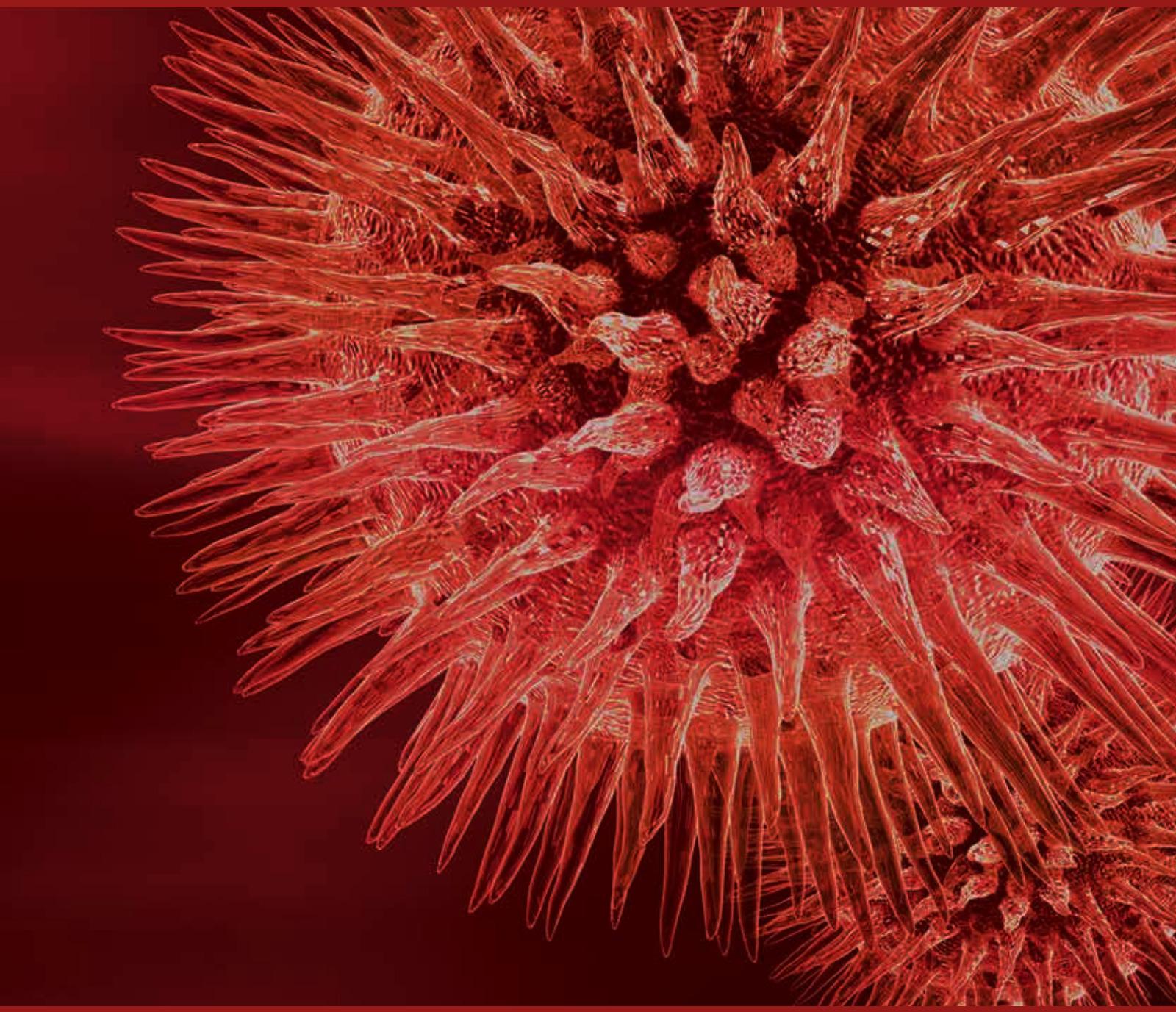
BioMed Research International

Parasites: From Source to Vector and Human

Guest Editors: Veeranoot Nissapatorn, Nongyao Sawangjaroen, Rogan Lee, and Subhash Chandra Parija



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Editorial

Parasites: From Source to Vector and Human

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Parasites cause a significant burden of disease globally and the majority of parasitic infections fall into the category of Neglected Tropical Diseases (NTDs). The emergence of parasitic diseases, including zoonotic infections, is associated with the process of rapid urbanization, exponential population growth, borderless trade between countries and climate change. To adopt new methods of control, a number of important issues must be addressed such as environmental-based interventions, novel drug discovery, the next generation sero-markers and development of new strategies for parasite control, and so forth. In this special issue on parasites: from source to vector and human, we have invited a few papers that address such issues.

The first paper of this special issue addresses serine proteinase activities in excretory-secretory products of *Toxocara canis* second stage larvae (TES) related to the known sequences of TES components for example, TES26 and MUC4. The authors use protein modelling to identify molecules that can be incorporated into diagnostic and therapeutic techniques ultimately leading to better methods of control. The second paper on Chagas' disease describes extensively on aspects of transmission, association with pregnant women and congenital infection, clinical manifestations, diagnosis, treatment, control strategies, case follow-up and disease complications in Latin America.

The third paper of this special issue presents a brief overview of the helminth-associated immune response, the currently available helminth secretome data, some major secretome-derived immunomodulatory molecules, their potential mode of actions and the applicability of helminth-derived therapeutic proteins in the treatment of allergic and autoimmune inflammatory disease. The fourth paper addresses the greater risk of exposure to tick borne pathogens (*Anaplasma phagocytophilum* and *Ehrlichia chaffeensis*) among rural residents using serological mass screening in China. Significant risk factors associated with infection are: having contact with animals, planting crops, having more employment time and having a history of fever. This study also proposes safety measures required to reduce zoonotic transmission.

The fifth paper shows the effectiveness of different concentrations of two fractions of Curcuma longa cortex rich in turmerones and their respective liposomal formulations for treating promastigote forms of *Leishmania amazonensis*. The hexane fraction from the turmeric cortex, incorporated in liposomal formulation (LipoRHIC), presents a promising result for a new anti-leishmanial agent. The sixth paper uses climate modelling for global warming to indicate geographical spread, increasing number of seasonal generations and epidemiological changes of *Dirofilaria* spp. In addition to dirofilariosis, it also covers other mosquito-borne diseases in Russia and its neighbouring countries. The final paper of this special issue reports on the next generation sero-marker for toxoplasmosis with improved specificity and sensitivity using *Toxoplasma gondii* dense granular protein-5 (GRA5) on human serum samples.

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Research Article

Proteinases in Excretory-Secretory Products of *Toxocara canis* Second-Stage Larvae: Zymography and Modeling Insights

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Components released in excretory-secretory products of *Toxocara canis* larvae (TES) include phosphatidylethanolamine-binding proteins (TES26), mucins (TES120, MUC2-5), and C-type lectins (TES32, TES70) and their biochemical, immunological, and diagnostic properties have been extensively studied albeit proteinase activities towards physiological substrates are almost unknown. Proteolytic activities in TES samples were first analyzed by gel electrophoresis with gelatin as substrate. Major activities of ~400, 120, and 32 kDa in TES were relatively similar over a broad pH range (5.5–9.0) and all these were of the serine-type as leupeptin abolished gelatinolysis. Further, the ~400 kDa component degraded all physiological substrates tested (laminin, fibronectin, albumin, and goat IgG) and the 120 kDa component degraded albumin and goat IgG while proteinases of lower MW (45, 32, and 26 kDa) only degraded laminin and fibronectin, preferentially at alkaline pH (9.0). By protein modeling approaches using the known sequences of TES components, only TES26 and MUC4 displayed folding patterns significantly related to reference serine proteinases. These data suggest that most of serine proteinase activities secreted *in vitro* by infective larvae of *T. canis* have intriguing nature but otherwise help the parasite to affect multiple components of somatic organs and bodily fluids within the infected host.

1. Introduction

Toxocariasis is caused in dog (definitive) and human (paratenic) hosts by infection with the larvae of the ascarid worm *Toxocara canis*. Puppies infected at the intestine with the adult stage of *T. canis* shed in feces large number of infective eggs into the environment. When these are accidentally ingested by a patient, the infective larva (L2) hatches, penetrates the intestinal wall, and through the circulatory system targets various organs [1]. In dogs, the prevalence of *T. canis* ranged from 5.5% to 64.7% [2–4]. In humans, recent data show a widespread prevalence of toxocariasis, which is probably the most prevalent helminthiasis in industrialized countries although a neglected and underestimated health problem [5–7]. It is clear that the hatching of infective/juvenile larvae from embryonated eggs and the migration of second- or third-stage larvae (L2-L3) through host tissues are critical steps triggering infection and pathogenesis by *T. canis*. For the hatching process, we have reported the presence of three bands of 91, 68, and 38 kDa with aspartic-type proteinase and acidic (pH < 5) activities that were induced and released into the egg perivitelline fluid (EPF) [8]. For the migratory phase, pathologies in human toxocariasis are due to *larva migrans* syndromes observed in overt (visceral and ocular) or covert (gut-, lung-, or brain-targeted) presentations [9–13]. Larvae migrating throughout bodily fluids (i.e. blood) and somatic organs shed large amounts of lipids and immunogenic glycoproteins known together as *Toxocara* excretory-secretory products (TES) which have been proposed to serve a strategy to

escape the immune attack of the host [14]. Nevertheless the *larva migrans* status in toxocariasis is associated with worms lacking anchoring apparatus (oral appendages, stylets, hooks, or scolex); hence other biological functions have to be considered for TES in the completion of *Toxocara* life cycle.

Excretory-secretory products from migratory larvae of nematodes contain several kinds of components. Besides their likely active role in hatching, proteinases are considered a decisive factor for tissue invasion and extracellular digestion of host proteins [15]. In spite of the extensive studies on the reliability of using TES for immunological/molecular diagnosis and treatment assessment strategies for toxocariasis [16–19], TES components are still being characterized at further detail. Major TES components include mucins (TES120/MUC1, MUC2–5), phosphatidylethanolamine-binding proteins (TES26), and C-type lectins (TES32, TES 70) of which nucleotide and amino acid sequences have been determined by expression sequence tag and DNA cloning strategies from *T. canis* genome [20–25]. In this context, in a classical study by Robertson et al. [26], the release of two serine proteinase activities with MW of 120 and 32 kDa in TES from L2s under *in vitro* culture that displayed higher activity at pH 9 as assessed by acrylamide-gelatin gels and radio gelatin microplate assays was reported. Total enzyme activity in TES was shown to degrade elastins, collagens, and glycoproteins contained in an extracellular matrix of a rat smooth muscle cell line. It is thus conceivable not only that different kinds of proteinase activities are developmentally secreted by hatching and migratory L2s as they encounter distinct microenvironments within the infected host but also that individual proteinases have preferential activities towards distinct physiological substrates, for example, immunoglobulins and carrier proteins (albumin) in bodily fluids and fibronectin and elastin in extracellular matrix of somatic organs.

In this work we identified by gel electrophoresis the proteinases released into culture medium by *T. canis* larvae (TES) and analyzed their (physiological) substrate- and pH-dependent activities.

2. Materials and Methods

2.1. Parasite Collection and TES Preparation. *T. canis*-infected puppies were euthanized and adult worms were collected from intestines, washed with sterile saline (SS), and stored at 4°C. Egg collection from female uteri, induction of hatching, and larvae recovery were carried out as previously described [8]. To obtain TES, 10,000 larvae/mL of RPMI 1640 medium (supplemented with 1% [w/v] glucose, 100 μg/mL HEPES, and 100 IU/mL penicillin-streptomycin) were incubated in 40 mL culture bottles at 37°C in a humidified atmosphere containing 5% (v/v) CO_2. Supernatants were collected at four weekly intervals, replacing the sampled medium each time. TES samples were filtered with a Millex filter unit (0.2 μm pore size), concentrated using an ultrafiltration device (Amicon), and stored at −20°C until needed. The protein concentration in these preparations was determined by a micro-Bradford technique [27].

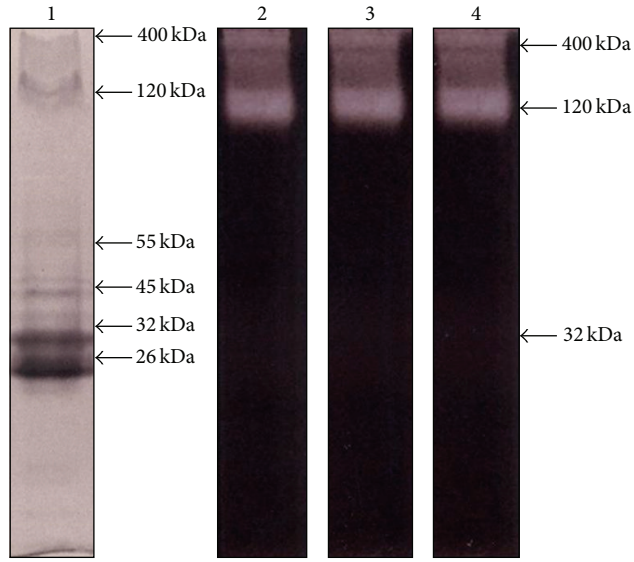

Figure 1: Electrophoretic detection of bands with proteolytic activity in TES of *T. canis* at different pH values.Samples of TES were loaded at a quantity of 10 μg/well in slab gels of 10% (w/v) acrylamide copolymerized with 0.1% (w/v) gelatin. After electrophoretic separation, proteinase activity was developed using acetate buffer (pH 5.5, *lane 2*), phosphate buffer (pH 7.6, *lane 3*), or glycine buffer (pH 9.0, *lane 4*) and then gels were stained with Coomassie blue. In other assays, electrophoresis of TES was performed in 10% (w/v) acrylamide gels and protein bands were directly stained with Coomassie blue (*lane 1*). Molecular weight of bands with gelatinolytic activity is indicated *at the right* of the corresponding lanes.

2.2. Gelatin and Substrate Gel Electrophoresis. Electrophoretic analysis of proteolytic activity contained in TES was carried out as described elsewhere [8] in SDS-10% (w/v) polyacrylamide slab gels containing 0.1% (w/v) gelatin in a Mini-Protean II Electrophoresis Unit (BioRad). Other substrates used were 0.5 mg/gel laminin, 0.5 mg/gel fibronectin, 0.1% (w/v) bovine albumin, and 0.1% (w/v) goat IgG. Samples (10 μg/well) were loaded in Laemmli's buffer devoid of reducing agents (dithiothreitol or β-mercaptoethanol) and electrophoresis was run for 90 min at 140 V at 4°C. To develop and visualize proteolytic activity, gels were then incubated in a 0.25% (v/v) Triton X-100 solution for 1 h at room temperature (RT), soaked 3 times in distilled water for 5 min each, and transferred to acetate buffer (pH 5.5), phosphate buffer (pH 7.6), or glycine buffer (pH 9.0). After further incubation for 18–20 h at 37°C in buffer, gels were stained with Coomassie blue (0.1% [w/v] in 40% [v/v] methanol-10% [v/v] acetic acid solution). Molecular weight determination was done with reference to prestained markers (BioRad).

2.3. Determination of Proteinase Types in TES. Samples of TES were electrophoretically separated as mentioned and gels were preincubated for 30 min at RT with one

Table 1: Summary of bands from TES with proteolytic activity in SDS-acrylamide gels copolymerized with the indicated substrate at different pH values.

Substrate	pH		
	5.5	7.5	9.0
Gelatin	400, 120, and 32 kDa	400, 120, and 32 kDa	400, 120, and 32 kDa
Laminin	400 and 120 kDa	400 and 120 kDa	400, 120, 45, 32, and 26 kDa
Fibronectin	400 and 120 kDa	400 and 120 kDa	400, 120, 45, and 32 kDa
Albumin	400 and 120 kDa	400 and 120 kDa	400 and 120 kDa
Goat IgG	400 and 120 kDa	400 and 120 kDa	400 and 120 kDa

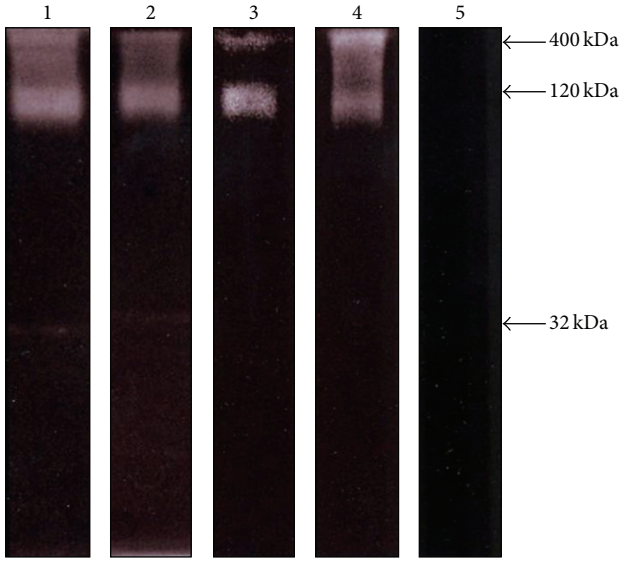

Figure 2: Proteolytic activity of TES components of *T. canis* analyzed by acrylamide-gelatin gel electrophoresis. Effect of proteinase inhibitors. TES components were preincubated for 30 min at RT with one of the following compounds: control without inhibitor (*lane* 1); 2 μM *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)-butane (E64) to identify cysteine proteinases (*lane 2*); 0.1 μM pepstatin A for aspartic proteinases (*lane 3*); 1 mM ethylenediaminetetraacetic acid (EDTA) for metalloproteinases (*lane 4*); and 0.1 μM leupeptin for serine proteinases (*lane 5*). Samples (10 μg/well) were electrophoretically separated in slab gels of 10% (w/v) acrylamide copolymerized with 0.1% (w/v) gelatin. Proteinase activity was developed using phosphate buffer (pH 7.6) and then gels were stained with Coomassie blue. Molecular weight of bands with activity is indicated *at the right*.

of the following compounds: 2 μM *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)-butane 1 (E64) to identify cysteine proteinases; 0.1 μM leupeptin for serine proteinases; 0.1 μM pepstatin A for aspartic proteinases; and 1 mM ethylenediaminetetraacetic acid (EDTA) for metalloproteinases. Then the proteolytic activity was developed and visualised as described above. In some cases, TES samples were separated in SDS-10% acrylamide gels without substrate and protein bands were visualized with Coomassie blue followed by destaining in the same solution lacking the dye.

2.4. Densitometry Analyses. Images of TES samples separated and developed by substrate gel electrophoresis were captured in Tag Image File Format (TIFF). Lanes displaying representative bands of proteolytic activity were manually analyzed using image analyzer software (Kodak Digital Science 1D v 3.0.0). In these, each band with noticeable proteolytic activity was delimited for individual determination of density and surrounding background zones were analyzed by single determinations of density. Data were collected as arbitrary density units and represented in graphics using Microsoft Excel 2010 software.

2.5. Protein Modeling. Amino acid sequences from *T. canis* components of TES were retrieved from GenBank or otherwise indicated and included Tc120/MUC1 (AAB05820.1), TcMUC2 (AAD49339.1), TcMUC3 (AAD49340.1), TcMUC4 (AAD49341.1), TcMUC5 (AAD49342.1), TcTES26 (UniProtKB/Swiss-Prot: P54190.1), TcTES32 (AAB96779.1), and TcTES70 (AAD31000.1). Sequences in FASTA format were used to raise protein structure models using the I-Tasser server [28, 29] that performs structural alignments between query sequences and known templates in the protein databank (PDB) library. This platform retrieves specific parameters for constructed models as the TM score (range 0-1), an index reflecting the accuracy of alignment for two given structures, and the root-mean-square-deviation (RMSD) score that indicates a measure of the differences between values predicted by retrieved models and the values actually observed in PDB templates. As recommended, we only considered significant structure alignments when TM > 0.5. The Cscore (range −5 to 2) is an index that ponderates TM and RMSD scores and allows ranking of the degrees of similarity between two given protein structures.

2.6. Ethics Statement. Experiments performed in this study are evaluated by official Mexican regulations (NOM-062-ZOO-1999) according to recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, USA. The study was approved by the Internal Committee for the Care of Experimental Animals of the Postgraduate Program of Animal Production and Health (UNAM, Mexico). Adult *T. canis* worms were obtained from necropsies of dogs humanely sacrificed using an overdose of sodium pentobarbital in the canine control centers of Mexico City.

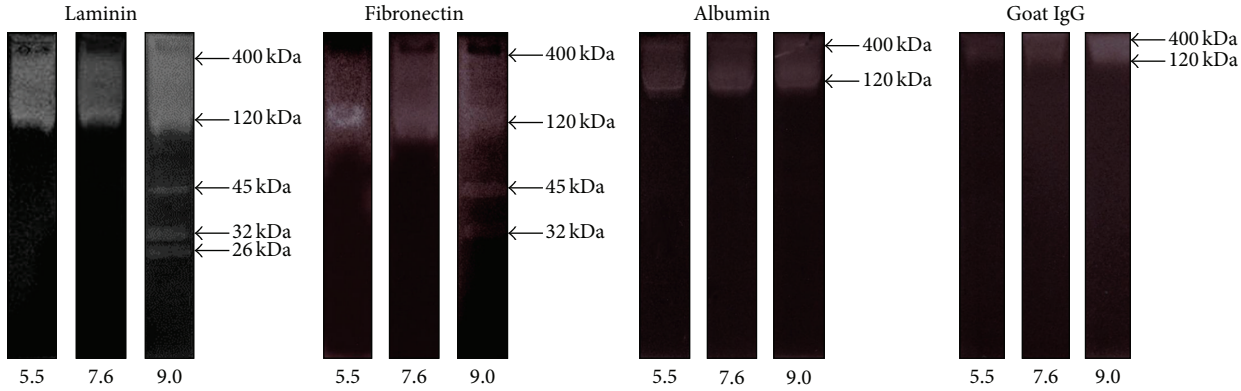

Figure 3: Proteolytic activity towards different substrates by TES components from *T. canis* at different pH values. TES samples (10 μg/well) were electrophoretically separated in slab gels of 10% (w/v) acrylamide copolymerized with the following substrates: 0.5 mg/gel laminin, 0.5 mg/gel fibronectin, 0.1% (w/v) bovine serum albumin, and 0.1% (w/v) goat IgG. After electrophoresis, proteinase activity was developed using acetate buffer (pH 5.5), phosphate buffer (pH 7.6), or glycine buffer (pH 9.0) and then gels were stained with Coomassie blue. Molecular weight of bands with activity is indicated *at the right* of the panels indicated *at the top*. The pH values used are indicated *at the bottom* of each lane.

3. Results

TES samples collected from second-stage larva cultured in RPMI 1640 medium gave yields of protein production similar to that of 8 ng/day/larvae previously reported [30]. Initial electrophoretic analyses of TES were done in polyacrylamide gels without proteinase substrate. In these, up to eight main proteins with MW of ~400, 120 (doublet), 55, 45, 32, and 26 kDa were clearly visualized (Figure 1, *lane* 1). A band of 70 kDa was much less visible. When gels were copolymerized with gelatin, proteolytic (gelatinolytic) activities developed at the three different pH values employed (5.5, 7.6, and 9.0; Figure 1, *lanes* 2–4, resp.) and only the ~400 and 120 kDa bands displayed a significant activity that was similar at the different pH values evaluated. There was a very faint activity detected at Mr of 32 kDa. In order to determine the type of proteinases contained in TES, samples were subjected to electrophoretic separation using representative substrate (gelatin) and pH 5 (7.6) conditions and proteolytic activity was developed by previous treatment of gels with proteinase inhibitors with selectivity for cysteine, serine, aspartyl, and metalloproteinases. As shown in Figure 2, gelatinolytic activity in TES was virtually unaffected by E64 and EDTA (*lanes* 1 and 2), with pepstatin A exerting a partial inhibition on the ~400 and 120 kDa bands (*lane* 3). However leupeptin abolished all proteolytic activity in TES (*lane* 4) suggesting the presence of serine-type proteinases in these samples.

The presence of proteinases in TES with similar activity at an ample pH range (5.5–9.0) on gelatin, an hydrolyzed form of collagen that is a very abundant protein of host's connective tissue, prompted us to determine if these enzymes could have the same activity on a series of physiologically important proteins (i.e., albumin, IgG, fibronectin, and laminin) that second-stage larvae eventually encounter while migrating across host tissues. In these substrate gel electrophoresis assays, the bands from TES with activity towards these different substrates and at different pH values are shown in Figure 3 and summarized in Table 1. The ~400 and 120 kDa bands were similarly active towards albumin and goat IgG at all the pH values tested, except for the case of goat IgG where these two bands displayed higher activity at pH 9. Of note, bands of 400 and 120 kDa displayed a significant difficulty to be separated in gels copolymerized with these two substrates. Otherwise some distinct bands with different pH-dependent activity were observed in gels copolymerized with laminin and fibronectin. The ~400 and 120 kDa bands appeared more separated to each other and displayed activity at all pH values while bands of 45 and 32 kDa displayed proteolysis only at basic pH (9.0). Moreover a 26 kDa proteinase was detected only with laminin at pH 9.0. Because a smear in the ~400–120 kDa range was observed using laminin and fibronectin as substrates, further densitometry analyses of these smears allowed defining reliable activity bands only by the ends of the aforementioned MW range (Figures 4(b) and 4(c)). As expected, the ~400- and 120 kDa sized proteinases could be detected separately from each other by densitometry when albumin and goat IgG were tested (Figure 4(d)). In additional assays in which proteinase inhibitors were used, as expected, only leupeptin abolished proteolysis of all these four protein substrates by TES components (not shown).

In next analyses, we looked for the sequence or structural basis to explain the serine-type proteinase activities displayed by TES components. At the primary sequence level, the 8 major TES components that have been already sequenced and reported by Rick Maizels' group [20–25] show well defined domains as are the ShKT domain, also called NC6 (nematode six-cysteine) domain, SXC (six-cysteine) domain or ICR (ion channel regulator) that is present in a 2-repeat module pattern in MUC1–5 and TES26, and the C-type lectin domain (CTL) or carbohydrate-recognition domain (CRD) in TES32 and TES70. These two domains have been used as isolated sequences to determine their folding pattern using sea anemone [24] and mammalian [22] counterparts as templates, respectively. Therefore we retrieved whole-molecule models for these major TES components and searched possible serine-type proteinase homologs in PDB.

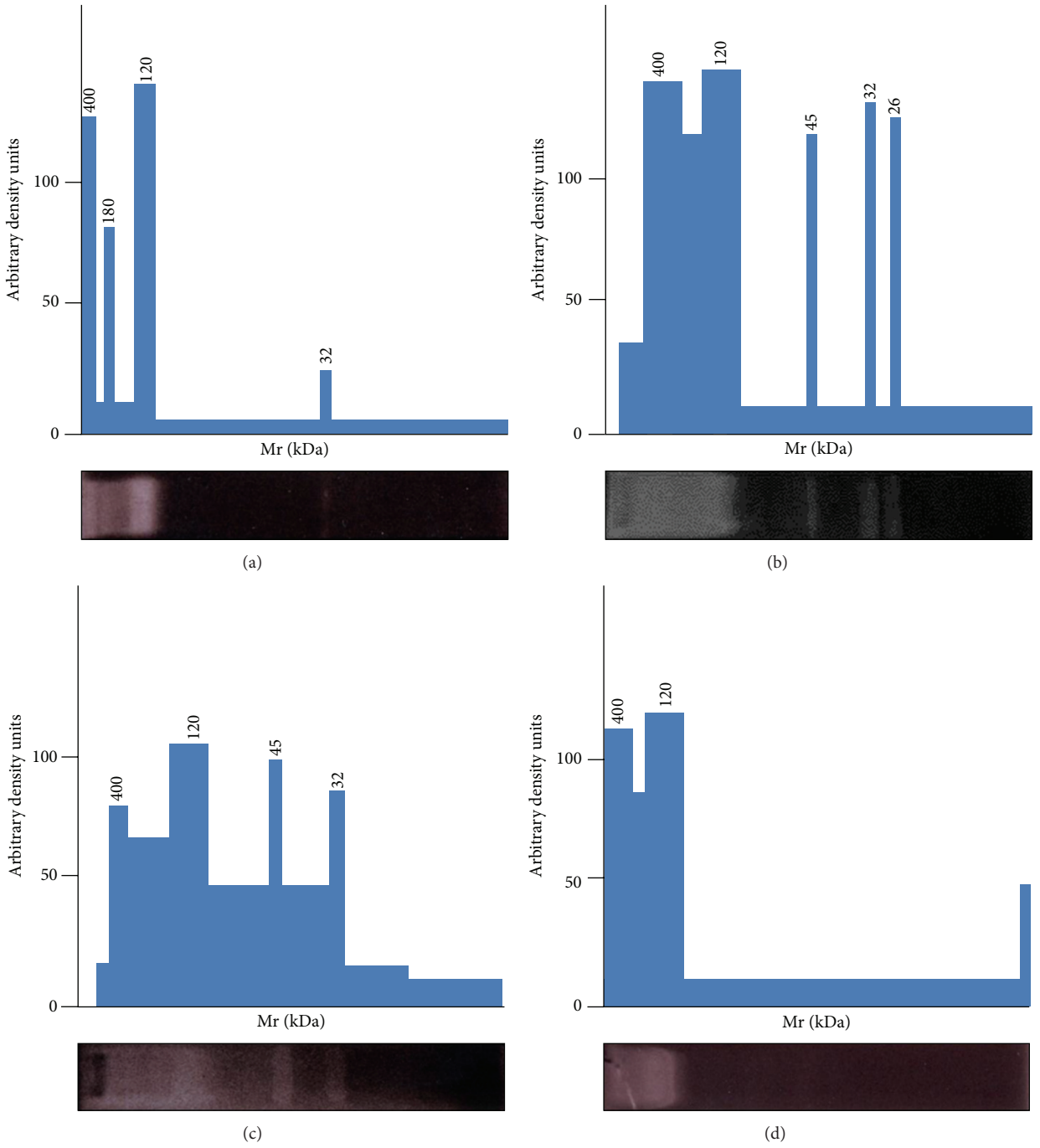

Figure 4: Densitometry analyses of representative proteolytic activities towards different substrates by TES components from *T. canis* L2s. TES samples previously separated by substrate gel electrophoresis were selected and densitometry analyses were carried out on tag image format file (TIFF) images delimiting each band with noticeable proteolytic activity from surrounding background zones and separate density determinations were obtained. Samples shown correspond to TES components resolved in 10% (w/v) acrylamide copolymerized with (a) 0.1% (w/v) gelatin at pH 7.6, (b) 0.5 mg/gel laminin at pH 9.0, (c) 0.5 mg/gel fibronectin at pH 9.0, and (d) 0.1% (w/v) bovine serum albumin at pH 9.0. The MW (in kDa) of each peak of density is indicated.

In the case of serine proteinase activities observed at 400 and 45 kDa, none encoding sequence has been conclusively proposed. In the case of TES120/MUC1 there were no obvious homologs (TM score = 0.24, RMSD = 16.1, and Cscore = −4.52) and searches in PDB retrieved only a serine-type proteinase with low homology (human aminopeptidase A, PDB 4KxTH, Tm score = 0.443, RMSD = 5.12). For MUC2, 3, and 5 the homologs found were basically of the mucin family. Interestingly, the folding of MUC2 and MUC5 models was strikingly distinct from the remainder mucins as it

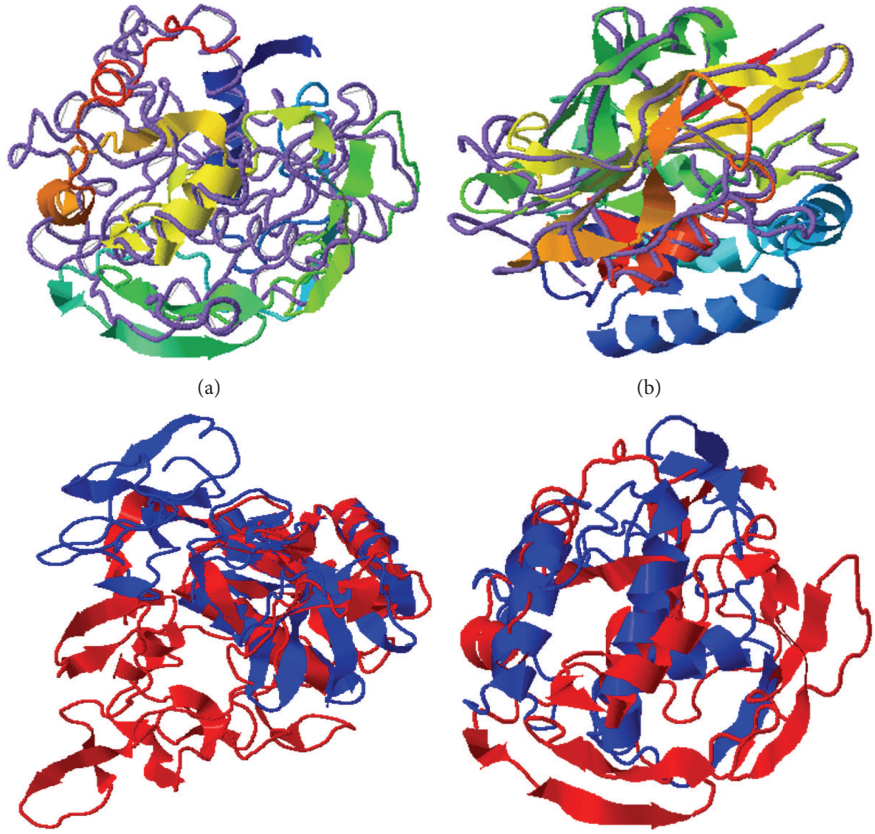

Figure 5: Protein modeling of *T. canis* excretory-secretory components (TES) and superimposition with homologs from other organisms ((a)-(b)) and between TES proteins with high (c) and low (d) structure homology. The following structures were superimposed using the I-Tasser platform coupled to the TM-align tool: (a) TcMUC4 (cartoon) and lipase with serine proteinase triad from *Rhizomucor miehei* (PDB 1TGL, backbone trace in purple); (b) TcTES26 (cartoon) and carboxypeptidase Y from *S. cerevisiae* (PDB 1WPX, backbone trace in purple); (c) TcTES32 (cartoon in blue) and TcTES70 (cartoon in red); (d) TES120/MUC1 (cartoon in blue) and TcMUC4 (cartoon in red).

was spindle-shaped while the others were globular-shaped. On the other hand, MUC4 had a reliable serine proteinase homolog which was the lipase with serine proteinase triad Ser/His/Asp from *Rhizomucor miehei* (PDB 1TGL, TM score = 0.554, RMSD = 4.90 with a sequence coverage of 0.90) (Figure 5(a)). In the primary amino acid sequence, the typical lipase serine active moiety VAVTGH**S**LGG is present as VVA**S**QAA in TcMUC4. Besides MUC4, TES26 was the only other component with a serine proteinase homolog retrieved, which was the carboxypeptidase Y from *Saccharomyces cerevisiae* (PDB 1WPX, TM score = 0.557, RMSD = 2.47 with a sequence coverage of 0.63) (Figure 5(b)). At primary sequence, the archetypal serine active moiety FHIAGESYAG is present as FNLGSPYAG in TcTES26 while the histidine-containing active moiety FTYLRVFNGG**H**MVPFDVP is apparently a divergent PSTPAANTGV**H**RYVFLVY sequence in TcTES26. Regarding TES32 and TES70, both retrieved homologs bearing the typical C-type lectin domain mediating binding to oligosaccharides; nevertheless in pair-matching comparison of protein structures among these 8 TES components, TES32/TES70 had the highest structural homology (TM score = 0.605, RMSD = 3.0) (Figure 5(c)). As reference, superimposition of TES120/MUC1 to MUC4 did not give significant homology (TM score = 0.288, RMSD = 4.88) (Figure 5(d)).

4. Discussion

The adaptive response to host stimuli by eggshell-enclosed larva and migratory larva of *T. canis* involves developmentally regulated processes as are the secretion of enzymes needed to facilitate larval emergence (in EPF) and tissue invasion (in TES), respectively. On the basis of the previous [8, 26] and the present *in vitro* studies, it is clear that *T. canis* larvae secrete aspartic proteinases in EPF and serine proteinases in TES.

As far as major TES components are concerned, these have been largely identified as proteins bearing several kinds of carbohydrate moieties and major bands have been identified at the sequence level as mucins (TES-120 as a member of a mucin family along to MUC2–MUC5) [21, 24, 25, 31], C-type lectins (TES-32, TES-70) [22, 23], and phosphatidylethanolamine-binding protein analogues (TES-26) [20]. A high MW component of TES (TES-400) is

a glycoprotein resistant to tryptic or peptic cleavage but sensitive to staphylococcal V8 proteolysis and is also detected in TES but not in L2 surface [30–32], raising the possibility that it would be an extracellular complex of other TES. Taking into account this information, it is intriguing the appearance of serine-type proteinase activity in bands with MW of 400 (glycoprotein complex), 120 (mucin complex of MUC1 to MUC3 [33]), 45 (unknown), 32 (lectin), and 26 (phosphatidylethanolamine-binding) kDa as these components have been reported to be of distinct nature and alignments and blast searches comparing these molecules with typical serine-type proteinases at the nucleotide and amino acid levels did not reveal significant similarities. Likely explanations for this issue are that a catalytically competent serine proteinase is trapped or contained within these complexes or that the macromolecular array among individual proteins in these complexes could assemble as and resemble functionally to catalytic moieties of serine-type proteinases. On the basis of the present protein modeling studies, it is conceivable that the latter possibility would occur for TES400 and TES120 complexes and that TES26 (exhibiting proteolytic activity against laminin at pH 9) and MUC4 (supposed to be scarcely secreted by larvae along to MUC5) have a serine proteinase-like folding structure and hence activity while TES32 (broadly active against gelatin and active towards laminin and fibronectin at pH 9) could have a still undefined serine-type catalytic region as other mannose-binding proteins (MASP family) have [34]. Further proteomic approaches will shed important information in these aspects.

All of the TES-n molecules aforementioned were observed in our TES preparations stained with Coomassie blue but only the ~400 and 120 kDa components along with a novel band of 180 kDa displayed serine-type activity. This is in part consistent with the 120 (doublet) and 32 kDa serine proteinases previously reported in L2 TES (Figure 2 of [26]) though in that work a significant activity in the range of >120 kDa was also detected. Accordingly TES components have shown higher proteolytic activity at pH 9.0 which correlated partially with our observations on the appearance of higher proteolytic activity by other bands (45, 32, and 26 kDa) when pH was raised to 9.0 using laminin and fibronectin as substrates but not with albumin or goat IgG. At this point, it is remarkable that the two latter substrates, contained mainly in a bodily fluid as blood, were not degraded by the low MW (<120 kDa) serine proteinases of TES while the other two extracellular matrix-associated substrates did. Remarkably the 120 kDa-associated serine proteinase activity apparently degraded substrates at the inverse pattern. The partial substrate selectivity of the 120 kDa and low MW serine proteinases contained in TES deserves future studies. In other helminthes, serine proteinases have a developmental role in the degradation of different substrates: in *Trichuris muris* two proteinases of 85 and 105 kDa have certain specificity for collagen-like substrates [35]; in the microfilaria *Onchocerca volvulus* only male worms secrete serine/metalloproteinase activities in ES products that degrade collagen type IV, fibronectin, and laminin [36] while only L3 but not the adult stage secretes a 43 kDa-sized serine elastase [37]. Likewise the proteolytic activity of the ~400 and 120 kDa TES components towards standard (gelatin) and physiological substrates (laminin, albumin, and goat IgG) over a broad pH range (5.5–9.0) extends previous observations with elastins and collagens [26] and reinforces two in-force hypotheses: firstly, the proposed relevance of TES components for larval migration and counteraction of host immune responses, and, secondly, that proteinases released by parasites often exhibit a pH profile broader than most of its mammalian counterparts in a consistent way with their explicit extracellular function (digestion, degradation, or processing of extracellular matrix proteins) within a potentially changing microenvironment. Indeed these molecules are not as redundant as mammalian ones could be [15, 38]. In addition, it remains to be determined if any proteinase(s) contained in TES might act towards hemoglobin, a major blood component, albeit hemoglobinolytic activities in helminths extracts are more easily detected using *in solution* assays than *in gel* ones and aspartic-type proteinases are more likely related to these activities [39]. However the virtually exclusive presence of serine proteinases in TES would be related to the restricted host specificity of *T. canis* (dogs and humans) as compared to other nematode organisms with lack of host specificity such as *Trichinella spiralis* where the ES products from infective larvae contain serine, aspartic, cysteine, and metalloproteinases [40].

5. Conclusions

Here, we describe the presence of up to five serine-type proteinases in excretory-secretory products of the second-stage larvae of *T. canis* (400, 120, 45, 32, and 26 kDa), three of them with proteolytic activity not reported before, that would contribute to the larval migratory process that implies its distribution by circulatory fluids and invasion of somatic organs affected in the different presentations of toxocariasis. The degrading activity of these enzymes on representative blood (albumin and IgG) and extracellular matrix (fibronectin and laminin) components not only extends previous observations using elastin and collagen [26] but also reveals the partial substrate selectivity of the 120 kDa complex and the low-MW proteinases in TES. Also, the availability of amino acid sequences for the most relevant TES components [20–25] allowed through protein modeling approaches obtaining important insights into the nature of the serine proteinase activities observed and providing basis for future studies. These proteinases remain by themselves attractive targets to improve strategies for control of toxocariasis including drug design, drug evaluation, diagnosis, and prophylaxis.

Conflict of Interests

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

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Review Article

Harnessing the Helminth Secretome for Therapeutic Immunomodulators

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Helminths are the largest and most complex pathogens to invade and live within the human body. Since they are not able to outpace the immune system by rapid antigen variation or faster cell division or retreat into protective niches not accessible to immune effector mechanisms, their long-term survival depends on influencing and regulating the immune responses away from the mode of action most damaging to them. Immunologists have focused on the excretory and secretory products that are released by the helminths, since they can change the host environment by modulating the immune system. Here we give a brief overview of the helminth-associated immune response and the currently available helminth secretome data. We introduce some major secretome-derived immunomodulatory molecules and describe their potential mode of action. Finally, the applicability of helminth-derived therapeutic proteins in the treatment of allergic and autoimmune inflammatory disease is discussed.

1. Introduction

During the last centuries living conditions in western countries changed extremely and social and economical structures shifted dramatically. As a suggested consequence of the resulting improvements in hygiene, antiparasite treatments, and the reduced exposure to pathogens and childhood infections, the occurrence of chronical inflammatory diseases and allergies increased rapidly [1, 2]. In 1989, David Strachan was the first one to link these two developments and enunciated the "*Hygiene Hypothesis.*" According to this thesis, the observed increases in certain inflammatory disorders were due to the decreased early-life exposure to microorganisms and other eukaryotic infectious agents including helminths [3].

Worm-like parasites that belong to unrelated phyla, namely, the plathelminthes (trematodes and cestodes) and the nematodes, were already present in early Hominidae. This long coexistence between humans and helminths must have had a fundamental impact on the constitution and regulation of the immune system [4–6].

As an advancement of the "*Hygiene Hypothesis,*" the "*Old Friend Hypothesis*" was put forward by Graham Rook. He hypothesized that numerous harmless pseudocommensals, including the helminths, were tolerated by the immune system due to their abundant presence [6]. In this way, the tolerance of helminths reduces the negative impact on the host's fitness, since it decreases the tissue damage or other fitness costs [8].

Recently, William Parker extended this hypothesis to the "*Lost Friends Theory*" or the "*Biome Depletion Theory.*" This theory describes the consequences of separating us from our partners in coevolution. Accordingly, the reduced pattern of exposure to microorganisms and helminths and their depletion from the human ecosystem lead to an unstable and unbalanced immune state [9]. Since the loss of components of our biome is partly responsible for epidemics of immune-related diseases such as autoimmune and allergic diseases,

Table 1: Overview of the most common human pathogenic helminths.

Organism	Number of people infected (in millions)	Disease pathology
Nematoda		
Ascaris lumbricoides	807–1121	Impaired digestion, anemia, iron deficiency, poor growth, cough, fever, abdominal discomfort, and passing of worms
Trichuris trichiura	795–1050	
Necator americanus	740–1300	
Ancylostoma duodenale		
Strongyloides stercoralis	30–100	
Wuchereria bancrofti, Brugia malayi, Brugia timori	120	Chronic lymphoedema, elephantiasis of limbs, and hydrocele
Onchocerca volvulus	37	Dermal pathology characterized by pruritus, altered pigmentation, atrophy, and lymphadenitis. Ocular lesions leading to sclerosing keratitis, chorioretinitis, optic nerve disease, and blindness
Schistosoma mansoni, Schistosoma haematobium, Schistosoma japonicum	207	Intestinal schistosomiasis characterized by abdominal pain, diarrhoea, and liver enlargement
Trematoda		
Fasciola hepatica, Fasciola gigantica	2.4–17	Fascioliasis characterized by fever, abdominal pains, and hepatomegaly
Paragonimus spp.	23	Chronic cough, chest pain with dyspnoea, and fever
Opisthorchis viverrini	10	Palpable liver, obstructive jaundice, cirrhosis, and cholangitis
Clonorchis sinensis	15.3	Clonorchiasis characterized by fever and colic pain
Cestoda		
Taenia solium, Taenia saginata	Not determined	Cysticercosis characterized by infection of the central nervous system
Echinococcus multilocularis, Echinococcus granulosus		Alveolar echinococcosis and cystic echinococcosis

Modified according to Perbandt et al. 2014 [7] and CDC report 2013.

the most reasonable solution would be the restoration of the biome [10]. Hence exposure to helminth parasites could again establish and maintain the normal immunological balance in humans. However, colonization with intestinal helminths as immune therapy is problematic due to various physiological side effects. Furthermore, the induced immune hyporesponsiveness could affect immune reactions to concomitant infections and vaccination efficacies [4, 11]. An alternative approach therefore is to identify the immune modulatory molecules produced by helminths that can alter immune functions.

2. Helminths

Infections with helminth parasites have great impact on global health and it has been estimated that at least one-third of the human population is infected with these parasites, prompting helminth infections to be termed the "*Great Neglected Tropical Diseases*" [4, 12]. Although highly parasitized individuals can suffer from severe pathology, helminths usually cause asymptomatic or subclinical chronic infections, with little evidence of an inflammatory response or overt tissue destruction. As such, many helminths can survive within their host for decades.

About one-third of mankind in the tropics and subtropics are chronically infected with one or more helminths [4, 12]. According to the WHO, more than 1.5 billion people or 24% of the world's population are infected with soil-transmitted infections (WHO, report 2014). The most common helminthiases of humans are caused by soil-transmitted nematodes, namely, *Ascaris lumbricoides*, *Trichuris trichiura*, and the hookworms *Necator americanus* and *Ancylostoma duodenale*, followed by schistosomiasis (blood flukes of the genus *Schistosoma*) and lymphatic filariasis (*Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori*) [13] (Table 1). According to the CDC, approximately 807–1,121 million people are infected with *A. lumbricoides*, 604–795 millions with whipworms, and 576–740 millions with hookworms (CDC, report 2013).

While these helminths show a remarkable variety in their mode of life, their hosts, and life history stages, they induce a canonical host immune response pattern.

3. Helminth-Associated Immune Response

The human immune system responds to the invasion of helminths into the organism differently than to bacterial or viral infections. While microbial pathogens are usually

eliminated from the host with a rapid and inflammatory immune response, the immune response to helminths is less severe and has a strong regulatory character [14].

Worm infections elicit T_H2 cell responses associated with a significant production of IL-4, IL-5, IL-9, IL-13, IL-31, IL-25, and IL-10 [13, 15]. Furthermore, the worm infections are often associated with high levels of IgE, IgG1, and IgG4 and stable eosinophil and mast cell responses [16]. Eosinophils become activated in helminth-infected sites and secrete proinflammatory cationic proteins, oxygen radicals, lipids, and other mediators like cytokines. Eosinophils and mast cells release their cytotoxic products during degranulations at infected sites [17]. The release of mediators leads to blood vessel enlargement, increased mucus production, and cell contraction of smooth muscle cells [18]. It is assumed that the primary role of eosinophils lies in the defence against large organisms which cannot be phagocytosed. Eosinophils can bind to carbohydrate ligands and fixed antibodies on the parasites surface, degranulate and release their cytotoxic agents to harm the parasite [19], and then get phagocytosed by macrophages after their response [17, 18].

Within 24 h after penetration into the host organism most helminths trigger an immediate production of T_H2 cytokines [14]. The protective effect of helminths against allergy and autoimmunity strongly depends on worm species (age, state of infection, and parasite burden) [20, 21]. Individuals infected with filarial nematodes like *W. bancrofti* and *Onchocerca volvulus* or with trematodes like *Schistosoma mansoni* and *Schistosoma japonicum* develop a strong T_H2 immune response [22]. Nevertheless, three helminth stages are known, which do not induce a T_H2 response immediately after infection: the cercariae of schistosomes, the microfilarial stage of *B. malayi,* and the nematode *Trichuris muris* [14].

In case of helminth and *Mycobacterium tuberculosis* coinfection, a dramatic reduction of protective immune responses can be observed [22]. However, some infections with parasitic worms like *Nippostrongylus brasiliensis* and *Toxocara canis* with *Mycobacterium bovis* or *M. tuberculosis* do not lead to an impaired protective immune response [22–24].

Although allergy-associated T_H2 responses and antihelminthic T_H2 responses are very similar, they also differ as follows: (1) larger amounts of polyclonal, non-parasite-specific IgE antibodies are produced that do not cause allergic reactions and (2) during helminth infection an induction of strong inflammatory regulatory immune responses occurs [25, 26]. In worm infections the Fcε receptors on mast cells can be saturated with non-worm-specific IgE; thereby, a binding of worm-specific IgE is averted. This occupation of receptor-binding sites suppresses the immediate hypersensitivity responses and the degranulation of mast cells (IgE blocking hypothesis) [18]. The IgE blocking hypothesis is still a matter of discussion. Larson and colleagues have shown that in mice the suppression of basophil responsiveness by chronic helminth infections was found to be dependent on host IL-10 [27]. IL-10 downregulates key-IgE signaling molecules [27] causing the level of serum IgE to decrease. This in turn influences the production of IgE receptors on basophils and mast cells [28–30]. Additionally, Mitre and coworkers demonstrated that the blocking of FcER1 on mast cells and basophils by parasite-induced polyclonal IgE does not mediate the protection against atopy, since the ratio of polyclonal IgE to allergen-specific IgE is too low to saturate the receptors and to suppress degranulation of mast cells and basophils [28].

Furthermore, Larson and colleagues compared the release of histamine from basophils in helminth-infected children before and after anthelminthic drug treatment and observed the suppression of basophil responsiveness during the intestinal helminth infection. They proposed that this inhibition of basophils, which are involved in the development of T_H2 responses and function as effector cells for allergy, leads to protection against allergic diseases [31].

Helminth parasites have developed a lot of strategies to evade or modulate the host immune responses with advantages on both sides [32]. Thus, there is a shift in the T_H2 response towards immunosuppression, immunological tolerance, or modified T_H2 response [16]. In case of immunosuppression an upregulation of regulatory T cells takes place which suppresses protective T_H2 as well as inflammatory T_H1 responses. During immunological tolerance development, effector T_H2 cells enter a state of anergy and fail to develop specific T effector cells which mediate resistance. Finally, in the modified T_H2 response, downstream effects of the normal T_H2 responses are muted and result in an increase of noncomplement fixing IgG4 and IL-10 [16, 33, 34]. In case of asymptomatic parasitic infections, the concentration of the T_H2-dependent isotype IgG4 is increased. A differential stimulation of IgG4 is promoted by IL-10 which is formed at high concentrations during chronic helminth infections [18]. Furthermore, many studies have shown that these helminth-mediated T_H2 responses can also prevent the often harmful inflammatory T_H1 responses by inducing suppressive regulatory T cells which contribute to the formation of IL-10 and TGF-β. Thus, helminths are able to regulate the immune responses and ensure homeostasis under various disease conditions such as autoimmune diseases, inflammations, cancer, and microbial infections [13, 15, 35].

Affected by IL-4, IL-13, and IL-21, the differentiation of alternative activated macrophages (AAMs) occurs that can inhibit the proliferation of other cells and support an increased intracellular growth of bacteria [13]. In addition to their recruitment to sites of infection and various effector functions, they also have strong anti-inflammatory properties. These are manifested by the secretion of IL-10 and TGF-β and the expression of certain genes that are involved in the repair of the extracellular matrix, fibrosis, and wound healing [13, 15]. Thus, AAMs serve tissue homeostasis, act as effector cells against parasites, and downregulate the adaptive immune system [16].

In summary, chronic helminth infections result in a downregulation of proinflammatory responses, an enhanced T_H2 response, and repair mechanisms [13, 32].

Figure 1 describes the interactions in the immune response to helminths.

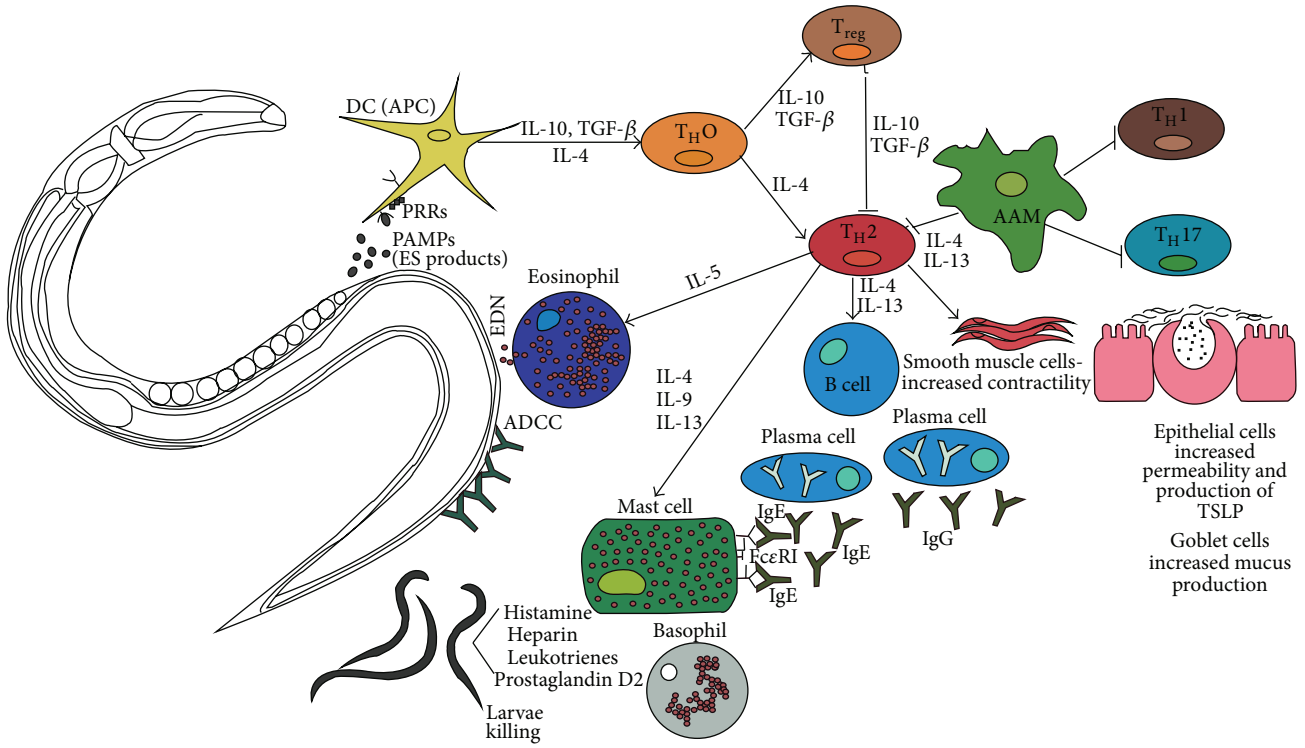

FIGURE 1: Cellular interactions in the immune response against helminths. Helminth-secreted excretory/secretory (ES) products are capable of inhibiting *in vitro* generated dendritic cells (DCs). They can inhibit the maturation of DCs and induce the expansion of functional T_{regs} [35, 36]. The helminth-induced T_H2 response starts with the recognition of parasitic pathogen-associated molecular patterns (PAMPs) by certain pattern recognition receptors (PRRs) that are expressed on the DCs of the host [13, 37]. Through contact with the antigen, the DCs become activated, allowing them to act as antigen-presenting cells (APC) after the migration to the adjacent lymph nodes, with the ability of processing and presenting the antigen to T cells to initiate an immune response [16]. The helminth-induced host immune response is focused on the protection of the host organism and is mediated by T_H2 cells. This response includes IL-4, IL-5, IL-13, and IL-10 secretion and production of IgG4 and IgE by B cells, as well as the activation of effector cells such as mast cells, eosinophils, and basophils [35]. Affected by IL-4 and IL-13 occurs the differentiation of alternatively activated macrophages (AAMs) which can inhibit the proliferation of other cells like T_H1, T_H2, and T_H17 cells. Thus, these cells have strong anti-inflammatory properties, which are manifested by the secretion of IL-10 and TGF-β as well as the expression of additional genes [13, 16, 32]. Furthermore, IL-4 and IL-13 lead to an increased contractility of smooth muscle cells and a hypersecretion of mucus for expulsion of intestinal helminths [38]. Immune complexes of IgE bind to high affinity IgE receptors (FcεRI) on mast cells and basophils; this leads to an activation of these cells and a secretion of inflammatory mediators like histamine, heparin, leukotrienes, and prostaglandin D2 [16, 38–40]. PAMPs: pathogen-associated molecular patterns; PRRs: pattern recognition receptors; ES: excretory/secretory; IL: interleukin; Ig: immunoglobulin; AAM alternatively activated macrophages; T_H: T helper cells; TGF-β: transforming growth factor-β; ADCC: antibody dependent cellular cytotoxicity; EDN: eosinophil derived neurotoxin; DC: dendritic cell; APC: antigen-presenting cell; T_{reg}: regulatory T cell.

4. Therapeutical Use of Helminths

Since there was such mounting evidence that helminth infections can modulate the mammalian immune response, treatment of immune dysregulatory diseases with live worms was considered to possess therapeutic capability, even though the suppression of an ongoing dysregulated immune response is probably more difficult to achieve than the prevention of its development. Because of the predicted lack of pathogenicity of certain helminth species, these were used in a series of clinical trials. For ethical reasons only individuals were treated who already suffered from immune dysregulatory diseases and in most studies the helminth dose was much lower than in natural infection [41, 42].

In the beginning, in a small trial three patients suffering from ulcerative colitis were treated with ova from the pig whipworm *Trichuris suis* [43]. In a clinical trial carried out by Summers et al., *T. suis* ova (TSO) were administered to 29 patients suffering from Crohn's disease. 79.3% improved significantly and 72.4% experienced remission [44, 45]. Similar results were obtained in a larger trial where patients with ulcerative colitis were treated. A decrease of pathological symptoms was observed among 43.3% of the 54 patients treated with TSO [46]. Further double-blinded placebo-controlled clinical trials using TSO are currently conducted by Coronado Biosciences and Falk Pharmaceutical company [47] (http://www.clinicaltrials.gov). A different approach, using 50 live *N. americanus* larvae, was executed by Croese and colleagues with 9 patients suffering from Crohn's disease. Following the treatment, a decrease in pathology was recorded for two patients [48]. Correale and Farez conducted studies with multiple sclerosis patients that had also been

affected by parasites. They were able to show that in these patients the disease pattern was weaker than in the control group [49, 50].

Nacher et al. observed that malaria patients with an additional gastrointestinal helminth infection, notably *Ascaris*, rarely showed acute renal failure or cerebral malaria in comparison to other malaria patients [51]. In mice infected with *Helicobacter pylori*, helminth infections were shown to reduce the tissue-damaging inflammation [52]. Recent epidemiological studies have clearly demonstrated that helminth, for example, *Schistosoma* spp., infected children had a reduced prevalence of allergic disorders. Other studies have shown that chronic infections with helminths protect people against allergic sensitization. The same results were achieved by infecting mice with *Strongyloides stercoralis* [25, 26]. Here, anthelmintic treatment led to loss of immune suppression and to an increase in atopic reactivity to allergens. Furthermore, the relationship between suppression of allergies and *Schistosoma* infection has been shown in both infected humans and mouse models [53].

A suppression of lung inflammation was shown in *S. stercoralis*-infected mice [54]. Also, extracts of the porcine parasite *Ascaris suum* inhibit IgE antibody production against unrelated antigens or antigens without reference and the generation of ovalbumin-specific T_H2 responses in a murine model of asthma [25, 55]. Infection with the rodent intestinal nematode *N. brasiliensis* is another example of suppression of T_H2 type allergic reactions, which inhibits the development of allergen-induced airway eosinophilia [56]. ES products of *N. brasiliensis* (NES) elicit a T_H2 response by affecting DCs. But besides the regulation of T_H2 response, NES also affect the proinflammatory T_H1 responses by suppressing mitogen-dependent IFN-γ release as well as DCs produced and LPS induced IL-12p70 [57–59].

The trematode *Fasciola hepatica* causes liver fluke disease in sheep and cattle. *F. hepatica* infected mice, which were experimentally coinfected with *Bordetella pertussis,* showed a reduced bacterial-specific T_H1 response. Furthermore, the mice were unabled to eliminate the microbe [60, 61]. This might be triggered by *F. hepatica* tegumental antigens that inhibit mast cells [62]. Contrariwise, *F. hepatica* did not suppress the IFN-γ-driven T_H1 response triggered by *Toxoplasma gondii* infection [63].

As described before, helminths can downregulate harmful T_H1 responses which are upregulated during autoimmune diseases. A therapeutic use of helminths could lead to a modified T_H2 response and to an induction of T_{regs}. This could result in a simultaneous reduction of T_H1/T_H17 responses and thereby reduce the pathology of autoimmune diseases [64–66].

In summary, all these studies support the concept of bystander immunoregulation by chronic helminthic infections being able to control allergen-specific or other inflammatory responses [67]. Since the dampening of the systemic immune response of the host is beneficial in transplantation, recent publications even suggest the use of helminthic therapy or helminth product therapy to enhance the allograft tolerance [68]. Despite these promising trials, the use of helminths within the therapeutical range is currently not possible due to various reasons: the breeding of helminths in the required amounts is not feasible and there are safety factors that need to be considered. Since there is evidence that only chronic but not acute infections are protective, parasite loss over time needs to be monitored [37]. The parasitic modes of action within the host are hardly explored and in some cases even completely unknown, so that possible side effects like diarrhea and intestinal pain are unpredictable [41, 69]. Unfortunately, most of the current experiments were performed with animal models and the assignability on humans cannot be guaranteed [70]. Furthermore, the psychological burden of the patients needs to be considered here as well [11, 25, 26].

The most potent anti-inflammatory response observed in humans is caused by chronic helminth infections, such as with *Schistosoma* spp. or *O. volvulus* and not by a transient infection. Therefore, it is obvious that only chronic infections with long-living helminths offer great therapeutic and preventive antiallergic effects [25, 26]. But not only live parasites can modulate or suppress the immune response. Glycans of the cuticula as well as helminth eggs or soluble extracts of worms can have the same effect. For example, *S. mansoni* egg soluble antigen (SEA) has the ability to prevent autoimmune type 1 diabetes by inducing a stronger T_H2 and T_{reg} cell response as well as functional changes in APCs [65, 71–73]. However, the repeated use of helminth antigens might also induce neutralizing antibodies, thereby preventing long-term protection. In order to avoid the possibly critical therpeutic infection with a parasite, one major research aim is to identify and characterize helminth-derived molecules that are capable of modulating the immune system and to implement therapeutic approaches based on such molecules and thus replicate the protective effect already observed in helminth therapy. These immunomodulators could lead to the generation of novel strategies for anti-inflammatory drug development [41, 58, 70, 74, 75].

5. Excretory/Secretory (ES) Products

The immunomodulatory potency of helminths appears to be largely achieved by their surface or ES products [25]. Secretory products are substances with certain biological functions that are secreted from cells or glands. Contrariwise, excretory products are unnecessary metabolic products that are released from the body. Both, however, are sometimes difficult to distinguish from one another. The composition of these products varies significantly from parasite to parasite, but in general all of them contain different glycoproteins, proteins, and smaller peptides; nonprotein components include glycans, glycolipids, and bioactive lipids, like the eicosanoid inflammatory mediators, prostaglandins, and leukotrienes [76, 77]. The term ES products describes both substances that are actively secreted by helminths and products that are released within the course of physiological processes, for example, digestion or egg-laying [58, 78]. Furthermore, varying compositions of ES products at different life cycle stages can be expected [78, 79].

Given below are a few examples of ES products that exert the antiallergic and anti-inflammatory effects of helminth

infections. In a chemically induced colitis mouse model the ES products of the canine hookworm *Ancylostoma caninum* reduced the inflammatory response and expression of proinflammatory cytokines while inducing the production of IL-4 and IL-10 [32, 75]. Furthermore, the ES products of the hookworm *Ancylostoma ceylanicum* can protect against chemically induced colitis by downregulating T_H1 and T_H17 cytokines [80]. Similar protection against inflammation was also obtained by using recombinant ES protein rTsP53 from *T. spiralis* in a colitis model [81]. Hsieh and associates also describe a secretory protein from *N. americanus* which binds to natural killer cells and stimulates the production of interferon-gamma [82]. The secreted protease inhibitor cystatin from *Acanthocheilonema viteae*, Av17, modulates macrophage-mediated inflammation in a murine model of colitis and significantly reduces inflammatory infiltrations and epithelial damage. As immunomodulatory strategy, the enhancement of IL-10 production by macrophages is proposed [83]. The immunomodulatory effect of ES products has also been shown for the cestode *Taenia crassiceps*. *T. crassiceps* ES products regulate DC activity by binding multiple receptors (e.g., MGL, MR, and TLR2), thereby downregulating TLR-mediated DC maturation and secretion of IL-12 and TNF-α. This results in T_H2 polarization [84].

There are a growing number of helminth mediators identified in the secretome that have the potential to be used in new therapeutic strategies against inflammatory diseases. Furthermore, the identification of the mechanisms and pathways these mediators utilize to redirect the immune system might reveal further key mechanisms that have evolved in host-parasite coevolution. Below we provide some examples of immunomodulatory proteins found in the secretome of parasitic nematodes.

6. Proteins Found in the Secretome of Parasitic Helminths

The secretome contains functionally diverse classes of molecules that are involved in different vital processes. While some proteins are secreted by exocytosis via the classical pathway using a hydrophobic signal peptide, other alternative pathways include exosomes, lysosomes, and microvesicles. Exosome-like vesicles have been described in the trematodes *Echinostoma caproni* and *F. hepatica*. These extracellular vesicles are internalized by an unspecific endocytic pathway or by specific ligand-receptor recognition mechanisms [85]. Transmembrane flipping and translocation can also result in the release of proteins. Finally, proteins can shed their extracellular domains, while other parts remain inside [86].

Parasitic nematodes secrete a wide range array of proteins and obviously not all of them interact locally and systemically with host immune cells; for example, there are proteolytic enzymes that are secreted to help parasites penetrate the host skin, enable tissue migration, or are involved in feeding. Furthermore, detoxifying enzymes or stress-related proteins are released to assist parasite survival in inflamed tissues. Acetylcholinesterases (AChe) are utilized that potentially interfere with secretion processes of the intestinal mucosa involved in the expulsion of pathogens [87]. Recently, it has been shown that acetylcholine is capable of modulating the activity of macrophages and attenuating local and systemic inflammation [88], making the secretion of AChe by parasites even more intruiging.

Parasitic nematodes include pathogens from plants and animals. Ectoparasitic plant parasites feed on the roots, while endoparasites penetrate the root. The obligate root-knot *Meloidogyne* species have evolved a highly sophisticated relationship with their hosts. Here, secretory proteins play an important role during migration through the roots and the formation and maintenance of proliferating cells [89]. Besides this, just like in animal-infecting parasites, molecules are secreted that are involved in the suppression or evasion of the innate immune system of the host plant. Here, antioxidant proteins coat the surface of the nematode or jasmonic acid-dependent responses are blocked. Furthermore, plant cells are reprogrammed to form multinucleate giant cells as a permanent feeding structure by the induction of nuclear division without cytokinesis [90].

Most secretory proteins of parasitic plant nematodes are produced in the oesophageal, amphidial, and rectal glands, as well as in the hypodermis and intestine [90, 91]. Common secretome components include cell-wall-degrading enzymes and expansins, venom allergen homologues (VAL), SXP/RAL-2 protein, MAP-1, SEC-2, and cuticle collagens [90].

Unlike the previously mentioned nematodes, the pine wood nematode *Bursaphelenchus xylophilus* does not establish permanent feeding sites but kills quickly by feeding on parenchymal cells after migrating through the resin canals of the tree. Following the death of the plant cells, the nematode feeds on fungal growth [79]. Due to this special feeding habit, ES products of the parasite include cell-wall-degrading enzymes like cellulases, pectate lyase, expansin-like, and venom allergen-like proteins. Furthermore, cysteine and aspartic peptidases are two of the most abundantly secreted peptidase groups found in the *B. xylophilus* secretome [79]. These could be beneficial for the parasite in several ways: it either allows the degradation of host molecules for their own nutritional purposes or serves as a defense against host responses [79]. Besides peptidases, 47 peptidase inhibitors were found that could battle against host plant peptidases. Interestingly, expression of host peptidases was significantly increased during *B. xylophilus* infection [79].

In general, animal parasitizing helminths secrete two sets of protease inhibitors that have immunomodulatory properties, cystatins, and serpins. The varying properties of cystatins from parasitic nematodes with respect to their free-living relatives point to the acquisition of anti-inflammatory properties during the coevolution of the parasites and their hosts. Cystatins have been shown to interfere with the host immune cell signaling pathways. They inhibit cysteine proteases such as cathepsins and aspartyl endopeptidase which are important for the processing and presentation of antigens by APCs. Thereby, they inhibit T cell activation. Furthermore, cystatins also prevent T cell proliferation and trigger the decrease in costimulatory molecule expression by APCs [58]. Serpins on the other hand are inhibitors of serine proteases and are able to inhibit neutrophil proteinases

and elastase and cathepsin G [92]. The serpin SPN-2 is the most abundant member of secreted proteins from *B. malayi* microfilariae; however, its function is still not clear [93].

To survive within their host, nematodes secrete a battery of diverse antioxidant systems that detoxify oxygen radicals produced by infection-stimulated host phagocytes. These proteins include peroxiredoxin, catalase, glutathione peroxidase, superoxide dismutase, thioredoxin, thiroredoxin peroxidase, and many more [7, 94]. Secretory glutathione S-transferases (GSTs) are thought to participate in the protection of parasite membranes from peroxidation [95]. Interestingly, the secretory GST-1 from *O. volvulus* has prostaglandin D2 activity, thereby contributing to the production of parasite-derived prostanoids [96].

The nematode *Haemonchus contortus* belongs to the order of the Strongylida and can infect both cattle and humans worldwide. This blood feeding nematode elicits haemorrhagic gastritis, anemia, oedema, and associated symptoms by nurturing on capillaries of gastric mucosa [97, 98]. *H. contortus* has a large set of secreted peptidases and peptidase inhibitors that function in host penetration, blood feeding, and blood-digestion [97–100].

Similar to the ES products of other parasitic nematodes, *H. contortus* releases substances influencing the host-parasite interaction as well as the host immune response, resulting mostly in a T_H2 response. ES products also include sugar-binding proteins that act as receptors for glycoprotein ligands. These C-type lectins and galectins mimic host molecules and might facilitate evasion by competing with host lectins for the binding to ligands that are involved in inflammation [58, 98, 101]. Interestingly, galectin-9 from the canine gastrointestinal nematode *Toxascaris leonina* was shown to suppress dextran sulfate sodium-induced intestinal inflammation in mice and elevated levels of IL-10 and TGF-β were observed [102].

Other types of molecules that mimic host molecules are IFN-γ, TGF-β, and the macrophage migration inhibition factors (MIFs) [103]. The cytokine MIF is an early mediator of innate and aquired immune responses and is rapidly upregulated in various inflammatory conditions [104]. Besides having cytokine activity, MIFs also have oxidoreductase and tautomerase activity. The filarial MIF homologue from *B. malayi* promotes alternative activation of macrophages in a T_H2 environment. This activation can be directly linked to its oxidoreductase activity [105, 106].

ES products from the murine gastrointestinal parasite *Heligmosomoides polygyrus* were shown to have a wide range of immunomodulatory activities including the suppression of airway allergic inflammation [41]. Also, the calcium-binding chaperone calreticulin was shown to induce a T_H2 response and at the same time interact with the mammalian scavenger receptor type A on DCs [107]. The proteins VAL-1 and AChe-1 are prevalent in L4 and adult ES products. They are considered as antigenic targets, since they induce protective immunity in mice; however, their mode of action is still unknown. While ES products from L4 and adults also seem to have TGF-β activity, released molecules from the egg stage appear to be less important in immunomodulation [108]. The Sushi domain protein family and the ShK/SXC domain toxin family are highly prevalent in the L4 secretome [108]. Sushi-like proteins are prevalent in mammals and regulate complement activation. The conserved ShK/SXC domain that shows similarity to cnidarians toxins is also extensively expressed by other nematodes including *T. canis* [108, 109]. Proteins of this family are able to inhibit calcium-dependent lymphocyte activation [110].

The *A. suum* secretome comprises about 750 molecules and contains many peptidases used for penetration and degradation of host tissue and molecules which serve to escape or modulate the host immune response. Secreted peptidases such as astacin, serine-, cysteine-, and metalloproteases ensure migration and feeding of the worm [111]. Besides this, these proteases are involved in the modulation of the host immune response [111–113]. In a murine air pouch model, the *A. suum*-derived protein PAS-1 inhibits the inflammatory leukocyte migration and reduces the synthesis of proinflammatory cytokines. Furthermore, the suppressive effect of PAS-1 in OVA-induced lung allergic inflammation was shown to be attributed to the induction of $CD4^+CD25^+$ T cells and $CD8^+$ T cells [114].

The secretome from the canine filarial parasite *Dirofilaria immitis* contains a 15 kDa antigen (DiAg) that can induce antigen-nonspecific IgE production in rats through increased generation of T_H2-related cytokines. Interestingly, DiAg suppresses the immediate dermal response to allergen-IgE interactions. This supports the IgE blocking hypothesis mentioned above [115].

In *Teladorsagia circumcincta*, an astacin-like metalloprotease and cathepsin F were identified as the most abundant ES products. These proteins are known to digest host proteins; however, the astacin-like metalloprotease additionally stimulates the immune responses during the early phase of the infection [116, 117].

Carbohydrates that are linked to proteins and lipids of nematodes have been shown to have immunogenic and immunomodulatory properties [118]. ES proteins of *A. suum* that are homologous to helminth-secreted peptides with important immunogenic or immunomodulatory roles in host animals are mostly O-linked glycosylated proteins. These glycans are unusual and structurally distinct from host glycans and induce a glycan-dependent cytokine response biased toward Th2 cells [111].

The major antigenic determinant phosphorylcholine (PC) is a small hapten that is often linked to carbohydrate epitopes in gastrointestinal and filarial nematodes [119]. PC-bearing antigens are able to interfere with key proliferative pathways in B and T cells, DC maturation, and mast cell degranulation [120]. The rodent filarial parasite *Acanthocheilonema viteae* secretes the aminopeptidase ES-62, which is the most intensely studied PC-substituted protein. ES-62 exerts its effect on various immune cells, where its anti-inflammatory action depends on the PC-moiety. It has the ability to inhibit B cell, T cell, and mast cell proliferation, promotes the alternative activation of macrophages, and is responsible for the T_H2 response through inhibition of IL-12p70 production by DCs [121]. In a mouse model for rheumatoid arthritis, ES-62 was able to significantly reduce the severity of developing collagen-induced arthritis and suppress further progression of an already established disease

Table 2: Overview of the proteomic analyses of helminths secretome.

Organism	Order	Principal host	Analyzed stage	Number of identified proteins	Approach used	References
Nematoda						
Ascaris suum	Ascaridida	Pig	Adults, female	775	Bioinformatics	[111]
Brugia malayi	Filariida	Human	Adults, mixed sex	193	Proteomics, bioinformatics	[125]
			Adults, mixed sex	82		[126]
			L3;	3		[127]
			L3/L4 molting stage;	3		
			microfilaria;	36		
			adults, male;	9		
			adults, female	12		
Dirofilaria immitis	Filariida	Dog	Adults, mixed sex	110	Proteomics, bioinformatics	[128]
Ancylostoma caninum	Rhabditida	Dog	Adults, mixed sex	105	Proteomics, bioinformatics	[129]
Heligmosomoides polygyrus	Rhabditida	Rodents	L4;	214	Proteomics, bioinformatics	[108]
			egg released material;	209		
			adults, mixed sex	364		
Ostertagia ostertagi	Rhabditida	Cattle	Adults, mixed sex	2	Proteomics, bioinformatics	[130]
			L4 and adults, mixed sex	15	Bioinformatics	[131]
Haemonchus contortus	Strongylida	Sheep, goat	Mixed stages;	1,457	Proteomics	[98]
			adults, mixed sex	107		
Nippostrongylus brasiliensis	Strongylida	Rat	Adults, mixed sex	3	Proteomics, bioinformatics	[58]
Strongyloides ratti	Strongylida	Rat	Adults, mixed sex	2572	Bioinformatics	[132]
			iL3;	196	Proteomics, bioinformatics	[133]
			parasitic female;	79		
			free-living stage	35		
Teladorsagia circumcincta	Strongylida	Sheep, goat	Larval stages;	18	Proteomics	[117, 134]
			L4;	15		
			adults, mixed sex	13		
Trichinella pseudospiralis	Trichocephalida	Bird	Larval stages	9	Proteomics, bioinformatics	[135]
Trichinella spiralis	Trichocephalida	Mammals	L1	13	Proteomics, bioinformatics	[136]
Trematoda						
Dicrocoelium dendriticum	Plagiorchiida	Ruminants	Adult (exosome-like vesicles);	84	Proteomics, bioinformatics	[137]
			adult (surface);	113		[138]
			adult (ESP);	29		
			tegument	43		
Fasciola hepatica	Prosostomata	Cattle, sheep	Larval stages;	22	Proteomics, bioinformatics	[139]
			adults, mixed sex;	26		
			mollusc-dwelling larva;	8		
			adults, mixed sex;	160	Proteomics	[140]
			dormant larvae	26		
Schistosoma mansoni	Strigeidida	Human	Cercaria;	72	Proteomics, bioinformatics	[141–143]
			egg;	188		
			cercaria	23		

[122] Furthermore, its anti-inflammatory action was also observed in human rheumatoid arthritis-derived synovial tissue cultures [123].

Here we have given a few examples of proteins found in the secretome of parasitic nematodes, some with known functions in immune modulation and some with as-yet hypothetical functions.

Helminth secretomes are a rich source of novel drug and vaccine targets, diagnostic markers, and immunomodulatory proteins. While the analysis of secreted proteins from different life stages of helminths is still quite challenging, numerous secretome analyses of helminths exist by now (Table 2). The combination of the existing data towards a more integrated view of ES products from helminths will be the next logical step. Existing difficulties, such as the lack of genomic sequence information, can be dealt with by using RNA-sequence assembly as reference for the identification of ES products. More challenging, however, are low protein concentrations due to high dilutions of cultivation media, is contamination of normally nonsecreted proteins due to cell lysis and death, or is that most developmental stages cannot be cultivated *in vitro* [117]. Here enrichment methods could be applied that are based on posttranslational modifications of secreted proteins, for example, glycosylation [124].

7. Conclusion

Helminthic infections have a large impact on global health and can cause severe forms of helminthiasis. Nevertheless, they have proven to have immunomodulatory and immunoregulatory effects on the host's immune system which can be exploited in the treatment of immune dysregulatory diseases. While helminths have independently evolved various strategies to gain entrance to host tissues and to actively evade or even manipulate the signaling network of the immune system, the host developed strategies to limit pathology by shifting the T_H2 response towards immunosuppression instead of triggering an inflammatory tissue-damaging response.

A number of promising clinical trials were performed using live worms to treat immune dysregulatory diseases. However, the major research aim is to identify and characterize helminth-derived modulators which can foster anti-inflammatory drug development.

Abbreviations

AAM: Alternative activated macrophages
ACE: Acetylcholinesterase
AcES: *Ancyostoma canium* ES products
APC: Antigen-presenting cell
DCs: Dendritic cells
DiAg: *Dirofilaria immitis* antigen
ECM: Extracellular matrix
ES: Excretory/secretory
FcεRI: High affinity IgE receptors
GST: Glutathione S-transferase
IBD: Inflammatory bowel disease
IFN-γ: Interferon-gamma
Ig: Immunoglobulin
IL: Interleukin
LF: Lymphatic filariasis
LPS: Lipopolysaccharide
MGL: Macrophage galactose C-type lectin
MHC: Major histocompatibility complex
MIF: Macrophage migration inhibitory factor
MR: Mannose receptor
NES: *N. brasiliensis* ES products
NK: Natural killer cells
OVA: Ovalbumin
PAMPs: Pathogen-associated molecular patterns
PAS-1: Protein from *A. suum*
PC: Phosphorylcholine
PRRs: Pattern recognition receptors
RELM-α: Resistin-like molecule-alpha
SEA: *S. mansoni* egg soluble antigen
TGF-β: Transforming growth factor-beta
T_H: T helper
TLR: Toll-like receptor
TNF-α: Tumor necrosis factor-alpha
T_{regs}: Regulatory T cells
TSLP: Thymic stromal lymphopoietin
TSO: *Trichuris suis* ova
VAL: Venom allergen/*Ancylostoma* secreted protein-like.

Conflict of Interests

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

Authors' Contribution

Dana Ditgen and Emmanuela M. Anandarajah contributed equally to this work.

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Research Article

Regional Warming and Emerging Vector-Borne Zoonotic Dirofilariosis in the Russian Federation, Ukraine, and Other Post-Soviet States from 1981 to 2011 and Projection by 2030

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We analyze through a climatic model the influence of regional warming on the geographical spreading and potential risk of infection of human dirofilariosis in Russia, Ukraine, and other post-Soviet states from 1981 to 2011 and estimate the situation by 2030. The model correctly predicts the spatiotemporal location of 97.10% of 2154 clinical cases reported in the area during the studied period, identified by a retrospective review of the literature. There exists also a significant correlation between annual predicted *Dirofilaria* generations and calculated morbidity. The model states the progressive increase of 14.8% in the potential transmission area, up to latitude 64°N, and 14.7% in population exposure. By 2030 an increase of 18.5% in transmission area and 10.8% in population exposure is expected. These findings strongly suggest the influence of global warming in both geographical spreading and increase in the number of *Dirofilaria* generations. The results should alert about the epidemiological behavior of dirofilariosis and other mosquito-borne diseases in these and other countries with similar climatic characteristics.

1. Introduction

Climatic change strongly affects human and animal health increasing the risk of infections by many vector-borne parasitic, bacteria, and virus diseases [1, 2]. The temporal and spatial changes in temperature, rainfall, and humidity influence the distribution and seasonality of vectors and the extrinsic incubation of pathogens [3]. There is currently a considerable concern in the European institutions about the emergence or reemergence in the continent of some mosquito-borne diseases like malaria, leishmaniosis, dirofilariosis, West Nile virus, and chikungunga, among others, as a consequence of the climatic change [3, 4].

Dirofilariosis is a zoonotic disease mosaic transmitted by culicid mosquitoes, affecting dogs, cats, and humans, caused mainly by the filarid nematodes *D. immitis* and *D. repens* [5]. While animal dirofilariosis may result from a benign to a severe and potentially fatal disease [6], human dirofilariosis manifests itself as benign subcutaneous or pulmonary nodules that mimic malignant tumors [7] caused by immature worms. With increasing frequency in humans, fully developed *D. repens* adult worms are detected in conjunctival, retroocular, and intravitreal locations, responsible for loss of vision or other permanent ocular alterations [8, 9]. In addition, surgical removal of the nodules, very aggressive in pulmonary and intraocular/retroocular cases, can cause considerable damages and treatment costs [10, 11].

In Western Europe dirofilariosis has been historically considered endemic in the Mediterranean countries [12, 13], but during the last 14 years a rapid expansion into central

and northern countries has occurred, mainly attributed to global warming [9]. In Eastern European countries dirofilariosis also exists but information on the epidemiological situation in canine populations is partial and limited. However, information is relatively abundant on human dirofilariosis, since many of the clinical cases recently reported in the world have been diagnosed in Ukraine and Russia, almost all attributed to *D. repens* [5, 14, 15]. Models to predict diseases patterns have become very valuable tools helping the design of appropriate control strategies [2, 16]. Unlike in other infectious diseases, in which predictions are difficult to assess [3, 17], changes predicted on dirofilariosis in Western Europe [18] were repeatedly confirmed, since its emergence in some central and northern European countries, previously free of dirofilariosis, has been already demonstrated [5]. Thus, dirofilariosis appears as a good model to evaluate the impact of global warming on the spread of mosquito-borne diseases. Furthermore, the influence of the changing climatic factors on the dynamic and trends of dirofilariosis has not been yet analyzed in the European far East, in spite of the strong thermal anomalies recently observed [19] and the recognition of dirofilariosis as an emerging disease [14, 15] in some post-Soviet states.

In the present work we developed a spatiotemporal geographic information system (GIS) model of *Dirofilaria* transmission in the former USSR based on the accumulated heat necessary to complete L3 development. Its validation was assessed by the spatial and temporal referentiation of human clinical cases obtained by an exhaustive retrospective review of different sources from 1981 to 2011 and derived morbidity. Moreover, a projection of the situation by 2030 is presented.

2. Materials and Methods

2.1. Data. We used daily temperature data recorded by a network of 421 meteorological stations of the Russian Federation and neighboring countries from 1981 to 2011 and temperatures foreseen by 2030 by the Russian Committee of Hydrometeorology [20].

Population data of each administrative unit of the former USSR were obtained from the current Russian State Federal Statistic Committee, State Statistics Committee of Ukraine, and the World Bank web site. Because the whole population appeared stable from 1981 to 2011 with a slight increase of 0.1%, we assumed the population data of 2011 for all calculations.

An exhaustive retrospective review of clinical cases of human dirofilariosis reported in the territory of the former USSR between 1981 and 2011 was carried out with the objective of the spatiotemporal referencing (year and administrative unit of occurrence) of each autochthonous case. The search was made using the following sources: (1) national and local medical journals from Russia and neighboring countries; (2) the archives of the Ukrainian Healthcare Ministry since 1997; (3) PubMed database to corroborate that no cases of human dirofilariosis have been published in non-Russian language journals was also consulted. Cases without date and/or geographical reference were excluded (8 cases). Clinical cases together with population data were used to calculate morbidity (number of cases/year/100000 people).

2.2. Model. The model predicts the spatiotemporal distribution of the number of generations of infective L3 of *Dirofilaria* that can yearly develop in the mosquito vectors (directly related to the annual length of the transmission period), considering temperatures calculated as indicated below. Full development of L3 needs 130 growing degree-days (GDDs) [16] accumulated in 30 consecutive days, the estimated mean life expectancy of a mosquito vector. Each day accumulates a number of GDDs resulting from the difference between the mean daily temperature and the threshold temperature for extrinsic incubation of *Dirofilaria* (L3 development), which has been already experimentally calculated in 14°C [21, 22]. Thus, the mean annual accumulated GDDs were used to calculate the number of annual generations of *Dirofilaria* developed in the vectors.

A GIS for the area studied was generated using the georeferenced meteostations previously indicated, the former Soviet Union regional administrative boundaries (oblasts and republics), and the model output databases. Given the high correlation between the indexes based on the GDD and altitude and latitude, the map of generations was produced following the methodology previously described [23–25]. In brief, considering the average GDDs previously calculated to each meteostation, for every 10-year period: 1981–1990, 1991–2000, 2001–2011, and on 2030, regressions were established to calculate the influence of altitude and latitude on GDD.

The GDD matrix was calculated using an image calculator module in accordance with the obtained regression formula, where corresponding latitudinal and altitudinal matrices were used as substitutes for latitude and altitude values. SRTM digital elevation models were used as altitudinal matrices [26]. The difference between calculated and real GDD values was the remainder. It was calculated as the difference between the regression calculated GDD and the real GDD for every meteorological station and interpolated over the whole analyzed territory by linear Kriging procedure. The interpolated remainder's layer was added to the calculated layer. The procedure was separately repeated for the coastal and continental stations. The coastal meteostations were located less than 30 km to the sea and the continental meteostations were located more than 30 km to the sea. The calculations were done separately because coastal and continental temperatures have different values and dynamic. After that, the coastal and continental GDD layers were joined through coastal 30 km buffer. The resulting GDD layers describing situation for 1981–1990, 1991–2000, 2001–2011, and 2030 have spatial resolution 10 km and presented in Albers Conic projection.

To validate the model two factors were analyzed: (1) the concordance between the predicted transmission areas and the spatiotemporal distribution of cases obtained by the retrospective review and (2) the statistical correlation between the number of predicted generations of *Dirofilaria* and the observed morbidity. This statistical correlation was evaluated globally and by decades using the Pearson

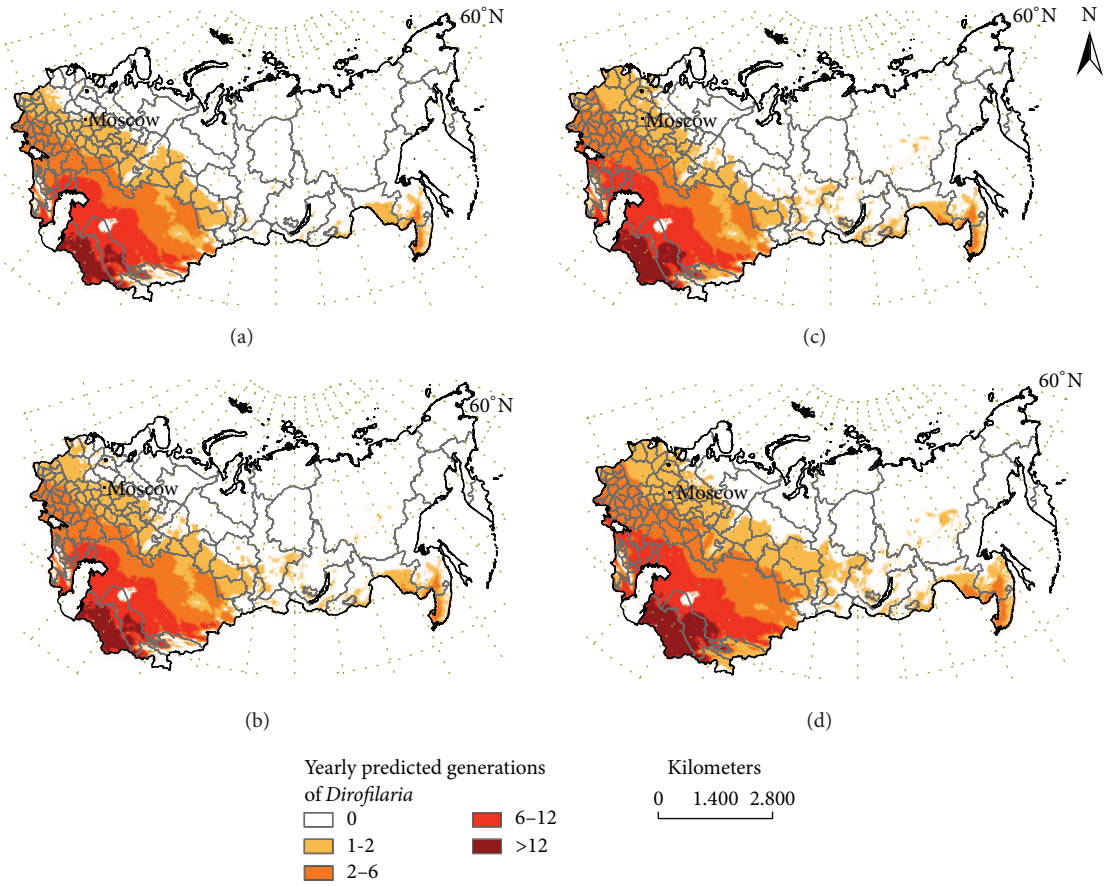

Figure 1: Retrospective prediction model of *Dirofilaria* transmission in the former USSR. Distribution of annual predicted generations: 1981–1990 (a), 1991–2000 (b), and 2001–2011 (c) and projection of the future scenario by 2030 (d).

correlation analysis, considering a significant correlation when $P < 0.01$ (highly significant).

Projection by 2030 scenario predicts the distribution of the transmission areas, including the number of predicted generations of *Dirofilaria,* and estimates the person-year of exposure using the same methodology described for the retrospective model.

3. Results

The model generated retrospectively predicts the existence of two different potential transmission areas of dirofilariosis, one in the South West and other in the Asian Far East of the territory studied, where a different number of annual generations of *Dirofilaria* exist. Moreover, the model predicts significant changes over time (Figures 1(a), 1(b), and 1(c)). From 1981 to 1990, 1/3 of the territory studied becomes a suitable area for transmission. The Southwestern area covered from Ukraine to the Eastern borders of the Asian republics, excluding the Caucasus, Urals, and Tien Shan/Pamir ranges. In the northern part of this area, with the boundary in latitude 53–57°N in the European part of the Russian Federation and 61°N in Western Siberia, summer temperatures are low, optimal conditions for extrinsic incubation of *Dirofilaria* appear only sporadically, and 1 to 2 annual generations are predicted. In the Southern side high temperatures allow *Dirofilaria* extrinsic incubation during long periods each year, including two zones with 6–12 and more than 12 annual predicted generations, respectively. Between these zones there is another zone where annual predicted generations range from 2 to 6. In the Far East area 1 to 2 annual generations of *Dirofilaria* in most of the territory with a small zone with 2–6 annual generations are predicted. During the following two periods the boundary of the Southwestern area moved progressively until latitude 60°N in the European Northwestern side while it turned back slightly in Western Siberia. Both the zones with 2–6 and 6–12 predicted generations clearly extend to the Northwest. Scattered small areas with 1 to 2 predicted generations appear in Siberia as far as latitude 64°N (near the Polar Circle), along the Yenisei and Lena river basins, the Far East area remaining almost stable. Moreover, an altitudinal spreading also occurs, since some low lands of the Tien Shan/Pamir ranges become included in the predicted area. The increase of the area suitable for transmission (5.8% in 1991–2000 and 9% in 2001–2011) is accompanied by rises in the estimated population exposure of 7% and 7.4%, while person-year of exposure, respectively, rises 3.3% and 14.3% in the same periods (Table 1).

Table 1: Estimated area, population exposure, and transmission of *Dirofilaria* in the former USSR.

Annual predicted generations of *Dirofilaria* intervals	Area (×1000 km^2)	1981–1990 Population exposure (millions)	PYE (millions)	Area (×1000 km^2)	1991–2000 Population exposure (millions)	PYE (millions)	Area (×1000 km^2)	2001–2011 Population exposure (millions)	PYE (millions)	Area (×1000 km^2)	2030 Population exposure (millions)	PYE (millions)
0	14908	77.15	0	14467 (−3%)	62.83 (−18.6%)	0 (0%)	13740 (−5%)	46.54 (−25.9%)	0 (0%)	12115 (−11.8%)	21.49 (−53.8%)	0 (0%)
0–2	2936	92.45	138.68	3321 (13.1%)	103.69 (12.2%)	155.54 (12.2%)	3641 (9.6%)	100.70 (−2.9%)	151.05 (−2.9%)	4388 (20.5%)	103.13 (2.4%)	154.70 (2.4%)
2–6	2498	62.19	248.76	2534 (1.4%)	65.27 (5%)	261.08 (5%)	2619 (1.6%)	76.69 (17.5%)	306.76 (17.5%)	3153 (20.4%)	96.27 (25.5%)	385.08 (25.5%)
6–12	1735	38.98	350.82	1733 (−0.1%)	38.98 (0%)	350.82 (0%)	1918 (10.7%)	40.79 (40.6%)	367.11 (40.6%)	2068 (7.8%)	41.68 (2.2%)	375.12 (2.2%)
>12	455	11.87	142.44	477 (4.8%)	11.87 (0%)	142.44 (0%)	614 (28.72%)	17.91 (50.9%)	214.92 (50.9%)	808 (31.6%)	20.05 (11.9%)	240.6 (11.9%)
Total affected area	7624	205.48	880.70	8065 (5.8%)	219.80 (7%)	909.88 (3.3%)	8792 (9%)	236.09 (7.4%)	1039.84 (14.3%)	10417 (18.5%)	261.49 (10.8%)	1155.5 (11.1%)

PYE: person-year of exposure. % increase shown in parentheses.

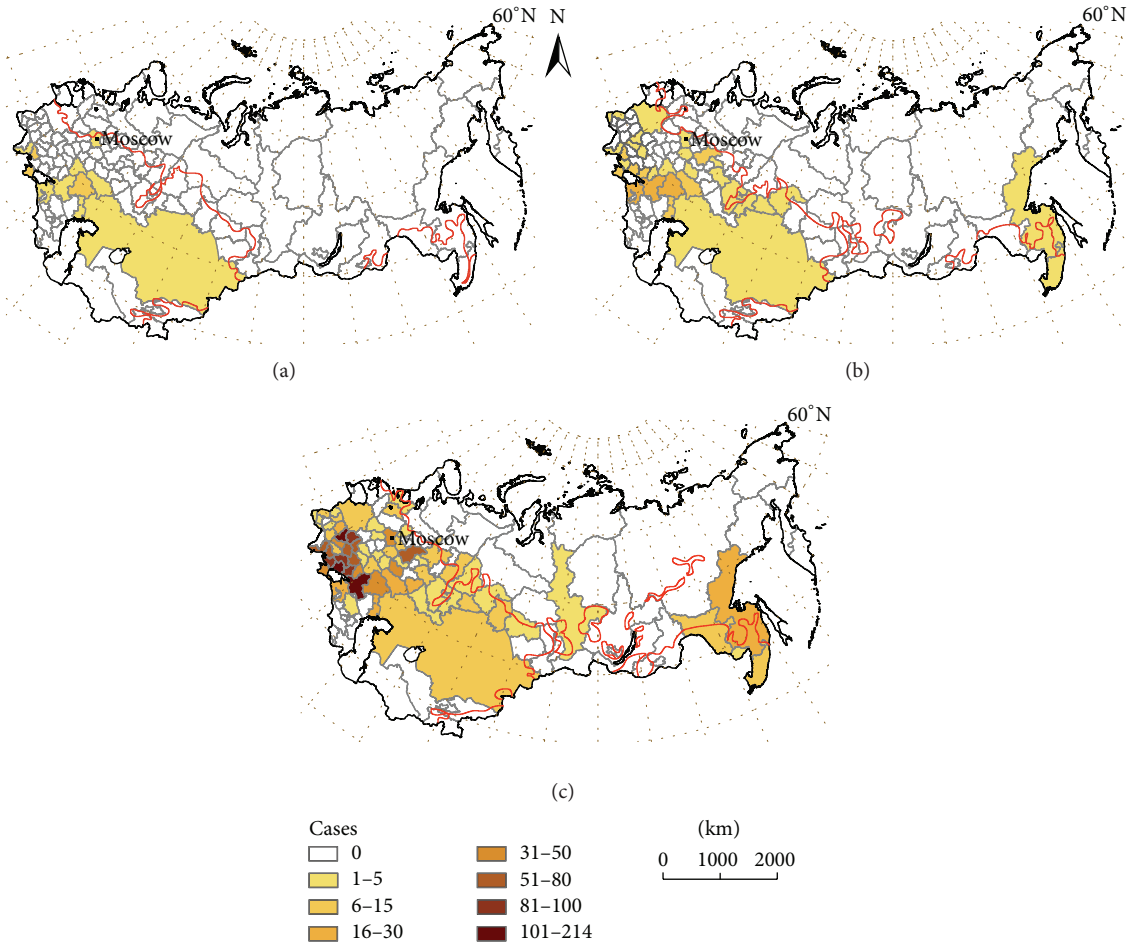

Figure 2: Administrative units of the former USSR where human dirofilariosis cases have been reported: 1981–1990 (a), 1991–2000 (b), and 2001–2011 (c). The red lines indicate the northern boundary of the predicted risk area of each lapse.

The retrospective review revealed 2154 cases of human dirofilariosis reported in the former USSR from 1981 to 2011. Data of geographical location, incidence, and morbidity are presented in Table 2. Fifty-eight, 196, and 1900 cases were, respectively, reported in 1981–1990, 1991–2000, and 2001–2011, most of them diagnosed in Ukraine and the Russian Federation. Cases appeared between latitudes 42°N (Kazakhstan) and 60°N (St. Petersburg) in 68 out of the 125 administrative units of the former USSR. Until 1996 there was a low annual incidence (2–11 cases/year), while from 1997 to 2011 incidence strongly rises from 22 to 365 cases/year. Morbidity rises over time in all administrative units where clinical cases have been referenced, except in Krasnodar (Southwestern Russia Federation), where reported cases decreased from 1991–2000 to 2001–2011 periods. Highest increases occurred in the Ukrainian and Russian administrative units near the Black and Azov seas, along the Dnieper, Don, and Volga river basins and in some regions of Southwestern Siberia. In relation to the validation of the model, the temporal and spatial distribution of cases reported in the literature review (by decades and administrative units) is shown in Figure 2. The concordance analysis between the predicted transmission areas and the spatiotemporal distribution of clinical cases shows that the model correctly predicts 97.10% of cases with a confidence interval (CI) of 92.42–100%. By periods the concordances are, respectively, 100% with CI of 96.15–100%, 96.97% with CI of 89.61–100%, and 98.53% with CI of 94.93–100%. The Pearson coefficient to assess the correlation between the number of predicted yearly generations and morbidity (Figure 3) shows a significant correlation at the global level (0.560, $P < 0.01$). By periods, correlations were 0.457 (1981–1990), 0.546 (1991–2000), and 0.510 (2001–2011), with $P < 0.01$ in all cases. The analysis revealed the existence of exceptional situations in very few administrative units, where higher or lower morbidities than expected appeared. Comparative dynamics of yearly appearance of the threshold of 130 GDDs allowing extrinsic incubation and incidence of cases was assessed in Moscow, as representative of the administrative units close to the North boundary of predicted area (Figure 4). From 1981 to 2000, when only 8 out of 20 years reached the threshold of 130 GDDs, 3 cases were recorded. From 2001 to 2011, with 8 out of 11 years reaching the threshold, 40 cases were reported in a yearly consecutive series. This series began after 4 consecutive years (1997–2000) reaching the threshold.

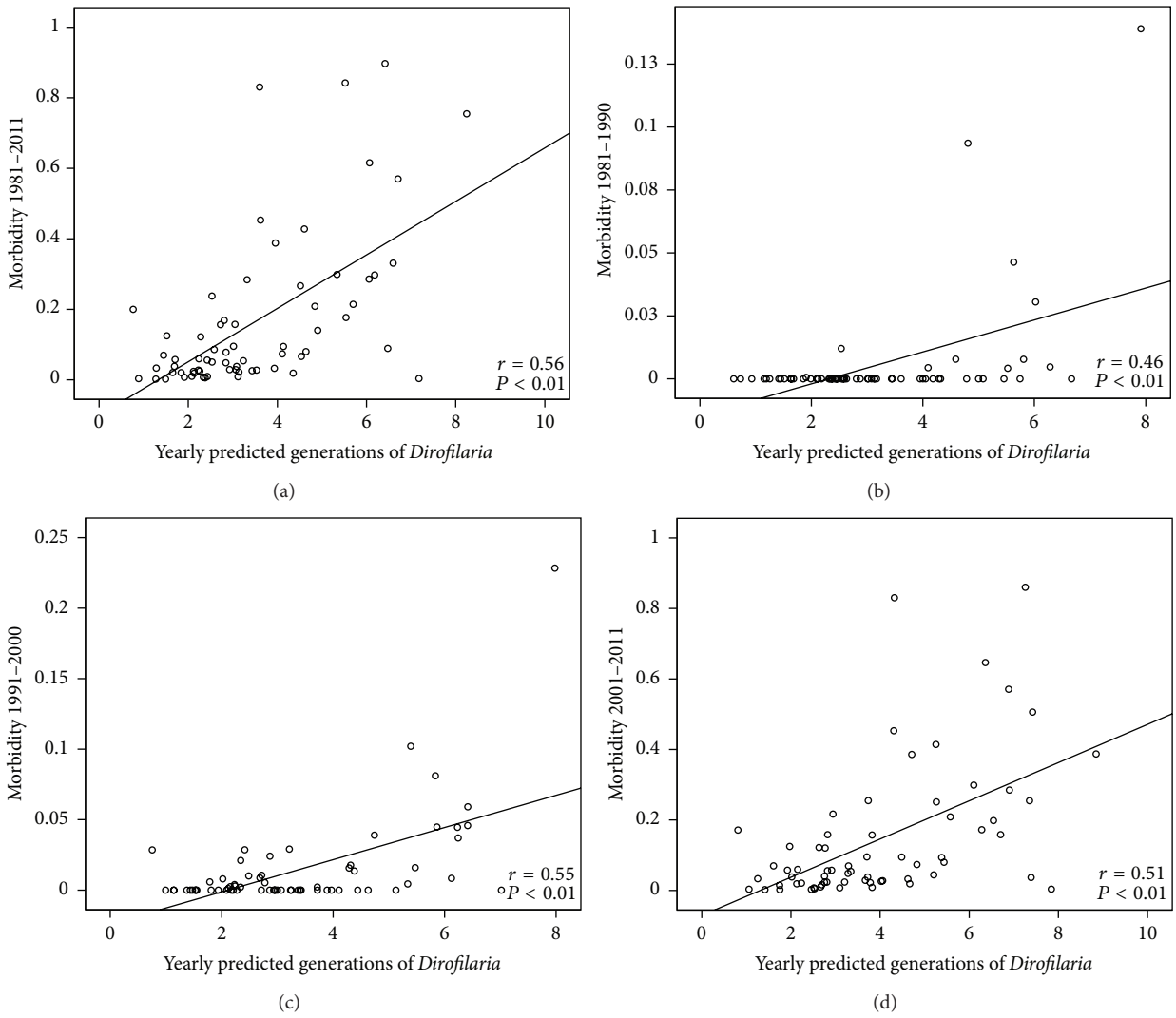

Figure 3: Statistical correlation (Pearson correlation coefficient) between yearly predicted generations of *Dirofilaria* and morbidity. From 1981 to 2011 (a), from 1981 to 1990 (b), from 1991 to 2000 (c), and from 2001 to 2011 (d).

Projection by 2030 (Figure 1(d)) predicts extensive latitudinal and altitudinal changes in the potential transmission area (18.5% of surface area increase), with a 46% of the total territory and 110 out of 125 administrative units of the former USSR becoming included. These changes mainly occur in the northern zone with low number of predicted generations, the following being the most important: (1) the northward shift of the boundary exceeding latitude 60°N in all the Southwestern area; (2) the spreading of Siberian areas which have previously appeared; (3) the slight increase of the Far East area; (4) the continuing altitudinal spreading in low lands of the Urals and Tien Shan/Pamir ranges. Population exposure in 2030 will rise to 10.8%, affecting 92.4% of the total former USSR population, while the person-year of exposure will increase 11.1%, in respect of estimations of 2001–2011 period (Table 1).

4. Discussion

The effects of climatic change on the vector-borne diseases are greatest at the temperature range extremes at which transmission occurs (14°–18°C and 35°–40°C) [27]. Thus, important modifications in the distribution and seasonality of these diseases in temperate and cold areas where changes occur are expected [3]. The former USSR can be considered a paradigm of this fact because of its vast size and climatic variety, the strong thermal anomalies observed there in the last years [19, 28], the current recognition of human dirofilariosis as an emerging disease [14, 15], and precedents which have already demonstrated the emergence of different zoonotic diseases in the Russian Arctic [29].

In the present work a spatiotemporal model, based on climatic forecast using the concept of growing degree days [16], retrospectively predicts a latitudinal and altitudinal spreading of the potential transmission area of *Dirofilaria*, mainly in the Russian Federation and Ukraine, near the boundaries where temperatures are close to the threshold for extrinsic incubation of parasite larvae. Moreover, strong increases in the population exposure and person-year of exposure are also predicted, as a consequence of both the spreading trends into highly populated zones and the rise

Table 2: Reported cases and morbidity of human dirofilariosis in the former USSR. Morbidity is expressed as (number of cases/year/100000 habitants).

(a)

	Population	1981–1990		1991–2000		2001–2011	
		Cases	Morbidity	Cases	Morbidity	Cases	Morbidity
Russia							
St-Petersburg	4600276	0	0.000	0	0.000	1	0.002
Krasnoyarsk	2893926	0	0.000	0	0.000	1	0.003
Perm	2701174	0	0.000	0	0.000	9	0.033
Kirov	1391059	0	0.000	0	0.000	8	0.058
Sverdlovsk	4393797	0	0.000	0	0.000	1	0.002
Novgorod	640613	0	0.000	0	0.000	8	0.125
Yaroslavl	1306320	0	0.000	0	0.000	5	0.038
Tyumen	3430313	0	0.000	2	0.006	5	0.015
Ivanovo	1066541	0	0.000	0	0.000	13	0.122
Mari-El	698176	0	0.000	2	0.029	4	0.057
Nizhni-Novgorod	3323600	0	0.000	7	0.021	72	0.217
Omsk	2012092	0	0.000	0	0.000	12	0.060
Vladimir	1430084	0	0.000	0	0.000	8	0.056
Moscow	17315765	1	0.001	2	0.001	40	0.023
Kurgan	947566	0	0.000	1	0.011	15	0.158
Tatarstan	3778504	0	0.000	2	0.005	9	0.024
Novosibirsk	2649871	0	0.000	0	0.000	5	0.019
Smolensk	965986	0	0.000	0	0.000	2	0.021
Khabarovsk	1400425	0	0.000	4	0.029	24	0.171
Chelyabinsk	3508447	0	0.000	1	0.003	1	0.003
Bashkortostan	4065993	0	0.000	1	0.002	2	0.005
Kaluga	1001559	0	0.000	0	0.000	1	0.010
Mordovia	826526	0	0.000	0	0.000	4	0.048
Ryazan	1151439	0	0.000	1	0.009	8	0.069
Amur	860686	0	0.000	0	0.000	6	0.070
Samara	3170141	0	0.000	0	0.000	30	0.095
Penza	1373236	0	0.000	4	0.029	35	0.255
Altay	2490714	3	0.012	6	0.024	30	0.120
Tambov	1088437	0	0.000	0	0.000	3	0.028
Lipetsk	1157852	0	0.000	0	0.000	3	0.026
Orenburg	2112910	0	0.000	0	0.000	4	0.019
Saratov	2564835	2	0.008	10	0.039	24	0.094
Kursk	1148610	0	0.000	0	0.000	1	0.009
Voronezh	2261628	1	0.004	4	0.018	10	0.044
Belgorod	1530124	0	0.000	0	0.000	5	0.033
Volgograd	2589887	12	0.046	21	0.081	41	0.158
Biribidzhan	185039	0	0.000	0	0.000	1	0.054
Rostov	4229505	2	0.005	27	0.064	214	0.506
Astrakhan	1007113	14	0.139	23	0.228	39	0.387
Krasnodar	5160656	4	0.008	23	0.045	19	0.037
Vladivostok	1981970	0	0.000	2	0.010	8	0.040
Stavropol	2711198	0	0.000	0	0.000	1	0.004

(a) Continued.

	Population	1981–1990		1991–2000		2001–2011	
		Cases	Morbidity	Cases	Morbidity	Cases	Morbidity
Ukraine							
Chernihiv	1083827	0	0.000	0	0.000	90	0.830
Sumy	1147749	0	0.000	0	0.000	52	0.453
Rivne	1156009	0	0.000	0	0.000	11	0.095
Volyn	1040606	0	0.000	0	0.000	3	0.029
Zhytomyr	1270939	0	0.000	0	0.000	20	0.157
Kiev	4536061	0	0.000	1	0.002	175	0.386
Lviv	2539031	0	0.000	1	0.004	6	0.024
Kharkiv	2732086	0	0.000	0	0.000	57	0.209
Khmelnytskyi	1318377	0	0.000	0	0.000	5	0.038
Poltava	1472541	0	0.000	2	0.014	61	0.414
Cherkasy	1274125	0	0.000	2	0.016	32	0.251
Crimea	1963770	6	0.031	9	0.046	50	0.255
Luhansk	2263676	0	0.000	1	0.004	39	0.172
Vinnytsia	1631305	0	0.000	0	0.000	12	0.074
Ivano-Frankivsk	1381184	0	0.000	0	0.000	1	0.007
Kirovohrad	999285	0	0.000	0	0.000	8	0.080
Zakarpattia	1252608	0	0.000	1	0.008	2	0.016
Dnipropetrovsk	3312064	0	0.000	0	0.000	99	0.299
Chernivtsi	905189	0	0.000	0	0.000	2	0.022
Donetsk	4387702	0	0.000	7	0.016	87	0.198
Zaporizhia	1786905	0	0.000	8	0.045	102	0.571
Mykolaiv	1175598	11	0.094	12	0.102	76	0.646
Odessa	2387282	1	0.004	2	0.008	68	0.285
Kherson	1081336	0	0.000	4	0.037	93	0.860

(b)

Post-Soviet states	Population	1981–1990		1991–2000		2001–2011	
		Cases	Morbidity	Cases	Morbidity	Cases	Morbidity
Belarus	9457500	0	0.000	2	0.002	7	0.007
Kazakhstan	16004800	1	0.001	1	0.001	10	0.006

(c)

Total cases	1981–1990	1991–2000	2001–2011	Total
Russia	39	143	732	914
Ukraine	18	50	1151	1219
Post-Soviet states	1	3	17	21
USSR	58	196	1900	2154

of the predicted yearly generations in Southwestern zones where dirofilariosis was already endemic. One of the most important problems related to prediction models is their correct validation, due to the frequent lack of suitable tools [17, 30]. To validate our model we used the clinical cases of human dirofilariosis identified by a retrospective review and derived morbidity in the studied territory from 1981 to 2011. In spite of clinical cases being only "the tip of the iceberg" of human dirofilariosis [31] their existence and dynamic of appearance can be good markers of the disease because, in endemic areas, human infections appear in parallel to canine dirofilariosis [32]. The retrospective review revealed that approximately 65% of the total cases of human dirofilariosis published in the world until 2011 were reported in the former USSR. Despite of this relatively abundant information, validation must be carefully analyzed. There exists a very high congruence between the transmission areas predicted by the model and the real spatiotemporal distribution of cases reported between 1981 and 2011 that confirms the spreading trend of the disease towards the Northwest of the area studied. Moreover, a significant statistical correlation between the number of annual predicted generations and morbidity exists. Nevertheless, there are some exceptions to the concordances observed, like the higher morbidity than

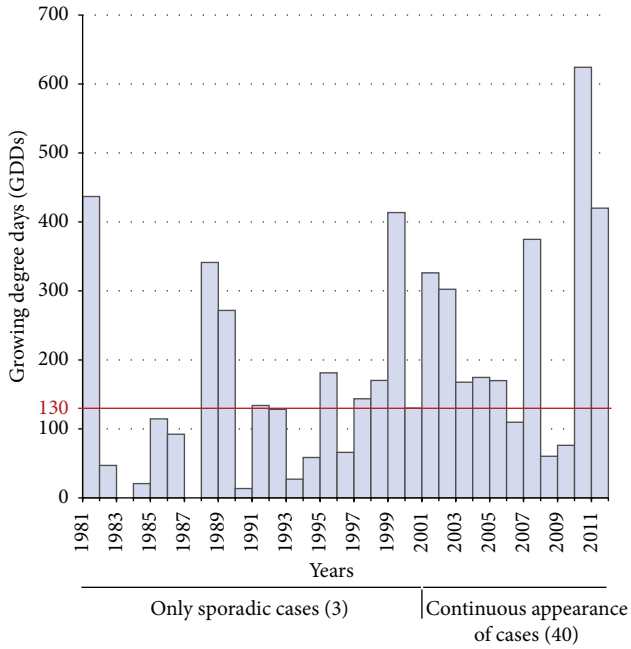

Figure 4: Appearance dynamics of human dirofilariosis cases and GDDs in Moscow administrative unit.

expected in some Ukrainian and neighboring Russian administrative units and the lower morbidity than expected in some other Russian administrative units and Southern Asian republics. In the first case the situation can be attributed both to appropriate conditions for transmission and the awareness and wide experience of physicians in the differential diagnosis of the disease, which has led to numerous case reports. Moreover, human dirofilariosis is a notifiable disease from 1997 in Ukraine, a fact making these data very reliable. Otherwise, Asian republics have a very low hydrothermal coefficient (less than 1) [24] limiting the development of mosquito populations in most of their territory, which consist mainly of populated areas concentrated near the water bodies. Moreover, human dirofilariosis is not habitually included in the differential diagnosis of subcutaneous or pulmonary nodules in these countries. As a consequence, in spite of the high number of predicted generations of *Dirofilaria*, human cases are detected sporadically, but they will probably emerge when they become appropriately diagnosed, near the water bodies. Spreading of human dirofilariosis along the Dnieper, Don, and Volga river basins is consistent with the need of humidity as a key factor related to the abundance of mosquito populations. This fact also appears in the spreading predicted in Siberia where new risk areas emerge along the Yenisei and Lena river basins.

Emergence of human dirofilariosis in administrative units located closer to the northern boundary of the predicted area could be delayed in respect to the real introduction time, since consecutive appearance of cases seems to occur only after some years consecutively reaching the threshold allowing extrinsic incubation, as has been observed in Moscow. Otherwise morbidity rises have been detected between 2001 and 2011 in this and other administrative units near the northern boundary (see Table 2). Taken together, these two facts suggest that in spite of the apparent slow emergence of human dirofilariosis when suitable conditions for transmission occur sporadically or during short periods of time, once introduced and with suitable conditions, the disease can present a strong spreading potential.

Projection by 2030 maintains the geographical spreading pattern shown retrospectively by the model as well as the increase in the person-year of exposure, which occurs mainly in zones of the Southwestern area where 2–6 annual predicted generations are predicted. These predictions are consistent with the increase in temperatures foreseen by that time by the Russian Committee of Hydrometeorology. This agency predicts more moderate thermal increases than other organisms; thus, different scenarios could be taken in consideration. With these findings we can assume that human dirofilariosis morbidity will increase in the future, emerging in many northern cold administrative units of the European Russia, in low lands of the ranges and in Siberia as far as latitude 64°N. Moreover, morbidity will strongly increase in Southwestern zones with medium number of predicted yearly generations.

5. Conclusion

Our model suggests that regional warming is clearly associated with the long-term spreading and emergence of dirofilariosis in extensive territories of the former USSR confirmed by the spatiotemporal appearance pattern of cases from 1981 to 2011. Nevertheless, factors influencing the dynamic of vector-borne diseases are complex, so prediction models necessarily must simplify the real situation. Our analysis suggests the involvement of other factors not included in the model like humidity, population distribution, knowledge of and interest in the disease by the scientific community, and probably pets management (there is a huge stray dogs population lacking preventive measures in the former USSR acting as reservoirs), among others. Our findings, together with the demonstrated severity of many cases, suggest that human dirofilariosis has become a serious medical problem that will increase in the future; thus, control of reservoir infections is urgently needed. Our model can help to design appropriate preventive and control strategies in the post-Soviet states. Moreover, these results can alert about the epidemiological behavior of this and other mosquito-borne diseases, not analyzed until now in different areas of the world.

Conflict of Interests

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

Authors' Contribution

Vladimir Kartashev performed the retrospective review. Alexandr Afonin and Luis Simón developed the GIS model. Rosa Sepúlveda conducted the statistical analyses. Javier González-Miguel, Rodrigo Morchón, and Fernando Simón processed and analyzed the data. Javier González-Miguel

and Fernando Simón wrote and organized the paper. Fernando Simón designed the project. All authors approved the final version of the paper.

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Research Article

Liposomal Formulation of Turmerone-Rich Hexane Fractions from *Curcuma longa* Enhances Their Antileishmanial Activity

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Promastigote forms of *Leishmania amazonensis* were treated with different concentrations of two fractions of *Curcuma longa* cortex rich in turmerones and their respective liposomal formulations in order to evaluate growth inhibition and the minimal inhibitory concentration (MIC). In addition, cellular alterations of treated promastigotes were investigated under transmission and scanning electron microscopies. LipoRHIC and LipoRHIWC presented lower MIC, 5.5 and 12.5 μg/mL, when compared to nonencapsulated fractions (125 and 250 μg/mL), respectively, and to ar-turmerone (50 μg/mL). Parasite growth inhibition was demonstrated to be dose-dependent. Important morphological changes as rounded body and presence of several roles on plasmatic membrane could be seen on *L. amazonensis* promastigotes after treatment with subinhibitory concentration (2.75 μg/mL) of the most active LipoRHIC. In that sense, the hexane fraction from the turmeric cortex of *Curcuma longa* incorporated in liposomal formulation (LipoRHIC) could represent good strategy for the development of new antileishmanial agent.

1. Introduction

Leishmaniasis is a parasitic disease and about 12 million people are infected worldwide with increasing numbers of new cases each year [1]. Also in Brazil new cases of leishmaniasis are reported annually [2]. The chemotherapeutic agents used for the treatment of leishmaniasis such as sodium stibogluconate, *N*-methylglucamine antimoniate, pentamidine, and amphotericin B are not orally active and require a long-term parenteral administration. These agents also present severe side effects such as cardio and renal toxicities and they are expensive [3]. Additionally, parasites of the genus *Leishmania* are increasingly resistant to the available antileishmanial agents; thus there is an urgency to find and identify new compounds that could be active against these parasites. Some advances have taken place in this field, for example, miltefosine, an alkylphospholipid, was registered in India for the treatment of visceral leishmaniasis (kala-azar) in 2002 [4]. Also there has been an increase in the use and diversity of drug delivery systems for the treatment of various diseases, such as cancer [5–8] and parasitic diseases [9–11]. Delivery systems like liposomes, nanoparticles, emulsions, and others have provided important advantages in terms of increasing the solubility of hydrophobic compounds and bioavailability, among other benefits [12].

Curcuma longa L. (*C. domestica* Valeton; *Amomum curcuma* Jacq.; *Stissera curcuma* Raeusch.), popularly known as "turmeric" (Zingiberaceae), is a herbaceous plant with laterally branched rhizomes of Asian origin that has a growing

reputation for its miraculous powers in the treatment and prevention of various diseases [13]. Several pharmacological properties have been described to this rhizome and pure substances derived directly from it, including anti-inflammatory, antibacterial, antioxidant, and antiparasitic activities [14, 15]. In the treatment of human parasitic diseases, turmeric is one of the most widely used species. Its action spectrum includes uses against schistosomiasis, helminthiasis, bebesiosis, scabies, coccidiosis, giardiasis, malaria, trypanosomiasis, and leishmaniasis as recently reviewed [16]. Some researchers have described the activity of curcuminoids isolated from *C. longa* against *Leishmania major* with IC_{50} values from 22 to 60 μM [16–18]. Thus, the aim of this work was to study the *C. longa* nonpolar fractions, turmeric cortex, and turmeric without cortex, separately, and to investigate their efficacy as hexane fractions and liposomal forms against *Leishmania amazonensis* strains.

2. Materials and Methods

2.1. Leishmania Culture. The MHOM/BR/76/Ma-5 Raimundo strain of *Leishmania (L.) amazonensis* was axenically maintained by weekly subculturing (passage each 5 or 6 days) in PBHIL medium supplemented with 10% fetal bovine serum (FBS), at 26°C as previously described [19]. The infectiveness of the promastigotes was assured by periodical infection of mice peritoneal macrophages.

2.2. Evaluation of Minimum Inhibitory Concentration (MIC). This assay was carried out in a 96-well microtiter plate where the extracts and their liposomal preparations were serially diluted in duplicates to final test concentrations (1–500 μg/mL). Then 5.0×10^5 promastigote forms of *L. amazonensis* were harvested at the stationary phase and added to each well and the plate was incubated at 26°C for 120 h. After the incubation period, resazurin solution (5 mg/100 mL of phosphate buffer saline, PBS, pH 7.2) was prepared and 25 μL added to each well and incubation continued for a further 2–4 h as described [20]. MIC was considered the lowest concentration of the extracts and their liposomal preparations that completely prevented the growth of *Leishmania in vitro*. Alternatively, parasites treated for 120 h were centrifuged, washed in PBS, and then reincubated in fresh culture medium in order to evaluate the leishmanicidal effect. The lowest concentration able to inhibit parasites growth was considered the MLC. The IC_{50} was determined by logarithmic regression analysis of the data obtained as described above.

2.3. Leishmanicidal Activity of Liposomal Preparations. Promastigotes of *L. amazonensis* (10^6 parasites/mL) were incubated in PBHIL medium in the presence of various concentrations of liposomal preparations, and parasite survival and cell morphology were evaluated daily by optical microscopy. Parasite viability was assessed before and after incubation by motility and by trypan blue exclusion, using a hemocytometer chamber. Growth was determined by counting the cells after 24 and 48 hours of treatment.

2.4. Scanning Electron Microscopy (SEM). Parasites were harvested at the early stationary phase of growth, washed twice with PBS, and treated with MIC and sub-MIC of the liposomal preparations for 24 hours. Cells were washed twice in cacodylate buffer pH 7.0 and then fixed in a solution containing 2.5% glutaraldehyde, 4% paraformaldehyde, and $CaCl_2$ (25 mg/mL), placed on the poly-L-lysine-coated coverslip and dehydrated in growing concentrations of ethanol. Parasites were then critical point dried in CO_2, sputter-coated with gold, and observed in a JEOL 6490LV scanning electron microscope.

2.5. Transmission Electron Microscopy (TEM). Parasites were obtained as described above. After treatment with MIC and sub-MIC for 24 hours, cells were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer containing 3.5% sucrose (pH 7.4 at 4°C for 60 min), postfixed with a 1% osmium tetroxide and potassium ferrocyanide solution for 1 hour, dehydrated sequentially in acetone, and then embedded in Epon 812. Thin sections were cut using an LKB ultramicrotome and collected on copper grids. Sections were stained with uranyl acetate and lead citrate and examined in a JEOL JEM1011 transmission electron microscope.

2.6. Plant Material and Extraction Method. The plant rhizome was purchased from the local market and authenticated by comparison with a voucher deposited at the Herbarium of INPA (Amazonas state), under number 224169. The turmeric cortex and turmeric without cortex (120 and 180 g, resp.) were separated and exhaustively macerated in methanol. The extracts were filtered and the solvents were evaporated under reduced pressure to yield 7.4% of methanol crude turmeric cortex extract and 8.2% of methanol turmeric without cortex crude extract. The methanol crude extracts from the turmeric cortex and turmeric without cortex of *C. longa* were submitted to liquid-liquid partition in hexane, to yield, relative to the vegetal material (p/p), 0.43% of the hexane fraction from the turmeric cortex (hexRHIC) and 1.19% of the hexane fraction from the turmeric without cortex (hexRHIWC).

2.7. GC-MS Analyses

Sample Preparation. The hexane fractions were analyzed by gas chromatography coupled to mass spectrometry (GC-MS). For sample analyses and identification of the substances a gas chromatograph (Agilent 6890N) coupled to a quadripolar mass spectrometer (Agilent 5973N) was used with ionization by electronic impact (70 eV). The apparatus was fitted with a HP-5MS column (internal diameter: 0.25 mm, length: 30 m, film thickness: 0.25 μm). The carrier gas was helium at a flow of 0.5 mL/min. The injector port temperature was 250°C and split ratio was 20 : 1. The transfer line temperature was 280°C, ion-source-heating was 230°C, and the scan-range was 40–700 *m/z*. The GC oven program was as follows: 40°C held for 5 min and then ramped at 4°C/min to 300°C, with a final hold time of 10 min. Interpretation and identification of the fragmentation mass spectrum was carried out by comparison with the Wiley NBS mass spectrum data base. Results were expressed as the relative percentage of peak area in the chromatogram.

Table 1: Chemical composition of *C. longa* hexane fractions.

Compounds	$RI^{lit.}$	$RI^{cal.}$	HF composition (%)	
			Turmeric cortex	Turmeric without cortex
trans-β-Farnesene	1456	1450	—	0.4
ar-Curcumene	1480	1476	4.5	5.0
α-Zingiberene	1493	1489	13.6	24.1
β-Bisabolene	1505	1504	2.0	3.3
β-Sesquiphellandrene	1522	1519	12.5	16.3
E-iso-γ-Bisabolene	1529	1525	0.9	0.8
ar-Tumerone	1669	1667	15.8	14.6
β-Turmerone	1677	1678	17.5	10.7
Germacrone	1693	1690	6.8	4.9
Curlone	1701	1699	7.2	5.5
Curcumenol	1734	1731	0.7	0.6
(6R,7R)-Bisabolone	1742	1741	2.1	1.9
Dehydrocurdione	1891	1889	6.8	5.3
Identified components			90.4	93.4

$RI^{cal.}$: retention index based on a homologous series of normal alkanes. $RI^{lit.}$: retention index from literature. HF: hexane fractions.

2.8. Preparation of Liposomes (LipoRHIC and LipoRHIWC). Liposomes were prepared as previously described by Sinico et al. [21] with some modifications. Briefly, 5.0 mg of hexane fractions (hexRHIC or hexRHIWC), 20 mg of phosphatidylcholine (Sigma Aldrich), 2.6 mg of cholesterol (Sigma Aldrich), and 0.3 mg of Tween 20 (Sigma Aldrich) were dissolved in 10 mL of chloroform (Tedia) in a 50 mL round-bottomed flask. The solvent was removed by evaporation, and a lipid film was formed on the inner wall of the flask. The lipid film obtained was hydrated with 2.0 mL of distilled water and a combination of both sonication and homogenization was performed for 30 min (5.0 min intervals) at room temperature for better drug loading. Finally, liposomes of the turmeric cortex (LipoRHIC) and of the turmeric without cortex (LipoRHIWC) were separated by ultracentrifugation at room temperature and 15,000 rpm for 7.0 min and filtered through a 0.20 μm filter (Whatman). LipoRHIC and LipoRHIWC were stored at 4°C for no longer than 15 days.

2.9. Determination of Entrapment Efficiency of LipoRHIC and LipoRHIWC. The entrapment efficiency of the liposomes hexRHIC and hexRHIWC was calculated in relation to turmerone (Sigma Aldrich) present in the vesicles. This percentage encapsulated was determined after lysis of the prepared liposomes with ethanol/chloroform (1 : 1) and sonication for 10 minutes. The concentration of turmerone in the liposomes was determined spectrophotometrically at 235 nm using a UV-visible spectrophotometer (model UV-601 PC, Shimadzu). This procedure was performed three times. A calibration curve was traced with five levels of triplicate analysis of standard solutions containing turmerone at concentrations of 0.5 to 3.0 mg. Samples and standard solutions were diluted in methanol. The equation of the regression (Absorbance = 0.061 × Concentration) showed linear fit in the concentration range studied (R^2 = 0.996). Variations among analyzes of replicates were less than 0.1%. Blanks containing liposomes without turmerone were evaluated under the same conditions and did not show any absorbance at 235 nm. The entrapment efficiency was expressed as follows: entrapment percentage (EP%) = entrapment sample related to turmerone/total turmerone × 100.

3. Results and Discussion

Curcuminoids isolated from *Curcuma longa* have a long history as antimicrobial agents, including against *Leishmania* [22, 23]. Although there are various reports about curcuminoids as the main molecular targets of *Curcuma* species, other constituents can be considered unique or coadjuvant molecular target of the curcuminoids in different therapies [24]. The essential oil of turmeric has been pharmacologically studied as an anti-inflammatory, anticancer, and antimicrobial agent among other activities. Its main constituents, associated to various species of *Curcuma*, are sesquiterpenoids like turmerones [25–30]. In this work, the hexane fractions (hexRHIC and hexRHIWC) obtained from the crude methanol extract of the turmeric cortex and turmeric without cortex of *C. longa*, respectively, were analyzed by GC-MS and the constituents are listed in Table 1. All substances detected in the extracts have already been described for the *Curcuma* species [31–34]. The comparison of the chromatographic profiles of the fractions, hexRHIC and hexRHIWC, showed a similarity in the composition, and the main difference was the relative percentage of each constituent. The main constituents of both fractions were the turmerones with total amounts of 40.50 and 30.80%, respectively.

The antileishmanial activity of the hexRHIC and hexRHIWC extracts from *C. longa* was evaluated against the promastigote forms of *L. amazonensis*. These fractions showed activity against the strain at minimal inhibitory concentrations of 125 and 250 μg/mL (IC50 = 35.4 and 83 μg/mL), respectively. Considering that ar-turmerone is one of the major components of both fractions, it was deemed necessary to test the antileishmanial activity of this substance alone.

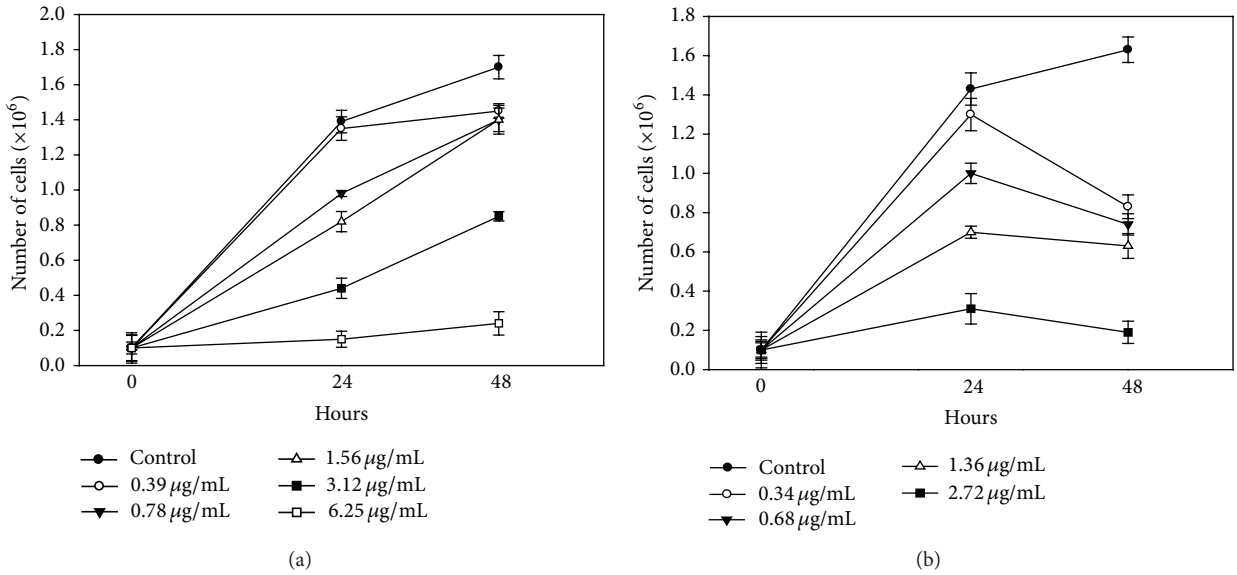

Figure 1: Growth inhibition effect of hexane fractions in liposomal formulations on *L. amazonensis* promastigotes. Parasites were counted at 24-hour intervals using a Neubauer chamber. (a) Promastigotes treated with LipoRHIWC; (b) promastigotes treated with LipoRHIC. Each bar represents the mean ± standard error of at least three independent experiments, which were performed in duplicate.

The MIC value of ar-turmerone was 50 μg/mL (IC_{50} = 11 μg/mL) against the promastigote forms of *L. amazonensis*. This result showed that it was more active than the hexane extracts and could be considered one of the target substances against *Leishmania*.

Solubility can be a problem when plant extracts and fractions are assayed, especially those with nonpolar characteristics, considering the need to disperse them in an aqueous media. Low solubility can influence the bioactivity of these samples directly by decreasing it or even inhibiting it completely. During the assays, some material precipitation was observed in the samples, which could be associated with the low activity of the extracts. Recently, much attention has been given to the search of novel drug delivery systems for drug-candidates in the combat of leishmaniasis. Liposomes as a drug delivery system have an interesting approach, because they are able to reduce the toxicity, prolong the action, and improve biodistribution and the stability of the drugs [35]. Thus, in order to improve solubility and antileishmanial activity of the *C. longa* extracts, liposomes, LipoRHIC, and LipoRHIWC, were prepared from the hexRHIC and hexRHIWC extracts and tested against *L. amazonensis* promastigotes. The percentage entrapment efficiency of the liposomes based on turmerones was 46.5% and 43.6%, respectively, and an increase in the activity of the two fractions was observed. However, attempts to incorporate turmerone in the liposomal form are not promising because of the volatility of this sesquiterpenoid. Subinhibitory concentrations (sub-MIC) of LipoRHIC and LipoRHIWC were able to affect parasite growth after 48 h treatment. Figure 1(a) shows a strong inhibition of the promastigote forms in the presence of LipoRHIWC at 6.25 μg/mL (IC_{50}/48 h = 2.9 μg/mL), when compared to control parasites. Despite the antileishmanial activity of LipoRHIWC, better results were observed when parasites were treated with LipoRHIC (Figure 1(b)). After 48 h treatment, LipoRHIC inhibited parasites growth at 2.75 μg/mL (IC_{50}/48 h = 0.4 μg/mL). The combination of the plant fractions and liposomal formulations has been successfully described in other studies. Lupane, a triterpene isolated from *Combretum leprosum* previously described as a leishmanicidal agent against *L. amazonensis* promastigotes [36], was recently incorporated into liposomes in a study conducted by Barros et al. [37]. Liposomal-lupane (6.0 μg/mL) reduced the number of parasites in murine macrophages by 61.7%. Another strategy using liposomes against a protozoan infection was demonstrated by Aditya et al. [38] with curcuminoids obtained from *C. longa* loaded into soybean phosphatidylcholine. These liposomes were able to reduce parasitemia and increase the survival of murine models with a *Plasmodium berghei* infection. Lala et al. [39] reported the antileishmanial activity of several vesicular delivery systems, including liposomes, incorporated with β-carboline alkaloid (harmine) isolated from *Peganum harmala*. Free harmine displayed an effective (IC_{50}) dose of about 25 μg/mL against *L. donovani* promastigotes, but when incorporated into liposomes and administered to infected hamsters, it showed a decrease of about 61% of the parasite load in the spleen when compared with untreated animals.

Since LipoRHIC was the most effective against *L. amazonensis* promastigotes, cellular alterations of these parasites were evaluated through scanning and transmission microscopy. Parasite photomicrographs revealed serious morphological alterations after 24 h exposure to LipoRHIC (Figure 2). At the MIC concentration (5.5 μg/mL), promastigotes showed "blebs" scattered over the flagella and rounded shapes could be observed (Figure 2(c)) in contrast to untreated parasites (Figure 2(a)-2(b)). LipoRHIC-treated promastigotes displayed significant alterations during the mitotic process,

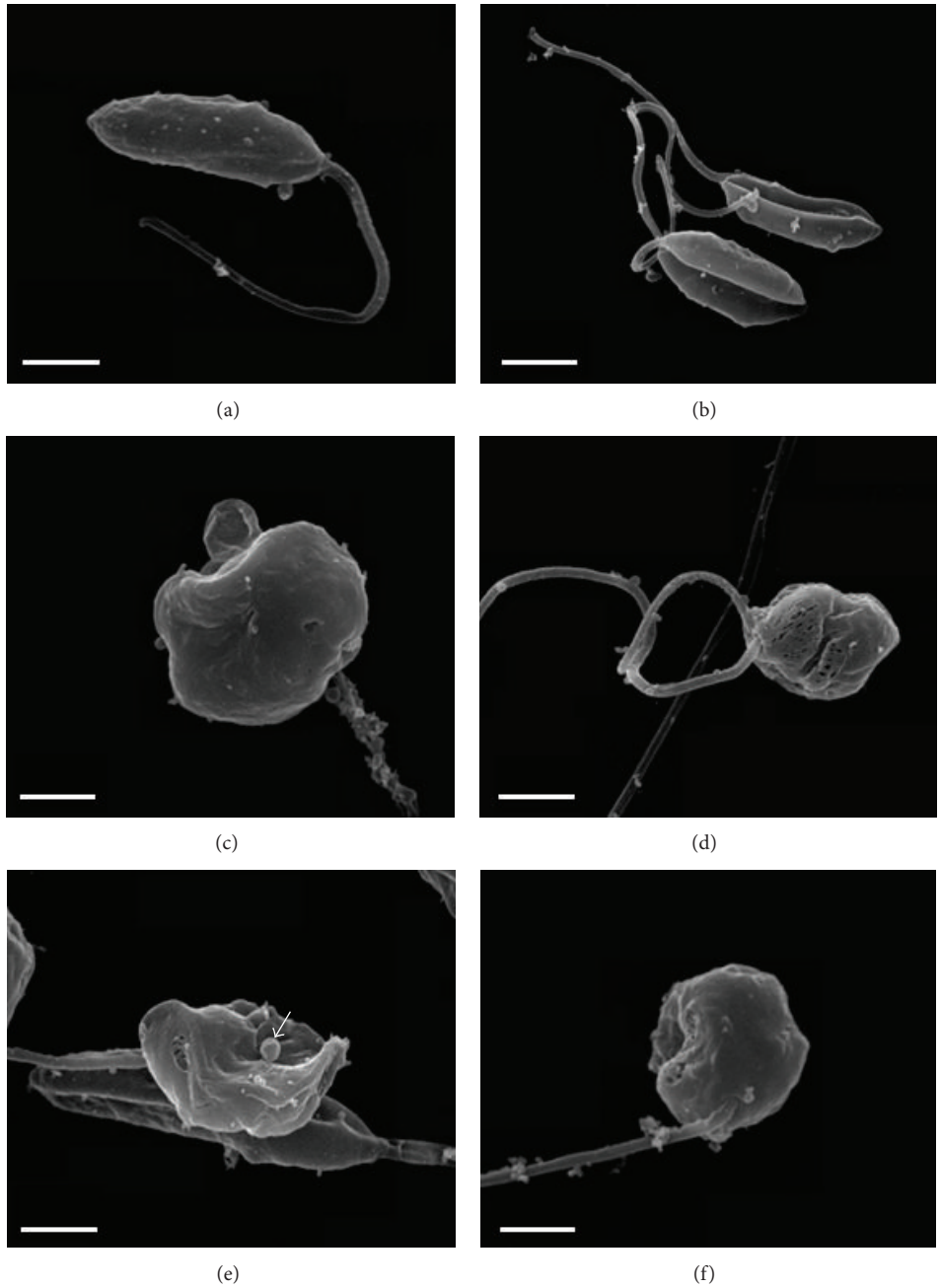

Figure 2: Morphological alterations of *L. amazonensis* promastigotes treated with LipoRHIC. ((a)-(b)) Untreated parasites; (a) *L. amazonensis* displaying the characteristic morphology of promastigotes; (b) promastigotes under mitotic process; ((c)-(d)) promastigotes treated with LipoRHIC liposomal at MIC concentration (5.5 μg/mL) for 24 h; (c) parasite showing "blebs" scattered over the flagella and rounded shape; (d) promastigotes under abnormal mitotic process, significant cell shrinkage and flagella reduced in size. Note the presence of pores on membrane surface; ((e)-(f)) promastigotes treated with LipoRHIC at a sub-MIC concentration (2.75 μg/mL); (e) some parasites showing "blebs" scattered over the plasma membrane (arrow); (f) parasite showing rounded shape. Bars = 1 μm.

including cell shrinkage and flagella reduced in size (Figure 2(d)). Furthermore, even when parasites were treated with a subinhibitory concentration (2.75 μg/mL) for 24 h, the alterations, such as membrane "blebs" and rounded bodies, became visible (Figure 2(e)-2(f)). In addition, the effects of LipoRHIC against *L. amazonensis* promastigotes were observed using transmission electron microscopy. Ultrastructural changes in promastigotes treated with LipoRHIC at the MIC concentration (5.5 μg/mL) showed significant alterations (Figure 3). After 24 h of treatment, parasites displayed a rounded body and had an abnormal membrane projection (Figure 3(c)) and mitochondrion swelling with the presence of several vacuoles inside the organelle (Figure 3(d)). Figures 3(e) and 3(f) show that most of the cells presented complete intracellular disorganization, as well as autophagic structures at the end of the treatment. Similar mitochondrion alterations

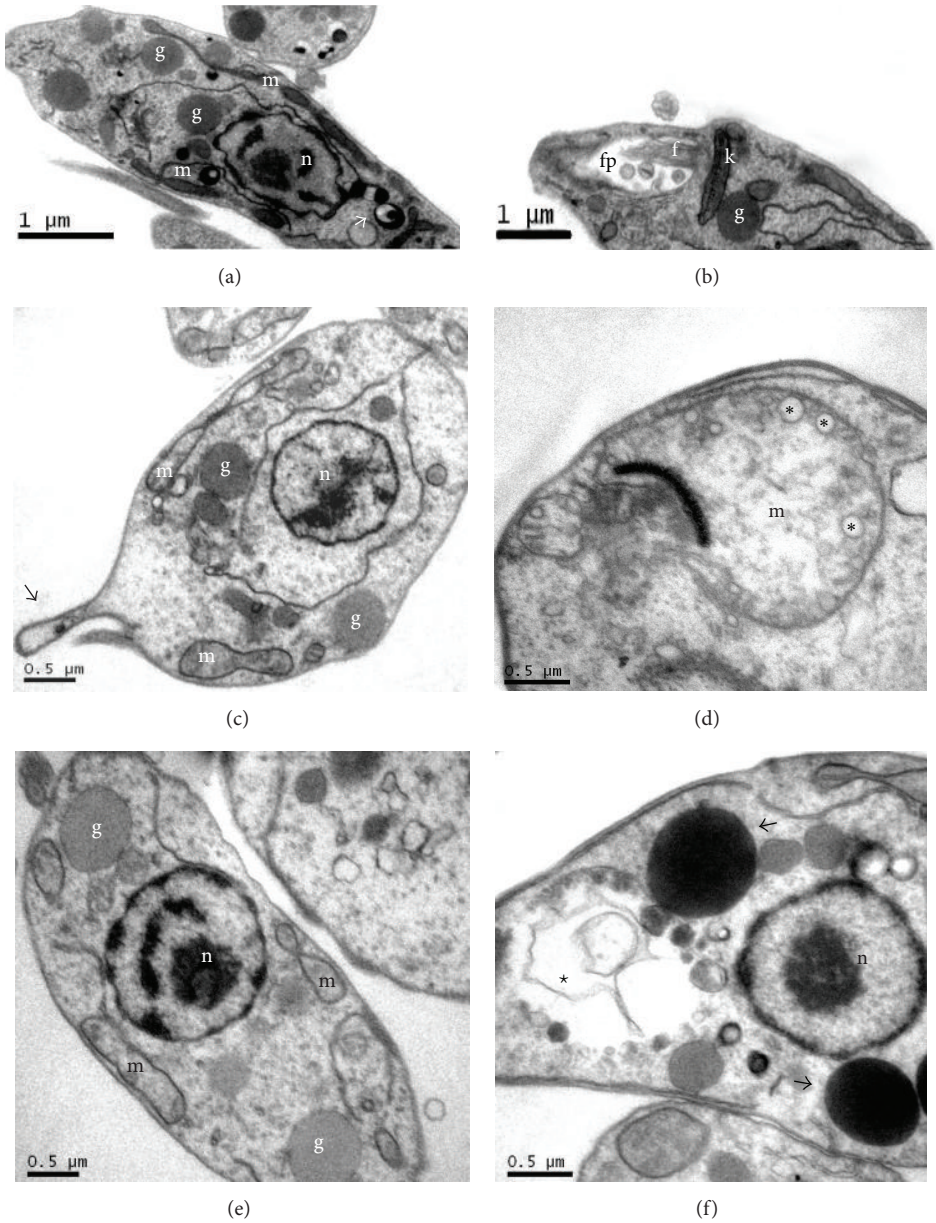

Figure 3: Ultrastructure alterations induced by LipoRHIC in *L. amazonensis* promastigotes. ((a)-(b)) Sections of untreated promastigote forms showing the main structures observed under transmission electron microscopy. (a) Promastigote presenting typical elongated body. The nucleus is rounded and the mitochondrion is branched. White arrow shows acidocalcisome in the cytoplasm; (b) normal flagellum and flagellar pocket are observed in the anterior portion of the parasite. Mitochondrion containing the kinetoplast can also be noted. ((c)–(f)) Parasites treated for 24 h with MIC concentration (5.5 μg/mL) of LipoRHIC. (c) Parasite presenting rounded body and an abnormal membrane projection (black arrow); (d) detail of mitochondrion swelling and the presence of several vacuoles (*); ((e)-(f)) complete intracellular disorganization although the nucleus membrane remained intact. (f) Autophagic structure ($\star$) and electron-dense granules (black arrows). n: nucleus; m: mitochondrion; k: kinetoplast; f: flagellum; fp: flagellar pocket; g: lipid.

and the presence of autophagic structures have been reported by previous studies as a possible consequence of sterol biosynthesis inhibition [40–43]. Some enzymes belonging to the Trypanosomatidae family that directly participate in sterol biosynthesis, such as $\Delta^{24(25)}$-sterol methyltransferase, are not expressed in mammalian cells [44]. Therefore such enzymes could be interesting targets for new drug candidates against trypanosomatids, including *Leishmania* [45].

In conclusion, despite the fact that the antileishmanial activity of curcuminoids has been extensively studied by various laboratories worldwide, the results reported here highlight the positive influence of the volatile constituents (enriched in turmerones) not hitherto associated with antileishmanial activity, reinforcing the scientific evidence of *C. longa* as the botanic species of the century. In addition, the incorporation of the hexane fractions into liposomes was

demonstrated to be an interesting approach for the study of new antileishmanial agents from natural sources.

Conflict of Interests

The authors declare that they have no conflict of interests regarding the publication of this paper.

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Research Article

Recombinant Dense Granular Protein (GRA5) for Detection of Human Toxoplasmosis by Western Blot

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Toxoplasma gondii infects all warm-blooded animals, including humans, causing serious public health problems and great economic loss for the food industry. Commonly used serological tests require costly and hazardous preparation of whole *Toxoplasma* lysate antigens from tachyzoites. Here, we have evaluated an alternative method for antigen production, which involved a prokaryotic expression system. Specifically, we expressed *T. gondii* dense granular protein-5 (GRA5) in *Escherichia coli* and isolated it by affinity purification. The serodiagnostic potential of the purified recombinant GRA5 (rGRA5) was tested through Western blot analysis against 212 human patient serum samples. We found that rGRA5 protein was 100% specific for analysis of toxoplasmosis-negative human sera. Also, rGRA5 was able to detect acute and chronic *T. gondii* infections (sensitivities of 46.8% and 61.2%, resp.).

1. Introduction

Toxoplasmosis is a parasitic disease caused by *Toxoplasma gondii* (*T. gondii*) which belongs to phylum Apicomplexa [1]. It is an obligate intracellular protozoan parasite capable of infecting all warm-blooded domestic animals as well as human beings [2]. The infection is globally distributed affecting up to one-third of the world's human population [3]. Infection of *T. gondii* involves the transmission within and between hosts by zoites [4]. Three infectious stages of the parasite are tachyzoite, bradyzoite, and sporozoite [5]. Humans get infected with such disease through congenital transmission, consumption of raw or undercooked meat contaminated with *T. gondii* tissue cysts, or uptake of water contaminated with sporulated oocysts from the infected cat feces [6].

Toxoplasmosis in immunocompetent individuals is often asymptomatic [7] but can cause severe clinical outcome to immunocompromised patients leading to multisystem organ failure or even death [2]. Meanwhile, primary infection in pregnant women will likely transmit the parasite to the fetus vertically causing congenital toxoplasmosis and might eventually bring about miscarriage in pregnant women [8, 9]. Besides pregnant women, similar infection does occur in sheep and goats [10], giving rise to similar consequence. Abortions in these animals contribute to great economic loss in livestock industry. Therefore, it is crucial to conduct a rapid, highly accurate, and early diagnostic test for *T. gondii* infected patients/hosts for prevention or early treatment.

In general, there are few methods available for conducting laboratory diagnosis of toxoplasmosis including serologic assays (antibody detection), polymerase chain reaction (PCR; specific gene detection), histologic examination, and isolation of the parasite followed by inoculation into peritoneal cavities of mice (*in vivo*) or tissue cultures (*in vitro*) from biopsy tissue and blood/body fluids, respectively, of the infected patients [11]. However, the most commonly used diagnostic test would be the serological test which relies on the *Toxoplasma* lysate antigens (TLAs) from tachyzoites propagated *in vivo* or *in vitro*. There are several disadvantages pertaining to the usage of antigens originating from tachyzoites: high production cost, time consuming, inconstant quality, contamination with extraparasitic components, and exposure of the staff to the harmful living parasites [12]. To overcome this, recombinant DNA technology plays an important role in producing a larger quantity of recombinant

antigenic proteins for serodiagnosis of *T. gondii* infection in a safer manner with lower production cost. Besides, recombinant tachyzoite proteins production either through prokaryotic or eukaryotic systems can reduce the variation of quality, enabling the development of a more specific and standardized serological assay.

Previous studies have reported the potential of various specific antigens of *T. gondii* such as the surface proteins [13, 14], microneme proteins [15], rhoptry proteins [16, 17], and dense granule proteins [18, 19] as seromarkers, either as single or multiantigen for detection of *T. gondii*-specific serum antibodies against acute (recently acquired) or/and chronic (distant past) *Toxoplasma* infection.

Dense granule (GRA) proteins are proteins with high immunogenicity [20]. They are found abundantly in both tachyzoites and bradyzoites [21] and make up most of the circulating antigens in the blood stream of an infected host which can be detected as early as a few hours postinfection (acute phase) [22]. GRA proteins were also found during the chronic stage of *T. gondii* infection [20]. As a result, the immunogenicity and prolonged expression of GRA proteins make them one of the promising candidates for recombinant protein production.

A total of 12 GRA proteins with the molecular weight ranging from 21 to 41 kDa have been identified [4, 21, 23–25]. Diagnostic performance of GRA antigens such as GRA2, GRA6, GRA7, and GRA8 has been investigated via ELISA for discriminating acute from chronic *Toxoplasma* infections [18, 19, 26–28]. Recombinant GRA7 was also shown to detect acute *T. gondii* infection more strongly compared to chronic infection [29]. Meanwhile, in our previous study, sensitivity and specificity of recombinant GRA2 for serodiagnosis of *Toxoplasma*-infected patients' sera have also been evaluated through western blot which is capable of discriminating present from past infection [30]. More diagnostic candidates capable of detecting the early acquired phase of toxoplasmosis ought to be determined to improve the efficacy of serodiagnosis especially of pregnant women in order to reduce the risk of transplacental transmission.

Dense granule antigen 5 (GRA5) is a 21 kDa hydrophobic protein consisting of a N-terminal hydrophobic signal peptide and a hydrophobic transmembrane domain [31]. It was reported that GRA5 appears in both soluble and hydrophobic forms [32]. GRA5 is secreted into the parasitophorous vacuole (PV) by *T. gondii* as a soluble form during the host cell invasion [33] followed by transmembrane insertion into the parasitophorous vacuole membrane (PVM) with its N-terminal projecting into the host cell cytoplasm, while C-terminus remains in the vacuole lumen [32]. A yeast two-hybrid analysis with GRA5 [34] showed that this antigen binds to calcium modulating ligand (CALMG) for regulation of intracellular calcium concentration which helps to inhibit apoptosis [35] and further allows for long-term survival of *T. gondii*. Besides playing an important role in host cell invasion, maintenance of the PV, and long-term survival of the parasite, GRA5 was found to exist in all life stages of the parasite [36].

However, only limited studies were done on the evaluation of the potential of GRA5 as a diagnostic marker in *Toxoplasma* infection, thus making it a protein of interest to be studied in this research. Only one study has been conducted showing the suitability of the full-length recombinant GRA5 for use as a component of an antigen cocktail for the detection of anti-*T. gondii* IgG antibodies [37]. This research study was aimed at the production of recombinant GRA5 (designated rGRA5) antigen in bacteria and at evaluation of its immunogenic properties as a potential single-antigenic diagnostic candidate through western blot. At the same time, we will also find out if GRA5 can detect the early acute stage of human toxoplasmosis through this study.

2. Materials and Methods

2.1. Parasite. *T. gondii* tachyzoites (RH strain) were maintained by serial intraperitoneal passage in BALB/c mice and were harvested from the peritoneal fluids after 3 to 4 days of infection. The tachyzoites were washed and subsequently resuspended in sterile phosphate buffered saline (PBS) prior to usage.

2.2. Construction of Recombinant Plasmids. The *T. gondii GRA5* gene sequence (corresponding to nucleotides 76–360), which encodes the GRA5 antigen, was obtained from Genbank (accession number: EU918733.1). DNA was extracted from tachyzoites of *T. gondii* (RH strain) and used as the template for PCR amplification of the *GRA5* gene with forward (5′-GCGGAATTCGGTTCAACGCGTGAC-3′) and reverse (5′-GACGAATTCCTCTTCCTCGGCAACTTC-3′) primers, which introduced *Eco*RI restriction sites (underlined) to facilitate cloning. The PCR product was purified and cloned into the pRSET B prokaryotic expression vector (Invitrogen, USA) at the EcoRI site. The resulting recombinant GRA5-pRSET B construct permitted expression of an N-terminally polyhistidine- (His-) tagged rGRA5 (amino acid residues 26–120), lacking its putative N-terminal signal sequence. Both the GRA5-pRSET B construct and the non-recombinant pRSET B plasmid were transformed into the prokaryotic expression host, *Escherichia coli* (*E. coli*) BL21(DE3)pLysS. The recombinant clones were screened and sequenced for verification purposes.

2.3. Optimization of Heterologous Protein Expression in E. coli. Optimal conditions for rGRA5 protein expression in *E. coli* were determined prior to scaling up the protein production protocol for further study. A single GRA5-pRSET B-containing colony was picked and inoculated into 5 mL of Luria-Bertani (LB) broth supplemented with ampicillin (100 μg/mL) and chloramphenicol (34 μg/mL). The culture was grown overnight at 37°C (200 rpm) and then diluted to a final volume of 10 mL with LB broth to yield an optical density of 0.1 at 600 nm (OD_{600}). The culture was then grown at 37°C (~250 rpm) until reaching an OD_{600} of 0.5, at which point protein expression was induced by addition of different concentrations (0.1, 0.5, and 1.0 mM) of isopropyl β-D-thiogalactopyranoside (IPTG; Invitrogen, USA) for various incubation periods (0, 2, and 4 h). The cells were harvested every hour by centrifugation at 5,000 ×g for 10 min

before assessing protein expression using dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.4. Expression and Purification of rGRA5. Large-scale protein production was achieved by inducing the culture with 1 mM IPTG and incubating it for 2 h before harvesting by centrifugation. The Probond Purification System (Invitrogen, USA) and nitrilotriacetic acid-nickel (Ni-NTA; Qiagen, Germany) resin were then used to purify rGRA5, according to the manufacturers' instructions. Briefly, cell lysate was prepared under denaturing conditions prior to the purification steps. The cell pellet was resuspended in guanidine lysis buffer (6 M guanidine hydrochloride, 500 mM sodium chloride, and 20 mM sodium phosphate, pH 7.8) and rocked slowly for 5 to 10 min at room temperature to ensure thorough cell lysis, followed by sonication on ice with three 5-second pulses (high intensity). After sonication, the lysate was separated from cellular debris by centrifugation at 3,000 ×g for 15 min, added to a column with resin, and allowed to bind for 30 min. Once the resin settled, the supernatant was aspirated, and the column was washed two times with each of the following: denaturing binding buffer (8 M urea, 500 mM sodium chloride, and 20 mM sodium phosphate, pH 7.8), denaturing wash buffer (8 M urea, 500 mM sodium chloride, and 20 mM sodium phosphate, pH 6.0), and denaturing wash buffer (8 M urea, 500 mM sodium chloride, and 20 mM sodium phosphate, pH 5.3). The supernatant was aspirated after each washing step. After the last wash, the rGRA5 protein was eluted from the Ni-NTA resin with denaturing elution buffer (8 M urea, 500 mM sodium chloride, and 20 mM sodium phosphate, pH 4.0). *E. coli* carrying the empty pRSET B vector was used as a negative control for both expression and purification. The concentration of purified rGRA5 protein was measured with the Bradford Assay Kit (Bio-Rad, USA). The identity of the expressed and purified rGRA5 protein was confirmed by matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry (MS).

2.5. In-Gel Tryptic Digestion of rGRA5. Affinity purified rGRA5 was resolved by SDS-PAGE using 12% polyacrylamide gels, which were stained with Coomassie Brilliant Blue R-250 (Bio-Rad, USA) for 2 h and then incubated with destaining solution (7% acetic acid, 5% methanol) overnight at room temperature. The rGRA5 protein band was then excised from the Coomassie-stained gel (based on size) and further destained with 50 μL of 50% acetonitrile (ACN) in 50 mM ammonium bicarbonate (NH_4HCO_3). This step was repeated several times (15–20 min washes, discarding the destaining solution after each wash) until the excised gel was completely destained. The rGRA5-containing gel plug was then incubated with 150 μL of 10 mM dithiothreitol (DTT) in 100 mM NH_4HCO_3 for 30 min at 60°C. The gel was subsequently cooled to room temperature, the DTT solution was discarded, and the band was incubated with 150 μL of 55 mM iodoacetamide (IAA) in 100 mM NH_4HCO_3 in the dark for 20 min. The gel plug was then washed four times with 50% ACN in 50 mM NH_4HCO_3 (500 μL washes, 20 min each), dehydrated via incubation with 50 μL of 100% ACN for 15 min, and subjected to speed vacuum for 15 min at ambient temperature to remove the ACN. The gel plug was then incubated with 25 μL of trypsin (6 ng/μL) in 50 mM NH_4HCO_3 at 37°C. Following overnight digestion, 50 μL of 50% ACN was added to the gel plug, and it was incubated for 15 min in order to disintegrate the trypsin enzyme and extract protein from the gel plug. The resulting liquid (containing the digested protein) was transferred into a new tube (Tube A), and the gel plug, which remained in the old tube, was further incubated with 50 μL of 100% ACN for 15 min. Subsequently, this liquid was also transferred to Tube A. The protein-containing solution in Tube A was then dried completely via speed vacuum. Prior to MALDI-TOF MS analysis, the protein sample was reconstituted in 10 μL of 0.1% formic acid and desalted using a Zip-Tip (Millipore, USA). For this, the Zip-Tip membrane was wetted and equilibrated with 50% ACN and 0.1% formic acid, respectively. The protein sample was bound onto the Zip-Tip membrane, which was then washed with 0.1% formic acid. Finally, the protein was eluted with 0.1% formic acid in 50% ACN and analyzed by MALDI-TOF MS.

2.6. MALDI-TOF MS Analysis. The Zip-Tip-eluted protein sample was mixed at a 1 : 1 ratio. The matrix was provided by UMCPR staff before spotting onto the MALDI plate. The analysis was carried out by University Malaya Center for Proteomics Research (UMCPR).

2.7. SDS-PAGE and Western Blot Analysis. Purified rGRA5 protein was resolved by SDS-PAGE on 12% polyacrylamide gels and transferred onto methanol-activated polyvinylidene difluoride (PVDF; Bio-Rad, USA) membranes, which were then cut into vertical strips. The membranes were incubated with blocking solution (5% nonfat skim milk in Tris Buffered Saline (TBS)) for 2 h at room temperature with constant shaking and were subsequently probed with diluted human serum samples (1 : 200) for 2 h. The membrane strips were washed and then incubated for 1 h with biotinylated goat anti-human IgM/IgG (KPL, USA; 1 : 2500) secondary antibody. Lastly, the membrane strips were washed and incubated with streptavidin-alkaline phosphatase (KPL, USA; 1 : 2,500) at room temperature for 1 h followed by detection using 5-bromo-4-chloro-3-indolyphosphate/nitro blue tetrazolium (BCIP/NBT; Sigma, USA).

2.8. Evaluation of Sensitivity and Specificity of rGRA5. Diagnostic sensitivity and specificity of rGRA5 protein were evaluated by western blot analysis using sera from both toxoplasmosis-diagnosed patients and toxoplasmosis-negative individuals. Toxoplasmosis cases were divided into three groups: (1) patients with early acute toxoplasmosis (n = 44; IgM positive, IgG negative); (2) patients with acute toxoplasmosis (n = 47; IgM positive, IgG positive); and (3) patients with chronic toxoplasmosis (n = 85; IgM negative, IgG positive). A fourth group was comprised of toxoplasmosis-negative control patients (n = 24; IgM negative, IgG negative). These human serum samples were grouped based on results obtained from Novalisa *Toxoplasma gondii* IgG and *Toxoplasma gondii* IgM enzyme-linked immunosorbent assay

Table 1: Immunoreactivities (sensitivity and specificity) of the rGRA5 antigen to serum samples from toxoplasmosis-positive and toxoplasmosis-negative patients.

Serum samples group	Number of human serum samples	Immunoreactivities			
		Positive		Negative	
		Number	%	Number	%
1 (Early acute: IgG−ve, IgM+ve)	44	0	0	44	100
2 (Acute: IgG+ve, IgM+ve)	47	22	46.8	25	53.2
3 (Chronic: IgG+ve, IgM−ve)	85	52	61.2	33	38.8
4 (Toxoplasmosis-negative: IgG−ve, IgM−ve)	24	0	0	24	100
Other infections	12	1	8.3	11	91.7
Amoebiasis	3	0	0	3	100
Cysticercosis	3	0	0	3	100
Filariasis	3	0	0	3	100
Toxocariasis	3	1*	33.3	2	66.7

*One out of three toxocariasis-positive sera samples reacted with the rGRA5 antigen. This particular toxocariasis-positive serum sample was shown to be IgG positive for toxoplasmosis based on the commercial kits.

(ELISA) kits (NovaTec, Germany). In addition, specificity of rGRA5 was determined using serum samples from patients diagnosed with amoebiasis (3 samples), cysticercosis (3 samples), filariasis (3 samples), and toxocariasis (3 samples). These sera had given positive results in serological tests for their respective infections. All serum samples were obtained from the Diagnostic Laboratory at the Department of Parasitology, University of Malaya. Sensitivity (number of true positives/[number of true positives + number of false negatives]) and specificity (number of true negatives/[number of true negatives + number of false positives]) were calculated and tabulated in Table 1.

3. Results

3.1. Cloning of the GRA5 Gene Fragment. We PCR-amplified a fragment of *T. gondii GRA5* gene, which encoded amino acids 26–120 of the GRA5 protein (excluding the putative hydrophobic signal peptide). The resulting ~285 bp product was cloned into the pRSET B vector in order to permit prokaryotic expression of N-terminally His-tagged rGRA5, which could be purified using a nickel resin column. Sequence analysis confirmed that the insert within the GRA5-pRSET B plasmid shared 100% identity with the published *GRA5* gene.

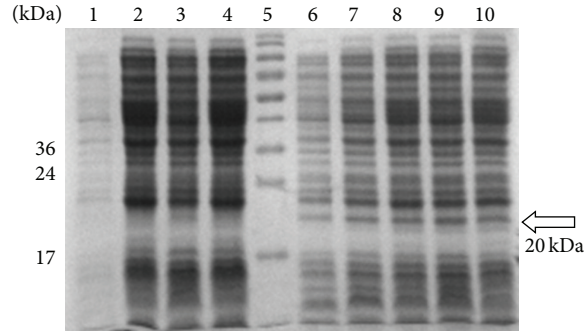

Figure 1: SDS-PAGE analysis on the optimized expression of rGRA5 protein in *E. coli* BL21 pLysS (DE3), Coomassie blue stained. Lane 5: prestained broad range protein marker. Lane 1, cell pellet fractions of pRSET B clone as negative control before induction (0 hr). Lane 2: cell pellet fractions of pRSET B clone after induction with 0.5 mM IPTG (4 hr). Lanes 3 to 4: cell pellet fractions of pRSET B clone after induction with 1.0 mM IPTG (2, 4 hr). Lane 6: cell pellet fractions of GRA5 clone before induction (0 hr). Lanes 7 to 8: cell pellet fractions of GRA5 clone after induction with 0.5 mM IPTG (2, 4 hr). Lanes 9 to 10: cell pellet fractions of GRA5 clone after induction with 1.0 mM IPTG (2, 4 hr). The GRA5 protein band of interest was observed at molecular weight of 20 kDa (arrow) compared to the negative control. The band intensity increased from 0 to 2 hr after induction and remained constant at the 4th hr with 1.0 mM IPTG, the optimum condition for maximum expression of the protein.

3.2. Optimization of rGRA5 Expression in E. coli. Production of rGRA5 protein was optimized by altering various parameters, and expression levels were analyzed by SDS-PAGE as shown in Figure 1. Upon induction of rGRA5 expression from GRA5-pRSET B-containing *E. coli*, we observed a 20 kDa band of increasing intensity, which was absent in the negative control (empty pRSET B). Expression of this protein increased up to two hours after induction and remained constant after four hours. Three different IPTG concentrations were tested, and 1.0 mM was found to result in maximum rGRA5 expression. Taken together, these data suggested that optimal rGRA5 expression was achieved following induction with 1.0 mM IPTG for 2 hours. The same conditions were applied to larger scale production of rGRA5.

3.3. Expression and Purification of rGRA5 Protein. Following optimization of rGRA5 expression in *E. coli*, a nickel resin column was used to purify the recombinant protein (Figure 2(a)), which could be detected by western blot analysis using serum from a *Toxoplasma*-infected patient (Figure 2(b)). This further suggested that the induced 20 kDa band observed prior to purification corresponded to rGRA5 (Figure 1).

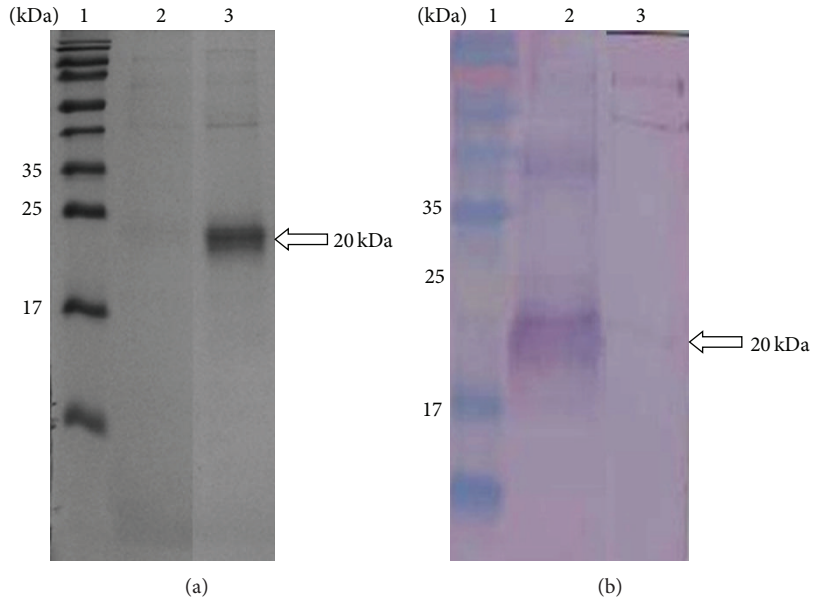

Figure 2: SDS-PAGE analysis on purified rGRA5 protein. (a) Coomassie blue stained. Lane 2: purified pRSET B. Lane 3: purified rGRA5 and (b) western blot probed with toxoplasmosis-infected patient's serum. Lane 2: purified rGRA5. Lane 3: purified pRSET B. Lane 1 (panel a and panel b) is the prestained broad range protein marker. The 20 kDa purified rGRA5 was detected (arrow).

3.4. Confirmation of rGRA5 Protein. Next, we confirmed the identity of our expressed and purified recombinant protein by MALDI-TOF MS analysis. Indeed, the results indicated that the isolated protein was *T. gondii* GRA5.

3.5. Western Blot Analysis of rGRA5 Protein with Human Serum Samples. The purified rGRA5 protein was tested for its diagnostic sensitivity and specificity through western blot analysis with serum samples from toxoplasmosis-positive (Groups 1, 2, and 3) and toxoplasmosis-negative (Group 4) patients. In addition, specificity was tested using sera from patients infected with other parasites, including amoebiasis, cysticercosis, filariasis, and toxocariasis. We observed that the rGRA5 protein had sensitivities of 0% (0 out of 44 sera), 46.8% (22 out of 47 sera), and 61.2% (52 out of 85 sera) for early acute, acute, and chronic infections, respectively (Table 1). In contrast, 0 out of 24 control sera from the toxoplasmosis-negative patients reacted with rGRA5 (100% specificity). In Figure 3, five example results are shown for each group (positive results for Groups 2 and 3; negative results for Group 4). Also, only 1 (toxocariasis) out of the 12 sera from patients infected with other parasites (data not shown) reacted with the rGRA5 protein (91.7% specificity).

4. Discussion

A fragment of the *T. gondii GRA5* gene was successfully cloned into a prokaryotic expression vector and transformed into *E. coli*. Full-length recombinant GRA5 protein (rGRA5) was subsequently expressed and purified, yielding a 20 kDa protein. However, the predicted molecular weight of GRA5 is 16 kDa. While this discrepancy between the calculated and observed molecular weights can be partially explained by the presence of the His-tag in rGRA5, it is also possible that this difference stems from common features of GRA proteins, such as proline residue composition [4]. Even though we observed this size discrepancy, the identity of our purified protein was verified by immunoblotting with *Toxoplasma*-infected sera and MALDI-TOF MS analysis.

Identification of rGRA5 via MALDI-TOF MS involved careful processing, which allowed for reliable confirmation of the purified protein. Briefly, the rGRA5-containing band was excised from a stained SDS-PAGE gel, followed by an in-gel digestion protocol that included seven major steps: (1) destaining of the gel plug, (2) reduction of the protein, (3) alkylation of the protein, (4) dehydration, (5) tryptic digestion of the protein, (6) extraction of the digested protein, and (7) desalting of the digested protein using a Zip-Tip. Reduction and alkylation (aminocarboxymethylation) of the protein at cysteine residues with dithiothreitol (DTT) and iodoacetamide (IAA), respectively, were important for permanent disruption of disulfide linkages, enabling overnight trypsin digestion.

It was demonstrated that the expression of predicted immunodominant epitopes of GRA5 failed to show any immunoreactivity with a pool of *T. gondii*-positive human sera [13]. Therefore, full-length rGRA5 was constructed and produced in this study. Our evaluation of rGRA5 immunoreactivity revealed high specificities when testing sera from toxoplasmosis-negative patients or from those infected with other parasites (100.0% and 91.7%, resp.). In addition, our findings indicate the sensitivities of 46.8% and 61.2% when analyzing serum samples from patients with acute and chronic *Toxoplasma* infections, respectively. However, none of the serum samples from the early acute phase patients

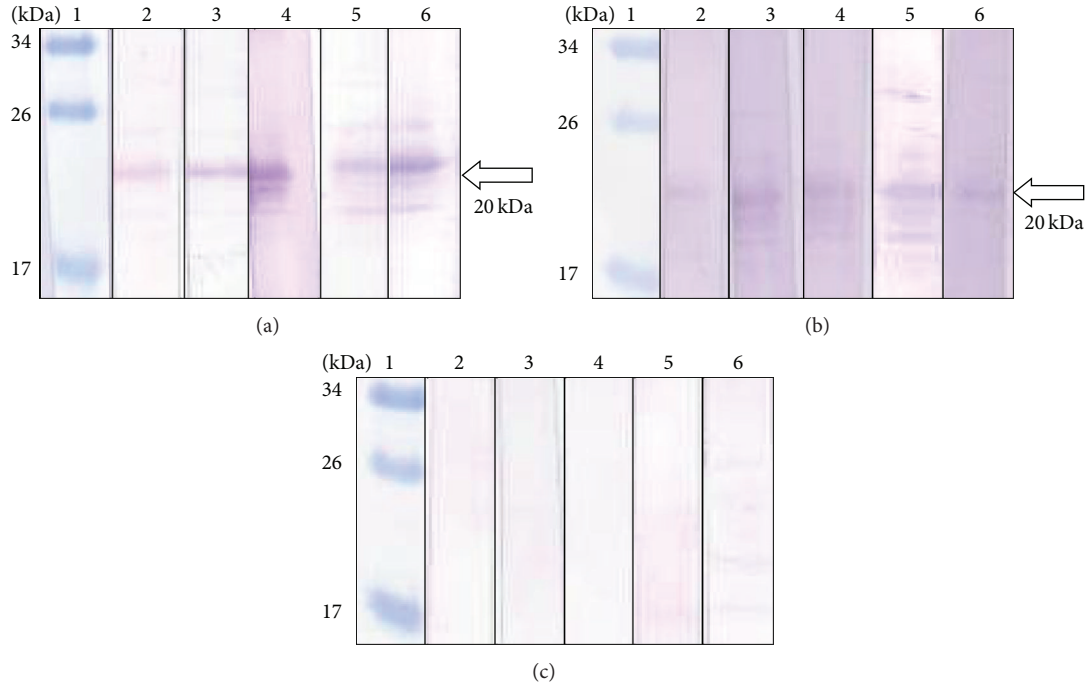

Figure 3: Western blots of purified rGRA5 protein with sera of toxoplasmosis and toxoplasmosis-negative patients. Lane 1 (panels a–c): the prestained broad range protein marker. Lanes 2 (a) to 6 (a): results of 5 sera from chronic-profile patients (Group 3: IgG +ve, IgM −ve). Lanes 2 (b) to 6 (b): results of 5 sera from acute-profile patients (Group 2: IgG +ve, IgM +ve). Lanes 2 (c) to 6 (c): results of 5 sera from toxoplasmosis-negative patients (Group 4: IgG −ve, IgM −ve). The 20 kDa purified rGRA5 was detected by toxoplasmosis-positive sera (arrow).

reacted with the rGRA5 protein. In fact, data from the present study are in agreement with previous results obtained from analysis of rGRA5 antigen-mediated detection of IgG antibodies using ELISA [37]. Specificity of the aforementioned study was shown to be 100.0%, whereas sensitivities of 63.0% and 75.0% were reported for sera from acute and chronic infections, respectively. Thus, it strongly suggested that rGRA5 yields a much higher reactivity towards IgG antibodies in sera from chronically infected patients compared to those with acute infection. Notably, this protein shows no sensitivity towards IgM antibodies found in sera from early acute stage patients. Our study involved the same expression host, BL21(DE3)pLysS, for the expression of full-length rGRA5 as the above mentioned study. In contrast, different expression vectors and evaluation techniques were used. Due to its higher specificity, western blot was chosen to evaluate rGRA5 protein in this study instead of the commonly used ELISA. Also, the chances of obtaining false-positive results via western blot are much lower compared to ELISA [38]. In fact, it has been reported that western blot analysis is superior to ELISA for screening sera samples because this technique gives more information, is less affected by sample degradation, produces results of high confidence with direct visualization of antibodies bound to specific diagnostic antigens, and offers improved determination of diagnostic antigen purity [39].

With regard to the future development of diagnostic tests for *T. gondii*, the western blot results obtained in this study should be reliable for predicting the efficacy of using rGRA5 antigen in immunochromatographic tests (ICT) due to similarities between the two assays (i.e., western blot and ICT are both immunoassays utilizing nitrocellulose membranes and direct visualization of results). Indeed, ICT is a better serological test for diagnosis of infections (including toxoplasmosis) compared to ELISA, which is commonly used due to its simplicity. However, ICT is a rapid test with high accuracy but lower cost compared to ELISA, which is time consuming and laborious [40]. In addition, ICT can be used in field conditions [40] especially for the diagnosis of farm animals.

Based on our results (Table 1), cross-reactivity was not observed in sera samples from patients infected with amoebiasis, cysticercosis, and filariasis. However, one out of three toxocariasis-positive sera samples reacted with the rGRA5 antigen. This particular toxocariasis-positive serum sample was shown to be IgG positive but IgM negative for toxoplasmosis based on findings from Novalisa *Toxoplasma gondii* IgG and IgM antibodies ELISA kits. This indicates that there was probably a coinfection of *T. gondii* and *Toxocara* spp. in this infected patient [41]. Although *T. gondii* (a protozoan) and *Toxocara* spp. (helminths) are two different parasites, they both can be acquired through soil ingestion. Therefore, the chances of coinfection between these two parasites are highly possible [41].

5. Conclusions

Our findings show that rGRA5 lacks sensitivity for detecting IgM antibodies and displays a much lower reactivity towards

IgG antibodies in sera from patients with acute infection compared to those with chronic toxoplasmosis (46.8% versus 61.2%). These data indicate that rGRA5 protein is unable to distinguish between current and past infections. Nevertheless, this protein can be combined with other *T. gondii* antigens (cocktails) in order to improve its sensitivity against toxoplasmosis-positive serum samples [37]. Last but not least, these findings should contribute to the future development of an ICT incorporating this antigen (either alone or in combination with other potential ESA) for diagnosis of *T. gondii* infection.

Ethical Approval

The authors declared that the experiments comply with the current laws of the country in which they were performed (Malaysia).

Conflict of Interests

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

Acknowledgments

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Review Article

Chagas' Disease: Pregnancy and Congenital Transmission

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Chagas disease is a chronic infection that kills approximately 12,000 people a year. Mass migration of chronically infected and asymptomatic persons has caused globalization of Chagas disease and has made nonvectorial infection, including vertical and blood-borne transmission, more of a threat to human communities than vectorial infection. To control transmission, it is essential to test all pregnant women living in endemic countries and all pregnant women having migrated from, or having lived in, endemic countries. All children born to seropositive mothers should be tested not only within the first month of life but also at ~6 months and ~12 months of age. The diagnosis is made by identification of the parasite in blood before the age of 6 months and by identification of the parasite in blood and/or positive serology after 10 months of age. Follow up for a year is essential as a significant proportion of cases are initially negative and are only detected at a later stage. If the condition is diagnosed and treated early, the clinical response is excellent and the majority of cases are cured.

1. Introduction

Chagas' disease (CD) is a zoonotic infection caused by the hemoflagellate protozoan parasite *Trypanosoma cruzi*. The infection is transmitted to mammalian hosts by a group of hemipteran insects belonging to the family Reduviidae, subfamily Triatominae. In endemic areas, the main mode of transmission is vectorial, by domestic, peridomestic, or sylvatic triatomines. Infection can be also acquired by blood transfusion, organ transplant, congenital infection, and oral transmission from food contaminated with insect faeces. CD is widely associated with poor rural areas, and it is considered as a neglected tropical disease by World Health Organization (WHO). In the early 1990s, the disease was ranked by the World Bank as the most serious of the parasitic diseases in Latin America, with a socioeconomic impact (measured as DALYs—disability-adjusted life years) considerably greater than that of the combined effects of all other parasitic infections [1]. Endemic countries, with the support of the Pan American Health Organization (PAHO), decided to establish regional programmes for the prevention and control of CD. The first program, the Southern Cone Initiative (INCOSUR), was created in 1991 by Argentina, Bolivia, Brazil, Chile, Paraguay, and Uruguay. Later on, the Central American Initiative (IPCA, 1997), the Andean Countries' Initiative (IPA, 1998), the Initiative of the Amazon Countries (AMCHA, 2003), and the Mexican Initiative (Iniciativa para la Vigilancia y el Control de la Enfermedad de Chagas en la República Mexicana, 2004) were created [2]. The main objectives of these initiatives were the control of the vector and the prevention of the transmission of infection by blood transfusion [3]. These multinational initiatives have led to substantial reductions in transmission by *Triatoma infestans*, the principal vector in the Southern Cone countries (Argentina, Brazil, Chile, Paraguay, Bolivia, and Uruguay), and by *Rhodnius prolixus* in Central America. In addition, the risk of transmission by blood transfusion has been substantially reduced throughout Latin America [1]. Estimated annual deaths globally decreased from 45,000 in 1990 to around 11,000 in 2008. The estimated number of infections decreased from 30 million in 1990 to 8 million in 2006 and the annual incidence during this 16-year period fell from 700 000 to 56 000. The burden of CD has been reduced from 2.8 million disability-adjusted life years to less than 500 000 [2].

However, mass migration of chronically infected and asymptomatic persons has caused globalization of CD and it has now been reported in 19 nonendemic areas including the United States, Canada, Europe, Japan, and Australia. In 2010, the World Health Assembly Resolution 63.20 on "Chagas' disease: control and elimination" urged Member States to reduce the burden of CD in nonendemic countries [4]. The resolution also called upon the Director-General to consider an initiative for the prevention and control of CD in nonendemic regions. With this purpose, a group of experts on CD from those European countries, where *T. cruzi*-positive cases had been detected, joined efforts to estimate the prevalence of CD in this region [5]. Their research revealed that, by 2009, 4,290 cases had been diagnosed in Europe, compared with an estimated 68,000 to 122,000 expected cases with an index of underdiagnosis between 94% and 96%. In the Western Pacific Region, an informal consultation held in Nagasaki, Japan, estimated that there were over 1500 infected individuals in Australia and over 3000 in Japan, recognizing the need for local surveillance programmes [6].

In 2004, PAHO focused its attention on the congenital transmission of CD and organized a specific consultation [7]. The advisory group emphasised that, in those regions where achievements or advancements had been made in controlling vectorial and transfusional *T. cruzi* transmission, congenital transmission constituted the main and most persistent form of the parasitosis among the human population. It also recommended that CD should be incorporated to the perinatal information system of the Latin American Centre for Perinatology/Women's and Reproductive Health (CLAP/SMR). CD is now part of the standardized electronic format for the perinatal medical history of CLAP/SMR [8]. PAHO also emphasized the need to consider congenital *T. cruzi* infection as a public-health problem and recommended that each endemic country should elaborate a protocol directed to the prompt detection and specific treatment of detected cases according to the capabilities of the local health services and their epidemiological situation. Vector control programs and serological screening of blood donors are the most effective ways for prevention of congenital infection [9, 10].

The number of cases of congenital CD has been estimated at 14,385 per year in Latin America, at 66–638 per year in the United States, and about 20 to 183 per year in Europe [5, 11, 12]. A systematic review of the literature estimated that in pregnant women with antibodies to *T. cruzi* the global rate of congenital transmission was 4.7% and that countries where the parasite is endemic had a higher rate of congenital transmission compared with countries where it is not endemic (5.0% versus 2.7%) [13]. This difference in prevalence probably reflects the diverse pool of immigrants that the nonendemic countries have, as it has been found that the prevalence of *T. cruzi* infection among immigrant populations normally mirrors the prevalence of the parasite in their countries and regions of origin [14].

2. Fertility and Outcome of Pregnancy

Very little is known about the effects of CD in human fertility. In a longitudinal study of the impact of CD in Chile in the 80s, no difference in fertility was identified between seropositive and seronegative women [15]. In animal studies, the majority of *T. cruzi* strains studied had no effect in fertility; however, experimental infection with certain strains has been associated with marked reductions in fertility [16–19]. Further studies are needed to define the overall risk of infertility in CD and its relationship with specific *T. cruzi* strains or lineages.

Evidence for an overall increased risk of abortion or prematurity in seropositive women is inconclusive [20]. However, several studies suggest that maternal chronic infection has no effect on the outcome of pregnancy or on the health of newborns as long as there is no maternal transmission of parasites to the unborn child [21–23]. These studies demonstrate that, when the child is infected, there is an increased risk of premature delivery, low birth weight, and more premature ruptures of the amniotic membranes, effects that may be related to inflammation of the placenta seen in these cases [24–26]. An increased risk of polyhydramnios has also been reported [27].

3. Risk Factors for Congenital Transmission

The risk factors for CD congenital transmission are as follows:

mothers living or migrating from endemic areas,

mothers living or migrating from areas with high rate of transmission,

precedent of siblings with congenital infection,

mother with detectable parasitemias,

mothers with decreased T-cell-mediated responses to *T. cruzi,*

coinfection with HIV or Malaria.

The *T. cruzi* infection prevalence among pregnant women varies between different countries, distinct geographical areas, and rural and urban localities from <1% to 70.5% [14]. In recent studies the prevalence rates rose with the increasing maternal age, particularly within those older than 20 years of age, reflecting the success of vector control programmes [28]. However in some rural areas of Bolivia prevalence remains as high as 70.5% and it is expected that the risk of infection will remain elevated in these areas [28–31]. The rate of transmission in endemic countries shows important geographic differences that range between 0% and 18.2% [13, 14].

Congenital transmission of CD may occur during any phase of maternal disease. During the first trimester of pregnancy (weeks 1–12), transmission is probably rare, since the placental intervillous space is not open due to endovascular trophoblast plugging of the spiral arteries. Maternal blood supply becomes continuous and diffuse in the entire placenta only after the 12th week of gestation. Therefore, transmission of blood parasites probably occurs most frequently during the second and third trimesters of pregnancy (prenatal transmission) and perhaps also closer to delivery and during labor (perinatal transmission) through placental breaches/tears [32, 33]. However, the stage of pregnancy when

the risk of infection is greatest has not been fully examined as this would entail the systematic evaluation of all pregnancies (including abortions and still births). Furthermore, as most pregnant women acquired the infection prior to conception it is impossible to accurately determine the exact moment of parasite transmission. In studies in which all pregnancies were evaluated, the risk of transplacental transmission was greatest below 34 weeks of gestation, occurring mainly between weeks 22 and 26 [34, 35]. When abortions or stillbirths were not included, congenital transmission occurred predominantly in newborns with a gestational age of 26 to 37 weeks [36]. Three cases of acute infection during pregnancy have been published in detail [37]; in these cases two of the children had no congenital disease and their mothers acquired the disease at weeks 28 and 32 of gestation; the third child was infected and the mother acquired the infection at week 20 of gestation. In another study, two of four women in acute phase of the disease transmitted the disease [38].

Factors that have been implicated in determining the risk of transmission include both maternal factors, such as maternal phase of the disease, immunological status, and obstetrical history, and parasite factors such as the *T. cruzi* strain or the parasitic load.

Infected mothers may transmit the parasite, in one, some, or all their gestations, and may also infect some or all of the siblings in multiple deliveries [21]. Not surprisingly, the clustering of cases within families has been reported [23]. The reasons why some mothers transmit the infection to their offspring and others do not or why one mother can transmit the infection in one pregnancy while not in other pregnancies are not known [14].

Parasitemias may recur with reactivation of chronic disease usually associated with immunosuppression [39]. Pregnancy is known to induce a transient depression of maternal cell-mediated immunity, to prevent rejection of the fetus. An increase in the levels of *T. cruzi* specific IgM has been found in chronically infected pregnant women, suggesting recrudescence of the disease as this antibody is usually found only in the acute phase of the disease [20]. It has been postulated that activation of innate immune defences in pregnant women might contribute to the limitation of the occurrence and severity of congenital infection. Mothers that gave birth to healthy offspring produced higher levels of IL1β, IL6, and TNFα under stimulation with *T. cruzi* or LPS/PHA than uninfected control mothers and this maternal cellular activation upregulated the capacity of their uninfected neonates to produce such cytokines [40]. Differences in immune responses between transmitting and nontransmitting mothers have been identified. Chronically infected nonpregnant women have increased levels of circulating TNF-α and these levels remain increased during pregnancy in women that did not transmit the disease [41]. In contrast, pregnant women that transmitted the parasite had a downregulation of the TNF response. Also, the spontaneous release of TNF by peripheral blood leukocytes was higher in nontransmitting *T. cruzi*-infected pregnant women [42]. As their mothers, noninfected neonates had higher circulating levels of TNF than congenitally infected children. The circulating levels of the soluble receptor TNF-R1 (a TNF regulator) were increased in nontransmitting and transmitting mothers and in infected and noninfected neonates. However, the circulating levels of soluble receptor TNF-R2 were ~60% higher in infected than in noninfected neonates [42]. A difference in IFN-γ response has also been associated with vertical transmission. Mothers that transmitted the infection had decreased production of IFN-γ after activation of blood cells with *T. cruzi* lysate and their CD14-positive monocytes expressed less HLA-DR (involved in antigen presentation) and CD54 (involved in cellular adhesion) than infected pregnant women with healthy offspring [43].

Maternal coinfection with *T. cruzi* and HIV results in increasing frequency and severity of congenital CD. Also coinfection with *Plasmodium vivax* results in increased levels of congenital transmission [32].

A high maternal parasitic load has been proposed as a risk factor for transmission. Parasitemias are high during the acute phase of infection and, therefore, transmission rates are expected to be higher in cases of acute disease acquired during pregnancy. In fact, of fifteen reported cases of acute CD during pregnancy, 8 (53%) transmitted the disease to their offspring [20, 37, 38], when the overall rate of transmission is 5% [13]. In contrast, parasitemias are known to be low and recurrent during the indeterminate or chronic phases of infection. However, there is evidence that parasitemias increase during pregnancy [44, 45]. The reported prevalence of parasitemia in pregnant women varies enormously depending on the diagnostic technique employed and on the number of samples taken [28, 29, 44, 45]. Overall rate of parasitemias at some point during pregnancy when more than one sample was examined was 29% when examination of the buffy coat of blood was used [44] and 60.4% when hemoculture was used [45]. The prevalence rate when a single sample was evaluated was 63% using quantitative real time PCR [28]. The time of pregnancy when parasitemia is highest is controversial. In one transversal study, the proportion of positive hemocultures in pregnant women was higher in the first trimester and decreased in women in later stages of pregnancy [45]. In contrast, in a longitudinal study the prevalence of maternal parasitemia was significantly higher during the third trimester of pregnancy than during the first two trimesters [44]. Nevertheless, a direct correlation between high levels of maternal parasitemia and increased risk of transmission has been reported [28, 43, 46, 47].

The role of different genotypes in the risk of congenital infection is unclear. *T. cruzi* parasites have been classified into six different lineages (TcI to TcVI; reviewed in [48]), all of which, with the exception of TcIV, have been identified in human cases of congenital CD [19]. The prevalence of specific *T. cruzi* lineages in cases of congenital disease probably reflects the prevalence of the lineages of the endemic area where they were born [46, 49–51]. The presence of mixed infections is known to occur and therefore the risk of transmitting more than one parasite lineage exists and, in fact, has been reported [49, 52, 53]. As the mixed lineages identified in the newborn are the same as

those found in the mother, it is likely that the different *T. cruzi* lineages have a similar potential of crossing the placental barrier [53]. However, animal models have proved that different strains may have different rates of placental invasion and of congenital transmission [17, 54], supporting the idea that the specific genotype of the strain involved is important.

Not surprisingly, factors that determine increased prevalence of chronic Chagas infection such as living in a rural environment, low education, poverty, and poor quality of housing are also factors for increased risk of congenital transmission [26–31].

4. Physiopathology of Congenital Infection

Parasites appear to reach the fetus mainly via the hematogenous route across the placenta or through the marginal sinus of the placenta [32, 33]. Less frequently, congenital *T. cruzi* transmission can also occur via the oral route through ingestion of infected amniotic fluid or via the hematogenous route through placental breaches and tears that may occur during delivery [32, 33].

There is evidence that placental innate immune responses can be activated when exposed to *T. cruzi* and that the activation of these responses might reduce or prevent maternal-fetal transmission of the parasites [32, 33]. However, excessive levels of inflammation can be deleterious rather than protective [32, 33].

In cases of aborted infected fetuses, all the placentas showed intense and extensive inflammatory infiltrate along with presence of the parasite; the fetuses also displayed inflammatory infiltrates in all organs studied, demonstrating the presence of catastrophic infection [55, 56]. In one case of maternal acute CD, where the placenta was examined, granulomatous changes, inflammatory infiltrates, and focal necrosis in the chorionic villi were observed. The fibrinoid layer was thicker in some modified villi in which syncytial modifications such as edema and calcification foci were present. Vascular thromboses were also seen. Interestingly, the mother did not transmit the disease [37]. Histopathological differences have been observed between placentas from children born with congenital disease compared to placentas from uninfected children born to seropositive mothers. Chorionitis, chorioamnionitis, and cord edema with lymphocyte infiltration were present in placentas of infected children, whereas such lesions were infiltrated only with polymorphonuclear cells in placentas of noninfected children. Parasites were found in the placentas of infected children, the fibroblasts and macrophages of chorion, membranes, and chorionic plate, mainly in the area of membrane insertion, as well as in cells of Wharton jelly and myocytes of umbilical cord vessels [57]. The authors propose that these results suggest that the maternofetal transmission of parasites occurs mainly through the marginal sinus, spreading into the chorionic plate infecting fibroblasts and macrophages until they reach a fetal vessel, inducing a fetal infection by hematogenous route.

5. Clinical Manifestations of Congenital Chagas' Disease

The severity of disease varies enormously from asymptomatic cases to fatal infection and it is related to the level of parasitemia at birth [58]. The reported prevalence of asymptomatic congenital infection varies from 40% to 100% [22, 59–61]. Clinical manifestations can be present at birth or appear within days or weeks after birth [32]. If left untreated, children enter the indeterminate phase of disease with some of them developing chronic disease with typical gastrointestinal and cardiac manifestations [62]. Occasionally, these late symptoms are the first indication of the disease [63].

Many of the signs and symptoms observed in newborns with congenital CD are not specific and may occur with other congenital infections such as toxoplasma or cytomegalovirus (TORCH syndrome). Congenitally *T. cruzi*-infected newborns are frequently premature, have a low birth weight for their gestational age, and have growth retardation, and their APGAR scores are lower than noninfected children [21, 22, 36, 59, 61]. Respiratory distress syndrome is frequently present and can be related to either immaturity of pulmonary function in premature babies and/or pneumonitis associated with parasitism of the alveolar wall [14, 21, 22]. Hepatomegaly, splenomegaly, and jaundice are also common [14, 22, 32, 59, 61].

In severe cases, one or more organs can be affected, most commonly the brain (meningoencephalitis that may be associated with microencephaly) and/or heart (acute myocarditis with cardiomegaly and arrhythmias) [21, 32, 64]. Purpura and oedema (anasarca/fetal hydrops in severe cases) can also occur [14, 32]. The more frequently haematological alterations are anaemia and thrombocytopenia [14, 32]. In children born to mothers infected with HIV and *T. cruzi*, infections were more severe and frequently fatal [65, 66].

In rare occasions, the digestive tract and the eye may be involved. Megaesophagus and megacolon may occur early in congenital disease and can be present at birth [21]. When the gastrointestinal tract is involved, disease is severe and has a high mortality rate [67, 68]. Ocular involvement, with chorioretinitis and opacification of the vitreous body, has also been reported [21, 69, 70].

Mortality rates of approximately 5%, mainly due to myocarditis and meningoencephalitis, have been published [14]. Torrico et al. [22] described mortality rates of up to 13% in a cohort of infected infants studied between 1992 and 1994, while the mortality rate dropped to 2% 6 years later, probably as a result of the improvement of the socioeconomic environment in Bolivia. Mortality was higher in infected children born prematurely with severe clinical manifestations.

6. Diagnosis

Symptomatic congenital CD should be considered in any newborn with clinical findings suggestive of a vertically

transmitted infection such as toxoplasma or rubella whose mother has positive serology for *T. cruzi* or has a sibling with CD.

Criteria for suspicion of symptomatic congenital Chagas' disease are as follows:

signs and symptoms of vertically transmitted infection:

prematurity,
small for gestational age,
low APGAR score,
respiratory distress syndrome,
hepato-/splenomegaly,
jaundice;

T. cruzi seropositive mother (by 2 different standard tests);

sibling with congenital Chagas' disease;

evidence of myocarditis or meningoencephalitis.

Increased awareness of the possibility of congenital transmission is essential as failure to test for and treat this infection may lead to the death of the infant [71].

Congenital infection is frequently asymptomatic; therefore, all children born to seropositive mothers should be tested for CD. Unfortunately, this is not routinely done even in endemic areas. A study in Argentina showed that only 17% of multiparous seropositive pregnant mothers that had been sent to a reference health centre had offspring that had been tested for CD [72]. In endemic areas it has been estimated that for each diagnosed case there is at least 6 undiagnosed cases [73, 74]. In nonendemic areas the rate of underdiagnosis is over 90% [5]. Routine testing of older children born to seropositive mothers can identify some of these cases [75].

According to the Technical Group on "Prevention and Control of Congenital Transmission and Case Management of Congenital Infections" (IVa) of WHO Programme on Control of CD, the gold standard for diagnosis of congenital infection is the detection of blood parasites at any time after birth or a positive *T. cruzi*—specific serology in infants aged >8 months (when previous transmission by vectors and blood transfusion has been ruled out) [76]. In the experience of Instituto Nacional de Parasitología Dr. Mario Fatala Chaben, a referral centre for pregnant seropositive women and for the diagnosis of congenital CD, a positive diagnosis could only be established within the first month of life in 44.6% of the babies, in 24.3% of cases diagnosis was not made until the 5th month, and in 31.1% diagnosis could not be confirmed until 6 to 12 months of age [77]. Therefore, it is essential to follow up children born to seropositive mothers and perform routine testing for both searching for parasite in blood and monitoring their antibody response until the end of the first year of life [73, 78]. It is regrettable that more than 55% of children are lost to followup after the age of 6 months and that less than one in two congenital cases is correctly identified and treated [61, 77–79].

7. Identification of the Parasite

Early diagnosis in children born to seropositive mothers depends on the detection of blood parasites, when maternal *T. cruzi* antibodies could still be present. The microscopical observation of fresh blood between slide and coverslip can easily disclose the presence of the parasites because of their motility. Thin- and thick-stained blood smears allow detection of the morphological characteristic of the parasite. When the parasite load is low, a concentration method is required, either by a Strout test (where serum is spun and the resulting pellet is examined) or by examination of the white buffy coat (the leucocyte layer that separates red cells from plasma in a haematocrit test, also known as microhematocrit method) [80, 81]. These methods are particularly useful when there are high levels of parasitemia such as cases of acute infection or during the first months of life in cases of congenital infection. It is worthwhile to test 4 microhematocrit tubes at a time, as this modified procedure increases the sensitivity of the test to detection of parasitemia levels as low as 50 parasites per milliliter [58].

Indirect methods for the identification of the parasite are generally used when the parasitic load is low (chronic disease) and require the expansion of the parasite population under laboratory conditions that are not available everywhere. There are two ways of growing the parasite, by hemoculture or by xenodiagnosis (technique that exposes suspected infected tissue to a vector and then examines the vector for the presence of the particular pathogen). These tests may take several weeks to become positive but are very sensitive [47, 82, 83].

The histopathological analysis of the placenta is not considered a good diagnostic test either because the presence of parasites may be missed or because its presence, although being suggestive, does not necessarily indicate congenital infection. Placentas of uninfected newborns from infected mothers can present with parasites and severe histological changes without being associated with fetal infection [14, 21, 32, 64]. Also placentas of children with congenital infection may not show abnormalities [64].

8. Molecular Diagnosis

The identification of parasite antigens or DNA in blood can suggest the presence of infection. The detection of *T. cruzi* soluble antigens in urines and serum by capture ELISA assays has been proposed for diagnosis of congenital cases. However, these tests did not detect all infected cases [32].

The amplification of *T. cruzi* nuclear or kinetoplastic DNA is considered "under evaluation" by the World Health Organization but has been used to detect low levels of parasitemia in congenital cases, and results can be obtained in a short time [76]. However, the presence of parasite DNA in the blood of the newborn does not necessarily indicate active infection as it does not prove that the parasites are viable [14, 32]. It has been suggested that the high rates of congenital infection reported by some authors may be due to the amplification of trace amounts of DNA from the

mother and not the presence of live parasites [32]. Therefore, it is recommended that the test should be confirmed with subsequent samples at approximately 3 and 9 months after birth [78, 84]. Although this technique has not been fully validated, it has a good predictive value and has the advantage of not needing a specially trained observer [78].

9. Serological Diagnosis

In the first months of life conventional serology is not useful due to the transference of maternal antibodies through the placenta. It has been suggested that a test using shed acute phase antigen (SAPA) only detects acute or congenital infection, as this antigen is not present in the chronic phase of disease [85, 86]. However, antibodies against this antigen were detected in ~80% of patients with intermediate disease [87]. More studies are needed to determine the value of this test.

After the first 9 months maternal antibodies have disappeared and therefore the presence of specific anti-*T. cruzi* antibodies with conventional serological test is considered diagnosis. As with chronic cases the use of two different serological tests is recommended (IHA, IF, or ELISA). In some cases, diagnosis is only made by a positive serology [47, 78]. Failure to detect parasites in these children is probably due to low parasitemias and reflects the need to develop more sensitive assays and the importance of performing a serological test at the age of 12 months [73, 78].

10. Antenatal Screening

According to the recommendations of the Technical Group IVa on "Prevention and Control of Congenital Transmission and Case Management of Congenital Infections," serological testing is recommended for pregnant women (i) who are living in disease-endemic areas, (ii) who are living in disease nonendemic areas and have occasionally received blood transfusion in disease-endemic areas, and (iii) who are living in disease nonendemic areas and are born or have lived previously in disease-endemic areas or whose mothers were born in such areas [76].

This group also recommended that CD should be systematically investigated in siblings and relatives of infected mothers (serological investigation), and positive cases should be clinically evaluated and treated accordingly [76].

11. Treatment

Treatment is generally successful and without the adverse reactions seen in adults if administered within the first year of life [76, 88]. All children must be followed up after treatment to ensure that they have eliminated the parasite. The long term prognosis has not been well studied, but in a communication of their 30-year experience, Moya et al. (2005) reported that, for children treated before the age of three, they were cured and that, at 13–15 years of age, they had no evidence of cardiac abnormalities [64]. Failure to diagnose and treat the infection may result in chronic symptomatic disease [63].

Treatment should be given according to the guidelines established by the Technical Group IVa on "Prevention and Control of Congenital Transmission and Case Management of Congenital Infections" [76].

All cases of congenital *T. cruzi* infection should be treated as soon as the diagnosis has been confirmed with either benznidazole or nifurtimox.

The recommended dose of benznidazole in infants, as in adults, is 5–7 mg/kg per day; doses of benznidazole up to 10 mg/kg per day can be used in neonates and infants aged <1 year. Benznidazole is manufactured by Laboratório Farmacêutico do Estado de Pernambuco (LAPEFE, Brazil) and is available in tablets of 100 mg through "Masters" (Davie, Florida, United States; Elstree, Hertfordshire, United Kingdom), the WHO, and the PAHO. This technical group suggests that dispersal tablets of 12.5 mg should become available to facilitate the preparation of paediatric suspensions.

The recommended doses of nifurtimox in neonates and infants are 10–15 mg/kg per day. Nifurtimox is manufactured by Bayer and is available in tablets of 120 mg through WHO and PAHO.

Treatment with either drug, should be administered orally in one dose in low-weight neonates or, preferably, in divided doses of two to three subdoses; precautions should to be taken to obtain appropriate dosage of active drug, since the currently available tablets have to be crushed and used as a suspension.

The recommended duration of treatment is 60 days and should not be <30 days.

12. Chagas' Disease and Breastfeeding

CD can be acquired through the ingestion of contaminated food or water. Therefore, the possibility of transmission through breastfeeding may be particularly relevant, particularly because such transmission could be preventable. The risk of transmission through this route has been recently reviewed [89]. In mice, oral transmission of *T. cruzi* infection through human milk contaminated with trypomastigotes is possible, although natural transmission through breastfeeding has not been clearly demonstrated. In humans, contamination of milk with trypomastigotes has been described; however, except for some dated and inconclusive cases, transmission through breastfeeding has not been reported [89]. More studies are needed to fully evaluate the risk of infection through breastfeeding.

Exclusive breastfeeding is an ideal way to provide nutrition during the first 6 months of life, and interruption of breastfeeding in resource-poor settings does not seem feasible unless the risks clearly outweigh possible benefits. Therefore, the discontinuation of breastfeeding by mothers with chronic CD is not recommended [89, 90]. However, breastfeeding by mothers, with acute CD or with fissures and bleeding nipples, should be avoided.

13. Concluding Remarks

Congenital CD may occur in any part of the world and the lack of well-established surveillance programs means that the diagnosis is largely missed. Identification of all affected children involves testing all pregnant women at risk of infection, that is, either living in an endemic area or having migrated from an endemic area. All children born to seropositive mothers should be tested not only within the first month of life but also at ~6 months and ~12 months of age. The diagnosis is made by identification of the parasite using standardized micromethods before 6 months and by a positive serology after 10 months of age. Followup for a year is essential as a significant proportion of cases are initially negative and are only detected at a later stage by either detection of blood parasites or by seroconversion. The success of the followup depends on establishing good followup routines in primary care settings and on extensive counselling of the mothers emphasizing the relevance of control even in asymptomatic and apparently healthy children [77]. Early diagnosis is important because, within the first year of life, the response to treatment is almost 100% and well tolerated. Siblings of children with congenital infection should also be studied.

In their first report on neglected diseases the WHO recognized that the movement of CD to areas previously considered nonendemic, resulting from increasing population mobility between Latin America and the rest of the world, represents a serious public-health challenge (report of neglected diseases) [91]. It also expressed a preoccupation that, in places where health professionals have little knowledge or experience of the disease and its control, the diagnosis of CD will be missed and left untreated. Therefore, it is important that developed country researchers establish bilateral and multilateral CD collaborations to help health care professionals in their regions to learn from the decades of experience of Latin American scientists and to provide the resources and a collaborative platform to advance the search for better ways to diagnose, treat, and prevent the disease [92].

Conflict of Interests

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

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Research Article

Rural Residents in China Are at Increased Risk of Exposure to Tick-Borne Pathogens *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis*

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As emerging tick born rickettsial diseases caused by *A. phagocytophilum* and *E. chaffeensis*, anaplasmosis and ehrlichiosis have become a serious threat to human and animal health throughout the world. In particular, in China, an unusual transmission of nosocomial cases of human granulocytic anaplasmosis occurred in Anhui Province in 2006 and more recent coinfection case of *A. phagocytophilum* and *E. chaffeensis* was documented in Shandong Province. Although the seroprevalence of human granulocytic anaplasmosis (former human granulocytic ehrlichiosis, HGE) has been documented in several studies, these data existed on local investigations, and also little data was reported on the seroprevalence of human monocytic ehrlichiosis (HME) in China. In this cross-sectional epidemiological study, indirect immunofluorescence antibody assay (IFA) proposed by WHO was used to detect *A. phagocytophilum* and *E. chaffeensis* IgG antibodies for 7,322 serum samples from agrarian residents from 9 provinces/cities and 819 urban residents from 2 provinces. Our data showed that farmers were at substantially increased risk of exposure. However, even among urban residents, risk was considerable. Seroprevalence of HGA and HME occurred in diverse regions of the country and tended to be the highest in young adults. Many species of ticks were confirmed carrying *A. phagocytophilum* organisms in China while several kinds of domestic animals including dog, goats, sheep, cattle, horse, wild rabbit, and some small wild rodents were proposed to be the reservoir hosts of *A. phagocytophilum*. The broad distribution of vector and hosts of the *A. phagocytophilum* and *E. chaffeensis*, especially the relationship between the generalized susceptibility of vectors and reservoirs and the severity of the disease's clinical manifestations and the genetic variation of Chinese HGA isolates in China, is urgently needed to be further investigated.

1. Introduction

Anaplasmosis and ehrlichiosis are emerging tick-borne rickettsial diseases (TBRDs) caused by the obligate intracellular bacteria *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis*, respectively [1–3]. These two rickettsiae are both in the family Anaplasmataceae but are in different genera. Both bacteria are transmitted by hard ticks, and certain wild rodents and animals are reservoirs. In China, *A. phagocytophilum* bacteria had been isolated from *Apodemus agrarius, Tscherskia triton,* and sheep, respectively, and these animals might be reservoirs hosts of *A. phagocytophilum* [4]. Moreover, a recent national investigation assessing the epidemiological status of *A. phagocytophilum* among domestic animals in 10 provinces/cities in China showed that some domestic animals including dogs, goats, and cattle might be important reservoirs hosts of *A. phagocytophilum* [5].

Although the diagnosis of anaplasmosis and ehrlichiosis is difficult, the annual numbers of infections reported throughout the world have steadily increased [6, 7] since the first recognition of *E. chaffeensis* in 1986 [8] and of *A. phagocytophilum* in 1990 [9]. Seroepidemiological data from the United States suggest that infection rates of *A. phagocytophilum* in endemic areas are as high as 15–36% [10, 11]. In China, an investigation in Tianjin City, one of the largest municipalities and the largest trade port in the northern part of China, revealed that the average seroprevalence in farmers was 8.8% in 2006 [12]. In the United States, the incidence rate of *E. chaffeensis* increases from 0.8 to 3.0 cases/10^5/year during 2000–2007, the hospitalization rate is 49.0%, and the case-fatality rate is 1.9% [7]. A recent investigation assessing the seroepidemiological status of *E. chaffeensis* among rural residents in Beijing indicated that the seroprevalence was 16.4% [13].

However, both anaplasmosis and ehrlichiosis are often underrecognized in China because epidemiological, ecological, clinical, and microbiological information about these two bacteria is very limited, and both diseases are often misdiagnosed due to their clinical manifestation's similarity to hemorrhagic fever with renal syndrome (HFRS) [14–16]. A typical example of misdiagnosis is the unusual cluster of nosocomial transmission of human granulocytic anaplasmosis (HGA) in a hospital in Anhui province in 2006. The index patient was originally misdiagnosed with HFRS, and five relatives of the patient and four medical workers were secondarily infected with HGA due to contact with blood or respiratory secretions, while the index patient experienced extensive hemorrhage and underwent endotracheal intubation [17].

Despite serological, molecular, and even etiological evidence demonstrating the nationwide distribution of *A. phagocytophilum* infections in humans, domestic animals, ticks, and rodents [4, 12–19], large-scale laboratory-based serological investigations among rural residents who may be at an increased risk of occupational and residential exposure are limited. Thus, it is crucial to obtain epidemiological data on geographical, occupational, and residential risk factors that could increase disease exposure. Herein, a cross-sectional epidemiological study of people residing in rural and urban areas was undertaken during March–May, 2009.

2. Materials and Methods

2.1. Ethics Statement. The study and the collection protocol were approved by the China CDC Institutional Review Board (no. 201103). Written consent was obtained before the blood sampling of participants. Parents provided written informed consent on behalf of all child participants. Preparations of positive rabbit sera used for quality control of antigen slide in the study were produced by rabbit immunization, and all experimental procedures were conducted to conform to the National Institutes of Health Guide for Care and Use of Laboratory Animals (J. Derrell Clerk, Ed., National Academy Press, Washington, DC, USA, 1996.) The Animal Ethics Committee of the Chinese Center for Diseases Control and Prevention approved a document on the experimental procedures (201104).

2.2. Study Area and Time. The nine provinces Zhejiang, Anhui, Jiangsu, Henan, Yunnan, Hainan, Xinjiang, Jilin, and Heilongjiang and the two independent municipalities Beijing and Tianjin were chosen based on the availability of information on recorded rickettsial infections for each province/city from March to May, 2009. The investigation time and order for each province/city were determined based on the breeding peak of ticks in the local areas. For each province or city, three or five rural counties were selected based on geographic location, for example, the eastern, southern, western, northern, and central areas of each province, to identify the investigation sites. In the same way, three to five villages were chosen based on their geographic locations in each county.

2.3. Study Population. Considering the age distribution and the accordance of labor style, the family as unite was investigated and sampled. Local permanent residents were selected from among the local government-registered families; for example, families were selected based on the last digit (odd or even) of the registration number of their registered permanent residence. For each selected family, every individual, including spouses and children, was included in the study. A standard questionnaire was used according to the "Guideline for the Control and Prevention of Human Granulocytic Anaplasmosis" issued by the Ministry of Public Health of the People's Republic of China in 2008 (the Ministry of Public Health of the People's Republic of China, 2008, No. 18). The demographic data collected included general information, such as sex, age, place of residence (plain areas or hilly regions), occupation (planting crops, planting fruit trees, or the unemployed, including retired people, students, and preschool children), the length of working time per day, the length of service time, and past medical history. The participants had to answer whether they could recognize ticks, whether they had been bitten by ticks, and how frequently they had been bitten by ticks within the last year.

All participants were asked whether they had a fever on the day of the survey and whether they had had a fever (temperature of ≥38.0°C) during the preceding 12 months. If so, they were asked about clinical manifestations, such as myalgia or headache.

In addition to the rural residents mentioned above, a total of 819 samples from urban residents, including 566 sera from Daqing city, Heilongjiang province, and 253 sera from Yanbian city, Jilin province, were collected during 2007-2008 and included in the study. The demographic data were recorded in the same way as for the rural residents.

2.4. Sampling and Laboratory Detection. A 2 mL sample of nonanticoagulated blood was collected from each participant after written informed consent was obtained. Samples were temporarily stored in a cooler and then transferred to the local county CDC for serum separation. The blood samples were centrifuged at 1,500 rpm for 10 min, and the separated sera were stored at −20 or −40°C at the local CDC and then transferred to the Department of Rickettsiology, National Institute for Communicable Disease Control and Prevention, China CDC, by air within 48 or 72 hours and stored at −80°C until laboratory testing.

Immunofluorescence assays (IFA) were performed for IgG antibody detection according to the reference methods proposed [20]. To reduce laboratory errors, testing of all samples was performed within a limited time frame (from May to August 2009). *E. chaffeensis* (Arkansas strain) antigen was provided by Dr. Robert Massung at the United States CDC. The *A. phagocytophilum* strain Webster was kindly provided by J. S. Dumler at the Johns Hopkins University School of Medicine. These two antigens were spotted in different rows in the same slide to reduce laboratory errors.

HGA-positive human serum from the Focus Diagnostics kit (Cypress, CA) served as a positive control for testing human sera, and three or five different diluted concentrations of rabbit sera were used for quality control of antigen slide because of the limited HGA-positive human sera. Rabbit sera against *A. phagocytophilum* and *E. chaffeensis* were prepared by immunizing rabbits with *A. phagocytophilum* (Webster strain) and *E. chaffeensis* (Arkansas strain), respectively. These two positive rabbit sera were serially twofold diluted and then assayed in parallel with human HGA-positive control sera from a Focus Diagnostics kit using the antigen slice prepared by our laboratory and a slide from Focus, respectively. Based on the quality control methods recommended by Focus Diagnostics, three or five different concentrations of diluted rabbit sera were screened as positive controls. The rabbit sera were frequently standardized using the Focus Diagnostics kit, especially when new batches of antigens were prepared. Two negative controls were selected for each IFA: PBS-milk and mixed healthy human sera (from five workers at our institute who were not members of our laboratory).

The IFAs were performed as follows. The serum samples were diluted 1 : 80 in PBS containing 3% nonfat powdered milk, and 25 μL of the diluted serum was placed in a slide well and incubated for 60 min in a moist chamber at 37°C. After washing in PBS to remove unbound antibody, the slides were labeled with FITC-conjugated rabbit anti-human immunoglobulin (IgG; Sigma Co., NY, New York State, United States) as a secondary antibody, which was diluted 1 : 400 with Evans blue, for another 60 min in a moist chamber at 37°C. The slides were then washed in PBS to remove unbound secondary antibody. The slides were air dried at 37°C and examined using a fluorescent microscope (Nikon, Tokyo, Japan). Samples were interpreted as reactive if there was strong green fluorescence corresponding to bacterial morulae within the cells on the slide. Samples reactive at the 1 : 80 screening dilution were considered to be positive [10, 12, 13]. If a serum sample had dual reactivity with *A. phagocytophilum* and *E. chaffeensis*, further dilution and titration were conducted, and a twofold or higher titer increase was read as positive.

2.5. Statistical Analysis. Statistical analysis was conducted using SAS software (version 9.1, SAS Institute, Inc., Cary, NC). Age was converted into a categorical variable (<10, 10–19, 20–29, 30–39, 40–49, 50–59, and >60 years of age). χ^2 and Fisher's tests were used to compare distributions of seropositivity or to examine associations between pairs of categorical measures. Logistic regression analyses were used to calculate odds ratios (ORs) for seropositivity among variables. The survey questions regarding the variables "living in plains areas," "living in hilly region," "crop planting," "planting fruit trees," "livestock breeding or contact with domestic animals," "length of working hours per day," and "length of service time" were created to be associated with presumed risk among permanent residents of rural areas andto develop the explanatory variables used in the logistic regression. All tests were two-sided, and significance was set at less than 0.05.

3. Results

3.1. Study Areas and Population. A total of 7,322 rural residents from 57 villages in 33 rural counties in Zhejiang, Anhui, Jiangsu, Henan, Yunnan, Hainan, and Xinjiang provinces and from the cities of Beijing and Tianjin were investigated during March–May, 2009 (Figure 1). The mean age of the participants was 44 years (range, 2–81 years). Males accounted for 3,493 of the participants, with a mean age of 43 years (range, 2–80 years), and females accounted for 3,829 of the participants, with a mean age of 45 years (range, 2–81 years). In the rural areas investigated, 72% of participants lived in plains areas, and 28% of people lived in hilly regions. In total, 57% of residents were engaged in planting crops; 23% of residents were engaged in planting fruit; 4% of residents were engaged in feeding domestic animals; and 16% of investigated individuals were preschool children, students, or retired people. Additionally, 95% of residents had contact with domestic animals or livestock. Although 94% of people could recognize ticks and 12% of people recalled that they had been bitten by ticks before, nobody could tell the species of the ticks. In total, 6% of residents recalled fever (≥38°C), headache, or myalgia during the past year. Only 15% (65)

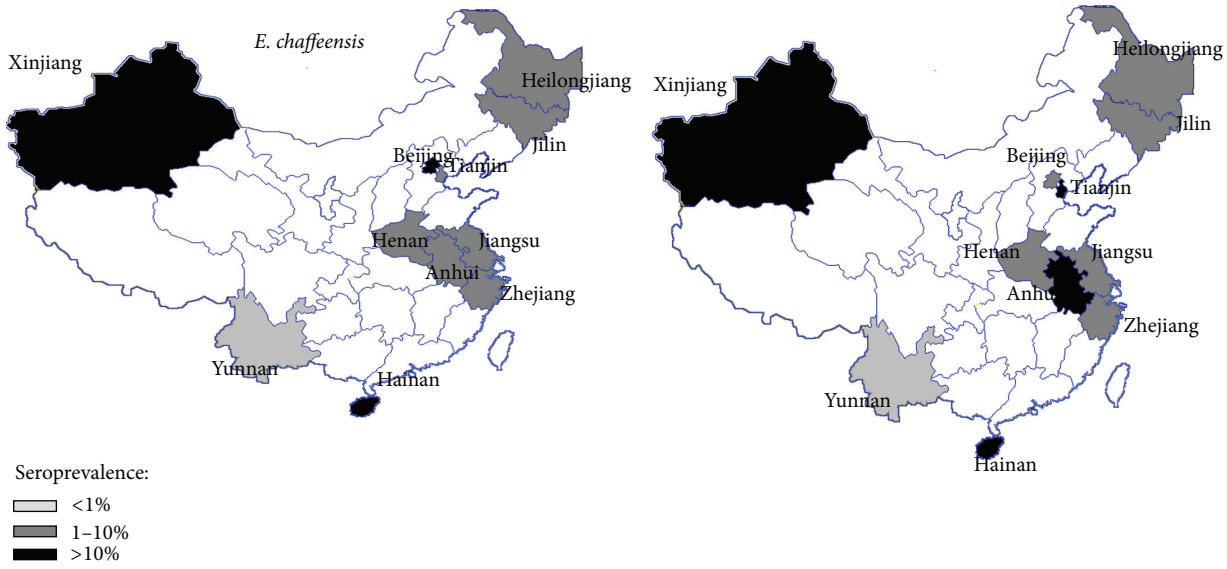

Figure 1: The seroprevalence distribution of *E. chaffeensis* and *A. phagocytophilum* from 11 provinces/cities in China from 2007 to 2009. The triangles on the map indicate the investigated counties in each province.

