoles (PVs) of macrophages [22, 23].

Depending on the transmission cycle, VL and CL are considered to be either anthroponotic (AVL/ACL) or zoonotic (ZVL/ZCL). Anthroponotic VL is caused by *Leishmania donovani* and is mainly distributed in the Indian subcontinent where it accounts for 70% of the burden of VL with estimated annual incidence of 500,000 and 50,000 deaths each year [24], a death toll that is surpassed among the parasitic diseases only by malaria.

Both figures are approximations as VL is frequently not recognized or not reported [25, 26]. The majority (>90%) of cases occur in just six countries—Bangladesh, India, Nepal, Sudan, Ethiopia, and Brazil (Figure 1). Severe VL epidemics have been reported in the past in southern Sudan; in context of civil war and famine, VL killed an estimated 100,000 people out of a population of 280,000 between 1984 and 1998 [27]. As India, Nepal, and Bangladesh harbor an estimated 67% of the global VL disease burden [28], the commitment of the government of these countries to launch regional VL elimination programme is welcome. The target of this programme is to eliminate VL as a public health problem by 2015, by using a local approach to reduce the annual incidence of VL to less than one case per ten thousand individuals. Visceral leishmaniasis (VL) results in death if not treated, the majority of leishmaniasis deaths go unrecognized, and even with treatment access, VL may result in case-fatality rates of 10–20% [29–31]. In East Africa, it causes around 50,000 annual cases, in the form of epidemic outbreaks distributed in scattered displaced populations with a high death rate. Post-kala-azar dermal leishmaniasis (PKDL), which is developed in 5–50% of AVL patients depending on geographical areas, requires lengthy and costly treatment with a low efficacy [32, 33]. The most relevant factors behind the spread of AVL are the increasing transmission in urban areas with large numbers of immigrants living in poor conditions, the breakdown of social and health structures, malnutrition inducing weakening of the immune system, and finally HIV-*Leishmania* coinfection. The HIV-VL coinfection is characterized by frequent relapses and a high fatality rate, and cases are considered to constitute an important infectious reservoir [34]. Zoonotic VL is caused by *L. infantum* and is widely distributed in Central Asia, Middle East, the Mediterranean, and Brazil. Up to 50,000 annual cases may be caused by this form worldwide, with a scattered distribution.

CL is commonly known as oriental sore. Its causative agents are *Leishmania major*, *L. tropica*, *L. aethiopica*, and *L infantum* in old world and *L. mexicana*, *L. venezuelensis*, *L. amazonensis*, *L. braziliensis*, *L. panamensis*, *L. guyanensis*, *L. peruviana,* and *L. chagasi* in new world. It produces skin lesions mainly on the face, arms, and legs. It is frequently self-healing in the old world but when the lesions are multiple and disabling with disfiguring scars, it creates a lifelong aesthetic stigma. In Central Asia, the Middle East, North Africa, and some sub-Saharan countries, ZCL caused by *L. major* accounts for 500,000 cases every year. Outbreaks are typical in rural areas and depend on fluctuations in the rodent population. ACL caused by *L. tropica* is transmitted in urban zones and affects around 400,000 patients annually. Massive outbreaks have occurred in overcrowded suburbs with poor housing and deteriorated environmental conditions [35]. ZCL caused by *L. aethiopica* is present in Ethiopia and is the most neglected form of CL despite 50,000 annual cases and a potential serious clinical progression, including diffuse CL and to a lesser degree mucocutaneous leishmaniasis. In South America, around 300,000 new cases of ZCL occur annually. *Leishmania braziliensis* is responsible for nearly 90% of all CL cases. Species belonging to the subgenus *Viannia* (*L. braziliensis*, *L. panamensis, L. peruviana,* and *L. guyanensis*) are capable of causing mucocutaneous leishmaniasis. No or

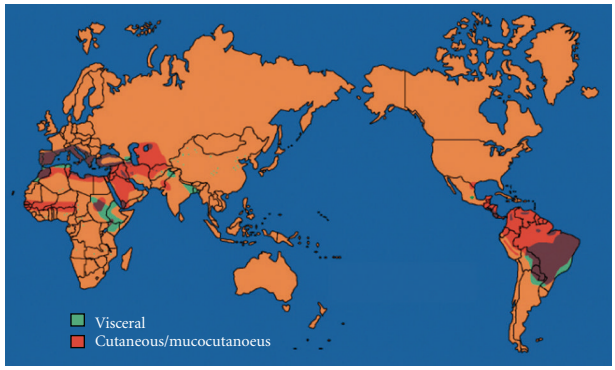

Figure 1: Geographical distribution of leishmaniasis worldwide [37].

incomplete treatment of CL is associated with the subsequent development of mucocutaneous leishmaniasis. It is estimated that there are 4000 new mucocutaneous leishmaniasis cases every year [36]. The subgenus *Leishmania* groups comprise two main species, *L. mexicana*, causing a form of CL that heals spontaneously but can sometimes cause necrosis of the external ear (the "chiclero" ulcer), and *L. amazonensis*, which can in some cases manifest as diffuse CL in patients with weak immune systems.

Although leishmaniasis affect 98 countries in the world, it should be stressed that 90% of VL cases occur in India, Bangladesh, Sudan, Brazil, Nepal, and Ethiopia, and 90% of CL cases occur in Afghanistan, Algeria, Ethiopia, Sudan, Iran, Iraq, Saudi Arabia, Syria, Brazil, and Peru [6]. Apart from VL and CL, diffused cutaneous leishmaniasis is also a type which is difficult to treat due to disseminated lesions that resemble leprosy and do not heal spontaneously. This form is especially related to a defective immune system and it is often characterized by relapses after treatment.

Mucocutaneous leishmaniasis is also called "espundia" in South America. Causative agents of MCL in old world are *Leishmania aethiopica* (rare) *L. major* and in new world are *L. mexicana*, *L. amazonensis*, *L. braziliensis*, *L. guyanensis,* and *L. panamensis*. The parasite invades the mucocutaneous region of the body and spreads to the oronasal/pharyngeal mucosa. The soft tissues and cartilage of the oronasal/pharyngeal cavity undergo progressive erosion. In contrast to cutaneous leishmaniasis, these lesions do not heal spontaneously. Suffering and mutilation are severe, and death occurs as a result of bronchopneumonia or malnutrition. There is always a large danger of bacteria infecting the already open sores. Reconstructive surgery of deformities is an important part of therapy [36].

The classification of *Leishmania* was initially based on ecobiological criteria such as vectors, geographical distribution, tropism, antigenic properties, and clinical manifestations [38–41]. However, biochemical and molecular analysis showed that pathological and geographical criteria were often inadequate and thus, other criteria such as the patterns of polymorphism exhibited by kinetoplastid DNA (k-DNA) markers, proteins, or antigens came to be used to classify *Leishmania* [42–49].

The genus *Leishmania* comes under subkingdom Protozoa, order Kinetoplastida, and family Trypanosomatidae. Initially, species classification was based on various extrinsic criteria such as clinical, geographical, and biological characteristics, for example, *L. guyanensis* (isolated in Guyana), *L. peruviana* (isolated in Peru), *L. infantum* (isolated from a child in Tunisia), and *L. gerbilli* (isolated from gerbils). Since the 1970s, intrinsic criteria such as immunological, biochemical, and genetic data have been used to define species of *Leishmania.* Use of these molecular techniques led to the publication of a taxonomic scheme by the World Health Organization [50]. New methods of detection, isolation, and genetic identification resulted in a massive increase in the number of species are described. Today, 30 species are known and approximately 20 are pathogenic for humans. These species generally present different epidemiological and clinical characteristics related to different genetic and phenotypic profiles. The validity of the classification scheme, considered by some workers as too arbitrary, has been questioned several times. Debate has centered on *L. panamensis*, *L. peruviana*, *L. chagasi*, *L. infantum*, *L. archibaldi*, *L. garnhami*, *L. pifanoi*, *L. venezuelensis,* and *L. forattinii* [15, 51, 52]. Different studies have already clarified the status of some of these species; for example, *L. chagasi* is accepted as a synonym of *L. infantum* [51] and *L. peruviana* has been validated as an independent species [53].

Very recently, Fraga and his team [54] used sequences of the highly conserved 70-kDa heat shock protein (hsp70 gene) to analyze isolates and strains of different geographic origin, showing that only eight monophyletic groups were detectable against the 17 examined (Table 1).

2. Diagnosis

The reason behind the diagnostic challenges for leishmaniasis is the wide spectrum of clinical manifestations that they present: ulcerative skin lesions developing at the site of the sand fly bite (localized cutaneous leishmaniasis); multiple nonulcerative nodules (diffuse cutaneous leishmaniasis); destructive mucosal inflammation (mucosal leishmaniasis (ML)); disseminated, potentially fatal, and visceral infection (visceral leishmaniasis (VL)) [55]. However, differential diagnosis is important because diseases of other etiologies with a clinical spectrum similar to that of the leishmaniases (e.g., leprosy, skin cancers, and tuberculosis for CL and malaria and schistosomiasis for VL) are often present in areas of endemicity. These main manifestations may themselves deviate, complicating definitive clinical diagnosis even further. Cutaneous leishmaniasis (CL) lesions, for example, may vary in severity (e.g., in lesion size), clinical appearance (e.g., open ulcer versus flat plaques versus wart-like lesions), and duration (e.g., in time of evolution or in time to spontaneous cure). Patient management, screening of asymptomatic infections, surveillance including verification of elimination, and epidemiological studies are some of the areas where diagnostic tests play a major role.

In all cases, it is desirable to have the diagnosis of leishmaniasis confirmed by the finding of the etiological

Table 1: Nomenclature of *Leishmania* species based on heat shock protein 70 (hsp 70) gene sequences [54].

Name of the organism	Geographical distribution
L. donovani	China, Indian subcontinent, Ethiopia, Sudan, Kenya, Iran, Saudi Arabia, and Yemen
L. infantum/L. Chagasi As given [51]	Albania, Algeria, France, Greece, Italy, Morocco, Portugal, Spain, Syria, Tunisia, Turkey, Yemen, Argentina, Bolivia, Brazil, Colombia, Ecuador, El Salvador, Guadalupe, Guatemala, Honduras, Martinique, Mexico, Nicaragua, Paraguay, Suriname, and Venezuela
L. archibaldi	India, Sudan, Ethiopia, Lebanon, and Israel
L. tropica	Afghanistan, Algeria, Azerbaijan, Greece, Iran, Iraq, Israel, Morocco, Tunisia, Turkey, and Yemen
L. aethiopica	Ethiopia, Kenya
L. major	Afghanistan, Algeria, Chad, Iran, Iraq, Israel, Libya, Mauritania, Morocco, Syria, and Sudan
L. mexicana	Belize, Colombia, Costa Rica, Dominican Republic, Ecuador, Guatemala, Honduras, Mexico, Panama, and Venezuela
L. amazonensis	Bolivia, Brazil, Colombia, Costa Rica, Ecuador, French Guyana, Panama, Peru, and Venezuela
L. garnhami	Venezuela
L. guyanensis	Brazil, Colombia, Ecuador, French Guyana, Peru, Suriname, and Venezuela
L. panamensis	Belize, Colombia, Costa Rica, Ecuador, Honduras, Nicaragua, Panama, and Venezuela
L. naiffi	Brazil, French Guyana, Ecuador, and Peru
L. braziliansis	Argentina, Belize, Bolivia, Brazil, Colombia, Costa Rica, Ecuador, Guatemala, Honduras, and Nicaragua
L. peruviana	Peru

agent or its antigen or molecule in the sample obtained from the lesion. When these approaches fail, immunological tests are used to provide indirect parameters for the diagnosis.

Ideally, a test should make the distinction between acute disease and asymptomatic infection, as most of the antileishmanial drugs are toxic. Moreover, such tests should be highly sensitive and specific, simple and affordable, but unfortunately some commonly used serological tests like DAT carry some significant drawbacks: the inability to differentiate between clinically active and asymptomatic infections and showing positive long after cure. Molecular diagnostic tools like PCR and real-time PCR are quite sensitive and specific but are cumbersome to perform and have a high cost. DNA-based tests are available in strip formats but these cannot be used in the field [56]. Compared to other diagnostic techniques available, the molecular approaches remain expensive and require technological expertise, and efforts should be made to make PCR platforms more user-friendly and cost-effective, especially in remote areas where leishmaniasis is endemic.

2.1. Parasitological Methods. Demonstration of the amastigote form of the parasite by light microscopic examination of tissue aspirates from spleen, bone marrow, or lymph nodes is regarded as the most suitable diagnostic instrument. In preparations stained with Giemsa or Leishman stain, the cytoplasm appears to be pale blue, with a relatively large nucleus that stains with red. In the same plane as the nucleus, but at a right angle to it, is a deep red or violet rod-like body called a kinetoplast. The sensitivity of the direct examination is low, in case of cutaneous and mucocutaneous leishmaniasis, with a range of approximately 15–70% in the old and the new world [57, 58]. In case of visceral leishmaniasis, the specificity of this technique is high, although the sensitivity varies depending on the tissue used, being higher for spleen (93–99%) than for bone marrow (53–86%) or lymph node (53–65%) aspirates [59]. However, the procedure for splenic aspiration is risky for fatal internal bleeding, so the results are totally dependent on technical expertise and quality of prepared slides/reagents, both of which are often not available in field settings [60].

The microculture method (MCM) is a good method for the diagnosis of visceral leishmaniasis (VL) with samples from both the bone marrow (BM) and peripheral blood (PB). The MCM is superior to the traditional culture method (TCM) as determined by its higher sensitivity in the detection of promastigotes and the more rapid time for emergence of promastigotes. The sensitivity of MCM (100% in BMs and 77.8–100% in PB) is considerably higher than that of the TCM (37.5–100% in BMs and 0–100% in PB) according to decreasing parasite density ($P < 0.05$) [61].

2.2. Serological Methods. The serological diagnosis is based on the presence of specific humoral response for which identification of antibodies in the sera of patients is done. Serum-based direct agglutination tests (DATs) using lyophilized promastigotes or urine-based latex agglutination tests (LATs) are used for determining antileishmanial antibodies or antigens in leishmaniasis patients. DAT has a high sensitivity (90–100%) and specificity (95–100%) [62, 63]. However, the major disadvantage of DAT is the need of multiple pipetting, relatively long incubation time, high cost of antigen, and limited production facility of quality controlled antigen. As with any antibody-based test, DAT remains positive for a long time after the disease is cured, thus cannot be used as a test of cure or for diagnosis of relapses [64].

Detecting antigen directly is an excellent method of diagnosing an infection and is more specific than antibody-based immunodiagnostic tests. Agglutination test to detect the antigen has been evaluated extensively in clinical trials,

using urine collected from well-defined cases and controls from endemic and nonendemic regions. This test showed 79.1–94.1% specificity and sensitivity of 60.4–71.6% in India [65].

Several surface antigens, ribosomal proteins, nuclear proteins, histones, and kinesin-related proteins elicit specific humoral immune responses in VL patients. Recombinant antigens have considerably improved the sensitivity and specificity of immunological diagnosis over crude/total antigens. They are more preferred than other antigens both in immunoblotting as well as ELISA.

Immunochromatographic strips using K39 antigen have been a quite promising method that has been tested widely. K39 (recombinant) antigen contains 39 amino acids encoded in the highly conserved kinesin region of *L. chagasi*. Using its recombinant product, an immunochromatographic-based strip test is used in which rK39 is fixed on a nitrocellulose paper, and colloidal gold-protein A is used for detection. In the initial clinical evaluation, 100% sensitivity and 98% specificity was observed. ICT suffers from the same disadvantage as DAT, being positive in a significant proportion of healthy individuals in endemic regions and for long periods after cure of VL.

ELISA has also been used in the serodiagnosis of all types of leishmaniasis but the sensitivity and specificity of ELISA depends upon the antigen used. Most promising results are shown by antigen RK39 with sensitivity and specificity of 100% and 96%, respectively. The antibody titres to this antigen directly correlate with active disease and have potential in monitoring the chemotherapy and in predicting the clinical relapse [66]. Due to the requirement of skilled personnel, sophisticated equipment, and electricity, ELISA is not used in the endemic regions for the diagnosis of VL [67].

2.3. Molecular Methods. PCR-based assays form the mainstay of molecular diagnosis of leishmanial infection as they enhance sensitivity, reliability, and rapidity for the benefit of researchers and health professionals. The primers target several multicopy sequences for the diagnosis of human and canine VL which include ribosomal RNA genes [68]; kinetoplast DNA (kDNA) [69]; miniexon-derived RNA (med RNA) genes and genomic repeats [70], the β-tubulin gene region [71], glycoprotein 63 (gp63) gene locus [72], and internal transcribed spacer (ITS) regions [73].

The PCR-ELISA has shown promising results for diagnosing visceral leishmaniasis (VL) in blood samples. However, the method has been validated mostly with HIV-positive patients who are known to have high levels of parasitaemia. As far as blood samples are concerned, PCR-ELISA is more sensitive (83.9%) than conventional PCR (73.2%) and demonstrated 100% and 87.2% specificity when healthy controls who had never travelled to a VL-endemic area and controls from a VL-endemic area as references, respectively, were used [74].

RT-PCR simply refers to amplification of DNA (by PCR) that is monitored while the amplification is occurring. The benefit of this RT-PCR is that it allows the researcher to better determine the amount of starting DNA in the sample before the amplification by PCR. Also real-time PCR can distinguish specific sequences from a complex mixture of DNA. However, its application requires the availability of primers and probes that must be selected according to very rigid conditions, which cannot always be easily applied [75]. Table 2 depicts the properties of few generally used diagnostic tools for leishmaniasis and PKDL.

2.4. Developments in Antileishmanial Drugs. Pentavalent antimonials were developed in 1945; the generic sodium stibogluconate (pentosan) and branded meglumine antimoniate, and so forth, are the first choice in the treatment of both visceral and cutaneous leishmaniases over more than five decades where resistance is not reported [91]. To overcome the challenge of clinical resistance to antimony, pentamidine has been tried for the treatment of visceral leishmaniasis and was the first drug to be used for patients refractory to pentavalent antimony (SbV) [92]. This drug is associated with serious adverse events like insulin-dependent diabetes mellitus, shock, and hypoglycaemia and death in significant proportion of patients. The declining efficacy, resistance, and serious toxicity associated with the drugs have made it unsuitable as a viable alternative to SbV for kala-azar patients [93, 94]. Amphotericin B is one of the most effective antileishmanial drug, which induces high cure rates. Use of formulation of amphotericin B, a polyene antifungal drug for treatment of leishmaniasis is biochemically rational because the target of the drug is ergosterol, which is the major membrane sterol of *Leishmania* species. Due to high affinity of amphotericin B for sterols, aqueous pores are formed in the membrane leading to increased membrane permeability and killing of *Leishmania* [95]. The need to develop less toxic, more effective formulation of amphotericin B has led to new clinical formulation of amphotericin B in which deoxycholate has been replaced by other lipids. Miltefosine, an alkyl phospholipids developed as an antitumor agent, has an excellent antileishmanial activity.Paromomycin is an aminoglycoside antibiotic with unique antileishmanials activity. Paromomycin is currently in phase IV clinical trials against leishmaniasis [96]. Sitamaquine, a primaquine analogue (8-aminoquinolene), is another orally administrable compound (Table 3). To date, little is known about its efficacy and toxicity. However, there is a need to go for combitorial chemotherapies which are under process and provide better outputs.

3. New Potential Drug Targets

The irony of the disease leishmaniasis is that it is the only tropical disease, which is being treated by nonleishmanial drugs [97]. Much of the focus till recent times was being made only on drug trials/combination therapy of available nonleishmanial drugs, evaluation of diagnostic and prognostic capability of available tools, and very little emphasis was being paid on other aspects by leishmanial biologists and researchers. Recently much emphasis has been given on novel control strategies in terms of new drug targets and vaccine candidates.

Table 2: Diagnostic methods for leishmaniasis and PKDL.

Diagnostic methods	Test name	Clinical specimen	Sensitivity	References
Parasitic detection methods	LD bodies	Lymph node, bone marrow, splenic, and slit aspirates	spleen (93–99%) than for bone marrow (53–86%) or lymph node (53–65%) aspirates	[59, 76]
	Culture (MCM)	Blood, lymph node, bone marrow, splenic, and slit aspirates	100% in bone marrow and 77.8–100% in peripheral blood	[77]
	Culture (TCM)	Blood, lymph node, bone marrow, splenic, and slit aspirates	37.5–100% in bone marrow and 0–100% in peripheral blood	[77]
Serological methods	DAT	Serum, urine	94.8%	[78–81]
	ELISA/immunoblotting soluble antigens	Serum, urine	100%	[82–84]
	Recombinant antigens (rK39, rK26, rHSP70)	Blood, serum, and antigens	100%	[85–88]
Molecular methods	PCR	Blood, serum, urine, lymph node, bone marrow,splenic, and slit aspirates	73.2%	[89, 90]
	PCR-ELISA	Blood, serum	83.9%	[90]
	Real-time PCR	Lymph node, bone marrow, splenic, and slit aspirates	90–100%	[75]

The route to drug target identification has been through comparative biochemistry of host and parasite enzymes, metabolites or protein identified in parasite. Biochemical analysis, genome sequencing of three *Leishmania* species (*L. major, L. braziliensis,* and *L. infantum*) has identified potentially useful target enzymes, transporters, metabolites, and hypothetical proteins that are distinct to parasite and their mammalian host. The genome mining will also aid in large scale proteomics studies generating expression profiles of *Leishmania* parasites and gene targets for treatment development. Search of new potential drug targets mainly focuses on biochemical and metabolic pathways essential for parasite survival. The target enzymes of these pathways should have significant structural and functional differences from their mammalian counterparts for selective inhibition of target sites. Further, strategies to target more than one enzyme of a metabolic pathway simultaneously may prove more usefulness and effectiveness. Biological studies for the function of 50% of *Leishmania* genes are lacking. The comparative genome study would provide a route to find those that might be essential to each species [98].

4. Thiol Metabolism

The enzymes of thiol metabolism/thiols, of parasitic protozoa, are different from those of mammals in many ways. *Trypanosoma* and *Leishmania* are most remarkable in that they have trypanothione reductase (TR) instead of glutathione reductase (GR). This enzyme is responsible for maintaining the parasites, reducing intracellular milieu by keeping trypanothione [N^1, *N8-bis-*(glutathionyl) spermidine] in the dithiol state. The crucial role of TR for thiol homeostasis and its absence from mammalian cells suggest that it might be well suited as a target molecule for rational drug development. The trypanothione system, which replaces the nearly ubiquitous glutathione/glutathione reductase (GR) system, protects the parasites from oxidative damage and toxic heavy metals and delivers the reducing equivalents for DNA synthesis. The parasite system is far less efficient than mammalian glutathione peroxidases in detoxifying hydroperoxide, but has the advantage of much broader substrate specificity, with lipid hydroperoxides also being reduced. The relatively low activity of the tryparedoxin peroxidase system is in accordance with the high sensitivity of the parasites to oxidative stress. Trypanosomes and *Leishmania* have superoxide dismutase (SOD), but lack catalase and glutathione peroxidase. Thus, the trypanothione system seems to be the only mechanism to detoxify hydrogen peroxides.

Trypanothione is kept reduced by the flavoenzyme TR. Several reverse genetic approaches have undoubtedly shown that TR is essential in different *Leishmania* species as well as in bloodstream of *T. brucei* [99] and is thus an attractive target molecule for structure-based drug design. Castro-Pinto et al. [100] have reported the cloning, sequencing, and expression of the TR encoding gene from *L. (L.) amazonensis*. A 3D homology model for L. amazonensis TR was constructed based on the previously reported *Crithidia fasciculata* structure. Within the past 15 years, numerous compounds have been elucidated that inhibit TR, but not human GR, which is the closest related host enzyme. Despite

Table 3: Current scenario of available chemotherapy drugs.

