**Clinico-Seroepidemiology and Molecular Characterization of Brucellosis in Animals**


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**A B S T R A C T**

The present study reports clinico seroepidemiological observations of Brucellosis including molecular epidemiology. During the period of study a total of 6410 sera were collected, which included cattle (2723), buffaloes (894), sheep (1072), goats (1281), camel (438) and equine(02). These sera were screened for the presence of Brucella antibodies by RBPT and i-ELISA. The overall seroprevalence detected in animals were 12.85% (824 out of 6410) and 11.87% (761 out of 6410) by RBPT and i-ELISA, respectively. Clinical conditions wise seroprevalence was recorded in heifer 4.65% and 6.97%, clinically healthy animals 7.23% and 6.56%, animals with the history of abortion 25.58% and 29.09%, hygroma 13.33% and 11.66%, pregnant 5.45% and 4.21%, non-pregnant 7.81% and 6.17%, status unknown 7.74% and 6.16%, still birth 16.00% and 20.00%, retention of placenta 14.22% and 11.71%, repeat breeding 19.70% and 14.35%, orchitis 34.34% and 18.18%respectively by RBPT and i-ELISA. Out of 744 milk samples of aborted animal, 152 milk samples found positive for Brucella antibody by MRT. A total l110 clinical samples were processed by genus specific PCR using B4/B5 (223bp) primer. Of these 15 samples positive for Brucella organism. Out of 15 genus specific PCR positive samples, 12samples positive by B. abortus +IS711 (498bp) species specific PCR and 3 samples positive by B. melitensis omp31 (723bp) species specific PCR. All the 15 samples also confirmed positive by Bruce ladder PCR.

**Keywords**

Brucellosis, Seroprevalence, PCR, B. abortus, B. melitensis, Molecular detection.

**Article Info**

Accepted: 02 March 2017
Available Online: 10 April 2017

**Introduction**

Brucellosis is caused by various species of the genus *Brucella*, which is the second most widely spread zoonosis worldwide (Dawood, 2008). *Brucella* can affect almost all domestic species and cross transmission can occur between cattle, sheep, goat, camel and other species (Ghanem et al., 2009). Brucellosis is characterized by abortion and birth of non-visible offspring in females, orchitis and epididymitis in males (Radostits et al., 2007) (Fig. 1) It may also cause chronic inflammation of joints, tendon sheath and synovial bursa especially at the carpus (Abbas and Agab, 2002). There are many factors that can affect the prevalence of brucellosis in various species of livestock. Prevalence may vary according to climatic conditions, geography, species, sex, age and diagnostic tests used (Gul and Khan, 2007). Proper treatment and prevention of disease requires
prompt and accurate diagnosis. Molecular diagnostics provides excellent platform for accurate and prompt diagnosis of diseases while maintaining safety of the personnel (Sola et al., 2014) and detection of Brucella organisms in various clinical, blood and serum samples of livestock and human employing PCR, Real time PCR, speciation by species specific PCR and Bruce ladder multiplex PCR. The disease has been reported from various species in different parts of India. Therefore, present study was planned for overall seroprevalence and molecular epidemiological characteristics of Brucellosis in animals.

Materials and Methods

Seroprevalence

A total 6410 Serum samples Cattle (2723), Buffaloes (894), Sheep (1072), Goats (1281), Camels (438), and equine (02) were collected from various district of Gujarat for detection of Brucella antibodies.

Rose Bengal Plate Test (RBPT)

One drop (30 µl) of known Brucella antigen (IAHVB, Hebbal, Bangalore) was taken on a glass slide by micropipette then add equal amount of suspected serum samples and mixed thoroughly. The result was read immediately. Definite clumping/agglutination was considered as positive reaction, whereas no clumping/agglutination was considered as negative.

Indirect ELISA

Indirect ELISA kit was procured from National Institute of Veterinary Epidemiology and Disease Informatics (NIVEDI) Bangalore, and used as per manufacturer’s protocols. The kit detects the antibodies against Brucella lipopolysaccharide (LPS) in serum samples of B. abortus and B. melitensis.

Milk Ring Test (MRT) / Abort Bang ring test (ABR)

A total of 744 milk samples were collected in sterila vial after thorough mixing 3 ml milk samples were taken in a test tube. Add 3 drops of ABR antigen and gently mixed (ABR-antigen prepared by IAHVB, Hebbal, Bangalore). The tubes were incubated at 37°C for one hour. Then keep at room temperature for 30 min. Blue colored ring of cream layer at the top and absence of color in milk layer is considered as a positive. If whole milk retains blue is considered as a negative.

