MALDI-TOF Mass Spectrometry Based Identification of *Brucella abortus* in Bovines of Western Rajasthan

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ABSTRACT

The present investigation was conducted to study the prevalence of bovine brucellosis in Western Rajasthan via cultural, biochemical, molecular and mass spectrometry based techniques and to evaluate the efficacy of MALDI-TOF-MS as a screening tool. A total of 120 samples including 89 vaginal swabs and 31 vaginal discharges from 87 cattle and 33 buffaloes manifesting reproductive failures, were cultured for isolation of *Brucella* organisms. A total of five isolates were obtained from vaginal swabs 4/89 (4.49%) and vaginal discharges 1/31 (3.23%) with 4.17% (5/120) overall rate of isolation. Further characterization of the five *Brucella* isolates was done based on molecular techniques including Bruce-ladder multiplex PCR and real-time PCR, including SYBR green based as well as Taqman probe based real-time PCR. For the precise biochemical identification, VITEK 2 Compact Automated Identification System was utilized and all the five clinical isolates were identified as *B. abortus* with a probability of 99% and excellent confidence level. All the five purified isolates were also loaded into the VITEK MS MALDI-TOF and the spectral profiles created by the VITEK MS RUO, were matched against the superspectra of the *Brucella* reference library previously created in the SARAMIS database. The VITEK MS identified all the isolates to the species level. Peak matches yielded the identification results as *Brucella abortus* with confidence values exceeding 99%. Based on the results, it can be suggested that MALDI-TOF-MS, has the potential to become a rapid first-line screening tool for the identification of bovine brucellosis with minimal time, labor and cost.

Table 1. *Oligonucleotide primers used for the identification of Brucella abortus*

Type of PCR	Primers denomination	Oligonucleotide sequences	Size of amplicons (bp)	References
Multiplex Bruce-ladder assay	BMEI0998 (F)	ATCCTATTGCCCCGATAAGG	1,682	22
	BMEI0997(R)	GCTTCGCATTTTCACTGTAGC		
	BMEI0535(F)	GCGCATTCTTCGGTTATGAA	450	
	BMEI0536(R)	CGCAGGGGAAAACAGCTATAA		
	BMEII0843(F)	TTTACACAGGCAATCCAGCA	1,071	
	BMEII0844(R)	GCGTCCAGTTGTTGTTGATG		
	BMEII436(F)	ACGCAGACGACCTTCGGTAT	794	
	BMEII435(R)	TTTATCCATCGCCCTGTCAC		
	BMEII0428(F)	GCCGCTATTATGTGGACTGG	587	
	BMEII0428(R)	AATGACTTCACGGTCGTTCG		
	BR0953(F)	GGAACACTACGCCACCTTGT	272	
	BR0953(R)	GATGGAGCAAACGCTGAAG		
	BMEI0752(F)	CAGGCAAACCCTCAGAAGC	218	
	BMEI0752(R)	GATGTGGTAACGCACACCAA		
	BMEII0987(F)	CGCAGACAGTGACCATCAAA	152	
	BMEII987(R)	GTATTCAGCCCCCGTTACCT		
SYBR green based real-time PCR	B4 (F)	TGGCTCGGTTGCCAATATCAA	NA	5
	B5 (R)	CGCGCTTGCCTTTCAGGTCTG		
TaqMan probe based real-time PCR	IS711 (F)	GCTTGAAGCTTGCGGACAGT	NA	17
	IS711 (R)	GGCCTACCGCTGCGAAT		
	Probe	FAM-AAGCCAACACCCGGCCATTATGGT-TAMRA		

(F) = Forward primer; (R) = Reverse primer; NA = Not applicable

INTRODUCTION

Bovine brucellosis, usually caused by *Brucella abortus*, is manifested by reproductive disorders and results in huge economic losses to dairy farmers throughout India. It has been recognized by OIE as the second most important zoonotic disease in the world after rabies. This widespread reproductive disease commonly causes abortion, death of young ones, stillbirth, retained placenta or birth of weak calves, delayed calving, male infertility, and marked reduction in milk yield.[4,8,15,34,35] The overall seroprevalence of 15.28% and 9.84% in cattle and buffaloes, respectively has been recorded in Western Rajasthan by i-ELISA.[34,35]

A precise diagnosis of *Brucella* spp. infection is important to control the disease in bovines, and consequently in man. Despite the vigorous attempts for more than one century to come up with a definitive diagnostic technique for brucellosis, the diagnosis still relies on the combination of several tests to avoid false-negative results.[33] The newer approaches including the proteomics based techniques, for e.g. Matrix-assisted laser desorption/ ionization-time of flight mass spectrometry (MALDI-TOF-MS) are being evaluated to cover the sensitivity and specificity related challenges. The technique, combined with reference peptide databases and advanced software, has revolutionized microbial identification.[41] Its quickness and reliability makes it fit for counter-bioterrorism, epidemiological tracing of field strains and detection of food contamination.[39]

