Austin Journal of Veterinary Science & Animal Husbandry

Open Access

Austin Publishing Group

Research Article

Antimicrobial Susceptibility Pattern of *Brucella* Isolates from Abortion Cases in Animals in Northern India

Singh BR[1]*, Singh KP[2], Singh SV[1], Agrawal RK[3] and Agri H[1]

[1]Division of Epidemiology, 438-MLB, Indian Veterinary Research Institute, Izatnagar-243 122, India

[2]Division of Pathology, ICAR-Indian Veterinary Research Institute, Izatnagar, India

[3]Division of Livestock Products Technology, ICAR-Indian Veterinary Research Institute, Izatnagar, India

***Corresponding author:** Singh BR, Head Division of Epidemiology, 438-MLB, Indian Veterinary Research Institute, Izatnagar-243 122, India

Received: August 22, 2019; **Accepted:** October 16, 2019; **Published:** October 23, 2019

Abstract

Brucellosis is a common animal disease in India and many other neighboring countries and due to the non-culling policy of carrier cows, the problem is an ever-spreading zoonosis. To find the treatment options, the present study was undertaken to determine the antimicrobial sensitivity of 42 *Brucella* strains isolated from different animals to antibiotics and herbal antimicrobials. The study revealed the existence of multiple drug resistance among strains of *B. melitensis* as well as *B. abortus*. A total of 6, 25, 5, 3, 30, 9, 4, 30 and 37 strains were resistant to tetracycline, doxycycline, streptomycin, gentamicin, co-trimoxazole (sulphamethoxazole+trimethoprim), ciprofloxacin, chloramphenicol, azithromycin and erythromycin, respectively. All the *B. melitensis* and 55.6% of *B. abortus* strains were classified as MDR. All strains were sensitive to imipenem and tigecycline. Irrespective of species all *Brucella* strains were sensitive to ajowan (*Tachyspermum ammi*) oil, carvacrol, and cinnamaldehyde and 93.5% to cinnamon (*Cinnamomum verum*) oil indicating the potential of herbal antimicrobials for future alternative drug development.

Keywords: *Brucella melitensis*; *Brucella abortus*; MDR; Carvacrol; Cinnamaldehyde; Tigecycline; Imipenem; Antibiotic Sensitivity

Introduction

Brucellosis is one of the three most devastating diseases of bovines in India. The estimates of losses due to Haemorrhagic Septicaemia (HS), Foot and Mouth Disease (FMD), Brucellosis, *Peste* des *Petits* Ruminants (PPR), Classical Swine Fever is in tune of Rs. 52.55 billion (2014), Rs. 200 billion (2016), Rs. 204 billion (2015), Rs. 24.17 billion (2016), and Rs. 4.29 billion (2016), respectively [1-4]. The Ministry of Animal Husbandry, Dairying and Fisheries have identified brucellosis and FMD as two priority diseases for immediate control. In animals, brucellosis rarely causes an apparent illness but it causes infertility in both sexes, abortions in females, orchitis in adult males, and hygromas of knee joints in calves [5,6]. Once infected the animal remains a lifelong carrier and continues to disseminate the disease to susceptible animals and humans [7]. Almost 1% of Indian population suffers from the Pyrexia of Unknown Origin/ aetiology (PUO) and *Brucella* is considered as a prime cause of PUO [8]. Although controlled in many parts of the world, it is still hyperendemic in Africa, the Mediterranean, Middle East, parts of Asia and Latin America [9]. It is hyperendemic in South East Asia and detected in 2.87% buffaloes, 2.66% cattle, 3.15% goats, and 2.31% sheep in Bangladesh [10], in 4.5-5.5%, cattle and 3.5-4.2% buffaloes in Sri Lanka [11] and in 5-13.5% cattle and 3% buffaloes in India [8,12]. In India, in Gujarat, seroprevalence of brucellosis in humans varied with the occupation as seropositivity with I-ELISA was 14.28%, 35.0%, 7.31% and 6.0% in veterinary officers, para-veterinarians, other staff related with animal husbandry activities and patients with PUO, respectively [13]. In Karnataka, seroprevalence of brucellosis in humans was 5.1% [14]. In Northern India, studies indicated that 9.94% of PUO patient were positive for brucellosis [14].

