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Isolation, Identification and Molecular Characterization of *Brucella abortus* from Bovines

M.D. Shrimali, N.M. Shah, B.S. Chandel, H.C. Chauhan, S.S. Patel, K.B. Patel, B.K. Patel, A.G. Bhagat, S.I. Patel, A.I. Dadawala, J.D. Shah, Manish Rajgor, R.P. Pandya, A.C. Patel, M.A. Patel, J.K. Kala and M.G. Patel

Department of Veterinary Microbiology, Veterinary College, S.D.A.U., Sardarkrushinagar, India.

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Brucellosis is an important zoonosis and a significant cause of reproductive losses in animals. Abortion, placentitis, epididymitis and orchitis are the most common clinical manifestations in animals. The present study a total of 168 clinical samples were collected from buffaloes (87) and cattle (81). All clinical samples were processed by cultural isolation on Brucella agar medium (BAM) with selective antibiotic supplements and genus specific PCR using B4/B5 (223bp) and F4/R2 (905bp) primer. Out of 168 clinical samples 15 samples yielded *Brucella* isolates by cultural isolation and 19 samples positive for *Brucella* organism by genus specific PCR. All genus specific PCR positive 19 samples also positive by Species specific PCR based on *omp31*, *B. abortus* +IS711 (498bp) primer. Amplicon of 498bp by *B. abortus* +IS711 primers indicates that all nineteen samples of cattle and buffaloes were found to be *Brucella abortus*.

Keywords: *Brucella abortus*, Cultural isolation, Molecular characterization, PCR.

Brucellosis is an infectious disease caused by Gram negative, facultative, intracellular bacterial organisms of the genus Brucella that are pathogenic for a wide variety of animals and human beings. The disease has a considerable impact on human and animal health, as well as socioeconomic impacts, especially in which rural income relies largely on livestock breeding and dairy products. It causes significant reproductive losses in sexually mature animals[5,27]. The disease is manifested by late term abortions, weak calves, still births, infertility and characterized mainly by placentitis, epididymitis and orchitis, with excretion of the organisms in uterine discharges and milk[4].

Diagnosis of Brucellosis by cultural isolation, serology and nucleic acid amplification has been explored for the rapid detection and confirmation of *Brucella*. Cultural isolation and identification of the agent is the gold standard test for *Brucella* diagnosis, however, this process is risky, time consuming, laborious and not suitable for disease surveillance. Moreover, it is a high risk biological pathogen that requires laboratories with qualified staff and facilities and class 3 personal protective equipment[13,22]. The Brucellosis diagnosis and surveillance by serological tests, The major drawback of these assays they are not always specific, can cross react with other gram negative bacteria[2] and antibodies are not produced at the acute stage of infection[17]. So, Confirmatory diagnosis by molecular techniques. A number of nucleic acid sequences have been targeted for the development of *Brucella* genus specific PCR assays, including 16S rRNA[24], IS711 genetic element, omp2[15] and bcsp31.

* To whom all correspondence should be addressed.
E-mail: drsandipvety@gmail.com

MATERIALS AND METHODS

Collection of Sample

A total of 168 clinical samples like deep vaginal swabs, placental cotyledon, vaginal discharge, aborted fetal materials, milk and blood from cattle and buffaloes.

Isolation

Each sample collected from an animal was separately streaked on Brucella agar medium (BAM) (Hi-media) with selective antibiotic supplements and incubated at 37°C aerobically in an atmosphere of 5 per cent CO_2 in CO_2 incubator for minimum of 15 days. The plates were observed at every 24 hours interval for the growth. The suspected colonies so obtained were streaked on Blood Agar (BA) and MacConkey Agar (MA).

Identification

The isolates suspected to be of *Brucella* were subjected to Gram staining and Modified Ziehl-Neelsen (MZN) staining for confirming the purity of cultures and morphological characters. Identification of Brucella organism by agglutination and biochemical test.

Rapid slide agglutination test

One drop (0.03 ml) of known *Brucella* positive serum (I.V.R.I., Izatnagar) was taken on a glass slide by micropipette. A loopful of culture from suspected single colony was mixed thoroughly with the spreader and then the slide was rotated for four min. The result was read immediately. Definite clumping/agglutination was considered as positive reaction, whereas no clumping/agglutination was considered as negative.