Keeping in view the importance of

diagnosing the brucellosis promptly and accurately for a better prognosis and treatment of the disease, it becomes imperative to take into account various diagnostic criteria including cultural, biochemical, molecular, and mass spectrometry based techniques to assess the exact scenario of bovine brucellosis in certain geographical locations in Rajasthan; as well as to evaluate the efficacy of MALDI-TOF-MS as a screening tool.

MATERIAL AND METHODS

Sample Collection

Samples including the vaginal swabs and discharges were collected from cattle and buffaloes with a clinical history of reproductive failures viz. abortion, stillborn or weak calves, neonatal mortality, retained placenta, delayed conception, and/or impaired fertility. The samples were collected from organized farms as well as rural households from the various parts of Western Rajasthan including Barmer, Bikaner, Jaisalmer, Jalore, Nagaur and Sirohi districts. In the present study, a total of 120 vaginal samples including swabs and discharges were collected from 87 cattle and 33 buffaloes. All the samples were collected in sterile vials in transport media i.e. *Brucella* broth supplemented with equine serum and *Brucella* selective supplement as per manufacturer's instructions. Collected samples were transported to the laboratory in insulated cold boxes containing ice packs.

Isolation and Purification of *Brucella* spp.

Brucella spp. were isolated and purified as per the standard protocols.[28] The vaginal discharges and vaginal swabs collected in *Brucella* selective broth as transport media were directly streaked on the *Brucella* agar supplemented with equine serum and *Brucella* selective supplement. All the plates were incubated anaerobically at 37°C and examined regularly after 2, and up to 14 days. The plates were examined for detailed colonial morphology and the colonies typical of *Brucella* spp. were sub-cultured to obtain pure bacterial isolates. The smears were prepared from the purified colonies and Gram's staining, as well as modified Ziehl-Neelsen staining, was done. The isolates obtained in the present study were further identified by biochemical tests. Methods described by[3] were used for biochemical identification. Tests like catalase, oxidase, urease, nitrate reduction, indole, H_2S production and motility test were performed taking single isolated colonies.

Identification of *Brucella abortus* Isolates

After the primary identification of *Brucella* spp. by cultural and staining characteristics, the isolates were confirmed upto species level by Vitek 2 compact, Vitek MS besides the molecular techniques including Bruce-ladder multiplex PCR and real-time PCR, including SYBR green based as well as TaqMan probe based real-time PCR.

PCR Based Detection of *Brucella* spp.

DNA was extracted from the bacterial culture by using commercial DNA extraction kit, ie, QIA amp DNA minikit (Qiagen) as per the manufacturer's protocol. The DNA extracted from each of the suspected bacterial isolates was screened by Bruce-ladder multiplex PCR which can identify and differentiate all of the *Brucella* species and the vaccine strains in the same test.[22] The isolates were further confirmed by real-time PCR including SYBR green based as well as TaqMan probe based real-time PCR. Various oligonucleotide primers used for screening of samples using the multiplex PCR and real-time PCR are shown in Table 1.

Multiplex Bruce-ladder Assay for Speciation of *Brucella*

For the single-step identification and differentiation of all of the *Brucella* species as well as the vaccine strains, the multiplex Bruce-ladder PCR was performed. The DNA extracted from each of the suspected bacterial isolates was subjected to PCR in a final reaction volume of 25 µl, with 1 µl test DNA per reaction. Reaction mixture in 24 µl volume was prepared on ice using 2.5 µl PCR buffer (10X) without MgCl2, 3 µl MgCl2 (25 mM), 1 µl of 10mM dNTP mixture (dATP, dCTP, dTTP, dGTP), 1 µl of Bruce-ladder eight pair primer cocktail (12.5 µM), and 1.5 µl of Taq polymerase. The thermocycling conditions used were

initial denaturation at 95°C for 7 min, then 25 cycles of denaturation at 95°C for 35 s, annealing at 64°C for 45 s, and extension at 72°C for 3 min. This was followed by final extension for 6 min at 72°C and soaking at 4°C. The electrophoresis was performed at 80-100 V in Tris Acetate EDTA (TAE) buffer (pH 8.0) as the running buffer for 45-60 min. The gel (2% agarose containing ethidium bromide) was visualized to analyze the size of bands under UV trans-illuminator using gel documentation system.

