Comparative Immunology, Microbiology and Infectious Diseases 47 (2016) 1–6

Contents lists available at ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

journal homepage: www.elsevier.com/locate/cimid

Apparent seroprevalence, isolation and identification of risk factors for brucellosis among dairy cattle in Goa, India

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ARTICLE INFO

Article history:
Received 12 May 2015
Received in revised form 4 May 2016
Accepted 23 May 2016

Keywords:
Brucella
Seroprevalence
Strain identification
AMOS PCR
Risk factors

ABSTRACT

Brucellosis is a highly contagious zoonotic infection affecting livestock and human beings. The disease has been reported worldwide except in few countries where it has been eradicated. The prevalence of brucellosis among cattle from 11 farms having a history of abortions was studied. A total of 481 samples comprising of blood, milk, vaginal swabs, vaginal discharges, placental tissues and fetal tissues were collected from 296 animals. Clinical samples were processed for the isolation of *Brucella*. Serum samples (n = 296) were tested by Rose Bengal Plate Test (RBPT) and indirect ELISA. A total of 90 (30.40%) and 123 (41.55%) samples were positive by RBPT and indirect ELISA, respectively. Also 27.02% samples were positive by both the tests. *Brucella* isolates (n = 8) were recovered from clinical samples using *Brucella* selective media. All the isolates demonstrated PCR amplification for the *bcsp31* and *IS711* genes. Amplification of *Brucella abortus* specific primer was demonstrated by all the isolates in AMOS PCR indicating isolates to be of either *B. abortus* biotype 1, 2 or 4. Risk factors for transmission of brucellosis among cattle population were studied by field surveys. It was observed that lack of awareness about brucellosis (OR = 8.739, P = 0.138) and inadequate floor space (OR = 0.278, P = 0.128) were crucial risk factors for transmission of bovine brucellosis.

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1. Introduction

Brucellosis is the most common zoonosis reported worldwide and is responsible for considerable economic losses due to abortion and culling of infected animals [1]. Bovine brucellosis is usually caused by *Brucella abortus* and rarely by *Brucella melitensis* [2]. The disease is characterized by abortions generally in last trimester of pregnancy, retention of placentae, endometritis, birth of weak or dead calves and reduced milk yield in bovines [3]. Transmission in animals occurs by contact with secretions of infected animals, licking of aborted fetuses and placenta, inhalation of aerosols and ingestion of fodder contaminated with *Brucella* [2,4]. Venereal transmission is not a major route of infection under natural conditions, but artificial insemination with contaminated semen is a potential source of infection [5,6]. The most common routes of human infection are through consumption of unpasteurized milk products and close contact with infected animals [1].

Laboratory diagnosis of bovine brucellosis involves serological testing, isolation of the pathogen from clinical material and its identification. A battery of serological tests including Milk Ring Test (MRT), Fluorescence polarization assay (FPA), intradermal test, Rose Bengal plate test (RBPT), complement fixation test (CFT), Coombs test and ELISA are extensively used for diagnosis of bovine brucellosis [7,8]. Currently, there is no diagnostic test sufficiently sensitive and specific to detect all stages of infection in live animals [9,10]. Previously, Standard Tube Agglutination Test (STAT) was used for determination of titers, however, it was observed that the test might give false positive reaction due to cross-reacting antibodies. Serological tests are reliable but sometimes false positivity due to cross reacting antibodies against *Yersinia enterolitica* and some other zoonotic pathogens may reduce the sensitivity [11].

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http://dx.doi.org/10.1016/j.cimid.2016.05.004
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The introduction of new animals in the herds has been identified as the main risk factor for seropositivity [12] besides, abortion in animals, age of the animal and awareness about brucellosis [13].

Isolation of *Brucella* from aborted fetuses, vaginal secretions, blood and milk of infected animal confirms the presence of disease. *B. abortus* has seven recognized biovars, the most reported are biovars 1, 2, 3, 4 and 9 [14,15]. The identification of *Brucella* species and biovars is based on a microbiological methods including testing for CO_2 requirement, H_2S production, urease activity, agglutination with monospecific sera (A and M), selective inhibition of growth on media containing dyes such as thionin or basic fuchsin, and phage typing [16,17]. Currently, AMOS PCR is used to identify species and selected biotypes within genus *Brucella* [18].

Bovine brucellosis is endemic in India [13,19,20]. The disease has been reported from almost every state with significant seropositivity in livestock. Various risk factors including lack of farm hygiene, overcrowding of animals and movement of animals for trade purpose have been recognized in India [13,21]. Singh et al. [22] reported that brucellosis in livestock was responsible for a median loss of 3.4 billion US dollars to India. Lack of awareness about calf hood vaccination is a major setback which increases severity of problem. The objective of this work was to study the prevalence of brucellosis among dairy farms in Goa using serological, bacteriological and molecular methods. The study also focused on identification of risk factors for spread of this infection among dairy cattle in Goa.

2. Materials and methods

2.1. Study area

Goa is a small coastal state of India located on the West coast between the co-ordinates 14°53′ to 15°47′ North latitude and 73°40′ to 74°20′ East longitude with a geographical area of 3702 sq. km. It bounded by Arabian sea in the West and the Western Ghat region runs along the eastern length of the state and consists of a wide belt of rich forest. The intermediate region that lies between the high Western Ghat region and the coastal plains, the mid land region, have distinct geographical and ecological characteristics. The climate of the region is humid tropical. The monthly maximum temperature varies between 24–33 °C (mean 27 °C). The relative humidity varies from 65 to 100% and annual rainfall varies from 2500 to 3400 mm. The state is not a traditional livestock growing area. However, the state has a sizable livestock population comprising about 76 thousand cattle mainly non-descript, 37 thousand buffaloes and 88 thousand pigs. The state does not have a recognized cattle market. So, the farmers have to purchase new stock of animals from neighbouring states. This is resulting in introduction of new animals with variety of problems. Having contracted with severe production losses, the local Animal Husbandry and Veterinary Services department requested to undertake a survey of the state to know the reproductive status of the animals. The bovine population of the state is very sparse. Herd size ranges from two to 130 animals.

2.2. Samples

A total of 481 samples comprising of blood (296), milk (60), vaginal swabs (80), vaginal discharges (20), placental tissues (10) and fetal tissues (15) were collected from 11 dairy farms located in Goa, India. The farms were selected on basis of incidences of abortions reported to the state veterinary officials. Blood samples from every adult cattle on each farm were collected irrespective of their health status. Data containing number of cattle with and without reproductive disorders related to brucellosis was recorded. The disorders included abortion, endometritis, retention of placenta, infertility, and reduced conception rate. All the clinical samples were transported to laboratory under chilled conditions. Bacteriological samples were processed for isolation of the pathogen. Serum was separated by centrifugation at 3000*g* and stored at −20 °C till further testing.

2.3. *Isolation of* Brucella

The clinical samples including vaginal swabs, vaginal discharge, milk, placental tissues and fetal tissues were processed for isolation of *Brucella*. Vaginal swabs and vaginal discharges were inoculated in *Brucella* broth (M348, HiMedia) with *Brucella* selective supplement (FD005, Himedia Laboratories, Mumbai, India) containing Polymyxin B sulphate (2500 IU), Bacitracin (12500 IU), Nystatin (500000 IU), Cycloheximide (50 mg), Nalidixic acid (2500 mg) and Vancomycin (10 mg). Samples were incubated for 72 h at 37 °C for enrichment. After enrichment vaginal swabs and discharges were spread on *Brucella* agar with selective supplements (Himedia Laboratories). Milk was centrifuged at 7000 rpm for 5 min. The upper cream and bottom deposit layers were spread on *Brucella* agar (M822, Himedia Laboratories Mumbai) with selective supplements. Placental tissues and fetal tissues were minced with sterile scalpel and directly placed on *Brucella* agar with selective supplements for isolation. The plates were incubated under 5% CO_2 at 37 °C till the growth appeared. The plates were first observed after 72 h for growth of characteristic small, circular, elevated, honey colored colonies with entire margin.

2.4. Identification

The isolates were tested by biochemical and PCR based methods for identification of *Brucella* spp. For biochemical identification, the isolates were tested by catalase, oxidase, urease production, H_2S production, CO_2 requirement, nitrate reduction, methyl red, Voges Proskeur test, Gram staining, Modified Ziehl-Neelsen staining and selective inhibition of growth on tryptic soy agar containing dyes, thionin and basic fuchsin at 10 μg/ml (1:25000) and 20 μg/ml (1:50000) concentrations as described by Alton et al. [16]. For molecular identification by PCR, genomic DNA was extracted by phenol: chloroform method and subjected to amplification of the *bcsp31* (B4/B5) and *IS711* genes [23,24]. The quantity and purity of extracted DNA were determined by using Nano-Drop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Genomic DNA from *Brucella abortus* S19 was used as positive control, and DNA from *E. coli* ATCC 8739 was used as negative control. The primers and other reagents were procured from Sigma Aldrich, Co., (St. Louis, MO, USA). The reaction mixture (25 μl) consisted of 12.5 μl of Ready Mix Taq buffer with $MgCl_2$ (Sigma Aldrich, Co., St. Louis, MO, USA), 0.5 μl forward primer (10 pmole/μl), 0.5 μl reverse primer (10 pmole/μl), 10 μl of nuclease free water. To this mixture 1.8 μl of template DNA was added. The PCR was performed using thermal cycler (Eppendorf Master Cycler, Germany). For detection of the bcsp31 gene initial denaturation was done at 95 °C for 3 min followed by 35 cycles of denaturation at 95 °C for 45 s, annealing at 60 °C for 45 s and extension at 72 °C for 2 min. Final extension was performed at 72 °C for 10 min. For detection of the *IS711* gene, the PCR conditions were same as the *bcsp31* except the primer annealing was done at 55 °C. The PCR products were analyzed by gel electrophoresis using 1.5% agarose gel containing ethidium bromide. The gel was visualized under UV illumination (AlphaImager, USA). Isolates showing amplification of both the *bcsp31* and *IS711* genes were regarded as belonging to *Brucella* spp.

AMOS PCR was performed for identification of species and selected biotypes within genus *Brucella*. The reaction was performed according to Bricker and Halling [18] with suitable

modifications. The primers and other reagents were procured from Sigma Aldrich, Co., (St. Louis, MO, USA). The reaction mixture (25 μl) consisted of 12.5 μl of Ready Mix Taq buffer with $MgCl_2$ (Sigma Aldrich, Co., St. Louis, MO, USA), 0.5 μl of each of 3 primers for *B. abortus*, *B. melitensis* and *B.suis* (10 pmole/μl), 2 μl of *IS711* reverse primer (10 pmole/μl) and 7 μl of nuclease free water. To this mixture 2 μl of template DNA was added. The PCR was performed using thermal cycler (Eppendorf Master Cycler, Germany). Initial denaturation was performed at 95 °C for 4 min followed by 35 cycles of denaturation at 95 °C for 1.15 min, annealing at 55.5 °C for 2 min and extension at 72 °C for 2 min. Final extension was performed at 72 °C for 5 min. The PCR products were analyzed by gel electrophoresis using 1.5% agarose gel containing ethidium bromide. The gel was visualized under UV illumination (AlphaImager, USA).

2.5. Determination of apparent seroprevalence

The serum samples were tested by Rose Bengal Plate Test (RBPT) and Indirect ELISA (i-ELISA) for detection of antibodies against *Brucella* spp.

The RBPT antigen was procured from Indian Veterinary Research Institute, Izatnagar, India. The test was performed according to Alton et al. [16]. Formation of clear clumps with antigen within 3 min of mixing was recorded as positive result.

Protein G based indirect ELISA kit for diagnosis of bovine brucellosis developed by National Institute of Veterinary Epidemiology and Disease Informatics, Bengaluru, India was used for this test. The test was performed according to manufacturer's instructions. The controls and samples were tested in duplicates. The mean absorbance values were determined. Percent positivity (PP) was determined according to method provided by manufacturer {PP = Average (OD value of test serum/Median OD value of Positive Control) × 100}. Samples with PP values more than 65% were considered strong positive while those with values between 55% to 65% were considered moderate positive. Samples showing PP values below 55% were considered negative for the test. Results were interpreted as negative, moderate positive and strong positive based on PP values of samples. Only the apparent seroprevalence was calculated.

2.6. Identification of risk factors

A questionnaire which provided epidemiological data related to bovine brucellosis was prepared. Data related to herd size, breed, reproductive disorders, animal density, housing type and farm hygiene were collected from farm owners (Table 2) by personal interview at the farm. Seropositivity was determined as number of animals positive for both RBPT and ELISA, divided by total number of animals tested. Odds ratio for all the variables was calculated using PAST software (version 3.01).

3. Results and discussion

Brucellosis has been reported as a significant cause of infectious abortions and economic losses worldwide. In this study, of the 296 serum samples tested, 90 (30.40%) were found to be positive by RBPT and 123 (41.55%) by Indirect ELISA (Table 1). Of the 123 samples positive by indirect ELISA, 105 samples were strongly positive and 18 were moderately positive. A total of 80 (27.02%) samples were positive by both RBPT and Indirect ELISA. Of these 30 (37.5%) samples were obtained from cattle demonstrating brucellosis symptoms. While 50 (62.5%) samples were belonging to apparently healthy cattle.

Previous studies across the world showed variable seroprevalence rates. An estimated true seroprevalence of 18.1% was reported among cattle herds in Jordan [12]. Sanogo et al. [25] observed 10.3% seropositivity among cattle in Ivory Coast. Kashiwazaki et al. [26] reported 21.5% prevalence in 3 districts of west Uganda and 3.4% in 2 districts of east Uganda by RBPT and indirect ELISA. A prevalence of 1.4% was observed among cattle in southeast Ethiopia [27] and 9.6% in northern plateau of north central Nigeria [28]. Boukary et al. [29] observed 1.3% animal level prevalence by using indirect ELISA in Niger. The variation in seroprevalence rates might be attributed to animal husbandry practices, the tests employed, vaccination programmes and hygiene at the farm [1,7].

Bovine brucellosis is common in most of the states in India. Long term serological survey across 23 states confirmed the presence of antibodies in 19 states with variable prevalence rates [19]. Earlier study in Goa [30] reported seropositivity of 37.38%, 36.45% and 40.18% in cattle against *Brucella* by RBPT, STAT and AB-ELISA, respectively. Recently, Chand et al. [13] observed 22.34% and 34.15% individual animal seroprevalence by RBPT and Indirect ELISA in Haryana and Punjab states of India, respectively. Jagapur et al. [31] found 31.74% animals to be positive for brucellosis, which included 27.21% cattle and 36.34% buffaloes among the three states of India. Similarly, Trangadia et al. [20] observed 22.18% and 13.78% seropositivity among bovines by ELISA and RBPT, respectively.

Isolation of *Brucella* spp. from aborted fetal tissues and secretions of infected animals is a gold standard for diagnosis. Isolation of pathogen from the farms on which abortions had occurred indicated that disease was actively circulating among animals. In this study, on bacteriological analysis of the clinical samples, 8 *Brucella* isolates were obtained. Of these, 4 isolates were obtained from vaginal swabs, three from placental tissues and one was obtained from fetal tissues. Biochemically, the isolates were confirmed as *Brucella abortus* biovars 1 or 3. The isolates grew on trypticase soy agar containing thionin and basic fuchsin. All the isolates exhibited amplification of the *bcsp31* and *IS711* genes.

In an earlier study, Verma et al. [32] isolated *B. abortus* biotype 3 from 2 of 110 samples from cows, while, *B. melitensis* biotype 1 was recovered from 3 of 115 does and 4 of 163 samples collected from ewes. Kaur et al. [33] reported the presence of *B. abortus* biotype 3 among cattle. An outbreak of brucellosis in an organized dairy farm leading to abortions, retained placenta and stillbirths among 24 of the 290 animals in the farm was reported [34] and *B. abortus* biotype 1 was isolated from placentae, uterine discharges, vaginal swabs and fetal stomach contents collected from infected animals. Similarly, Chand and Chhabra ([13] investigated abortion storms in cattle and buffalo and recovered 29 *B. abortus* isolates from morbid materials in Punjab and Haryana, India. A total of 30 *B. abortus* biovar 1 isolates were recovered from milk (n = 5), aborted fetuses (n = 13), and vaginal swabs (n = 12) from cattle in Pakistan [35].

AMOS PCR is reliable method for identification of four classical species within genus *Brucella* namely, *B. abortus*, *B. melitensis*, *B. ovis* and *B. suis*. As the assay can identify three biovars (1, 2, and 4) of *B. abortus*, all three biovars of *B. melitensis*, biovar 1 of *B. suis*, and all *B.ovis* biovars, it can be used to determine biotype of field isolates [18]. In this study, only 3 primers (abortus, melitensis and suis) were used since ovine or caprine brucellosis due to *B. ovis* has not been reported in this region. All the isolates demonstrated amplification of *B. abortus* specific amplicon in AMOS PCR (Fig. 1) indicating that the isolates belonged to either biotype 1, 2 or 4. Conventional biotype identification methods showed that isolates were belonging to biotypes 1 or 3. We have not used the primers specific to biovar 3, therefore, its presence could not be confirmed by AMOS PCR. Rodríguez-Hidalgo et al. [36] recovered 10 isolates from lymph node and milk samples of cattle which were confirmed as *Brucella abortus* by AMOS PCR.

Biochemical tests can identify pathogen at biovar level but molecular methods are more reliable and less time consuming. Polymerase chain reaction (PCR) assays can be used to detect *Brucella* DNA in pure cultures and in clinical specimens. Several PCR

Table 1
Results of serological tests and bacteriological methods used for detection of brucellosis in dairy cattle farms.

Farm	No. of Animals	Samples		RBPT positive	ELISA positive	RBPT & ELISA positive	Isolation
		Blood	Clinical				
Farm 1	37	11	6	3	5	1	1
Farm 2	77	25	10	7	3	2	0
Farm 3	112	40	20	21	24	18	2
Farm 4	46	15	15	9	9	8	1
Farm 5	54	18	12	3	4	3	0
Farm 6	79	39	30	13	13	9	2
Farm 7	98	39	30	5	23	4	1
Farm 8	48	11	12	9	10	8	0
Farm 9	62	20	14	1	5	0	0
Farm 10	53	25	14	2	10	0	0
Farm 11	130	53	22	17	17	17	1
Total		296	185	90	123	80	8

Table 2
Risk factors related to brucellosis seropositivity among dairy farms in Goa.

Risk Factor	Variable	No. of farms	Seropositivity (%)	OR	95% CI	P value
Herd size	<20	4	6.75	1.716	0.38–7.7	0.4821
	>20	7	20.27			
Breed	Local	2	40.90	0.120	0.00–0.60	0.610
	Cross	9	22.26			
Abortions on the farm	Present	8	26.01	1.035	0.22–4.7	0.963
	Absent	3	1.01			
Floor space	Adequate	4	3.71	0.278.	0.05–1.455	0.128
	Inadequate	7	23.31			
Grazing	Present	5	7.43	2.197	0.512–9.429	0.289
	Absent	6	19.59			
Farm hygiene	Proper	5	13.83	0.793	0.194–3.239	0.7472
	Improper	6	13.17			
Awareness about brucellosis	Present	2	0.67	8.739	0.496–153.9	0.138
	Absent	9	26.35			
Use of milking machines	Yes	3	6.75	1.126	0.2303–5.507	0.883
	No	8	20.27			
Purchase of unscreened animals	Yes	10	26.68	0.123	0.00–6.76	0.305
	No	1	0.33			
Separation of pregnant animal	Yes	2	4.05	1.260	0.196–8.105	0.8074
	No	9	22.97			

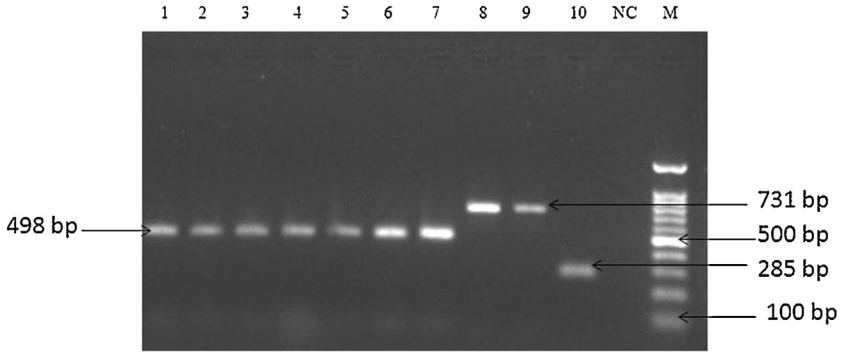

Fig. 1. Gel showing PCR products of AMOS PCR.
Lane 1–5: *Brucella* isolates, Lane 6: *Brucella abortus* S19, Lane 7: *Brucella abortus* S99, Lane 8: *Brucella melitensis*, Lane 9: *Brucella melitensis* Rev1, Lane 10: *Brucella suis* 2330, Negative control—*E. coli* ATCC 8739, M: 100 bp DNA ladder.

based methods have been developed based on the detection of specific sequences of *Brucella* spp., such as the 16S–23S genes, the *IS*711 insertion sequence and the *bcsp*31 gene encoding a 31-kDa protein [37,38]. Previously, Ghodsara et al. [39] used primers targeting the *bcsp31*, *omp2*, *omp*31 and 16 S rRNA genes for confirmation of 10 *Brucella* isolates. Nagalingam et al. [40] used Bruce ladder PCR and AMOS PCR assays to characterize 47 *Brucella* isolates of Indian origin. Of these, 28 isolates showed the amplification of *B. abortus* specific primer in AMOS PCR.

This study detected overall apparent seroprevalence of 27.02% among dairy cattle in Goa. The pathogen was isolated from clinical samples indicating that the disease is actively circulating in this region. In India, risk factors such as rapid movement of livestock for trade purpose, improper farm hygiene, lack of awareness about brucellosis has been found related to seropositivity among animals [13]. In this study, lack of awareness about brucellosis (OR = 8.739, P = 0.138) and inadequate floor space (OR = 0.278, P = 0.128) were significant risk factors for brucellosis in bovines. Seropositivity was observed on the farms having inadequate floor space as overcrowding might have facilitated the transmission of disease. Most of the farm owners were not aware of brucellosis and its zoonotic potential. Improper sanitation and hygiene were observed at almost every farm. Risk behaviors involving overcrowding of farms, introduction of new animals without testing, improper disposal of aborted materials were observed.

Chand and Chhabra [13] observed that the most crucial risk factor which facilitated intra-herd spreading of brucellosis was abortion or delivery of an infected animal on the farm in Punjab, India. Age of animal had been found to be significantly associated with seropositivity for brucellosis in cattle in Niger [29]. While, Mohammed et al. [41] observed that age, sex, location, and herd size played role in the epidemiology of brucellosis among cattle herds in Jigawa state, northwestern Nigeria. In this study, herds with a history of abortions were found to be associated with seropositivity. Lindahl et al. [42] reported that large herds with more than eight cattle and the number of calvings were more likely to be related for higher seropositivity compared to smaller herds with one to two cattle and having younger cows with one to two calvings in Tajikistan. Brucellosis seropositivity was associated with age of animal, history of abortion and herd size [25,43].

Goa, India is a non-traditional region for dairy farming. Trading of animals from neighbouring states is promoted under government schemes as there are no recognized cattle markets in the state. Available reports indicated that seroprevalence was high among animals as well as humans in this region of the country [19,44]. Unscreened infected animals might be introduced to clean herds which could be a potential threat in spreading the infection to a healthy herd and in turn to humans. Prevalence of brucellosis among occupationally exposed persons including dairy farmers, veterinarians and animal handlers and cases of pyrexia of unknown origin has been reported in this region [45]. Screening of animal herds for brucellosis prior to purchase is not a usual practice in Goa, mostly due to economic reasons. Lack of proper veterinary services and vaccination facilities in Goa could be major reason for high seroprevalence.

4. Conclusions

The study reported the high seropositivity among the dairy cattle in Goa region. Bacteriological analysis of the clinical samples revealed isolation of *B. abortus* biovars 1 or 3. The isolates were confirmed by PCR using the *bcsp31* and *IS711* genes. All the isolates demonstrated amplification of *B. abortus* specific amplicon in AMOS PCR. Lack of awareness about brucellosis and inadequate floor space at farms were significant risk factors for brucellosis in bovines. Live attenuated *Brucella abortus* strain 19 vaccine is recommended for calf- hood vaccination. The high prevalence of brucellosis in cattle is potential threat to human health.

Acknowledgements

The authors are thankful to the Director, ICAR Research Complex for Goa, Old Goa for providing necessary facilities. The research was supported by the grants from Department of Biotechnology, Government of India to SBB and NVK.

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