Molecular detection of Brucella

A total of 1110 various clinical samples were collected from cattle, buffaloes, sheep, goats, camel and equine for molecular characterization of Brucella.

DNA extraction

The genomic DNA from clinical samples was extracted using DNeasy Blood and Tissue Kit (Qiagen, USA) following manufacturers protocols.

Detection of Brucella using Genus-Specific, Species-specific and Bruce-ladder PCR

These samples processed for genus PCR, species specific PCR and Bruce-ladder PCR for confirmation using primer pair (Table 1) and conditions of thermal cycling for different primer pairs in PCR (Table 2) and PCR reaction mixture was prepared as per table 3.

PCR amplified product was checked by running samples over 2.0% agarose gel.

Results and Discussion

Seroprevalence

Out of 6410 sera sample, overall seroprevalence recorded in animals was
12.85% (824 out of 6410) and 11.87% (761 out of 6410) by RBPT (Fig. 2) and i-ELISA (Fig. 3) respectively (Table 4). Aulakh et al., (2008) reported overall seroprevalence of 18.26% in cattle and buffaloes from Punjab by ELISA. Gumber et al., (2004) reported overall seroprevalence of 22.5% positive in cattle and buffaloes in Punjab, by RBPT. Panchasara et al., (2015) reported overall seroprevalence was 10.66%, 10.29% and 9.38% by RBPT, STAT and i-ELISA, respectively in North Gujarat. However, in contrast to the present study higher rate of overall seroprevalence 28.00% was reported by Ahmed et al., (2010) by RBPT. Kushwaha et al., (2015) also reported very high seroprevalence rate of 33.85%, 32.61% and 30.90% by ELISA, RBPT and STAT, respectively in Pakistan. Clinical symptoms wise seroprevalence was 4.65% to 34.34% by RBPT and 4.21% to 29.09% by i-ELISA (Table 5).

**Table 1** List of primers

<table>
<thead>
<tr>
<th>Primer Forward/ Reverse</th>
<th>Sequence (5’-3’)</th>
<th>Product size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>B4 (F) B5 (R)</td>
<td>TGG CTC GGT TGC CAA TAT CAA</td>
<td>223bp</td>
<td>Bailey et al., (1992)</td>
</tr>
<tr>
<td></td>
<td>CGC GCT TGC CTT TCA GGT CTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Species specific primers (B. abortus):</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IS711(F) IS711(R)</td>
<td>GAC GAA CGG AAT TTT TCC AAT CCC TGC CTA CTT AAG GGC CTT CAT</td>
<td>498 bp</td>
<td>Bricker and Halling, (1994).</td>
</tr>
<tr>
<td><strong>Species specific primers (B. melitensis):</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Omp31(F) Omp31(R)</td>
<td>TGACAGACTTTTTTCGCCGAA</td>
<td>723bp</td>
<td>Vizcaino et al (1996)</td>
</tr>
<tr>
<td></td>
<td>TATGGATTCGCAGCAACGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Bruce Ladder Primers:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMEI0998 (F) BMEI0997(R)</td>
<td>ATCCTATTGCCCCCGATAAGG</td>
<td>1682bp</td>
<td>Lopez-Goniet et al., (2008)</td>
</tr>
<tr>
<td>BMEI0535(F) BMEI0536(R)</td>
<td>GCCAGGGGAAAAACAGCTATAA</td>
<td>450bp</td>
<td></td>
</tr>
<tr>
<td>BMEI0843(F) BMEI0844(R)</td>
<td>TTTACACAGCAATCCAGCA</td>
<td>1071bp</td>
<td></td>
</tr>
<tr>
<td>BMEI436(F) BMEI435(R)</td>
<td>AGCGAGACGACCTCGGTAT</td>
<td>794bp</td>
<td></td>
</tr>
<tr>
<td>BMEII0987(F) BMEII0987(R)</td>
<td>TTTATCCATCGGCCTGTCAC</td>
<td>152bp</td>
<td></td>
</tr>
<tr>
<td>BMEII0428(F) BMEII0428(R)</td>
<td>GCCGCTATTATGACTTGG</td>
<td>587bp</td>
<td></td>
</tr>
<tr>
<td>BMEII0285(F) BMEII0285(R)</td>
<td>AATGACCTTACGCTGCCTTG</td>
<td>272bp</td>
<td></td>
</tr>
<tr>
<td>BR0953(F) BR0953(R)</td>
<td>GGAACACTACGCCCATTTG</td>
<td>1218bp</td>
<td></td>
</tr>
<tr>
<td>BMEI0847(F) BMEI0847(R)</td>
<td>CGCAGACGAGTGACCATCAA</td>
<td>152bp</td>
<td></td>
</tr>
<tr>
<td>BMEII0428(F) BMEII0428(R)</td>
<td>GCCGCTATTATGACTTGG</td>
<td>794bp</td>
<td></td>
</tr>
<tr>
<td>BR0953(R)</td>
<td>GATGGAGCAAACGCTGAAG</td>
<td>1218bp</td>
<td></td>
</tr>
<tr>
<td>BMEII0847(F) BMEII0847(R)</td>
<td>CGCAGACGAGTGACCATCAA</td>
<td>152bp</td>
<td></td>
</tr>
<tr>
<td>BMEII0428(F) BMEII0428(R)</td>
<td>GCCGCTATTATGACTTGG</td>
<td>794bp</td>
<td></td>
</tr>
<tr>
<td>BR0953(R)</td>
<td>GATGGAGCAAACGCTGAAG</td>
<td>1218bp</td>
<td></td>
</tr>
<tr>
<td>BMEII0847(F) BMEII0847(R)</td>
<td>CGCAGACGAGTGACCATCAA</td>
<td>152bp</td>
<td></td>
</tr>
<tr>
<td>BMEII0428(F) BMEII0428(R)</td>
<td>GCCGCTATTATGACTTGG</td>
<td>794bp</td>
<td></td>
</tr>
<tr>
<td>BR0953(R)</td>
<td>GATGGAGCAAACGCTGAAG</td>
<td>1218bp</td>
<td></td>
</tr>
<tr>
<td>BMEII0847(F) BMEII0847(R)</td>
<td>CGCAGACGAGTGACCATCAA</td>
<td>152bp</td>
<td></td>
</tr>
</tbody>
</table>
Table 2: Steps and conditions of thermal cycling for different primer pairs in PCR