Biochemical characterization of isolates

Oxidase test

Standard oxidase discs (HiMedia Laboratories Ltd., Mumbai) containing 1% NNN'N' –tetramethyl-p-phenylene diamine dihydrochoride were used to perform the test. The loopful of culture from single colony was just touched on the disc. Development of blue colour within 10 seconds was considered as positive test.

Catalase test

This test was performed by taking 2-3 drops of 3% H_2O_2 on clean grease-free sterile glass slide and single colony from BAM plate was mixed with the help of a wire loop. Immediate development of gas bubbles was considered as positive test.

Triple Sugar Iron Agar (TSI) Test

In Triple Sugar Iron Agar test a test colony was taken with a sterilized straight inoculation needle and inoculated first by stabbing through the center of the medium to the bottom of the tube and then streaking the surface of the agar slant. Then tube with loose cap was incubated at 35°C for 18 to 24 hours and observed for color changes and gas production.

Molecular Detection of *Brucella*

DNA extraction

Tissues samples were cut into small pieces and triturated along with sterile sea sand in mortar and pestle then homogenates by tissue homogenizer. DNA extraction was carried out from samples using DNeasy Blood and Tissue Kit (Qiagen) following manufacturers protocols.

Detection of *Brucella* using Genus-Specific B4/ B5 primer

A PCR was standardized in a total reaction volume of 25 µl, containing 12.5 µl of 2 x PCR Master mixture, 10 pmol of forward (5'TGG CTC GGT TGC CAA TAT CAA3') and reverse (5'CGC GCT TGC CTT TCA GGT CTG3') primers each 1 µl , Template DNA 2 µl and nuclease free water upto 25 µl. The reaction was standardized in a thermal cycler (Eppendorf, Germany). with initial denaturation at 93ºC for 5 min, followed by 35 cycles at 90ºC for 60 s, 64ºC for 30 s and 72ºC for 60 s. Final extension was carried out at 72ºC for 10 min. The amplified product (223 bp) was electrophoresed in 2% agarose gel stained with ethidium bromide (0.5 µg/ml) and image was documented by gel documentation system (Mini BiS BioImaging System).

Detection of *Brucella* using Genus-Specific F4/ R2 primer

A PCR was standardized in a total reaction volume of 25 µl, containing 12.5 µl of 2 x PCR Master mixture, 10 pmol of forward (5' TCG AGC GCC CGC AAG GGG 3') and reverse (5' AAC CAT AGT GTC TCC ACT AA 3') primers each 1 µl , Template DNA 2 µl and nuclease free water upto 25 µl. The reaction was standardized in a thermal cycler (Eppendorf, Germany). with initial denaturation at 95ºC for 5 min, followed by 30 cycles at 95ºC for 30 s, 54ºC for 90 s and 72ºC for 90 s. Final extension was carried out at 72ºC for 6 min. The amplified product (905 bp)

was electrophoresed in 2% agarose gel stained with ethidium bromide (0.5 µg/ml) and image was documented by gel documentation system (Mini BiS BioImaging System).

Detection of *brucella* using Species-specific *B. abortus* + IS711primer

A PCR was standardized in a total reaction volume of 25 µl, containing 12.5 µl of 2 x PCR Master mixture, 10 pmol of forward (5' GAC GAA CGG AAT TTT TCC AAT CCC 3') and reverse (5' TGC CGA TCA CTT AAG GGC CTT CAT 3') primers each 1 µl, Template DNA 2 µl and nuclease free water upto 25 µl. The reaction was standardized in a thermal cycler (Eppendorf, Germany). with initial denaturation at 95ºC for 5 min, followed by 35 cycles at 95ºC for 90 s, 57ºC for 120 s and 72ºC for 120 s. Final extension was carried out at 72ºC for 5 min. The amplified product (498 bp) was electrophoresed in 2% agarose gel stained with ethidium bromide (0.5 µg/ml) and image was documented by gel documentation system (Mini BiS BioImaging System).