SYBR Green Based Real-Time PCR

A final reaction volume of 25 µl was prepared on ice with 23 µl of standard SYBR green dye based reaction mixture and 2 µl of template DNA extracted from the suspected bacterial isolates. The reaction mixture was prepared using 12.5 µl of SYBR green master mix (1X) and 1.0 µl each of *BCSP31* based forward (B4) and reverse (B5) primers (10 pg/ µl). The thermocycling conditions used were initial activation at 95°C for 5 min; then 40 cycles of denaturation at 90°C for 1 min, annealing at 64°C for 30 s, and extension at 72°C for 1 min, followed by 1 cycle of melting curve analysis at 95°C for 15 s, 60°C for 1 min, and 95°C for 30 s. The data obtained using SYBR Green master mix in combination with the *BCSP31* genus-specific primers for *Brucella* spp, in the form of amplification curves was analyzed. The specificity of the amplified PCR product was assessed by performing a melting curve analysis.

TaqMan Probe Based Real-Time PCR

The PCR reaction was set up by mixing 2.5 µl of DNA template with 12.5 µl of TaqMan Universal PCR Master Mix, 1 µl each of forward and reverse primers (300 nm), 2.5 µl probe (200 nm), 0.5 µl Internal Positive Control template (50X), and 2.5 µl TaqMan Exogenous Internal Positive Control Mix (10X) into each well of multiwell plate. List of TaqMan probe based real-time PCR primers and probe targeting the IS711 insertion element are given in Table 1. The thermocycling conditions used were initial activation at 60°C for 1 min, followed by 40 cycles of denaturation at 95°C for 10 min, annealing at 95°C for 30 s, and extension at 60°C for 50 s. The amplification curves, thus obtained, were analyzed.

VITEK 2 Compact Automated Identification System

The VITEK 2 Compact (bioMérieux) is an automated microbiology system utilizing growth-based technology. The GN colorimetric reagent card which is available for the identification of Gram-negative fermenting and non-fermenting bacilli was used for the identification and confirmation of *Brucella* isolates. The reagent cards have 64 wells that can each contain an individual test substrate. Substrates measure various metabolic activities such as acidification, alkalinization, enzyme hydrolysis, and growth in the presence of inhibitory substances.

For the preparation of inoculums, at least 3 ml of sterile saline was aseptically transferred into clear polystyrene 12×75 mm test tube. Using sterile cotton swabs, a homogenous organism suspension was prepared by transferring several pure isolated colonies from the plates to the saline tube. The suspension was adjusted to the McFarland standard, i.e., 0.5-0.63 required for the GN reagent, using a calibrated V2C Densi-CHEK Plus Meter. The test tube containing the microorganism suspension was placed into the VITEK 2 Cassette at the Smart Carrier Station of the instrument, with the GN identification card placed in the neighbouring slot while inserting the transfer tube into the corresponding suspension tube. The VITEK 2 Card and sample were linked via barcode.

Once the Cassette was loaded, the subsequent steps for incubation and reading were handled by the instrument. Then, the VITEK system analyzed the data results and the identity of the test microbes i.e. *Brucella* were determined based on colorimetric tests (biochemical reactions) and numerical probability calculations for proximity to the respective database taxa.

VITEK MS MALDI-TOF

VITEK MS MALDI-TOF identification

was performed with the VITEK MS RUO (Research Use Only), also known as the Axima Assurance mass spectrometer, with the SARAMIS (Spectral Archive and Microbial Identification System) database (bioMérieux). VITEK MS analyses were performed according to the manufacturer's instructions. Isolates were tested simultaneously, in duplicate. FlexiMass target slides (disposable 48 wells target slide divided into three acquisition groups) were inoculated by picking up a freshly grown colony with a 0.001 ml calibrated disposable plastic loop and then adding 1 ml matrix solution, a-cyano-4-hydroxycinnamic acid (CHCA). For each acquisition group, a standard (E. coli ATCC 8739) was included to calibrate the instrument and validate the run. Once dry, the target plate was loaded into the Axima Assurance mass spectrometer, where mass spectra were generated in a linear positive mode with a laser frequency of 50 Hz and with a molecular mass range of 2–20 kDa, using software version 3.5.1.3.