Drug	Properties and administration	Comments	Reactions in patients with CL/VL
Pentavalent antimonials	Polymeric organometallic complexes, intravenous, or intramuscular	For VL and CL. Drug resistance in Bihar, India. Variable response in different forms of CL. Generic sodium stibogluconate (SSG) has made treatment cheaper	Pain, erythema, oedema, abdominal pain, nausea, and thrombocytopenia
Amphotericin B fungizone	Polyene antibiotic, Fermentation product of Streptomyces nodus, intravenous	For VL, CL, and complex forms of CL, for example, MCL. first-line drug for VL in India where there is antimonial resistance	Infusion related, azotemia, anemia, or hypokalemia
Amphotericin B ambisome	Unilamellar liposome, intravenous	Most effective lipid formulation for VL and available at $18/50 mg ampoule via WHO used for complex forms, such as PKDL and MCL	Hypotension, anorexia, nausea, vomiting, and headache generalized weakness.
Miltefosine	Hexadecylphosphocholine, oral	First oral drug for VL. Effective against some forms of CL contraindicated in pregnancy	Nausea, vomiting and/or diarrhea, raised creatinine, and raised LFT's
Amphotericin B formulations	Lipidic formulations, intravenous	Other lipid formulations, including Abelcet, Amphocil, Amphomul, and multilamellar liposomes have been in clinical studies, mainly for VL	Shaking chills, nausea, hypotension, anorexia, headache, and vomiting,
Paromomycin	Aminoglycoside (also known as aminosidine or monomycin), fermentation product of *Streptomyces rimosus*. Supplied as sulphate. Intramuscular for VL and topical for CL	Registered for VL in India, completed Phase III trials for VL in East Africa where less effective in Sudan. Topical formulation (12%) with methyl benzethonium chloride available for CL. Topical with gentamicin and surfactants in Phase III trial	Pain, erythema, oedema, blisters, and ototoxicity
Pentamidine	Diamidine, as isethionate salt, intramuscular	For specific forms of CL in South America only	Nausea, vomiting, diarrhea, hyperglycemia, and cardiotoxicity
Sitamaquine	8-aminoquinoline analog, orally active	Tested in VL patients in Kenya and Brazil with limited success	Abdominal pain, headache, vomiting, dyspepsia, and cyanosis

knowledge of the three-dimensional structure of the protein and of complexes with its substrates and an inhibitor, as well as several high-throughput and virtual screening approaches, inhibitors of TR that are suitable to enter the clinical phase are still elusive. This lack of success might be attributable to several factors. The extremely wide active site of the parasite enzyme represents an obstacle for a structure-based drug design. In addition, the pharmacokinetic properties of the potential inhibitors are crucial because insufficient uptake, rapid extrusion, or metabolism plays significant roles in determining the *in vivo* efficacy of a drug. Recently, the biological activities of a series of mesoionic 1,3,4-thiadiazolium-2-aminide derivatives were evaluated to determine their effect on trypanothione reductase (TryR) activity in *Leishmania* sp. and *Trypanosoma* cruzi. MI-4-NO(2) showed enzyme inhibition effect on extracts from different cultures of parasites, which was confirmed using the recombinant enzyme from *T. cruzi* (TcTryR) and *Leishmania infantum* (LiTryR) [101]

Thioredoxin reductase (TrxR) is a pyridine nucleotide-disulphide oxidoreductase as GR, TR, and lipoamide dehydrogenase. TrxR maintains the levels of reduced thioredoxin, a protein involved in the activity of ribonucleotide reductase, transcription factors, and cell signaling and the detoxification of reactive oxygen species. Most studies to date on TrxR of parasitic protozoa have concerned the enzyme of *P. falciparum*. Current evidence suggests that it is a promising drug target, although validation is awaited. The absence of this pathway in mammalian host and trypanosomatids sensitivity towards oxidative stress, trypanothione reductase, and enzymes of trypanothione metabolism is an attractive drug target for antileishmanial drug designing [102]. Homology modeling of *Leishmania infantum*, TR, and mammalian glutathione reductase shows a remarkable difference in their three-dimensional and catalytic active sites. Hence, specific inhibitors designed against TR may be an ideal drug that will stop parasite growth without altering host glutathione reductase (GR) activity.

4.1. Sterol Biosynthetic Pathway. The enzymes of this pathway are attractive targets for the specific treatment of leishmaniasis, because the etiological agents for the disease, that is, the leishmanial parasites have a strict requirement for specific endogenous sterols (ergosterol and analogs) for survival and growth and cannot use the abundant supply of cholesterol present in their mammalian host. There are

differences in the enzymes in the biosynthetic pathways of ergosterol and cholesterol. A number of enzymes in the ergosterol biosynthetic pathway have been investigated as potential drug targets for these organisms and have shown great promise. Thus, C14-demethylase, sterol 24-methyltransferase, 3-hydroxy-3-methylglutaryl CoA reductase, squalene epoxide, squalene synthase, and farnesyl pyrophosphate synthase have been studied both individually and in combination, with varying degrees of success [103, 104]. Ergosterol biosynthesis inhibitors with potent *in vitro* activity and special pharmacokinetic properties in mammals can induce radical parasitological cure in animal models of several forms of leishmaniasis [105].

Trypanosomatids contain predominantly ergostane-based sterols, which differ from cholesterol, the main sterol in mammalian cells, in the presence of a methyl group in the 24 position. The methylation is initiated by S-adenosyl-L-methionine: Delta (24 (25))-sterol methenyltransferase, an enzyme present in protozoa, but absent in mammals. The importance of this enzyme is underscored by its potential as a drug target in the treatment of the leishmaniasis [106]. The C-24 transmethylation reactions involving S-adenosyl-L-methionine as the methyl donor and a $\Delta^{24(25)}$-sterol or $\Delta^{24(24')}$-sterol substrate can be inhibited by various azasterols with a nitrogen substitution in the side chain and such compounds have been tested against trypanosomatids [107].

4.2. Polyamine Biosynthetic Pathway. The polyamine pathway of protozoan parasites has been successfully targeted in antiparasitic therapies. Polyamines are ubiquitous organic cations found in all eukaryotic cells and play critical role in key cellular processes such as growth, differentiation, and macromolecular biosynthesis. During the course of inhibition of any of the polyamine, the parasite cannot synthesize trypanothione, a conjugate of spermidine and glutathione that is unique to *Trypanosoma* and *Leishmania*. Trypanothione is a reducing agent with many protective and regulatory functions and consequently its depletion proves detrimental to the parasites. Recent studies on polyamine supplementation show that *L. donovani* lacks an intact back conversion pathway, thus the pathways operating in promastigote stage of parasite differ crucially from that in the host. Inhibitors of polyamine biosynthetic pathway have shown antileishmanial activity. Adomet DC inhibitor cures animal leishmanises but has not been tested on humans and seeks further experimental studies [108]. Arginase provides a building block for production of polyamines so it has been touted as a potential antileishmanial drug target, because N(omega)-hydroxyarginine, an inhibitor of arginase that is produced by the macrophages during the formation of nitric oxide, can reduce polyamine levels in *Leishmania* amastigotes and lowers parasitic loads [109, 110]. Other enzymes of this pathway which are under study as antileishmanial target are ornithine decarboxylases, S-adenosylmethionione, and Spermidine synthase.

4.3. Glycosomal Machinery. In all kinetoplastida studied so far the majority of the glycolytic enzymes are localized in organelles called glycosomes, whereas in other organisms these are cytosolic. As a result of this compartmentation, many regulatory mechanisms operating in other cell types cannot work in trypanosomes as reflected by the insensitivity of the glycosomal hexokinase (HK) and phosphofructokinase (PFK) to compounds that act as activity regulators in other cell types [111, 112]. Blocking of parasite enzyme without producing damage to glycolysis in host remains challenging. Several approaches have been considered—(1) exploitation of metabolic differences; (2) exploitation of differences in 3D structure; (3) exploitation of unique reactive residue in or near the active site of the parasite enzyme

Leishmania like other trypanosomatids depends solely on its host for carbon source to fulfill its energy requirements. The amastigotes uptake blood glucose from mammalian blood stream and receive other essential components like fatty acids, amino acids from phagolysosome of macrophages. Due to the result of the metabolic activities of glycosomes, superoxide radicals are generated as side products in large amount. To protect glycosomal enzymes from superoxide radical toxicity, Fe-superoxide dismutases (FeSods) are evolved in *Leishmania* species. More importantly, FeSod is absent in mammalian counterpart, so it could be used as effective drug target.

4.4. Cyclin-Dependent Kinases. Cyclin-dependent kinases (cdks) play crucial role in cell division cycle, transcription, apoptosis, and differentiation. In *Leishmania mexicana*, disruption of cdk-related kinase 3 (CRK3) leads to change ploidy level of the cell, though it was avoided when extra copy of CRK3 was expressed from episome, ensuring that CRK3 is essential [113]. The chemical inhibitors of class indirubin of CRK3 hampers the parasite viability within macrophage, proving the validity of CRK3 as potential drug target [114]. In *Leishmania donovani*, it was recently shown that glycogen synthase kinase (LdGSK3) is also involved in cell cycle control and apoptosis as validations based on indirubin test [115], exploiting the LdGSK3 as potential drug target in combination with CRK3. Likewise, other cdk may also be explored as possible targets.

4.5. Folate Metabolism. Dihydrofolate reductase (DHFR) is a key enzyme in folate metabolism, linked to the production of thymidine. DHFR reduces dihydrofolate to tetrahydrofolate using NADPH as cofactor. Therefore, inhibition of DHFR prevents biosynthesis of thymidine and as a consequence, DNA biosynthesis. Fortunately, this enzyme from *Leishmania major* and *Trypanosoma cruzi* has been crystallized and the structural data can be used to exploit structural difference between parasite and human enzymes that may help to design selective DHFR inhibitors [116, 117]. An approach to discover novel parasite DHFR inhibitors using database mining has also been made to search the Cambridge structural database but DHFR as drug target requires more attention [118, 119]. In addition, it has been shown that the enzyme dihydrofolate reductase-thymidylate (DHFR-TS) that catalyzes conversion of dihydrofolate from methyhylene

tetrahydrofolate (M-THF) and thymidine, which is related to parasite survival and the parasite lacking this enzyme is not able to survive in animals [120].

5. Mitochondria

The unique mitochondrial features of *Leishmania* make this organelle an ideal drug target while minimizing toxicity. *Leishmania* has a single large mitochondrion which is distributed in branches under the subpelicullar microtubes and a specialized region rich in DNA called the kinetoplast. Fonseca-Silva et al. demonstrated that the effect of the quercetin is associated with ROS production leading to mitochondrial dysfunction, ultimately causing parasite death [121]. The effects of several drugs that interfere directly with mitochondrial physiology in parasites such as *Leishmania* are under study. The unique mitochondrial features of *Leishmania* make this organelle an ideal drug target while minimizing toxicity.

6. Conclusions

All the neglected tropical diseases together take the toll of millions of lives every year. Leishmaniasis is a fatal disease which affects the people living below poverty line. The recent years have witnessed extraordinary progress in diagnosing and treating *Leishmania* infection. Lack of efficiency, high price, and growing resistance of the current antileishmanials imply the importance of search for new targets to be focused for making drugs against *Leishmania*. The challenge is to convert such studies in effective strategic programmes aimed to control and eradicate the disease.

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Research Article

Specific and Rapid Detection of *Mycobacterium tuberculosis* Complex in Clinical Samples by Polymerase Chain Reaction

Anamika Singh[1,2] and Vijendra Kumar Kashyap[1,3]

[1] *National DNA Analysis Center, Central Forensic Science Laboratory, 30 Gorachand Road, Kolkata 700014, India*
[2] *Sol Sherry Thrombosis Research Center, Temple University School of Medicine, 3400 N. Broad Street, Philadelphia, PA 19140, USA*
[3] *Directorate of Forensic Science, MHA, Government of India, CGO Complex, New Delhi 110 003, India*

Correspondence should be addressed to Anamika Singh, anamikas@temple.edu

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Background. Tuberculosis, a global health problem and highly prevalent in India, has always been a serious problem with respect to definitive diagnosis. Polymerase chain reaction (PCR) techniques are now widely used for early detection and species differentiation of mycobacteria, but mostly with their own limitations. We aim to detect and differentiate *Mycobacterium tuberculosis (Mtb)* infections by choosing appropriate target sequences, ideally present in all mycobacterial species (*MTB* complex) and absent in others. *Methods*. Amplification of three target sequences from unrelated genes, namely, *hsp 65* (165 bp), *dnaJ* (365 bp), and insertion element *IS 6110* (541 bp) by PCR was carried out in clinical samples from suspected cases of tuberculosis/mycobacterioses and healthy controls. *Results*. The sensitivity of this method ranged from 73.33% to 84.61%, and the specificity was 80%. The PCR method was significantly better ($P = 0.03$ and $P = 0.009$) than both smear and culture methods. *Conclusion*. Our trimarker-based PCR method could specifically detect *M. tuberculosis* and *MTB* complex infection from that of major pathogenic NTM and nonpathogenic mycobacteria. This method, by well distinguishing between *MTB* complex and NTM, presented a fast and accurate method to detect and diagnose mycobacterial infections more efficiently and could thereby help in better patient management particularly considering the increase in mycobacterial infections due to emergence of NTM over the past decades.

1. Introduction

Tuberculosis is a major public health problem with a total of 9.2 million new cases and 1.7 million deaths from tuberculosis (TB) in 2006. India accounts for one-fifth of the global TB burden (WHO 2008), which has been on the rise due to multidrug-resistant and highly virulent strains of *Mycobacterium tuberculosis* (*Mtb*) [1] and combined effect of HIV. An accurate diagnosis of tuberculosis is desirable before the start of anti-tuberculosis therapy [2]. The laboratory diagnosis of *Mtb* depending on acid-fast bacillus (AFB) smear can yield a result within 24 h. However, smear is not very specific for *Mtb* and also requires 10^3 to 10^4 organisms per mL of sputum. Bacterial culture is superior to AFB smear, both in terms of sensitivity and specificity. But, since mycobacteria have very strict growth requirements, culture-based diagnostic methods are slow. Further, diagnoses involving radiological examinations and Tuberculin test help to detect the disease to some extent, but are not very reliable in case of extrapulmonary tuberculosis. The BACTEC system however, gives a very quick result compared to culture, but it is expensive and involves handling of radioisotopes (BACTEC 460). The fluorometric BACTEC MGIT 960 is fast, efficient, and sensitive but expensive to be used in poor economies. In view of these, accurate and early diagnosis of tuberculosis (TB) is a critical step in the management and control of TB. Polymerase chain reaction has so far been a very useful tool for rapid detection of mycobacterial DNA in clinical specimens such as sputum, bronchial lavage, cerebrospinal fluid (CSF), and ascitic fluid. However, in-house tests vary widely in their accuracy and factors that contribute to heterogeneity in test accuracy,

are not well characterized. Definitive diagnosis of tuberculosis and mycobacterioses (nontuberculous mycobacteria (NTM) infections) has always been a serious problem. These methods also have their own limitations due to region-specific variations in the genome of mycobacteria [3–5]. Our study describes a PCR assay using *MTB*-complex-specific DNA markers encompassing 165, 365, and 541 bp target fragments of three unrelated genes, namely, *hsp 65, dnaJ* and insertion element *IS 6110*, respectively, of *M. tuberculosis* in tuberculosis suspected patients from Kolkata, India. Considering the increase in mycobacterial infections resulting from the emergence of NTM over the past decades, there is also need for a method that can detect virtually any deep-seated mycobacterial organism present in small numbers in suspected clinical samples irrespective of the mycobacterial species affiliation.

2. Materials and Methods

2.1. Subjects. The study samples were suspected cases of pulmonary tuberculosis visiting the Chest Outpatient Department of Calcutta National Medical College and Hospital (CNMCH). This study was approved by the Ethical Committees of the institutions involved, and all the subjects signed informed consent documents before entering into this study. A total of 60 subjects, including suspected cases and healthy controls were studied. The suspected TB cases were further categorized into three groups (Group I–III) based on laboratory diagnostic results (AFB-smear, culture and antero-posterior chest X-ray), clinical symptoms, and past history. The clinical and demographic data on studied subjects is presented in Table 1. Five to ten mL of early morning sputum samples were collected from all the subjects. All the samples were smeared and screened with conventional microbiological test such as Ziehl-Neelsen acid fast staining for recording smear positivity and further cultured on Lowenstein-Jensen slants according to standard method. Group I ($n = 26$) had cases, which were positive by smear Ziehl-Neelsen staining and also culture positive, while Group II ($n = 15$) had smear negative but culture and radiologically positive samples. In some patients ($n = 9$), the laboratory diagnosis for *Mtb* infection was negative but based on their clinical symptoms, they were considered as having other respiratory diseases (than PTB) or mycobacterioses (Group III). However, these patients did not have any past history of tuberculosis and subsequent treatment. The healthy control subjects (designated as Gr IV, $n = 10$) were with no clinical symptoms or history and were diagnosed negative for *Mtb* infection (bacteriologically negative). All the 60 samples were further analyzed by PCR method.

2.2. Isolation of DNA from Sputum Samples. The sputum samples were decontaminated followed by DNA isolation according to the previously published method [6].

2.3. PCR Amplification. The PCR amplification targeted two conserved regions and one insertion element of mycobacterial genome. A 165 bp conserved region of a gene coding for 65 k Dalton antigen protein of *M. tuberculosis* was amplified using primer set published previously [7]. Similarly, a 365 bp region (between sequence position 1377–1741) of *dnaJ* gene of *M. tuberculosis* was amplified using genus-specific primers described earlier by Takewaki et al. (1993) [8]. The 541 bp region within insertion element IS6110 of *M. tuberculosis* was amplified using oligonucleotides designated as Pt-8 and Pt-9 by Kox et al. 1994 [9]. Ten microliters of the amplified PCR product were fractionated electrophoretically on a 2% agarose gel, stained with ethidium bromide and visualized under UV-transilluminator for the accuracy and specificity of PCR amplification, using PhiX174 HaeIII digest as gene ruler. All the PCR amplifications were subjected to internal laboratory standardization using template DNA from *M. tuberculosis* reference strain H37Rv as positive control (Figure 1). Briefly, 50–100 ng of purified total cellular DNA was amplified with thermostable Taq DNA polymerase in Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA). To establish positivity, we added 50 uL of reaction mixture containing 10 mm Tris-HCl (pH 8.3), 50 mm KCl, 1.5–2.5 mm $MgCl_2$, 0.01% (w/v) gelatin, 20–50 pmol of respective primers (described earlier), 2.0–2.5 nmol of each of the four deoxynucleoside triphosphates (dATP, dCTP, dGTP and dTTP), 1 U of Taq DNA polymerase (Applied Biosystems, Foster City, CA). The PCR cycle conditions were slight modifications of the previously published one for 165 bp hsp gene fragment (94°C for 20 s to denature the DNA, then cooled to 63°C for 20 s, heating to 72°C for 1 min for extension, cycle repeated 30 times with final incubation at 72°C for 10 min), for 365 bp *dnaJ* gene fragment (94°C for 30 s to denature the DNA, then cooled to 65°C for 60 s, heating to 72°C for 2.5 min for extension, cycle repeated 35 times with final incubation at 72°C for 10 min) and for 541 bp IS6110 sequence (94°C for 60 s to denature the DNA, then cooled to 65°C for 1.5 min, heating to 72°C for 3.5 min for extension, cycle repeated 38 times with final incubation at 72°C for 10 min) of mycobacterial DNA. Figure 2 shows the amplification of all the above mentioned genetic markers in clinical samples.

2.4. Statistical Analysis. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) and Chi-square tests were calculated using ACASTAT Software [10]. The results of 2×2 Chi-square tests and P values were uncorrected ones, unless otherwise indicated. A P value ≤ 0.05 was considered statistically significant.

3. Results

Out of 60 studied samples, 26 were positive by smear Ziehl-Neelsen staining and were also culture positive (Gr I). We detected 22 samples of Gr I as positive by PCR (84.62%). Out of 15 samples of Group II (smear negative but culture and radiologically positive), 11 samples (73.33%) turned out positive by PCR method. Hence, our trimarker system-based polymerase chain reaction detected 33 (80.5%) positive out of 41 patients (combined Gr I & II). We also found 7 samples (77.78%) out of 9 from the IIIrd Group as positive for 365 bp *dnaJ* gene (but not for rest two genes) indicating that they might be cases with mycobacterioses but not tuberculosis. In

Table 1: Characteristics of clinical samples and controls based on conventional diagnostic methods and other clinical symptoms.

Category	Status of the samples				Samples (n)	Age (Range)	Sex (Male/Female) (n)
	*AFB staining (Z-N)#	Culture	X-ray	Other			
Group I	+	+	+	+	26	14–72	19/7
Group II	−	+	+	+	15	14–65	10/5
Group III	−	−	−	+	9	14–75	4/5
Group IV	−	−	−	−	10	15–60	8/2

*AFB: acid fast bacilli; #Z-N: ziehl-neelsen; +: positive; −: negative.

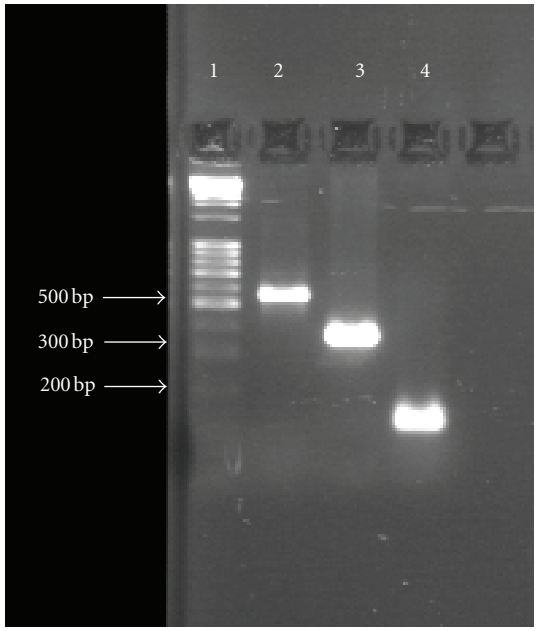

Figure 1: PCR showing 165, 365, and 541 bp fragment of *M. tuberculosis* reference strain H37Rv (positive control). Lane 1: gene marker, Lane 2: 541 bp amplicon, Lane 3: 365 bp amplicon, and Lane 4: 165 bp amplicon.

the controls (Gr IV, $n = 10$), none was PCR positive for any of the 3 markers used in this study. However, there were 4 samples in Gr I and 2 in Gr II, which could not be amplified for *IS6110,* possibly due to the presence of PCR inhibitors. Similarly, 2 samples in Gr II did not amplify for *hsp 65* gene marker. The PCR amplification results for Gr III clearly demonstrated infection with nontuberculous mycobacteria. The specificity of this study was 80%, and sensitivity range was 73.33% (Gr.II) to 84.61% (Gr.I). Also, the PPV and NPV were 77.78–91.67% and 66.67–80%, respectively. When compared to the smear and culture methods, the results were statistically significant ($P = 0.030$ and $P = 0.009$, resp.), showing that the PCR technique had a significantly higher ability to detect tuberculosis caused by *Mycobacterium tuberculosis* complex. The results of this study are presented in Tables 2, 3, and 4.