Fig. 1. Growth of *Brucella* organisms on BBL agar

RESULTS AND DISCUSSION

Isolation

Out of 168 clinical samples, 15 (8.92%) samples produce round, glistening and smooth or

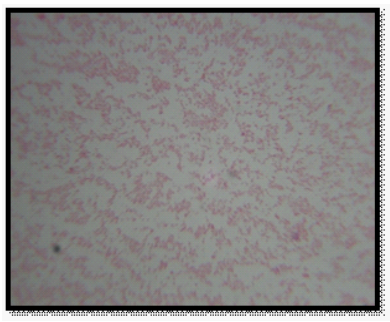


Fig. 2. Gram'sstaining- Gram negative cocco bacilli

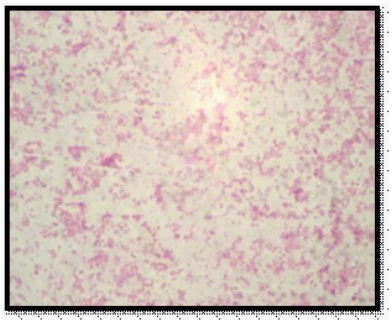


Fig 3. MZN staining of Brucella isolates

Table 1. Isolation of Brucella On Brucella agar medium

	Animal	Cattle	Buffalo	Total
Vaginal swabs	Tested	21	25	46
	Positive	03(14.28%)	01(4.00%)	04(8.69%)
Vaginal discharge	Tested	08	06	14
	Positive	02(25.00%)	01(16.66%)	03(21.42%)
Placenta	Tested	07	06	13
	Positive	02(28.57%)	01(16.66%)	03(23.07%)
Aborted fetus	Tested	06	11	17
	Positive	01(16.66%)	04(36.36%)	05(29.41%)
Milk	Tested	20	17	37
	Positive	00	00	00
Blood	Tested	19	22	41
	Positive	00	00	00
Total	Tested	81	87	168
	Positive	8(9.87%)	7(8.04%)	15(8.92%)

mucoid colonies on Brucella agar medium (BAM) (Fig 1, Table 1). These all 15 isolates produce Non-haemolytic colonies on blood agar (BA) but no growth could be obtained on MacConkey agar (MA). In the present finding was in agreement with earlier studies which reported 4% to 8% overall isolation rate[6,11]. However, in contrast to these findings overall isolation rate between 20 to 39 % [3,19]

Identification

Morphological and staining characters of isolates

The all 15 isolates were subjected to Gram's staining and Modified Ziehl-Neelsen's (MZN) staining. In Gram's staining pink, gram negative, coccobacillary rods (Fig 2). while in MZN staining they appeared to be red coccobacillary organisms (Fig 3). Similarly, morphology of organism observed by some other authors[1,6,11,19].

Rapid Slide Agglutination Test

All the colonies presumed to be of Brucella organism were tested for agglutinatibility with known positive anti Brucella serum. All the isolates revealed clear agglutination, indicative of Brucella abortus.

Biochemical characterization of isolates

All these 15 isolates gaved positive reaction in Catalase (Fig 4) and Oxidase test (Fig 5). On TSI slant, organism showed reaction as Slant (yellow), Butt (black) indicative as *Brucella abortus* (Fig 6). Pal and Jain (1985)[19] and Rhyan *et al.* (1994)[23] reported catalase and oxidase positive for *B. abortus*,

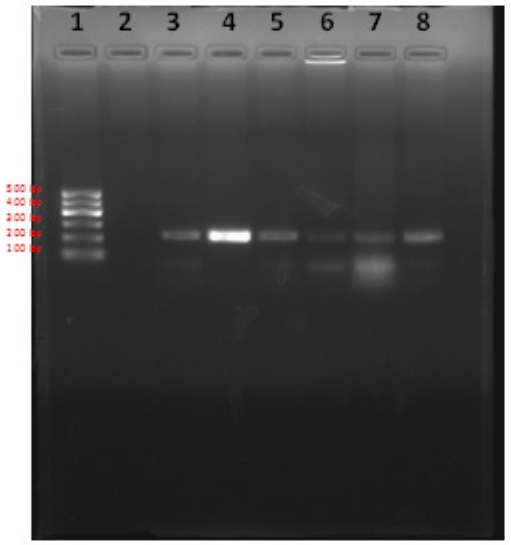
Molecular Detection of *Brucella*

In PCR study targeting 16S rRNA gene, Out of 168 clinical samples nineteen samples were found positive to give specific amplicon of 223bp region of the sequence encoding a 31 kDa immunogenic bcsp31 by Brucella genus specific primer pairs B4/B5 (Fig 7) and 905bp region of