Mass spectra were compared with the SARAMIS database version 4.09 (originally developed by AnagnosTec, which contains ReferenceSpectra for 1161 bacteria, and 263 mycota and yeast, and SuperSpectra for 552 bacteria, and 139 mycota and yeast[23]. Each spectrum of the tested sample was matched against the spectra of the *Brucella* reference library previously created in the SARAMIS database. Peak matches that yielded identification results with confidence values exceeding 80% were considered significant and displayed. Saramis database results were evaluated according to a colored index:

- green for percentages equal to or above 90%
- yellow for those between 85 and 89.9%, and
- white for those below 85%

All of the identifications to the genus or species level that fell into the green zone, with a score above 90%, were considered reliable. Scores between 80 and 90% were also considered for acceptable identifications. A cutoff of 90% was chosen for Vitek MS.

RESULTS AND DISCUSSION

In our survey, a total of 87 cattle and 33 buffaloes were found to manifest the clinical symptoms apparent in suspect cases of brucellosis. The cattle and buffaloes had to meet any of the following criteria, either alone or in combination, to be considered as diseased animal exhibiting clinical manifestations:

- abortion, stillborn or weak calves
- neonatal mortality
- retained placenta
- delayed conception
- impaired fertility

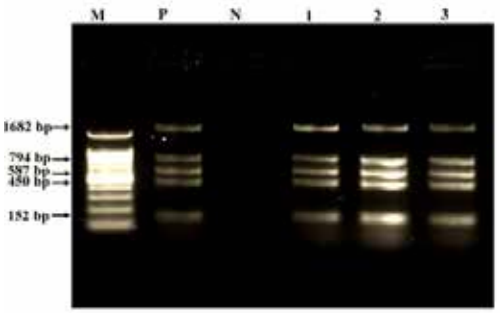
During the sample collection for the conduction of the present study, various factors were observed which might be aiding in the rapid spread of brucellosis in the herds. It was noticed that most of the animal owners disposed the afterbirth to open dump. Also, the provision of separate parturition pens was not there in most of the farms. It was also found out that most of the owners were dependent on the selling of the known *Brucella* infected animals.

Isolation of Aetiological Agent

A total of 89 vaginal swabs and 31 vaginal discharges from bovines were cultured for isolation of *Brucella* organisms. The colonies presumed to be of *Brucella* spp. were observed on the *Brucella* agar supplemented with equine serum and *Brucella* selective supplement after 4 days of incubation, as pinpoint, round, convex with smooth margin, translucent, and pale honey in colour. The culture smear showed Gram-negative coccobacilli in Gram's staining and red stained coccobacilli in modified Ziehl-Neelsen staining. All the isolates were positive for catalase, oxidase, and urease, but none of the isolates produced indole, H2S and all were found to be non-motile. All the isolates were found to reduce nitrate.

Of the 120 clinical samples cultured in the present study 4.17% (5/120) overall rate of isolation was found and all the five isolates were *B. abortus* based on the confirmatory tests presented in the subsequent sections. *B. abortus* was isolated from vaginal

Figure 1. *Multiplex Bruce-ladder PCR assay for speciation of Brucella. Lane 1 to 3 are test samples. PCR positive samples (lane 1,2,3) show amplicons of approximately 152 bp, 450 bp, 587 bp, 794 bp and 1682 bp sizes. N is negative control. P is positive control. M is 100 bp plus marker.*



swabs 4/89 (4.49%) and vaginal discharges 1/31 (3.23%). All the five isolates obtained were from cattle and none from buffaloes.

Like the present study, *Brucella* spp. has been recovered by many investigators from the vaginal swabs and vaginal discharges from cows having history of reproductive failures. A total of 248 clinical samples of animals were collected, having history of recent abortion and reproductive disorders and ten *Brucella* isolates were obtained including three from cows and two from buffaloes, four from goats and one from dog.[16] Samples comprising of blood, milk, vaginal swabs, vaginal discharges, placental tissues and fetal tissues were collected by[31] from 296 animals and eight isolates were recovered using *Brucella* selective media.

Although the isolation of *brucella*e by culturing blood and clinical specimens is essential for confirmation of the diagnosis, isolation of *Brucella* is not always successful due to fastidious nature of organism and presence of lower numbers of viable organisms in clinical specimens. During the present study, pure colonies of *Brucella abortus* were recovered and no contaminating organisms were grown except fast growing organisms especially fungal organisms, which were observed in many plates at the last stage of incubation. This might have been due to the inefficiency of *Brucella* selective supplement to prevent the growth of fungi. This might be responsible for the loss of many of the isolates and the resulting lower recovery rate of *Brucella* isolates. Similar problems were also faced by[20] and[30] while isolating *Brucella* using *Brucella* agar medium.

