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ABOUT COVER

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ORIGINAL ARTICLE

Basic Study

Three-dimensional models of antigens with serodiagnostic potential for leprosy: An *in silico* study

Bianca Luiza Melo de Assis, Rafaela Viana Vieira, Ian Theodoro Rudenco Gomes Palma, Matheus Bertolini Coutinho, Juliana de Moura, Gabrielle Caroline Peiter, Kádima Nayara Teixeira

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Abstract

BACKGROUND

Leprosy is a disease caused by *Mycobacterium leprae (M. leprae)*, an intracellular pathogen that has tropism and affects skin and nervous system cells. The disease has two forms of presentation: Paucibacillary and multibacillary, with different clinical and immunological manifestations. Unlike what occurs in the multibacillary form , the diagnostic tests for the paucibacillary form are nonspecific and not very sensitive, allowing the existence of infected individuals without treatment, which contributes to the spread of the pathogen in the population. To mitigate this contamination, more sensitive diagnostic tests capable of detecting paucibacillary patients are needed.

AIM

To predict the three-dimensional structure models of *M. leprae* antigens with serodiagnostic potential for leprosy.

METHODS

In this *in silico* study, satisfactory templates were selected in the Protein Data Bank (PDB) using Basic Local Alignment Search Tool to predict the structural templates of ML2038, ML0286, ML0050, and 85B antigens by comparative modeling. The templates were selected according to general criteria such as sequence identity, coverage, X-ray resolution, Global Model Quality Estimate value and phylo-

genetic relationship; Clustal X 2.1 software was used in this analysis. Molecular modeling was completed using the software Modeller 9v13. Visualization of the models was made using ViewerLite 4.2 and PyMol software, and analysis of the quality of the predicted models was performed using the QMEAN score and *Z*-score. Finally, the three-dimensional moels were validated using the MolProbity and Verify 3D platforms.

RESULTS

The three-dimensional structure models of ML2038, ML0286, ML0050, and 85B antigens of *M. leprae* were predicted using the templates PDB: 3UOI (90.51% identity), PDB: 3EKL (87.46% identity), PDB: 3FAV (40.00% identity), and PDB: 1F0N (85.21% identity), respectively. The QMEAN and Z-score values indicated the good quality of the structure models. These data refer to the monomeric units of antigens, since some of these antigens have quaternary structure. The validation of the models was performed with the final three-dimensional structure - monomer (ML0050 and 85B antigens) and quaternary structures (ML2038 and ML0286). The majority of amino acid residues were observed in favorable and allowed regions in the Ramachandran plot, indicating correct positioning of the side chain and absence of steric impediment. The MolProbity score value and Verify 3D results of all models indicated a satisfactory prediction.

CONCLUSION

The polarized immune response against *M. leprae* creates a problem in leprosy detection. The selection of immunodominant epitopes is essential for the development of more sensitive serodiagnostic tests, for this it is important to know the three-dimensional structure of the antigens, which can be predicted with bioinformatics tools.

Key Words: Antigens; Leprosy diagnosis; *Mycobacterium leprae*; Molecular modelling; Serological test; *In silico* study

Core Tip: Leprosy is a disease with high clinical and epidemiological impact, because it causes irreversible and disfiguring sequelae and has a high incidence in endemic countries. Its variability of manifestations, with different immune responses and the difficulty of cultivating *Mycobacterium leprae (M. leprae)* in the laboratory, makes it difficult to develop sensitive and specific tests for the diagnosis of the disease, thus emphasizing the importance of *in silico* studies to solve this problem. In this sense, this study aimed to obtain three-dimensional models of *M. leprae* antigens, which have stood out in previous studies as candidates for the serological diagnosis of leprosy.

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INTRODUCTION

Leprosy is a chronic transmissible and infectious disease caused by the bacillus *Mycobacterium leprae (M. leprae)*, which persists as a major public health problem in Brazil. The etiological agent of this disease mainly affects the skin, peripheral nerves, and eyes, presents slow evolution, and causes deformity and physical disability, when not correctly diagnosed and treated[1].

According to the World Health Organization (WHO)[1], in 2019, there were 202 185 cases of *M. leprae* infection worldwide, 93% of which were reported in the Americas. In addition, according to the WHO, Brazil contributed 27 864 new cases of the disease in 2019, placing it in second place among the countries with the highest number of leprosy cases, behind only India.

Clinically, leprosy may manifest in two different forms, which differ mainly by the immune response developed by the host against the pathogen. The current classification of leprosy came into being in 1982, from the WHO Committee, which proposed a simplified and operational classification of paucibacillary and multibacillary individuals based on the likely relationship between clinical form and smear [2].

The manifestations of leprosy are distinguished both by clinical presentations and characteristics of the immune response. Such presentations are considered antagonistic, which guide the understanding of the pattern of dual response observed in Th1 and Th2 Lymphocytes. In leprosy, the paucibacillary form is characterized by the Th1 response, which confers some resistance of the organism to the pathogen, thereby this is a milder form of the infection; the multibacillary form, a more severe infection, is characterized by the development of the Th2 pattern, which does not have an effective mechanism against the pathogen[3].

Due to the variety of clinical manifestations of leprosy, the WHO[4] recommends that the diagnosis be based on loss of sensitivity of a hypopigmented or reddish skin area, visualization of thickening or augmentation of a peripheral nerve, accompanied by weakness of muscle tissue supplied by the nerve, which can only be observed or visualization of acid-alcohol fast bacilli by means of the intradermal sample smear technique. In addition, enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) tests can be used to complement the diagnosis, especially in suspected multibacillary form. Thus, it is noted that the recognition of the disease can be based both on factors arising from the clinical examination (anamnesis and physical examination) and complementary tests, requiring the expertise of the professionals who will perform and interpret the procedure, especially with regard to histological techniques, and reliability of the tests available.

However, the tests used to diagnose the multibacillary form, such as serological tests by ELISA have become ineffective since the paucibacillary patient presents low titers of antibodies. Due to the high production of pro-inflammatory cytokines, there is little bacterial proliferation, which makes it difficult to find bacilli at the site of the lesion by the intradermal smear technique. Consequently, when applied to patients with paucibacillary leprosy, the recommended tests have low sensitivity and specificity, which may lead to false-negative results, contributing to the spread of the disease[5]. Thus, tests aimed at improving these parameters and diagnosing both severe and mild forms of leprosy are necessary.

To support the development of diagnostic tests with greater sensitivity for any type of clinical manifestation of leprosy, Santana[6] mapped epitopes of *M. leprae*, constructed by the spot synthesis technique, using serum from leprosy patients, based on a previous study[7]. Of 12 selected proteins, seven were promising candidates for immunodominant antigens. Bioinformatics tools were used to verify molecular mass, isoelectric point, hydrophobicity, and acid-basic characteristics[5]; however, none of the proteins has experimentally resolved three-dimensional structure, which hinders a complete biochemical characterization.

The three-dimensional structure of a protein is fundamental to determine the selection of the best candidates in order to obtain a functional epitope given that the peptides hidden in the protein core are not of interest, since the antibodies do not have access to this region. In turn, antigenic determinants actively participate in antigen-antibody binding, which, when established, generate conformational and structural changes, which are important in the process of antigenic neutralization [6].

In this study, the three-dimensional structures of *M. leprae* antigens selected by Santana[6] were predicted with bioinformatics tools in order to provide support for the elaboration of possible serological diagnostic tests for both multibacillary and paucibacillary clinical forms of leprosy.

MATERIALS AND METHODS

Template selection

The FASTA sequences of antigens ML2038, ML0286, ML0050, and 85B (GenBank WP_010908683.1, WP_010907650, WP_010907488.1, and CAA43269.1, respectively) available at The National Center for Biotechnology Information (ncbi.nlm.nih.gov) were used to select templates for molecular modeling at Protein Data Bank (PDB) (rcsb.org), through local alignment using the BLAST (Basic Local Alignment Search Tool)[8], aiming to find templates with (1) three-dimensional structures resolved; (2) high sequence identity and coverage values as much as possible; (3) high X-ray resolution; and (4) phylogenetic proximity to the target proteins. The choice of templates also took into account the Global Model Quality Estimate (GMQE) value, which predicts the overall quality of the model and ranges from 0 to 1, with higher numbers indicating higher quality. The identity between primary sequence and template, as well as sequence coverage, were verified through global alignment using ClustalX 2.1 software[9].

Molecular modeling and validation

The antigens whose template was satisfactory were modeled by the comparative modeling methodology using the Modeller 9v13[10] software. The selection of the models released by Modeller was performed using the Discrete Optimized Protein Energy (DOPE) method, which predicts the lowest energy models, therefore the most stable ones. The selected three-dimensional models were visualized and analyzed with the ViewerLite 4.2 (Accelrys Inc.) and PyMol (Schrödinger Inc.) software, and the quality was verified using the QMEAN score[11] and *Z*-score. The validation of the models was performed using the MolProbity[12] and Verify 3D platforms[13].

RESULTS

Selected templates

Templates were selected for target proteins ML2038, ML0286, ML0050, and 85B antigens - 3UOI (90.51% identity and 1.90 Å resolution), 3EKL (87.46% identity and resolution of 1.51 Å), 3FAV (40.00% identity and 2.15 Å resolution), and 1F0N (85.21% identity and 1.80 Å resolution), respectively. In addition, a 100.00% coverage was obtained for ML2038 and ML0050 proteins, 99.00% for ML0286, and 87.00% for 85B antigen (Table 1).

Comparative molecular modeling

The primary sequences of the target proteins were submitted to comparative molecular modeling using the Modeller 9v13 software which generated the three-dimensional structures as shown in Figures 1 and 2. The comparative modeling of ML0050 was performed with a pipeline designed for low identity templates[14]. QMEAN scores for the structural models were 0.13 (ML2038), 0.56 (ML0286), -0.62 (ML0050), and -0.39 (85B antigen); this score typically ranges from 0 to 4.0, and values closer to 0 indicate a good model. QMEAN score refers to the monomeric subunits that were modeled; however, the ML2038 and ML0286 antigens are quaternary proteins. ML0050 can dimerize, but functionally, the protein is a monomer. Validation of the predicted structural models was performed with the functional structure of each antigen, *i.e.*, ML0050 and 85B antigens in monomeric form and ML2038 and ML0286 in quaternary form. The *Z*-scores of all structure models were > 0.5 but < 1.0. Figure 3 shows the *Z*-score plot which indicates the quality of the three-dimensional models obtained.

Validation of three-dimensional models

Validation was performed using MolProbity software; parameters related to the geometry of the angles of the chemical groups of amino acid residues were evaluated in the models obtained by molecular modeling. The MolProbity score, outlier residues, and the percentage of residues in favorable regions in the Ramachandran plot were considered (Table 2). On Verify3D software, 3D/1D score for all models was more than 80%; it means that the models have good quality.

DISCUSSION

The leprosy infection caused by the *M. leprae* induces marked lesions on the skin and peripheral nerves and, when not properly treated, leads to irreversible and disfiguring sequelae in the patient, such as destruction of cartilage and leonine facies[15]. In addition, the great social stigma related to the disease is added, which contributes even more to the worsening of the psychological issue of these patients[16]. It is also worth noting the high prevalence of this pathology in the Americas, especially in Brazil, which in 2017 was responsible for 92.3% of the cases reported on the continent, evidencing the clinical and epidemiological importance of the pathology for the country[15]. Thus, there is a need for tests to identify the disease early, to prevent its spread and enable the early institution of the recommended therapy, avoiding or reducing the associated sequelae.

However, there are variations in the clinical manifestation of leprosy. In the multibacillary/lepromatous form, there is a Th2 response with great dissemination of the pathogen in the organism and an increase in the number of lesions, while in the paucibacillary/tuberculoid form, there is a predominance of the Th1 response, better fight against the pathogen and fewer lesions, which hinders the development of specific and sensitive diagnostic tests for its identification.

Moreover, as there is little presence of initial signs and symptoms, and there may be asymptomatic presentation and similarity with other diseases, making the pure clinical diagnosis of leprosy difficult[5, 15].

In addition, there is also the difficulty of growing *M. leprae* in artificial or cellular media, constituting one of the major obstacles to leprosy research. The available forms of Hansen's bacillus cultivation are inoculation in captive armadillo and in the paw pads of immunocompetent mice (Shepard method) and immunodeficient mice (Prabhakaran method). The use of armadillos for leprosy research is limited by the difficult management of the animal, while research with different types of mice is extensive, taking up to 8 mo for adequate immunological manifestation[17-19].

Therefore, *in silico* studies aimed at the identification and mapping of B-cell epitopes[20] which predict the type of interaction and the affinity energy between the epitope and the Fab region of the antibodies, are valid for the development of serological tests for the diagnosis of leprosy. This kind of study allows the recognition of immunodominant protein domains and their selection for the preparation of immunohistochemical tests, as well as for the manufacture of synthetic peptides, which are less expensive and easy to manipulate[15,21]. Thus, there is a decreased possibility of cross-reactivity with antigens from other pathogens, increasing the specificity and sensitivity of the test.

This study aimed to predict the three-dimensional structure models of *M. leprae* antigens that, according to previous studies[6,7], have the potential to be used in serodiagnostic tests for both paucibacillary and multibacillary leprosy. One of these antigens, ML2038, is the Bacterioferritin protein or Major

Table 1 Selected templates for comparative modeling

Antigen	Template (PDB)	Organism	Identity (%)	X-ray resolution (Å)	GMQE
ML2038	3UOI	*M. tuberculosis*	90.51	1.90	0.96
ML0050	3FAV	*M. tuberculosis*	40.00	2.15	0.74
ML0286	3EKL	*M. tuberculosis*	87.46	1.51	0.96
85B Ag[a]	1F0N	*M. tuberculosis*	85.21	1.80	0.81

[a]85B antigen; GMQE: Global Model Quality Estimate; PDB: Protein Data Bank.

Table 2 Validation of three-dimensional models of the *Mycobacterium leprae* antigens

Antigen	Identification	Functional structure	MolProbity Score	Ramachandran plot[a] (%)	Outliers (%)
ML2038	Bacterioferritin	Homopolymer	1.31	98.48	0.08
ML0050	ESAT-6-like protein esxB	Monomer	0.77	98.61	0.00
ML0286	Fructose-bisphosphate aldolase	Homotetramer	1.32	96.47	0.59
85B Ag	85B antigen	Monomer	1.85	93.95	0.00

[a]Residues in favorable regions.

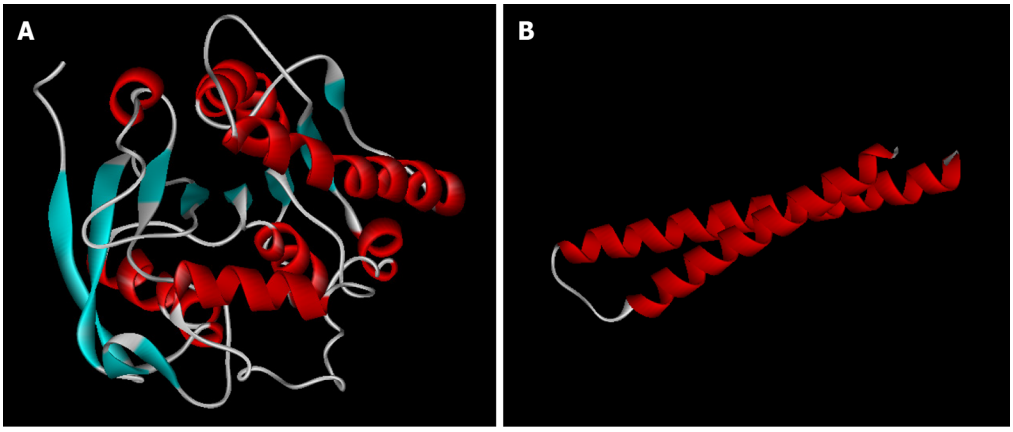

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Figure 1 Three-dimensional structure models of *Mycobacterium leprae* antigens predicted *in silico*. A: 85B antigen; B: ML0050. Blue: β-sheets; red: α-helices; white: Loops.

Membrane Protein II (MMP-II), which is encoded by the *BFRA* gene. This protein is present in the cell membrane of *M. leprae* and is responsible for iron storage in restricted situations. In addition, MMP-II has a high identity with its homologue found in *M. tuberculosis* and has the ability to activate CD4+ and CD8+ T cells[6,15,22].

Due to the high primary sequence identity of the template (91.14%), it was possible to predict the three-dimensional structure of the ML2038 antigen by comparative modeling. The quality of the model was evaluated by the QMEAN score, a parameter used to analyze the structure obtained and compare it with others already known, in relation to physicochemical and evolutionary characteristics, in order to identify problematic regions for subsequent correction[23,24].

The QMEAN score of the three-dimensional structure of ML2038 was 0.56. This value was applied to the *Z*-score plot, a tool that combines the QMEAN score with those of proteins with structure deposited in the PDB, demonstrating that the model obtained by the study is similar to what is expected for native proteins with molecular mass similarity [24,25,26].

Using these data, it was possible to correct the errors found in the configuration of amino acid residues using tools of the MolProbity platform, for subsequent validation of the three-dimensional models. Thus, correcting the outliers, the MolProbity score equal to 1.31 was obtained. This score evaluates the log of the clashscore, percentage of amino acids in unfavorable regions in the

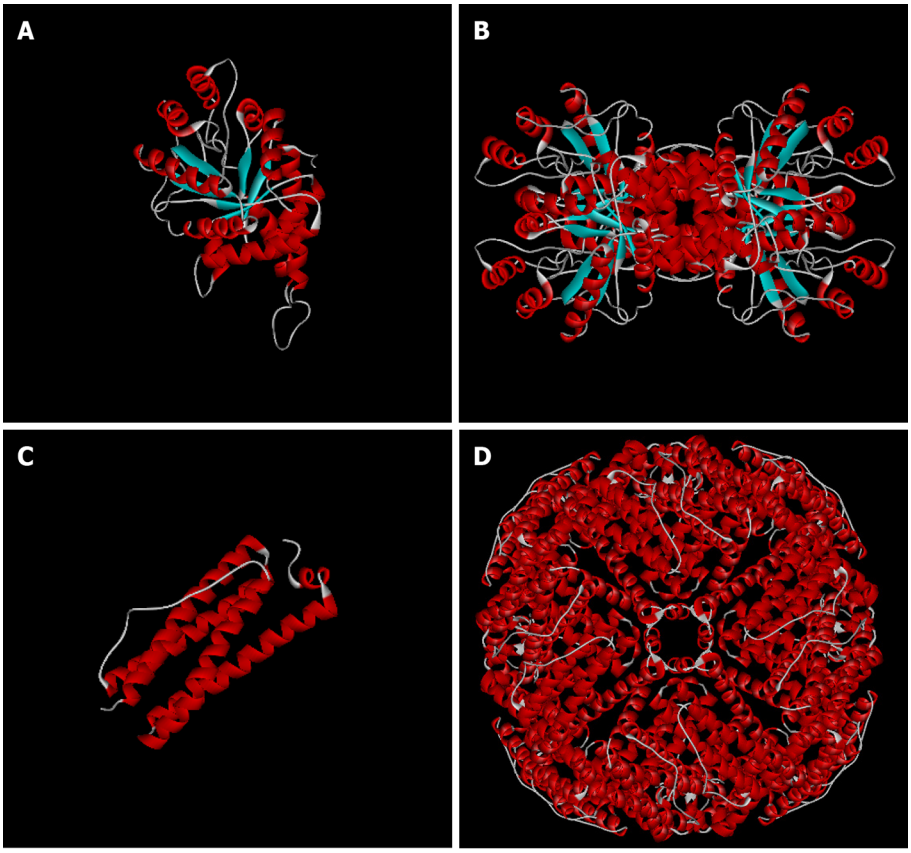

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Figure 2 Three-dimensional models of the monomers and quaternary structures of *Mycobacterium leprae* antigens predicted *in silico*. A: ML0286 monomer; B: ML 0286 quaternary structure (tetramer); C: ML2038 monomer; D: ML2038 quaternary structure (24 subunits). Blue: β-sheets; red: α-helices; white: Loops.

Ramachandran chart, and bad rotations of the lateral chains; MolProbity score values lower than the crystallographic resolution of the template (1.90 Å) indicate three-dimensional model quality[27].

The results of the Ramachandran plot also contribute to the reliability of the three-dimensional organization of the ML2038 model, which predicts that 98.48% of residues are in permitted regions, indicating the stability of the predicted structure.

In a study conducted by Maeda *et al*[22], in which sera from paucibacillary and multibacillary patients, sera from patients with tuberculosis, and sera from healthy individuals vaccinated with Bacillus of Calmette-Guérin (BCG) were tested, and a better sensitivity of the serological test was observed for individuals with the multibacillary form (82.4%, 95.0% confidence interval [CI]: 71.8-90.3) than those with the paucibacillary form of leprosy (39.0%, 95.0%CI: 28.8-50.1), when screened for antibodies against MMP-II. Furthermore, healthy individuals or individuals with tuberculosis showed high specificity, indicating the low influence of homologous *M. bovis* antigens, and ancestry between *M. leprae* and *M. tuberculosis* on the diagnostic test result.

