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Short Communication

Antimicrobial susceptibility testing of *Brucella melitensis* isolates of human and livestock origin

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Abstract

Brucellosis, a pervasive zoonotic disease caused by various *Brucella* species, poses a substantial threat to global public health with significant economic impact. In India, where brucellosis is endemic, identification and antimicrobial susceptibility testing of *B. melitensis* isolates is paramount for effective management. This study aimed to identify a set of 24 nos. of *B. melitensis* isolates of human (n=20) and livestock origin (n=4) by molecular techniques and assess the antimicrobial susceptibility against six antibiotics by employing Kirby Bauer disk diffusion method. The isolates were initially identified by biochemical tests followed by molecular confirmation using AMOS PCR. Antimicrobial susceptibility testing revealed 100% sensitivity to ciprofloxacin and cotrimoxazole, but highlighted intermediate resistance to other antibiotics. Intermediate resistance to doxycycline, gentamicin, streptomycin, and rifampicin was observed in 87.5%, 75%, 29.1%, and 16.67% of the isolates. A host-wise difference in susceptibility pattern was observed with human isolates demonstrating higher intermediate resistance to doxycycline, gentamicin and streptomycin than animal isolates. These findings underscore the imperative for sustained surveillance, prudent antibiotic usage, and innovative treatment modalities to combat brucellosis effectively. Further investigation into the mechanisms of antimicrobial resistance in *B. melitensis* isolates holds promise for devising targeted therapeutic strategies and enhancing public health outcomes.

Keywords: AMOS PCR, Antimicrobial resistance, Disc diffusion method, Public health

Highlights

- Kirby Bauer disc diffusion test was employed to assess the antibiotic sensitivity of 24 *B. melitensis* isolates.
- All isolates displayed 100% sensitivity to ciprofloxacin and cotrimoxazole, suggesting their efficacy in treating *B. melitensis* infections.
- Intermediate resistance was observed against doxycycline, gentamicin, streptomycin, and rifampicin in 87.5%, 75%, 29.1%, and 16.67% highlighting potential challenges in treatment options.
- Human isolates demonstrated higher level of intermediate resistance to doxycycline, gentamicin and streptomycin than animal isolates.

Brucellosis, caused by various species of the genus *Brucella*, stands as a significant zoonotic disease worldwide (Pappas *et al*., 2006), with an annual incidence of 2.1 million cases in humans (Laine *et al.*, 2023). The disease, recognized since the discovery of *B. melitensis* in 1887 (Godfroid *et al*., 2005), remains a persistent threat to both animal and human health. Initially described with six species, the genus *Brucella* has expanded to include 12 species, each exhibiting distinct mammalian host preferences (Corbel, 2006; Saavedra *et al*., 2019).

Among the *Brucella* species, *B. melitensis*, *B. suis*, and *B. abortus* pose significant zoonotic risks, with *B. canis* exhibiting lower potential for human infection. The modes of transmission to humans primarily involve direct contact with infected animals, consumption of contaminated animal products, and inhalation of airborne agents. Consumption of unpasteurized milk and cheese remains a common route of infection, particularly from sheep and goats. Notably, occupations involving close contact with animals, such as abattoir workers, shepherds, and veterinarians, are at heightened risk of contracting brucellosis (Pappas *et al*., 2005).

Traditional microbiological identification of *Brucella* involves colonial morphology, staining characteristics, and serological tests. However, due to the high similarity among *Brucella* species, biochemical profiling for identification may not always be conclusive. Consequently, PCR-based methods are being increasingly employed in diagnostic laboratories for confirming pure cultures and differentiating species

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and biovars (Bricker and Halling, 1994; López-Goñi *et al*., 2008; López-Goñi *et al*., 2011).

Antimicrobial resistance (AMR) in *Brucella* spp. is a growing concern, potentially compromising treatment efficacy. While *Brucella* isolates have historically been susceptible to commonly used antibiotics, reports of resistance have emerged, necessitating periodic surveillance. Treatment of brucellosis typically involves a combination of antibiotics for an extended duration. However, the development of resistance, particularly to rifampicin and cotrimoxazole, has been documented, leading to treatment failures and relapses (Yousefi-Nooraie *et al*., 2012; Trott *et al*., 2018).

In India, brucellosis remains endemic, impacting both animal and human populations. With the country boasting the world's largest livestock population, brucellosis presents a significant public health and economic challenge. While bovine brucellosis is predominant, small ruminant brucellosis is equally concerning. Human cases are frequently reported among individuals with occupational exposure to livestock and their products (Pandit and Pandit, 2013).

Given the escalating antimicrobial resistance in *Brucella* spp., there is an urgent need for systematic surveillance and characterization of Indian *B. melitensis* isolates. This study aims to identify and assess the antimicrobial susceptibility of *B. melitensis* strains isolated in India from both humans and livestock to provide crucial insights for effective management and control strategies.

The initial step in the study involved the identification and confirmation of *B. melitensis* isolates. The isolates of human origin (n = 20) were received in tertiary care hospitals. All the ethical guidelines were duly followed by the tertiary care hospitals involved in collection and processing of samples from human subjects under informed consent. The isolates of animal origin (n = 4) were isolated from aborted foetus of goat (n = 1) and sheep (*n* = 3) that were collected from farmers field during outbreak investigations in Bareilly district of Uttar Pradesh. At the beginning, an approach combining traditional biochemical tests and molecular techniques were employed. Gram staining was performed to evaluate the morphological characteristics of the isolates and to detect any potential contamination. Biochemical tests, including the dye test comprising Basic Fuschin and Thionine tests, were utilized to assess the characteristic biochemical properties of the isolates. Basic fuchsin/thionine and 5% horse serum were added to the melted and cooled TSA medium to give three different concentrations of 1:25000, 1:50000 and 1:100000. The plates were incubated at 37°C for 48 h with or without CO_2 (5-10%) as per the requirement of isolates (Alton *et al*., 1975). Molecular confirmation of the *B. melitensis* isolates was performed using AMOS-PCR (AMOS refers to the *Brucella* species identified i.e., *B. abortus*, *B. melitensis*, *B. ovis*, and *B. suis*) a highly specific and sensitive technique for the identification of *Brucella* spp. (Bricker and Halling, 1994). This multiplex PCR method utilizes a panel of species-specific primers targeting distinct genetic loci within the *Brucella* genome. *B. melitensis* 16M (ATCC 23456; NCTC 10094) was used as a control for the validity of the molecular identification process.

The antimicrobial susceptibility testing of confirmed *B. melitensis* strains was conducted through the Kirby-Bauer disc diffusion method, adhering to the CLSI, 2021 guidelines. The bacterial cultures were cultivated on trypticase soy agar at 37°C for 48 hours. Following this incubation period, the inoculum was suspended in sterile NSS to match with 0.5 McFarland standard and transferred onto cation-adjusted Muller Hinton agar plates, ensuring uniform distribution across the agar surface. Antibiotic discs (Table 1) each impregnated with a specific concentration of antimicrobial agent, were systematically applied onto the surface of the inoculated agar plates. The choice of antibiotics was aligned with recommendations set forth by the World Health Organization (WHO) for the treatment of human brucellosis, encompassing a spectrum of six distinct antibiotics: doxycycline, rifampicin, streptomycin, cotrimoxazole, gentamicin, and ciprofloxacin (Corbel, 2006). These antibiotics were carefully selected based on their efficacy against *Brucella* spp. and their relevance in clinical practice. The discs were sourced from BD BBL, and susceptibility testing was done in duplicate to ensure result reliability and consistency. Following the application of antibiotic discs, the agar plates were incubated under optimal conditions at 37°C for a duration of 42 h. This incubation period allowed for the formation of distinct zones of inhibition around each antibiotic disc, indicative of the susceptibility of the bacterial strains to the respective antimicrobial agents. The diameter of these inhibition zones was measured using standardized techniques, the breakpoints for *Brucella* spp. against the studied antimicrobial agents were determined using procedures established for slow-growing bacteria (*Haemophilus* spp.), as previously described (Elbehiry *et al*., 2022).

The identification process of the isolates initially relied on traditional microbiological methods,

including Gram staining and assessment of colony morphology. Colonies exhibited typical characteristics such as a smooth, convex, raised morphology with translucent appearance and intact edges, consistent with *Brucella* spp. Microscopic examination further supported this classification, revealing the presence of Gram-negative coccobacilli. All isolates showed growth in the presence of specific concentrations of Basic Fuchsin and Thionine, which further reinforced their categorization as *B. melitensis*. Subsequent confirmation was achieved through AMOS PCR, which produced characteristic bands at approximately 730 base pairs specific to *B. melitensis* (Fig. 1).

Antimicrobial susceptibility testing revealed 100% sensitivity to ciprofloxacin and cotrimoxazole among both animal and human isolates. Intermediate resistance to doxycycline, gentamicin, streptomycin, and rifampicin was observed in 87.5%, 75%, 29.1%, and 16.67% of the isolates, respectively (Table 1). Isolates of human origin showed higher level of intermediate resistance to doxycycline, gentamicin and streptomycin than animal isolates (Table 2, Fig. 2).

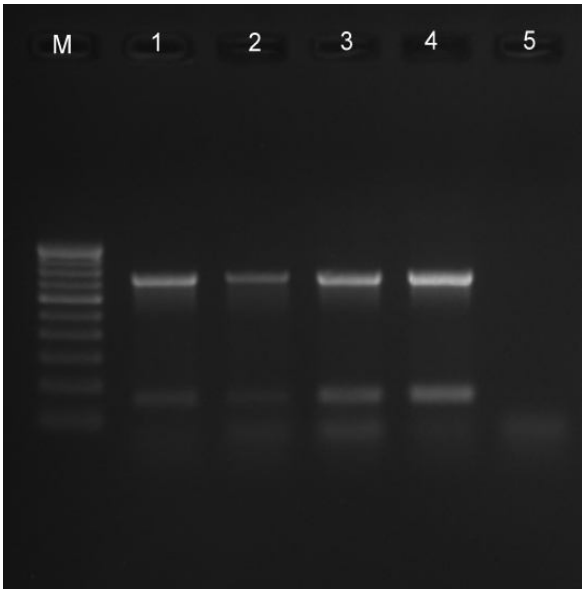

Fig. 1. Gel electrophoresis image of AMOS PCR

[Lane M: 100 bp ladder; Lanes 1-3: Field isolates *B. melitensis* (730 bp amplicon); Lane 4: Positive control: *B. melitensis* 16M (730 bp amplicon); Lane 5: No template control]

Table 1. Susceptibility pattern of *B. melitensis* isolates using Kirby Bauer disc diffusion assay

Antibiotic	Concentration (μg/mL)	Range (mm)	Degree of susceptibility (*B. melitensis*)					
			Sensitive		Intermediate		Resistant	
			No.	%	No.	%	No.	%
Doxycycline	30	32-49	3	12.5	21	87.5	0	0
Ciprofloxacin	5	27-44	24	100	0	0	0	0
Cotrimoxazole	1.25/23.75	16-56	24	100	0	0	0	0
Gentamicin	10	25-51	6	25	18	75	0	0
Streptomycin	10	24-50	17	62.9	7	29.1	0	0
Rifampicin	5	18-27	20	83.33	4	16.67	0	0

Table 2. Host-wise pattern of intermediate resistance in *B. melitensis* isolates

Antibiotic	Degree of susceptibility (*B. melitensis*)			
	Animal (n=4)		Human (n=20)	
	No.	%	No.	%
Doxycycline	2	50	19	95
Ciprofloxacin	0	0	0	0
Cotrimoxazole	0	0	0	0
Gentamicin	2	50	16	80
Streptomycin	0	0	8	40
Rifampicin	2	50	2	10

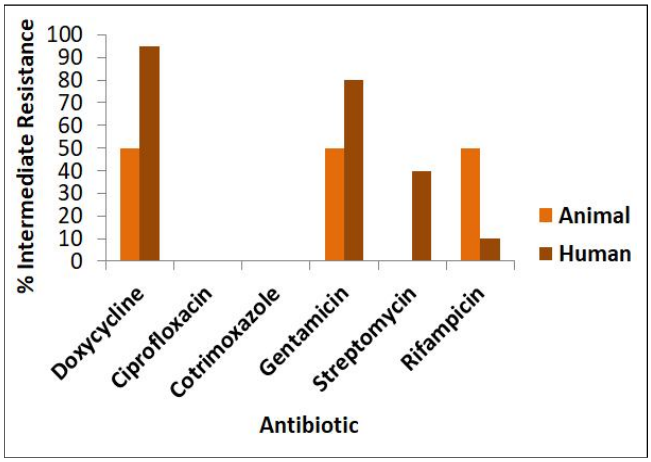

Fig. 2. Comparison of intermediate resistance pattern in *B. melitensis* among human and animal isolates

Brucellosis in humans remains underreported in India, mostly classified as pyrexia of unknown origin (Gupta *et al*., 2023). The combined approach of

integrating traditional microbiological techniques in the current study with molecular assays ensured the reliability and accuracy of the identification process, validating the initial morphological and growth-based observations. This comprehensive method provided robust confirmation of the isolates' identity as *B. melitensis.* Brucellosis treatment in humans usually involves a combination of doxycycline with streptomycin or rifampicin or cotrimoxazole (Alavi and Alavi, 2013). Treatment failure and relapse, observed in many cases, may partly be explained by the emergence of multidrug-resistant *Brucella* spp. strains in endemic areas (del Pozo and Solera, 2015). The susceptibility of *B. melitensis* to antimicrobials varies across different countries, reflecting regional disparities. Antimicrobial susceptibility testing in this study revealed 100% sensitivity to ciprofloxacin and cotrimoxazole, indicating their potential effectiveness in treating *B. melitensis* infections. This finding aligns with observations made by Dadar *et al.* (2023) regarding cotrimoxazole. However, Elbehiry *et al.* (2022) reported a contrasting result, with approximately 36% of isolates being resistant to cotrimoxazole. Similar patterns were observed in studies by Alamian *et al.* (2019), reporting 100% sensitivity to cotrimoxazole in *Brucella* spp. isolates. Concerning trends emerged with other antibiotics. A substantial proportion of isolates showed intermediate resistance to doxycycline, gentamicin, streptomycin, and rifampicin. Most of the previous studies reported 100% sensitivity to doxycycline, gentamicin and streptomycin (Deshmukh *et al.*, 2015; Johansen *et al.*, 2018; Khan *et al.*, 2019; Gültekin *et al.*, 2021; Wareth *et al.*, 2021). A host-wise difference in susceptibility pattern was observed with human isolates demonstrating higher level of intermediate resistance to doxycycline, gentamicin and streptomycin than animal isolates. Resistance to rifampicin has previously been reported (Doimari *et al.*, 2019; Khan *et al.*, 2019). Since both brucellosis and tuberculosis are endemic in India, it is imperative to explore the emergence of community-acquired resistance to rifampicin. The potential overuse or misuse of rifampicin for treatment of tuberculosis may be the reason for higher rifampicin resistance (Ranjbar, 2015). Our study findings emphasize the importance of continuous monitoring of antimicrobial susceptibility patterns in *Brucella* spp. isolates to guide empirical therapy and public health interventions effectively. Additionally, the emergence of intermediate resistance highlights the need for judicious antibiotic use and the development of alternative treatment strategies to combat brucellosis effectively. Further research focusing on the mechanisms underlying antimicrobial resistance in *B. melitensis* isolates would provide valuable insights for the development of targeted therapeutic approaches and strategies for antimicrobial stewardship.

Conflict of interest: Authors have no conflict of interest in this study.

Author contributions: HA, MSK, RM, PT: Conception and design; HA, VS, RM: Acquisition of data; HA, MSK, PT, VS, HD: Performed statistical analysis of data; HA, MSK, VS, KNB: Performed interpretation of the results.

Data availability statement: All the data supporting the research findings have been presented in this paper. The corresponding author is willing to provide the raw data upon reasonable request.

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