To prevent brucellosis, strategic management involves the calf-hood vaccination. Many countries have eradicated brucellosis through calf-hood vaccination. However, the vaccination remains ineffective if test and removal of reactors policy is not implemented. The severity of the problem in India has lead to the National *Brucella* Control Program, which was launched in 2010-11 [15], but little could be achieved due to non-implementation of culling policy for the reactors. For control of brucellosis culling of the positive reactors is the best policy, however, it cannot be practiced in India in light of various cow protection Acts. Therefore, to contain the disease, therapeutic interventions are urgently required in India. Though potentially effective therapies have been experimented in a small group of animals [16], till now, there are no validated treatments for brucellosis affected animals. Brucellae being intracellular parasites are rarely in reach of conventional antimicrobial therapies and proper understanding of pharmacokinetics of antibiotics acting on intracellular bacteria is required [17]. Developments in nanomedicine and better on-target drug delivery systems may modify the outcome of antimicrobial chemotherapy, control and treatment of brucellosis not only in human beings but also in animals.

In literature [18], only five antibacterials including doxycycline, tetracycline, streptomycin, gentamicin, and cotrimoxazole (sulpham ethoxazole+trimethoprim) have been recommended for treatment of burcellosis in humans but relapses after treatment are common. In the present study, we examined the susceptibility of *Brucella* isolates to several potentially useful antimicrobials and herbal antimicrobials, so that better options can be chosen for the therapeutic purpose.

Materials and Methods

Brucella isolates

A total of 32 isolates of *B. abortus* from abortion cases of 4

Citation: Singh BR, Singh KP, Singh SV, Agrawal RK and Agri H. Antimicrobial Susceptibility Pattern of *Brucella* Isolates from Abortion Cases in Animals in Northern India. Austin J Vet Sci & Anim Husb. 2019; 6(3): 1062.

Table 1: Susceptibility (% sensitive) of *Brucella* strains of different species and origin to conventional antimicrobials used.

Name of antimicrobial	*B. abortus* (36)	*B. meli-tensis* (10)	Buffaloes (*B. abortus* 4, *B. melitensis* 1)	Cattle (*B. abortus* 25, *B. meli-tensis* 9)	Mithun (*B. abortus* 3)	Reference (*B. abortus* 2 strains each of Strain 99 and Strain 19)	All (46)	MIC µg/mL
Tetracycline	83.3	100	80	85.3	100	100	87	0.1->256
Doxycycline	58.3	0	80	47.1	0	25	45.7	NT
Streptomycin	86.1	100	100	85.3	100	100	89.1	NT
Gentamicin	91.7	100	80	94.1	100	100	93.5	NT
Cotrimoxazole	44.4	0	40	26.5	100	50	34.8	NT
Azithromycin	41.7	10	100	32.4	0	0	34.8	NT
Chloramphenicol	75	100	80	79.4	100	75	80.4	NT
Ciprofloxacin	88.9	100	80	97.1	33.3	100	91.3	0.1- 6.0
Erythromycin	25	0	0	14.7	66.7	50	19.6	0.1-12.0
Amoxycillin+ clavulanic acid	63.9	10	60	44.1	100	75	52.2	NT
Amoxycillin	63.9	10	60	44.1	100	75	52.2	NT
Amoxycillin+ sulbactam	69.4	10	80	47.1	100	75	56.5	NT
Ampicillin	19.4	0	0	20.6	0	0	15.2	1.024->256
Aztreonam	50	30	0	58.8	33.3	0	45.7	NT
Cefotaxime	94.4	100	100	94.1	100	100	95.7	NT
Cefoxitin	75.0#	0	20	88.2	NT	NT	71.4	NT
Ceftazidime	55.6*	0	20	64.7	NT	NT	52.6	NT
Ceftriaxone	94.4	100	100	94.1	100	100	95.7	0.125-256
Imipenem	100	100	100	100	100	100	100	<1.0
Meropenem	94.4	100	100	94.1	100	100	95.7	<1 – 8
Nitrofurantoin	80.6	90	60	88.2	33.3	100	82.6	NT
Piperacillin	85.0**	NT	80	88.2	NT	NT	85	0.75-96
Piperacillin Taztobactam	90.0**	NT	100	88.2	NT	NT	90	NT
Tigecycline	100	100	100	100	100	100	100	0.064-1.0
MARI	0.282	0.26	0.358	0.325	0.286	0.222	0.24	
MDR	55.6	100	40	67.6	100	50	65.2	

MARI: Multiple Antimicrobial Resistance Indexes; NT: Not Tested; MIC: Minimum Inhibitory Concentration with E-test strips (BioMerieux, France); MDR, resistant to three or more of tetracycline, doxycycline, gentamicin, streptomycin, ciprofloxacin, cotrimoxazole, chloramphenicol, erythromycin and azithromycin; *only 19 isolates were tested; # only 21 isolates were tested; ** only 20 isolates were tested.

buffaloes, 25 cattle and 3 mithuns, (*Bos frontalis*), 10 isolates of *B. melitensis* from abortion cases of a buffalo and 9 cattle, available as glycerol stocks in Epidemiology Laboratory and two strain each of reference *B. abortus* Cotton strain-19 and Strain-99 available in the Institute (in two different facilities) were revived, tested for purity and confirmed as per standard protocols [19,20]. All 42 *Brucella* strains were cultured on blood agar and stored at 4°C until tested.

Antimicrobial susceptibility assay

Characterised *Brucella* (n=42) isolates were tested for their sensitivity to different conventional antimicrobials including amoxycillin, amoxycillin+clavulanic acid, amoxycillin+sulbactam, ampicillin, azithromycin, aztreonam, cefotaxime, cefoxitin, ceftazidime, ceftriaxone, chloramphenicol, ciprofloxacin, cotrimoxazole, doxycycline, erythromycin, gentamicin, imipenem, meropenem, nitrofurantoin, piperacillin, piperacillin-tazobactam, streptomycin, tetracycline and tigecycline through disc diffusion assay as per guidelines of CLSI [18]. All antimicrobial discs were purchased from BBL, Diffco.

The Minimum Inhibitory Concentration (MIC) of selected antibiotics was determined using E-test strips (Biomerieux, France) as per the instructions of the manufacturer. For all isolates, Multiple Antibiotic Resistance (MAR) indices were calculated and interpreted as per CLSI guidelines [18]. All incubations were carried out under 5±0.5% CO_2 at 37°C.

Antimicrobial susceptibility testing for herbal antimicrobials

All *Brucella* strains were also tested for their susceptibility to herbal antimicrobials using disc diffusion assay as described earlier [21]. For making discs of herbal antimicrobials >98% pure herbal compounds were used to make 6mm discs cut from Whatman filter paper No.-3, each disc contained 1mg of herbal compound [21]. In the study, discs were prepared for carvacrol, cinnamaldehyde, lemongrass (*Cymbopogon citrates*) oil (from Sigma, USA), guggul (*Commiphora wightii*) oil (from ICAR-Indian Institute of Natural Resins and Gums,

Namkum, Ranchi), agarwood (*Aquilaria malaccensis*) oil, ajowan (*Tachyspermum ammi*) oil, cinnamon (*Cinnamomum verum*) oil, holy basil (*Ocimum sanctum*) oil, patchouli (*Pogostemon cablin*) essential oil, sandalwood (*Santalum album*) oil, Indian pepper (*Zanthoxylum rhetsa*) essential oil (all from Shubh Flavours and Fragrance Ltd, New Delhi). A reference sensitive *E. coli* strain (E-382) available in the laboratory was used as control.

For determining Multiple-Drug-Resistance (MDR), nine antibiotics (tetracycline, doxycycline, gentamicin, streptomycin, co-trimoxazole, azithromycin, erythromycin, chloramphenicol and ciprofloxacin) recommended for treatment of brucellosis were considered. The isolates resistant to three or more of the mentioned drugs were considered MDR [18,22,23]

Determination of Minimum Inhibitory Concentration (MIC) of herbal antimicrobials

The MIC of herbal compounds for *Brucella* strains was determined using agar well diffusion assay [24]. To determine MIC, nine wells of 6mm diameter were cut in suitable MHA plates under sterile environment and bottoms of wells were sealed with the same medium. The test strain prepared for antimicrobial sensitivity assay was swab inoculated and wells were filled with 50µL of serially diluted herbal antimicrobials in sterile dimethyl sulphoxide (DMSO, SDFCL, India) so that well numbered one to nine contained 10, 20, 40, 80, 160, 320, 640, 1280µg and 2560µg of the herbal antimicrobials, respectively. Plates were incubated under appropriate growth conditions for 2h in upright position to get contents of the well absorbed in the medium and then overnight under inverted position. Measurable zone of growth inhibition around the well containing the highest dilution of herbal antimicrobial was marked as MIC value for the microbe. Tests were conducted in triplicate for confirmation.

The results were analysed with Microsoft Excel 2007 worksheet using shorting and filter tools.