| Primers (Forward and Reverse) | Cycling conditions | | | | | Cooling |
|-------------------------------|--------------------|----|---|---|---|
|                              | Initial denaturation | Denaturation | Annealing | Extension | Final extension | |
| B4 (F) B5 (R)                | 93°C 5 min          | 90°C 1 min | 64°C 30 sec | 72°C 1 min | 72°C 10 min     | 4°C |
| IS711 (F) IS711 (R) (B. abortus) | 95°C 2 min.         | 95°C 1.15 min | 55.5°C 2 min. | 72°C 2 min. | 72°C 2 min.     | 1 cycle |
| Omp31 (F) Omp31 (R) (B. melitensis) | 94°C 5 min.         | 94°C 1 min. | 58°C 1 min. | 72°C 2 min. | 72°C 10 min.    | 1 cycle |
| Bruce Ladder Primers         | 95°C 7 min          | 95°C 35 second | 64°C 45 sec | 72°C 3 min. | 72°C 6 min      | 1 cycle |
|                              | 1 cycle             | Repeated for 35 cycles | 1 cycle | |
|                              | 1 cycle             | Repeated for 35 cycles | 1 cycle |
|                              | 1 cycle             | Repeated for 25 cycles | 1 cycle |

Table 3: Various components used in PCR

<table>
<thead>
<tr>
<th>Components</th>
<th>Genus/Species specific PCR</th>
<th>Components</th>
<th>Bruce ladder PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR Master Mix (2X)</td>
<td>12.5 μl</td>
<td>PCR Master Mix (2X)</td>
<td>12.5 μl</td>
</tr>
<tr>
<td>Forward Primer (10 pmol/μl)</td>
<td>1 μl</td>
<td>Bruce-ladder eight pair primer cocktail (12.5μM)</td>
<td>1 μl</td>
</tr>
<tr>
<td>Reverse Primer (10 pmol/μl)</td>
<td>1 μl</td>
<td>Template DNA</td>
<td>2 μl</td>
</tr>
<tr>
<td>Template DNA</td>
<td>2 μl</td>
<td>Nuclease free water</td>
<td>8.5 μl</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>8.5 μl</td>
<td>Total</td>
<td>25 μl</td>
</tr>
<tr>
<td>Total</td>
<td>25 μl</td>
<td>Total</td>
<td>25 μl</td>
</tr>
</tbody>
</table>