4. Discussion

Pulmonary tuberculosis is one of the highly infectious diseases and mostly prevalent in India, but it still bears with it the problem of definitive diagnosis. PCR amplification of mycobacterial DNA is a highly sensitive and specific technique to detect Tuberculosis. This also performs better than smear (which needs >10,000 bacilli/mL sample for detection) as well as culture method (which has restricted growth conditions). In these cases, PCR helps to diagnose whether the positivity (smear or culture) is due to *MTB* complex bacteria or other nontuberculous infection. In case of negative Ziehl-Neelsen staining, PCR helps to confirm the diagnosis. Our Data clearly showed that there is a significant difference between smear and PCR ($P = 0.030$) and between culture and PCR ($P = 0.009$) methods, further signifying that the PCR method has proved better than smear and culture methods in this study. However, many of the mycobacterial PCR assays employing species-specific primers allow for the detection of a single or limited number of mycobacterial species [11–16]. The efficiency and success of any rapid diagnostic PCR method depend on the appropriate DNA target sequences chosen for amplification. In this study, the marker system chosen is ideally applicable to most of the mycobacterial species (*M. tuberculosis, M. bovis, M. bovis BCG, M. africanum, and M. microti*) of *MTB* complex. The Success of this method for the detection and identification of mycobacteria further increased due to inclusion of one target sequence (*dnaJ* gene) for NTM. A meta-analysis published recently [17] demonstrated that use of *IS6110* as a target of amplification was significantly associated with increased accuracy of the PCR-based detection test. However, although this sequence is present in multiple copies in the bacterial genome, some strains from certain parts of the world lack it [18, 19], and this may cause false-negative results further affecting the sensitivity of the assay. A possible solution to this problem would be to amplify more than one target sequences. Hence, we have also used 165 and 365 bp target DNA sequences in our study. The 365 bp *dnaJ* gene marker is a genus-specific, one which has a broader spectrum of detection and can be amplified from all mycobacterial (including nontuberculous and non-pathogenic) species, while the 541 bp *IS6110* and 165 bp 65 kDa antigen protein gene sequences are amplifiable from almost all the mycobacterial species of *MTB* complex (but not from NTM) [7, 9], thus accounting for their higher sensitivity. So far no single target sequence has provided 100% sensitivity and a total absence of false positive when used alone. It was also observed that multiplex PCR did not contribute to increase the diagnostic accuracy in a large meta-analysis study [17]. Our trimarker based PCR method

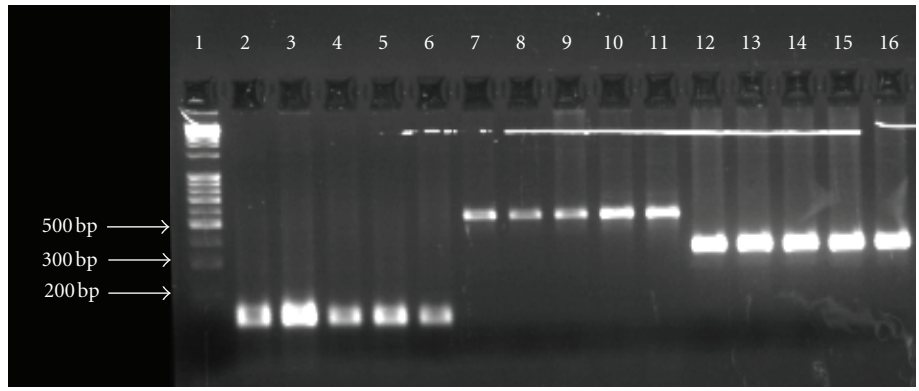

Figure 2: PCR showing 165, 365, and 541 bp target sequence amplified in case samples. Lane 1- gene marker, Lanes: 2–6, 165 bp amplicon, Lanes: 7–11; 541 bp amplicon, Lanes: 12–16, 365 bp amplicon.

Table 2: Detection of mycobacterial infection by polymerase chain reaction.

Category	Samples (*n*)	PCR results			PPV (%)	NPV (%)	No PCR- amplification		
		Positive *n* (%)	Sensitivity (%)	Specificity (%)			For (*n* [%])		*dnaJ*
							IS6110	*hsp* 65	
Group I	26	22 (84.62)*	84.6	80	91.67	66.67	4 (15.38)		
Group II	15	11 (73.33)*	73.33	80	84.62	66.67	2 (13.33)	2 (13.33)	
Group III	9	7 (77.78)#	77.78	80	77.78	80			2 (22.22)
Group IV	10	0 (0)	—	—	—	—	10 (100)	10 (100)	10 (100)

*Indicates positive result for all three markers; #indicates positive result for only *dnaJ*; PPV: positive predictive value; NPV: negative predictive value.

Table 3: Comparison of PCR and smear methods for detection of mycobacterial infections.

Samples	Smear *n* (%)	PCR *n* (%)	*P*
Positive	26 (43.33)	42 (70)	0.030*
Negative	34 (56.66)	18 (30)	

* *P* value < 0.05.

Table 4: Comparison of PCR and culture methods for detection of mycobacterial infections.

Samples	Culture *n* (%)	PCR *n* (%)	*P*
Positive	41 (68.33)	42 (70)	0.009*
Negative	19 (31.67)	18 (30)	

* *P* value < 0.05.

could sensitively and specifically detect clinically important mycobacterial infections such as those of *M. tuberculosis*, *M. tuberculosis* complex other than *M. tuberculosis* (especially *M. bovis*) and differentiate from major pathogenic NTM and nonpathogenic mycobacteria. Hence, this method presented a fast and efficient technique to diagnose tuberculosis infection and differentiate it from other mycobacterial infections in order to help better patient management. However, since some samples could not be amplified properly for certain DNA sequences in this study, it is important to consider PCR inhibitors and the sensitivity of the assay could further improve with effective control of such inhibitors.

5. Conclusions

The study presents a trimarker based PCR technique, which also applies to a wide variety of clinical samples and hence evaluated as a useful technique in the diagnosis of pulmonary tuberculosis. This method is very useful in cases where Ziehl-Neelsen staining and/or culture results are negative. By including one relatively less-specific marker (365 bp *dnaJ* gene), this PCR method also facilitates discrimination of *MTB* complex-specific infection from nontuberculous or nonpathogenic mycobacterial infections. The fact that DNA amplification can detect mycobacterial DNA sequences in the presence of excess amounts of human DNA makes it especially useful when quick results are required in certain clinical circumstances. This may also be useful when large-scale screening of mycobacteria is required, such as in parts of the countries where tuberculosis is still endemic and remains a major public health problem.

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Review Article

Cardiac Autonomic Control Mechanisms in the Pathogenesis of Chagas' Heart Disease

Diego F. Dávila, Jose H. Donis, Gabriela Arata de Bellabarba, Vanesa Villarroel, Francisco Sanchez, Lisbeth Berrueta, Siham Salmen, and Barbara Das Neves

Instituto de Investigaciones Cardiovasculares, Departamento de Fisiopatología, Instituto de Inmunologìa Clinica, Facultad de Medicina, Hospital Universitario de Los Andes, Universidad de Los Andes, Mérida 5101, Venezuela

Correspondence should be addressed to Diego F. Dávila, diegod@ula.ve

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Primary abnormalities of the autonomic nervous system had been postulated as the pathogenic mechanisms of myocardial damage, in patients with Chagas disease. However, recent investigations indicate that these abnormalities are secondary and amenable to treatment with beta-adrenergic blockers. Moreover, muscarinic cardiac autoantibodies appear to enhance parasympathetic activity on the sinus node. Therefore, the purpose of this paper is to analyze how knowledge on Chagas' disease evolved from being initially considered as a primary cardioneuromyopathy to the current status of a congestive cardiomyopathy of parasitic origin.

1. Introduction

The natural history of Chagas disease is characterized by an acute phase, followed by an indeterminate or transitional stage and a terminal arrhythmic-congestive phase. This disease appears to evolve from localized myocardial damage to a clinical form of congestive cardiomyopathy, with diffuse myocardial damage [1–4]. Several hypotheses have been postulated in order to explain the mechanisms responsible for the progression of myocardial damage. The proposed mechanisms are (1) microvascular disturbances, (2) immune-mediated myocardial injury, (3) parasite-dependent myocardial aggression, and (4) primary abnormalities of the parasympathetic and sympathetic divisions of the autonomic nervous system. Microcirculatory disturbances and immune-mediated myocardial injury are prominent peculiarities of Chagas cardiomyopathy. However, the roles of these two proposed mechanisms of myocardial damage are very likely ancillary rather than fundamental to the pathogenesis of disease progression. Concerning parasite-dependent myocardial damage, due to the diversity of *Trypanosoma cruzi* populations isolated from patients presenting the same clinical form of the disease an association between the parasite's genotype and the clinical manifestations of the disease is still not definitively established. Moreover, the available data are considered insufficient to justify trypanocidal therapy as a therapeutical alternative aimed at modifing clinical outcomes. Nonetheless, the BENEFIT trial (Benefit Evaluation for Interrupting Trypanosomiasis), currently in progress, will undoubtedly provide definitive answers for this crucial therapeutic dilemma [1–4].

The parasympathetic abnormalities were initially attributed to a direct action of the parasite on the postganglionic cardiac parasympathetic neurons. This hypothesis was postulated by Koberle in the 1950s [5, 6]. A cardiac autoimmune response, aimed at the sympathetic postganglionic fibers and to the cardiac muscarinic receptors, was proposed in the 1990s by Iosa et al. and Goin et al., respectively [7–9]. The autoantibodies, with adrenergic and cholinergic activities, would be responsible for an early sympathetic and parasympathetic dysautonomias. These two primary abnormalities of the autonomic nervous system would precede and contribute to the progression of myocardial damage and cardiac dysfunction. Recent investigations have shown, on the contrary, that these autonomic abnormalities are indeed secondary and characterized by an impairment of cardiac parasympathetic control and activation of the sympathetic nervous system [2, 10]. Moreover, muscarinic cardiac autoantibodies appear to enhance parasympathetic control of

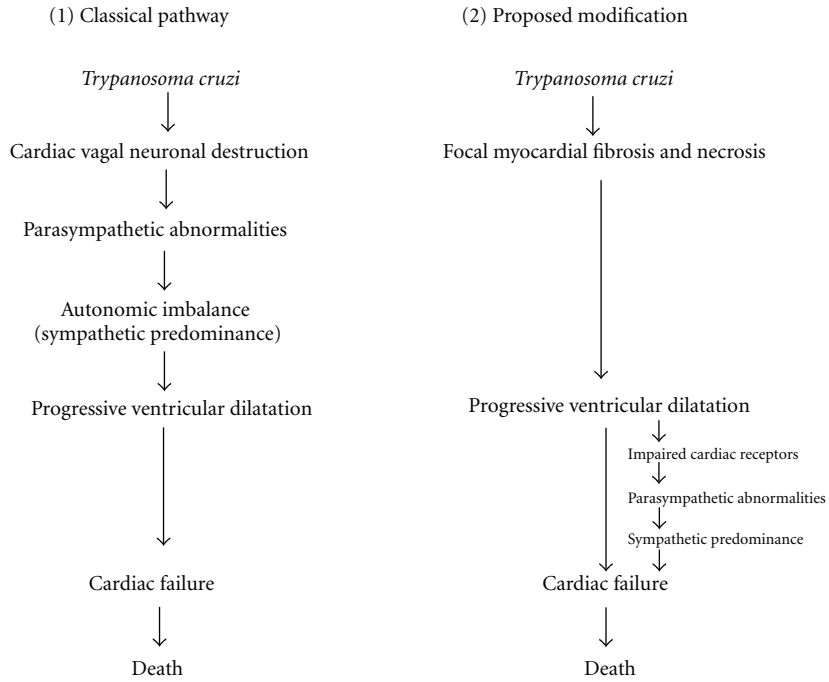

Figure 1: The neurogenic hypothesis on the pathogenesis of myocardial damage in chagas disease. According to the classical pathway, sympathetic activation, due to an early and selective destruction of the cardiac postganglionic neurons, would precede and induce cardiac ventricular dilatation. The proposed modification states that myocardial damage of certain extent and sequelae of the acute phase of the disease leads to progressive ventricular dilatation with impairment of cardiac receptors, parasympathetic abnormalities, and sympathetic activation (see [15]; 5 : 327-29, adapted with permission from Elsevier).

heart rate and therapeutic strategies, which antagonize the cardiotoxic effects of catecholamines, increase quality of life and survival of these patients [11, 12]. Therefore, the main purpose of this paper is to analyze how knowledge on Chagas disease evolved from being initially considered as a primary cardioneuromyopathy to the current status of a congestive cardiomyopathy of parasitic origin.

2. Chagas Heart Disease as a Primary Cardioneuromyopathy

2.1. The Neurogenic Hypothesis on the Pathogenesis of Chagas Heart Disease. According to the neurogenic hypothesis, the parasite would irreversibly and selectively destroy the postganglionic cardiac parasympathetic neurons during the acute phase of the disease. Sympathetic predominance would follow, exposing the myocardium to the toxic effects of catecholamines. However, Koberle's hypothesis was based on autopsies of patients who had died in advanced stages of the disease (i.e., congestive heart failure). Cardiac neuronal counts in patients, with no macroscopic evidence of myocardial damage, were either normal or minimally decreased [5, 6, 13]. Moreover, functional tests of autonomic function were normal in asymptomatic patients with no electrocardiographic evidence of heart disease and could also be normal or impaired, in those patients with abnormal electrocardiograms [14]. The results of these functional and morphological studies clearly indicated that the abnormalities of the parasympathetic nervous system were not homogenous. According to the neurogenic hypothesis most if not all Chagasic patients should have functional evidence of an impaired parasympathetic control of heart rate and abnormal neuronal counts, in the early stages of the chronic phase of the disease (i.e., intederminate stage). The presence and extent of myocardial damage, in most clinical studies carried out between 1960 and 1980, had been assessed by means of surface electrocardiography and chest X-rays [14]. We, at the Institute of Cardiovascular Research of the University of the Andes in Mérida, Venezuela, were puzzled by the heterogeneity of the results of the functional and morphological studies on the autonomic nervous system of Chagasic patients and postulated an alternative explanation for the autonomic abnormalities (Figure 1) [15]. To test this alternative hypothesis, we began a series of experimental [16–21] and clinical studies [22–24], on the functional status of the autonomic nervous system, in acutely infected laboratory animals and in Chagasic patients who were in the different stages of the natural history of the disease (acute, indeterminate, and congestive stages). The presence and extent of the myocardial damage were assessed by autopsy in the former and by left ventricular cine angiography in the latter. The results of these investigations were congruent in pointing out the following.

(1) Acutely infected laboratory animals, with unequivocal evidence of chagasic myocarditis, had normal functional tests of the efferent and afferent components of the cardiac parasympathetic nervous system. (2) Asymptomatic Chagasic patients, with abnormal ectrocardiograms, could have

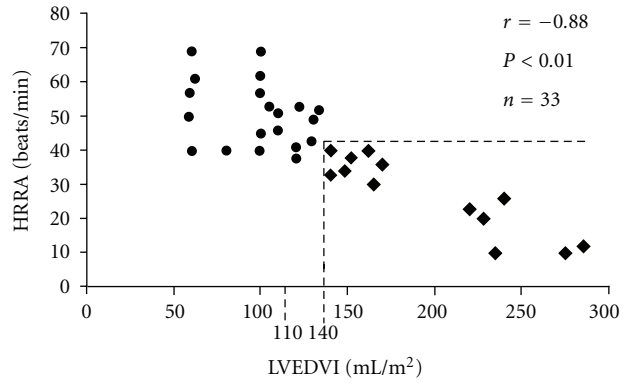

Figure 2: Parasympathetic abnormalities and cardiac remodelling in chagas disease; the heart rate response to atropine (HRRA) is indirectly related to left ventricular end-diastolic volume (LVEDI). Thus, parasympathetic impairment is secondary to cardiac remodelling and dysfunction (• = patients with segmental myocardial dysfunction, normal HRRA, and no ventricular dilatation; ♦ = patients with abnormal HRRA and progressive ventricular dilatation). (see [22], adapted with permission form Elsevier).

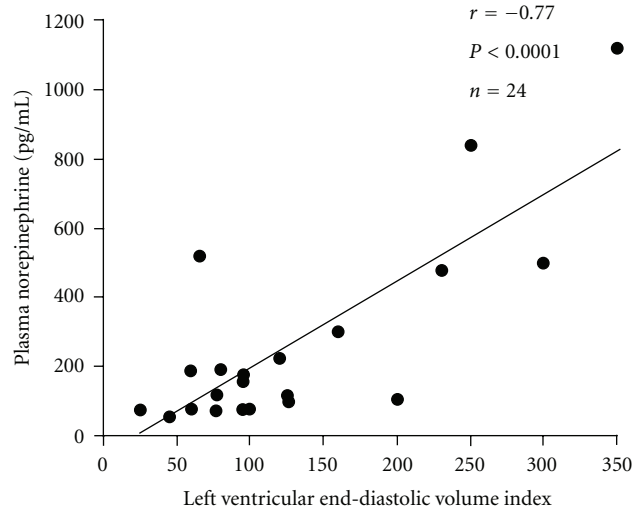

Figure 3: Sympathetic activation and cardiac remodelling in chagas disease. Norepinephrine serum levels are directly related to the left ventricular end-diastolic volume index. Patients with normal-sized left ventricles have no increased serum levels of norepinephrine. Thus, sympathetic activation is secondary to the process of cardiac remodelling (see [32], adapted with permission from Elsevier).

either segmental of diffuse myocardial damage and variable degrees of left ventricular systolic dysfunction. (3) The heart rate response to atropine and to the Valsalva maneuver was normal in patients with localized myocardial damage, but these tests were impaired in the presence of diffuse myocardial damage. Furthermore, the heart rate response to atropine was significantly and inversely related to the degree of cardiac remodelling (i.e., left ventricular end-diastolic volume and ejection fraction) (Figure 2) [22]. All of these studies consistently questioned the primary nature of the cardiac parasympathetic abnormalities. It was now absolutely necessary to determine when, in the different stages of the natural history of Chagas disease, the activation of the sympathetic nervous system occurred [25, 26].

2.2. Abnormalities of the Sympathetic Nervous System. Iosa et al. [7, 8], Goin et al. [9], and Sterin-Borda and Borda [27] considered that the sympathetic and the parasympathetic nervous systems of Chagasic patients were primarily and irreversibly damaged by the presence of cardiac autoantibodies. Chagas disease was, therefore, a primary cardioneuromyopathy. This hypothesis was based, in part, on the norepinephrine serum levels of Chagasic patients, with clinical evidence of congestive heart failure, which were found to be significantly lower than those of non-Chagasic patients [7]. At the time, we reasoned that since blood samples had been drawn from a systemic source in the former and from the coronary sinus in the latter; the methodological design of the study could be influencing the results of the investigation. Furthermore, we asked how could a patient with a primary abnormality of the sympathetic nervous system reach the stage of congestive heart failure? In the presence of low output heart failure, congestion and tissue perfusion pressure are absolutely dependent on the activation of the sympathetic and other neurohormonal systems [28]. The parasympathetic and sympathetic balance shifts towards a predominance of the latter, in non-Chagasic patients and in experimental models with asymptomatic and symptomatic left ventricular systolic dysfunction [29–31]. Consequently, Chagasic patients with impaired parasympathetic function and cardiac remodelling should also have evidence of a late and secondary activation of the sympathetic and other neurohormonal systems, as their cardiac disease progressed and congestive heart failure ensued [26]. We and other investigators found that indeed neurohormonal activation was present in the advanced stages of chagas heart disease [32, 33]. Systemic norepinephrine correlated directly and significantly with the degree of cardiac dilatation (Figure 3) [32] and coronary sinus norepinephrine was similar to that of patients with non-chagasic heart failure, due to left ventricular systolic dysfunction [33]. It was now clear to us that the abnormalities of the parasympathetic and sympathetic divisions of the autonomic nervous system were late and secondary events, in the natural history of chronic chagas disease. Despite the infectious nature of Chagas disease [6, 34, 35], the overwhelming clinical and neurohormonal similarities between chagasic and non-chagasic cardiac patients lead us to an utmost pertinent and relevant question ((Tables 1 and 2) [36–38]): should we use beta-adrenergic blockers in Chagasic patients with congestive heart failure secondary to left ventricular systolic dysfunction?

When considering the risk factors for cardiac morbidity and mortality, prospective studies [39] provided direct and indirect evidences of neurohormonal activation in symptomatic Chagasic patients. These prospective studies indicated that a heart rate above 90 beats/min was predictive of a poor prognosis. Based on these studies and the above information already described, we decided to test the hypotheses that Chagasic patients, with congestive heart failure, would tolerate and benefit from beta-adrenergic blockers [26]. The

Table 1: Clinical, radiologic, and echocardiographic characteristics of non-Chagasic and chagasic patients with advanced systolic heart failure. There was no difference in the baseline heart rate, blood pressure, and functional class (NYHA). The degree of ventricular dysfunction and dilatation was also similar in both groups of patients (see [36], with permission from Elsevier).

Characteristic	Non-Chagasic patients ($n = 7$)	Chagasic patients ($n = 6$)	P value
Male	6 (86%)	5 (83%)	
Female	1 (14%)	1 (17%)	
Age	54 ± 9	47 ± 10	NS
Baseline heart rate (beast/min)	116 ± 16	115 ± 16	NS
Baseline systolic pressure (mmHg)	111 ± 14	115 ± 16	NS
Baseline diastolic pressure (mmHg)	75 ± 13	83 ± 12	NS
Functional class			
III	5 (71%)	3 (50%)	
IV	2 (29%)	3 (50%)	
Cardiothoracic index	>60.0	>60.0	
Two-dimensional echocardiogram			
Left ventricular diastolic diameter (cm)	5.9 ± 0.39	6.49 ± 1	NS
Left ventricular ejection fraction	0.17 ± 0.04	0.21 ± 0.06	NS

Values are mean ± S.D., except for sex and functional class.

Table 2: Baseline serum levels of norepinephrine, plasma renin activity, and aldosterone in chagasic and non-chagasic patients with advanced congestive heart failure. The baseline serum levels of norepinephrine (NE), plasma renin activity (PRA), and aldosterone (ALDOST) of the chagasic patients were not different from those of the non-chagasic patients (see [36], with permission from Elsevier).

	Non-Chagasic patients ($n = 7$)			Chagasic patients ($n = 6$)		
	NE	PRA	ALDOST	NE	PRA	ALDOST
	2698	2.33	255	1570	6.30	166
	824	6.40	326	890	10.50	363
	1211	6.57	636	2800	16.87	518
	2149	2.20	107	4827	35.57	457
	1052	3.40	918	2000	1.08	147
	1291	9.70	—	1486	—	—
	975	—	—			
Mean ± S.D.	1.457 ± 695*	5.10 ± 2.96	448 ± 325**	2262 ± 1404*	14 ± 13*	330 ± 168**
Confidence interval (95%)	942; 1972	5; 10	312; 583	1705; 2818	9; 19	194; 465

NE: plasma norepinephrine (pg/mL); PRA: plasma renin activity (ng/mL per h); ALDOST: plasma aldosterone (pg/mL). *$P < 0.001$ as compared to normal controls. **$P < 0.05$ as compared to normal controls.

short-term effects of the selective beta-blocker metoprolol were studied in Chagasic patients with severe congestive heart failure [40]. Patients were receiving conventional treatment and had sinus tachycardia, low systolic blood pressure, and echocardiographic evidence of severely depressed left ventricular systolic function. Mild sympathetic activation was present and most of them were in functional class IV. At the end of the fifth week of treatment with metoprolol (25 mg), the heart rate and blood pressure showed favorable and significant changes. The left ventricular ejection fraction, however, increased significantly only at the end of the tenth week of treatment with metroprol (50 mg). Similar clinical benefits had been previously shown in the 1960s by Luquez et al. in Argentina [41] and more recently by Botoni et al. [42]. However, all of these were short-term studies which did not assess the effects of beta-adrenergic blockers and mortality and rate of hospital readmissions.

Long-term clinical investigations have been recently carried out by Brazilian investigators [43, 44]. Theodoropoulos et al., [43] followed-up patients with congestive heart failure of chagasic etiology. The design of the investigation explicitly excluded comorbidities that could contribute to the genesis of the underlying heart disease. The study was aimed to determine the predictors of all-cause mortality. To this purpose, one hundred and twenty-seven patients, who fulfilled established criteria for congestive heart failure and had received conventional treatment plus angiotensin converting enzyme inhibitors (90%) and beta-adrenergic blockers (34%) were followed up for 25 ± 19 months. multivariate cox regression survival analysis indicated that, no treatment with a beta-adrenergic blocker was a more important independent predictor of mortality than hyponatremia, left ventricular ejection fraction, and functional class. The probability of survival was significantly diminished in

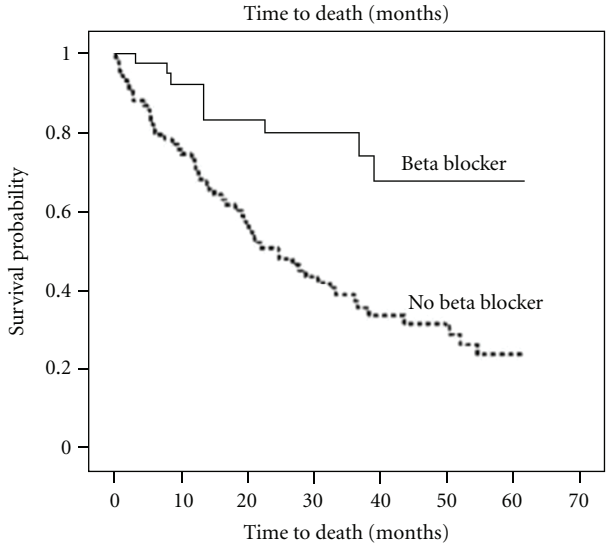

Figure 4: Survival probability according to use or no use of beta-adrenegic blockers. The probability of survival was significantly diminished in Chagasic patients who were not treated with beta-adrenegenic blockers. (see [43], adapted with permission from Elsevier).

Chagasic patients who were not treated with beta-adrenergic blockers (Figure 4). Similar findings were more recently reported by Issa et al., [44] who compared the survival of Chagasic patients treated and untreated with beta-adrenergic blockers with that of non-Chagasic patients. These two clinical investigations are congruent in pointing out that the increased mortality of Chagasic patients, who are in the arrhythmic-congestive phase, is due to lack of treatment with neuropharmacologically active drugs. The low proportion of Chagasic patients on beta-blocker therapy may be due, in part, to the administration of targeted doses of ACEI, particularly captopril. This therapeutic strategy may lower systolic pressure and preclude the use of beta-adrenergic blockers [45]. Nonetheless, these results strongly suggest that secondary neurohormonal activation is the main underlying mechanism of disease progression, in chronic Chagasic patients [11]. Therefore, the neurogenic hypothesis [5, 6], although incorrect regarding the timing of appearance of the cardiac parasympathetic abnormalities, did envision the pathogenic role of an enhanced cardiac sympathetic drive [3, 15].

2.3. Parasympathetic Dysautonomia and Cardiac Muscarinic Autoantibodies. The parasympathetic division of the autonomic nervous system is also affected by the autoimmune response to the presence of the parasite in the hearts of patients with Chagas disease [46]. A cardiac autoimmune response arises in Chagasic patients because of antigenic mimicry between the parasite and cardiac muscarinic receptors [47]. The second extracellular (o2) and the third intracellular loops (i3), of these receptors, are considered as autoimmune epitopes, in patients with chronic chagas disease [48, 49]. The autoimmune response occurs early in the natural history of the disease and it is considered to be responsible for the abnormalities of parasympathetic control of heart rate [50–54]. These investigations have demonstrated that the chronotropic responses to cardiac autonomic tests are apparently impaired in the indeterminate form of the disease. However, we [22–26] and other investigators [55] have found that Chagasic patients, who are in different stages of natural history of the disease, may have normal, abnormal [2], or even enhanced responses to conventional cardiac autonomic tests [56, 57]. Moreover, the frequency and time domain indexes of parasympathetic modulation may be depressed in the supine position, but become similar to controls in the standing position and while performing isometric exercise [58–60]. Since, muscarinic autoantibodies indirectly correlate with the high frequency component of heart rate variability [52] are highest in the presence of chronotropic insufficiency [55] and may behave as positive allosteric modulators of parasympathetic activity [61], an alternative explanation, for the "impaired" chronotropic responses to the cardiac autonomic tests, would be over stimulation with saturation of the parasympathetic system [62–65]. As stated by Benchimol-Barbosa, a continuous cholinergic effect of anti-M2 antibody, by acting on the muscarinic receptor of the sinus node cells, could slow heart rate and limit acute heart rate variations. Therefore, adjustments of heart rate in response parasympathetic efferent activity are expected to be apparently "impaired" [62]. Consequently, we have studied the cardiac chronotropic responses to the Valsalva maneuver and to dynamic exercise and correlated them with the serum levels of cardiac muscarinic autoantibodies, of Chagasic patients who were in the chronic phase of the disease [12]. Our clinical investigation included asymptomatic Chagasic patients, who were in the indeterminate and cardiac forms of the disease and had normal two-dimensional echocardiograms. Heart rate acceleration, during the early phases of the Valsalva maneuver and of dynamic exercise, was significantly diminished in the Chagasic patients. However, the heart rate changes, during the late phases of the Valsalva maneuver and of dynamic exercise, revealed a normal and enhanced response, respectively. These apparently "discordant" results would indirectly suggest the following. (1) The diminished initial heart rate acceleration during the early phases of both of these tests is indicative of reduced or impaired resting parasympathetic activity; (2) the normal or augmented heart rate recovery, at the end of these two same tests, would indicate, on the other hand, that parasympathetic reactivation is normal and even enhanced. Thus, chronotropic responses to parasympathetic withdrawal would be apparently impaired, but the chronotropic responses to parasympathetic reactivation are normal or even accentuated. How can one reconcile these apparently "discordant" results, with current knowledge on cardiac parasympathetic function in chronic chagas heart disease?

Antimuscarinic autoantibodies are detected eary in the natural history of chagas disease [52]. However, the serum levels of these autoantibodies do not differentiate the various forms of chagas heart disease and do not correlate with the parameters of left ventricular function. Therefore, the

role of the autoantibodies in the pathogenesis of myocardial damage and disease progression is questionable [54]. Moreover, muscarinic receptors are known to be upregulated by *Trypanosoma cruzi* infection and muscarinic cardiac autoantibodies potentiate the chronotropic effects of acetylcholine on the cardiac muscarinic receptors of the sinus node [61–66]. Therefore, the postsynaptic muscarinic receptors, which mediate the negative chronotropic effects of parasympathetic activity, are numerically increased and positively influenced by *Trypanosoma cruzi* infection. In this particular context, we have found that the serum levels of the cardiac muscarinic autoantibodies correlated directly with the magnitude of cardiac deceleration, following cessation of exercise. Therefore, the more prominent heart rate recovery of the Chagasic patients could be an expression of a positive allosteric effect of the muscarinic autoantibodies on the membrane muscarinic receptor. Alternatively, these results could be due to a direct agonist effect of the autoantibodies on the muscarinic receptor and thereby potentiate early heart recovery [12]. The results of this investigation provide a plausible explanation for the heterogeneity of heart rate responses to conventional cardiac autonomic tests [55–60, 67]. A continuous cholinergic effect of anti-M2 antibody, by acting on the muscarinic receptor of the sinus node cells, may limit acute heart rate variations (i.e., heart rate responses to parasympathetic withdrawal) and simultaneously potentiate responses to parasympathetic reactivation [62–64].

In summary, the clinical and experimental investigations discussed indicate that the abnormalities of the parasympathetic and sympathetic divisions of the autonomic nervous systems are secondary and amenable to treatment with beta-adrenergic blockers. This therapeutic strategy, although not directed at the parasite, improves quality of life and survival of patients with chagas heart disease. The cardiac muscarinic and adrenergic autoantibodies may not have a direct role in the pathogenesis of the cardiac damage [54]. Moreover, the former appears to enhance parasympathetic control of heart rate. Consequently, knowledge on Chagas disease has evolved from being initially considered as a primary cardioneuromyopathy to the current status of a congestive cardiomyopathy of parasitic origin [11, 43–45].

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Review Article

Ophthalmic Parasitosis: A Review Article

Amal R. Nimir,[1] Ahmed Saliem,[1] and Ibrahim Abdel Aziz Ibrahim[2]

[1] *Division of Basic Medical Sciences, Faculty of Medicine, Cyberjaya University College of Medical Sciences, 63000 Selangor, Malaysia*
[2] *Department of Pharmacology, Faculty of Medicine, University Technology MARA, 40100 Shah Alam, Malaysia*

Correspondence should be addressed to Amal R. Nimir, aralmadi@yahoo.com

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Ocular parasitosis in human is more prevalent in geographical areas where environmental factors and poor sanitary conditions favor the parasitism between man and animals. Lesions in the eye can be due to damage directly caused by the infectious pathogen, indirect pathology caused by toxic products, or the immune response incited by infections or ectopic parasitism. The epidemiology of parasitic ocular diseases reflects the habitat of the causative parasites as well as the habits and health status of the patient. An ocular examination may provide clues to the underlying disease/infection, and an awareness of the possibilities of travel-related pathology may shed light on an ocular presentation. This paper is a comprehensive review of the parasitic diseases of the eye. The majority of the clinically important species of parasites involved in eye infection are reviewed in this paper. Parasites are discussed by the disease or infection they cause.

1. Introduction

Ocular parasitosis in human is more prevalent in geographical areas where environmental factors and poor sanitary conditions favor the parasitism between man and animals. In recent years, population shift and rapid transport have facilitated the spread of certain parasitic diseases from endemic to nonendemic areas. The routes of infection to man vary with species of the parasite and the animal hosts they infest. Lesions in the eye can be due to damage directly caused by the infectious pathogen, indirect pathology caused by toxic products, immune response incited by infections, or ectopic parasitism of the preadult or adult stages.

The epidemiology of parasitic ocular diseases reflects the habitat of the causative parasites as well as the habits and health status of the patient. Additional consideration must include local sanitation and the presence of a vector for transmission as well as the more complicated life cycles of the parasites and definitive hosts. Dietary history should be considered since most parasitic transmission is through food and water contamination. Travel history to endemic areas is important to determine the source of infection. An awareness of these is therefore important to the clinician evaluating this group of patients.

An ocular examination may provide clues to the underlying disease, and an awareness of the possibilities of travel-related pathology may shed light on an ocular presentation. The eye is involved both in a variety of systemic infections and may be the primary focus of other pathologies. The majorities of conditions affecting the eyes—other than injuries—are infectious.

In some occasions, the ophthalmic lesions occur as a result of antiparasitic treatment as it has been noticed in the prophylactic and therapeutic attempts to treat malaria [1, 2]. Drugs such as hydroxychloroquine and chloroquine can damage vision because of their toxic effects, which is due to slow accumulation of the drugs in the retinal epithelium that results in irreversible visual loss. Much debate and confusion have taken place over the type and frequency of ocular examination in patients taking these drugs.

Despite improved understanding of the clinical features of inflammatory eye diseases and advances in diagnostic testing, clinicians should maintain a high index of suspicion for infective parasitic diseases in patients thought to have inflammatory eye involvement.

Because of this somehow more complex scenario, and the tendency for the parasites to cause a wider variety of pathologic lesions, the various parasitic etiologies of

ocular diseases will be addressed individually, including epidemiology, pathology, diagnosis, and treatment.

2. Protozoan Eye Infection

2.1. Acanthamoeba Keratitis. *Acanthamoeba* spp. is ubiquitous free-living protozoa that have been isolated from several habitats, including soil, bottled water, eyewash stations, and air.

There are two stages in the life cycle of this environmental ameba: the motile trophozoite (8–40 μm) and the dormant cyst (8–29 μm) [3]. By encysting, *Acanthamoeba* spp. can evade extreme environmental conditions such as hyperosmolarity, glucose starvation, desiccation, extreme temperatures, and extreme pH [4].

The leading risk factors for *Acanthamoeba* keratitis are contact-lens wear and corneal trauma [5, 6]. Although >80% of the cases of *Acanthamoeba* keratitis occur in contact lens, in other countries such as India, keratitis that is not related to contact lenses commonly occurs after corneal trauma or exposure to contaminated water [7]. One clinical manifestation of *Acanthamoeba* keratitis is radial neuritis and severe pain that is not commensurate with the extent of tissue damage. It typically presents as a unilateral central or paracentral corneal infiltrate, often with a ring-shaped peripheral infiltrate. Other characteristic symptoms (which appear in the early phases of infection) include eyelid ptosis, conjunctival hyperemia, epithelial ulcers, and lack of discharge. These symptoms are often followed by the appearance of a ring-like stromal infiltrate in the later stages of disease. *Acanthamoeba* keratitis can progress to scleritis, and, in severe cases, uncontrolled infections require the removal of the affected eye [8]. Interestingly, the pathogenic cascade of *Acanthamoeba* keratitis has interesting parallels with the amebic colitis caused by *E. histolytica* [4].

A provisional diagnosis of AK can be made using the clinical features and confocal microscopy although a definitive diagnosis requires culture, histology, or identification of *Acanthamoeba* deoxyribonucleic acid by polymerase chain reaction [9]. Trophozoites and cysts can be identified in Giemsa or periodic-acid-schiff-stained smears from scrapings or corneal biopsy specimens. Culture of *Acanthamoeba* spp. requires growth on nutrient agar plate seeded with bacteria. Treatment of amebic keratitis is difficult and disappointing. Long-term topical application of agents such as propamidine, miconazole, and neomycin has been successful in only few instances.

2.2. Chagas' Disease. Chagas' disease, or American trypanosomiasis, results from infection by *Trypanosoma cruzi*. Infection occurs when an infected reduviid triatomine bugs bites a human. Once introduced, the trypomastigotes circulate throughout the body with a preference for invading muscle cells, neural tissue, and the reticuloendothelial system. If the initial bite is near the orbit, the patient may experience significant palpebral and periorbital oedema (Romana's sign). The edema is usually painless and is frequently followed by constitutional symptoms of fever, malaise, and anorexia.

The diagnosis of acute Chagas' disease is made by the detection of trypomastigotes in the bloodstream by direct examination of uncoagulated blood or buffy coat preparation. Direct culturing of blood on Novy, MacNeal, Nicolle's medium (NNN) or other suitable media may result in positive cultures in 7 to 10 days [10]. The technique of xenodiagnosis may be used for diagnosis, if available. Serologic testing is of little value in the diagnosis of acute Chagas' disease as antibodies do not usually appear for 2 to 40 days following the onset of symptoms. Additionally, serologic studies may falsely detect the cross-reactivity of antibodies to nonpathogenic *Trypanosoma rangeli* [11].

Therapy of Chagas' disease with antitrypanosome therapy is most successful in the acute stage. Two medications are available: nifurtimox and benznidazole. Therapy is usually extended for a period of months, and parasitologic cure rates are somewhat disappointing. Both medications carry a long list of significant side effects.

2.3. Giardiasis. The protozoan disease giardiasis can cause ocular complications, including "salt and pepper" retinal changes. One study showed that asymptomatic, nonprogressive retinal lesions are particularly common in younger children with giardiasis. This risk does not seem to be related to the severity of the infection, its duration, or the use of metronidazole but may reflect a genetic predisposition [12].

Diagnosis is confirmed by finding the cyst stage in the fecal smear. Treatment is the same as for intestinal infection, that is, metronidazole.

2.4. Leishmaniasis. *Leishmania* spp. is obligate intracellular protozoans which infect an estimated 12 million persons. There are numerous species within the genus, and disease manifestation is, in part, species specific.

Once injected into humans during the sandfly blood meal, the promastigote develops into an amastigote after being engulfed by tissue macrophages. Within these cells, the amastigotes replicate and may spread either systemically or cutaneously.

Visceral leishmaniasis, or that which represents systemic disease, is known as kala-azar. The ocular manifestations of kala-azar are relatively uncommon and include chorioretinitis, central retinal vein thrombosis, iritis, papillitis, and keratitis [13]. Additionally, flame-shaped retinal hemorrhages have been described. Glaucoma has been reported to develop after the successful treatment of kala-azar.

Ocular findings in cutaneous leishmaniasis represent a local phenomenon resulting from the initial site of infection near the eye with occasional spread to the lacrimal duct. Ptosis may be a presenting complaint [14]. If the initial bite occurs on the conjunctival mucosa, the disease is termed mucocutaneous leishmaniasis. This state may lead to severe ulceration and possible loss of the eye.

The diagnosis of leishmaniasis is made by direct demonstration of organisms on tissue smears or biopsy. Amastigotes are usually demonstrated fairly easily in the case of cutaneous or mucocutaneous ocular disease. However, amastigotes have not been directly identified in cases of ocular disease associated with kala-azar. When present, *Leishmania* spp. may

be cultured on Novy, MacNeal, Nicolle's medium (N.N.N.) as well as Schneider's *Drosophila* medium supplemented with 30% fetal bovine serum. While available, serologic testing is not particularly useful for diagnosing cutaneous and mucocutaneous disease due to cross-reactivity with *T. cruzi* and *Mycobacterium leprosum*.

Treatment of choice is pentavalent antimony, sodium stibogluconate 15–20 mg per kg per day IM or IV for 15–20 days. A second or even a third treatment course with pentavalent antimonal can be given over 6–8 weeks if healing is not progressive.

2.5. Malaria. Caused by the *Plasmodium* species and transmitted via the bite of the female anopheles mosquito, this sometimes fatal infectious disease has characteristic findings in the eye. Signs of *falciparum* malaria in the eye include retinal whitening, retinal haemorrhage, papilloedema, and cotton wool spots [15, 16]. Much research done in endemic areas has shown a correlation between papilloedema or extramacular retinal oedema (retinopathy) and poor outcome in children with cerebral malaria. Studies show that retinopathy was associated with subsequent death, and the increasing severity of retinal signs was related to increasing risk of fatal outcome [17]. Other studies have shown that retinal changes related to microvascular obstruction were common in adults with severe falciparum malaria and correlated with disease, severity and comma [18, 19]. It is important to emphasise that whilst these signs give a pointer to the severity of disease they do not alter the drug management of malaria. The outcome in terms of vision in patients with ophthalmological findings and severe malaria is usually good [20]. Insights from retinal investigations have furthered the understanding of cerebral malaria [21, 22].

Quinacrine and chloroquine are molecules with the same alkyl side chain but different nuclei. The photobiological effects of quinacrine and chloroquine are similar in model systems; thus, development of a bull's-eye maculopathy with quinacrine ingestion is an unsurprising potential side effect.

The definitive diagnosis of malaria is made by microscopic identification of the parasite in the blood smear. A thin blood film should be examined for at least 15 minutes, whereas a 5-minute search of a thick film should reveal parasites if present. The thick film is the most efficient method of detecting malarial parasites, but interpretation requires an experienced worker.

Antimalarial drugs may be classified as (1) suppressive, by acting upon asexual blood cell stages and preventing the development of clinical symptoms; (2) therapeutic, by also acting on asexual forms to treat the acute attack; (3) radical cure, for destruction of the EE forms; (4) gametocytocidal, for destroying gametes; (5) sporoniticidal, for drugs that render gametocytes noninfective in the mosquito.

2.6. Microsporidiosis. Two genera appear to be important in the pathogenesis of ocular disease: *Encephalitozoon* and *Nosema*. Another genus, *Microsporidium*, is classified as *Nosema*-like. Recently, *Septata* spp. has been implicated in keratoconjunctivitis [23]. It should be noted that knowledge of microsporidiosis seems to be rapidly expanding, given its important role as an opportunistic infection in patients with AIDS. The life cycle is somewhat complex, involving three general stages: infection, merogony, and sporogony.

Ocular infection is presumed to occur either by direct inoculation into eye structures or by dissemination systemically, with the latter proposed to be the pathogenesis in patients with AIDS. Ocular findings are generally limited to the conjunctiva and cornea.

With respect to diagnosis, spores have been demonstrated in most cases in which corneal scrapings or biopsy specimens are examined by light or electron microscopy. Where available, serologic testing may assist in the diagnosis of microsporidiosis.

Current recommendations for treatment include the use of albendazole, which has shown some promise in the treatment of corneal disease. Historically, severe, progressive cases of ocular microsporidiosis have resulted in enucleation.

2.7. Rhinosporidiosis. Rhinosporidiosis, caused by *Rhinosporidium seeberi*, is a mucocutaneous disease that involves the palpebral conjunctiva in ~15% of all cases of rhinosporidiosis [24]. It is an infrequent cause of disease in India and tropical South America. Reproduction of *R. seeberi* in tissue produces polypoid or papillary growths that arise from mucous epithelium. Recent investigations of RNA genes from this microorganism disclose that it may be more closely related to fish parasites than to fungi [25], and it is, therefore, included in protozoan diseases of the eye. The etiologic agent, *Rhinosporidium seeberi,* has never been successfully propagated in vitro.

At present, the treatment for rhinosporidiosis is the surgical excision. Some authors proposed a medical therapy with dapsone [26], but the results are not convincing. Antimicrobial therapy is ineffective, and the disease may recur after months or years.

2.8. Toxoplasmosis. Toxoplasma gondii is a protozoan parasite, the lifecycle of which passes through cats. It represents the commonest cause of uveitis worldwide [27]. Human infection occurs through ingestion of food or water contaminated with cat faeces. Toxoplasmosis may be acquired at any age but most commonly during childhood. The majority of infections are asymptomatic and the prevalence of antitoxoplasma IgG (indicating past infection) ranges from 15 and 20% in Northern Europe to 80% or more in parts of the developing world. South America has a particularly high rate of ocular disease from toxoplasmosis.

Some patients may present with a glandular fever-like systemic febrile illness with adenopathy. Most cases of adult infection will not present with eye signs, those that do usually present with a focal necrotising retinitis occasionally associated with vascular occlusion [28]. Toxoplasmosis in immunosuppressed patients, for example, with AIDS can present with multiple, widespread lesions of differing chronicity and look different from classical toxoplasmosis. Vitritis is a common feature of toxoplasmosis which often leads to symptomatic haze and floaters that lasts for months

after the resolution of the acute attack. Lesions are usually self-limiting, but where they threaten sight—around the macula or optic nerve—treatment with a combination of corticosteroids, pyrimethamine, and sulfadiazine is usually advocated. However, therapeutic trials suggest that there is little evidence that drug therapy alters the natural history of the disease [29]. Relapses are relatively common, occurring in around 80% of patients followed up for more than 5 years [30]. The most serious consequences of toxoplasmosis are seen when acquisition occurs in pregnancy leading to congenital infection of the newborn. Involvement of the macula is common in the developing foetus and has devastating effects on central vision. Bilateral ocular involvement is common, and both maculae can be affected.

Serologic tests are very important in the diagnosis of toxoplasmosis. Because of the common occurrence of antibodies to the parasite in the general population, diagnosis by serologic means requires the demonstration of a significant increase in antibody titers.

Drug treatment for ocular and cerebral toxoplasmosis is the same and lesions will continue to grow without therapy. Clindamycin and azithromycin are now commonly used as first line treatment. Azithromycin has been shown to be effective in reducing the number of attacks in Brazil (see Tables 1 and 2).

3. Helminthic Eye Infection

3.1. Eye Infection Caused by Round Worms

3.1.1. Angiostrongyliasis. Ocular angiostrongyliasis, caused by *Angiostrongylus cantonensis*, is a nonfatal disease; however, it can cause permanent damage to an affected eye. It was first reported in Thailand in 1962 [31] and since then has rarely presented in tropical countries [32]. According to the *A. cantonensis* life cycle, the human is an accidental host. Most larvae usually live in the subarachnoid space or brain parenchyma. Only a small number of worms remigrate to pulmonary arteries or move randomly to other tissues such as cranial nerves or orbits.

Although blood eosinophilia is demonstrated in most cases of eosinophilic meningitis [33], it has not been noticed in ocular angiostrongyliasis without eosinophilic meningitis. Hence, it may indicate that ocular angiostrongyliasis occurs because a worm moves randomly from the bloodstream to an eye without invasion of the brain or meninges. In cases of ocular involvement ocular symptoms usually present between 2 weeks and 2 months after snail ingestion.

Although a wide range of initial visual acuity was reported, from finger count to 6/6, five cases had visual acuity less than 2/60. The duration of visual impairment varied from 4 days to 8 weeks, mostly 2-3 weeks. Additionally, indirect ophthalmoscopy should be recommended in any individual presenting with a history of eating raw *Pila* spp. snails and blurred vision either with or without eosinophilic meningitis. No dominant affected eye has been reported because of a nonspecific pattern of parasite movement [34].

Any types of laser, surgical removal, and corticosteroid treatment did not improve visual acuity. Alteration of the retinal pigment epithelium or retinal inflammation caused directly by parasites was the main reasons for poor vision at presentation. Furthermore, it produced permanent damage to an affected eye and gave a poor outcome. Although corticosteroids and albendazole have been reported to be effective in ocular cysticercosis and neurocysticercosis [35], there are no specific anthelmintic therapies in ocular angiostrongyliasis even after a definite diagnosis has been made.

3.1.2. Bancroftian and Brugian Filariasis. Human ocular infestation by live filarial worm is a rare occurrence and has been reported mostly from South-East Asia. It involves the eyelids, conjunctiva, cornea, anterior chamber, and uvea. Ocular filariasis can present in an otherwise asymptomatic patient without any constitutional symptoms. Inflammation of the retinal pigment epithelium and retinal vasculitis decreased vision, and panuveitis with secondary glaucoma can occur.

Indirect ophthalmoscopy showed vitritis with plenty of vitreous membranes, and subretinal yellow lesions in the peripheral retina along with retinal pigment epithelial tracts [36]. An aqueous tap and a peripheral blood smear isolate microfilariae of *W. bancrofti*. Therapy with diethyl carbamazine citrate along with systemic steroids provides symptomatic relief.

3.1.3. Baylisascariasis. *Baylisascaris procyonis* is the common intestinal raccoon roundworm in North America and is found in 82% of raccoons in Illinois. It is a known cause of neural larva migrans in animals [37]. It was identified in seven childhood cases manifesting as diffuse unilateral subacute neuroretinitis and choroidal infiltrates in association with neurologic disease. Those children had a history of pica and raccoon exposure. Differences in inoculum level are likely responsible for isolated ocular larva migrans versus neural larva migrans in humans [38].

Identification of the worm in the eye is the definitive diagnosis. Indirect immunofluorescence assays on serum, and cerebrospinal fluid is usually positive or serially positive and increasing [39]. Treatment is with albendazole and corticosteroids, and prognosis is usually poor.

3.1.4. Dirofilariasis. Dirofilaria are parasitic nematodes that are common in domestic and wild animals. Dirofilarial zoonotic infections are caused by mosquito vectors that carry the parasites from their animal hosts to people. Although these infections remain rare, they are increasing in incidence and human dirofilariasis may be considered an "emergent zoonosis [40].

As the worm matures, it elicits a host inflammatory response that ultimately produces the clinical presentation of a subcutaneous nodule. These nodules are most often found on areas with exposed skin [41]. Subcutaneous dirofilariasis appears as a small subcutaneous nodule that gradually grows over periods of weeks or months. The consistency of the nodule is hard and elastic with marked erythema. When the

Table 1: Ocular parasitosis caused by protozoa (*geographical distribution & ocular findings*).

Disease/Infection	Causative agent	Geographical distribution	Ocular findings
Acanthamoebic keratitis	*Acanthamoeba* spp	Worldwide, soil and water	Conjuctival oedema, sever pain, ring infiltrate around the cornea, hypopyon, hyphema, uveitis, loss of vision
Chagas' disease	*Trypanosoma cruzi*	Central and South America	Palpebral and periorbital oedema
Giardiasis	*Giardia lamblia*	Southeast Asia, Europe, USA and South Africa	Salt and pepper retinal changes, chorioretinitis, retinal haemorrhage and uveitis
Leishmaniasis	*Leishmania* spp	Africa, Mediterranean region, Middle East, parts of Asia and Central and South America	*Visceral*: conjunctivitis, uveitis and retinal haemorrhage *Cutaneous*: lesions on eyelid, blepharoconjunctivitis *Mucocutaneous*: severe ulceration, loss of the eye
Malaria	*P. falciparum*	Africa, Central and South America, Oceania and Asia	Retinal haemorrhage, papilloedema, cotton wool spots
Microsporidiosis	*Microsporidia* spp	Worldwide	Conjunctival hyperemia, punctate epithelial keratitis, hyphema, necrotizing keratitis, corneal ulcer
Rhinosporidiosis	*R. seeberi*	South America and Africa	Cojuctival granuloma
Toxoplasmosis	*Toxoplasma gondii*	Worldwide, South America	Congenital: strabismus, nystagmus and blindness Acute aquired: Primarily; necrotizing chorioretinitis Virtitis is common Secondary findings include; scotoma, photophobia, blindness, Glaucoma, ↑ IOP, necrotizing inflammation, loss of central vision

location is ocular, the worms are situated in the conjunctiva and can be extracted by incision.

The diagnosis of dirofilariasis is established histopathologically. Both the gross and microscopic features of *D. tenuis* have been well described [42]. Once diagnosed, the recommended treatment is complete removal of the nematode. If the nematode is not removed, it eventually degenerates, and the mature granulomatous response results in either calcification or abscess formation with subsequent purulent expulsion of the parasite.

3.1.5. Loiasis. The agent of loiasis is *Loa loa*. Infection is acquired by humans through the bite of the tabinid flies of the genus *Chrysops*. When humans are bitten, larvae pass from the fly to the human, where they develop over 1 year into mature adult worms [43]. These adults migrate through cutaneous and deep connective tissue, producing microfilariae. Ocular disease may be due to both the presence of microfilaria and the presence of the adult worm.

The diagnosis of loiasis is generally made by the detection of circulating microfilariae. In cases of conjunctival involvement, extraction of an adult worm confirms the diagnosis. Therapy of loiasis involves the manual removal of adult worms present in the conjunctiva in addition to the use of diethylcarbamazine (DEC). Severe hypersensitivity responses may occur due to the killing of both microfilariae and adult worms.

3.1.6. Onchocerciasis (River Blindness). It appears that humans are the main reservoir of onchocerciasis, with infection occurring from the bite of an infected female blackfly, *Simulium* spp. that require fast-flowing water for their breeding and development. The disease is restricted to areas adjacent to river systems. An estimated 37 million people in 34 countries in Sub-Sahara Africa and South America are affected by it [44]. After biting an infected person and ingesting microfilariae, the microfilariae mature to the larval stage as they migrate to the proboscis of the fly. There, the larvae may be injected into a human with the next bite, resulting in the formation of an adult worm capable of producing microfilariae. These microfilariae migrate throughout skin and connective tissue, where they die after several years. Adult worms may live in the subcutaneous tissue for years, with a female producing one-half to one million microfilariae yearly. The site of the adult worm is usually found over a bony prominence and may develop into a firm, nontender nodule, or onchocercoma.

Table 2: Ocular parasitosis caused by protozoa (*diagnosis and treatment*).

Disease/Infection	Diagnosis	Treatment
Acanthamoebic keratitis	Corneal scrapings, culture	Propamadine (0.1% solution) + antibacterial preparation, polyhexamethylene biguanide (0.02% solution), chlorhexidine (0.02% solution), keratoplasty.
Chagas' disease	Blood smear, Buffy coat*, culture, Xenodiagnosis*	Nifurtimox, benznidazole
Giardiasis	Diagnosing intestinal disease and exclusion	Metronidazole, albendazole, paromomycin
Leishmaniasis	Tissue smears or biopsy, culture in NNN medium	Antimonials, amphotericin B, Paromomycin, Fluconazole, zinc sulfate
Malaria	BFMP*, Buffy coat, PCR, serological	Chloroquine, Primaquine, Dapsone, Mefloquine, artemisinin derevatives
Microsporidiosis	Corneal scrapings, biopsy, serological	Albendazole
Rhinosporidiosis	Histopathologic demonstration	Dapsone, amphotericin B
Toxoplasmosis	Serology (IgM, IgG), PCR	Clindamycin+ azithromycin, pyrimethamine+ sulfadiazine,

*Buffy coat: The thin layer of concentrated white blood cells that forms when a tube of blood is spun in a centrifuge.
*Xenodiagnosis: a method of animal inoculation using laboratory-bred bugs and animals.
*BFMP: Blood Film for Malaria Parasite.

It is the migration of microfilariae through skin and connective tissue which is responsible for the majority of clinical findings in onchocerciasis. Ocular onchocerciasis is due to the presence and/or migration of microfilariae in and through ocular structures as well as the host's response to the migration [45]. There are five predominant ocular findings that correlate with the location of microfilariae: punctate keratitis, sclerosing keratitis, iridocyclitis, chorioretinitis, and optic atrophy. Other findings may include distortion of the pupil, which may also be covered with exudate.

Wolbachia and Wolbachia-derived molecules are bacterial symbionts of *O. volvulus* that is implicated in the pathogenesis. Experiments using Wolbachia-containing extracts of *O. volvolus* in a mouse model of onchocercal keratitis demonstrated that the presence of the bacteria was essential for neutrophil-mediated inflammation, opacity, and corneal haze [46].

The diagnosis of onchocerciasis is accomplished by a combination of clinical symptoms and signs with histopathologic examination of specimens. Slit lamp examination may confirm the presence of microfilariae in the anterior chamber. A sclerocorneal punch biopsy may aid in the diagnosis as well [47]. Rarely, microfilariae are demonstrated in blood and/or urine samples. PCR may aid in the diagnosis of disease associated with a low burden of microfilariae. Xenodiagnosis, using laboratory-bred blackflies, may provide a clue as well.

Traditional therapy has centered on the use of DEC, but this is active only against microfilariae, allowing adult worms to repopulate the microfilariae in several months.

Ivermectin is the treatment of choice and mass distributed by the WHO Onchocerciasis Control and the Onchocerciasis Elimination Programme for the Americas. This had led to dramatic improvements in disease control to the extent that elimination has become a realistic target [48].

3.1.7. Thelaziasis. Transmission of eyeworms occurs via nonbiting diptera that feed on the ocular secretions, tears, and conjunctiva of animals. The disease, thelaziasis, is characterized by a range of subclinical to clinical signs such as epiphora, conjunctivitis, keratitis, corneal opacity, and ulcers [49]. The adult and larval stages are responsible for eye disease. Asymptomatic, subclinical thelaziasis occurs mainly when only the male nematodes parasitize animals, whereas evident symptoms have been more frequently registered in the presence of gravid females [50]. The lateral serration of the *Thelazia* cuticle causes mechanical damage to the conjunctival and corneal epithelium.

Collected nematodes are identified based on morphologic key [51]. *T. callipaeda* nematodes have a serrated cuticle, buccal capsule, mouth opening with a hexagonal profile, and 6 festoons. For treatment of human cases, the removal of the worm is suggested. Topical treatment with thiabendazole has also been reported to kill the worms.

3.1.8. Toxocariasis. Larva migrans in man are a disease characterized by inflammatory reaction around or in the wake of migrating larvae, most commonly larvae of nematode parasites of other animals. For some of the larva migrans producing larvae, man is merely an accidental but more or less normal intermediate or paratenic host.

Toxocariasis is an important cause of unilateral visual loss and leukocoria in infants, and as a differential diagnosis of retinoblastoma. *Visceral larva migrans* are best known in the form produced by the larvae of *Toxocara canis*, these having been identified in autopsy specimens of lungs, liver, brain, and in several enucleated eyes [52]. Human infection by a spiruroid form of nematode *Gnathostoma spinigerum* has been reported sporadically from Thailand, the Philippines, China, Japan, and India. The high prevalence may be increasing in areas whereby freshwater raw fish is customary. Palpebral oedema with conjuctival erythema developed when lesions developed near the eye. Intraocular parasites occur so rarely that they are considered as ophthalmological curiosities, nevertheless, it can cause intraocular hemorrhage, uveitis, and loss of vision within 2 days [53]. Following surgical removal, treatment is with albendazole and topical corticosteroids.

3.1.9. Trichinosis. Trichinosis is a parasitic disease which probably presents itself for diagnosis not infrequently. Because of its varied symptomatology trichinosis is, unless by chance, almost as frequently undiagnosed. This is evidenced by the comparatively few cases reported in the literature. Ocular trichinosis can manifest itself as oedema of the face especially around the eyes, conjunctivitis, and exophthalmoses. Diagnosis is only confirmed by finding the worm in a section of the excised muscle (see Tables 3 and 4).

3.2. Eye Infection Caused by Flat Worms

3.2.1. Cysticercosis. This helminthic infection caused by the larval cysts of the pork tapeworm (*Taenia solium*). Infection is often asymptomatic though neurological symptoms—predominantly seizures—are the most common manifestation. Ocular involvement is well recognised and includes orbital, intraocular, subretinal, and optic nerve lesions [54–57]. Cysticercosis can be evident as a free-floating cyst with amoeboid movements within the vitreous or anterior chamber of the eye. Gaze palsies may also occur secondary to intramuscular cysts or cranial nerve lesions from intracerebral cysts.

Diagnosis depends on imaging with ultrasound, MRI, and CT scanning all being useful, depending on the location of the cysts [58, 59]. Serology can be useful but in cases of isolated cysts may be negative.

Treatment is largely with the antihelminthic albendazole. Antihelminthic therapy may lead to an increased inflammatory reaction around the lesions, and for this reason corticosteroids are often used when treating neurological or ocular disease. Spontaneous extrusion of cysts from the orbit may occur, and surgery may be required for isolated ocular lesions when they are growing and causing visual loss.

3.2.2. Fascioliasis. Fasciola hepatica is a zoonotic helminth that is prevalent in most sheep-raising countries. In 1989, an outbreak of human infestation of more than 10 000 cases living in Guilan Province, Iran was reported [60]. The biliary duct of the liver is the main site of establishment of the parasite. However, immature flukes may deviate during migration, entering other organs, and causing an ectopic infestation [61]. In humans, ectopic locations in the orbit [62, 63] have been reported. Identification of the route of entry of the parasite larva into the anterior chamber of the eye is difficult. One possible route can be via the central retinal artery into the vitreous, causing vasculitis and endophthalmitis [64]. Severe intraocular reaction, haemorrhage, diffuse vasculitis, and retinal ischaemia of the patient may be caused as a result of the presence or irritation of the parasite. Early vitrectomy and removal of the parasite resulted in a rapid response, with reasonable final visual acuity.

3.2.3. Hydatid Cyst. Hydatid cysts are most commonly seen in the liver (60–70%) and lungs (20%) [65]. Hydatid disease involving the orbit represents <1% of all cases of hydatid disease [66] and requires surgical treatment.

Definitive preoperative diagnosis is difficult [67]. Laboratory and immunologic tests are generally unhelpful. From the literature and our own observations, orbital hydatid cysts usually appear as a well-defined, thin-walled, oval shape lesions with fine peripheral rim enhancement of their fibrous capsule after contrast medium administration [68].

3.2.4. Schistosomiasis. Various theories have been postulated as to the different routs by which the schistosoma ova or even the adult worms can reach the systemic circulation and then after lodged in ectopic sites such as the eyes. Cercariae (the infective stage) develop to maturity and lay their eggs in the veins directly under the skin or the mucous membrane through which they have penetrated if the part is richly vascularised [69]. The presence of schistosomal eggs in the eye can produce granuloma formation and inflammatory sequelae [70]. Considering how common the infection is in endemic areas, involvement of the eye is incredibly rare.

Effective treatment, using the drug praziquantel, has been available for 25 years, but the growth of human populations in high-risk areas, as well as the high probability of rapid reinfection after treatment, has thwarted efforts to control the number of human infections worldwide [71] (see Tables 5 and 6).

4. Ocular Infestation Caused by Ectoparasites

4.1. Myiasis. Ocular myiasis is the result of invasion of the eye by larvae of flies. Genera important to human myiasis include *Dermatobia*, *Gasterophilus*, *Oestra*, *Cordylobia*, *Chrysomia*, *Wohlfahrtia*, *Cochliomyia*, and *Hypoderma*. Ophthalmomyiasis may be categorized into three categories: ophthalmomyiasis externa, ophthalmomyiasis interna, and orbital myiasis [72].

Ophthalmomyiasis externa is usually seen in areas of shepherding and is typically due to larvae of the sheep nasal botfly, *Oestra ovis* [73]. A crawling or wriggling sensation accompanied by swelling and cellulitis may be seen in palpebral myiasis.

Table 3: Ocular parasitosis caused by round worms (*geographical distribution & ocular findings*).

Disease/Infection	Causative agent	Geographical distribution	Ocular findings
Angiostrongyliasis	*Angiostrongylus cantonensis*	Southeast Asia, Pacific region, eastern Australia	Blurred vision and poor visual acuity
Bancroftian and Brugian filariasis	*W. bancrofti, B. malayi*	Southeast Asia	Retinal vasculities, decreased vision and panuveitis with secondary glaucoma
Baylisascariasis	*Baylisascaris procyonis*	Few records in US, Japan, Germany	Vision loss, transient visual obscuration, and diffuse unilateral subacute neuroretinitis
Dirofilariasis	*Dirofilaria repens*	Europe, Asia and Africa	Pain, oedema, and congestion of the conjunctiva, diplopia, foreign body sensation in the eye
Loiasis	*Loa loa*	Central and West Africa	Conjunctival congestion and pain with movement of the eye. May affect vision transiently. Retinal hemorrhages and perivascular inflammation
Onchocerciasis	*Onchocerca volvulus*	Tropical Africa, South America, and the Arabian peninsula	Chorioretinitis, keratitis, uveitis, corneal opacification, neovascularisation, blindness
Thelaziasis	*Thelazia callipaeda*	Asian Pacific region	Epiphora, conjunctivitis, keratitis, corneal opacity and ulcers
Toxocariasis	*Toxocara canis* and *Toxocara cati*	Wide spread	Peripheral white mass is often visible in affected eyes
Trichinosis	*Trichinella spiralis*	Central and eastern Europe, united States	Oedema around the eye, conjunctivitis and exophthalmoses

Table 4: Ocular parasitosis caused by round worms (*diagnosis and treatment*).

Disease/Infection	Diagnosis	Treatment
Angiostrongyliasis	Identification of *Angiostrongylus cantonensis* in the eye Direct and indirect immunofluorescent	Oral and topical prednisolone, laser treatment, surgical removal of the parasite
Bancroftian and Brugian filariasis	An aqueous tap and a peripheral blood smear isolate microfilariae or adult worm	Carbamazine citrate along with systemic steroids
Baylisascariasis	Exclusion of other known causes of OLM*	Steroids and antihelminthic agents
Dirofilariasis	Excision biopsy	Surgical excision of the adult worm, DEC*
Loiasis	Extraction of adult worm or microfilaria	Manual removal of adult worm or microfilaria present in the conjunctiva and DEC
Onchocerciasis	Slit lamp, sclerocorneal punch biopsy, Xenodiagnosis	Manual removal of adult worms, ivermectin or mebendazole
Thelaziasis	Eggs or larvae can be seen when tears or other eye secretions are examined under light microscope	Surgical
Toxocariasis	Serology	Cryopexy and photocoagulation, albendazole and corticosteroid
Trichinosis	Muscle biopsy	Thiabendazole, mebandazole, steroids

*OLM: ocular larva migrans.
*DEC: diethylcarbamazine.

Table 5: Ocular parasitosis caused by flat worms (*geographical distribution & ocular findings*).

Disease/Infection	Causative agent	Geographical distribution	Ocular findings
Cysticercosis	*Cysticercus cellulosae*	Mexico, Central America, Indian subcontinent, Far East and Africa	Subconjunctival and eyelid masses, papilloedema, cranial nerve palsies, vitritis and optic neuritis
Fascioliasis	*Fasciola hepatica*	Africa and Asia	Painful red eye, and there may be visual defect
Hydatid cyst	*Echinococcus granulosus*	South America, Australia, Middle East and Mediterranean countries	Orbital swelling, exophthalmus and proptosis
Schistosomiasis	*S. mansoni, S. haematobium, S. japonicum*	Sub-Sahara Africa, China, South Asia	Uveitis and subretinal granuloma

Table 6: Ocular parasitosis caused by flat worms (*diagnosis and treatment*).

Disease/Infection	Diagnosis	Treatment
Cysticercosis	Imaging with ultrasound, MRI and CT Serology can be useful	Albendazole, corticosteroids
Fascioliasis	Adult worm in the eye	Vitrectomy and removal of the parasite
Hydatid cyst	Imaging	Surgical removal
Schistosomiasis	Eggs in the feces, urine or eggs/cercariae in the eye	Praziquantel

Ophthalmomyiasis interna is most commonly caused by a single larva of the *Hypoderma* spp. Infection is due to invasion of the tissues, leading to uveitis. More serious complications may include lens dislocation and retinal detachment [74].

Orbital myiasis may be due to a number of fly species and is generally seen in patients who are unable to care for themselves [75].

Diagnosis of ophthalmomyiasis is made by demonstration of maggots, and histologic examination may show granuloma formation. Anticholinesterase ointment may help kill or paralyze the larvae. Steroids and antibiotics may be necessary to control inflammation and secondary bacterial infection.

4.2. Phthiriasis Palpebrum. Lice belong to the order Anoplura. Of these, medically important species include *Pediculus humanus* var. *corporis*, the human body louse; *Pediculus humanus* var. *capitis*, the human head louse; *Phthirus pubis*, the crab louse. Depending on the species, eggs, or nits, are laid and glued to body hairs or clothing fibers. Following this, nymphs emerge to feed on the host, giving rise to symptoms of pruritis. Of the species mentioned, *P. pubis* is most likely to involve the eyebrows and eye lashes. In addition to pruritis, small erythematous papules with evidence of excoriation may be present. Involvement of the eyelash may cause crusting of the lid margins. In this case, diagnosis is relatively simple as nits are easily seen at the base of the eyelash [76].

Eyelid disease is treated with a thick layer of petrolatum twice a day for 8 days, or the application of 1% yellow oxide of mercury four times a day for 2 weeks [77].

4.3. Tick Infestation. Ticks are arthropods belonging to the class Arachnida. There are a number of different species of ticks which may cause disease in humans and animals. Ticks exist in three life stages—larva, nymph, and adult—all of which requires blood meals. Most tick bites are uncomplicated, and prompt removal of the tick is all that is necessary [78]. Ticks have been reported to attach to ocular structures. In one such case, the nymph was associated with a stinging sensation [79]. Following the removal of the tick, a firm nodule, representing a tick bite granuloma, may remain for several weeks. This granuloma likely represents retained tick material and generally resolves spontaneously.

5. Search Strategy and Selection Criteria

This is a comprehensive paper of the parasitic diseases/infections of the eye. The majority of the clinically important species of parasites involved in eye infections are reviewed in this paper. Parasites are discussed by the disease or infection they cause. Emphases have been placed on literatures published within the past decade, but prior noteworthy reviews and case reports are included.

We searched the MEDLINE database via PubMed and identified articles by cross-referencing the terms ocular, eye, ophthalmic, retinitis, endophthalmitis, conjunctivitis, and uveitis to specific infectious diseases in adults. We

Table 7: Ocular parasitosis caused by ectoparasites (*geographical distribution & ocular findings*).

Disease/Infection	Causative agent	Geographical distribution	Ocular findings
Myiasis	Larvae of flies	Shepherding places	Uveitis, lens dislocation and retinal detachment
Phthiriasis palpebrum	*Phthirus pubis*	Cosmopolitan	Crusting of the eyelid margins
Tick infestation	Hard and soft ticks	Cosmopolitan	Stinging sensation

Table 8: Ocular parasitosis caused by ectoparasites (*diagnosis and treatment*).

Disease/Infection	Diagnosis	Treatment
Myiasis	Demonstration of maggots, histological examination	Manual removal of the maggots, anticholinesterase ointment
Phthiriasis palpebrum	Nits seen at the base of eyelashes	Manual removal of the lice and nits, thick layer of petrolatum, 1% yellow oxide of mercury
Tick infestation	Biomicroscopy may reveals ticks embedded in conjunctiva, eyelid margins or crawling on the eyelashes	Removal by conjunctival excision

searched the Cochrane database for systematic reviews on the treatment of specific parasitic ocular infections. Additionally, we reviewed texts for completeness and to obtain other references of eye complications of systemic infections (see Tables 7 and 8).

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Research Article

Prevalence and Risk Factors of *Toxoplasma gondii* Infection in Domestic Cats from the Tropics of Mexico Using Serological and Molecular Tests

Virgen J. Castillo-Morales,[1] Karla Y. Acosta Viana,[2] Eugenia del S. Guzmán-Marín,[2] Matilde Jiménez-Coello,[2] José C. Segura-Correa,[1] A. J. Aguilar-Caballero,[1] and Antonio Ortega-Pacheco[1]

[1] *Departamento de Salud Animal y Medicina Preventiva, CA Salud Animal, Facultad de Medicina Veterinaria y Zootecnia, Universidad Autonoma de Yucatán, Km. 15.5 Carretera, Merida-Xmatkuil, Apd. 4-116, Merida, YUC, Mexico*
[2] *Laboratorio de Biología Celular, CA Biomedicina de Enfermedades Infecciosas y Parasitarias, Centro de Investigaciones Regionales, Centro de Investigaciones Regionales "Hideyo Noguchi", Universidad Autónoma de Yucatán, Avenida Itzáes 490, 97000 Mérida, YUC, Mexico*

Correspondence should be addressed to Antonio Ortega-Pacheco, opacheco@uady.mx

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The aim of this study was to determine the prevalence and risk factors associated with *Toxoplasma gondii* infection in domestic cats using an indirect-ELISA (IgM and IgG) and PCR. Samples collected from 220 cats from Merida, Yucatan, Mexico, were analyzed. Cases were reported as acute or chronic. Cases when positive to IgM and IgG and PCR were considered as reactivated chronic infection. Risk factors (sex, age, body condition, diet access to hunting, and number of cats in home) were assessed with a multivariate analysis, 75.5% (166/220) of the cats were IgM and 91.8% (202/220) IgG-seropositive and 79% were PCR-positive (173/220). Number of cats per household and low body condition score were associated with reactivated chronic infection ($P < 0.05$). It is concluded that *T. gondii* is scattered in the studied population with several periods of reinfection, and therefore an environmental contamination with infecting oocysts exists and there are intrinsic associated factors in cats that increase the risk of becoming infected.

1. Introduction

Toxoplasmosis is a major parasitic zoonoses of worldwide distribution caused by the intracellular protozoa *Toxoplasma gondii*. Felines, in particular domestic cats, have an important role in the epidemiology of the disease because the sexual (and asexual) reproduction takes place in them and they excrete a large number of infective and environmentally resistant oocysts [1, 2]. This parasite infects most mammals (including humans) and birds which are intermediary hosts. Among vertebrates, food animals are particularly important because they are intended for human consumption [3, 4]. Cats primarily become infected when they ingest the encysted tissue cyst from the intermediate hosts like rodents and birds. Human acquire infection when they ingest sporulated oocysts from the soil, contaminated water sources, tissue cysts from raw or undercooked meat, or transplacentally [2, 5]. Infection in cats frequently takes place as subclinical or asymptomatic disease and is very rare to develop evident clinical signs [6]. Toxoplasmosis in cats is reported worldwide with seroprevalences ranging from 60 to 90%. It is probably due to different diagnostic techniques used in each of the reported studies, cultural factors and environment of every region promoting the persistence of infective oocysts [2, 7]. In Mexico, the reported prevalence of cat toxoplasmosis varies from 9.3% to 70.8% [8–11] depending on the region. Associated risk factors for infection in cats included feeding with raw meat [8, 10], age and sex

[10], and access to hunting [12]. In Mexico the seroprevalence of human toxoplasmosis is estimated to be 25% [13]. In Yucatan, Mexico, no epidemiological information about toxoplasmosis in cats is available despite the presence of the infection in humans, with a seroprevalence of 57.5% [14] and the tragic consequences associated with toxoplasmosis in AIDS patients [15].

There are several diagnostic methods for determining infection of *T. gondii*. The most widely used serological test for diagnosis of toxoplasmosis is the indirect ELISA (IgM and IgG). Moreover, the polymerase chain reaction (PCR) is a molecular test which allows detection of parasite DNA; it is highly specific and sensitive and very useful together with serological tests to differentiate the chronic, acute or reactivated infections. PCR is also an important diagnostic method when the immune system of the patient is compromised or when antibody titers have no reach threshold levels of detection [16, 17]. In domestic cats, several diseases may affect the overall health and cause immunosuppression such as infection with Bartonella spp. feline immunodeficiency virus (FIV) and feline leukemia virus (FeLV) [18, 19] that may increase the effects of acute toxoplasmosis [20, 21].

The information on the prevalence of toxoplasmosis in cats is useful for evaluating environmental contamination with the protozoa and to evaluate the risk it poses to public health. The aim of this study was to estimate the prevalence of toxoplasmosis in domestic cats in Merida, Yucatan, Mexico, using serological and molecular methods, and to determine possible risk factors (sex, age, diet, hunting, body condition, and number of cats per household).

2. Material and Methods

2.1. Study Area and Population. A cross-sectional study was performed in the city of Merida, Yucatan, (19° 30′ and 21° 35′ north latitude, 87° 30′ and 90° 24′ west longitude). The city's climate is characterized by warm subhumid with summer rainfall and average temperature of 26°C. The relative humidity is maximum 83% and minimum 61% with a height of 6 meters above sea level [22]. The population of interest were cats (*Felis silvestris catus*) living in Merida. The inclusion criteria were domiciled cats, of any race or sex, and older than 3 months of age.

2.2. Sample Size. A pilot study in 40 cats measuring IgG antibodies specific for *T. gondii* using an indirect ELISA test showed a seroprevalence of 82.5% which was used as the expected prevalence in the calculation of sample size. It was considered an infinite population of cats, a confidence level of 95% and an absolute accuracy of 5%, calculated a sample size of 222 cats [23].

2.3. Collection of Samples. A total of 231 blood samples (1–3 mL) were taken by puncturing the jugular vein and deposited in two vacutainer tubes, with and without anticoagulant, the first for the PCR test and the second for serological testing. DNA extraction was performed with the commercial kit DNeasy Blood and Tissue (QIAGEN, cat no. 69 506). Before extraction, a prelysis of blood was conducted as suggested by Jalal et al. [24]. The mean volume of DNA extraction from each cat was 100 μL. Samples were stored at −20°C until further PCR assay. From the collected samples, 11 were not viable for diagnostic testing (negative samples by PCR for beta-globin constitutive gene) and were discarded leaving a total of 220 samples.

2.4. Risk Factors. Through a questionnaire survey to each owner, information was obtained to assess the following risk factors.

Age. Age was determined based on the review of the animal or based on their dentition [25, 26]. The animals were considered in two groups: ≤1 year and >1 until 7 years.

Sex. Male or female.

Type of diet. Two categories were considered: cats fed only with commercial food and cats fed with commercial food and/or raw meat.

Access to the hunting. If the cat had any access to hunting.

Number of cats per household. Categorized as one or more than one cat at home.

Body condition. It was divided into two groups: cats with good body condition and body condition regular to bad [27].

2.5. Indirect ELISA Test. The presence of specific IgM and IgG antibodies against *T. gondii* was determined separately by the use of indirect ELISA tests (Human-GmbH, Wiesbaden, GER), The technique used was adapted to that described by Figueroa et al. [28], but using anti-IgM and IgG cat antibodies labeled with horseradish peroxidase (HRP) (Santa Cruz Inc., CA, USA) on 96-well plate coated with sonicated parasite proteins from tachyzoites of *T. gondii*. Serum samples were diluted to a ratio of 1 : 100 in phosphate-buffered saline (PBS; pH 7.2). The secondary goat anti-IgG and anti-IgM cat antibody HRP labeled were used at a dilution of 1 : 5,000. Sera from cats showing high anti-IgG antibodies titer by ELISA (1 : 1024) and positive results to PCR against *T. gondii* were used as positive controls, and sera pool from 10 healthy cats previously tested by triplicate with ELISA IgM, IgG and PCR, were used as negative controls. On the basis of the indirect ELISA results, subjects were diagnosed as either positive/negative for specific IgG and IgM antibodies to *T. gondii*. The optical density (OD) was measured in a spectrophotometer at 450 nm (Multiskan Multisoft Primary EIA) and was used to compute the percent positivity (PP) using the formula mean OD (sample or negative control) divided by the mean OD value positive control multiplied by 100. Percent positivity of 15% or above was considered as positive.

2.6. Polymerase Chain Reaction. The PCR was performed as described by [24]. Primers Tg1 (5′-AAAAATGTGGGAATG-AAAGAG-3′) and Tg2 (5′-ACGAATCAACGGAACTGT-AAT-3′) that amplify a fragment of 469 base pairs (bp) from the B1 gene of *T. gondii* were used. Each PCR reaction was carried out at a final concentration of 0.4 mM dNTPs, 2.5 μmol of each primer, 1.5 mM $MgCl^2$, and 1U of GoTaq

(PROMEGA) DNA polymerase with its corresponding colorless buffer (1X), 3 μL of DNA from each cat evaluated were used and the reaction was completed in a final volume of 25 μL. Amplification conditions were 95°C for 10 minutes for denaturalization, followed by 35 cycles of 94°C for 1 minute, 52°C for 30 seconds and 72°C for 1 minute, and a final extension cycle of 72°C for 7 minutes. The PCR products were analyzed by electrophoresis on agarose gels and stained with 1.8% ethidium bromide. As a positive PCR control a plasmid clone pMOSBlue/Toxo 469/3, containing a sequence of 469 pb, amplification product of Tg1 and Tg2 primers of parasite genome was used.

2.7. Statistical Analysis. Prevalence for each serological or molecular result and its combinations was estimated. Because of the small number of cases, Chi-square test and binomial logistic regression were carried out only for IgM, IgG and PCR positive cases. Significant risk factors, under the Chi-square tests, were further investigated using binomial logistic regression. Values were considered significant when $P < 0.05$. Odds ratio (OR) and confidence interval (CI) at 95% were also estimated. Statistical analyses were carried out using the SPSS package v 17.0 (SPSS Inc., Chicago, Il, USA).

3. Results

3.1. Prevalence. Serological prevalence of *T. gondii* was 91.8% and 75.5% for IgG and IgM respectively. One hundred seventy-three samples of DNA (79%) tested amplified a 469 bp fragment corresponding to the expected size of the B1 gene sequence specific for *T. gondii.* An example of amplification is shown in Figure 1. The result of the different serological, molecular, and combination of positive and negative cases is shown in Table 1. Cases were categorized as acute when were only PCR positive or when the patient showed positive titers of IgM + PCR. Chronic cases were considered when patients were only IgG positive, IgG + PCR positive, IgM + IgG positive, or IgM + IgG + PCR positive. Positive IgM + IgG + PCR cases (61.4%), positive IgM + IgG (11.8%) and positive IgG + PCR (13.2%) were the most frequent cases.

3.2. Risk Factors. The binomial logistic regression for cats with positive IgM + IgG + PCR cases showed association with body condition (fair—poor) and the presence of more than one cat at home (Table 2). However, there was no association of the variables sex and type of diet with cats infected with *T. gondii*. Also preliminary Chi-square tests showed not association of the factor cat having access to hunting and toxoplasmosis infection.

4. Discussion

The results of this study indicate a high rate of infection in cats with *T. gondii*, which suggests a high environmental contamination with infective oocysts. There is a wide variation in reported prevalence of feline toxoplasmosis, which is probably due to environmental, cultural, and diversity

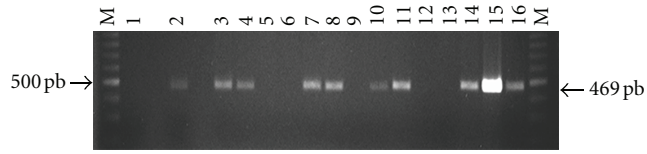

Figure 1: Electrophoresis in 1.8% agarose and ethidium bromide staining of amplified products with primers Tg1 and Tg2 of *T. gondii*. Lanes; (M) molecular weight marker (Promega, 100 bp DNA Ladder), (1) negative control, (2) positive control, (plasmid pMOSBlue/Toxo469/3), and samples from positive cats (3, 4, 7, 8, 10, 11, 14, 15, 16) and from negative cats (5, 6, 9, 12, 13).

of diagnosis techniques and type of molecule measured in each study (IgA, IgM, IgG, genome, etc.). In tropical countries like Colombia, Thailand, and Brazil, reported IgG seroprevalences of toxoplasmosis in cats are 45.2% [29], 11.0% [30], and 40% [31], respectively. In some regions of Mexico the reported seroprevalence in cats is 28.8% in Colima [9], 21.9% in Mexico City [10] and 70.8% in Guadalajara [8]. This study has recorded the highest seroprevalence (91.8%) of IgG antibodies towards *T. gondii* in cats, even higher than that found in Tehran, Iran, where an overall prevalence of 63% ($n = 100$ cats) reported and from which feral cats had a 90% infection [32]. In Parana, Brazil, it was reported that 84.4% of cats were seropositive for IgG [33], which is close to the results obtained in this study. The rainfall and subtropical climate conditions with hot summers of Parana is similar to the tropical climate of the city of Merida, which is favorable to maintain viable oocysts in the environment for long periods of time.

In this study the prevalence of cats seropositive for IgM was 75.5%. In a study by Kodym et al. [34] it was reported that IgM antibodies against *T. gondii* may remain in circulation for 12–18 months, so that the titers of IgM cannot by themselves be indicators of recent infection, which is contrary to what is believed. In our study, the IgM and IgG serological status was complemented with molecular studies for a better determination of the infection status.

The percentage of acute cases of toxoplasmosis found in this study (5.9%) considering only PCR positive cases (1.8%) plus IgM, PCR positive cases (4.1%) are lower than the 8.3% found by Galván et al. [8] in Guadalajara, Mexico, considering only IgM antibodies. Acute cases are not commonly found in survey studies [35] and their presence indicates a constant dynamic of the disease and the high risk of contact of cats with the protozoan. This prevalence of cases defined as acute may depend on the chance of cats to be in contact with infecting cyts through the hunting infected prey or food provided to them by their owners, hence the importance consider adequate food for cats. Early-acute cases are those that were positive by PCR before the production of any immune response. In this study 79% of cats were positive to PCR, but only 1.81% were in the initial stage of infection where there is not production of antibodies [36] or those cats that may have compromised immune system. Suh and Joo [37] reported 5.3% of cats PCR positive to *T. gondii*. However, Lee et al. [38] more recently reported

Table 1: Type and frequency of *T. gondii* infection in cats according to serological and molecular results.

Type	IgM	IgG	PCR	*n*	%
Acute	−	−	+	4	1.8
	+	−	+	9	4.1
Chronic	−	+	−	12	5.5
	−	+	+	29	13.2
	+	+	−	26	11.8
	+	+	+	135	61.4
Negative	−	−	−	5	2.2
Total				220	100

Table 2: Binomial logistic regression to evaluate the association between studied variables and *T. gondii* infection cases from chronically reactivated cats. Chronically reactivated cases (positive to IgM, IgG, and PCR).

	β	SE	OR	CI	*P*
Age (>1–7 Years)	0.489	0.294	1.63	0.916–2.903	0.097
Cats per household (>1)	−0.714	0.292	0.49	0.276–0.869	0.015
Body condition (Regular-bad)	0.928	0.338	2.53	1.306–4.904	0.006

SE: standard error. OR: odds ratio. CI: confidence interval. *P*: *P* value.

47.2% of positive cats by using nested PCR both in South Korean conditions.

A high number of chronic cases should be expected while studying cat populations. Traditionally, cats seropositive for IgG are considered as chronically infected [6]. In this study 91.9% of cats were in the chronic stage of infection but with different serological and/or molecular status. When considering a constant exposure to the protozoa the immune response and circulation of the genome in the hot may differ giving as result the variety of diagnostic situations here reported.

Cases when cats were IgG positives but with high levels of IgM are an indicator of revival [21]. But they also may indicate a switching from IgM to IgG weeks after infection. Reactivation in chronically infected stages involves reactivation of cysts and conversion of bradyzoites to tachyzoites and not necessarily involves a new enteroepitelial phase and excretion of oocysts [6]. However, IgM not necessarily indicates a recent infection but the outcome of the combination of different antibodies and molecular studies may be useful to confirm the chronically reactivated stage; the recirculation of the parasite in blood and serological tests indicate that there was a previous immune response.

Most cats in the chronic stage found in this study were IgM + IgG + PCR positive and are proposed to be named as chronic reactivated cases; this means that not only the parasite is circulating in the environment producing constant contact with the cats, but there are periods of reactivation with or without excretion of oocysts depending on its immune system. In chronically infected hosts with tissue cysts, these may be rupture and release bradyzoites to the circulation [39] which are eventually destroyed by the immunocompetent host or reactivate the infection in the immunosuppressed animal [40].

It is expected that cats having access to the streets be more likely to hunt and become infected with *T. gondii*. However, in the present study no association was found between cats gaining access to the streets and hunting than those kept indoors. The nocturnal gecko *Hemidactylus frenatus* is commonly found inside all households in the area of the study which are predated by cats and ten infected; these in part may explain the results founded.

In chronic reactivated cases, a significant protective association was found with more than one cat per household. This result is difficult to explain; however, it indicates that when living with other cats, they are less likely to be infected and reinfected with *T. gondii*, similarly as reported in cats from México city which indicates a change in environmental conditions and different immune status of cats living together [10].

Body condition has been used as an indicator of poor nutrition in small animals and consequently a weakened immune status, increased susceptibility to disease and longer periods of parasitemia including those caused by protozoa [40, 41]. In the present study regular-to-poor body condition was significantly associated with chronic reactivated cases, indicating some degree of release and circulation of *T. gondii* oocysts. Cats with immunosuppressive diseases, poor body condition are those suffering with periods of *T. gondii* reactivation [42, 43].

Finally, it is expected that with age, the risk of contact with the agent and infection occurs, but results from the present study indicates that cats become infected very early during their life.

It is concluded that *T. gondii* is widely widespread in the domestic owned cat population from Merida city with various periods of reinfection and therefore there is a high environmental contamination with infective oocysts. Cats over one year old, with a poor body condition, increase the risk of becoming infected with *T. gondii*.

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Review Article

Leprosy: An Overview of Pathophysiology

Ramesh Marne Bhat and Chaitra Prakash

Department of Dermatology, Father Muller Medical College, Karnataka, Mangalore 572002, India

Correspondence should be addressed to Ramesh Marne Bhat, rameshderma@gmail.com

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Leprosy, also known as Hansen's disease, is a chronic infectious disease caused by *Mycobacterium leprae*, a microorganism that has a predilection for the skin and nerves. The disease is clinically characterized by one or more of the three cardinal signs: hypopigmented or erythematous skin patches with definite loss of sensation, thickened peripheral nerves, and acid-fast bacilli detected on skin smears or biopsy material. *M. leprae* primarily infects Schwann cells in the peripheral nerves leading to nerve damage and the development of disabilities. Despite reduced prevalence of *M. leprae* infection in the endemic countries following implementation of multidrug therapy (MDT) program by WHO to treat leprosy, new case detection rates are still high-indicating active transmission. The susceptibility to the mycobacteria and the clinical course of the disease are attributed to the host immune response, which heralds the review of immunopathology of this complex disease.

1. Introduction

Leprosy, also known as Hansen's disease, is a chronic infectious disease caused by *Mycobacterium leprae*, a microorganism that has a predilection for the skin and nerves. Though nonfatal, leprosy is one of the most common causes of nontraumatic peripheral neuropathy worldwide. The disease has been known to man since time immemorial. DNA taken from the shrouded remains of a man discovered in a tomb next to the old city of Jerusalem shows him to be the earliest human proven to have suffered from leprosy. The remains were dated by radiocarbon methods to 1–50 A.D. [1]. The disease probably originated in Egypt and other Middle Eastern countries as early as 2400 BCE. An apparent lack of knowledge about its treatment facilitated its spread throughout the world. *Mycobacterium leprae*, the causative agent of leprosy, was discovered by G. H. Armauer Hansen in Norway in 1873, making it the first bacterium to be identified as causing disease in humans [2, 3]. Over the past 20 years, the WHO implementation of MDT has rendered leprosy a less prevalent infection in 90% of its endemic countries with less than one case per 10,000 population. Though, it continues to be a public health problem in countries like Brazil, Congo, Madagascar, Mozambique, Nepal, and Tanzania [4].

2. *Mycobacterium leprae*

M. leprae, an acid-fast bacillus is a major human pathogen. In addition to humans, leprosy has been observed in nine-banded armadillo and three species of primates [5]. The bacterium can also be grown in the laboratory by injection into the footpads of mice [6]. Mycobacteria are known for their notoriously slow growth. With the doubling time of 14 days, *M. leprae* has not yet been successfully cultured in vitro [7, 8]. The genome of *M. leprae* has been sequenced in totality [9]. It presents with less than 50% coding capacity with a large number of pseudogenes. The remaining *M. leprae* genes help to define the minimal gene set necessary for in vivo survival of this mycobacterial pathogen as well as genes potentially required for infection and pathogenesis seen in leprosy.

M. lepromatosis is a newly identified mycobacterium which is described to cause disseminated leprosy whose significance is still not clearly understood [10, 11].

3. Genetic Determinants of Host Response

Human genetic factors influence the acquisition of leprosy and the clinical course of disease [12]. Single-nucleotide

polymorphism (SNP) association studies showed a low lymphotoxin-α (LTA)-producing allele as a major genetic risk factor for early onset leprosy [13]. Other SNPs to be associated with disease and/or the development of reactions in several genes, such as vitamin D receptor (VDR), TNF-α, IL-10, IFN-γ, HLA genes, and TLR1 are also suggested [14–17]. Linkage studies have identified polymorphic risk factors in the promoter region shared by two genes: PARK2, coding for an E3-ubiquitin ligase designated Parkin, and PACRG [18]. A study also suggests that NOD2 genetic variants are associated with susceptibility to leprosy and the development of reactions (type I and type II) [19].

4. Transmission

Two exit routes of *M. leprae* from the human body often described are the skin and the nasal mucosa. Lepromatous cases show large numbers of organisms deep in the dermis, but whether they reach the skin surface in sufficient numbers is doubtful [20]. Although there are reports of acid-fast bacilli being found in the desquamating epithelium of the skin, there are reports that no acid-fast bacilli were found in the epidermis, even after examining a very large number of specimens from patients and contacts [21]. However, fairly large numbers of *M. leprae* were found in the superficial keratin layer of the skin of lepromatous leprosy patients, suggesting that the organism could exit along with the sebaceous secretions [22]. The quantity of bacilli from nasal mucosal lesions in lepromatous leprosy ranges from 10,000 to 10,000,000 [23]. Majority of lepromatous patients show leprosy bacilli in their nasal secretions as collected through blowing the nose [24]. Nasal secretions from lepromatous patients could yield as much as 10 million viable organisms per day [25].

The entry route of *M. leprae* into the human body is also not definitively known. The skin and the upper respiratory tract are most likely; however, recent research increasingly favours the respiratory route [26, 27].

5. Incubation Period

Measuring the incubation period in leprosy is difficult because of the lack of adequate immunological tools and slow onset of the disease. The minimum incubation period reported is as short as a few weeks and this is based on the very occasional occurrence of leprosy among young infants [28]. The maximum incubation period reported is as long as 30 years, or over, as observed among war veterans known to have been exposed for short periods in endemic areas but otherwise living in nonendemic areas. It is generally agreed that the average incubation period is between three and ten years [29].

6. Risk Factors

Those living in endemic areas with poor conditions such as inadequate bedding, contaminated water, and insufficient diet, or other diseases that compromise immune function are at highest risk for acquiring *M. leprae* infection. There has been concern that coinfection with HIV might exacerbate the pathogenesis of leprosy lesions and/or lead to increased susceptibility to leprosy as it is seen with tuberculosis. However, HIV infection has not been reported to increase susceptibility to leprosy, impact on immune response to *M. leprae*, or to have a significant effect on the pathogenesis of neural or skin lesions to date [30, 31]. On the contrary, initiation of antiretroviral treatment has been reported to be associated with activation of subclinical *M. leprae* infection and exacerbation of existing leprosy lesions (type I reaction) likely as part of immune reconstitution inflammatory syndrome [32–34].

7. Interaction of *M. leprae* with Schwann Cells and Macrophages

Schwann cells (SCs) are a major target for infection by *M. leprae* leading to injury of the nerve, demyelination, and consequent disability. Binding of *M. leprae* to SCs induces demyelination and loss of axonal conductance [35]. It has been shown that *M. leprae* can invade SCs by a specific laminin-binding protein of 21 kDa in addition to PGL-1 [36, 37]. PGL-1, a major unique glycoconjugate on the *M. leprae* surface, binds laminin-2, which explains the predilection of the bacterium for peripheral nerves [37]. The identification of the *M. leprae*-targeted SC receptor, dystroglycan (DG), suggests a role for this molecule in early nerve degeneration [38]. *Mycobacterium leprae*-induced demyelination is a result of direct bacterial ligation to neuregulin receptor, ErbB2 and Erk1/2 activation, and subsequent MAP kinase signaling and proliferation [39].