Results and Discussion

The antibiotic susceptibility assay of *Brucella* isolates in the study revealed (Table 1) that drug resistance is not uncommon in *Brucella* isolates specifically for the drugs recommended for use in antimicrobial therapy of the infections caused by *Brucella*. Of the 46 isolates tested 6, 25, 5, 3, 30, 9, 4, 30 and 37 were resistant to tetracycline, doxycycline, streptomycin, gentamicin, co-trimoxazole (sulphamethoxazole+t rimethoprim), ciprofloxacin, chloramphenicol, azithromycin and erythromycin, respectively (Table 1). However, none of the isolates was resistant to imipenem and tigecycline. Of the 46 isolates tested 30 (>65%) were resistant to three or more of the recommended drugs. All the *B. melitensis* isolates had MDR, being resistant to doxycycline, co-trimoxazole and erythromycin. However, all the 10 isolates were sensitive to tetracycline, streptomycin and gentamicin. In contrast to our results, none of the 48 isolates of *B. melitensis* in Iran had resistance to any of the commonly used antibiotics [25]. In a recent study in Kazakhstan [26], all the 329 isolates of *B. melitensis* from human cases were susceptible to streptomycin, tetracycline and doxycycline and only 2.7% were resistant to gentamicin. Similar to our observations, all 50 isolates of *B. melitensis* in turkey were sensitive to tetracycline, streptomycin and ceftriaxone [27]. Similar observations on ciprofloxacin sensitivity of *B. melitensis* have been reported in Peru [28] but sensitivity of the isolates to azithromycin, doxycycline and co-trimoxazole was in contrast to our observations. In concurrence to the observations of present study, most of the isolates tested in China were resistant to doxycycline and all to co-trimoxazole [29]. The azithromycin, doxycycline and co-trimoxazole resistance of *B. melitensis* isolates observed in the study might be a trait specific to isolates in the geographic region of the study or due to their origin from animals or due to overuse of these antibiotics in animals in India and need more studies on a larger number of isolates.

Several *B. abortus* strains in the study were resistant to one or more of the commonly used antibiotics and none of the recommended antibiotic effectively killed all the *B. abortus* isolates. The MIC of strains varied for different strains and antibiotics (Table 1). Six of the *B. abortus* were resistant to tetracycline and had MIC 8->256µg/mL. In the study >40% isolates were resistant to doxycycline (Table 1). However, in Brazil (2015), 100% sensitivity was reported to doxycycline and only one of 147 strains, was resistant to ciprofloxacin, two strains each were resistant to streptomycin and sulfamethoxazole-trimethoprim and five were resistant to gentamicin [30]. Resistance in *Brucella* strains was less common in Brazil than in the present study (8.3%). The difference may be attributed either to difference in antimicrobial use in animals in the two regions or circulation of more resistant strains in Northern India, as in Brazil only 2 of the 147 strains were classified as MDR [30] but in this study, 55.6% strains were classified as MDR strains.

Treatment of brucellosis is always difficult and is considered as a challenge to clinicians due to relapses of the disease even after long antimicrobial therapy [22]. Probably due to the need of long-term antimicrobial therapy and economy of the treatment, brucellosis therapy has rarely been advocated in livestock even though sizeable numbers are suffering and disseminating the disease as lifelong carriers [1-3]. Due to the impracticability of antimicrobial therapy for brucellosis in animals, antibiotic sensitivity pattern of *Brucella* spp. isolates from animals is rarely reported [17]. However, for treatment of brucellosis in humans, several antibiotics have been recommended as first-line of drugs including gentamicin, streptomycin in a combination of rifampin, doxycycline or tetracycline, azithromycin and erythromycin, co-trimoxazole, chloramphenicol and ciprofloxacin [18,22,23]. Introduction of new antibiotics like tigecycline and imipenem-cilastatin or other carbapenems may also be seen as alternatives [22]. Though in earlier reports, rifampin and co-trimoxazole combinations have been reported to be better than others [31], we have not tested *Brucella* isolates for rifampin sensitivity because rifampin is not permitted to be used in animals.

In the study, of the 25 *B. abortus* strains causing abortion in cattle, two were resistant to all therapeutically used antibiotic combinations for treatment of brucellosis (tetracycline or doxycycline with streptomycin or gentamicin, erythromycin with streptomycin and azithromycin with gentamicin), i.e., 8% chances of failure of therapy existed if gentamicin was used in place of streptomycin but with the combinations of the latter drug chances increased to 12% in case of zoonotic brucellosis contracted from cattle. Most of the time, instead of single-drug therapy combination of two or more antibiotics is recommended for treatment of brucellosis in humans [22,23]. In earlier studies too, relapse rates have been reported in up to 30%

Table 2: Susceptibility (% sensitive) of Brucella strains of different species and origin to herbal antimicrobials.