Fig. 4. Catalase test positive

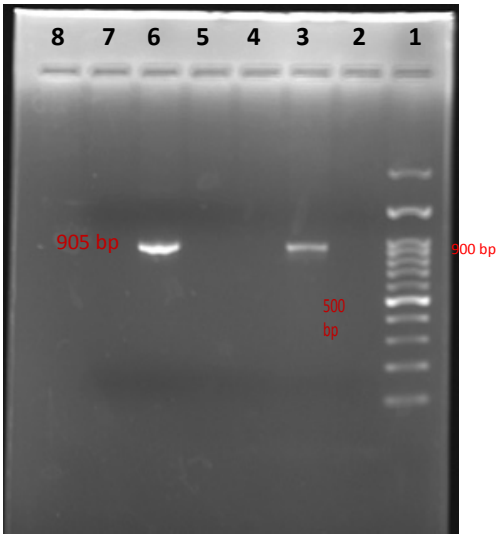
Fig. 5. Oxidase test

Fig. 6. TSI test



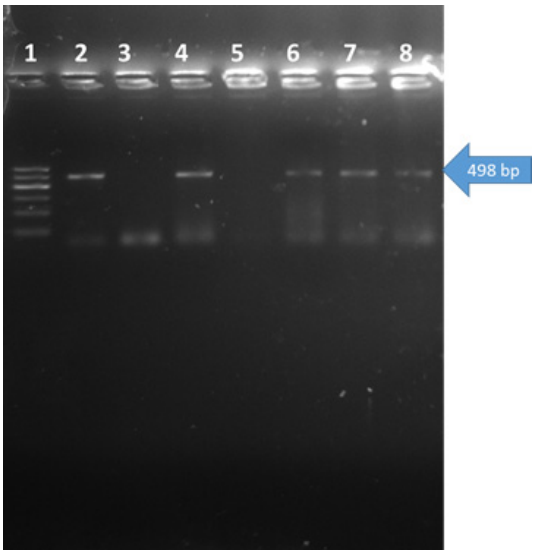
1- Ladder (100 bp) 2-Negative Control
3- Positive Control 4- Sample (positive)
5-Sample (positive) 6- Sample (positive)
7- Sample (positive) 8- Sample (positive)

Fig. 7. Agarose gel showing genus specific PCR amplification products of 223bp by using primer pairs B4/B5 (*bcsp31*)



1- Ladder (100 bp) 2- Negative control
3- Sample (positive) 4- Sample (negative)
5-Sample (negative) 6- Sample (positive)
7- Sample (negative) 8 -Sample (Negative)

Fig. 8. Agarose Gel electrophoresis of showing genus specific PCR amplification products of 905bp with F4/R2 primers



1- Ladder (100 bp) 2-Positive Control
3- Negative control 4- Sample (Positive)
5-Sample (Negative) 6,7,8- Samples (Positive)

Fig. 9. Agarose Gel electrophoresis of showing species specific PCR amplification products of 498 bp PCR product with primer IS711

the sequence 16S rRNA of *B. abortus* by Brucella genus specific primer pairs F4/R2 (Fig 8). All genus specific positive nineteen samples yielded an amplicon of 498bp in +IS711 primers indicate species as *Brucella abortus* (Fig 9). Similarly, Kanani (2007)[9] and Jung *et al*. (1998)[11] detection of *Brucella* by using bcsp31 gene based B4/B5 primer. Navarro *et al*. (2002)[18] and Varasada (2003)[26] using same primer pair for diagnosis of human brucellosis. Earlier Navarro *et al*. (2002)[18], Kanani (2007)[9] and Patel (2007)[21] used same three primer pairs for molecular detection of *Brucella abortus*. Patel *et al.,* (2015)[22] and Karthik *et al.,* (2014)[12] used species specific +IS711 primers for detection of *Brucella abortus* and they yielding 498 bp band when electrophoresed through 2 per cent agarose gel.

Comparative evaluation of cultural and molecular methods for detection of brucella infection

A total 168 clinical samples are collected for cultural isolation and molecular detection of Brucella organism by PCR. Of these, 19 samples detected positive for Brucella which were further identified as *B. abortus*. When these samples were processed for the isolation only 15 samples yielded *B. abortus*.[8,10,14,16,25] Detected more number of positives samples by PCR assay than cultural methods.

CONCLUSIONS

Present study indicated that *B. abortus* is widely prevalent in five districts of Gujarat (viz. Banaskantha, Patan, Sabarkantha, Surat and Katchchh) as a cause of Bovine Brucellosis. The isolation results showed the presence of *B. abortus* in clinical samples which is of public health importance because it is zoonotic disease. There is need to educate about how to prevent and control of brucellosis due to it cause high socioeconomic loss to the farmer.

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