Identification and Confirmation of *Brucella abortus* Isolates

Multiplex Bruce-ladder Assay for Speciation of Brucella

All the five isolates obtained in the present investigation were found to be *B. abortus* as per the Bruce ladder multiplex PCR. The multiplex PCR assay (Bruce-ladder) was developed by[22] which could differentiate in a single step all of the classical *Brucella* species, including those found in marine animals and the S19, RB51 and Rev.1 vaccine strains. Further characterization of the five *Brucella* isolates was done based on Bruce ladder multiplex PCR. The five bands of 152 bp, 450 bp, 587 bp, 794 bp and 1682 bp sizes were obtained in all the five isolates confirming the species as *Brucella abortus* (Figure 1). No vaccine strains were found in the isolates screened.

Figure 2. *Amplification plot of SYBR green based real-time PCR.*

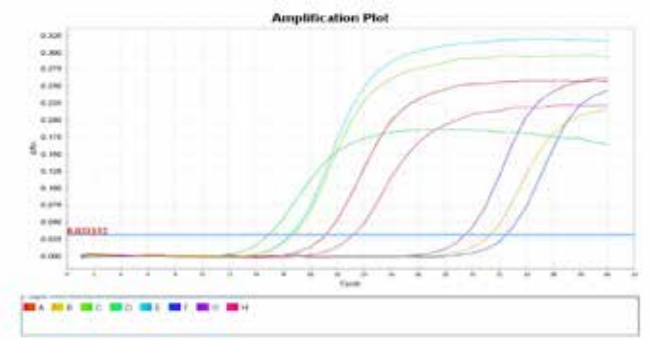
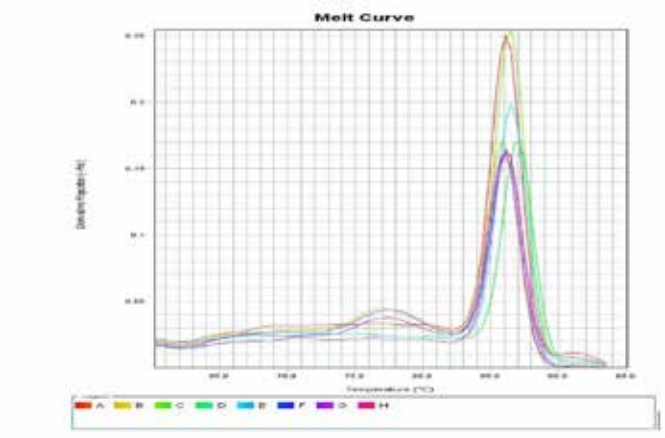


Figure 3. *Melt curve of SYBR green based real-time PCR.*



Because of its speed, Bruce-Ladder PCR has served as a useful method in the typing of *Brucella* species isolated from animal and humans. The multiplex PCR Bruce-ladder based typing of 153 *Brucella* strains isolated from different regions of Mexico was reported by[25]. The same assay was used by[29] for the identification and differentiation of *Brucella* species viz., *Brucella abortus*, *B. suis*, *B. ovis*, *B. melitensis* in the field as well as slaughter house samples in Brazil.

SYBR Green Based Real-Time PCR

In the present study, SYBR green based real-time PCR for detection of *Brucella* species were carried out using the species-specific primers targeting the *bcsp31* gene. After the completion of cycling parameters, data analysis was done based on the amplification curves obtained (Fig. 2). All five isolates were confirmed as positive by generating a fluorescent signal during real-time PCR which matched the reference strain of *B. abortus*. All isolates of *B. abortus*, as well as the positive reference isolate, showed specific amplification from approximately the 12th to 18th cycle. The mean Ct ranged between 15 and 21. Additionally, the specificity of the amplified PCR products was also confirmed by performing a melting curve analysis (Fig. 3). The Tm calling value of *B. abortus* reference strain and isolates was generated at 86°C. The Tm values of all the five isolates were almost identical to the reference *B. abortus* strain.

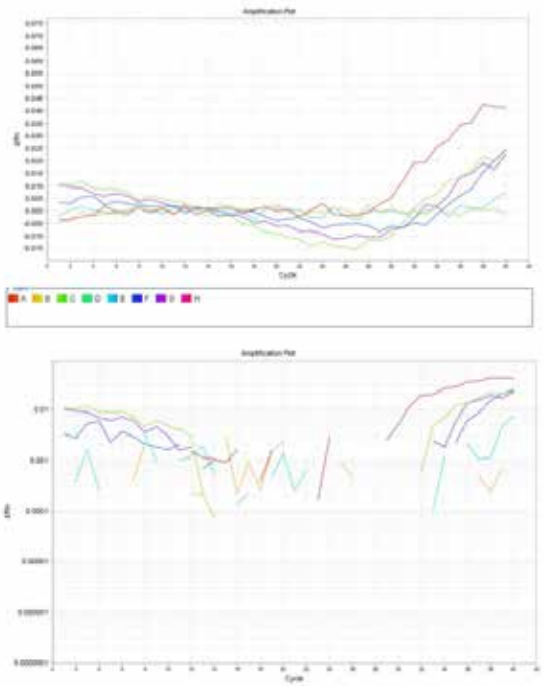
The use of a simple SYBR Green format can be justified by multiple reasons including high specificity of 223 bp target, clear distinction of specific products from artefacts apart from the simplicity and cost-effectiveness. The LightCycler-based real-time PCR (LCPCR) assay was developed by[38] for the detection of *Brucella* based on a 223-bp gene sequence encoding an immunogenetic membrane protein (*BCSP31*) specific for the *Brucella* genus. To evaluate the various previously published real-time PCR assays targeting *bcsp31*, per, IS711, alkB/IS711 and BMEI1162/ IS711, an in-house assay was developed by[1] using 248 *Brucella* strains representing the biotypes of all species and a large panel of clinically relevant, phylogenetically related and serologically cross-reacting bacteria. It was concluded that assays targeting the *bcsp31* gene can be recommended to screen for *Brucella*.

TaqMan Probe Based Real-Time PCR

A comprehensive approach was made by[17] who used unique genetic loci of the six classical species to develop seven individual reactions for detection of the *Brucella* genus and the differentiation between the six species to be used in conventional as well as real-time PCR assay based on the *Brucella*-specific insertion sequence IS711.

In the present investigation, the TaqMan probe based real time PCR with the target sequence of IS711 was carried out with the probe of *Brucella* genus Hinic Probe (IS711)[18]. The filter used was FAM 465-510 nm filter. The five isolates that were detected positive by Bruce ladder PCR and SYBR green PCR, were also detected to be positive by IS711 based real time PCR and were giving Ct values between 28 to 32. Amplifica-

Figure 4. *Amplification plots of TaqMan probe based real-time PCR.*



tion plots were observed as shown in Fig.4.

The real-time PCR assays using such TaqMan probes have been used to study the prevalence of *Brucella* by various investigators, like[12] who performed TaqMan analysis on 425 bovine blood samples in southwest Iran and found 9, 69 and 5 of these samples to be positive for *B. melitensis*, *B. abortus*, and both bacteria respectively. A similar real-time assay was carried out by10 who found 281/892 (31.5%) positive results for *Brucella* species in the bovine foetuses by conventional PCR and the TaqMan analysis confirmed that 45/281 and 231/281 were positive for B. meltensis and *B. abortus*, respectively. The high sensitivity and specificity of this real-time PCR assay, together with its speed and versatility make this technique a very useful tool for the diagnosis of brucellosis.

Identification with VITEK 2 Compact

All the five clinical isolates were identified as *B. abortus* with a probability of 99% and excellent confidence level using the GN colorimetric reagent card of Vitek 2 Compact Automated Identification System. The biochemical and metabolic profiles obtained in the VITEK system are depicted in Table 2.

The Vitek 2 Compact is a fully automated microbial identification system which has also been used successfully for the identification of the isolates of *Brucella* spp. in the present investigation as well as by various other researchers recently including.[2,7,11,13,19,24,32,43] It provides more precise and rapid identification with minimum handling of cultures by simultaneous application of a multitude of biochemical tests, followed by the precise prediction of *Brucella* upto species level.

Identification with VITEK MS MALDI-TOF

All the five purified isolates were loaded into the VITEK MS MALDI-TOF and the spectral profiles created by the VITEK MS RUO, were matched against the superspectra of the *Brucella* reference library previously created in the SARAMIS database. The VITEK MS identified all the isolates to the species level. Peak matches yielded the identification results as *Brucella abortus* with confidence values exceeding 99%. The graphs depicting the matching mass spectral profile of the superspectra and the five tested isolates are presented in Fig. 5-9 (VS 1-5).

As no reference library for *Brucella* has been incorporated into the main databases because of the issues derived from their potential bio-terrorist use, the *Brucella* reference libraries are created prior to their routine use for the accurate identification of *Brucella* spp. up to species level and even up to certain biovar level in some species. The same approach has been used in the present study as well as in other studies worldwide for *Brucella* spp. identification via VITEK MS MALDI-TOF.