Table 1: The overall seroprevalence of *E. chaffeensis* and *A. phagocytophilum* in rural and urban residents, China, 2007–2009.

Pathogens	Rural		Urban		Odds ratio (95% CI)	*P* value
	Number positives/total number	Seropositivity rate % (95% CI)	Number positives/total number	Seropositivity rate % (95% CI)		
E. chaffeensis	719/7322	9.8 (9.1–10.5)	20/819	2.4 (1.4–3.4)	4.4 (2.8–6.8)	<0.0001
A. phagocytophilum	1331/7321	15.4 (14.6–16.2)	12/819	1.5 (0.7–2.3)	12.7 (7.2–22.5)	<0.0001

CI: confidence interval.

of people remembered the name of the antibiotics that they had used, for example, a tetracycline (34), penicillin (15), a cephalosporin (13), or a macrolide (3).

The characteristics of the 819 urban residents were as follows: 310 sera (from males aged 18–25 years) were collected from people who were ready to be drafted into the army in Daqing city in 2007, Heilongjiang province, 98% of whom were senior high school students, and 2% of whom were young people waiting for job assignments. Another 256 sera (from females aged 23–67 years) from Daqing were collected from healthy people who had participated in a medical examination in 2008. In addition, 253 sera (from 121 males aged 18–76 years and 132 females aged 20–83 years) were collected from healthy enterprise employees at a medical examination center in Yanbian city, Jilin province, in 2008. All of the people mentioned above lived in cities and rarely engaged in work in the wild or were exposed to ticks.

3.2. Seroprevalence of E. chaffeensis. Values for the seroprevalence of *A. phagocytophilum* and *E. chaffeensis* in the studied areas are shown in Table 1 and Figure 1. The overall seroprevalence of *E. chaffeensis* was 9.8% (95% confidence interval (CI) 9.1–10.5%) in rural residents and 2.4% (95% CI 1.4–3.4%) in urban residents. Chi-square test analysis indicated that the seropositivity rates of rural residents were significantly higher than the rates of urban residents ($P = 0.0001$, OR 4.4, 95% CI 2.8–6.8%). In an IFA, 37 (0.45%) samples exhibited a cross-reaction with *A. phagocytophilum* and *E. chaffeensis* at a 1 : 80 cut-off, 16 (43%, 16/37) of which were confirmed to be reactive with *E. chaffeensis* by further titration. Among the seven provinces and two cities, the seropositivity rates of *E. chaffeensis* of rural residents in Xinjiang (43.2%, 95% CI 28.6–57.8%) and Hainan (44.6%, 95% CI 41.3–47.9%) provinces and Beijing city (19.4%, 95% CI 9.9–28.9%) were significantly higher compared with rates in other areas. The difference in serological prevalence between males and females in each province or city was not statistically significant (Table 2). Although age distribution differed across age strata, the seroprevalence in the 20-to-29-year-old (15.3%, 95% CI 13.5–17.1%) and 30-to-39-year-old (13.0%, 95% CI 11.4–14.6%) groups was significantly higher than in the other age groups ($P < 0.05$) (Table 3). However, no statistically significant difference was found between the two groups mentioned above ($P = 0.06$). Of the seven provinces and two cities in rural areas, the seroprevalence (44.6%, 95% CI 41.3–47.9%) was the highest in Hainan, followed by Xinjiang (43.2%, 95% CI 28.6–57.8%) and Beijing (19.4%, 95% CI 9.9–28.9%) (Table 2).

Regarding the variables associated with the presumed risk based on the questionnaires, a statistical analysis indicated

Table 2: The seroprevalence of *E. chaffeensis* and *A. phagocytophilum* by location and sex.

Area	Province/ sampling year	*E. chaffeensis*						*A. phagocytophilum*					
		M		F		Odds ratio (95% CI)	*P* value	M		F		Odds ratio (95% CI)	*P* value
		Number positives/total number	Seropositivity rate % (95% CI)	Number positives/total number	Seropositivity rate % (95% CI)			Number positives/total number	Seropositivity rate % (95% CI)	Number positives/total number	Seropositivity rate % (95% CI)		
Rural	Anhui/2009	13/244	5.3 (2.5–8.1)	20/352	5.7 (3.2–8.1)	0.9 (0.4–1.9)	0.9	101/244	41.4 (35.2–47.6)	100/352	28.4 (23.7–33.1)	1.8 (1.3–2.5)	0.001
	Hainan/2008	186/426	43.7 (35.8–51. 9)	193/424	45.5 (39.8–51.2)	0.9 (0.7–1.2)	0.6	172/427	40.3 (35.6–45.0)	162/425	38.1 (33.5–42.7)	1.1 (0.8–1.4)	0.5
	Henan/2009	21/348	6.0 (3.5–8.5)	26/389	6.7 (4.2–9.2)	0.9 (0.5–1.6)	0.7	30/348	8.6 (5.7–11.5)	36/389	9.3 (6.4–12.2)	0.9 (0.5–1.5)	0.8
	Jiangsu/2009	8/1161	0.7 (0.2–1.2)	33/1586	2.1 (1.4.–2.8)	3.6 (1.0–13.3)	0.08	143/1160	12.3 (10.4–14.2)	131/1586	8.3 (6.6–9.7)	1.5 (1.2–2.0)	0.0004
	Zhejiang/2009	3/279	1.1 (0.5–1.7)	4/300	1.3 (0.6–2.6)	0.8 (0.2–3.6)	1.0	1/279	0.4 (0.3–1.1)	2/300	0.7 (0.2–1.6)	0.5 (0.05–5.9)	1.0
	Yunnan/2009	1/151	0.7 (0.6–2.6)	0/175	0	0.5 (0.1–3.9)	0.5	0/151	0	1/176	0.6 (0.5–1.7)	1.0 (0.9–1.1)	1.0
	Xinjiang/2009	12/30	40.0 (22.5–57.5)	7/14	50.0 (23.8–76.2)	0.7 (0.2–2.4)	0.5	4/30	13.3 (7.9–20.1)	2/14	4.3 (10.5–16)	1.0 (0.1–5.7)	1.0
	Tianjin/2007, 2008/2009	45/581	7.7 (5.5–9.9)	38/301	12.6 (8.9–16.3)	0.6 (0.4–0.9)	0.02	253/578	43.8 (39.8–47.8)	114/301	37.9 (32.4–43.4)	1.3 (1.0–1.7)	0.09
	Beijing/2009	50/273	18.3 (13.7–22.9)	59/288	20.5 (15.8–25.2)	0.9 (0.6–1.3)	0.5	43/273	15.8 (11.5–20.1)	36/288	12.5 (8.6–16.3)	1.3 (0.8–2.1)	0.3
	Total	**339/3493**	**9.7 (8.7–10.7)**	**380/3829**	**9.9 (9.0–10.8)**	**1.0 (0.8–1.1)**	**0.8**	**747/3490**	**21.4 (20.0–22.8)**	**584/3831**	**15.2 (14.1–16.3)**	**1.5 (1.3–1.7)**	**<0.0001**
Urban	Jilin/2008	5/106	4.7 (0.7–8.7)	11/147	7.5 (3. 2–11.8)	0.6 (0.2–1.8)	0.4	2/106	1.9 (0.7–4.5)	2/147	1.4 (0.5–3.3)	1.3 (0.1–10.0)	0.7
	Heilongjiang/2008	2/310	0.7 (0.2–1.6)	2/256	0.8 (0.3–1.9)	0.8 (0.1–5.9)	1.0	4/310	1.3 (0.1–2.6)	4/256	1.6 (0.1–3.1)	0.8 (0.2–3.3)	0.8
	Total	**7/416**	**1.7 (0.5–2.9)**	**13/403**	**3.2 (1.5–4.9)**	**0.5 (0.2–1.3)**	**0.2**	**6/416**	**1.4 (0.3–2.5)**	**6/403**	**1.5 (0.3–2.7)**	**0.9 (0.3–3.0)**	**1.0**

Table 3: The seroprevalence of *E. chaffeensis* and *A. phagocytophilum* by age.

Age	*E. chaffeensis*		*A. phagocytophilum*		OR (95% CI)	*P* value
	Number positives/total number	Seropositivity rate % (95% CI)	Number positives/total number	Seropositivity rate % (95% CI)		
Rural						
2–19	105/937	11.2 (9.2–13.2)	139/936	14.8 (12.5–17.1)	0.72 (0.6–1.0)	0.02
20–29	241/1574	15.3 (13.5–17.1)	318/1574	20.2 (18.2–22.2)	0.71 (0.6–0.9)	0.0003
30–39	217/1670	13.0 (11.4–14.6)	423/1670	25.3 (23.2–27.4)	0.44 (0.4–0.5)	<0.0001
40–49	147/1770	8.3 (7.0–9.6)	246/1770	13.9 (12.3–15.5)	0.56 (0.5–0.7)	<0.0001
50–59	70/1137	6.2 (4.8–7.6)	124/1137	10.9 (9.1–12.7)	0.5 (0.4–0.7)	<0.0001
>60	11/234	4.6 (1.9–7.3)	16/234	6.8 (3.7–10.0)	0.67 (0.3–1.5)	0.3
Urban						
18–29	5/370	1.3 (0.2–2.5)	4/370	1.1 (0–2.2)	1.3 (0.3–4.7)	1.0
30–39	3/138	2.1 (0.3–4.5)	3/138	2.1 (0.3–4.5)	1.0 (0.2–5.0)	1.0
40–49	7/136	5.1 (1.4–8.8)	3/136	2.2 (0.3–4.7)	2.4 (0.6–9.5)	0.2
50–59	3/98	3.0 (0.3–6.3)	1/98	1.0 (0.9–3.0)	3.0 (0.3–29.9)	0.6
>60	2/77	2.5 (1.0–6.0)	1/77	1.3 (1.2–3.8)	2.0 (0.2–22.8)	1.0

that fever in the last 24 months and service time >2 years were associated with the exposure risk of *E. chaffeensis*. However, no association between seroprevalence and other specific demographic variables was observed (Table 4).

3.3. Seroprevalence of A. phagocytophilum. The overall seroprevalence of *A. phagocytophilum* was 15.4% (95% CI 14.6–16.2%) in rural residents and 1.5% (95% CI 0.7–2.3%) in urban residents. The seroprevalence in rural residents was significantly higher than in urban residents ($P < 0.0001$, OR 12.7, 95% CI 7.2–22.5%). The seroprevalence varied between investigated sites, and the highest seroprevalence (41.8%, 95% CI 38.5–45.1%) of *A. phagocytophilum* was in Tianjin, followed by Hainan (39.2%, 95% CI 35.9–42.5%), Anhui (33.7%, 95% CI 39.5–40.2%), and Beijing (13.6%, 95% CI 3.5–23.7%) (Table 2). Analysis of sex indicated that the total seroprevalence of *A. phagocytophilum* in males (21.4%, 95% CI 20.0–22.8%) was significantly higher than in females (15.2%, 95% CI 14.1–16.3%) ($P < 0.001$, OR 1.5, 95% CI 1.3–1.7%). Similarly, the seroprevalence of *A. phagocytophilum* in the 20-to-29-year-old (20.2%, 95% CI 18.2–22.2%) and 30-to-39-year-old (25.3%, 95% CI 23.2–27.4%) groups was higher than in the other age groups ($P < 0.05$), although the seroprevalence varied between individual age groups.

Regarding associations between demographic characteristics and seropositivity, our data showed that the seroprevalence in residents who were engaged in planting crops was significantly higher than in people who were employed predominantly in fruit tree planting ($P = 0.006$, OR 0.8, 95% CI 0.7–0.9%). Similarly, the seroprevalence in residents who had contact with domestic and livestock animals was significantly higher than in residents without contact with animals ($P < 0.0001$, OR 4.0, 95% CI 2.6–6.3%). In addition, the seroprevalence in people who had worked for more than 2 years was higher than in people who had worked for less than 2 years ($P < 0.0001$, OR 1.8, 95% CI 1.5–2.1%). Additionally, fever in the last 24 months was associated with a high seroprevalence of *A. phagocytophilum* ($P < 0.0001$, OR 0.04, 95% CI 0.03-0.04%). However, no association was observed between tick bites and human infection, although tick exposure and bites were major risk factors for *A. phagocytophilum* and *E. chaffeensis* infections ($P = 0.3$, OR 1.1, 95% CI 0.9–1.3%).

3.4. Comparative Distribution of A. phagocytophilum and E. chaffeensis. Comparing the distribution of *A. phagocytophilum* and *E. chaffeensis*, the seroprevalence (33.7%, 95% CI 39.5–40.2%) of *A. phagocytophilum* in Anhui was strikingly higher than that of *E. chaffeensis* (5.5%, 95% CI 3.8–7.3%) (OR 0.12, 95% CI 0.08–0.2%). The same tendency was observed in Tianjin (OR 0.14, 95% CI 0.2–0.2%) (Table 5). In contrast, the seroprevalence of *E. chaffeensis* in Hainan (44.6% versus 39.2%, $P = 0.03$, OR 1.2, 95% CI 1.0–1.5%) and Xinjiang (43.2% versus 13.6%, $P = 0.002$, OR 4.8, 95% CI 1.7–13.7%) provinces and in Beijing city (19.4% versus 14.1%, $P = 0.02$, OR 1.5, 95% CI 1.1–2.0%) was significantly higher than that of *A. phagocytophilum* (Table 5).

4. Discussion

Regarding emerging zoonotic infectious diseases, increasing numbers of HGA cases have been confirmed in China since the unusual transmission of nosocomial cases of HGA occurred in Anhui province in 2006 [14, 16, 21]. Specifically, a case of coinfection with *A. phagocytophilum* and *E. chaffeensis* was reported in Shandong province [22]. A recent nationwide etiological investigation of HGA indicated that a total of 46 confirmed and 16 probable HGA cases were recorded from 2009 to 2010, and these cases were broadly distributed in Hebei, Shandong, and Henan provinces and in Beijing and Tianjin cities [21]. In this report, 41.2% of patients were diagnosed with multiple organ dysfunction syndrome

Table 4: Analysis of the presumed risk of *E. chaffeensis* and *A. phagocytophilum* among rural residents.

Variables	*E. chaffeensis* Number (%) of residents						*A. phagocytophilum* Number (%) of residents					
	Total cohort $N = 7322$	IFA positive $N = 719$	IFA negative $N = 6603$	OR	95% CI	P value	Total cohort $N = 7321$	IFA positive $N = 1331$	IFA negative $N = 5990$	OR	95% CI	P value
Living in plains areas	5271 (72.0)	506 (70.4)	4765 (72.2)	0.92	0.8–1.1	0.3	5271 (72.0)	945 (71.0)	4326 (72.2)	0.96	0.8–1.1	0.6
Living in hilly areas	2051 (28.0)	213 (29.6)	1545 (27.8)				2050 (28.0)	386 (29.0)	1664 (27.8)			
Planting crops	4174 (57.0)	396 (55.1)	3778 (57.2)	0.95[a]	0.8–1.1[a]	0.6[a]	4173 (57.0)	720 (54.1)	3452 (57.6)	0.8[a]	0.7–0.9[a]	0.006[a]
Planting fruit trees	1684 (23.0)	168 (23.4)	1516 (23.0)	0.90[b]	0.7–1.1[b]	0.2[b]	1683 (23.0)	342 (25.7)	1340 (22.4)	0.9[b]	0.8–1.1[b]	0.4[b]
Nonplanting	1464 (20.0)	155 (21.6)	1309 (19.8)	0.94[c]	0.7–1.2[c]	0.6[c]	1464 (20.0)	266 (20.0)	1198 (20.0)	1.1[c]	1.0–1.4[c]	0.1[c]
Contact with farm animals	6956 (95.0)	682 (94.8)	6274 (95.0)	0.97	0.71–1.4	0.9	6955 (95.0)	1311 (98.5)	5644 (94.2)	4.0	2.6–6.3	<0.0001
Tick bite in last 24 months	879 (12.0)	90 (12.5)	789 (11.9)	1.1	0.8–1.3	0.7	879 (12.0)	172 (12.9)	707 (11.8)	1.1	0.9–1.3	0.3
Fever in last 24 months	439 (6.0)	46 (6.4)	393 (6.0)	0.006	0.004–0.008	<0.0001	439 (6.0)	85 (6.4)	354 (5.9)	0.04	0.03–0.04	<0.0001
Working time >3 hours per day	5415 (74.0)	535 (74.4)	4882 (74.0)	1.0	0.9–1.2	0.8	5414 (74.0)	985 (74.0)	4429 (73.9)	1.0	0.9–1.2	1.0
Service time >2 years	5418 (74.0)	560 (77.9)	4858 (73.6)	1.3	1.1–1.5	0.01	5417 (74.0)	1095 (82.3)	4321 (72.1)	1.8	1.5–2.1	<0.0001

[a] Planting crops versus planting fruit trees.

[b] Planting crops versus nonplanting.

[c] Planting fruit trees versus nonplanting.

Table 5: Comparison of distribution of *E. chaffeensis* and *A. phagocytophilum* among areas in the study.

Area	Sites	*E. chaffeensis*		*A. phagocytophilum*		OR (95% CI)	*P* value
		Number positives/total number.	Seropositivity rate% (95% CI)	Number positives/total number	Seropositivity rate % (95% CI)		
Rural	Anhui	33/596	5.5 (3.8–7.3)	201/596	33.7 (39.5–40.2)	0.12 (0.08–0.2)	<0.0001
	Hainan	379/850	44.6 (41.3–47.9)	337/852	39.2 (35.9–42.5)	1.2 (1.0–1.5)	0.02
	Henan	47/737	6.4 (4.6–8.2)	66/737	9.0 (6.9–11.1)	0.69 (0.5–1.0)	0.06
	Jiangsu	41/2474	1.5 (1.0–2.0)	274/2473	10.0 (8.9–11.1)	0.14 (0.1–0.19)	<0.0001
	Zhejiang	7/579	1.2 (0.3–2.1)	3/579	0.5 (0.1–1.1)	2.3 (0.6–9.1)	0.2
	Yunnan	1/326	0.3 (0.29–0.9)	1/327	0.3 (0.29–0.9)	1.0 (0.1–16.1)	1.0
	Xinjiang	19/44	43.2 (28.6–57.8)	6/44	13.6 (3.5–23.7)	4.8 (1.7–13.7)	0.002
	Tianjin	83/882	9.4 (7.5–11.3)	367/879	41.8 (38.5–45.1)	0.14 (0.1–0.2)	<0.0001
	Beijing	109/561	19.4 (9.9–28.9)	79/561	14.1 (11.2–17.0)	1.5 (1.1–2.0)	0.02
	Total	**719/7322**	**9.8 (9.1–10.5)**	**747/3490**	**15.4 (14.6–16.2)**	**0.4 (0.4–0.5)**	**<0.0001**
Urban	Jilin	16/235	6.3 (3.3–9.3)	4/253	1.6 (0.1–3.1)	4.2 (1.4–12.8)	0.006
	Heilongjiang	4/566	0.7 (0–1.4)	8/566	1.4 (0.4–2.4)	0.5 (0.1–1.7)	0.3
	Total	**20/819**	**2.4 (1.4–3.4)**	**12/819**	**1.5 (0.7–2.3)**	**1.7 (0.8–3.5)**	**0.2**

(MODS), and the fatality rate was as high as 8.1%. Four human HGA isolates were obtained from patients, and one tick isolate was obtained from the *Haemaphysalis longicornis* parasite on the bodies of the domestic animals owned by these patients. Among these HGA isolates, two human isolates and one tick isolate from Shandong Peninsula, where all of the patients exhibited severe clinical manifestations, were identical to each other, based on an analysis of 16S rRNA and the *ankA* and *msp2* genes but were different in sequence from sequences identified in other parts of the world. Moreover, the 16S rRNA gene of the five Chinese HGA isolates showed 99% identity with the strain China-C-Tt (GQ 412339) in *Tscherskia triton*, the strain China-C-Y (GQ412338) in domestic sheep, and the strain China-C-Aa (GQ 412337) in *Apodemus agrarius* from the northeastern areas of China [4]. Here, we had to address that some genetic groups of *A. phagocytophilum* identified in China were related to human infection, while others might be only associated with sylvatic or domestic animals but not able to infect humans. However, these nonpathogenic Anaplasma such as *A. platys*, *A. ovis,* and *A. bovis* might inflate the seropositivity in the study. Similarly, some isolates of *E. chaffeensis* and other related organisms such as *E. canis* that may not be pathogenic to humans might elicit anti-*Ehrlichia* antibodies. In addition, the genetic diversity of the key immunogenicity MSP2 proteins between Chinese HGA isolates and USA HGA isolates mentioned above might impact the seropositivity in the study.

In China, the free-range feeding of animals is a major part of livestock production, in contrast to livestock production in modern developed countries. Animals roam hills for feeding during daylight and return at sundown. In such a situation, animals can return with many ticks from wild fields. Moreover, most farm families own 2-3 dogs for guarding their animals and belongings, and these dogs also roam freely in and out of yards. Therefore, it is not surprise that contacting with domestic animals is regarded as a main exposure risk of *A. phagocytophilum*. A national investigation on domestic animals in 10 provinces/cities of China indicated that the PCR-positive rates for *A. phagocytophilum* 16S rRNA were 26.7% for goats, 23.4% for cattle, and 10.9% for dogs [5].

Phylogenetic analyses of the 16S rRNA genes identified in these animals and ticks indicated that the dominant group, which consisted of 59.2% of the sequences from the domestic animals and 67% of the sequences from the ticks, was grouped with the sequences of the two human Chinese HGA isolates from Shandong province, mentioned above [21]. Moreover, the sequences (EF211110) identified in a patient with a nosocomial infection in Anhui in 2006 and the sequences (EU982709) from a patient in Yiyuan county, Shandong province, in 2007, were classified into the group [5].

The geographic distribution of *A. phagocytophilum* was mainly in Hainan, Anhui, Tianjin, and Beijing (Table 2). Hainan province is the second largest island in China, and its climate characteristics are advantageous to vector-borne rickettsial diseases. A retrospective field investigation on rickettsioses in Chengmai county, Hainan province, revealed that 5% of farmers' houses contained ticks, and a tick blood trail could be observed on the walls of the houses. Additionally, 15% of local children (40/270) had a typical eschar or rash on their bodies. A total of 11 isolates of rickettsiae were isolated from 23 febrile patients, and seven isolates of rickettsiae were isolated from wild *Rattus flavescent,* which were captured in local areas. The field investigation indicated that the seroprevalence was 6.3% (51/812) for *A. phagocytophilum*, 12.5% (101/812) for *E. chaffeensis,* and 37.5% (305/812) for spotted fever rickettsia in the local population [23]. PCR amplification of the 16S rRNA genes of rickettsiae in tick samples indicated that the positive rates were 23.3% (7/30 sample pools) for *R. sanguineus*, 6.9% (2/29 sample pools) for *H. doenitzi,* and 12.7% (9/71 sample pools) for *R. microplus* [23]. Anhui province is located in the middle of the eastern

part of China and an unusual outbreak of anaplasmosis occurred in a hospital in this province in 2006. In a previous study, we focused on investigating Guangde county, where the index patient from the nosocomial transmission of HGA lived; Huaiyuan county; and Mingguang city. The results demonstrated that the average seropositivity rate of *A. phagocytophilum* among rural residents was 33.7% (201/596) [24]. Of the three sites investigated, Guangde county had the highest seroprevalence (76.5%, 153/200) and Huaiyuan county had the lowest (10.4%, 26/249). Tianjin CDC conducted a continuous seroepidemiological investigation of *A. phagocytophilum* among people at high risk of exposure (animal breeders, hand-milking workers, animal birth assistants, and cleaners) from 2006 to 2009. The results indicated that the total seroprevalence of *A. phagocytophilum* was 8.8% in 2006 [12], 44.4% (169/381) in 2007, 42.9% (110/256) in 2008, and 59.2% (147/249) in 2009 [25]. We proposed that this dramatic change might be associated with the different occupational structure of the participants each year (from ordinary rural residents in 2006 to people at high risk of exposure from 2007 to 2009). Another reason for such changes might be related to the different natural geographic characteristics of the sites investigated each year (from high-altitude areas in 2006 to low-altitude areas from 2007 to 2009). A recent etiological investigation of HGA in Tianjin revealed four laboratory-probable HGA cases among 24 undiagnosed febrile patients [21].

Because human monocytic ehrlichiosis (HME) is an emerging tick-borne infectious disease, seroepidemiological information about HME is limited in China. This study serologically demonstrated a higher prevalence rate of *E. chaffeensis* among farm residents in Hainan (44.6%, 95% CI 41.3–47.9%), Xinjiang (43.2%, 95% CI 28.6–57.8%), and Beijing (19.4%, 95% CI 9.9–28.9%) (Table 2). Additionally, Hainan and Xinjiang provinces shared a higher coprevalence of *E. chaffeensis* and *A. phagocytophilum*. Xinjiang province, located in central Asia and neighboring Mongolia, Russia, and Kazakhstan, is famous in China for numerous tick species. Many studies have identified *E. chaffeensis* in ticks collected from Xinjiang province [26] and other parts of China [27] and from countries neighboring China [28].

The sex distribution varied between the areas investigated, but male farm residents are at a higher risk of infection with *A. phagocytophilum* than females when considering the total population studied, although no differences were observed between males and females for *E. chaffeensis*. An analysis of the age distribution indicated that the highest seroprevalence of *E. chaffeensis* and *A. phagocytophilum* was identified in the 20-to-29-year-old and 30-to-39-year-old groups (Table 3). We presumed that this phenomenon was due to more outside activities among these young people.

Our current findings and previous data provide strong evidence that *A. phagocytophilum* and *E. chaffeensis* exist in China [4, 5, 12–19, 21–25, 29, 30] and in other Asian countries [28, 31, 32]. Not only rural residents but also urban residents were at a substantially increased risk of exposure to these pathogens. Although the seroprevalence of urban residents were lower than that of rural residents, we had to address that the results in the study might be affected by the geographic characterizes [33, 34]. The urban samples were collected from Daqing city of Heilongjiang and Yanbian city of Jilin provinces, which located in the northeast of China and shared cooler climate, especially Daqing areas characterized with sterile saline and alkali soil with rare vegetative cover was not conductive to the breeding of ticks.

More and more researches indicated that different climate and other environmental conditions determined the distribution of tick-borne pathogens [33, 34]. China is one of the largest countries in the world and had complex ecological system and each province had different geographic and climatic characteristics. It is reported that Xinjiang province alone had more than 50 species of ticks [35].

For analysis of proposal transmission vector of *A. phagocytophilum* in China, an epidemiological field investigation found that there were many ticks on the bodies of animals, and at least six species of ticks were identified. PCR amplification of *A. phagocytophilum* 16S rRNA showed that the positive rates were 58.3% for *Dermacentor silvarum*, 43.9% for *Haemaphysalis longicornis*, 12.5% for *Ixodes persulcatus*, 7.5% for *Rhipicephalus microplus,* and 5.2% for *Rhipicephalus sanguineus* [5]. More molecular investigations indicated that *Ixodes persulcatus, Dermacentor silvarum, Haemaphysalis concinna, Haemaphysalis longicornis, Rhipicephalus microplus, Rhipicephalus sanguineus*, and *Dermacentor nuttalli* might transmission of *A. phagocytophilum* in China [5, 23, 27, 29, 36]. Notably, such more species of ticks carrying *A. phagocytophilum* organisms in China were different from those found in USA. Whether this phenomenon was associated with some genetic variations of Chinese HGA isolates mentioned above remained to be further explored.

Additionally, some recent researches showed that several kinds of domestic animals including dog, goats, sheep, cattle, horse, wild rabbit, and some small wild rodents including *Apodemus agrarius*, *Tamias sibiricus, Apodemus peninsulae, Apodemus sylvaticus, Clethrionomys rufocanus, Niviventer confucianus, Niviventer coxingi, Niviventer anderson, Niviventer eha, Rattus nitidus, Al XII temus latronum, Apodemus chevrien, Apodemus draco, Eothenomys eleusis, Eothenomys custos, Eothenomys cachinus, Tamiops swinhoei,* and *Naaillus gracilis* were proposed to be the reservoir hosts of *A. phagocytophilum* [18, 19, 23, 24, 27–29, 37, 38].

Although the epidemiology data of *E. chaffeensis* is very limited in China, a case of coinfection with *A. phagocytophilum* and *E. chaffeensis* was documented in Shandong province [22]. Furthermore, 3.84% of coinfection rates of *A. phagocytophilum* and *E. chaffeensis* were found in *Gerbillus* sp. collected from Xinjiang province, which is the biggest desert in China where the *Gerbillus* spp. are the dominant rat [39]. Previously studies and recent investigations revealed that several species of hard ticks including *Ixodes persulcatus, Dermacentor silvarum,* and *Dermacentor nuttalli* [40] and *Rhipicephalus microplus* [41] might be associated with the transmission of *E. chaffeensis* in China.

As emerging tick born infection diseases, the distribution of vector and hosts of the *A. phagocytophilum* and *E. chaffeensis* and their role in the transmission of these pathogens are limited. Such information is urgently needed to be further

investigated to better understand the pathogenesis of these pathogens, especially the relationship between the generalized susceptibility of vectors and reservoirs, the severity of the disease's clinical manifestations, and the genetic variation of Chinese HGA isolates in China.

In summary, the tick born rickettsial diseases caused by *A. phagocytophilum* and *E. chaffeensis* have become a serious threat to human and animal health. Several measures should be taken to minimize the likelihood of becoming infected with these two zoonotic rickettsiae from direct contact with farm animals, especially among individuals who work with livestock. The health management department should emphasize the differentiation of these zoonotic infectious diseases from other febrile diseases, especially for the prevention and control of nosocomial human-to-human transmission, during treatment.

Conflict of Interests

No competing financial interests exist.

Authors' Contribution

Lijuan Zhang, Hong Liu, Bianli Xu, Zhilun Zhang, Yuming Jin, Weiming Li, Qunying Lu, Liang Li, Litao Chang, Xiuchun Zhang, Desheng Fan, Minghua Cao, Manli Bao, Ying Zhang, and Zengzhi Guan contributed equally to the epidemiological field investigation. Xueqin Cheng, Lina Tian, Shiwen Wang, Huilan Yu, Qiang Yu, Yong Wang, Yonggen Zhang, Xiaoyan Tang, Jieying Yin, Shijun Lao, Bin Wu, Juan Li, Weilong Li, Qiyi Xu, Yonglin Shi, and Fang Huang contributed equally to the laboratory assays in this study.

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