Assessment of haemato-biochemical parameters and therapeutics on Brucella infected cattle

Abstract
Brucellosis is a contagious systemic bacterial disease of livestock. Antibiotics commonly being used have limited efficacy against the disease. In the present study, therapy with antibiotics on Brucella infected cattle was performed and subsequently haemato-biochemical parameters, detection of anti-Brucella antibodies in both serum and milk by different diagnostics and shedding of Brucella in milk were assessed. Treatment with antibiotics; streptomycin and long acting tetracycline alone and in combination were given for four weeks. Hb, TEC, TLC, neutrophils, eosinophils, lymphocytes, monocytes count, AST, ALT and SD values were lower whereas, PCV, serum glucose, total protein, albumin and creatinine were higher in Brucella infected cattle. Treatment with combination therapy showed promising results in these animals. Haemato-biochemical parameters were become near to normal in most of animals that received combination therapy. However, not all animals were returned to normal after treatment but a significant numbers in combination therapy were reduced anti-Brucella antibody titre, both in serum and milk. The level of as shedding of Brucella in milk had also gone undetectable.

Keywords: brucellosis, cattle, haemato-biochemical parameters, oxytetracycline, streptomycin

Materials and methods
A total of 27 cattle which showed antibodies both in serum and milk as well as Brucella DNA in milk were selected for the study. ELISA, RBPT and STAT were employed on serum whereas, MRT and ELISA on milk to detect the anti-Brucella antibodies. Milk samples were processed for detection of pathogen itself in form of its DNA (16S rRNA) by PCR. Reference antigens and serum for RBPT, STAT and, for MRT, Abortus Bang Ring Antigen (ABR) were procured from the Indian Veterinary Research Institute, Izatnagar (India). AniGen B. Brucella Ab ELISA kit (Cat. No. EB43-01) (i-ELISA and Milk-ELISA) was procured from Bionote (Korea). Haemato-biochemical parameters, antibody titre in milk/serum and shedding of pathogen in milk were evaluated before and after the therapeutic trial.

Antibody detection
RBPT was performed according to the method prescribed in OIE. Equal volume of both antigen and serum sample was mixed, definite agglutination was taken as positive whereas, no agglutination as negative. STAT was performed as per the method described by Alton et al. All serum samples were tested up to minimum of five dilutions. Considering the special significance of 50% end point, a control tube was set up to simulate 50% clearing by mixing 0.5 ml antigen with 1.5 ml of phenol saline (0.5%, v/v) in an agglutination tube. All tubes
were incubated at 37°C for 20 hrs before result was observed. The highest serum dilution showing 50% or more agglutination (50% clearing) was considered as the titre of the serum.

MRT was performed on individual milk samples according to the method described in OIE. Dark pink ring above the white milk column was taken as positive whereas, pink colour of the underlying milk excels that of the cream layer as negative. i-ELISA and Milk-ELISA was performed by using AmiGen B. Brucella Ab ELISA kit. Method mentioned in manual provided with kit was followed. OD of ELISA plate was taken at 450nm to calculate the percent positivity (%P) of both serum and milk samples. Positive and negative samples were determined based on Percent positivity (%P) value. Sera which have %P value ≥25 were taken as positive, whereas, samples having %P value <25 were negative. %P of serum was calculated from OD as follow.

\[ \%P = \left( \frac{OD_{serum\ sample}}{Average\ OD\ of\ standard\ positive\ control} \right) \times 100 \]

The milk samples gave %P value ≥15 were taken as positive whereas, samples with <15 %P value as negative. It was calculated as follows:

\[ \%P = \left( \frac{OD_{milk\ sample}}{Average\ OD\ of\ standard\ positive\ control\ milk} \right) \times 100 \]

Polymerase chain reaction

Specific DNA sequence of 905 bp, which belong to 16S rRNA of B. abortus, was targeted for amplification. Oligonucleotides (F4: 5'-TCGAGCGCCCGCAAGGGG-3' and R2: 5'-AACCATAGTGTCCACTAA-3') required for amplification of earlier said sequence were taken from published literature. Methods described for DNA extraction from milk and PCR were followed as mentioned in their respective literatures. Agarose gel (0.8% w/v in 0.5X Tris-borate EDTA) containing 0.5μg/ml ethidium bromide was used to electrophorese the amplified PCR products in 0.5X Tris-borate EDTA at 40 Volt/cm.