Similar results were obtained in a study by Kai *et al*[28], in which paucibacillary and multibacillary patients tested positive, 47.6% and 85.1%, respectively when investigated for antibodies against ML2038, and 20.2% and 57.0% when investigated for antibodies against PGL-I (Phenolic Glycolipid-I), one of the first isolated *M. leprae*-specific antigens currently instituted in serological tests for leprosy[28,29].

In this way, Tsukamoto *et al*[30] conducted clinical research associated with the search for anti-MMP-II and anti-MMP-I antibodies. This association allowed the increase in the sensitivity of the test and the rescue of patients with false-negative results obtained when researched for antigens purely against ML2038. Thus, it is perceived that the literature converges in relation to the possibility and advantage of the use of Bacterioferritin in the diagnostic test of leprosy patients.

In the face of these studies, Santana[6] points out in a study aimed to identify antigen-antibody recognition for some antigens of the *M. leprae* that the ML2038, as well as ML0286 and ML0050, have no reactivity to the sera from leprosy patients. The author reports that the possible reasons for this non-recognition would be the presence of conformal immunodominant protein domains, which depend on the novel structure of the protein to act as epitopes, and the variability of sera from patients of different ethnicities and regions.

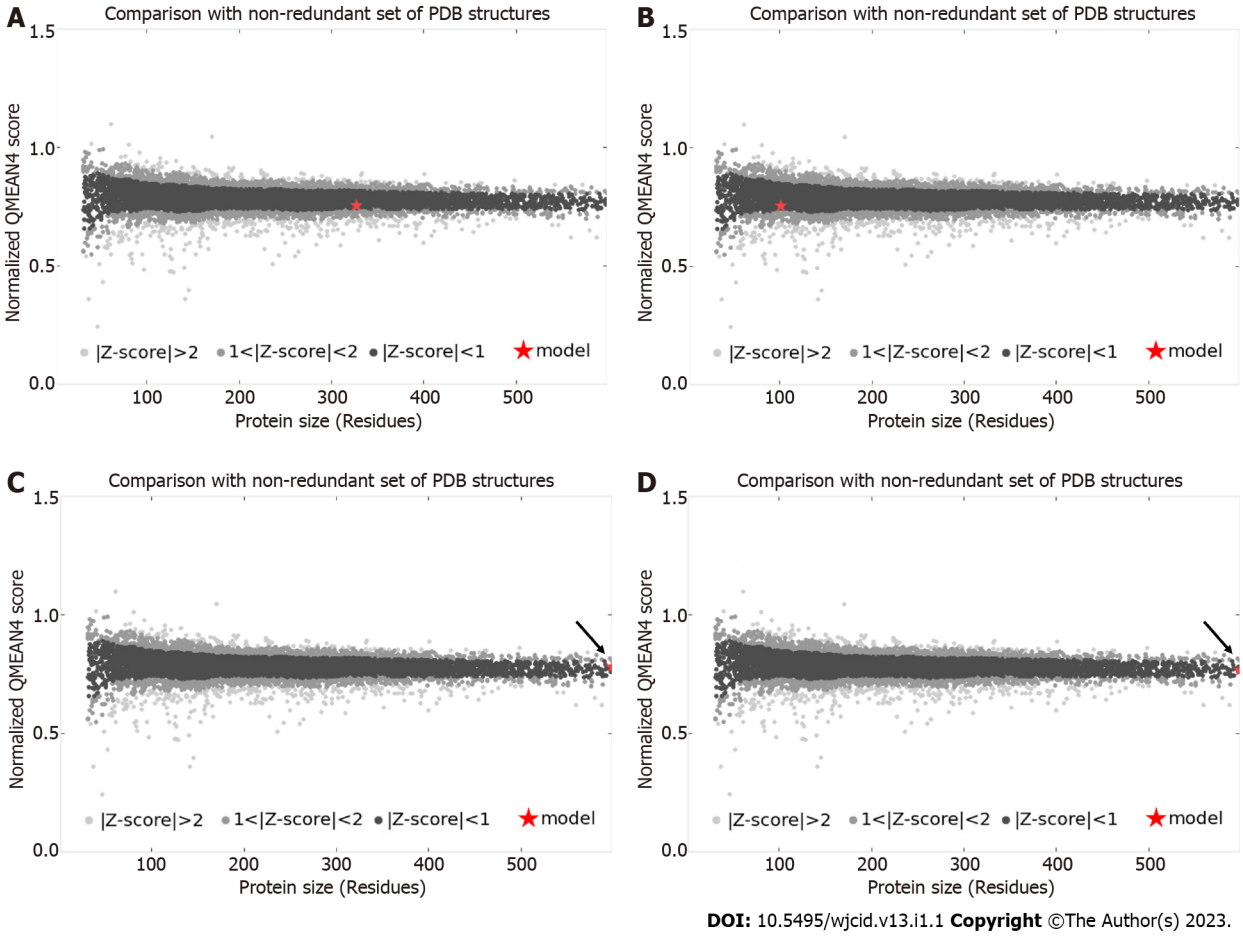

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Figure 3 Z-score plot for three-dimensional structure models of the monomers of *Mycobacterium leprae* antigens predicted *in silico*. A: 85B antigen; B: ML0050; C: ML0286; D: ML2038. The red star indicates the position of the model among the non-redundant structures deposited in the Protein Data Bank. Z-score < 1 indicates higher quality models. Arrow points the star position. PDB: Protein Data Bank.

The ML0286 antigen (Putative Fructose Bisphosphate Aldolase), encoded by the *FBA* gene, is a cytosolic enzyme, soluble and integral to the energy metabolism of *M. leprae*. This enzyme catalyzes the conversion of fructose 1-6-diphosphate into glyceraldehyde-3-phosphate and dihydroxyacetone-phosphate by metabolizing this sugar. Puckett *et al*[31] have suggested that this enzyme regulates the glycolytic and gluconeogenic metabolism of carbohydrates in mycobacteria, indicating participation in aminoglycoside resistance in strains resistant to these antibiotics. The overexpression of Rv0363c, another classification for this aldolase, can maintain the energy supply for resistant *M. leprae* strains, providing adenosine triphosphate (ATP) at the state of antimicrobial resistance. Homology modeling has revealed that ML0286 is a homotetramer, with 96.47% of residues in favorable regions, and is therefore feasible for the elaboration of serological tests for recognition of the protein epitope[7,32,33].

The ML0050 antigen, also called ESAT-6-like protein esxB or culture filtrate protein (CFP-10), acts on the virulence and pathogenicity of *M. leprae* together with ESAT-6 protein, which is secreted by the ESX-1 secretion system and improves the stability of other externalized antigens[15,19,34]. In addition, ML0050 is encoded by the *esxB* gene, having recognized its expression only in the *Mycobacterium* genus [19]. For such evolutionary conservation, it is understandable that there is homology among such proteins in distinct species of mycobacteria, such as between *M. leprae* and *M. tuberculosis*, increasing the chances of cross-reactivity in serological tests.

According to Geluk *et al*[35], although the specificity of ML0050 is high for the *Mycobacterium* genus, the absence of differentiation between both paucibacillary and multibacillary leprosy is a problem with the use of serological tests based on this antigen. Moreover, even if the authors point out the properties of this antigen as a measure of disease progression and effectiveness of leprosy treatment, it is noted that there are obstacles to its use.

The 85B antigen (MPT59), also called ML2028 or diacylglycerol acetyltransferase, is encoded by the *FBPB* gene, having function in cell wall synthesis through its mycolyl-transferase activity[15,36]. Studies have shown that the 85B antigen induces the proliferation and release of high levels of IFN-gamma in T lymphocyte cultures of immunized mice. According to Spencer *et al*[7], ML2028 may represent a biomarker of disease progression because a patient who developed leprosy had the strongest response to the antigen about 19 mo before clinical diagnosis; due to this incubation time and delay in epitope

reactivity, the authors report that this characteristic is dependent on bacterial burden.

The molecular modeling of the 85B antigen was possible due to the similarity between homologous proteins of *M. leprae* and *M. tuberculosis*. Thus, the identity rate between the two reaches 85.21%, with the antigen having 11 beta-sheet and 6 alpha-helix regions. According to Santana[6], the external location of reactive peptides facilitates the action of antibodies in the recognition of the antigenic target.

According to Serafín-López *et al*[37], the specificity of 85B antigen reaches 100%, so that control patients or tuberculosis patients showed no reactivity against the antigen. The results also showed a high degree of antigenicity in leprosy patients, regardless of the clinical classification, and it is therefore an important candidate for serological markers.

CONCLUSION

Regarding the effector mechanisms of the immune system for mycobacteria and other pathogens, it would be feasible that surface antigens and those secreted would be better candidates for serodiagnostic tests, since these would be more exposed to the components of the immune system. However, this placement needs to be better evaluated, at least in relation to *M. leprae*, in which a polarity of response is observed. Therefore, it is important to know the biological functionality of the antigen and also its three-dimensional structure in order to be able to evaluate the localization of epitopes within the structure, and thus try to understand the behavior of the antibody response against *M. leprae*, which is exploited by serological tests. In this context, the prediction of satisfactory structural models is able to collaborate with the development of these tests.

ARTICLE HIGHLIGHTS

Research background

Our research group has been trying to use bioinformatics tools as allies of experimental research in order to corroborate and confirm data. So far the results have been very satisfactory and have saved research time and financial costs.

Research motivation

This study was motivated by our group's previous studies on diagnostic tests for leprosy. Promising and relevant data have been achieved in experimental research with patient serum and have been confirmed by bioinformatics analyses. This study predicting *Mycobacterium leprae (M. leprae)* antigen models is only one step towards future research on the development of more sensitive diagnostic tests for leprosy.

Research objectives

The aim of this study was to provide reliable three-dimensional structure models for the analysis of immunodominant epitopes that can be tested later, in the form of synthetic peptides, as possible candidates for the development of diagnostic tests that can detect patients with paucibacillary leprosy. The structure and location of the epitope within the antigen structure is important to understand the behavior of the humoral response of patients.

Research methods

The methods used were classic methods of bioinformatics, which were well established and had proven reliability. Comparative modeling is the simplest methodology of molecular modeling, which was used in this study due to antigen conditions. Once the input data is well filtered, the results are very satisfactory, which can be proven by the similarity of the structures with the homologous ones.

Research results

The results obtained in this study were considered of good quality; no important parameters, such as steric impediment and lack of stability, were observed. Therefore, the structure models of *M. leprae* antigens are satisfactory for the research of immunodominant epitopes.

Research conclusions

The structural models of *M. leprae* antigens are considered high-quality models by validation parameters and can be used for the mapping of epitope candidates for serodiagnostic tests.

Research perspectives

The research perspective is to continue the study and map the epitopes and evaluate them through experimental studies.

FOOTNOTES

Author contributions: de Moura JF, Peiter GC, and Teixeira KN designed and coordinated the study and interpreted the data; Melo de Assis BL carried out the experiments, and acquired and analyzed the data; Vieira RV, Coutinho BM, and Palma ITRG reviewed the literature and wrote the manuscript; Teixeira KN reviewed the manuscript.

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MINIREVIEWS

Emerging leishmaniasis in southern Himalayas: A mini-review

Ashwani Sharma, Santosh Kumar, Prasan Kumar Panda, Sweety Yadav, Deepjyoti Kalita

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Abstract

Leishmaniasis is a vector-borne parasitic disease affecting millions of people worldwide. However, in the last decade, the number of cases has been reduced from well-documented endemic parts, but sporadic cases have been reported widely from various non-endemic areas, especially from the southern Himalayan zone. This raises concerns about the emergence of new ecological niches. This warrants a critical evaluation of key factors causing this rapid spread and possibly indigenous transmission. This mini-review article is aimed to briefly address the parasite, the vector, and the environmental aspects in the transmission of leishmaniasis in these new foci against a background of worldwide endemic leishmaniasis with a special focus on the southern Himalayan zone. As the lack of knowledge about the causative parasites, vectors, reservoir hosts, atypical presentations, and their management make the problem serious and may lead to the emergence of public health issues. The present works also reviewed the existing information regarding clinical variations, diagnostic methods, treatment, its outcome, and ignite for further research in these aspects of the disease.

Key Words: Anthroponosis; Kala azar sandfly; Sporadic transmission; Southern Himalaya

Core Tip: This mini-review article is aimed to briefly address the parasite, the vector, and the environmental aspects in the transmission of leishmaniasis in these new foci against a background of worldwide endemic leishmaniasis with a special focus on the southern Himalayan zone.

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INTRODUCTION

Leishmaniasis is a vector-borne parasitic disease that exists either as zoonosis (in most endemic parts of the world) or anthroponosis (endemic part of the Indian sub-continent) and is transmitted by S and fly. The latter entity is on the verge of elimination, efficiently with the help of assigned memorandum of understanding by the five most endemic countries: India, Nepal, Bangladesh, Bhutan, and Thailand[1]. But at present, a major challenge is the increasing emergence of new ecological niches having indigenous transmission. Recently World Health Organization (WHO) declares it as a category I disease (emerging and uncontrolled), and the World Health Assembly recognizes it as a major public health concern[2]. Leishmaniasis is a disease of low altitude. It does not occur at an altitude of more than 2000ft (600m)[3]. The Southern Himalayan regions (of countries like Pakistan, India, Nepal, and Bhutan) are considered as non-endemic regions probably because of the non-conducive environment for the growth of its vector, *i.e.*, Sand flies. But as several cases of leishmaniasis have been reported from the sea areas, the above observational facts are being indistinct. Most of these cases were found along with the upstream of Himalayas river belts (like Indus, Ganga, Yamuna, and the Brahmaputra) especially in the western part (Islamabad, Jammu & Kashmir, and Himachal Pradesh), the middle part (Uttarakhand), and the eastern part (Nepal and Bhutan) of Himalayas[3-33].

Here, a mini-/narrative review is done considering the available case reports/case series/observational studies from new emerging areas, regarding leishmaniasis disease profile (epidemiology, microbiology, patho-physiology, clinical variations, diagnostic methods, treatment, and outcome, including the entomological assessment of S and fly). This article also intends to focus on the difference between the disease profile of leishmaniasis in the southern Himalayan belt *vs* the world's endemic areas in a systematic manner.

METHODOLOGY

A mini-review of all published (PubMed/Medline, Embase, Cochrane database, Google Scholar) leishmania cases from the Himalayas regions of Pakistan, India, Nepal, and Bhutan were reviewed and analyzed with prime focus on the disease profiles of the cases reported in the lower Himalayan belt (Figure 1). For distinctive comparison and obtaining good inference, the Indian Himalayan belt is further divided into Jammu & Kashmir, Himachal Pradesh (Shimla, Chamba & Kinnaur), and Uttarakhand (Garhwal & Kumaon) regions. Leishmaniasis which was initially considered a disease of plain lower altitude areas along the banks of major rivers is now prevailing in higher altitudes. This ecological shift provides us with an excellent opportunity to study the epidemiological triad and also warranting a need to implement appropriate control measures. Hence, this review is done with the objective to identify the newly reported endemic areas on these hilly terrains related to the disease and multiple factors associated with it, especially in relation to river belts.

Selection: (1) Leishmania disease: Only records that concern the leishmania/Kala-azar in the Indian sub-continent or related topics are included in the selection; (2) Original records: We excluded letters, editorials and comments; and (3) English language: We excluded articles written in other language.

RESULTS

Across all literature and records available, 31 references were found which were relevant to our study (Table 1) among 51 qualitative syntheses (Figure 2). The sample size in these studies varied from a single case report to a study containing more than thousands of cases[3-33].

Epidemiology (demography)

The studies reviewed were specially chosen from the southern Himalayan region to emphasize the

Table 1 Findings of published articles on leishmaniasis in southern Himalayas w.r.t. agent-host-environment details

Ref.	Sample size	Location (if available district, state, country)	Agent factors	Host factors	Vector identified	Environmental factors	River body associated	Authors conclusion
Katakura *et al*[4]	> 1000	Different areas of Pakistan, India, and Nepal	In Pakistan Himalayas, *Leishmania tropica* followed by *Leishmania major* In India, *L. donovani* followed by *L.tropica* In Nepal, *L. major*	CP: Cutaneous leishmaniasis (CL) cases only; No descriptions Rx: Not known Outcome: Not known	In all Himalayas, *P. sergenti* followed by *P. argentipes* and *papatasi*	Altitude is not documented	Indus, Ganges	Microsatellite analysis of the parasites will be a powerful tool for population genetic and epidemiological studies of Leishmania species
Rab *et al* [5]	239 (1984-1992) and more cases in the past (before 1984)	Different areas of Northern areas of Pakistan (Bagh, Abbottabad, Chilas, and Baltistan)	*Leishmania* *infantum*	Clinical presentation (CP): *Visceral leishmaniasis* (VL – all cases); Not described Rx: Not known Outcome: Not known	Not documented	Altitude is not documented	Indus	The clinical pattern of VL in north Pakistan is akin to that in north-western China, with a marked predilection for young children, and a male preponderance. The infantile VL has risen 10-fold in the last decade from 0.2 to almost 2 per 100 000 population
Wani *et al* [6]	18	Different areas of Uri &Karnah belt, Jammu & Kashmir, India	Leishmania, species not identified	CP: Cutaneous leishmaniasis (CL); mostly nodulo-ulcerative, mostly on the face and single lesion Rx: Intravenous sodium Stibogluconate including two received intra-lesional Outcome: Survival for all cases	Not documented	Altitude is not documented. The hot and arid climate of these areas(Uri belt) is quite conducive to the growth and development of leishmania and the sand fly	Not documented	Any patient with nodular/nodulo-ulcerative lesion on exposed parts must be suspected for CL, especially if belonging to the Uri and Karnah region of the Kashmir Valley. The public health authorities should make every effort to contain this new infection in this Valley
Leherwal *et al*[7]	Three	Uri belt, Jammu & Kashmir, India	Leishmania, species not identified	CP: Cutaneous leishmaniasis (CL); solitary erythematous nodule on the face Rx: Not documented Outcome: Not documented	Not documented	Altitude is not documented	-do-	Focuses on the diagnostic part. FNAC may be the method of choice for suspected CL in cases of solitary nodular lesions
Mahajan *et al*[8]	One	Uri in South West Kashmir, Jammu & Kashmir, India	Leishmania, species not identified	CP: Visceral leishmaniasis (VL); 2months fever, weight loss, ascites, anemia, Hepato-splenomegaly, Rx: Intravenous sodium	Not documented	Altitude is not documented	-do-	This advice for further research into the epidemiology, geographic distribution, and inter-species interactions of the parasite

				Stibogluconate Outcome: Survived				
Sharma *et al*[9]	285	Nirmand village,Shimla & Kullu Districts of Himachal Pradesh, India	Among 14 cases, *Leishmania tropica* (3) and *Leishmania donovani* (11) Tissue smear positivity for amastigotes was 43%	CP: CL; mostly nodulo-ulcerative, mostly on extremities Rx: Intra-leisonal sodium Stibogluconate Outcome: Survival for all cases	Among 41 cases, *P. longiductus* (29), *P. major*(8), *P. kandelaki* (2), and 2 remained unidentified	Altitude is not documented The climate of the affected areas varies from temperate to subtropical	Satluj river	Different leishmania species and vectors compared to other parts of India are found in these Himalayas
Sharma *et al*[10]	161 new localized cases of LCL from May 2001 and December 2003	sub-alpine valley in the mountainous region of the Kinnaur District,Himachal Pradesh, India	*L. donovani* in eight cases and *L. tropica* in two cases	Histopathology showed non-caseating epitheloid cell granuloma in 77% of the cases. Lesions involved mainly the face	*Phlebotomus longiductus* is a possible vector	Altitude, 700-2,900 m above sea level	Satluj River	Intralesional sodium stibogluconate was effective in all patients
Raina *et al* [11]	18	Shimla, Kinnaur & Kullu Districts of Himachal Pradesh, India	Leishmania, species not identified	CP: VL - prolonged fever, weight loss, ascites, pancytopenia, hepato-spleno-megaly, lymphadenopathy, diarrhea, and epistaxis Rx: Intravenous sodium Stibogluconate Outcome: 14 Survives and 4 deaths	Not documented	Altitude, 924 - 2960 m above sea level The patients had never visited any of the endemic areas	Satluj and Beas river	Initial failure to suspect VL in this area might cause a diagnostic delay There is a favorable therapeutic response without recurrence of symptoms during 6 months of follow-up
Thakur *et al*[46]	Cases of CL During 2014–2018 in the study area	case reports came from Districts of Kinnaur, Shimla, and Kullu and the previously nonendemic districts of Mandi and Solan,Himachal Pradesh, India	*L. donovani* variants distinct from the viscerotropic *L. donovani* strain from northeast India	Coexistence of VL and CL	Not documented	Not documented	Not documented	The scenario appears somewhat similar to Sri Lanka and Kerala, where *L. donovani* parasites cause cutaneous disease, albeit with differences in the region-specific *L. donovani* variants
Thakur *et al*[47]	Sixty CL patients over the period from 2014 to 2018	Satluj river belt in Himachal Pradesh, Khaneri/rampur (location of medical college),Himachal Pradesh, India	Presence of *L. seymouri* co-infection in the unusual CL cases in Himachal Pradesh (HP) caused by *L. donovani* variants	Coexistence of VL and CL	Not documented	Not documented	Satluj river	Found the presence of *Leptomonas seymouri* in 38.5% (22/57) of the patients along with L. donovani detected in all the samples. *L. seymouri* is a monoxenous insect *trypanosoma*, generally incapable of infecting humans

Sharma *et al*[49]	None	Shimla, Kinnaur, &Kullu Districts of Himachal Pradesh, India	Not applicable	Not applicable	Among 62 cases, *Phlebotomus longiductus* (46), *P. major* (8), *P. kandelaki* (8)	Our patients reported having been out of the state or district during the three years the preceding onset of symptoms	Satluj river	*Phlebotomus longiductus* may be the primary vector for human leishmaniases in this endemic focus, however, it needs another study to prove the vector species corresponding to the type of leishmania species

growing concern of leishmania in newly endemic areas. Among all reviewed studies, one was conducted in north Pakistan, twenty-three in north India, four in Nepal, and two in Bhutan. Among Indian studies, three were in Kashmir, eleven in Himachal Pradesh (Shimla, Chamba & Kinnaur), and nine in Uttarakhand (Garhwal & Kumaon). One study was multi-centric, covering vast geographical areas falling in Pakistan, India, and Bhutan[4].

Considering the pivotal role of the environment in the natural history of disease meticulous scrutiny of various articles was done. The majority of studies included in this review have been conducted along the banks of major river-belts of the terrain (Figure 3). In northern Pakistan, the major river associated was Indus and its tributaries. In northern India, the Uri Belt of Jammu & Kashmir, the river belt of Satluj and Ravi in Himachal Pradesh, and the bank of the river Ganges in Uttarakhand were the major site of focus. In Nepal, a total of four studies have been reported which were conducted along the banks of river Budhi Ganga and Kailash. One study from the mid-west region of Nepal has not documented an associated river, but further search for location indicates the site belongs to the banks of river Karnali. Similarly, studies from eastern Bhutan have not specified associated rivers but the described areas are mainly located between the three major rivers-Drangme Chhu, Kuru Chhu, and Mangde Chhu, all are tributaries of the Brahmaputra river. A multi-national study from South and South-east Asia also reported Indus and the Ganges to be the major associated river[4]. Among all these studies none of them established a direct association between the presence of any major water bodies & ecological niche conducive for the vector species.

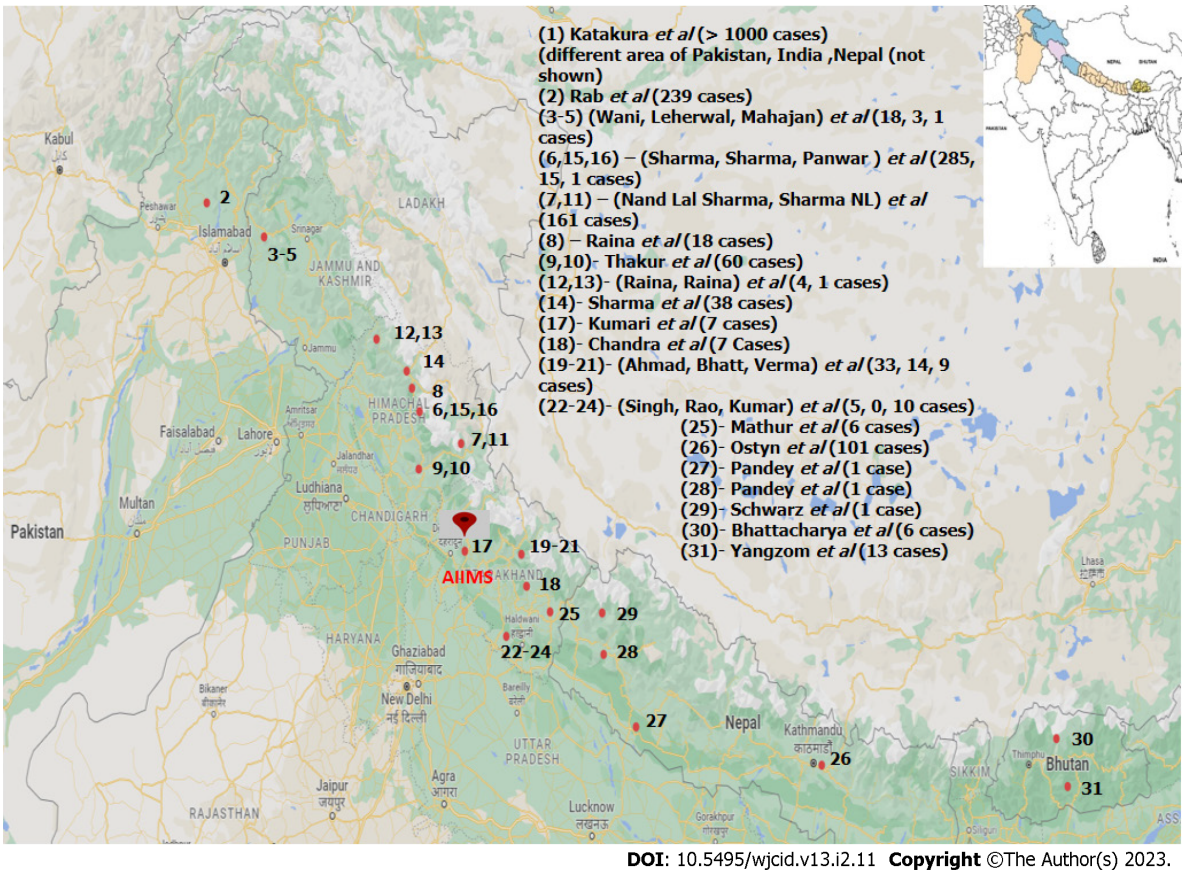
Entomological and parasitological findings (Table 1)

Although the majority of the reviewed studies did not identify the vector species, *Phlebotomus argentipes* was the pre dominant vector species among all the reported cases[5-7]. Few studies have also found some different species as a possible vector such as *P. longiductus*> *P.major*> *P. kandelaki* as a leading species of the vector in studies of Shimla & Kullu districts of Himachal Pradesh, India[8,9]. Similarly, one study from Bhutan has also reported four different phlebotomine species[10].

The existence of *L. donovani* was ubiquitous however the quest to identify the predominant causative leishmania species remains unresolved as the majority of the studies did not identify any. Among the studies included in our review, five studies have reported *L. donovani*[10-15], while two studies reported *L. infantum*[16], as the predominant leishmanias pecies. Few studies indicated the presence of dual-species like both *L. tropica* and *L. donovani*[4,8,9], were documented in three studies and both *L. infantum* and *L. Donovani*[17] were documented in a single study. It Is also recorded that *L. donovani* variants found in Himachal Pradesh, India were different from the viscera tropic leishmania strain predominant in north east India[11].

DOI: 10.5495/wjcid.v13.i2.11

Figure 1 Map of the southern Himalayas describing rivers.

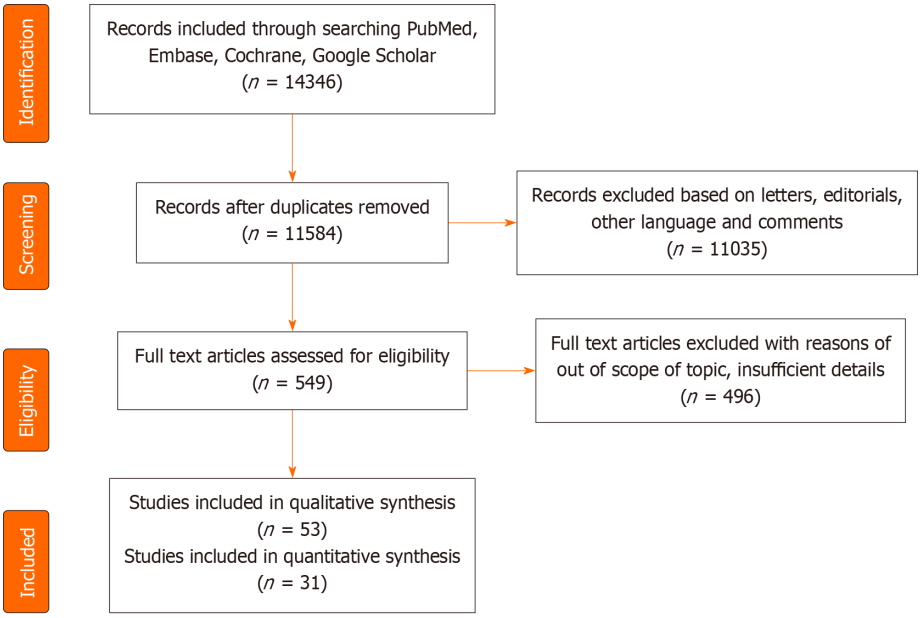


DOI: 10.5495/wjcid.v13.i2.11

Figure 2 Map of the southern Himalayas representing the magnitude of leishmaniasis.

Clinical presentation (Table 1)

The majority of the studies reported cases of visceral leishmaniasis (VL) with high-grade prolonged fever, malaise, abdominal discomfort[3,7,10,13,14,16,18-27]. Cutaneous leishmaniasis (CL) was reported in a few studies with clinical presentation of nodulo-ulcerative lesions or solitary erythematous nodule



DOI: 10.5495/wjcid.v13.i2.11

Figure 3 Preferred Reporting Items for Systematic reviews and Meta-Analyses flow diagram.

[4,8,9,12,28,29]. Three studies reported cases with both types (VL and CL) of leishmaniasis[11,17,30]. Another three studies did not identify the type of leishmaniasis however they described a clinical picture of hepatomegaly and weight loss as a common feature in their studies[15,31,32].

Laboratory diagnosis (Table 1)

Methods of laboratory diagnosis were not documented in any of the reviewed literature, however, smear-positive by Giemsa or Leishman technique for *Leishmania donovani* (LD) bodies are reported in most cases. LD bodies were demonstrated in the bone marrow in the case of VL and from the skin in the case of CL[13,18,28]. Some studies also found LD bodies in splenic aspirate, lymph node aspirate, duodenal and colonic mucosal biopsy in patients presenting with diarrhea[3,6,9,22]. Only in a few reference studies, there were records of other methods (mostly rK39 ICT) as an additional test. One case report of a pregnant lady was found rK16 test positive, rather than commonly used rK39 antigen[21]. Secondary hemophagocytosis lymphocytic syndrome (HLH) in VL cases was diagnosed either by 4 out of 6 criteria of HLH diagnosis or by bone marrow aspirate examination for hemophagocytosis[20,21,23]. Rarely polymerase chain reaction (PCR) for the leishmania kinetoplast mini circle gene was tested and found to be positive in a case of *L. donovani* infection which was confirmed on subsequent sequencing of the PCR – amplification method[27]. An age-old aldehyde test was found positive for five out of six cases of kala-azar, however, they confirm edit either by rK39 testing or by bone marrow aspiration examination for the L D bodies[33].

Treatment and outcome (Table 1)

Pharmacological therapies with sodium stibogluconate, amphotericin-B or miltefosine, either single or in various combinations had been reported in 21 reviewed studies. Studies were done in northern Pakistan and the Uri belt of Kashmir did not document the pharmacotherapy used and hence the subsequent outcomes[4,16,29]. In the case of VL, studies had reported intravenous sodium stibogluconate alone is sufficient for upto 84% of cases (19 survivals and 5 deaths, out of 24 cases)[8,18,25]. However, some studies were not clear about the route of stibogluconate therapy (intravenous or intralesional). Plain amphotericin-B showed > 90% recovery rate and liposomal showed upto 100% cure rate[7,13,20,21,22,31,32]. Various studies have a different outcome for the combinations of drugs, like, in one study, a combination of sodium stibogluconate and plain amphotericin-B resulted in 2 deaths out of 4 cases (50%cure rate), while three drugs combination (sodium stibogluconate + plain amphotericin-B+ miltefosine) for 33 cases resulted in all cure with one relapse which later treated with liposomal amphotericin-B (100% curerate)[23].

For CL diagnosed cases use of intra-lesional sodium stibogluconate alone showed recovery of all 285 cases (100% cure rate)[8]. Inspiring results were also seen in cases where the combination of intravenous and intra-lesional stibogluconate resulted in the survival of all 18 cases[28].

In case of relapse or failure, liposomal or plain amphotericin-B was most commonly used, this showed diverse efficacy in different studies. Like in one, out of 10 cases, 6 survived, 3 Lost to follow up and 1 resulted in death after the use of plain amphotericin-B[26]. While in another study, plain

amphotericin-B was sufficient for the relapsed case after initial sodium stibogluconate (intralesional or intravenous not explained)[10]. A similar instance was reported in a study where plain amphotericin-B was given after failed miltefosine therapy and the case survived[10].

The dose of all drugs was not available in studies, however, a single dose (10 mg/kg) of liposomal amphotericin-B was used with a 100% cure rate including one relapse case after use of plain amphotericin-B[13].

DISCUSSION

The thirty-one studies of southern Himalayas show emerging leishmaniasis in high-altitude areas. The disease profile is distinctive from typical endemic areas. This can be discussed under various aspects of disease profile.

Epidemiology (demography)

Leishmaniasis is prevalent mainly in the poor and marginalized communities of the world, predominantly of the Indian subcontinent like Bangladesh, India, and Nepal. However, recent studies are suggestive of the emergence of new endemic foci in various parts of the world as well. In 2017, 94% of new VL cases were reported in seven countries: Brazil, Ethiopia, India, Kenya, Somalia, South Sudan, and Sudan while the majority of CL cases reported from Afghanistan, Algeria, Brazil, Colombia, the Islamic Republic of Iran, Pakistan, Peru, Saudi Arabia, and the Syrian Arab Republic[34]. Some latest sporadic cases have also been reported from Bhutan and Thailand[32]. All these countries share a similar topography, ecological and environmental factors (high humidity, adequate rainfall, and surface dampness) which are favorable for the proliferation of Phlebotomes. Results of recent studies demonstrate that now leishmaniasis is not confined to a specified topography, rainfall, temperature, or vegetation, it has now continuously expanded its geographical distribution which can be explained by factors such as rapid growing globalization, global warming, deforestation, and urbanization. These facts can't be confirmed as very few epidemiological studies are available on this issue. Furthermore, reviewing the literature, it was observed that the majority of the cases have been reported along with the major river belts in these new areas. This observation is highly suggestive of possible up stream migration of vectors along the rivers. In the past 15 years of reporting, good numbers of cases were found in newly endemic areas of Bhutan, Nepal, India (Uttarakhand, Himachal Pradesh, Jammu and Kashmir),and Northern parts of Pakistan.

Entomological and parasitological findings

Sandfly, vector of VL and CL, includes many species of the genus *Phlebotomus* (in the Old World) and *Lutzomyia longipalpis* (in the New World)[35,36]. Although the majority of the reviewed studies have not mentioned the associated vectors, *Phlebotomus argentipes* was found to be the predominant vector among the reported cases except in Himachal Pradesh (India) where *P. longiductus* and *P. major* were identified in co-existence. Interestingly *P. argentipes* remain closely associated with the exclusive cases of VL while *P. longiductus* (most common) and *P. major* were associated with areas where both CL & VL forms were found (Table 2). Therefore, the associated area needs an entomological study to know the basic characteristics of the vector and associated factors.

L. donovani transmission in East Africa consists of both anthroponotic and zoonotic components[37]. In Sudan, rodents and dogs were found to be reservoirs; however, observation in the majority of outbreaks reflects anthroponotic predominant transmission[38,39]. While in SEAR countries, the human being is the only reported reservoir. In this review also, we found a similar finding of the human being as the sole reservoir for VL.

Major species of parasites of VL are reported as *L. donovani* in South Asia and *L. infantum* in the Mediterranean region along with some sporadic cases in Central Asia, China, Mexico and Central Brazil [35,40]. The central western area of Brazil which is considered an area of recent transmission for VL and is on the risk for CL, *L. longipalpis* was the widespread species discovered[40]. In the new world, the most common etiological agent is *L. infantum*. The current review also documents similar findings of *L. donovani* in the majority of studies but one study from the Himalayan areas of Pakistan reported *L. infantum* in the majority[16]. A study in Brazil documented to have detected for the first time the presence of either *L. infantum* or *L. braziliensis* circulating in the domestic host[41]. In India, VL is caused by *L. donovani* in the north eastern region, and CL is caused by *L. tropica* in the western Thar Desert region[42]. Himachal Pradesh is a more recently leishmaniasis endemic state in north-west India where VL and CL coexist. The incidence of CL is higher than that of VL and most cases are attributable to *L. donovani*[33,43]. One of the studies conducted in the same region reported an interesting presence of *Leptomonas seymouri* co-infection in CL with *L. donovani*[30]. Undoubtedly there may be some missing links and associations that are still unknown and undiscovered since no other areas around Himachal Pradesh of the southern Himalayan region reported any remarkable epidemiological studies. Therefore, this review may act as a catalyst to perpetuate epidemiological search in this region to establish various niches.

Table 2 Characteristics of Leishmaniasis in the southern Himalayan region

Sr No.	Geographical area	Causative agent	Vector	Clinical picture
1	Northern areas of Pakistan[2]	*LeishmaniaInfantum*	Not identified	Visceral leishmaniasis
2	Indian states of Jammu & Kashmir[3-5]	Not identified	Not identified	Cutaneous leishmaniasis most common with a single case study of visceral Leishmaniasis
3	Himachal Pradesh[6-16]	*L. donovani* & *L. tropica*	*P. longiductus* (most common) & *P. major*	Both cutaneous & visceral forms ofLeishmaniasis
4	Uttarakhand (Garhwal)[17-21]	*L. donovani*	*P. argentipes*	Visceral leishmaniasis
5	Uttarakhand (Kumaon)[22-25]	Not identified	*P. argentipes*	Visceral leishmaniasis
6	Nepal[26-29]	*L. donovani*	*P. argentipes*	Visceral leishmaniasis
7	Bhutan[30,31]	*L. donovani*	*P. argentipes*	Visceral leishmaniasis

CL in the New World is generally caused by *L. mexicana*, while CL of the Old World is caused by five species of Leishmania: *L. infantum* (more common), *L. tropica*, *L. major*, *L. aethiopica*, and *L. donovani*. However, a study in the Indian sub-continent documents *L. tropica* in Pakistan, *L. donovani* and *L. major* in Nepal are the most common organism causing CL[4]. PKDL is caused primarily by *L. donovani* both in India and Sudan with only a few cases by *L. infantum* or *L. chagasi*[28].

This shows the existence of different types of species for both VL and CL in different parts of the South Asian countries including the southern Himalayas of the Indian Sub-continent. The rationale behind this diversity and associated epidemiological factors needs to be studied further.

Clinical presentation

VL has different clinical features in the endemic, epidemic, or sporadic situation. It tends to be relatively chronic and mostly affects children in endemic areas. Both the VL and CL are endemic in Pakistan and India while only VL is endemic in Nepal and Bhutan (WHO updates). Study analysis revealed that the characteristics of the disease vary with the environment. Here we see the preponderance of VL in Bhutan, Nepal, and Uttarakhand (India) with the coexistence of CL and VL in the Indian states of Himachal Pradesh, Jammu and Kashmir and Pakistan (Table 1).

Most cases are asymptomatic, but some eventually develop VL on follow-up, more commonly in males[35]. Risk factors for progression to VL include malnutrition, genetic factor and other co-infections, mainly HIV. The major classical presentation is prolonged fever, fatigue, loss of appetite and weight, and left hypochondrium discomfort. There may be non-tender splenomegaly with or without hepato-megaly, pallor, and lymphadenopathy (especially in Sudan, commonly by *Viannia* subgenus species). The darkening of the skin is typical for the Indian variant (Hindi name, kala-azar). Clinically CL usually exhibits painless, multiple, round-to-oval crater-form dry nodular lesions, mostly at the site of inoculation. Usually, these cutaneous lesions heal spontaneously in 1year, often with disfiguring scars. PKDL is extremely rare, confined mainly in two regions endemic to kala-azar the Indian sub-continent and Sudan plus adjoining areas (up to 50% and 10% of patients with kala-azar respectively)[44-46].

Among all the studies reviewed none of them documented an asymptomatic period. The majority documented similar classical VL and CL symptoms except a few, which documented some atypical presentations like ascites, diarrhea, epistaxis, HLH syndrome, and hypergammaglobulinaemia (Table 1). Few cases of PKDL were reported from the hilly area of Uttarakhand too. The occurrence of PKDL after VL treatment in Nepal is also low as compared to neighboring countries[47].

Diagnosis

For diagnosis of Leishmaniasis many tests like dual path platform, a rapid immune-chromatographic test, and enzyme linked immune-sorbent assay (ELISA) are recommended by the Brazilian Ministry of Health[41]. Govt. of India recommends various tests for the detection of leishmania, including serology, aldehyde test, complement fixation test, indirect hem-agglutination test, ELISA, direct agglutination tests (DAT), spleen or bone marrow aspirates, and rK39[43]. The diagnostic policy for leishmaniasis is variable depending on the level of health systems. In first-line centers or rural hospitals of the highly endemic zone, the rK39 test is mostly used. Parasitological diagnosis is necessary for relapse identification. In low- endemic areas, more specific tests like PCR or parasitic demonstration are found necessary, as PCR is more sensitive than microscopic examination, therefore, can detect more asymptomatic infections. However, it is not available in most centers, and evaluation of its diagnostic accuracy and proper standardization is needed. For relapse, serological tests such as DAT, ELISA, and rK39 rapid test are usually positive and frequently used in majority areas but are of limited value, as a

positive result may be due to antibodies persisting after a past episode of VL, so better to show parasitological evidence for confirmation. A study in Brazil documented use of nested PCR (LnPCR) and PCR-restriction fragment length polymorphism for identification of Leishmania species[41]. A careful perusal of studies in this review showed a comprehensive use of various diagnostic procedures with no conclusive evidence towards any particular method. Future studies in these regions are need of the hour to formulate a diagnostic policy suitable for primary to tertiary health care levels.