Table 4: Overall seroprevalence

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of tested</th>
<th>Seroprevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>RBPT positive</td>
</tr>
<tr>
<td></td>
<td></td>
<td>i-ELISA positive</td>
</tr>
<tr>
<td>Cattle</td>
<td>2723</td>
<td>333 (12.22%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>307 (11.27%)</td>
</tr>
<tr>
<td>Buffalo</td>
<td>894</td>
<td>129 (14.42%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>116 (12.97%)</td>
</tr>
<tr>
<td>Goat</td>
<td>1281</td>
<td>123 (9.60%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>108 (8.43%)</td>
</tr>
<tr>
<td>Sheep</td>
<td>1072</td>
<td>170 (15.85%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>171 (15.95%)</td>
</tr>
<tr>
<td>Camel</td>
<td>438</td>
<td>69 (15.75%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>59 (13.47%)</td>
</tr>
<tr>
<td>Equine</td>
<td>02</td>
<td>0 (0.00%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 (0.00%)</td>
</tr>
<tr>
<td>Total</td>
<td>6410</td>
<td>824 (12.85%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>761 (11.87%)</td>
</tr>
</tbody>
</table>
Table 5 Clinical status wise seroprevalence

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>No. of tested</th>
<th>Seroprevalance</th>
<th>RBPT positive</th>
<th>i-ELISA positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heifer</td>
<td>172</td>
<td>08(4.65%)</td>
<td>12(6.97%)</td>
<td></td>
</tr>
<tr>
<td>Clinically healthy</td>
<td>2087</td>
<td>151(7.23%)</td>
<td>137(6.56%)</td>
<td></td>
</tr>
<tr>
<td>Abortion</td>
<td>1110</td>
<td>284(25.58%)</td>
<td>323(29.09%)</td>
<td></td>
</tr>
<tr>
<td>Hygroma</td>
<td>180</td>
<td>24(13.33%)</td>
<td>21(11.66%)</td>
<td></td>
</tr>
<tr>
<td>Pragnant</td>
<td>403</td>
<td>22(5.45%)</td>
<td>17(4.21%)</td>
<td></td>
</tr>
<tr>
<td>Non-pragnant</td>
<td>486</td>
<td>38(7.81%)</td>
<td>30(6.17%)</td>
<td></td>
</tr>
<tr>
<td>Status unknown</td>
<td>762</td>
<td>59(7.74%)</td>
<td>47(6.16%)</td>
<td></td>
</tr>
<tr>
<td>Still birth</td>
<td>50</td>
<td>08(16.00%)</td>
<td>10(20.00%)</td>
<td></td>
</tr>
<tr>
<td>Retension of Placenta</td>
<td>239</td>
<td>34(14.22%)</td>
<td>28(11.71%)</td>
<td></td>
</tr>
<tr>
<td>Repeat breeding</td>
<td>822</td>
<td>162(19.70%)</td>
<td>118(14.35%)</td>
<td></td>
</tr>
<tr>
<td>Orchitis</td>
<td>99</td>
<td>34(34.34%)</td>
<td>18(18.18%)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>6410</td>
<td>824(12.85%)</td>
<td>761(11.87%)</td>
<td></td>
</tr>
</tbody>
</table>

Table 6 Processing of milk samples by MRT

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of tested</th>
<th>MRT positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle</td>
<td>584</td>
<td>116(19.86%)</td>
</tr>
<tr>
<td>Buffalo</td>
<td>154</td>
<td>36(23.37%)</td>
</tr>
<tr>
<td>Camel</td>
<td>06</td>
<td>00(0.00%)</td>
</tr>
<tr>
<td>Total</td>
<td>744</td>
<td>152(20.43%)</td>
</tr>
</tbody>
</table>

Fig.1 Clinical symptoms

Abortion Retension of placenta
Hygroma Orchitis
Table 7 Direct detection of Brucella by PCR