Macrophages are one of the most abundant host cells to come in contact with mycobacteria. Phagocytosis of *M. leprae* by monocyte-derived macrophages can be mediated by complement receptors CR1 (CD35), CR3 (CD11b/CD18), and CR4 (CD11c/CD18) and is regulated by protein kinase [40, 41]. Nonresponsiveness towards *M. leprae* seems to correlate with a Th2 cytokine profile.

8. Disease Classification

Leprosy is classified within two poles of the disease with transition between the clinical forms [42]. Clinical, histopathological, and immunological criteria identify five forms of leprosy: tuberculoid polar leprosy (TT), borderline tuberculoid (BT), midborderline (BB), borderline lepromatous (BL), and lepromatous polar leprosy (LL). Patients were divided into two groups for therapeutic purposes: paucibacillary (TT, BT) and multibacillary (midborderline (BB), BL, LL) [43]. It was recommended later that the classification is to be based on the number of skin lesions, less than or equal to five for paucibacillary (PB) and greater than five for the multibacillary (MB) form.

9. Clinical Features (Table 1)

9.1. Indeterminate Leprosy. Indeterminate (I) is a prelude to the determinate forms of leprosy [44, 45]. It is characterized by an ill-defined, bizarre hypopigmented macule(s) with

Table 1: Clinical features of leprosy.

Characteristics	Tuberculoid	Borderline tuberculoid	Midborderline	Borderline lepromatous	Lepromatous leprosy
Number of lesions	Single or upto 3	A Few (up to 10)	Several (10–30)	Numerous, asymmetrical (>30)	Innumerable, symmetrical
Size	Variable, usually large	Variable, some are large	Variable	Small, some can be large	Small
Surface changes	Hypopigmented	Dry, scaly, look bright, and infiltrated	Dull or slightly shiny	Shiny	Shiny
Sensations	Absent	Markedly diminished	Moderately diminished	Slightly diminished	Minimally diminished
Hair growth	Nil	Markedly diminished	Moderately diminished	Slightly diminished	Not affected initially
Skin smear	Negative	Negative or 1+	1–3+	3–5+	Plenty, including globi (6+)
Lepromin test	Strongly positive	Weakly positive	Negative	Negative	Negative

a smooth or scaly surface. The sensations over the macule may or may not be impaired. The nerve proximal to the patch may or may not be thickened.

9.2. Polyneuritic Leprosy. Manifesting with only neural signs without any evidence of skin lesions, polyneuritic leprosy mostly well recognized in the Indian subcontinent. The affected nerves are thickened, tender, or both. Localized involvement of the nerves may form nerve abscesses [46].

9.3. Histoid Leprosy. Histoid leprosy is relatively uncommon, distinct clinical, and bacteriologic and histopathologic expression of multibacillary leprosy [47]. It may occur as a primary manifestation of the disease or in consequence to secondary drug resistance to dapsone following irregular and inadequate monotherapy. It manifests as numerous cutaneous nodules and plaques primarily over the back, buttocks, face, and bony prominences.

10. Histopathological Reactions

Histopathologically, skin lesions from tuberculoid patients are characterized by inflammatory infiltrate containing well-formed granulomas with differentiated macrophages, epithelioid and giant cells, and a predominance of $CD4^+$ T cells at the lesion site, with low or absent bacteria. Patients show a vigorous-specific immune response to *M. leprae* with a Th1 profile, IFN-γ production, and a positive skin test (lepromin or Mitsuda reaction).

Lepromatous patients present with several skin lesions with a preponderance of $CD8^+$ T cells in situ, absence of granuloma formation, high bacterial load, and a flattened epidermis [48]. The number of bacilli from a newly diagnosed lepromatous patient can reach 10^{12} bacteria per gram of tissue. Patients with LL leprosy have a CD4 : CD8 ratio of approximately 1 : 2 with a predominant Th2 type response and high titers of anti-*M. leprae* antibodies. Cell-mediated immunity against *M. leprae* is either modest or absent, characterized by negative skin test and diminished lymphocyte proliferation [49, 50].

11. Leprosy Reactions

Leprosy reactions are the acute episodes of clinical inflammation occurring during the chronic course of disease. They pose a challenging problem because they increase morbidity due to nerve damage even after the completion of treatment. They are classified as type I (reversal reaction; RR) or type II (erythema nodosum leprosum; ENL) reactions. Type I reaction occurs in borderline patients (BT, midborderline and BL) whereas ENL only occurs in BL and LL forms. Reactions are interpreted as a shift in patients' immunologic status. Chemotherapy, pregnancy, concurrent infections, and emotional and physical stress have been identified as predisposing conditions to reactions [51]. Both types of reactions have been found to cause neuritis, representing the primary cause of irreversible deformities.

Type I reaction is characterized by edema and erythema of existing skin lesions, the formation of new skin lesions, neuritis, additional sensory and motor loss, and edema of the hands, feet, and face, but systemic symptoms are uncommon. The presence of an inflammatory infiltrate with a predominance of $CD4^+$ T cells, differentiated macrophages and thickened epidermis have been observed in RR. Type II reaction is characterized by the appearance of tender, erythematous, subcutaneous nodules located on apparently normal skin, and is frequently accompanied by systemic symptoms, such as fever, malaise, enlarged lymph nodes, anorexia, weight loss, arthralgia, and edema. Additional organs including the testes, joints, eyes, and nerves may also be affected. There may be significant leukocytosis that typically recedes after the reactional state. Presence of high levels of proinflammatory cytokines such as TNF-α, IL-6, and IL-1β in the sera of ENL patients suggests that these pleiotropic inflammatory cytokines may be at least partially responsible for the clinical manifestations of a type II reaction [52, 53].

12. Immunology of Leprosy Reactions

Type I reaction is a naturally occurring delayed-type hypersensitivity response to *M. leprae*. Clinically, it is characterized

by "upgrading" of the clinical picture towards the tuberculoid pole, including a reduction in bacillary load. Immunologically, it is characterized by the development of strong skin test reactivity as well as lymphocyte responsiveness and a predominant Th1 response [54, 55]. RR episodes have been associated with the infiltration of IFN-γ and TNF-secreting $CD4^+$ lymphocytes in skin lesions and nerves, resulting in edema and painful inflammation [56, 57]. Immunologic markers like CXCL10 are described as a potential tool for discriminating RR [58]. A significant increase in FoxP3 staining was observed in RR patients compared with ENL and patients with nonreactional leprosy, implying a role for regulatory T cells in RR [59].

Pathogenesis of type II reaction is thought to be related to the deposition of immune complexes [60]. Increased levels of TNF-α, IL-1β, IFN-γ, and other cytokines in type II reactions are observed [61–63]. In addition, C-reactive protein, amyloid A protein, and α-1 antitrypsin have also been reported to be elevated in ENL patients' sera [64]. A massive infiltrate of polymorphonuclear cells (PMN) in the lesions is only observed during ENL and some patients present with high numbers of neutrophils in the blood as well. Neutrophils may contribute to the bulk of TNF production that is associated with tissue damage in leprosy. More recently, microarray analysis demonstrated that the mechanism of neutrophil recruitment in ENL involves the enhanced expression of E-selectin and IL-1β, likely leading to neutrophil adhesion to endothelial cells; again, an effect of thalidomide on PMN function was observed since this drug inhibited the neutrophil recruitment pathway [65]. Altogether, the data highlight some of the possible mechanisms for thalidomide's efficacy in treating type II reaction. TNF-α may augment the immune response towards the elimination of the pathogen and/or mediate the pathologic manifestations of the disease. TNF-α can be induced following stimulation of cells with total, or components of *M. leprae*, namely, lipoarabinomannan (the mycobacteria "lipopolysaccharide-" like component) a potent TNF inducer [66]. In addition, mycolyl-arabinogalactan-peptidoglycan complex of *Mycobacterium* species, the protein-peptidoglycan complex, and muramyl dipeptide all elicit significant TNF-α release [66].

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Research Article

Inhibited Production of iNOS by Murine J774 Macrophages Occurs via a *phoP*-Regulated Differential Expression of NFκB and AP-1

Scott D. Hulme, Paul A. Barrow, and Neil Foster

School of Veterinary Medicine and Science, The University of Nottingham, Sutton Bonington Campus, Sutton Bonington, Leicestershire, Nottingham NG7 2NR, UK

Correspondence should be addressed to Neil Foster, n.foster@nottingham.ac.uk

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Background. There are no reported data to explain how *Salmonella* suppress nitrite ion production in macrophages or whether this phenomenon is unique to typhoidal or non-typhoidal serovars. The aims of this study were, therefore, to investigate these phenomena. *Methods*. We measured survival of *S. typhimurium* 14028 and its phoP mutant in murine J774 macrophages, cultured with or without interferon gamma. We compared expression of inducible nitric oxide synthase (iNOS) mRNA and protein, and nitrite ion production and also examined binding of nuclear factor κB (NFκB) and activator protein 1 (AP-1) to macrophage DNA. *Results*. *S. typhimurium* 14028 inhibited binding of NFκB and AP-1 to DNA in murine J774. A macrophages via an intact phoP regulon. This correlated with increased survival and reduced iNOS expression. Suppression of NFκB activity was ameliorated in macrophages cultured with IFN-γ and this correlated with increased expression of iNOS mRNA and nitrite ion production, although IFN-γ had no effect on AP-1/DNA interaction. We show, that with one exception, suppression of iNOS is unique to typhoidal serovars. *Conclusion*. *S. typhimurium* inhibit NFκB and AP-1 interaction with macrophage DNA via the PhoP regulon, this reduces nitrite ion production and is principally associated with typhoidal serovars.

1. Introduction

S. typhimurium infection in mice is a standard laboratory model for human typhoid, and previous studies have shown that *S. typhimurium* mutants which are unable to survive in murine macrophages are avirulent [1]. Thus, survival of *Salmonella* in macrophages appears to be a critical step in the induction of typhoid. The *Salmonella phoP/phoQ* regulon regulates genes located on *Salmonella* Pathogenicity Island 2 (SPI-2) which encode proteins needed for survival of *Salmonella* inside of macrophages [2] and *Salmonellae* which have mutations in their *phoP/phoQ* regulon are avirulent in mice [3]. The affect of *phoP* on *salmonella* survival is multifaceted but studies by Svensson et al. [4] have shown that *phoP* mutation induces increased nitrite ion production by macrophages compared with nitrite ion production induced by the parent strain but this study did not investigate the mechanisms behind this phenomenon. Studies using iNOS$^{-/-}$ and NADPH$^{-/-}$ mice indicate that reactive nitrogen species (RNS) are important in controlling *Salmonella* later in the infection and this is preceded by a reactive oxygen species (ROS)-dependent control phase [5, 6] and it is also known that nitric oxide increases the sensitivity to cellular acid by *phoP* mutants [7]. Taken together these studies indicate that the ability of *Salmonella* to down-regulate nitrite ion production by host macrophages may be due to the effect of SPI-2 proteins under the control of *phoP* and that this confers survival advantage to the *Salmonella* at some point in the infection, but the underlying inductive mechanism has not been reported.

For example, nuclear factor kappa B (NFκB) and activator protein-1 (AP-1) are both known to transcribe the iNOS gene [8, 9] but nothing has been reported regarding the activity of these transcription factors in relation to iNOS

and in the context of wild type or *phoP* mutant *Salmonella*. Furthermore, there are no reported comparisons between typhoidal and nontyphoidal *Salmonella* serovars with regard to iNOS suppression.

The aim of this study was to investigate the effect of wild type *Salmonella* and *phoP* mutants on NFκB and AP-1 activity and their subsequent downstream effect using iNOS as a biological readout. We also compared the effect of serovars which cause typhoid in mice with those which do not.

2. Materials and Methods

2.1. Bacterial Culture and Strains. Bacteria were grown in Luria Bertani (LB) broth (Life Technologies Ltd, Paisley, UK) for 18 h at 37°C under agitation. The bacteria were then subcultured in fresh LB for 4 h to late log phase (established by conventional counts of cfu/mL). Prior to incubation with J774.2 cells, bacteria were adjusted to the multiplicity of infection (moi) of 10. During this study, the following strains/mutants were used: *S. typhimurium* 14028 (ATCC strain), *S. typhimurium* CS022 (*phoP* mutant of 14028, a gift from Dr S. I. Miller, University of Washington, USA), which does not survive in macrophages [10]. In a separate study, the effect of other murine typhoid-inducing (*S. typhimurium* 4/74, *S. enteritidis* KMS1977, *S. dublin* 2229, and *S. choleraesuis* A50) and nontyphoid inducing strains (*S. gallinarum* 9, *S. kedougou* GP, and *S. montevideo* KMS) was analysed. Growth curves for each serovar were obtained as previously stated.

2.2. Cell Culture. J774.2 cells were grown to confluence in 96 well plates (Nunc, Naperville, IL, USA) containing RPMI 1640 media at 37°C in CO_2 (5% v/v). The cells were then washed 3 times in phosphate-buffered saline (PBS), to remove media and nonadherent cells, and incubated in PBS at 22°C for 15 min prior to infection. Cell passages, between 4–16, were used throughout this study.

2.3. Measurement of Nitrite Ion Concentration. Nitrite ion concentration in J774.2 supernatants were measured by Griess reagent kit (Promega, Madison, WI, USA) as per manufacturer instructions. Briefly, 50 μL of supernatant were mixed with 50 μL of sulfanilic solution and incubated at 22°C in darkness for 10 min. 50 μL of napthyl ethylenediamine solution was added and incubated for a further 10 min and the reaction was read on a plate reader (Anthos Labtech Instruments, Hamburg, Germany) at 550 nm. The nitrite ion concentration was determined by comparison with a nitrite ion standard curve with a limit of 1000–5 μM.

2.4. Immunocytochemical Analysis of iNOS Expression. The activity of iNOS in infected and uninfected J774.2 cells was determined by standard immunocytochemical methods. *Salmonella*-infected cells, which had or had not been incubated with 100 U/mL IFN-γ, were washed free of media and permeabilised for 10 min at ambient temperature in 0.05% v/v Triton X-100 after 2, 7, 12, and 24 h postinfection. The cells were then fixed for 15 min in 5% v/v paraformaldehyde at ambient temperature and washed in PBS. Fixed cells were then incubated in the dark on an end-to-end shaker for 60 min at 4°C with mouse anti-iNOS IgG (Autogen Bioclear, UK) PBS-Tween 20 (0.02% v/v Tween, PBS-T) to give a final antibody concentration of 1/100. The cells were then washed three times in PBS-T and incubated in identical conditions but with secondary anti-mouse antibody conjugated to fluorescein isothiocyanate (FITC, Sigma). Samples were viewed on a TCS NT confocal laser scanning microscope equipped with an argon laser (Leica, Cambridgeshire, UK). Controls included J774.2 cells which had not been infected or incubated with IFN-γ and also uninfected cells which were incubated with 100 U/mL IFN-γ.

2.5. Western Blotting. Expression of iNOS protein in *Salmonella*-infected J774.2 cells was assessed by western blotting. J774 cells were lysed in cold water containing protease inhibitors (pepstatin 1 μg/mL; phenylmethylsulphonyl fluoride 1 mM and leupeptin 10 μM) (Sigma) and protein concentration was determined using a bicinchoninic acid (BCA) assay (Pierce, Cheshire, UK). 10 μg/mL J774.2 protein was electrophoresed by standard methods on two 10% acrylamide gels ran back-to-back. To test for parity in protein loading, one gel was stained with Coomassie blue whilst proteins on the second gel were transferred to Hybond-C nitrocellulose paper (Amersham, Buckinghamshire, UK) by a Trans-blot SD, semi-dry transfer cell (Bio-Rad, Hertfordshire, UK). The nitrocellulose was blocked at room temperature for 60 min in PBS-T containing bovine serum albumin (5% w/v, Sigma). After blocking, the nitrocellulose was washed 3 times for five min in PBS-T on an end-to-end shaker at room temperature. The nitrocellulose was then incubated for 60 min in PBS-T containing 1/200 dilution of anti-mouse iNOS (Autogenbioclear, UK), washed three times as before and incubated for 60 min at room temperature in PBS-T containing Rabbit anti-mouse IgG conjugated to horseradish peroxidase (Sigma, 1/12000 dilution). The nitrocellulose was then washed and developed using an enhanced 3′, 3′ diaminobenzidine (DAB) kit (Vector Laboratories, Burlingame, CA, USA).

2.6. iNOS mRNA Expression. iNOS expression in J774.2 cells infected with *Salmonella* was measured by reverse transcription polymerase chain reaction (RT-PCR) using a previously reported method [11]. Briefly, 6×10^6 J774 cells were suspended in 3 mL TRI reagent (Sigma) and stored at −70°C until required (used within 14 days). Samples were centrifuged at 12,000 g for 10 min in a bench top centrifuge at 4°C. The supernatants were transferred to separate tubes, and 200 mL chloroform was added per mL TRI reagent prior to incubation for 10 min at 22°C. The sample was then centrifuged at 12,000 g and 4°C for 15 min, the aqueous phase was removed, and an equal volume of propan-2-ol was added. The sample was centrifuged at 12,000 g for 10 min, and the RNA pellet was washed in a mixture of 1 vol 75% ethanol : 1 vol sterile water. The mixture was then centrifuged

for 10 min at 7,500 g, and, after removal of the supernatant, the pellet was allowed to air dry for further 10 min. The pellet was then resuspended in diethyl pyrocarbonate treated water. RNA purity was measured using an Ultraspec III spectrophotometer (Pharmacia, Hertfordshire, UK) and was found to have a typical 260/280 nm ratio of 1.9 to give yields of around 100 μg/mL. RNA concentration was adjusted to 1 μg/μL prior to the RT reaction. RT reactions were performed on a thermal cycler (Eppendorf, Hamburg, Germany) using the following previously reported [11] primer sequences:

Forward: 5′-GTA AAC TGC AAG AGA ACG GAG AAC-3′.

Reverse: 3′-GAG CTC CTC CAG AGG GGT AG-5′.

As a loading control, the following glyceraldehyde-3-phosphate dehydrogenase (GAPdh) primer sequences were used:

Forward: 5′-GTT CAG CTC TTG GAT GAC CTT GCC-3′.

Reverse: 3′-TCC TGC ACC ACC AAC TGC TTA GCC-5′.

2.6.1. Preparation of Nuclear Lysates. Nuclear lysates were prepared by scraping cells into 5 mL PBS and washing once with buffer I (HEPES 10 mM, KCl 10 mM, $MgCl_2$ 1.5 mM, pH 7.9). The cells were disrupted by freeze-thawing twice in 1 mL buffer I containing Nonidet P-40 (NP-40) (5%, Sigma). All subsequent procedures were carried out at 4°C. The lysate was centrifuged for 5 min at 2000 rpm in a bench-top microfuge. The pellet was then washed twice with buffer I and NP-40 before being centrifuged at 12,000 rpm for 5 min to obtain the nuclear pellet. Nuclear proteins were extracted from the pellet for 10 min with 30–40 μL extraction buffer (HEPES 20 mM, NaCl 420 mM, $MgCl_2$ 1.5 mM, EDTA 0.2 mM, 25% glycerol, pH 7.9). After mixing, the suspension was centrifuged twice as stated above. The supernatant was then diluted in 40–60 μL dilution buffer (HEPES 20 mM, KCl 50 mM, EDTA 0.2 mM, glycerol 20%, pH 7.9). Protease inhibitors (pepstatin 1 μg/mL, phenylmethylsulphonyl fluoride 1 mM, leupeptin 10 μM, Sigma) were added to the lysate which was then used immediately or stored at –70°C for up to 14 days.

2.6.2. Electromobility Shift Assay (EMSA). EMSA was used to determine DNA binding of NFκB (p50) and activating protein (AP)-1 (c-Jun) to J774.2 DNA following infection by *Salmonella* and/or incubation with IFN-γ (100 U/mL). EMSA reactions were performed using a kit as per manufacturer instructions (Promega, USA) using the following oligonucleotide sequences:

AP-1 (c-Jun)

5′-CGC TTG ATG AGT CAG AAG GAA-3′

3′-GCG AAC TAC TCA GTC GGC CTT-5′

NFκB (p50)

5′-AGT TGA GGG GAC TTT CCC AGG C-3′

3′-TCA ACT CCC CTG AAA GGG TCC G-5′

Kit controls included HeLa cell nuclear lysate with consensus oligo (positive control) and HeLa cell nuclear lysate without consensus oligo (negative control). As an additional negative control, J774.2 cells which had not been infected or incubated with IFN-γ were used.

Digital Image Analysis was performed using a Phoenix 1D analyser using a power scanner V.3 (Phoretix, Newcastle upon Tyne, UK).

2.7. Statistical Analysis. Mann-Whitney analysis (Minitab) was used to measure significant difference at the 95% confidence limit between different test groups and between the same test groups at different time points.

3. Results

3.1. The Effect of Salmonella phoP on Survival and Nitrite Ion Production in Murine J774.2 Cells. Nitrite ion production by macrophages was significantly ($P < 0.05$) lower when the cells were cultured with wild type *S. typhimurium* 14028 compared to nitrite ion production by macrophages cultured with 14028 *phoP* mutant. However addition of IFN-γ to culture media significantly increased ($P < 0.05$) nitrite ion concentrations in supernatants recovered from both wild type and *phoP*-cultured macrophages (Figure 1(a)).

The numbers of wild type 14028 recovered from macrophages were also between 1-2 logs greater after 12–24 h culture compared with the numbers of 14028 *phoP* mutants recovered from cells over the same period (Figure 1(b)). When macrophages were cocultured with IFN-γ, the numbers of wild type 14028 recovered from cells decreased and were comparable to the numbers of 14028 *phoP* mutants recovered over the same time period (Figure 1(c)).

3.2. The Effect of Salmonella phoP on iNOS mRNA and iNOS Protein Expression by J774.2 Cells. Increased iNOS mRNA expression was detected in macrophages cultured with 14028 *phoP* mutants when compared with 14028 wild type *Salmonella* and in both cases the iNOS mRNA signal was increased by coculture with IFN-γ (100 U/mL) (Figure 2(a)). However, when adjusted for GADPH expression, iNOS mRNA expression, in J774 cells, induced by IFN-γ and 14028 wild type, was still slightly lower than was induced by IFN-γ alone.

Confocal microscopy data also clearly showed that a *phoP* mutation (Figure 2(d)) caused a more intense iNOS protein signal in the cytoplasm of J774.2 cells when compared to uninfected control macrophages (Figure 2(b)) or wild type 14028-infected cells (Figure 2(c)). The intensity of iNOS signal was increased by co-culture of macrophages with wild type 14028 and IFN-γ (Figure 2(e)) and was even more intense in macrophages which were co-cultured with 14028 *phoP* mutants and IFN-γ (Figure 2(f)). The results obtained by confocal were also repeated by Western blotting (Figure 2(g)) and in this case J774 cells co-cultured with 14028 wild type and IFN-γ produced a greater amount of iNOS protein when compared to J774 cells cultured only with IFN-γ.