Haematological- biochemical parameters

Haematological parameters; Haemoglobin (Hb), Packed Cell Volume (PCV), Total Erythrocytes Count (TEC), Total Leucocytes Count (TLC) and Differential Leucocytes Count (DLC) were estimated as methods described by Jain. Biochemical parameters; Glucose, Total Protein, Albumin, Creatinine, Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST) and Sorbitol Dehydrogenase (SD) were estimated in serum by commercially available kits (Span Diagnostics and Sigma).

Therapeutic trial

Cattle which had anti-Brucella antibodies both in serum and milk as well as presence of pathogen in milk were chosen for therapeutic trial. Three groups were formed; each contained nine cattle and maintained for 4 weeks. Long acting oxytetracycline at a dose rate of 25mg/kg intramuscularly, every other day for four weeks was given in Group A. Similarly, streptomycin at a dose rate of 20mg/kg intramuscularly, every other day for 4 weeks was given in Group B. While in Group C, long acting oxytetracycline at a dose rate 25mg/kg combined with streptomycin at a dose rate 20mg/kg were injected intramuscularly every other day for two weeks and further, oxytetracycline at the same dosage without streptomycin for two weeks. A control group of three animals, all were negative for anti-Brucella antibodies both in serum and milk as well as no pathogen found in milk were maintained for 4 weeks without any treatment given.

Statistical analysis

Statistical analysis between respective means for various parameters was performed using ANOVA and t-test, as per the method described by Snedecor & Cochran at 5% level of significance.

Results

Cattle found positive for antibodies both in serum and milk as well as presence of pathogen in milk were selected for the therapeutic trail. Treatment efficacies were evaluated on the basis of haemato-biochemical parameters, detection of antibodies both in milk and serum and shedding of pathogen in milk, before and after the treatments. Brucella infected cattle showed; lower Hb, TEC, TLC, neutrophils, eosinophils, lymphocytes and monocytes count whereas, PCV was higher than the respective value from control group (Table 1). The biochemical study in the Brucella infected cattle showed higher level of serum glucose, total protein, albumin and creatinine but, AST, ALT and SD values were lower than the respective value from control group (Table 2). Group A, which received long acting oxytetracycline treatment, showed significant increase in level (p<0.05) of Hb, lymphocyte and AST (Tables 1 & 2). But, other haemato-biochemical parameters; PCV, TEC, TLC, glucose, total protein, creatinine, albumin, ALT and SD were not significantly altered (p>0.05) after the treatment. Furthermore, five cattle reduced the shedding of pathogen to undetectable level in milk, as it was confirmed by PCR (Table 3). Similarly, STAT and ELISA were not able to detect anti-Brucella antibodies in serum samples of four and two animals, respectively after the treatment. Furthermore, five cattle found positive for antibodies both in serum and milk after therapy. RBPT, STAT and ELISA were unable to detect respective pathogens in! (Table 3). Similarly, MRT and ELISA were given negative results respectively, in five and three cattle after the treatment (Table 3). Treatment with streptomycin was given in Group B; reduced the anti-Brucella antibodies levels in cattle to undetectable levels in some of them. RBPT and STAT were unable to detect anti-Brucella antibody in three cattle (Table 3). However, two cattle, as screened by ELISA, seem to be free of antibody. Screening of milk for presence of antibodies were not detected by MRT and ELISA respectively, in five and four cattle (Table 3). Furthermore, PCR failed to detect Brucella DNA in five cattle’s milk after therapy. All the haematological and biochemical parameters under study were not altered significantly except TEC (p>0.05) (Table 1 & 2).

In group C, there was reduction in anti-Brucella antibody level in serum and milk to such an extent, RBPT, STAT and ELISA were unable to detect respectively, in five, six and five animals (Table 3). Similarly, MRT and ELISA on milk from six and five animals were negative for antibody, respectively. Furthermore, six cattle shed undetectable amount of pathogen in milk, as it was confirmed by PCR (Table 3). Animals which had received combination therapy, significant increased its level (p<0.05) of Hb, TEC, TLC and lymphocytes counts (Table 1). However, alteration in PCV, neutrophils, eosinophils and monocytes count were non-significant (p>0.05). Biochemical parameters; glucose, total protein and albumin showed significant decrease (p<0.05) but, ALT, AST and SD increased significantly (p<0.05) after therapy (Table 2).