Name of herbal antimicrobial	*B. abortus* (36)	*B. melitensis (10)*	Buffaloes (*B. abortus* 4, *B. melit ensis* 1)	Cattle (*B. abortus* 25, *B. melitensis* 9)	Mithun (*B. abortus* 3)	Reference (*B. abortus* 2 strains each of Strain 99 and Strain 19)	All (46)	MIC µg/mL
Ajowan oil	100	100	100	100	100	100	100	10-640
Guggul oil	22.2	0	0	17.6	66.7	0	17.4	320->2560
Carvacrol	100	100	100	100	100	100	100	10-320
Cinnamon oil	91.7	100	100	91.2	100	100	93.5	20-1280
Holy basil oil	44.4	0	60	38.2	0	0	34.8	NT
Cinnamalede hyde	100	100	100	100	100	100	100	10-320
Lemongrass oil	36.1	80	60	44.1	33.3	50	45.7	NT
Sandalwood oil	47.2	0	60	26.5	100	50	37	10->2560
Zanthoxylum rhetsa essential oil	13.9	0	40	8.8	0	0	10.9	160->2560
Agarwood Oil	27.8	0	20	23.5	33.3	0	21.7	NT
Patchouli (*Pogostemon cablin*) oil	36.1	10	40	20.6	100	50	30.4	10->2560
MHARI	0.437	0.555	0.382	0.481	0.333	0.5	0.462	

MHARI: Multiple Herbal Antimicrobial Resistance Index; NT: Not Tested; MIC: Minimum Inhibitory Concentration determined by agar well diffusion assay; all resistant isolates had MIC >640µg/mL.

cases [22,31,32]. The lowest relapse rate of 4.6% has been reported for doxycycline+ streptomycin therapy [33]; however, in the present study three *B. abortus* isolates from cattle (12%) were resistant to this combination. Though, it appeared that observed drug resistance rates are in concurrence to earlier observations on antimicrobial therapy failure on brucellosis [22,23,25-27], are not in reality. In earlier studies [22,23,31-33] the cause of relapse has not been attributed to the development of drug-resistance or MDR but to the intracellular localization of the pathogen. Intracellular localization makes the pathogen a little less susceptible to aminoglycosides and if the pathogen is resistant to other drugs like rifampin, tetracycline, doxycycline etc. in combination, relapse is destined [17]. Tigecycline inhibited all the isolates of *Brucella* in the study and can reach intracellular [17] may be an approach for the treatment of brucellosis in human patients.

Besides the nine recommended antibiotics, the sensitivity of *Brucella* isolates was also determined to several other antimicrobials (Table 1), as in past two decades, many advances have been made for intracellular delivery of the antimicrobials and other drugs [17] irrespective of their capability to enter in cells and reach the target. Therefore, testing of other antimicrobials and finding their efficacy for intracellular pathogens may be useful in future for development of effective treatment of brucellosis. For the same reason, the sensitivity of *Brucella* isolates was also tested for herbal antimicrobials. In the study, all isolates were sensitive to ajowan oil, carvacrol, and cinnamaldehyde and 93.5% to cinnamon oil (Table 2) indicating that herbal antimicrobials may be seen as an alternative of antibiotics for development of therapies, if these herbal compounds are made deliverable systematically to reach in cytosol and phagosomes [34]. All the *Brucella* strains tested sensitive with disc diffusion assay for herbal antimicrobials had MIC ≤640µg/mL. Guggul oil and *Z. rhetsa* essential oil were the least effective herbal antimicrobials on *Brucella* strains and their MIC was always ≥320µg/mL and ≥160µg/mL, respectively (Table 2). Antimicrobial activity of carvacrol and cinnamaldehyde has been reported higher than any other herbal antimicrobials on other microbes [34] but it is rarely reported for *Brucella* isolates.

The study concludes that antimicrobial drug resistance is not rare in brucellae in India and may be a cause of concern for medical as well as veterinary doctors. However, several newer antibiotics like tigecycline and imipenem may be the option for therapy in human patients in India.

Acknowledgement

Authors are thankful to the staff of Epidemiology (Mr. HC Joshi, Mr. Pratap Singh, Mr. Laiqur Rahman, Mr. Ram Das, Mr. Laxmi Prasad and Mr. Ashok Kumar) for assisting in testing of samples reaching in the laboratory and for helping in systematic data management. The Director and Joint Directors of the Institute are also acknowledged for the grant of funds for extending the ABST-services.

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Austin J Vet Sci & Anim Husb - Volume 6 Issue 3 - 2019
ISSN: 2472-3371 | www.austinpublishinggroup.com

Citation: Singh BR, Singh KP, Singh SV, Agrawal RK and Agri H. Antimicrobial Susceptibility Pattern of *Brucella* Isolates from Abortion Cases in Animals in Northern India. Austin J Vet Sci & Anim Husb. 2019; 6(3): 1062.