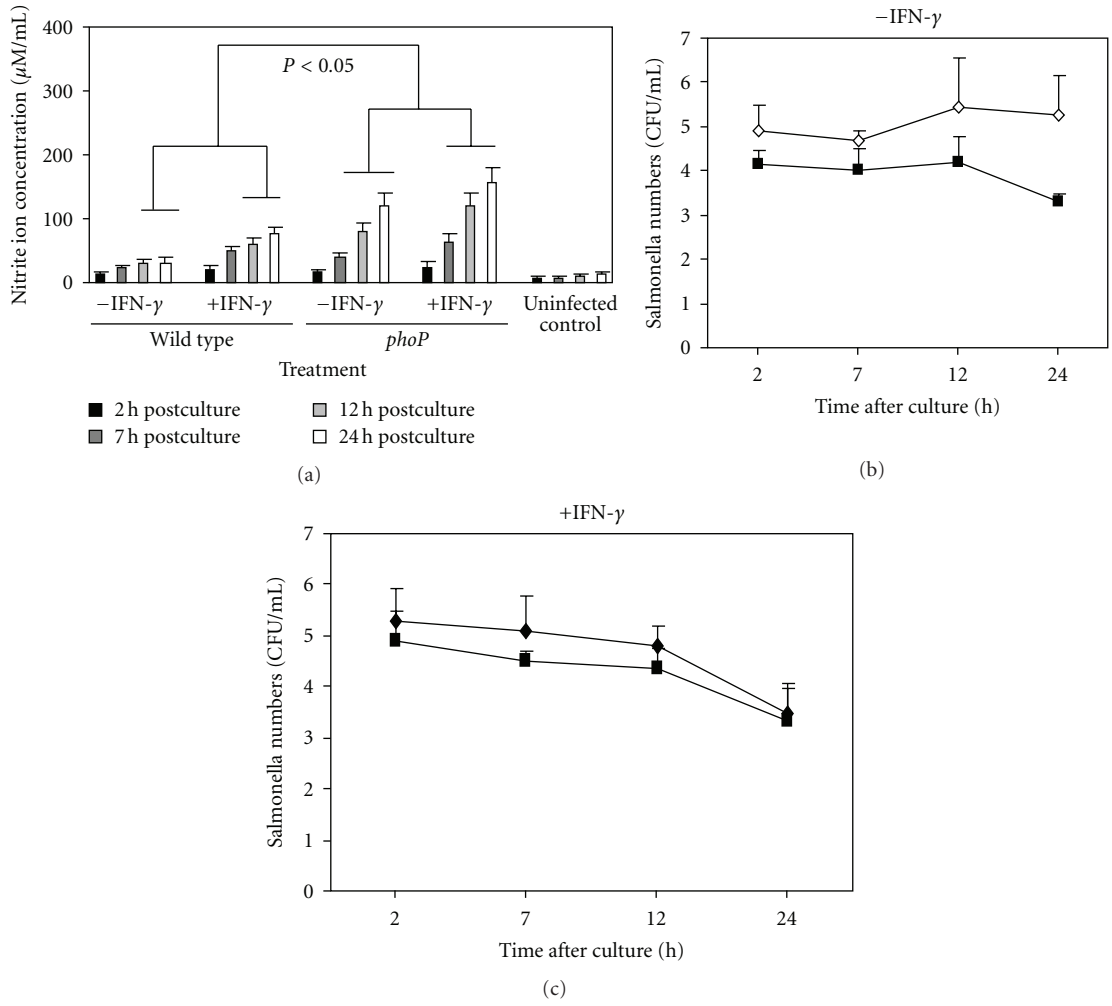

Figure 1: Wild type *S. typhimurium* 14028 suppresses macrophage nitrite ion production via the *phoP* regulon but is inhibited by IFN-γ. Histograms show nitrite ion concentrations measured in supernatants from macrophages cultured with 14028 wild type and *phoP* mutants and uninfected controls and with or without the addition of IFN-γ. *Significant increase ($P < 0.05$) in nitrite ion concentration in cell supernatants compared to uninfected controls at equivalent time points. Significant increase ($P < 0.05$) in nitrite ion concentrations recovered from macrophage supernatants cultured with 14028 *phoP* mutants compared to 14028 wild type is also shown. Each point (and standard deviations) is mean values calculated from triplicate cultures replicated on 5 separate occasions.

3.3. The Effect of phoP Mutation on NFκB and AP-1 Binding to Macrophage DNA. Our results show that mutation in the *Salmonella phoP* regulon increased the amount of NFκB and AP-1 bound to macrophage DNA after 2 h, when compared to wild type 14028 (Figure 3(a)). When compared to a positive control, NFκB/DNA interaction following wild type 14028 infection was reduced by a mean of 68% but when infected macrophages were co-cultured with IFN-γ (100 U/mL), NFκB/DNA interaction was reduced by 42% (Figure 3(a)). In comparison, when macrophages were infected with 14028 *phoP* mutants, NFκB/DNA interaction was only reduced by a mean of 22% (relative to the positive control) and this remained constant following co-culture of infected cells with IFN-γ (Figure 3(a)). After macrophages were cultured with wild type 14028 for 12 h, NFκB/DNA interaction was reduced by a mean of 66% but when infected macrophages were co-cultured with IFN-γ, NFκB/DNA interaction was reduced by 76% (Figure 3(b)). when macrophages were infected for 12 h with 14028 *phoP* mutants, NFκB/DNA interaction was only reduced by 40% (relative to the positive control) and this also remained constant following co-culture of infected cells with IFN-γ (Figure 3(b)).

In macrophages infected for 2 h with wild type 14028, results obtained for AP-1/DNA binding was equivalent to those obtained for a negative control which contained no oligonucleotide (92% reduction in DNA binding compared to the positive control) and this was altered very little by coculture with IFN-γ (88% reduction compared to the positive control) (Figure 4(a)). However, when macrophages were infected with 14028 *phoP* mutants for 2 h, AP-1/DNA binding was only reduced by 39% relative to the positive control and this level was maintained following co-culture with IFN-γ (Figure 4(a)).

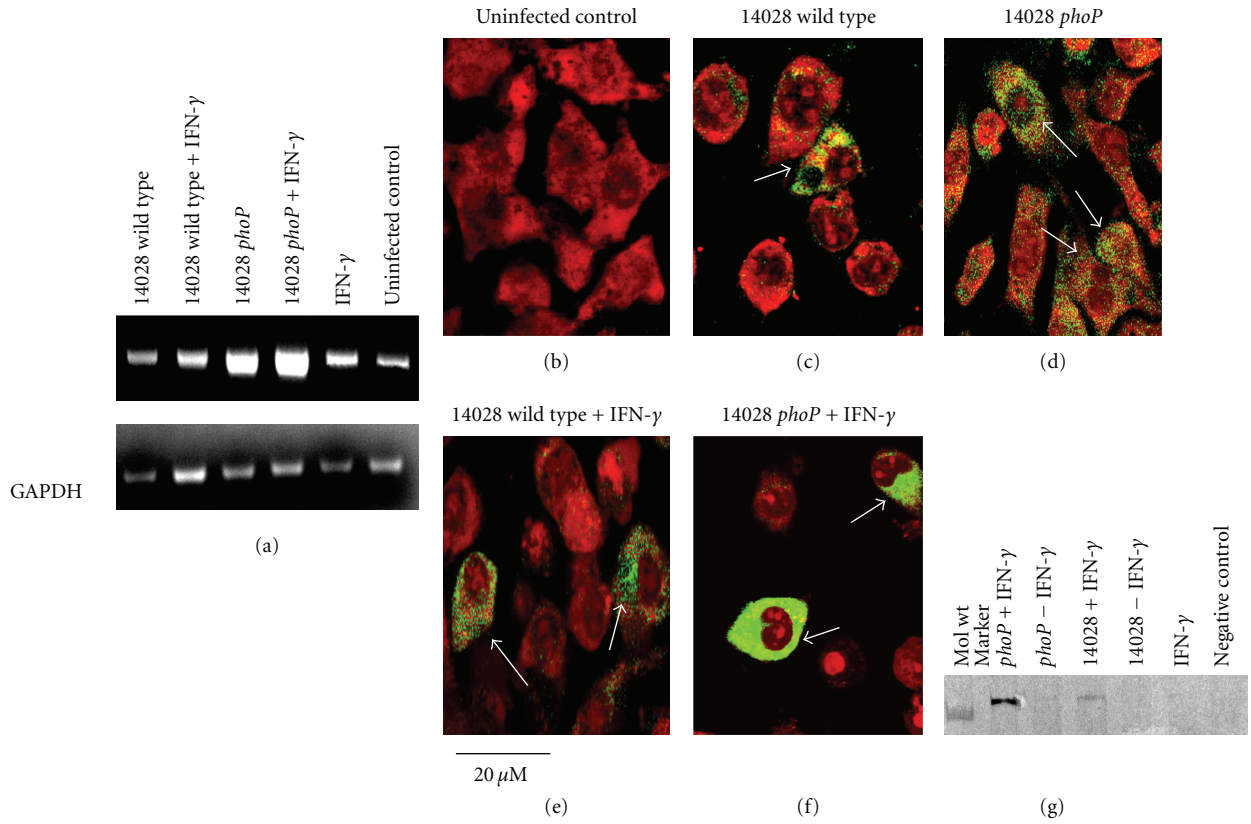

Figure 2: *S. typhimurium* 14028 *phoP* regulon suppresses iNOS mRNA and protein expression in murine macrophages but fails to suppress iNOS when co-cultured with IFN-γ. (a) PCR showing iNOS mRNA expression is reduced in macrophages cultured with 14028 wild type compared to 14028 *phoP* mutants. The addition of IFN-γ to culture media increased iNOS mRNA expression in macrophages infected with either wild type or *phoP* mutant. (b) Intracellular iNOS protein cannot be detected by immunocytochemistry in uninfected (control) macrophages. (c) Suppression of intra-cellular iNOS protein observed in macrophages cultured with wild type 14028 compared to macrophages cultured with *phoP* mutants (d). Addition of IFN-γ to cell culture media also increased intra-cellular iNOS in macrophages cultured with wild type 14028 (e) or *phoP* mutants (f). Arrows show iNOS positive cells, scale bar bottom left: 20 μm. (g) Western blot of iNos protein in whole cell preparations stimulated with wild type 14028 or 14028 *phoP* with or without IFN-γ. Lane 1, molecular weight marker: 130 kDa. Lane 7: Unstimulated J774 cells (negative control). All analyses are representative of data obtained on 3 separate occasions.

After macrophages were cultured with wild type 14028 for 12 h, AP-1/DNA interaction was increased with a reduction relative to the positive control of 62.9% and AP-1/DNA interaction was increased further following co-culture of infected macrophages with IFN-γ for 12 h, in which a mean reduction of 55% (relative to the positive control) was measured (Figure 4(b)). However, AP-1/DNA interaction in macrophages infected with 14028 *phoP* mutants for 12 h was reduced only by 53% compared to the positive control but, following co-culture with IFN-γ, AP-1/DNA interaction was increased with a mean reduction of only 21% relative to the positive control (Figure 4(b)).

3.4. Salmonella Serovars Which Induce Murine Typhoid Suppress Nitrite Ion Production. When macrophages were cultured with *Salmonella* serovars which are known to induce murine typhoid, nitrite ion concentrations were reduced in cell supernatants compared to supernatants isolated from macrophages cultured with nontyphoidal strains over the same time period (Figure 5). However *S. Choleraesuis*, which is known to induce murine typhoid, was an exception to this rule since it stimulated nitrite ion production by macrophages at similar levels to those measured in supernatants isolated from macrophages cultured with nontyphoidal serovars (Figure 5).

4. Discussion

Eriksson et al. [12] have previously reported that hypersurvival mutants of *S. typhimurium* TT16729, obtained by multiple passage through J774 cells, induced lower nitrite ion concentrations than did the parent strains, although an attenuated *Salmonella* mutant was not studied for comparison. However, Svensson et al. [4] reported that wild type *S. typhimurium* 14028 induced lower nitrite ion production in murine bone marrow derived macrophages when compared with an *S. typhimurium* constitutive *phoP*^{c} mutant but that the survival of the *phoP* was only marginally different (<0.5 Log) to the 14028 wild type. In contrast to this result, we show that there is a clear increase in survival rates of

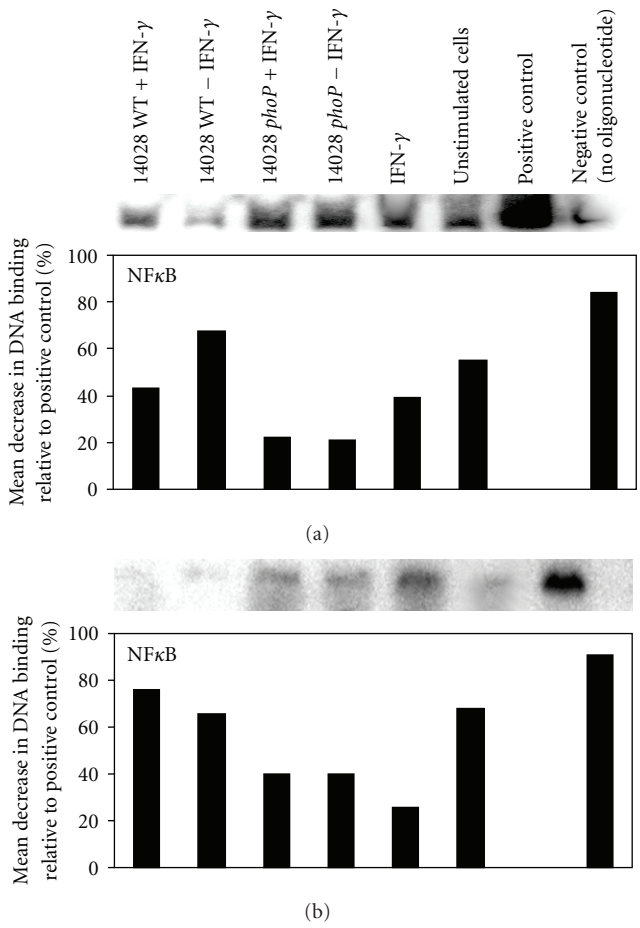

Figure 3: Wild type *S. typhimurium* suppress NFκB/DNA interaction in J774.2 cells 2 and 12 h afterculture via *phoP*. (a) 2 h postculture. (b) 12 h post-culture. Controls include, uninfected J774.2 cells, manufacturers kit positive control (HeLa cell nuclear lysate with oligonucleotide), and manufacturers kit negative control (HeLa cell nuclear lysate without oligonucleotide). Data is representative of EMSAa performed on 3 separate occasions.

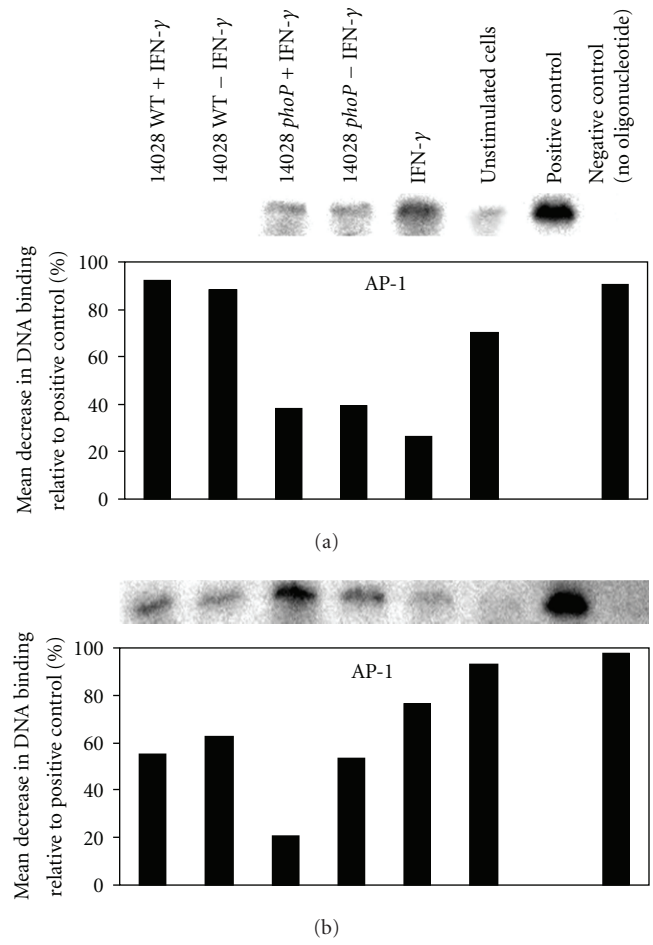

Figure 4: Wild type *S. typhimurium* suppresses AP-1/DNA interaction in J774.2 cells 2 and 12 h afterculture via *phoP*. (a) 2 h postculture. (b) 12 h postculture. Controls include, uninfected J774.2 cells, manufacturers kit positive control (HeLa cell nuclear lysate with oligonucleotide), and manufacturers kit negative control (HeLa cell nuclear lysate without oligonucleotide). Data is representative of EMSAa performed on 3 separate occasions.

wild type 14028 compared to its *phoP* mutant in J774.2 macrophages. However, the differences we observe may conceivably be due to differences in cell type or moi, since our moi was constant at 10 : 1 whereas the previous study used 14028 at an moi of 15 : 1 and $phoP^c$ at an moi of 17 : 1 [4]. In contrast to these studies, a study by Das et al. [13] has shown that in the murine macrophage cell line RAW264.7, wild type *Salmonella* inhibit IFN-γ-induced NO production via the virulence gene nirC and that this correlates with increased cellular survival. However our study shows that iNOS mRNA, iNOS protein, and nitrite ion production are increased when wild type 14028-infected J774 cells are co-cultured with IFN-γ but overall wild type infected cells elicit much weaker responses than do 14028 *phoP*-infected macrophages, and this occurs in the presence or absence of IFN-γ. We have also found that 14028 wild type and 14028 *phoP* have comparable sensitivity to exogenous nitrite ions, as shown by bacterial growth curves obtained at different nitrite ion concentration (data not shown). It is possible that the inhibition of NO production via iNOS suppression may have relevance later in the infection as shown by Mastroeni et al. [5] or it is also possible that the inhibition of iNOS per se (rather than downstream NO) may have immediate impact on other factors. For example, iNOS-dependant induction of 8-nitroguanosine 3′,5′-cyclic monophosphate (8-nitro-cGMP) has been shown to induce heme oxygenase 1 (HO-1) which has both cytoprotective and antimicrobial effects in murine Salmonellosis [14]. NO production may also enhance other antimicrobial pathways, for example, via interaction with reactive oxygen species [15].

Our study also attempted to relate changes in iNOS to the activity of two critical transcription factors (NFκB and AP-1) in infected J774 macrophages, with or without co-culture with IFN-γ. The iNOS gene has previously been shown to be transcribed by nuclear factor kappa B (NFκB) (p50/65) [16] which may translocate to the cell nucleus following stimulation by interferon gamma (IFN-γ) [17, 18] and

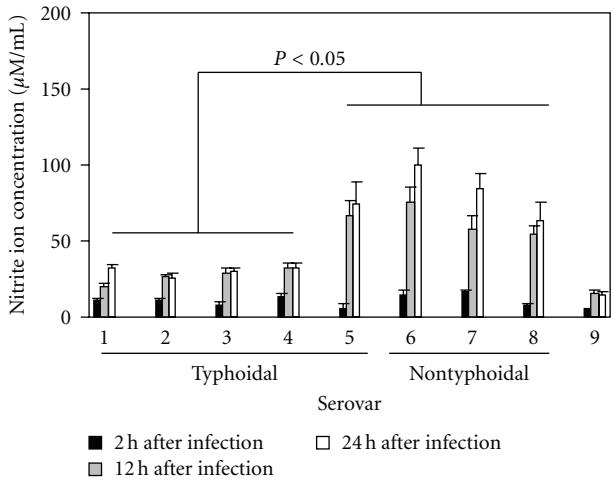

Figure 5: Nitrite ion production by J774 cells cultured with different typhoid-causing or non-typhoid-causing *Salmonella* serovars and co-cultured with IFN-γ. 1: *S. typhimurium* 14028; 2: *S. typhimurium* 4/74; 3: *S. enteritidis* KMS 1977; 4: *S. dublin* 2229; 5: *S. choleraesuis* A50; 6: *S. kedougou* GP; 7: *S. montevideo* KMS; 8: *S. gallinarum*; 9: uninfected cells. All experiments were replicated 3 times on at least 5 separate occasions. Statistical bars show significant different ($P < 0.05$) between nitrite ion production in macrophages infected with typhoidal and nontyphoidal serovars.

this may act synergistically with bacterial lipopolysaccharide (LPS), depending on relative concentrations [19].

As well as NFκB binding sites, promoter sequences on murine iNOS genes also contain binding sites for AP-1 and IFN-γ response elements (γ-IRE) ([8] reviewed [20]). However, AP-1 activation during *Salmonella* invasion of macrophages has not been comprehensively studied but temporal changes in the heterodimeric composition of AP-1 during culture of murine macrophages with *S. typhimurium* LPS or porins have been reported [21].

Our data was interesting for a number of reasons; firstly we show that wild type 14028 suppresses NFκB/DNA interaction within the first 2 h postinfection of J774 macrophages but this is not the case when *phop* mutation is induced. This suggests that the ability to suppress NFκB activity is, therefore, *phoP*-dependent. However, the inherent suppression of NFκB activity by wild type 14028 is overcome when the macrophages are co-cultured with IFN-γ, and this is consistent with one study which has shown that IFN-γ suppresses *phoP* transcription in wild type *Salmonella* [22]. Interaction of NFκB with macrophage DNA was also reduced further after 12 h culture with wild type 14028 and co-culture with IFN-γ had no effect, whereas *phoP* mutants maintained relatively strong NFκB/DNA interaction (although this was also reduced by about half when compared to NFκB/DNA interactions measured after 2 h). These results suggest that an intact *phoP* regulon promotes significant changes in the ability of NFκB to interact with DNA and this probably had a significant impact on the iNOS suppression we observed in wild type-infected cells. Saura et al. [23] have shown that IFN-γ induces nuclear translocation of interferon regulatory factor 1 (IRF-1) which then synergises with NFκB to transcribe iNOS. Therefore, it is possible that this is an additional mechanism by which IFN-γ increases iNOS transcription in our study, as well as the increased NFκB/DNA interaction we have shown.

However, our study also indicates that there is temporal separation between the induction of NFκB/DNA interaction and AP-1/DNA interaction in 14028 wild type infected macrophages but this was not observed in macrophages infected with 14028 *phoP* mutants. After 2 h postinfection with wild type 14028, no discernable interaction between AP-1 and macrophage DNA was measured but after 12 h post-infection AP-1/DNA interaction was measured and this was increased when the cells were co-cultured with IFN-γ, although this was on average 55–63% lower than that measured for the positive control. In contrast, when the macrophages were cultured with 14028 *phoP* mutants, AP-1/DNA interaction was only about 53% lower than the positive control but when the cells were co-cultured with IFN-γ, AP-1/DNA binding was only 21% lower than the positive control. These results also indicate that an intact *phoP* regulon prevents long-term interaction of AP-1 with DNA and IFN-γ-induced increase in AP-1/DNA interaction (as was the case with NFκB). We, therefore propose that the affect of the *phoP* regulon to prevent long-term and strong interaction of macrophage DNA with NFκB and AP-1 and IFN-γ-stimulated upregulation of this interaction will have a profound effect on iNOS expression and nitrite ion production via reduced exposure of iNOS promoter sequences within macrophage DNA to these essential transcription factors.

No previously published data exists which has compared iNOS activity and nitrite ion production in murine macrophages cultured with typhoidal and non-typhoid *Salmonella* serovars. However, Eisenstein et al. [24] have shown that *S. typhimurium* and *S. dublin* both inhibit nitrite ion production by murine splenocytes, and although this study at least considered different typhoid inducing serovars, a comparison between typhoid-inducing and non-inducing serovars was not reported. The one surprising exception in our study was *S. choleraesuis* which failed to down- regulate nitrite ion production by macrophages. *S. choleraesuis* causes typhoid-like systemic disease in a much wider range of mammalian hosts than do *S. typhimurium*, *S. Dublin,* or *S. enteritidis* [25] and we cannot, as yet, explain why this serovar does not down regulate iNOS. However, all other typhoid-inducing serovars were able to down-regulate nitrite ion production by murine macrophages whereas those which do not induce typhoid were unable to do so. This may suggest that the ability of typhoid-inducing *Salmonella* may allow dissemination to deeper tissues, via a *phoP*-induced suppression of NFκB and AP-1 and subsequent suppression of iNOS.

Our data may be relevant in the future treatment of Typhoid in humans, since it suggests a possible role for the adjunctive use of IFN-γ (and antibiotic) to overcome *phoP*-dependent iNOS suppression. Thus, increasing nuclear translocation of essential transcription factors needed for transcription of the iNOS gene and nitrite ion production.

Conflict of Interests

The authors have no conflict of interests.

Authors' Contributions

S. Hulme performed EMSA analyses and participated in all other experimental procedures. P. Barrow coconceived of the study and participated in its design and coordination and helped to draft the final paper. N. Foster performed all analyses (with the exception of EMSA), coconceived of the study, participated in its design and helped to draft the final manuscript. All authors read and approved the final paper.

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