Treatment and outcomes

Depending upon the sensitivity of drugs and the economic status, the treatment regime varies in different parts of the world. Liposomal amphotericin- B monotherapy (total dose of 20 to 21 mg/kg) is the preferred treatment in Europe, North America, and South America[48,49]. In East Africa, first-line therapy consists of a combination treatment of sodium stibogluconate and paromomycin for 17 d; the efficacy of liposomal amphotericin-B, miltefosine and paromomycin monotherapy are unacceptably low [50]. WHO Expert Committee and the Regional Technical Advisory Group of SEAR recommends liposomal amphotericin-B in a single dose of 10 mg/kg body weight as the first line treatment regimen for the Indian subcontinent within the current elimination strategy, given its high antimicrobial efficacy, safety, ease of use and assured compliance[1].

The majority of the studies in this review comply with the above standards and none of them documented parallel or supplemental pharmacotherapy other than the recommended regimen. However, agreeable documentation about the efficacy of the above drugs cannot be established in these emerging foci, as different studies had different outcomes. On summarizing the treatment outcomes liposomal amphotericin-B has emerged as the most effective therapy against the disease (with 100% cure rate achieved with single-dose). Furthermore, it is also found to be effective in VL-associated HLH and the explanations were that it inhibits macrophage function, reduces cytokine expression, and antigen-induced proliferation of T and B cells in vitro, causing a dual effect on both HLH and VL[13]. At last, the treatment regimen must follow national or regional guidelines, if applicable. Species identification usually is not critical to treatment decisions for VL (incontrast with CL)[51]. Multiple trial studies regarding drugs and doses should be done for the best suitable management protocol in these new niches.

Limitations

As said before, the availability of only a few studies related to the Himalayan regions is the major limitation of this review. Limited studies have covered the factors determining the transmission of VL in these new foci. The paucity of data limits the freedom to give any conclusive remarks on this new possible niche of leishmaniasis. A detailed analysis of these factors and the molecular characterization of vector species and leishmaniasis strain are still lacking. However, this mini-review aspires to highlight the surge of new cases in non-endemic areas as a matter of public health importance and research.

CONCLUSION

Despite substantial progress towards VL elimination in most endemic parts of the world, recently reported the emergence of new endemic foci in Southern Himalayas, forecast a great challenge for public health. Upstream river belts are a possible path of Sandfly spread towards these non-endemic areas, need a better environmental study to prove. In these areas, *P. argentipes* is found to be a predominant vector, *L. donovani* as a major parasite cause of VL, and *L. tropica, L. donovani*, and *L. major* as a major cause of CL in Pakistan, India, and Nepal respectively. Isolated VL is seen in Bhutan, Nepal, and the Uttarakhand state of India, while both VL and CL are seen in other Himalayan areas. Moreover, patients of these areas have a typical clinical presentations (ascites, diarrhea, epistaxis, HLH syndrome, and hypergammaglobulinaemia) so they need a high index of clinical suspicion, prompt diagnosis, and management. Single-dose liposomal amphotericin-B holds a 100% cure rate. As the a typical disease is recognized as a major threat to ongoing leishmaniasis elimination, so continuous monitoring of the disease type and associated parasitic variants and vector species should be implemented as part of the ongoing leishmaniasis elimination and maintenance programs. Studies on vector species and alternate reservoirs are also required for a better understanding of region-specific disease transmission and epidemiology.

FOOTNOTES

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CASE REPORT

Brucellosis, a diagnostic dilemma, presenting atypically in a child with terminal ileitis: A case report

Kokila Banerjee, Amitava Pahari, Subhendu Roy

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Abstract

BACKGROUND

Brucellosis is endemic in India with seropositivity rates as high as 10% in children in the eastern states, yet the disease is not on the radar when a differential diagnosis of pyrexia of unknown origin (PUO) is being considered, especially in children in urban set-up. This may be because of the non-specific multitude of systemic symptoms seen in this disease and the lack of awareness among clinicians.

CASE SUMMARY

We present a case of a 13-year-old boy, who came with a history of undulating fever for the past three and a half months, loss of appetite, and abdominal pain. The child had visited several pediatricians and was even admitted to a tertiary care hospital for PUO evaluation, but to no avail. He presented to us after three and half months of suffering and weight loss of more than 10% of body weight. His ultrasonography revealed thickening of the terminal ileum. His blood culture grew *Brucella melitensis*. A diagnosis of Brucellosis with terminal ileitis was made. *Brucella* serology by enzyme-linked immunoassay (ELISA) was positive for both IgG and IgM. He was treated with doxycycline and Rifampicin along with syrup multivitamin and zinc, for 6 wk. There was remarkable improvement with gain in 4 kg body weight within 2 mo of completing treatment. History revealed consumption of unpasteurized milk and contact with cattle.

CONCLUSION

Clinical suspicion, detailed history, appropriate laboratory investigations are the three pillars for diagnosing Brucellosis in patients presenting with vague symptoms.

Key Words: Pyrexia of unknown origin; Terminal ileitis; Brucellosis; Case report

Core Tip: Pyrexia of unknown origin has always been a diagnostic challenge for clinicians, in spite of development of most modern diagnostic techniques. The decision to choose the right investigation depends on the clinician's acumen which in turn is guided by detailed history-taking and knowledge of local disease prevalence, leading to timely diagnosis with prevention of mental, physical and financial agony. Our child suffering for three-and-half months, could have landed in the emergency department with acute abdomen, had there been a further delay in the diagnosis of his vague symptoms that were due to Brucellosis presenting atypically with terminal ileitis.

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INTRODUCTION

Diagnosing pyrexia of unknown origin (PUO) almost always poses serious challenges. Infections in developing countries, like India[1], and non-infectious inflammatory diseases in developed countries are the major causes[2]. Globally, 7%-53% of PUO cases go undiagnosed despite thorough workup and advancements in diagnostic techniques[3]. The initial diagnostic investigation protocol for PUO should at the very least be a thorough history taking and repeated physical examination, complete blood count with differential cell count, electrolytes, renal and liver function tests, protein electrophoresis, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), urine culture, chest X-ray, abdominal ultrasonography, and a tuberculin skin test[4-6]. The case described here is of a child with PUO that proved to be an immense diagnostic challenge, finally diagnosed as brucellosis, highlighting the need for collecting detailed history and keeping in mind the local infectious epidemiological data. Brucellosis is a major endemic zoonotic disease in developing countries including India, in which systemic generalized symptoms predominate, rather than gastrointestinal complaints.

CASE PRESENTATION

Chief complaints

The patient, a 13-year-old male child, presented to us with undulating fever, loss of appetite, abdominal pain, weight loss along with malaise and myalgia for the last 14 wk.

History of past illness

The child had ventricular septal defect repair in 2018. His current echocardiogram showed mild tricuspid regurgitation.

History of present illness

The child weighed 32 kg, 3 mo prior to coming to us when a local pediatrician was consulted for complaints of intermittently high-grade fever (102-104℉) for the past 5-7 d, malaise, and abdominal pain. His blood investigations showed Haemoglobin: 11.3G/dL, CRP: 11.8 (reference level: 6 mg/L) Total leukocyte count: 3700/cu mm, Neutrophils: 56%, Lymphocytes: 39%, ESR: 30 mm (1st hour), platelets: 210000/cu mm. Dengue NS1 and Malaria dual antigen were negative. The child was treated with amoxicillin-clavulanic acid for 7 d. However, the fever was persistent after an initial remission. The local pediatrician was again consulted after 5 wk. Routine investigations were repeated and found to be normal. Additionally, Widal was reactive at 1:80 for TO and TH antigens. Chest X-ray was normal, abdominal ultrasonography revealed mild splenomegaly.

As the fever was not subsiding, the child was admitted to a tertiary care hospital for PUO evaluation of > 8 wk duration with intermittently high-grade (102-104℉) fever. On general examination, the patient was alert and active, with pallor, but no history of bleeding from any source. The chest was clear and the spleen palpable. Routine blood investigations bore similar results. A sputum acid-fast bacillus smear for 3 consecutive days was negative. Mantoux test and GeneXpert for tuberculosis, from gastric aspirate, were negative. Weil-Felix and Scrub typhus IgM ELISA were non-reactive. Serial Automated blood cultures were done to rule out Infective endocarditis all three blood culture samples yielded no growth of any pathogens after 96 h of aerobic incubation. Routine urine examination revealed no abnormality. As all other investigations were non-suggestive, in the light of an older Widal report of TO: 1:80 and TH: 1:80 positivity, the patient was started on injection ceftriaxone 50 mg/kg body weight in two divided doses, intravenously for 5 d. The patient became afebrile and was discharged in a week.

However, after a few days of remission, the child had a relapse of fever (102-104°F) with chills, abdominal pain, with irregular bowel movements. One month after discharge from the hospital, he visited us in the Outpatient department.

Physical examination

On examination, a few cervical lymph nodes were found enlarged and pallor present. Hepatosplenomegaly (liver 2 cm, spleen 0.5 cm) and abdominal tenderness were noted. His current body weight was 27.7 kg (1st percentile) < 50 percentile (45 kg) body mass index - 13.1 (1st percentile), < 50 percentile (18.4).

Imaging examinations

Chest X-ray was normal. Ultrasonography of the abdomen showed thickening of the terminal ileum wall with no enlargement of abdominal lymph nodes. A Paediatric Gastroenterologist's opinion was taken. A differential diagnosis of Luminal Koch's and Small bowel Crohn's (IBD) was considered. Colonoscopy and computed tomography (CT) enterography were planned. On Colonoscopy no abnormalities in colon or caecum were noted. No ulcers, friability, granularity, polyp, or tumor seen. The terminal ileal mucosal surface looked normal, hence no biopsy was taken. During computed tomography (CT) enterography, the ileum was not accessible, due to suboptimal distension of loops. Splenomegaly was however noted. The differential diagnosis of luminal Koch's and inflammatory bowel disease (small bowel Crohn's disease) was ruled out based on these investigations.

Laboratory examinations

Routine blood investigations revealed a low Haemoglobin (10.5 g/dL) red blood cell (RBC): 4.23 million/cu mm, white blood cell (WBC): 10640/cu mm, and adequate platelets. The differential count was predominantly lymphocytic, (Neutrophils 45%, Lymphocytes 50%); however, the peripheral blood film showed normocytic normochromic anemia, no malaria parasite or abnormal cells seen. The ESR was 60 mm (1st hour). Liver function tests revealed normal bilirubin level, A: G ratio was 1, serum LDH level: 365 IU/L, and globulin level: 4.3 g/100 mL, the serological tests for dengue andmalaria were again negative. The Widal tube agglutination test was non-reactive, (titer: < 1:20, for antibodies to *Salmonella typhi* (TO, TH) and Paratyphi A (AH) and Paratyphi B (BH) Epstein-Barr virus IgM was negative. Automated Blood culture by BacTAlert revealed growth of *Brucella melitensis* after 5 d of aerobic incubation identified by Vitek 2 Compact. Serology for *Brucella* was done later by ELISA, and tested positive for *Brucella* IgG: 35.14 U/maleL (positive > 12 U/maleL) and IgM: 46.97 U/maleL (positive > 12 U/maleL). The *Brucella melitensis* isolate was tested on MALDI-TOF, identified as *Brucella spp*. The chronological investigations are enumerated in Table 1.

Personal and family history

During this time, the patient's history of contact with cattle during Bakri-Eid, and consumption of unpasteurized milk was gathered upon questioning. There was no significant family history or similar symptoms in any other family member.

FINAL DIAGNOSIS

The final diagnosis was brucellosis with an atypical presentation of terminal ileitis.

TREATMENT

The patient was treated with doxycycline (3-5 mg/kg body weight) in two divided doses daily for 6 wk, rifampicin (10 mg/kg body weight) once daily for 6 wk, and syrup multivitamin and zinc.

OUTCOME AND FOLLOW-UP

The patient turned afebrile on day 3 of treatment. On follow-up, two months after completion of the antibiotic course, the patient had drastically improved clinically, gained 4 kg body weight, hemoglobin level increased to 12.7 g/dL from 10.2 g/dL, the A: G ratio improved to 1.2 from 1.0, and liver enzymes came back within normal range.

DISCUSSION

Literature review

The gastrointestinal symptoms of brucellosis are frequently present but are usually restricted to loss of appetite. Ileal involvement in human brucellosis is extremely rare. Only seven cases in the literature have been reported to the best of our knowledge, and not a single case from India[7-13]. The first case of ileitis was reported by Petrella *et al*[7] from Texas in a child, in the year 1988, which was linked to the ingestion of unpasteurized goat milk cheese, during an outbreak of

Table 1 Laboratory investigations for diagnosis of pyrexia of unknown origin

Laboratory investigations		Results				
		First visit to a paediatrician (13 wk prior to visiting us)	Revisit to a pediatrician (8 wk before visiting us)	Tertiary care hospital admission (5 wk before visiting us)	At the time of visiting us	2 mo after treatment completion
Complete blood count	Haemoglobin (g/dL)	11.5	11.3	10	10.5	12.2
	RBC (million/cu mm)	-	-	3.82	4.23	-
	Total leucocyte count (/cu mm)	3700	9600	8100	10640	8430
	Erythrocyte sedimentation rate	30	40	56	60	28
	Neutrophil (%)	56	63	60	45	38
	Lymphocyte (%)	39	30	35	50	52
	Platelet count	Adequate	Adequate	Adequate	Adequate	Adequate
Liver function test		-	-	Serum glutamic-oxaloacetic transaminase -90, Serum Glutamic Pyruvic Transaminase -58	Globulin: 4.3 g/dL, LDH: 365 U/L	Serum glutamic-oxaloacetic transaminase -42, Serum Glutamic Pyruvic Transaminase -29
Serological examination	Dengue nonstructural protein (NS1) antigen	Negative	Negative	Negative	-	-
	C-Reactive protein (mg/dL)	1.18	0.45	0.69	0.503	-
	Brucella serology (IgG and IgM)	-	-	-	Positive	-
	Epstein-Barr virus IgM	-	-	-	Negative	-
	Dual antigen test for malaria	Negative	Negative	Negative	Negative	-
	Widal test	-	TO-1/80, TH- 1/80	-	-	-
Tuberculosis	Cartridge Based Nucleic Acid Amplification Test from the gastric aspirate	-	-	Negative	-	-
	Sputum for acid-fast bacilli	-	-	Negative	-	-
	Mantoux test	-	-	Negative	-	-
Scrub typhus	Weil-Felix test	-	-	Negative	-	-
Automated blood culture		-	-	Negative	*Brucella melitensis*	-
Routine urine examination		-	-	Normal	-	-
Chest X-ray		-	Normal	-	Normal	-
Ultrasonography whole abdomen		-	Mild splenomegaly	-	Splenomegaly and thickened ileum wall	-

Brucella melitensis[7]. The age and sex of this child and details of diagnosis and treatment are not available. A 15/male patient, from China, living in an endemic area, denied any contact with cattle, had a fever and diffuse abdominal pain of 4 wk duration, and demonstrated mucosal thickening on abdominal Ultrasonography[8]. Our patient too had presented with a three-month long history of undulating fever, and abdominal pain, with thickening of mucosa of terminal ileum, demonstrated on ultrasonography. Another patient, an adolescent, 17/male, from Turkey, presenting with features of terminal ileitis and epididymo-orchitis, had a fever for 3 d, whose family dealt in livestock, gave a history of abortion in cattle[9]. A 31/male patient from Jordan presenting with ileocolitis, had an 11-mo long history of fever, night sweats, abdominal pain, diarrhea, and bleeding per rectum. Serology was positive for *Brucella abortus* IgM (1:160). The patient was treated with Rifampicin and Cotrimoxazole for 6 wk. Blood culture was however negative[10]. A 68/female from

Table 2 Summary of brucellosis cases with terminal ileitis as reported in the literature

Ref.	Country	Age/sex	Presenting Symptoms	Mode of transmission	Blood culture report	Serology report	Radiology report	Treatment	Outcome
Petrella *et al*[7], 1988	Texas	Child	-	Unpasteurized goat milk cheese consumption	-	-	Ileitis	-	-
Wang *et al* [8], 2017	China	15/male	Fever, intermittent, diffuse abdominal pain	H/O eating barbecue	Negative	Positive	USG-thickened ileum	Rifampicin and minocycline for 12 wk	Drastic improvement
Oguz *et al* [9], 2018	Turkey	17/male	Abdominal pain and a fever for 3 d	Cattle contact, recent abortion in cattle	Negative	Positive	USG-terminal ileum was edematous	Rifampicin and doxycycline 6 wk	Drastic improvement
Tamini *et al*[10], 2019	Jordan	31/male	Fever, chills, night sweats, abdominal pain for 11 mo	-	Negative	Positive	USG-normal, computed tomography - ileocaecal thickening	Rifampicin and cotrimoxazole for 6 wk	Gradual resolution of fever in 4 wk
Santos *et al* [11], 2020	Portugal	68/female	Night sweats, abdominal pain, weight loss (10%) for 4 mo	-	Positive for *Brucella* sp.	Positive	USG-thickened ileum	Rifampicin and doxycycline for 10 wk	Remarkable improvement
Noureen *et al*[12], 2020	Pakistan	32/male	Fever for 1 d, acute abdominal pain for 1 d	Unpasteurized milk consumption	Negative	Positive	Biopsy- ileitis	Surgery for 2 times, followed by rifampicin and doxycycline for 6 wk	Gradual improvement
Alejandro *et al*[13], 2021	Mexico	56/female	Severe abdominal pain for 12 h	-	-	Positive	Biopsy- ileitis, colitis	Surgery followed by rifampicin + cotrimoxazole for 6 wk	Improvement after surgery

CTScan: Computed tomography scan; USG: Ultrasonography.

Portugal tested positive by both blood cultures which showed growth of *Brucella* sp. and serology with 4 mo long duration of symptoms[11]. Our patient was also positive for *Brucella melitensis* on blood culture and serology reactive for *Brucella* IgG and IgM. A 32/male from Pakistan, with fever for 10 d and acute abdominal pain for 1 d, history of unpasteurized milk consumption, had to be operated on twice for intestinal perforation repair. His biopsy revealed inflammation of Peyer's patches[12]. The latest and last reported case is from Mexico, 56/female, with antithrombin III deficiency, presented to the emergency with acute abdomen, and had to be operated on to relieve intestinal obstruction. She was reactive for Rose Bengal Plate agglutination test (RBPT) (1:100). Biopsy revealed ileitis and colitis[13].

A total of 42.8% of cases (3/7) in literature are under 18 years of age. 67% (4/7) are males. The duration of symptoms ranged from as short as 12 h to as long as 11 mo. 50% of patients (3/6) had symptoms of ≥ 4 wk duration. The commonest symptoms were abdominal pain: 100% (6/6), fever: 83.3% (5/6), malaise: 50% (3/6), significant loss of weight equivalent to 10% of body weight: 16.6%. History of contact with cattle was given by 1/7 patient (14.2%)[9], unpasteurized dairy product consumption by 28.4% (2/7) of patients[7,11] history of eating barbecue 14.2% (1/7 patients)[8] and one 15/male (14.2%) came from a province in North China that is endemic for Brucellosis. For two patients cattle contact or unpasteurized dairy product consumption history was not available.

Serology was positive in all 6/6 (100%) cases for which details are available. Only one patient had a blood culture positive for *Brucella* sp. (14.2%) The species identification, however, is not available in that case[11]. 5/7 cases (71.4%) gave radiographic evidence of ileitis, three on ultrasound of abdomen (42.8%)[8,9,11], and one patient by CT scan. His Ultrasonography was normal[10]. The Texas child had radiographic evidence of ileitis too, but the method was unknown [7]. 2/7 of the patients (28.5%) underwent emergency surgery to relieve intestinal obstruction and repair of ileal perforation. Both patients' biopsies (28.5%) revealed ileitis[12,13].

Antimicrobial therapy was with Rifampicin and Doxycycline in three of the patients, one each from Turkey[9], Portugal[11], and Pakistan[12] for 6, 10, and 6 wk respectively. All three patients improved drastically. One patient each from Jordan[10] and Mexico[13] was treated with Rifampicin and trimethoprim-sulfamethoxazole combination for 6 wk, due to allergy to tetracyclines. The 15-year-old boy from China was treated with Rifampin and Minocycline (due to unavailability of Doxycycline) for a duration of 12 wk[8]. Recovery was complete in all cases (100%). Duration of therapy was 6 wk in 4/6 patients (66.6%) It was 12 wk in the Chinese on Minocycline[8]. The patient from Portugal had colitis along with ileitis, and diarrhea had not responded at the end of 6 wk, so the treatment was prolonged by an additional 4 wk[11]. Treatment details of the first reported case from Texas are unavailable. Our patient responded very well and recovered fully, after treatment with Rifampin and Doxycycline for 6 wk. Table 2 enumerates the summary of brucellosis

cases with terminal ileitis as reported in the literature.

Brucella melitensis causes the most severe infections among all *Brucella* sp. in humans. It is transmitted mainly through oral route and gains entry through ingestion by infecting the Peyer's patches in the small intestine[14]. Unpasteurized dairy products, improperly cooked meat, or rarely airborne transmission through abortus are common modes[15]. Our patient had a history of consumption of unpasteurized milk as well as contact with cattle during Bakri-Eid.

It's a worldwide zoonotic disease, endemic in Mexico, the Middle East, China, India, and African and Southern European nations[16]. In India, serological studies showed 1.28% positivity in rural Nagpur[17], 4.96% in Jammu region [18], 6.02% in Goa region[19], 8.5% in Gujarat[20] and up to 10.6% in Eastern India[21]. Yet Brucellosis is a forgotten entity when a differential diagnosis of PUO is being considered especially in children in the urban set-up. It is not just a debilitating illness, it also contributes to a significant economic burden. It has been estimated that annual median losses in India, due to Brucellosis in the human population, is Rs 442.3 million among adults and Rs 185.0 million among children [22]. A timely diagnosis can lead to the prevention of both mental and physical agony for the patients and their families along with a much lower financial burden. Given that epidemiological factors and patient history give a significant clue to the etiology of PUO, laboratory testing based on local data and a diagnostic algorithm may be helpful in diagnosing a large proportion of such cases. Since *Brucella melitensis* is a difficult-to-isolate pathogen, timely and adequate volumes of sample collection for blood, and bone marrow cultures, along with awareness and expertise on the part of laboratory personnel are important to diagnose this rare isolate. Serology is an easier option to diagnose this disease as the majority of cases are culture-negative. Several serological tests are available. RBPT, with high sensitivity but low specificity and ease of doing the test, is a good screening test. Titers of > 1:8 or 1:16 in endemic areas need to be confirmed by the Standard Tube agglutination test (SAT). SAT titers above 1:320 in endemic areas are suggestive of Brucellosis[23]. ELISA assays are extremely sensitive and specific. Other tests like dipstick assays, and lateral flow assays are also available. PCR from samples like classical cerebrospinal fluid is a promising test but with limited availability[23].

CONCLUSION

Alertness and coordination amongst treating pediatrician, physician, and microbiologists can lead to timely diagnosis of this relatively easy-to-treat cause of PUO, preventing dire complications like intestinal perforation and intussusception. To ensure prompt and correct diagnosis, a high index of suspicion, knowledge of local epidemiological data, detailed history collection, rapid access, and an effective healthcare setting are needed.

FOOTNOTES

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MINIREVIEWS

Monkeypox in humans: Transmission, pathophysiology, diagnosis, treatment, prevention, and all recent updates

Taral Parikh, Ashish Goti, Kanica Yashi, Nilesh Dankhara, Sandhya Kadam, Ramesh Dihora, Kapil Paiwal, Narendrasinh Parmar

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Abstract

The Centers for Disease Control and Prevention (CDC) is monitoring an epidemic of monkeypox infection in the United States. The outbreak is now global and more than 6900 cases have already been reported. There are 83 confirmed cases among children and adolescents, as shown in the report published on November 3, 2022, in the USA. However, monkeypox in pediatric patients is still infrequent (< 0.3% of total cases). Among cases in the United States, 16 cases were in children < 5 years, 12 in the age group 5-12 years, and 55 cases in adolescents 13-17 years old. In the adolescent age group, 89% were male. For children < 12 years of age, close physical contact with an adult household with monkeypox was the primary exposure, but for adolescents, male-to-male sexual contact was found more frequently. The CDC advised United States healthcare providers to remain vigilant for patients with a rash resembling monkeypox, even if there is no history

of travel to a country with high risk. This article summarizes the history and epidemiology of monkeypox with a specific emphasis on clinical features and management in pediatric patients.

Key Words: Pediatric monkeypox; Smallpox; Monkeypox case definition; JYNNEOS vaccine; ACAM2000

Core Tip: This article describes current updates on the clinical features and management of pediatric monkeypox infection.

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INTRODUCTION

The monkeypox virus is an orthopoxvirus that causes monkeypox. Orthpoxviruses that infect humans range from lethal small poxviruses to highly contagious but benign molluscum contagiosum viruses[1]. Monkeypox has always been found in West and Central Africa. However, in May 2022, the United States and other countries reported cases of monkeypox, even though there was not previously documented monkeypox transmission[2]. There are two distinct monkeypox virus classes: The Congo basin clade, mainly in central Africa, and the West Africa clade[3]. The Congo basin clade is known to cause disease with a severe impact and causes more morbidity and mortality. Human-to-human transmission has also been reported more frequently with the Congo basin clade.

Monkeypox in non-human primates

The monkeypox virus was first discovered in 1958 from a monkey in Copenhagen, Denmark, at the Staten's Serum Institute - and that is how it got its name[4]; monkeypox virus-hosts also include dormice, pouched rats, rope squirrels, and tree squirrels. Like many other zoonoses, Pox virus is known to be transmitted accidentally to a human when dealing with infected animals.

Monkeypox in humans

The Dominican Republic (DR) of the Congo noted the first known human case of monkeypox in 1970. Six unvaccinated people from the DR of Congo, Liberia, and Sierra Leone presented with an illness similar to smallpox on clinical presentation[5]. The DR Congo reported the first pediatric case in a 9-month-old infant. Four other children from Bouduo and Liberia aged 4 to 9 years were also affected. Three children close to these cases also developed a rash in the following days, indicating possible exposure. There was also the case of the 24-year-old male reported in Sierra Leone who was reported to have removed the stomach and intestine from a red monkey, and after 3-4 wk, he felt ill. No one died of monkeypox.

In the United States, monkeypox cases were first reported in 2003[6]. Seventy-one people were infected by Gambian pouched rats and prairie dogs, when they received a shipment of these infected animals as pets. The Centers for Disease Control and Prevention (CDC) and Wisconsin Research Department mentioned this outbreak in which patients presented with febrile illness with vesiculopustular eruption between May and June 2003. The five male and six female patients were aged between 3 and 43 years. The possible epidemiology, clinical, and laboratory investigations in this outbreak were also summarized. Contact with ill pet prairie dogs exposed to sick rodents from West Africa and Ghana was identified in all these patients. The illness started with a fever with or without chills, skin rash, and excessive sweating. All patients reported papular skin rash and headache; many reported fevers, chills, sweating, or persistent cough, and approximately half of the patients had lymphadenopathy. The characteristic rash started as a papule followed by a vesiculopustular lesion surrounded by erythema. Lesions finally resolved with serous fluid and a hemorrhagic crust with a mean duration of 12 d (3-25 d). All cases had a mild disease course, and only four were hospitalized, but recovered quickly. This was the first time monkeypox was identified among humans in the Western world. Only five adults were vaccinated against smallpox, while others were too young to receive the vaccine.

An outbreak of human monkeypox occurred in Nigeria in 2017[7]. There were 38 suspected cases, of which 18 received laboratory confirmation, three cases were probable, and 17 did not meet the case definition. Most of the confirmed cases were male adults. There was an association with varicella, syphilis, and human immunodeficiency virus (HIV) in two confirmed cases, and one healthcare worker had a nosocomial infection.

In September 2018, the United Kingdom reported monkeypox transmission from a patient to a healthcare worker[8]. The possible source of infection was contaminated bedding. The hospital undertook all possible infection control measures to control the outbreak. Four of the 134 possible cases exposed became ill, but the clinical course was mild.

Transmission

Monkeypox infection resembles smallpox, and the illness may be initially diagnosed as smallpox as both illnesses share similar clinical features[9]. Monkeypox was identified after the eradication of smallpox. Monkeypox is a zoonosis, although human-to-human transmission can occur. Monkeypox can spread due to close or skin-to-skin contact. Direct contact with monkeypox rash and contact with the patient's saliva, upper respiratory secretions, and areas around the anus, rectum, or vagina can lead to infection. It is not as contagious as smallpox among humans. Although monkeys and other primates are the primary reservoirs, other animals, such as squirrels and other rodents, can also be reservoir hosts for this virus. Pox virus deoxyribonucleic acid (DNA) has been identified in anal and urethral swabs from persons who neither demonstrated clinical signs nor reported symptoms of illness at the time of specimen collection. Few cases remained asymptomatic despite having known or possible sexual exposure to infected personnel[10].

How monkeypox relates to smallpox

In 1980 smallpox was declared eradicated worldwide, and the last reported case was in 1977. However, Huang *et al*[11] reported that it had been over 40 years since all countries stopped administering the smallpox vaccine. Previous history of vaccination against smallpox can provide some protection against monkeypox, but it is uncertain how long this protection lasts. In the 2003 monkeypox outbreak and 2022 outbreak, multiple infected patients with monkeypox had a history of smallpox vaccination in past decades[12].

Pathophysiology

The monkeypox virus enters the body *via* routes such as the oropharynx, nasopharynx, or intradermal and replicates at the inoculation site, then spreads to local lymph nodes[13], followed by viremia and infection of organs. The incubation period typically ranges from 7 to 14 d, with a maximum of 21 d. Symptoms start with fever and lymphadenopathy 1-2 d before developing skin lesions. In the 2022 outbreak, it was noted that monkeypox spread from when symptoms appeared to the phase where the rash had healed completely, and a new layer of skin had formed[14].

Case definition and clinical features

Below is the Case definition by the CDC and European Centre for Disease Prevention and Control guidance[14,15] (Table 1).

Exclusion criteria

Another diagnosis is made or an individual with suspected monkeypox does not develop clinical symptoms or a rash within five days or suspicious clinical specimens fail to demonstrate orthopoxvirus infection or antibodies against the infection.

Clinical features

Monkeypox rash begins with macules followed by papules, vesicles, and pustules. Pustules are characteristically deep-seated, firm, and well-circumscribed. These lesions can progress to become umbilicated or confluent but ultimately progress to scabs[16]. The rash can also spread to other parts of the body. Lesions on a distinct body part are at the same stage in classic monkeypox.

Classic symptomatology during monkeypox infection includes fever with chills, malaise, sore throat, and lymphadenopathy, followed by a characteristic rash. However, in the 2022 outbreak, some patients developed perianal and genital lesions but no fever or other systemic symptoms.

Monkeypox rash can mimic other common illnesses in clinical practice such as syphilis, herpes simplex virus and varicella zoster infection, chancroid, and molluscum contagiosum, and these illnesses can frequently be associated with monkeypox. Therefore, it is necessary that the clinician remains vigilant, especially with patients who present with the characteristic rash and men who practice sex with men (Table 2).

How long is monkeypox contagious?

As shown by Guarner *et al*[1], the infected person is not contagious during the incubation period. However, humans can be infectious as soon as symptoms begin until all scabs on the pox lesions fall off.

Diagnosis

When monkeypox is suspected in the United States; the clinician should contact the health department to determine the availability of testing, and lesions should be thoroughly swabbed and sent to testing laboratories. The monkeypox virus can be detected by an orthopoxviral polymerase chain reaction (PCR) test at a designated laboratory, and a positive PCR is enough for the diagnosis of monkeypox. When complex cases or positive laboratory results do not meet epidemiological criteria, the CDC should be consulted so that additional tests such as viral-specific or clade-specific PCR and blood testing can be conducted.

Complications

Reported complications are encephalitis, secondary skin infections, conjunctivitis, keratitis, and secondary pneumonia. During outbreaks in epidemic areas, mortality can be between 0% and 11%, affecting significantly young children[17]. Severe monkeypox infection is common in immunocompromised patients. Patients with HIV infection suffered more during the 2017 Nigeria outbreak than HIV-negative patients, with severe skin lesions and genital ulcers. However, no

Table 1 Case definition and clinical features

Suspect case	New-onset typical rash
	Fulfill one of the epidemiologic criteria and have a solid clinical possibility of monkeypox
Probable case	No other possible orthopoxviral exposure (*e.g.*, vaccination), and evidence of the presence of
	orthopoxviral DNA by PCR in the patient's sample
	Presence of orthopoxvirus using immunohistochemical or electron microscopy testing methods
	Positive anti-orthopoxviral IgM antibody after onset of rash for a duration of 4 to 56 d. Men who practice sex with men
Confirmed case	Evidence of monkeypox virus DNA detected by PCR in a patient specimen or detection of virus in clinical specimen culture
	Epidemiological criteria: Within three weeks of beginning the illness: Possible exposure to a person with a characteristic rash or who was diagnosed with monkeypox or a probable case, or following intimate exposure to individuals with monkeypox-like symptoms. Travel to a monkeypox endemic country outside the United States or a country with a monkeypox outbreak, or contact with a dead or live wild animal or pet from an endemic African region or a product obtained from such animals

DNA: Deoxyribonucleic acid; PCR: Polymerase chain reaction; IgM: Immunoglobulin M.

Table 2 Monkeypox symptoms and treatment options

Monkeypox symptoms	Treatment options
Monkeypox symptoms: Itchy, painful pimple/blister-like rash with several stages	Oral antihistamines, creams, and lotions such as calamine lotion. Keep the rash covered, do not scratch, soak in a warm bath, use oatmeal
Fever, chills, lymph node swelling, body ache, URI symptoms	Symptomatic pain medications
Severe disease involving eyes, mouth, throat, genitals and anus	Antiviral tecovirimat

URI: Upper respiratory infection.

deaths were reported. Between September 2017 and June 2022, Nigeria reported 257 confirmed cases, with nine deaths; of the nine patients who died, five were immunocompromised[18]. Disfiguring scars and corneal damage can be frequent significant sequelae. It was noted that vaccinated patients experienced fewer complications, and the secondary case rate in such households was lower[19]. As shown by Mbala *et al*[20], pregnant patients had more complications, including preterm delivery, fetal death, or congenital diseases. An observational study was performed at the Hospital in Kole between 2007 and 2011, which showed that of four pregnant women with monkeypox, who were included in the study, one had a full-term, healthy baby, two experienced a stillbirth in the first trimester, and the remaining patient experienced fetal death.

Precautions

Monkeypox spreads from human to human *via* exposure to the rash, close contact, or articles contaminated with contagious inflammation or body secretions[21]. Standard care is required for all suspected monkeypox patients. People with monkeypox who are not hospitalized require isolation at home. For confirmed monkeypox, isolation must continue until the rash has healed, the scabs have fallen off, and skin is intact.

Treatment

As shown by Rizk *et al*[22], monkeypox does not require treatment in all patients. Immunocompromised patients, children under eight years of age, pregnant or breastfeeding women, and those with eczema or exfoliative skin lesions are considered high risk. Also, patients with severe complications or rashes involving the eyes, mouth, and private areas may qualify for treatment.

Unfortunately, there are no treatment protocols for pediatric patients with monkeypox; however, local public health officials can help with CDC consultation to initiate antiviral therapy.

Tecovirimat was developed to treat smallpox, which can be used for monkeypox and is currently the first-line treatment for children. An oral dose in children of more than 13 kg is possible, which can be taken as a capsule, or the capsule's content can be mixed with food. In children less than 13 kg, the intravenous formulation can be considered depending on clinical status. Monitoring renal function is recommended, especially in children under two years of age.

The CDC is also developing a protocol for intravenous immunoglobulin in patients with monkeypox, but its effectiveness has not been established.

Brincidofovir was Food and Drug Administration (FDA) approved for smallpox treatment, and cidofovir was FDA-approved for cytomegalovirus retinitis in acquired immunodeficiency syndrome in the pediatric population. However,

there is still a lack of data on the effectiveness of brincidofoir and cidofovir in treating pediatric monkeypox.

Post-exposure prophylaxis

The CDC is conducting studies to determine how long immunity lasts after vaccination. They are looking at specimen samples from infected patients to determine whether the virus has changed. The CDC works closely with local and state partners to determine how the virus spreads among monkeypox patients. Studies have been carried out to assess how many patients were vaccinated, if they were fully vaccinated, and when they were vaccinated. Close monitoring of those newly diagnosed with monkeypox after vaccination is ongoing.

Two vaccines can be given to people who have been in contact with a monkeypox patient[23]. Data on post-exposure prophylaxis (PEP) in children are limited. JYNNEOS is the only vaccine that can be used in pediatrics. The decision to vaccinate must be made according to the level of risk in terms of the patient's exposure and health conditions. While vaccination is preferred in most cases, immunoglobulin may be considered in an infant less than six months of age. There is the possibility of using anti-viral medication after consultation with the appropriate CDC facility for PEP.

JYNNEOS

This vaccine has not been extensively studied in pediatrics for monkeypox; it contains non-replicating vaccine virus. This vaccine has been used in pediatrics for illnesses such as tuberculosis, Ebola, and measles without major side effects. In 2018-2019, this vaccine was used in the United Kingdom in pediatrics following monkeypox exposure without any major side effects. In the current outbreak, JYNNOS is available for children and adolescents under 18 years of age, who are classified as having high-risk exposure according to the CDC[24]. The dose is 0.5 mL for each subcutaneous injection with a two-dose series, and ideally, the first dose should be given within 96 h post-exposure[24].

ACAM2000

As shown by Singhal *et al*[25], this vaccine contains replicating viruses associated with side effects such as uncontrolled viral replication and eczema vaccinatum. It is not a preferred vaccine for pediatrics and should only be considered if JYNNEOS is unavailable or contraindicated.

Immunoglobulin: Immunoglobulin is approved under the emergency authorization for the prevention of monkeypox and is preferred for infants less than six months old with high-risk exposure[26].

CONCLUSION

Monkeypox virus is a very contagious orthopoxvirus currently causing a global outbreak, and primarily affecting men who have sex with men. After discontinuing the smallpox vaccine, population immunity decreased and led to an increase in monkeypox cases. Furthermore, the increased number of cases outside Africa demonstrates the global spread of the disease. Obtaining control over this infection requires doctors, hospitals, and health care officials to work together and define appropriate diagnostic testing, contact tracing, and availability of medical care to the affected patient. It is very important that pediatric physicians should be aware of the clinical course and possible outcomes in pediatric patients. Monkeypox seems scary, but it is still a sporadic disease, especially in pediatrics. However, it is always good to be aware of health risks.

FOOTNOTES

Author contributions: Parikh T, Goti A, Yashi K, Dankhara N, Kadam S, Dihora R, Paiwal K, and Parmar N contributed equally to study conception and design, data collection, analysis and interpretation of the results, and manuscript preparation.

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ORIGINAL ARTICLE

Retrospective Study

Analysis of clinical characteristics and risk factors between elderly patients with severe and nonsevere Omicron variant infection

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Abstract

BACKGROUND

Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has led to millions of confirmed cases and deaths worldwide. Elderly patients are at high risk of developing and dying from COVID-19 due to advanced age, decreased immune function, intense inflammatory response, and comorbidities. Shanghai has experienced a wave of infection with Omicron, a new variant of SARS-CoV-2, since March 2022. There is a pressing need to identify clinical features and risk factors for disease progression among elderly patients with Omicron infection to provide solid evidence for clinical policy-makers, public health officials, researchers, and the general public.

AIM

To investigate clinical characteristic differences and risk factors between elderly patients with severe and nonsevere Omicron SARS-CoV-2 variant infection.

METHODS

A total of 328 elderly patients with COVID-19 admitted to the Ninth People's Hospital Affiliated to Shanghai Jiao Tong University School of Medicine from April 2022 to June 2022 were enrolled and divided into a severe group (82 patients) and a nonsevere group (246 patients) according to the diagnosis and treatment protocol of COVID-19 (version 7). The clinical data and laboratory results of both groups were collected and compared. A chi-square test, *t* test, Mann-Whitney U test, hierarchical log-rank test, univariate and multivariate logistic regression, and hierarchical analyses were used to determine significant differences.

RESULTS

The severe group was older (84 *vs* 74 years, $P < 0.001$), included more males (57.3% *vs* 43.9%, $P = 0.037$), had a lower vaccination rate ($P < 0.001$), and had a

higher proportion of comorbidities, including chronic respiratory disease (P = 0.001), cerebral infarction (P < 0.001), chronic kidney disease (P = 0.002), and neurodegenerative disease (P < 0.001), than the nonsevere group. In addition, severe disease patients had a higher inflammatory index (P < 0.001), greater need for symptomatic treatment (P < 0.001), longer hospital stay (P = 0.011), extended viral shedding time (P = 0.014), and higher mortality than nonsevere disease patients (P < 0.001). No difference was observed in the application of Paxlovid in the severe and nonsevere groups (P = 0.817). Oxygen saturation, cerebral infarction, and D-dimer were predictive factors for developing severe disease in patients with COVID-19, with D-dimer having an excellent role (area under the curve: 90.1%, 95%CI: 86.1-94.0%). In addition, D-dimer was a risk factor for developing severe COVID-19 according to multivariate stratified analysis.

CONCLUSION

The clinical course of severe COVID-19 is complex, with a higher need for symptomatic treatment. D-dimer is a suitable biomarker for identifying patients at risk for developing severe COVID-19.

Key Words: Coronavirus disease 2019; Omicron; Severe infection; Elderly patients; Clinical features; Risk factor

Core Tip: Since March 2022, the Omicron wave has affected Shanghai, China. Many elderly patients with severe and nonsevere Omicron severe acute respiratory syndrome coronavirus 2 variant infections have been admitted to our hospital. These patients have a precise diagnosis, complete examination, and clear treatment results. After China adjusts its coronavirus prevention and control policies in 2023, findings such as those in this article will no longer be available.

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INTRODUCTION

Currently, coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has led to millions of confirmed cases and deaths around the world. As of 6:32 pm Central European time, September 27, 2023, there were 770875433 confirmed cases of COVID-19 globally, including 6959316 deaths, reported to the WHO[1].

SARS-CoV-2 not only affects the respiratory tract, causing pneumonia, but it can also affect the gastrointestinal tract, nervous system, and cardiovascular system[2,3]. The severity of symptoms in COVID-19 patients varies from asymptomatic to life-threatening[4]. Among all age groups, elderly patients, defined as 60 years of age or older, are at higher risk of developing and dying from COVID-19[5,6]. In a multicentre study in the Netherlands, the in-hospital mortality of older hospitalized patients with COVID-19 was 38%[7]. From the perspective of epidemic transmission, many older people with disabilities and severe cardiovascular and neurological diseases live together in close contact in long-term care centres, which facilitates transmission of the virus and leads to infection as well as progression of severe COVID-19 in the elderly[8,9]. Based on analysis of global COVID-19 data, it was concluded that the causes of severe illness in elderly infected patients are closely related to their advanced age, decreased immune function, intense inflammatory response in the body, and comorbidities. In previous studies, hypertension, atrial fibrillation, type 2 diabetes, chronic respiratory disease, dementia, and depression were associated with hospitalization rates and mortality in elderly patients with COVID-19[10-12].

Previous studies have shown that excessive inflammation, cytokine storms, and coagulopathy are important pathological mechanisms of COVID-19[13,14]. The neutrophil-to-lymphocyte ratio (NLR) reflects the systemic inflammatory response and level of neutrophil-to-lymphocyte activation. The systemic inflammatory response index (SIRI) may also reflect the host's immune and inflammatory balance[15]. Additionally, white blood cell count, neutrophil percentage, C-reactive protein (CRP), procalcitonin (PCT), D-dimer, and lactate are closely related to the severity and mortality of COVID-19[16-19].

Shanghai has experienced a wave of infection with Omicron, a new variant of SARS-CoV-2, since March 2022. The Omicron variant, which was first identified in Botswana and South Africa in November 2021, accounted for 41% of all strains by August 20, 2022[20]. Omicron has several subvariants, including BA.1, BA.2, BA.3, BA.4, and BA.5, all of which have a high transmission rate and significant antibody avoidance, posing a great threat to the prevention and control of COVID-19[21-23]. This study retrospectively analysed the baseline clinical features and risk factors of older patients with severe and nonsevere Omicron infection to provide solid evidence for clinical policy-makers, public health officials, researchers, and the general public, to help to identify high-risk groups, and to promote appropriate remediation.

MATERIALS AND METHODS

Subjects

Clinical data for 328 elderly patients diagnosed with COVID-19 and admitted to the Ninth People's Hospital Affiliated to Shanghai Jiao Tong University School of Medicine from April 2022 to June 2022 were collected during hospitalization. Confirmed diagnosis of COVID-19 was based on positive results for a nasopharyngeal swab sample tested by real-time reverse transcription polymerase chain reaction using a SARS-CoV-2 ZC-HX-201-2 kit (Biogerm, Shanghai, China). Elderly patients were defined as those diagnosed at age 60 years or older[6]. The discharge criteria for patients were as follows: (1) Body temperature returned to normal for more than 3 d; (2) respiratory symptoms improved obviously; (3) pulmonary imaging showed obvious absorption of inflammation; and (4) nucleic acid tests were negative twice consecutively (sampling interval of at least 24 h)[24].

In this study, 15 people died, comprising 0 nonsevere disease patients and 15 severe disease patients, and the direct cause of death was comorbidity. This study was approved by the Ethics Committee of the Ninth People's Hospital Affiliated to Shanghai Jiao Tong University School of Medicine (Ethics Approval No: SH9H-2022-T139-1).

Methods

Baseline data, vaccination status, onset time, onset symptoms, viral shedding time, comorbidities, laboratory data, therapeutic drugs, length of hospitalization, and survival for the 328 elderly patients with COVID-19 were collected. Laboratory tests included routine blood tests, CRP, PCT, coagulation function, liver function, cytokines, lactic acid, and other indicators. According to the discharge diagnosis and clinical data during hospitalization, the study cohort was divided into mild, general, severe, and critical severe types according to the clinical classification criteria of the novel coronavirus pneumonia diagnosis and treatment protocol (trial version 7)[24]: (1) Mild type: Fever and cough, nasal stuffiness, and other respiratory tract clinical symptoms are mild; no imaging manifestations of pneumonia; (2) general type: with the above clinical manifestations and imaging manifestations of pneumonia; (3) severe: Conformed to any of the following articles, including shortness of breath, respiratory frequency acuity 30 times/min; oxygen saturation 93% or less in the resting state; arterial blood oxygen partial pressure ≤ 300 mmHg or less oxygen concentration (1 mmHg = 0.133 kPa); and progressively worsening clinical symptoms and lung imaging showing lesions that progressed significantly more than 50% within 24-48 h; and (4) critical severe: Cases meeting any of the following criteria: respiratory failure and requiring mechanical ventilation; shock; with other organ failure requiring intensive care unit care.

Among the 328 elderly patients in this study, mild and general types were included in the nonsevere group (246 cases in total), whereas severe and critical severe types were included in the severe group (82 cases in total). The baseline data at admission, differences in mortality risk, and risk factors for developing severe disease among the patients in the severe and nonsevere groups were analysed retrospectively to verify the ability and clinical significance of using laboratory indicators to identify severe infection.

Statistical method

SPSS Software 25.0 (SPSS Inc., Chicago, United States) was used for statistical analysis. Measurement data with skewed distribution are represented by the median (interquartile range), while measurement data with normal distribution or approximate normal distribution are represented by the mean ± SD. The chi-square test or Fisher exact probability test and *t* test and the Mann-Whitney U test were used for comparisons between groups. Count data are expressed as the number of cases (percentage). A risk accumulation curve was determined using a stratified log-rank test and univariate and multivariate analyses with logistic regression. A receiver operating characteristic curve (ROC) was used to analyse and calculate the area under the curve (AUC). The optimal critical value of D-dimer and the corresponding sensitivity and specificity were calculated. The layered analysis was drawn by GraphPad 8.0 (GraphPad Software, San Diego, CA, United States). All tests were bilateral. A $P < 0.05$ was considered statistically significant.

RESULTS

Comparison of general data between severe and nonsevere COVID-19 patients

Among the 328 patients with COVID-19, 155 were males and 173 females, with a median age of 77 (68, 86) years. The severe infection group was older than the nonsevere infection group (84 *vs* 74 years, $P < 0.001$), included more males (57.3% *vs* 43.9%, $P = 0.037$), and had lower vaccination rates ($P < 0.001$). In terms of comorbidities, severe disease patients had higher rates of chronic respiratory disease ($P = 0.001$), cerebral infarction ($P < 0.001$), chronic kidney disease ($P = 0.002$), and neurodegenerative disease ($P < 0.001$) than nonsevere disease patients, and the difference was statistically significant. In terms of symptoms, the severe group included more patients with fever ($P < 0.001$), cough ($P < 0.001$), nasal stuffiness ($P = 0.026$), and other symptoms (including impaired smell, poor appetite, and nausea) than the nonsevere group ($P < 0.001$). In terms of disease severity, the inflammatory indicators SIRI, NLR, tumor necrosis factor-α, interleukin (IL)-10, IL-1, PCT, CRP, white blood cell, neutrophil percentage, lactic acid, and D-dimer in severe disease patients were significantly higher than those in nonsevere disease patients ($P < 0.001$). The glomerular filtration rate in severe disease patients was lower than that in nonsevere disease patients, and the difference was statistically significant ($P = 0.039$). Severe disease patients had significantly higher demands for respiratory support, glucocorticoids, anticoagulation (low molecular weight heparin or ordinary heparin), and antibiotics than nonsevere disease patients ($P < 0.001$). Application of Lianhua Qingwen granules in patients with severe COVID-19 was significantly lower than that in patients with

nonsevere COVID-19 (P = 0.007). There was no difference in the application of Paxlovid between the severe and nonsevere groups (P = 0.817). The length of hospitalization (P = 0.011) and virus shedding time (P = 0.014) in severe disease patients were higher than those in nonsevere disease patients, and the difference was statistically significant. In terms of clinical outcome, the number of deaths was 15, among which the mortality rate of nonsevere disease patients was 0% and that of severe disease patients was 18.29%. Thus, the mortality rate of severe disease patients was significantly higher than that of nonsevere disease patients (P < 0.001) (Table 1).

In this study, the viral shedding times of severe and nonsevere COVID-19 patients were 10.95 ± 7.74 and 8.65 ± 4.87 d, respectively. During the viral shedding period, a total of 15 patients died, all of whom had severe COVID-19. The cumulative incidence of death risk during viral shedding was higher in severe disease patients than in nonsevere disease patients (log-rank test = 36.286, P < 0.001) (Figure 1).

Univariate and multivariate analyses of the development of severe disease in elderly patients with COVID-19

Univariate and multivariate logistic regressions were used to analyse risk factors for developing severe infection in COVID-19 patients (Table 2). In univariate regression analysis, only oxygen saturation [Odds ratio (OR) = 0.513, 95%CI: 0.369-0.714; P < 0.001] was a risk factor for developing severe COVID-19. In multivariate logistic regression analysis, oxygen saturation (OR = 0.573, 95%CI: 0.451-0.728; P < 0.001), cerebral infarction (OR = 4.26, 95%CI: 1.012-17.937; P = 0.048), and D-dimer (OR = 1.394, 95%CI: 1.000-1.944; P = 0.05) were predictors of severe infection.

ROC curve analysis of elderly patients with severe COVID-19

A ROC curve was used to analyse and calculate the AUC of neutrophil percentage, CRP, D-dimer, NLR, SIRI, lactic acid, white blood cell count, and PCT indicators to assess the ability of each indicator to identify severe infection in elderly patients with COVID-19. Among them, the AUC of neutrophil percentage was 0.895, that of CRP 0.900, that of NLR 0.883, that of SIRI 0.854, that of lactic acid 0.764, that of white blood cell count 0.775, and that of PCT 0.871. The AUC of D-dimer was 0.901 (P < 0.001). When the threshold was 1.020 mg/L, the AUC was 90.1% (95%CI: 86.1%-94.0%). The sensitivity and specificity of D-dimer to identify severe disease in elderly patients with COVID-19 were 85.5% and 81.7%, respectively (Figure 2).

Multivariate stratified analysis of D-dimer levels in elderly patients with COVID-19

Figure 3 shows multivariate stratified analysis of D-dimer levels in elderly patients with COVID-19. Overall, D-dimer was a risk factor for the development of severe disease in elderly patients with COVID-19 (OR = 1.839, P < 0.001). In further variable stratification analysis, D-dimer remained a risk factor for the development of severe COVID-19, including in female patients (OR = 1.621, P < 0.001), male patients (OR = 2.288, P < 0.001), patients younger than the median age of 77 years (OR = 2.506, P < 0.001), patients older or equal to 77 years old (OR = 1.583, P < 0.001), patients not vaccinated against COVID-19 (OR = 1.702, P < 0.001), patients not vaccinated against COVID-19 (OR = 3.148, P = 0.006), patients without chronic respiratory disease (OR = 1.771, P < 0.001), patients with chronic respiratory disease (OR = 11.525, P = 0.006), patients without hypertension (OR = 1.621, P < 0.001), patients with hypertension (OR = 1.621, P < 0.001), patients without diabetes mellitus (OR = 1.754, P < 0.001), patients with diabetes mellitus (OR = 3.270, P = 0.002), patients without coronary heart disease (OR = 1.856, P < 0.001), patients with coronary heart disease (OR = 1.793, P = 0.009), patients without cerebral infarction (OR = 1.746, P < 0.001), patients with cerebral infarction (OR = 6.158, P = 0.002), patients without chronic kidney disease (OR = 1.811, P < 0.001), patients without immune system disease (OR = 1.886, P < 0.001), patients without neoplastic disease (OR = 1.802, P < 0.001), patients with neoplastic disease (OR = 3.161, P = 0.030), patients without neurodegenerative disease (OR = 1.765, P < 0.001), patients without other comorbidities (OR = 2.329, P < 0.001), patients with other comorbidities (OR = 1.492, P < 0.001), patients without Paxlovid (OR = 2.176, P < 0.001), patients with Paxlovid (OR = 1.739, P < 0.001), patients given Lianhua Qingwen granules (OR = 1.834, P < 0.001), and patients not given Lianhua Qingwen granules (OR = 1.835, P < 0.001).

In addition, in stratified analysis for chronic kidney disease (OR = 1.621, P = 0.067) and neurodegenerative disease (OR = 4.068, P = 0.075), although it did not achieve statistical significance, the OR of D-dimer was still greater than 1.0. In patients with immune system diseases (OR = 0.847, P = 0.753), the OR of D-dimer was less than 1.0, but there was no statistical significance.

DISCUSSION

Pneumonia is often regarded as a terminal event that complicates long-term diseases, such as dementia, cardiovascular disease, and cancer, in the elderly[25], SARS-CoV-2 mainly causes pulmonary interstitial pneumonia changes, typical bilateral patchy ground glass shadows, and peripheral consolidation. Compared with other age groups, the elderly seem to be more susceptible to COVID-19, and severe disease is an important reason for the high mortality rate and intensive care unit hospitalization rate of elderly patients with COVID-19[26,27]. In previous reports, the case fatality rate of elderly patients with COVID-19 ranged from 8.0% to 37.5%, increasing with age[26,28,29]. In addition, the population characteristics include a higher male proportion, intense inflammatory response in the body, prolonged viral shedding time, and prolonged hospital stay[26,30].

This study found that elderly patients with severe COVID-19 were older and comprised a higher proportion of males than nonsevere COVID-19 patients. The inflammatory reaction in severe disease patients was more intense than that in nonsevere disease patients. In addition, levels of lactic acid and D-dimer in severe disease patients were significantly higher than those in nonsevere disease patients, and the estimated glomerular filtration rate was lower. The length of

Table 1 Comparison of clinical data between the severe and non-severe groups of elderly patients with coronavirus disease 2019

Variables	Total (*n* = 328)	Non-severe (*n* = 246)	Severe (*n* = 82)	*P* value
Age (yr)	77.0 (68.0, 86.0)	74.0 (64.0, 84.0)	84.0 (75.0, 89.0)	< 0.001
Male sex	155 (47.4)	108 (43.9)	47 (57.3)	0.037
Vaccinations (times)	0 (0, 0)	0 (0, 2)	0 (0, 0)	< 0.001
Comorbidities				
Chronic respiratory disease	37 (11.2)	20 (7.8)	17 (20.7)	0.001
Hypertension	185 (56.4)	135 (54.8)	50 (61.0)	0.332
Diabetes mellitus	65 (19.9)	44 (17.8)	21 (25.6)	0.129
Coronary heart disease	56 (17.0)	40 (16.1)	16 (19.5)	0.478
Cerebral infarction	64 (19.6)	34 (13.5)	30 (36.6)	< 0.001
Chronic kidney disease	16 (4.8)	7 (2.6)	9 (11.0)	0.002
Immune system disease	6 (1.9)	5 (2.2)	1 (1.2)	0.589
Neoplastic disease	31 (9.6)	23 (9.6)	8 (9.8)	0.96
Neurodegenerative diseases	23 (7.1)	8 (3.0)	15 (18.3)	< 0.001
Other comorbidities	99 (30.1)	69 (27.8)	30 (36.6)	0.138
Symptoms				
Fever	61 (18.6)	33 (13.0)	28 (34.1)	< 0.001
Pharyngodynia	58 (17.6)	41 (16.5)	17 (20.7)	0.39
Cough	149 (45.5)	98 (39.6)	51 (62.2)	< 0.001
Nasal stuffiness	31 (9.6)	18 (7.4)	13 (15.8)	0.026
Diarrhea	3 (1.0)	1 (0.4)	2 (2.4)	0.11
Other symptoms	21 (6.4)	9 (3.5)	12 (14.6)	< 0.001
Laboratory data				
D-dimers (mg/ml)	0.66 (0.30, 1.85)	0.42 (0.21, 0.82)	3.19 (1.33, 7.32)	< 0.001
eGFR (ml/min/1.73 m^2)	76.63 ± 23.62	78.59 ± 20.82	71.09 ± 29.47	0.039
TNF-α (pg/ml)	9.78 (7.47, 12.60)	8.85 (7.12, 11.10)	12.37 (10.90, 18.80)	< 0.001
IL-10 (pg/ml)	5.00 (5.00, 5.82)	5.00 (5.00, 5.00)	6.47 (5.24, 8.99)	< 0.001
IL-1β (pg/ml)	5.00 (5.00, 5.55)	5.00 (5.00, 5.00)	5.55 (5.00, 7.17)	< 0.001
PCT (ng/ml)	1.58 ± 6.76	0.14 ± 0.57	5.66 ± 12.31	< 0.001
CRP (mg/L)	23.41 ± 38.25	9.83 ± 21.20	61.96 ± 48.30	< 0.001
WBC (10^9/L)	6.82 ± 3.29	5.92 ± 1.93	9.36 ± 4.72	< 0.001
Neutrophil percentage	69.80 ± 14.30	64.44 ± 11.38	85.02 ± 10.22	< 0.001
Lactic acid (mmol/L)	1.80 (1.37, 2.30)	1.64 (1.25, 2.00)	2.47 (1.81, 3.20)	< 0.001
SIRI	3.45 ± 5.08	1.71 ± 1.93	8.36 ± 7.48	< 0.001
NLR	5.27 ± 5.58	3.19 ± 3.05	11.17 ± 6.79	< 0.001
Oxygen saturation	96.13 (93.00, 97.13)	96.60 (95.98, 97.73)	91.00 (89.00, 92.90)	< 0.001
Treatment				
Respiratory support	108 (33.01)	29 (10.44)	79 (96.34)	< 0.001
Paxlovid	196 (59.62)	148 (60.00)	48 (58.54)	0.817
Glucocorticoids	78 (23.72)	24 (8.70)	54 (65.85)	< 0.001
Anticoagulation (low molecular weight heparin or regular heparin)	98 (29.81)	42 (16.09)	56 (68.29)	< 0.001

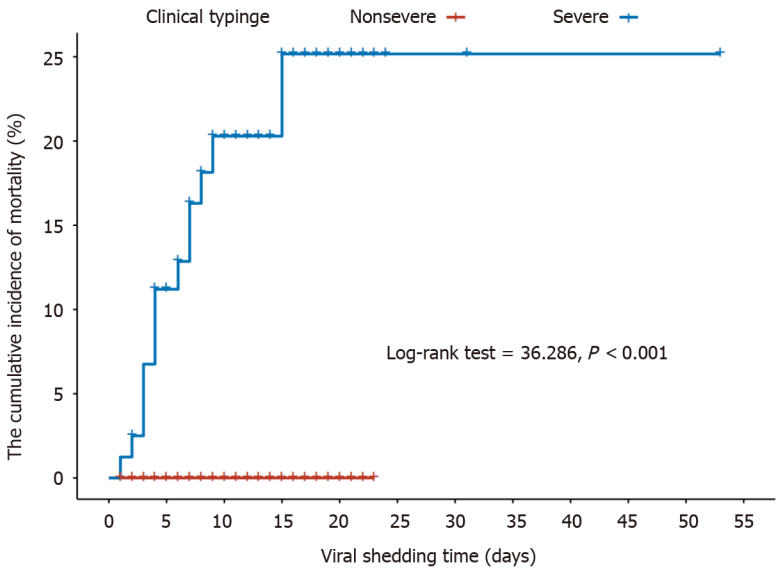
Lianhua Qingwen Granule	212 (64.74)	169 (69.13)	43 (52.44)	0.007
Antibiotics	113 (34.30)	46 (17.39)	67 (81.71)	< 0.001
Length of hospital stays (d)	8 (5, 11)	7 (5, 10)	8 (5, 14)	0.011
Viral shedding time (d)	9.25 ± 5.84	8.65 ± 4.87	10.95 ± 7.74	0.014
Death	15 (4.57)	0 (0.00)	15 (18.29)	< 0.001

Data are expressed as median (interquartile range), mean ± SD deviation, or number (percentage). eGFR: Estimated glomerular filtration rate; TNF: Tumor necrosis factor; IL: Interleukin; PCT: Procalcitonin; CRP: C-reactive protein; WBC: White blood cell; SIRI: Systemic inflammatory response index; NLR: Neutrophil to lymphocyte ratio.