<table>
<thead>
<tr>
<th>Type of sample</th>
<th>Species</th>
<th>No.of tested</th>
<th>Result</th>
<th>No. of sample positive in PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cattle</td>
<td>Buffalo</td>
<td>Sheep</td>
<td>Goat</td>
</tr>
<tr>
<td>Blood</td>
<td>231</td>
<td>102</td>
<td>178</td>
<td>111</td>
</tr>
<tr>
<td>Vaginal swab</td>
<td>73*</td>
<td>45*</td>
<td>37</td>
<td>31</td>
</tr>
<tr>
<td>Vaginal discharge</td>
<td>08**</td>
<td>05*</td>
<td>02*</td>
<td>00</td>
</tr>
<tr>
<td>Milk</td>
<td>12</td>
<td>08</td>
<td>04</td>
<td>06</td>
</tr>
<tr>
<td>Placenta</td>
<td>17**</td>
<td>09**</td>
<td>04*</td>
<td>01</td>
</tr>
<tr>
<td>Placental fluid</td>
<td>05</td>
<td>02</td>
<td>00</td>
<td>00</td>
</tr>
<tr>
<td>Hygroma fluid</td>
<td>02</td>
<td>01</td>
<td>00</td>
<td>00</td>
</tr>
<tr>
<td>Amniotic fluid</td>
<td>00</td>
<td>02</td>
<td>00</td>
<td>00</td>
</tr>
<tr>
<td>Orchitis fluid</td>
<td>00</td>
<td>00</td>
<td>03</td>
<td>01</td>
</tr>
<tr>
<td>Foetal intestine</td>
<td>00</td>
<td>00</td>
<td>03</td>
<td>01</td>
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<tr>
<td>Foetal intestine fluid</td>
<td>02</td>
<td>01</td>
<td>01</td>
<td>02</td>
</tr>
<tr>
<td>Foetal lung</td>
<td>09*</td>
<td>04</td>
<td>02</td>
<td>06</td>
</tr>
<tr>
<td>Foetal liver</td>
<td>09*</td>
<td>04</td>
<td>02</td>
<td>06</td>
</tr>
<tr>
<td>Foetalabomasal content</td>
<td>00</td>
<td>00</td>
<td>03*</td>
<td>02</td>
</tr>
<tr>
<td>Foetal stomach content</td>
<td>03*</td>
<td>01</td>
<td>03</td>
<td>05</td>
</tr>
<tr>
<td>Foetal heart</td>
<td>03</td>
<td>00</td>
<td>02</td>
<td>02</td>
</tr>
<tr>
<td>Foetal heart blood</td>
<td>06</td>
<td>04</td>
<td>02</td>
<td>04</td>
</tr>
<tr>
<td>Cotyledon</td>
<td>06</td>
<td>04</td>
<td>00</td>
<td>00</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>386</td>
<td>192</td>
<td>246</td>
<td>178</td>
</tr>
</tbody>
</table>

*indicate number of positive sample in PCR

Fig.2 Rose Bengal test
**Fig. 3** i-ELISA

Wells A1, B1, A2, B2: Negative control; Wells C1, D1, C2, D2: Moderately positive control
Wells E1, F1, E2, F2: Strong positive control; Wells A3, B3, C3, D3, C5, D5 etc. positive field sera reaction

**Fig. 4** Milk ring test

**Fig. 5** Genus specific PCR223bp PCR products with B4B5 primer

1-ladder
2-NTC
3- sample (positive) Vaginal discharge
4- sample (positive) Foetal lung
5- sample (positive) Placenta
6- sample (positive) Foetal liver
7- sample (positive) Foetal stomach content
Out of 744 milk samples, of these 152 (20.43%) samples were found positive for Brucella antibodies by MRT (Fig. 4; Table 6). Present finding was in agreement with Zowghi et al., (1990) which detected 25.21% Brucella antibody from milk by MRT. However, In
contrast to the present findings, Al-Mariri (2015) reported 9.16% and Gulluce et al., (1996) reported 56.32% Brucella antibody from milk by MRT.

**Molecular detection of Brucella from clinical samples**

In PCR study targeting 16S rRNA gene, Out of 1110 clinical samples fifteen samples (Table 7) were found positive to give specific amplicon of 223bp region of the sequence encoding a 31 kDa immunogenic bscp31 by Brucella genus specific primer pairs B4/B5 (Fig. 5). Out of 15 genus specific positive samples, 12 samples yielded an amplicon of 498bp in +IS711 primers indicate species as Brucella abortus (Fig. 6) and 3 samples yielded an amplicon of 723bp in omp31 primers indicate species as Brucella melitensis (Fig. 7). All 15 genus specific positive samples were also confirmed as positive by Bruce ladder PCR (Fig. 8).


**Acknowledgement**

We are highly thankful to DBT, Govt. of India for financial assistance for the project.

**References**


How to cite this article: