Original Article

Microbiology Section

Isolation, Identification and Antimicrobial Susceptibility of *Brucella* Isolates From Human Cases

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ABSTRACT

Introduction: Brucellosis is a worldwide zoonotic disease that remains an important public health problem in India. As clinical manifestations of human brucellosis are variable in nature, and no constellation of clinical findings can be considered, characteristic laboratory help is must in the diagnosis. In the laboratory, brucellosis is generally diagnosed by serological tests. Though many serological tests with different principles are available, serological testing does not provide direct evidence for the presence of the pathogen, hence isolation of *Brucella* spp. from the clinical specimen is considered to be the gold standard. *Brucella* is highly infectious and requires level 3 bio-containment facilities and technically skilled personnel, Brucella cultures are rarely performed. Due to the rampant use of rifampin for the treatment of tuberculosis and reports on development of resistance in virtually all organisms the sensitivity of *Brucella*e to the traditional drugs cannot be presumed.

Aim: To isolate, identify the *Brucella*e from blood culture and to assess the antimicrobial susceptibility of the isolates to WHO recommended anti-*brucella*r antibiotics by agar dilution method.

Materials and Methods: A total of 169 blood samples were collected for *brucella* culture, from 593 epidemiologically, clinically and serologically suspected cases of human brucellosis. Of the 169 blood cultures 77 yielded *Brucella*, of which nine were from the western Maharashtra and 68 from Northern Karnataka. The isolates were identified using conventional methods. The minimal inhibitory concentration (MIC) values for streptomycin, gentamicin, doxycycline, rifampin and trimethoprim-sulfamethoxazole were determined by using agar dilution method.

Results: Isolation rate for *Brucella* was 45.5%. Of the 77 isolates, 75 were identified as *B. melitensis* and two as *B. abortus*. All the isolates were sensitive to all the drugs tested.

Conclusion: Human brucellosis due to *B.melitensis* is fairly a common disease in this area. The current WHO recommended drug regimen for the treatment of human brucellosis continues to be effective as no drug resistance is noted in the study.

INTRODUCTION

Human brucellosis is the most common zoonotic disease with worldwide distribution [1]. Though it has been eradicated from many developed countries, it still remains hyper endemic in the Mediterranean basin, Middle East, Southwest Asia and parts of Latin America [1,2].Though Brucellosis was reported as early as in 1942, it still remains neglected and is endemic throughout India [3,4]. The disease has been reported in domestic animals like cattle, buffalo, sheep, goats, pigs, dogs and also in humans [4].

As *Brucella* is an intracellular bacterial pathogen, antibiotics that penetrate and function within the macrophages are required for the treatment of brucellosis [5]. Therefore, a limited number of antibiotics are effective against these organisms. Doxycycline, combined with either rifampicin or streptomycin is recommended for the treatment of human brucellosis by WHO [6].Though there is no drug resistance problem noted for antibiotics used to treat brucellosis, some workers have reported reduced susceptibility to rifampicin, streptomycin and co-trimoxazole [7-10].

Antibiotic susceptibility patterns of *Brucella* spp. differs geographically. Since, high level drug resistance has been reported in gram positive (*staphylococci*, *enterococci*) and gram negative bacteria (*pseudomonas* and *enterobactericeae* spp.), the sensitivity of *Brucella* to the standard drugs cannot be foreseen. Studies on antibiotic sensitivity for *Brucella* species from India are limited.

This study aimed to isolate *Brucella*e from blood samples, know the predominant species infecting humans in this part of India and determine the antimicrobial susceptibility to commonly used anti-*Brucella* agents.

MATERIALS AND METHODS

This cross-sectional study was carried out in the Department of Microbiology BLDEU'S Shri B M Patil Medical College Hospital and Research Center Vijayapur and Microbiology

Division DRDE Gwalior from October 2008 - December 2012.

Inclusion criteria: Patients with clinical and epidemiological history suggestive of brucellosis with significant Serum agglutination test (SAT) titres and who consented to participate in the study were included.

Exclusion criteria: Patients who had no clinical and epidemiological history suggestive of brucellosis and or had insignificant SAT titres (<160 IU) or denied to participate in the study were excluded.

Collection of sample: The entire experimental protocol was approved by the institutional ethical committee and utmost care was taken during the experimental procedure. Informed consent was taken from all the adults and from parents in paediatric age group (1.5-14 yrs), before collecting the sample.

Blood samples from 593 patients with clinical and epidemiological history suggestive of brucellosis were screened for *brucella* agglutinins, of which 209 showed positive RBPT and significant titres by SAT. Amongst the 209 cases blood culture was performed in 169 individuals (who gave consent for blood culture).

Serological study: 5ml of blood was collected from each patient. The blood was allowed to clot, serum was separated aseptically and the clot was preserved for further use. The serum samples were processed for Rose Bengal plate test (RBPT) and Serum Agglutination Test (SAT). The antigens for these tests were procured from the Division of Biological Products, Indian Veterinary Research Institute (I.V.R.I.); Izatnagar, Uttar Pradesh. The tests were performed according to the manufacturer's guidelines. Any degree of agglutination within four minutes was considered as positive for RBPT. Highest dilution of the serum showing agglutination equal to third control tube was considered as the end point for SAT and a titer of ≥160 IU was considered significant.

Blood culture: 10 ml blood was collected from each patient who fulfilled the inclusion criteria for culture. Of that, 5 ml was used for conventional culture and remaining 5 ml was processed by lysis centrifugation method. The preserved clot was used for clot culture. Castaneda's biphasic media prepared using *Brucella* selective agar and broth with *Brucella* selective supplement (Hi-Media) was used for all the three methods [11].

All the work related to culture, identification and antibiotic susceptibility was performed in biosafety level 3 (BSL-3) cabinet. The media were incubated at 37^0C with 10% CO_2 for a maximum of 45 days.

The bottles were observed daily for appearance of colonies. Sub-culturing was done if no growth was noted. For this the bottles were tilted allowing the blood broth mixture to flow over the solid phase.

Identification methods: Preliminary identification of the colonies was done based on colony morphology, Gram's

and Modified Ziehl-Neelsen's staining. Further speciation and biotyping was done by the following tests: requirement of added carbon dioxide for growth, catalase and oxidase test, urease and H_2S production, sensitivity to basic fuchsin and thionin (10-40 µg/ml). Provisionally the isolates were confirmed by slide agglutination test using absorbed monospecific antisera for *B. abortus* and *B. melitensis* using antisera procured from Murex Diagnostics, UK [11,12]. The isolates were confirmed at the Department of Microbiology, Defence Research and Development Establishment, Gwalior and IVRI Izatnagar.

Antimicrobial susceptibility testing: The procedure was performed according to the manual on antimicrobial susceptibility testing by Lalitha M.K. and the results were interpreted based on Clinical and Laboratory Standards Institute (CLSI) guidelines for slow growing bacteria [13,14]. All the isolates were subjected to antimicrobial susceptibility testing for: doxycycline (DOX), rifampicin (RIF), streptomycin (STR) and trimethoprim-sulfamethoxazole (TMP-SMZ). Minimum inhibitory concentrations (MIC) were determined by agar dilution method using Mueller-Hinton agar (Oxoid, Basingstoke, UK) supplemented with 5% sheep blood. The MIC results were interpreted after 48 hours of incubation at 37^0C in ambient air.

Controls: *B.abortus* 544 and *B. melitensis* 16M were used as controls for identification, biotyping and antimicrobial susceptibility testing. In addition to *Brucella* reference strains, *Escherichia coli* ATCC 25922, *Staphylococcus* aureus ATCC 29213 were used as the quality control strains [15].

Test	Number positive
Added CO_2 requirement	02
Oxidase test	77
Catalase test	77
H_2S production	13
Urease activity within 15 minutes	64
Urease activity within 2 hours	13
Growth in the presence of Thionin 1:25000 (40µg/ml)	00
Growth in the presence of Thionin 1:50000 (20µg/ml)	75
Growth in the presence of Thionin 1:100000 (10µg/ml)	75
Growth in the presence of Basic fuchsin 1:50000 (20µg/ml)	77
Growth in the presence of Basic fuchsin 1:100000 (10µg/ml)	77
Agglutination with *B. melitensis* mono-specific antiserum only	72
Agglutination with *B. abortus* mono-specific antiserum only	02
Agglutination with both *B. melitensis* & *B. abortus* mono specific antisera	03

[Table/Fig-1]: Biochemical and antigenic characters of *Brucella* isolates

Species	No of isolates	Biotype		
		1	2	3
Brucella melitensis	75	71	00	4
Brucella abortus	2	2	--	--
Total	77	73	00	4

[Table/Fig-2]: Species and biotypes of *Brucella isolates*

Antibiotic	Streptomycin		Doxycycline			Rifampin			TMP/SMZ		
MIC (µg/ml)	0.25	0.5	0.06	0.125	0.25	0.125	0.25	0.5	0.5/9.5	1/19	2/38
Agar dilution	0	77	02	02	73	0	75	02	0	75	02

[Table/Fig-3]: minimum inhibitory concentrations values for 77 *Brucella isolates*

RESULTS

Brucellae could be isolated from 77 individuals. The isolates were identified as *B. melitensis* and *B. abortus* depending on biochemical and antigenic characters as shown in [Table/Fig-1].The species and biotype of the isolates are shown in [Table/Fig-2]. Evaluation and interpretation of *in vitro* activities of DOX, STR, RIF and TMP-SMZ against the isolates according to the CLSI criteria are given in [Table/Fig-3].

DISCUSSION

Brucellosis is endemic in India and large numbers of human brucellosis cases occur annually. Definite diagnosis of brucellosis can be achieved by the isolation of *Brucellae* from the clinical specimen. However, cultivation of this organisms is difficult, time consuming and also expensive. Furthermore, as *Brucella* spp. are highly infectious, the attempts to isolate and identify the organism from clinical specimens is rarely performed. Consequently, epidemiological data of human brucellosis is inadequate and limited information is available about the prevalence and the predominant species infecting humans in India.

In this study, overall culture positivity was found to be 45.5% with 97.4% (75) isolates being *B.melitensis.* Of the 75 *B. melitensis* isolates, 71 were biotype-1 and 4, biotype-3. All the isolates from northern Karnataka were *B. melitensis*,whereas two of the nine isolates from western Maharashtra were *B.abortus*, indicating prevalence of both *B. abortus* and *B. melitensis* in this region. Most of the studies from Karnataka have shown the predominance of *B.melitensis* biotype-1, whereas predominance of *B. melitensis* biotype-3 has been reported from Turkey and Northern Greece [16-21].

Antibiotic sensitivity studies done in Turkey by Baykam N et al., has shown four *Brucella* strains to be non-susceptible to rifampin, one strain resistant to trimethoprim-sulphamethoxazole and the study by Ayaslioglu et al., has reported intermediate sensitivity to rifampin [7,10]. Also the study by Tanyel E et al., from the same country has demonstrated higher MIC values for streptomycin [9].

Whereas, Maves et al., from Peru have reported one isolate each with reduced susceptibility to RIF (MIC: 1.0 mg/l) and SXT (MIC: 0.64 mg/l) [8]. Kinsara et al., from Saudi Arabia have reported 62% resistance for co-trimoxazole [22]. In this study we did not come across any reduced susceptibility/high MIC values for usually recommended drugs.

LIMITATION

Antibiotic susceptibility testing was not performed for the other drugs that are being used to treat human brucellosis.

CONCLUSION

B. melitensis biotype-1 was the commonest isolate. There is no significant drug resistance problem in *Brucella* species from this region of India, WHO recommended treatment regimen still holds good. However, it is necessary to determine the sensitivity pattern of the local isolates of *Brucella* periodically to detect the emergence of resistance.

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