Table 2 Univariate and multivariate analysis of the development of severe infection in elderly patients with coronavirus disease 2019

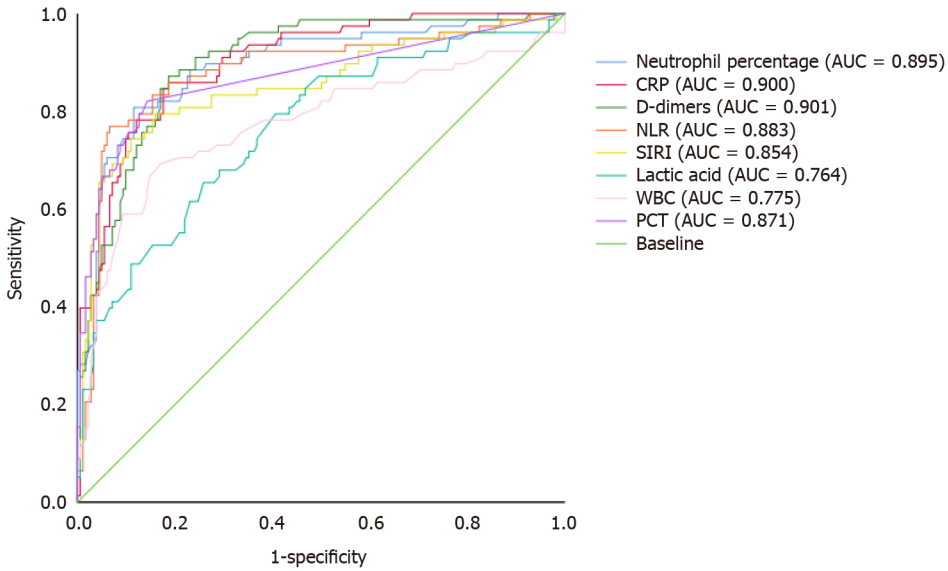
Variables	Univariate analysis OR (95%CI)	*P* value	Multivariate analysis OR (95%CI)	*P* value
Age	1.084 (0.978, 1.202)	0.126	-	-
Sex	0.575 (0.1, 3.322)	0.536	-	-
Vaccinations	0.636 (0.039, 10.385)	0.751	-	-
Chronic respiratory disease	0.559 (0.025, 12.453)	0.713	-	-
Diabetes mellitus	7.76 (0.446, 134.92)	0.16	-	-
Hypertension	3.267 (0.365, 29.275)	0.29	-	-
Coronary heart disease	0.142 (0.01, 1.926)	0.142	-	-
Cerebral infarction	7.757 (0.704, 85.443)	0.094	4.26 (1.012, 17.937)	0.048
Chronic kidney disease	0.057 (0.001, 4.578)	0.2	-	-
Neurodegenerative diseases	19.385 (0.149, 2527.003)	0.233	-	-
Neoplastic disease	0.527 (0.022, 12.846)	0.695	-	-
Immune system disease	0 (0, 203.169)	0.213	-	-
WBC	1.096 (0.669, 1.794)	0.716	-	-
Neutrophil percentage	1.125 (0.95, 1.331)	0.172	-	-
eGFR	1.044 (0.966, 1.128)	0.281	-	-
NLR	1.017 (0.636, 1.626)	0.943	-	-
SIRI	1.003 (0.534, 1.883)	0.993	-	-
CRP	1.023 (0.984, 1.063)	0.251	-	-
PCT	1.552 (0.67, 3.598)	0.305	-	-
Oxygen saturation	0.513 (0.369, 0.714)	0.000	0.573 (0.451, 0.728)	0.000
Lactic acid	0.768 (0.269, 2.194)	0.622	-	-
D-dimers	1.156 (0.754, 1.772)	0.507	1.394 (1, 1.944)	0.05
Viral shedding time	1.066 (0.892, 1.274)	0.484	-	-
Lianhua Qingwen Granule	0.486 (0.055, 4.302)	0.517	-	-
Paxlovid	2.505 (0.19, 33.049)	0.485	-	-

Bold letters represent significant predictors of the development of severe infection in elderly patients with coronavirus disease 2019. OR: Odds ratio; CI: Confidence interval; eGFR: Estimated glomerular filtration rate; NLR: Neutrophil to lymphocyte ratio; SIRI: Systemic inflammatory response index; CRP: C-reactive protein; PCT: Procalcitonin; WBC: White blood cell.



DOI: 10.5495/wjcid.v13.i4.37

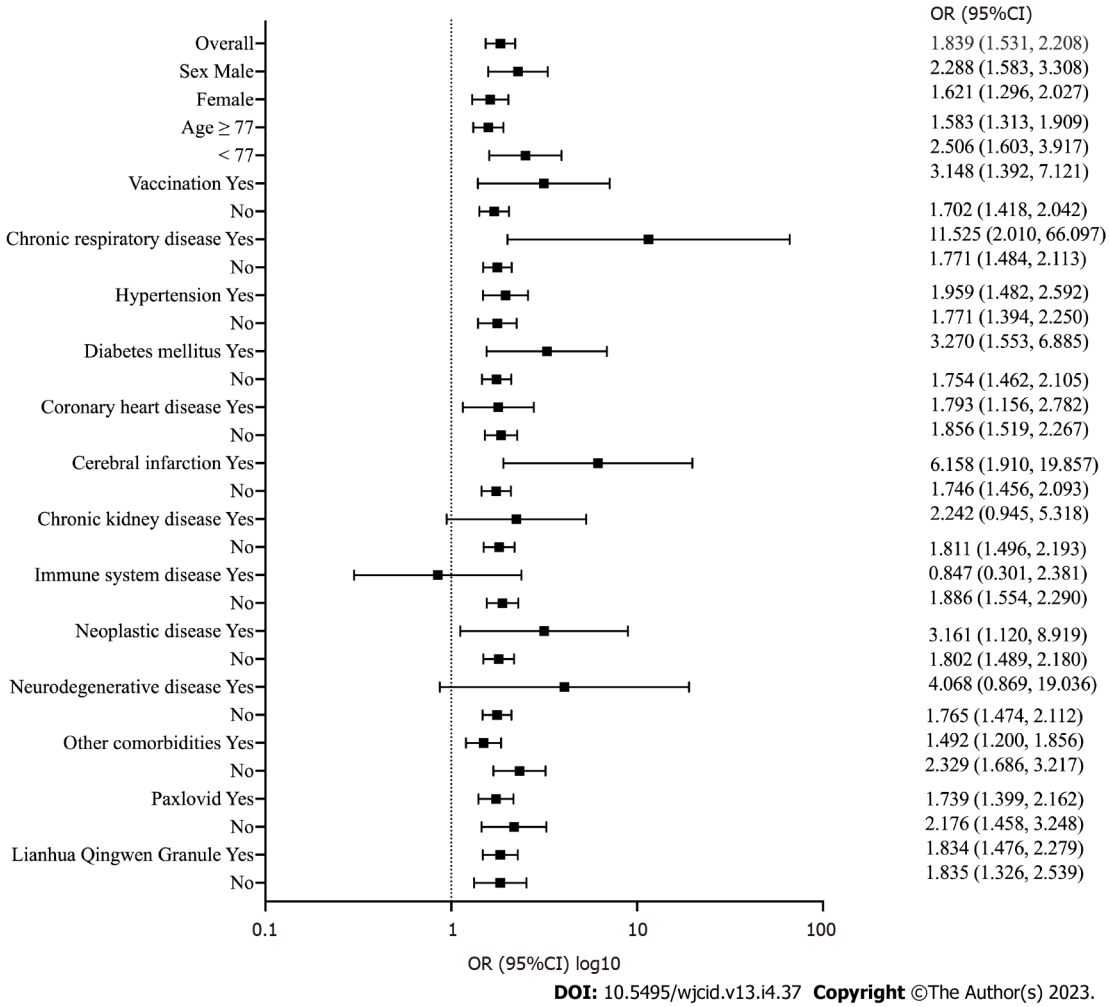
Figure 1 The cumulative incidence of mortality in elderly patients with severe and non-severe coronavirus disease 2019 during viral shedding time.



DOI: 10.5495/wjcid.v13.i4.37

Figure 2 Receiver operating characteristic curves in elderly patients with severe coronavirus disease 2019. CRP: C-reactive protein; NLR: Neutrophil to lymphocyte ratio; SIRI: Systemic inflammatory response index; WBC: White blood cell; PCT: Procalcitonin; AUC: Area under the curve.

hospitalization and viral shedding time of severe disease patients were longer than those of nonsevere disease patients. In this study, the severe infection group had lower vaccination rates than the nonsevere infection group; however, the vaccination status was not significant in univariate and multivariate analyses of the development of severe disease in elderly patients with COVID-19. This suggests that vaccination status is associated with a significantly lower risk of hospitalization for COVID-19 but is not associated with the development of severe COVID-19 in elderly patients, which was similar to a previous observational study[31]. Regarding the management and treatment of COVID-19 in this study, no difference was observed in the application of Paxlovid in the severe and nonsevere groups, suggesting that Paxlovid did not benefit patients in terms of avoiding the development of severe COVID-19 in this study. On the other hand, the need for respiratory support, glucocorticoids, anticoagulation (low molecular weight heparin or ordinary heparin), and antibiotic therapy was significantly higher in severe disease patients than in nonsevere disease patients. This is consistent with current research showing that COVID-19, similar to other community-acquired pneumonia, is considered to be a late-stage event that complicates long-term disease[26]. To personalize clinical management of COVID-19, researchers are also reflecting on better therapeutic strategies, including early adoption of non-steroidal anti-inflammatory drugs[32], application of broad-spectrum antimicrobials[33], and a personalized risk-benefit ratio for glucocorticoid use[34]. In terms of clinical outcome, 15 people died in this study; the mortality rate of nonsevere disease patients was 0%, and that of



DOI: 10.5495/wjcid.v13.i4.37

Figure 3 Multivariate stratified analysis of D-dimer levels in elderly patients with coronavirus disease 2019. OR: Odds ratio; CI: Confidence interval.

severe disease patients was 18.29%. Hence, the mortality rate of severe disease patients was significantly higher than that of nonsevere disease patients, which was also consistent with previous literature reports[26,30].

In addition to age, the presence and quantity of comorbidities are considered to be key factors in predicting the death of elderly patients. However, the significance of specific comorbidities, such as hypertension, coronary heart disease, and respiratory diseases, in the development of severe COVID-19 in elderly patients varied in previous research[35-38]. The results of this study also showed that the proportions of chronic respiratory diseases, cerebral infarction, chronic renal diseases, and neurodegenerative diseases were higher in severe disease patients than in nonsevere disease patients. Further analysis of the predictive factors of severe disease in elderly patients showed that among all comorbidities, cerebral infarction was the only risk factor for the development of severe disease in elderly patients with COVID-19 in this study.

In addition, studies have found that elderly patients from long-term care centres seem to have a higher rate of severe illness and fatality on admission than elderly patients from family care situations[26]. Indeed, staying in a long-term care centre is a strong risk factor for COVID-19 diagnosis and all-cause mortality[39]. Studies have suggested that this may be related to the fact that elderly COVID-19 patients living in long-term care centres usually have more comorbidities, are physically weaker, and are more susceptible to infection when in a closed environment[26]. In this study, cerebral infarction was a risk factor for severe COVID-19 in elderly patients. This can be explained by the fact that elderly people with cerebral infarction may need to stay in bed for a longer period and need increased daily nursing care. As a result, early identification of COVID-19 tends to be missed in this group of people, and they tend to receive insufficient nursing care after developing COVID-19, leading to severe infection in these patients. Therefore, the results of this study showed that elderly COVID-19 patients with cerebral infarction may be the most vulnerable group of elderly COVID-19 patients during the current wave of Omicron infection in Shanghai.

In previous studies, plasma D-dimer levels were directly related to the development of pulmonary embolism and vascular thrombosis complications during COVID-19 and correlated highly with adverse outcomes[40,41]. In this study, D-dimer was also a risk factor for the development of severe COVID-19 in elderly patients, which is consistent with previous literature reports[42]. In previous studies, NLR, CRP, and neutrophil percentage were demonstrated to be predictors of severe diseases, showing good recognition ability for severe COVID-19[17,37,43]. In this study, it was found that compared with white blood cell count, neutrophil percentage, CRP, PCT, NLR, SIRI, and lactic acid, the ROC curve

of D-dimer yielded the largest AUC, with good sensitivity and specificity and an outstanding ability to identify severe COVID-19. In multivariate stratified analysis, D-dimer was a risk factor for the development of severe COVID-19 in elderly patients both at the overall level and stratified by sex, age, vaccination, chronic respiratory disease, hypertension, diabetes mellitus, coronary heart disease, cerebral infarction, chronic kidney disease, immune system disease, neoplastic disease, neurodegenerative disease, other comorbidities, use of Paxlovid, and use of Lianhua Qingwen granules. This confirms the important role of D-dimer in the course and outcome of COVID-19 in elderly patients.

This study has at least two limitations. First, the sample size was small, especially regarding the number of patients in the severe disease group. This may be related to the relatively reduced pathogenicity of the Omicron subtype in the current wave of COVID-19 and the protective effect of the vaccine in reducing the risk of hospitalization for COVID-19, which is a result of active participation in receiving the vaccine in Shanghai. Second, this study was a single-center study, and the patients were limited to those diagnosed with COVID-19 and admitted to the Ninth People's Hospital Affiliated to Shanghai Jiao Tong University School of Medicine from April 2022 to June 2022. Because the outbreak is evolving rapidly around the world, follow-up studies with more patients are needed to improve the statistical power of these findings.

CONCLUSION

In conclusion, the results of this study suggest that COVID-19 complicates long-term illness in elderly patients. There are considerable differences in disease severity and adverse clinical outcomes between severe and nonsevere cases in older patients with COVID-19. Elderly people are vulnerable to severe illness and death due to their age and comorbidities, especially elderly patients with preexisting cerebral infarction. D-dimer is a risk factor for severe COVID-19 in elderly patients and has a good recognition function for severe disease. Therefore, a comprehensive assessment of the comorbidities of older patients with COVID-19 may help to establish risk stratification for admission of COVID-19 patients, and dynamic monitoring of D-dimer levels can provide valuable information for planning appropriate interventions at the health assistance level.

ARTICLE HIGHLIGHTS

Research background

Elderly patients are at higher risk of contracting and dying from coronavirus disease 2019 (COVID-19) due to advanced age, decreased immune function, intense inflammatory response, and comorbidities. Omicron, a new variant of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has a high transmission rate and significant antibody avoidance, posing a great threat to the prevention and control of COVID-19.

Research motivation

Previous studies have evaluated risk factors for severity or death among elderly people with COVID-19, though analyses of Omicron infection risk and protective factors among elderly people are relatively few.

Research objectives

To identify clinical features and risk factors for disease progression among elderly patients with Omicron infection to provide solid evidence for clinical policy-makers, public health officials, researchers, and the general public.

Research methods

A chi-square test, *t* test, Mann-Whitney U test, hierarchical log-rank test, univariate and multivariate logistic regression analyses, and hierarchical analyses were used to determine significant differences between elderly patients with severe and nonsevere Omicron SARS-CoV-2 variant infection.

Research results

The clinical course of severe disease patients is more complex, as both the need for symptomatic treatment and the risk of death are higher than those of nonsevere disease patients. Oxygen saturation, cerebral infarction, and D-dimer are risk factors for developing severe COVID-19. D-dimer also showed a suitable role in identifying severe infection.

Research conclusions

Elderly people are vulnerable to severe illness and death due to their age and comorbidities, especially elderly patients with preexisting cerebral infarction. D-dimer is a risk factor for severe COVID-19 in elderly patients and has a good recognition function for severe disease.

Research perspectives

A comprehensive assessment of the comorbidities of older patients with COVID-19 may help to establish risk stratification for admission of COVID-19 patients, and dynamic monitoring of D-dimer levels can provide valuable information for planning appropriate interventions at the health assistance level.

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FOOTNOTES

Author contributions: Liu XQ designed the research study, analysed the data, and wrote the manuscript; Lu GZ, Yin DL, Kang YY, Zhou YY, and Wang YH collected and analysed the data; Xu J designed the research study and reviewed and revised the paper.

Institutional review board statement: This study was reviewed and approved by the Ethics Committee of the Ninth People's Hospital Affiliated to Shanghai Jiao Tong University School of Medicine (Ethics Approval No.: SH9H-2022-T139-1).

Informed consent statement: As the study used anonymous and pre-existing data, the requirement for the informed consent from patients was waived.

Conflict-of-interest statement: We have no financial relationships to disclose.

Data sharing statement: No additional data are available.

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MINIREVIEWS

Helicobacter pylori infection in pregnant women: Gastrointestinal symptoms and pregnancy- related disorders

Luana Kauany de Sá Santos, Jonathan Santos Apolonio, Beatriz Rocha Cuzzuol, Bruna Teixeira da Costa, Vinícius Lima de Souza Gonçalves, Ronaldo Teixeira da Silva Júnior, Marcel Silva Luz, Fabian Fellipe Bueno Lemos, Samuel Luca Rocha Pinheiro, Fabrício Freire de Melo

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Abstract

Helicobacter pylori (H. Pylori) is a gram-negative, flagellated and spiral-shaped bacterial pathogen that impacts approximately 46% among pregnant women globally and has been associated with various maternal-fetal complications. Iron deficiency anemia, fetal growth restriction, cardiovascular diseases, and insufficient nutrient absorption can be observed in pregnant women, as well as miscarriages and pregnancy-specific hypertensive disease, such as pre-eclampsia. Thus, the evidence supports the influence of *H. pylori* infection on fetal implantation/placentation failure, and positive strains of the cytotoxin-associated gene A of *H. Pylori* were reported as the most prevalent in these conditions. However, current knowledge indicates a relationship between this infection and the occurrence of hyperemesis gravidarum, characterized by frequent nausea and vomiting. Regarding the diagnosis of this bacterial infection, non-invasive approaches such as stool antigen test, urea breath test, and serological tests are more accepted during pregnancy, as they are easy to carry out and cost-effective. Finally, the bacteria eradication therapy should consider the risks and benefits for the pregnant woman and her child, with pharmacological intervention depending on the clinical presentation.

Key Words: *Helicobacter pylori*; Pregnancy; Hyperemesis gravidarum; Iron deficiency anemia; Pre-eclampsia; Fetal growth restriction; Miscarriage

Core Tip: *Helicobacter pylori* infection during pregnancy is related to the development of disorders that may pose risks to maternal life and affect the proper development of the child. This bacterium has been associated with various complications such as hyperemesis gravidarum, iron deficiency anemia, pregnancy-specific hypertensive disease like pre-eclampsia, fetal growth restriction, and miscarriage. Therefore, this review provides a comprehensive overview of this condition, as well as its diagnosis and treatment, bringing together the most up-to-date information on the subject.

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INTRODUCTION

Helicobacter pylori (H. pylori) is a Gram-negative, spiral-shaped bacterial pathogen[1], which affects roughly half of the people worldwide[2]. Its virulence factors, such as the production of the enzyme urease, which aids in the hydrolysis of gastric urea into ammonia, providing for the neutralization of gastric pH, have been related to bacterial survival in the stomach acid and colonization of this region, contributing to the development of gastric disorders[3], such as gastritis, ulcers, dyspepsia, and carcinomas[2,4]. However, despite a high prevalence of this bacterium in the world's population, studies report that infection tends to be asymptomatic in approximately 90% of infected patients[5].

Epidemiological data has shown that, across all age groups, the prevalence of *H. pylori* prevalence is reduced in developed countries compared to developing countries[6], with the latter having approximately 80% of the population over 50 years old infected by *H. pylori*[3]. Moreover, some studies have shown that approximately 60% of the Brazilian population is infected by the bacterium. The transmission routes of *H. pylori* are not fully established, but available data suggests that transmission tends to occur, mainly before the age of 10, through fecal-oral contact, person to person, considered the most likely route, and through the consumption of contaminated food or water[7].

Furthermore, approximately 46% of the world's pregnant women are seropositive for *H. pylori*, and the implications of the infection are related to maternal and fetal life impairments. Among the main complications, anemia, fetal growth restriction, cardiovascular diseases, and insufficient nutrient absorption are the most reported, although there are also reports of miscarriages and the development of pre-eclampsia in these women[8,9].

This review aims to identify the main complications related to *H. pylori* infection during pregnancy and discuss the diagnosis and treatment associated with this condition.

GASTROINTESTINAL SYMPTOMS AND DISORDERS IN PREGNANCY

Gastrointestinal discomfort during pregnancy is common and associated with hormonal and mechanical factors. There is a direct connection with esophageal reflux due to the loss of sphincter tone. The high availability of steroids, as well as frequent vomiting, can alter the gastrointestinal pH. This adjustment would predispose to the development of *H. pylori* infection, which is supported by the higher seropositivity of the bacterium in pregnant women when compared to other populations[10-12].

Occasional nausea and vomiting are concerns in early pregnancy. On the other hand, hyperemesis gravidarum, characterized as continual and excessive nausea and vomiting starting prior to the completion of the 22nd week of pregnancy, accompanied in a decrease in body weight, dehydration, electrolyte, and metabolic disturbances, affects only 0.3%-2% of all pregnant women[13-15].

Current evidence indicates that *H. pylori* infection plays a role in the occurrence of occasional nausea and vomiting, suggesting that hyperemesis gravidarum is a consequence of different unrelated disorders, and *H. pylori* is one of the recently recognized factors for this condition[13,16].

One study proposes that the accumulation of bodily fluids, hormonal changes, and immune tolerance in a woman lead to a reduction in gastric acid production, which can trigger the activation of *H. pylori* infection and result in symptoms such as nausea and vomiting[17].

Another study suggests that colonization of *H. pylori* in the gastric mucosa leads to the production of toxins and induces mucosal damage, resulting in local inflammation. This scenario during pregnancy is responsible for the worsening of the clinical picture of hyperemesis gravidarum[16]. Cytotoxin-associated gene A (CagA) plays an important role in *H. pylori* virulence, generally associated with severe peptic ulceration and tissue damage. Thus, women with intense inflammatory response and CagA seropositive infection are associated with more severe hyperemesis gravidarum [16]. It has also been demonstrated that CagA seropositivity predominates in pregnant women with *H. pylori* infection [18].

A prospective population-based cohort study of pregnant women showed that *H. pylori* was positively associated with women with daily vomiting (64.4%), and CagA-positivity was predominant. On the other hand, 39.9% of women who did not experience vomiting or had occasional vomiting tested positive for *H. pylori*, while 62.4% of women experiencing

daily vomiting were negative[15].

A prospective study showed that 70% of pregnant women with hyperemesis gravidarum were seropositive for *H. pylori*, and the severity and recurrence of vomiting were higher among these women[13]. Another study demonstrated a prevalence of 75% seropositivity for *H. pylori* in women with hyperemesis gravidarum[19].

H. pylori positivity was also associated with a reduced total weight gain with daily vomiting, an increased risk of the fetus being small for gestational age, and reduced birth weight[14]. Therefore, the eradication of the bacterium should be the primary goal in reducing hyperemesis gravidarum, nausea, and vomiting[15].

It is common for dyspepsia symptoms to be confused with those of hyperemesis gravidarum. Patients with dyspepsia report frequent bloating and symptoms of gas and tightness; in addition, sensations of gastric pain, burning and early satiety. Postprandial nausea is common, but vomiting is rare and also a differential diagnostic indicator[10,11,20].

Dyspepsia symptoms in pregnant women seropositive for *H. pylori* were related to age. Those between 24 and 37 years were more likely to develop dyspeptic symptoms when compared to seronegative women. Factors such as obesity and other factors like parity and late stages of pregnancy did not show significance in seropositivity results[10]. In addition to this study, more recent works do not significantly correlate dyspepsia symptoms with *H. pylori* alterations, although dyspepsia symptoms have a slightly higher seroprevalence compared to patients not infected by the bacterium[11,17,21].

However, studies found an association between CagA seropositivity and the development of dyspepsia during pregnancy, in which patients with the virulence factor suffering much more from dyspeptic symptoms[17,21].

PREGNANCY-RELATED DISORDERS

Iron deficiency anemia

During the gestational period, there is a growing need for iron to meet maternal and fetal requirements, which typically cannot be provided by regular diets alone and, therefore, needs to be supplemented to meet physiological needs. Iron deficiency anemia is a significant clinical condition as it may contribute to approximately 40% of maternal deaths in developing countries, with *H. pylori* infection associated with its genesis[22].

Research has found a high prevalence of this bacterium in pregnant women with anemia, demonstrating that *H. pylori* infection may be related to alterations in iron metabolism[23]. Furthermore, studies have also shown that iron deficiency was more prevalent in patients seropositive for *H. pylori* infection than in those who were not seropositive and that during the early stages of pregnancy, women infected with *H. pylori* have lower hemoglobin levels and less capacity to regulate these levels over time, when compared to uninfected women[17].

Among the possible hypotheses for the development of anemia during *H. pylori* infection in pregnancy are chronic inflammation leading to gastric damage and peptic ulcers that can favor blood loss through hidden bleedings in feces, and the competition for iron between the gastric cells and the bacterium, leading to a decrease in the absorption of this mineral by the organism[24]. On the other hand, chronic gastritis can also contribute to the reduced release of ascorbic acid in gastric juice and stimulate the production of hepcidin, responsible for regulating iron absorption through binding to ferroportin, leading to alterations in iron metabolism[25].

Finally, a study conducted with 40 women demonstrated that iron supplementation in pregnant women after *H. pylori* eradication therapy was able to contribute to positive outcomes in improving cases of anemia. However, it is essential to assess the impact of infection treatment in a larger group of pregnant women, as well as long-term follow-up of the study population[22].

Pre-eclampsia and fetal restriction growth

Pre-eclampsia (PE) is a significant contributor of fetal and maternal morbidity and mortality, affecting 2%-8% of all pregnant women[26]. Its onset typically occurs after 20 wk of pregnancy and leads to intense maternal inflammatory reaction, elevated pro-inflammatory cytokines concentrations in the blood, and harm to the endothelial cells[27,28]. Moreover, PE can also lead to impaired placentation, vascular dysfunction, gestational hypertension, and proteinuria[26].

Studies have shown a strong association between *H. pylori* and PE, concluding that this bacterium is a potential risk factor for pre-eclampsia. One of the possible explanations for this association is that *H. pylori* infection induces endothelial dysfunction, which, combined with inflammation and oxidative stress, influences the development of PE[29,30]. *H. pylori* can also trigger the activation of cascades and secrete cytokines, such as TNF-alpha, and stimulates proinflammatory cytokines. This process can cause damage to blood vessels. Additionally, free radicals can lead to oxidative stress and increased lipid peroxidation, resulting in endothelial injury and elevated blood pressure[26,31]. A systematic review concluded that women infected with *H. pylori* have a higher risk of developing PE compared to seronegative women. Furthermore, two case-control studies also demonstrated a higher frequency of seropositivity for *H. pylori* among women with PE compared to controls[17].

CagA-positive strains also play an important role in the onset of PE. Some studies have shown that antibodies against CagA can recognize antigens in endothelial cells and cross-react with placental tissue, negatively impacting its invasiveness[27,32,33]. CagA may also be related to abnormal placentation and exhibits higher virulence, which may be associated with generalized inflammation and vascular damage[9,33]. Another study suggests that VacA-positive strains are not strongly associated with severe systemic inflammation on their own, but, when combined with CagA-positive strains, they are strongly associated with typical PE responses[27].

PE may also be associated with fetal growth retardation (FGR), as vascular disorders directly affect fetal growth[34]. FGR is characterized as a failure by the fetus not reaching its genetically predetermined growth capacity and corresponds to 3%-10% of infants[27]. Infections and PE are two of its possible causes. Researchers have observed an association

between *H. pylori* infections and low birth weight, noting that pregnancies seropositive for *H. pylori* showed more intrauterine growth restriction than women seronegative for *H. pylori*[34,35].

One possible mechanism behind this process is that *H. pylori* can induce dyspepsia, nausea, vomiting, and anemia, which can lead to reduced fetal absorption and growth[36]. The impact of CagA on placental invasiveness and abnormal placentation can also lead to FGR[36,37] reduced fetal absorption and growth. Moreover, one study reported that seropositivity for CagA and VacA was significantly higher in PE-FGR pregnancies. Seronegativity for CagA and VacA may be associated with a lower risk of developing PE and RCF[27,38].

Miscarriage

The rate of early loss among clinically recognized pregnancies, is estimated to be between 12% and 15%, affecting about 2% of the reproductive age population and resulting in fetal death before 23 wk of gestation[39,40]. Many factors are associated with miscarriage, such as anatomical, endocrine, genetic, infectious, and immunological disorders[41]. Among them, maternal infections by some etiological agents such as Chlamydia trachomatis, cytomegalovirus, Toxoplasma gondii, Mycoplasma hominis and Listeria monocytogenes, were related to single abortion, however, infections are less relevant compared to other etiological factors[17,42].

However, a study found an association between maternal infection with CagA-positive strains of *H. pylori* and early pregnancy loss in patients undergoing intracytoplasmic sperm injection[43]. The results findings indicated a notably greater prevalence of *H. pylori* in human immunodeficiency virus positive females within the group of primigravid women who experienced a miscarriage compared to the control group, meanwhile, the presence of maternal serum antibodies against *H. pylori* did not seem to correlate with recurrent miscarriages[44]. This evidence suggests a connection between *H. pylori* infection and implantation/placental failure, potentially as a result of the interaction between antibodies targeting *H. pylori* and placental cells[45].

DIAGNOSIS

Regarding *H. pylori* diagnosis, there are several tests capable of detecting the bacterium, which are selected taking into consideration its benefits, limitations and the clinical situation of the patient[46]. Generally, these diagnostic methods are divided into invasive and non-invasive tests, with the former including histology, culture, rapid urease test (RUT), and molecular methods, while the latter refers to urea breath tests, stool antigen, and serology[47,48].

Among invasive procedures, histology was the first and probably the most widely used method for diagnosing *H. pylori*, and consists of a way to analyze common inflammatory patterns during infection in tissue slides[48]. In addition, culturing gastric biopsy samples is a very specific method, although it is expensive, hard to perform and not as sensitive, while RUT is a simple, rapid and specific test, that works through the conversion of urea into carbon dioxide and ammonia by urease in a urea-rich medium, which increases the reagent pH[49]. Lastly, the polymerase chain reaction (PCR) is also used to detect *H. pylori* infection and may even be more accurate than RUT[50].

Regarding endoscopy as a diagnostic method for *H. pylori* infection in pregnant women, several studies indicate that this method is not suitable, impossible, or prohibited for this population[23,51,52]. However, some studies have performed this procedure to link hyperemesis gravidarum to *H. pylori* infection, highlighting the lack of protocols and studies to determine the best management for the diagnosis of this bacterium in pregnant women.

On the other hand, non-invasive approaches are more generally accepted during the prenatal period[17,53]. As mentioned above, among the various attempts to avoid endoscopic diagnostic methods, some procedures, such as the urea breath test (UBT), stool antigen test (SAT), and antibody-based tests, were developed as an alternative choice[1]. However, they do not provide data on antibiotic resistance, so further analysis is required[52].

Stool antigen and serologic exams are the primary preference for *H. pylori* infection analysis during pregnancy, due to the fact they are easy to carry out and low-cost non-invasive diagnostic exams[17]. Within this context, the SAT is an enzymatic immunoassay based on polyclonal antibodies identifying the effective presence of *H. pylori* antigen in human fecal samples and is the favored choice for assessing the pathogen's condition following eradication. Additionally, research has shown the possibility to diagnose *H. pylori* infection through a stool antigen test in amniotic fluid[54].

In contrast, serological analyses are generally based on identifying particular anti-*H. pylori* immunoglobulin G antibodies towards *H. pylori* provides insight into an immune reaction that can be associated with both existing infection and past contact, as they typically vanish for only a few months following the eradication of the microorganism[48].

Finally, and equally important, urea breath tests are not typically used throughout pregnancy, regardless of their reliability and safety. Because of *H. pylori's* urease activity, when a patient ingests urea labeled with either 13C or 14C, it is hydrolyzed in the stomach, resulting in labeled CO2, this labeled CO2 is then absorbed into the bloodstream and exhaled when the patient inhaling, allowing for the measurement of the labeled CO2[46].

In fact, it has been proven that the 13C-urea breath test, which employs the non-radioactive stable isotope 13C as a tracer, is not radioactive and secure also in kids and pregnant women[10]. Nonetheless, one downside of the UBT, despite its high sensitivity and specificity, is its cost. This test is expensive and requires specialized equipment and personnel[52].

In the case of a recognized pregnancy, the test should only be performed if the benefits outweigh the risks, despite the confirmation that the ionizing radiation dose associated with ^{14}C-urea breath tests is exceptionally low, less than the radiation exposure from naturally occurring sources, and a thousand times less than the fetal radiation threshold considered teratogenic[55-57]. Thus, in case of unintentional exposure throughout pregnancy, the pregnant women need to be pacified[58]

TREATMENT

It is well known that there are multiple options for the treatment of *H. pylori* infection. Since the Maastricht Consensus of 1997, followed by the Canadian *H. pylori* Consensus of 1998, triple therapy with clarithromycin (500 mg), metronidazole (500 mg) or amoxicillin (1000mg), and standard-dose proton pump inhibitors twice daily for 7 to 10 days has been employed as the first-line regimen for eradication of *H. pylori* in several countries[59,60]. However, due to increasing rates of clarithromycin resistance in many regions, the main guidelines for the treatment of *H. pylori* infection, produced by expert groups in Europe, United States and Canada, currently indicate quadruple bismuth therapy (QBT) as the first choice regimen[61,62]. The QBT comprises proton pump inhibitors (PPI) (standard dose, twice daily), bismuth (four times daily), metronidazole (400 mg, four times daily, or 500 mg, three to four times daily) and tetracycline (500 mg, four times daily) for a duration of 10-14 d. In regions where bismuth is not available, the guidelines recommend an alternative non-bismuth quadruple therapy involving PPI (standard dose, twice daily), amoxicillin (1000 mg, twice daily), metronidazole (500 mg, twice daily) and clarithromycin (500 mg, twice daily) for 10-14 d[63-65]. Nevertheless, in spite of these efforts to establish a standard treatment protocol for the infection, increasing resistance of *H. pylori* to multiple antibiotics has made eradication of the bacterium a major concern[66].

Despite the wealth of literature that includes evidence associated with *H. pylori* eradication therapies in the general population, there are currently no specific guidelines for the treatment of this infection in pregnant women[67]. Nevertheless, some studies have reported the improvement of gastrointestinal symptoms in pregnant women who tested positive for *H. pylori* after the use of antibiotics, especially in patients with hyperemesis gravidarum[11,68-70].

The applicability of eradication therapy in pregnant women should mainly take into account the dichotomy of fetal risk *vs* symptom relief and eradication of the bacterium. The potential risks of using medications during pregnancy are known, especially because of the fetal toxicity danger. The use of tetracycline in some therapeutic regimens may imply the inhibition of bone growth and discoloration of teeth[71], and some studies also report that the use of clarithromycin in the first trimester leads to an increased risk of miscarriage, without increasing the risk of congenital malformations[72]. The World Health Organisation also recommends avoiding, when possible, the use of metronidazole, as there are animal studies demonstrating possible carcinogenic effects.

In this regard, several authors have suggested that the viability of pharmacological intervention in these patients depends on the clinical presentation, and in the case of asymptomatic patients, treatment should be deferred[73]. Current evidence summarizes the current scheme, indicating that asymptomatic or mildly clinical patients should delay treatment until the period after pregnancy and breastfeeding. Yet, in the case of hyperemesis gravidarum, it is necessary to assess the risks and benefits. Generally, if the patient is in the first trimester, the use of amoxicillin, metronidazole and PPI is recommended. However, in the second trimester, triple therapy comprising clarithromycin, amoxicillin and PPI in standard dose is more suitable[71].

Additionally, for planned pregnancies, it is important to check the individual *H. pylori* status before potential conception. Choosing a non-invasive method for detecting *H. pylori* infection and preference for treatment before or after pregnancy. If *H. pylori* infection will be confirmed as a significant contributor to pregnancy complications, we recommend that traditional *H. pylori* eradication, specifically triple therapy, be ideally achieved several months prior to conception to achieve seronegativity. This strategy would help prevent interactions between antibodies against *H. pylori* and the antigens of host tissue[17]. In conclusion, it is essential to emphasize the importance of a medical decision based on discussion with the patient, especially drawing attention to the binomial fetal risk *vs* symptoms relief and bacterial eradication. This is particularly necessary considering the incipient literature on the treatment of *H. pylori* infection in pregnant women and the lack of specific guidelines in this regard (Table 1).

CONCLUSION

H. pylori infection is associated with gastrointestinal symptoms during pregnancy and some other serious pregnancy-related disorders. It may contribute to the development of these conditions *via* different mechanisms: including the reduction of micronutrients such as iron, the initiation of pro-inflammatory cytokine release at both local and systemic levels, and the generation of oxidative stress in gastrointestinal disorders and pre-eclampsia. Additionally, cross-reactivity can occur between particular anti-*H. pylori* antibodies and antigens found in placental and endothelial cells , which can be linked to conditions like pre-eclampsia, fetal growth restriction, and miscarriage (Figure 1). Furthermore, the influence of *H. pylori* infection on fetal implantation/placental failure and its correlation with strains positive for the cytotoxin-associated gene A is also described.

Diagnostic methods are divided into invasive and non-invasive tests, with the latter being preferable for diagnosing *H. pylori* infection in pregnant women. Similarly, it is preferable to treat the infection outside of pregnancy, although studies have shown an improvement in gastrointestinal symptoms in *H. pylori*-positive pregnant women after antibiotic therapy, especially in those with hyperemesis gravidarum. Therefore, the risk/benefit of treating the infection during pregnancy should be assessed due to the potential risks of antibiotic use during pregnancy, especially concerning fetal toxicity. Early diagnosis before pregnancy and preventive eradication of *H. pylori* are anticipated to decrease the occurrence of certain complications. Therefore, there is a need for more scientifically rigorous prospective investigations to evaluate the suitability of these treatments, focusing on the most encouraging new therapeutic protocols.

Table 1 Recommended schemes for *Helicobacter pylori* eradication

Regimen	Drugs (doses)	Duration (d)	Ref.
Clarithromycin triple therapy	PPI (standard dose, BID) + clarithromycin (500 mg, BID) + metronidazole (500 mg, BID) or amoxicillin (1000 mg, BID)	10-14	[63-65]
Bismuth quadruple therapy	PPI (standard dose, BID) + bismuth (QID) + metronidazole (400 mg, QID or 500 mg, TID-QID) + tetracycline (500 mg, QID)	10-14	[63-65]
Concomitant nonbismuth quadruple therapy	PPI (standard dose, BID), amoxicillin (1000 mg, BID), metronidazole (500 mg, BID) and clarithromycin (500 mg, BID)	10-14	[63-65]

PPI: Proton pump inhibitors; BID: Twice daily; TID: Three times a day; QID: Four times a day.

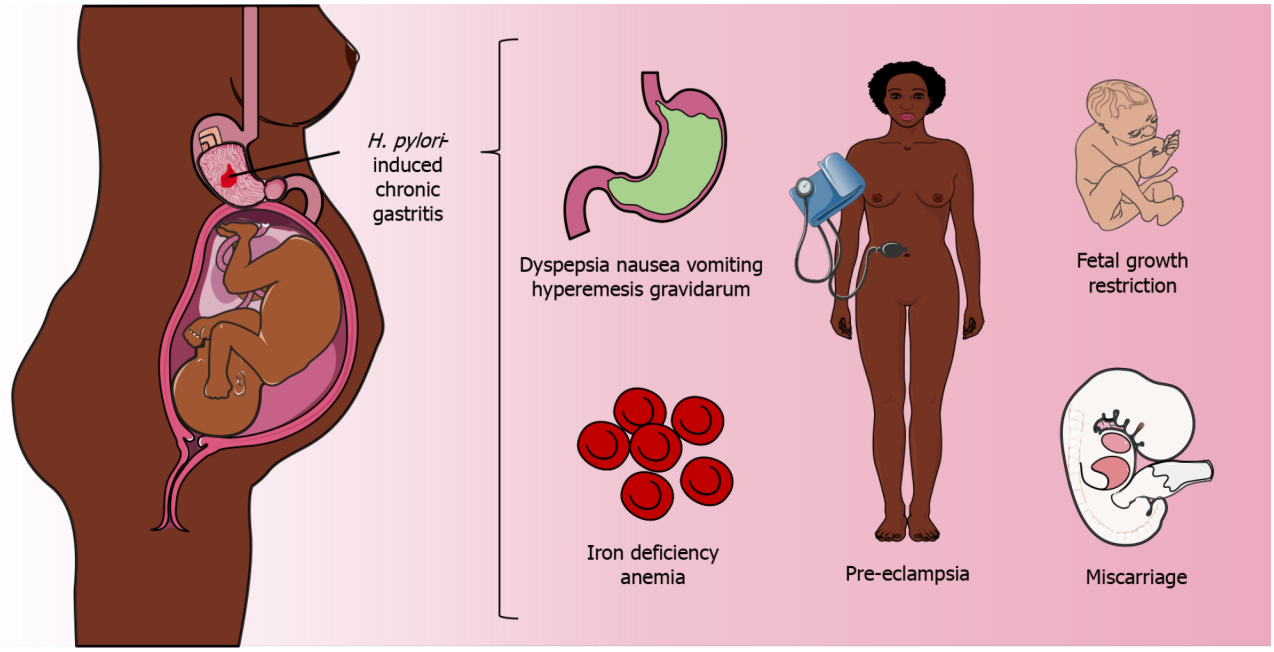

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Figure 1 Main disorders related to the infection by *Helicobacter pylori* during pregnancy. *Helicobacter pylori* (*H. pylori*) infection is able to cause damage to the gastric mucosa and stimulate local inflammation, which leads to the development of gastric disorders, such as dyspepsia, hyperemesis gravidarum, nausea and vomiting. Furthermore, the bacterium is responsible for modifying the capacity of absorption of micronutrients by the pregnant woman, contributing to the depletion of essential substances to the organism homeostasis. Among these nutrients, the reduction of organic iron reserves has been related to the emergence of anemia. In addition, the systemic inflammation stimulated by *H. pylori* can also be responsible for iron deficiency anemia. On the other hand, the immune response and the inflammatory process stimulated by *H. pylori* may cause placental and endothelial damages, which are able to promote the development of miscarriages and fetal growth restriction, which can also be caused by anemia, and pre-eclampsia.

FOOTNOTES

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