The accurate identification of *Brucella*

Figure 5. *Graph depicting the matching mass spectral profile of the superspectra and Brucella abortus isolate VS 1.*

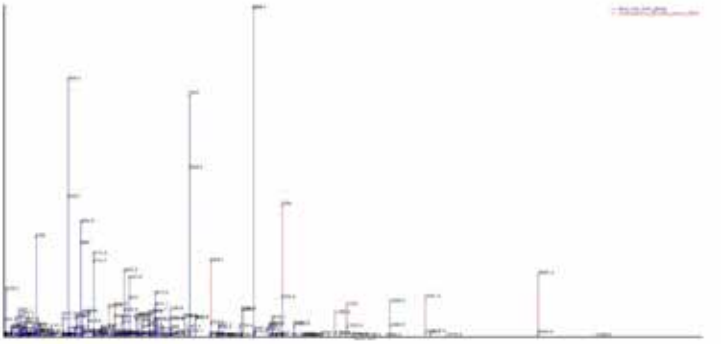


Figure 6. *Graph depicting the matching mass spectral profile of the superspectra and Brucella abortus isolate VS 2.*

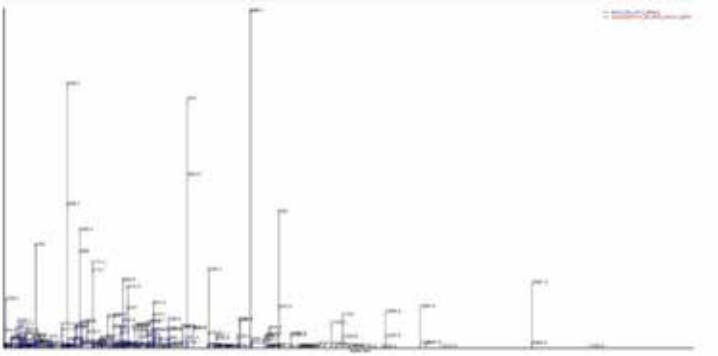
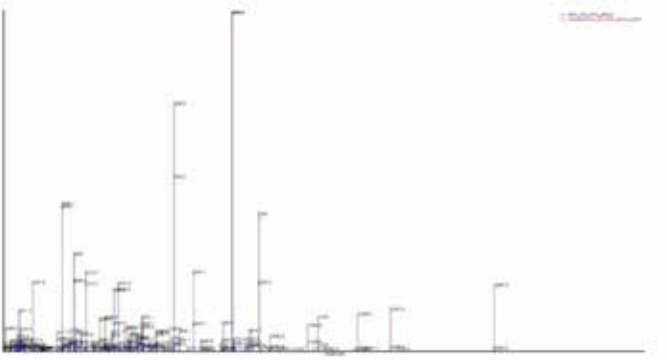


Figure 7. *Graph depicting the matching mass spectral profile of the superspectra and Brucella abortus isolate VS 3.*



upto species level was achieved by[21] by constructing a *Brucella* reference library and identifying the 99.3% of the 152 isolates at the species level, and *B. suis* biovar 1 and 2 at the biovar level. A home-made reference database was constructed by[9] and all the *Brucella* field isolates tested were correctly identified at the genus level and 94% at the species level. The MALDI-TOF-MS was used by[40] for biotyping of 124 *Brucella* isolates and enough resolution was achieved for binomial identification with good matching scores.

Still undisputable detection of *Brucella* is the classic isolation followed by genus and species identification with either phenotypic or nucleic acid recognition.[27] But the molecular diagnostic methods,

Figure 8. *Graph depicting the matching mass spectral profile of the superspectra and Brucella abortus isolate VS 4.*

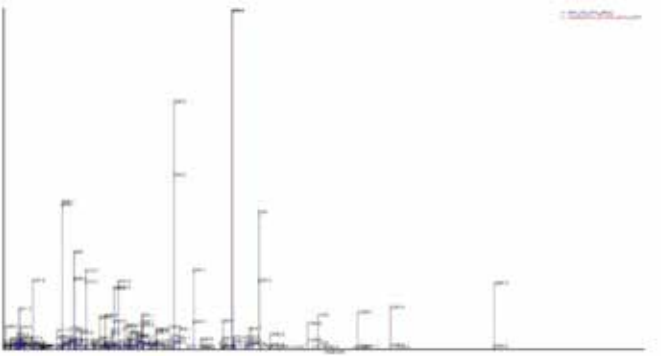
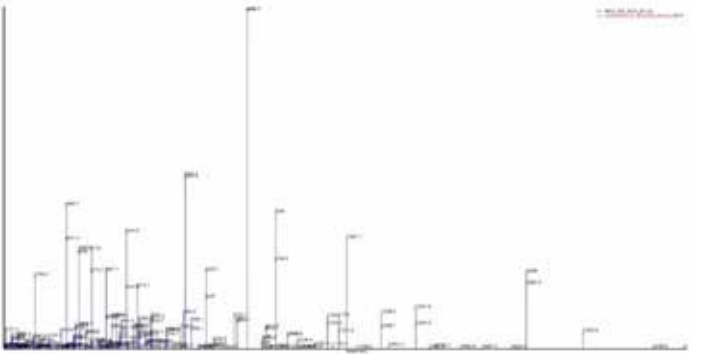


Figure 9. *Graph depicting the matching mass spectral profile of the superspectra and Brucella abortus isolate VD 5.*



mainly 16S ribosomal RNA sequencing or real-time PCR detection of selected genes remain complicated and costly and are not suited for use on the vast majority of routine samples. Further, the high genetic and phenotypic homology of *Brucella* renders its genospeciation an easier-said-than-done task. PCR based on specific genome sequences still suffer some inter-lab standardization problems.[44]

MALDI-TOF-MS technology has significant advantages over other conventional and molecular identification methods[26]. It is rapid and reliable, it takes only few minutes for correct identification[14]. In addition, MALDI technique is simple, does not require highly skilled personnel and is cost effective[41]. MALDI-TOF-MS works well for many bacterial species hence has the potential to replace conventional phenotypic identification for most bacterial strains isolated in clinical microbiology laboratories.[6] It is consistent with 16S rRNA gene sequencing.[42]

Further work is also being carried out to develop safe and MS-compatible protocols for inactivation of vegetative cells and spores of highly pathogenic organisms. Thus, the results from all the previous studies as well as the present investigation indicate that MALDI-TOF-MS assay is a quick as well as reliable approach to identify *Brucella* genus and species and an increasing number of different *Brucella* strains in the database could provide higher discriminatory power.

CONCLUSION

The present investigation highlights an urgent need to implement strict control measures to control the disease in Western Rajasthan, and entire India as a whole, because isolation from a single animal is sufficient evidence to establish the infection

Table 2. *Biochemical profile obtained by GN colorimetric reagent card in Vitek 2 Compact*

Well no.	Test	Result	Well No.	Test	Result
2	Ala-Phe-Pro-arylamidase enzyme activity	–	33	Saccharose/sucrose utilization	–
3	Acid production from adonitol	–	34	D-tagatose utilization	–
4	L-Pyrrolydonyl-arylamidase enzyme activity	–	35	D-trehalose utilization	–
5	L-arabitol utilization	–	36	Citrate (sodium) utilization	–
7	Acid production from D-cellobiose	–	37	Malonate utilization	–
9	β -galactosidase enzyme activity	–	39	5-keto-D-gluconate utilization	–
10	H2S production	–	40	L-lactate alkalinization	–
11	β -n-acetyl-glucosaminidase enzyme activity	–	41	Alpha-glucosidase enzyme activity	–
12	Glutamyl arylamidase para-nitroanilide activity	–	42	Succinate alkalinization	–
13	Acid production from D-glucose	–	43	β -N-acetyl-galactosaminidase activity	–
14	Gamma-glutamyl-transferase	–	44	Alpha-galactosidase enzyme activity	–
15	Fermentation of glucose	–	45	Phosphatase enzyme activity	–
17	β -glucosidase enzyme activity	–	46	Glycine arylamidase enzyme activity	+
18	D-maltose utilization	–	47	Ornithine decarboxylase enzyme activity	–
19	D-mannitol utilization	–	48	Lysine decarboxylase enzyme activity	–
20	D-mannose utilization	–	53	L-histidine assimilation	–
21	β -xylosidase enzyme activity	–	56	Courmarate utilization	–
22	β -alanine arylamidase para-nitroanilide activity	–	57	β -glucoronidase enzyme activity	–
23	L-proline arylamidase enzyme activity	+	58	O/129 resistance	–
26	Lipase enzyme activity	–	59	Glu-Gly-Arg-arylamidase enzyme activity	–
27	Palatinose utilization	–	61	L-malate assimilation	–
29	Tyrosine arylamilase enzyme activity	+	62	Ellman reaction	–
31	Urease enzyme activity (urea hydrolysis)	+	64	L-lactate assimilation	–
32	D-sorbitol utilization	–			

+ Positive and – Negative

status of a herd leading to the uncontrolled spread of disease even in the replacement stocks. Also, based on the results of present study, it can be suggested that MALDI-TOF-MS has the potential to become a rapid first-line screening tool for the identification of bovine brucellosis and can be used as a tool for epidemiological studies and outbreak investigation with minimal time, labor and cost, making it an attractive alternative to the relatively high investment required for other molecular settings.

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CONFLICT OF INTEREST

The authors declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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