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Table 1 Means of respective haematological parameter of animals from respective groups, before and after the therapeutic trial

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Before treatment</th>
<th>After treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (n=9)</td>
<td>Group A (n=9)</td>
</tr>
<tr>
<td>Hb (g/L)</td>
<td>135.3±11.00</td>
<td>98.0±10.48</td>
</tr>
<tr>
<td>PCV (L/L)</td>
<td>0.38±0.61</td>
<td>0.39±0.02</td>
</tr>
<tr>
<td>TEC (X 10^9/L)</td>
<td>7.9±0.85</td>
<td>6.4±0.53</td>
</tr>
<tr>
<td>TLC (X 10^9/L)</td>
<td>8.2±0.56</td>
<td>3.26±0.24</td>
</tr>
<tr>
<td>Neutrophil (X 10^9/L)</td>
<td>2.96±0.21</td>
<td>2.62±0.52</td>
</tr>
<tr>
<td>Eosinophil (X 10^9/L)</td>
<td>1.06±0.15</td>
<td>0.84±0.11</td>
</tr>
<tr>
<td>Lymphocyte (X 10^9/L)</td>
<td>3.63±0.38</td>
<td>1.97±0.40</td>
</tr>
<tr>
<td>Monocyte (X 10^9/L)</td>
<td>0.3±0.10</td>
<td>0.15±0.03</td>
</tr>
</tbody>
</table>

Figures having same superscripts are significant at 5% level of significance for respective parameters, n, number of animals; Hb, haemoglobin; PCV, packed cell volume; TEC, total erythrocytes count; TLC, total leucocytes count; DLC, differential leucocytes count.

Table 2 Means of respective biochemical parameters of animals from respective groups, before and after the therapeutic trial

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Before treatment</th>
<th>After treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (n=9)</td>
<td>Group A (n=9)</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>3.33±0.33</td>
<td>5.07±0.16</td>
</tr>
<tr>
<td>Total Protein (g/L)</td>
<td>74.67±4.93</td>
<td>92.96±6.15</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>29.2±6.5</td>
<td>37.77±1.07</td>
</tr>
<tr>
<td>Creatinine (µmol/L)</td>
<td>102.67±35.22</td>
<td>179.24±19.47</td>
</tr>
<tr>
<td>ALT (units/L)</td>
<td>15.26±3.24</td>
<td>7±2.10</td>
</tr>
<tr>
<td>AST (units/L)</td>
<td>91.3±4.53</td>
<td>61.53±7.73</td>
</tr>
<tr>
<td>SD (units/L)</td>
<td>9.9±1.25</td>
<td>2.92±0.33</td>
</tr>
</tbody>
</table>

Figures having same superscripts are significant at 5% level of significance for respective parameters, n, number of animals; ALT, alanine aminotransferase; AST, aspartate aminotransferase; SD, sorbitol dehydrogenase.

Table 3 Number of cattle remains positive for concerned attributes in respective diagnostics after the therapeutic trail

<table>
<thead>
<tr>
<th>Tests</th>
<th>Group A (n=9)</th>
<th>Group B (n=9)</th>
<th>Group C (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBPT</td>
<td>6</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>STAT</td>
<td>5</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>i-ELISA (on serum)</td>
<td>7</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>MRT</td>
<td>4</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Milk- ELISA</td>
<td>6</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>PCR on milk</td>
<td>4</td>
<td>4</td>
<td>3</td>
</tr>
</tbody>
</table>

N, number of animals; RBPT, rose Bengal plate test; STAT, standard tube agglutination test; i-ELISA, indirect-enzyme linked immunosorbent assay; MRT, milk ring test; ELISA, enzyme linked immunosorbent assay PCR, polymerase chain reaction.

Discussion

Brucellosis has wide socio-economic impact, especially in countries in which rural income relies largely on animal husbandry. In India, it causes approximately Rs. 350 million economic losses. Several studies have confirmed widespread prevalence in different States of India. High seroprevalence has been reported in Indian dairy herds. Long-term serological studies at national level indicated, 5% cattle infected with brucellosis. Despite the advances made in the diagnosis, vaccination and therapy to control the disease, it is still wide spread and prevalent in many developing countries. There are limited researches on treatment and haemato-biochemical parameters of infected animals. Lowered haematological values such as Hb, TEC, TLC, neutrophils, eosinophils, lymphocytes and monocytes counts were recorded in the present study. Most of earlier studies didn’t explore the all haematological parameters in Brucella...

infected animals. RAR\textsuperscript{30} reported lower Hb value than the reference value in the *Brucella* infected cattle. Intra-cellular position of the *Brucella* spp. might cause reduction of Hb percentage.\textsuperscript{30} Animals such as moose, infected experimentally with *B. abortus* showed slight lower Hb concentration.\textsuperscript{15} or within the range of reference value.\textsuperscript{30} Some worker has also reported higher Hb concentration in *Brucella* infected animals.\textsuperscript{32,33} Lymphocyte count was also lowered in *Brucella* infected animals.\textsuperscript{32,33} However, Sikder et al.,\textsuperscript{34} found higher values of neutrophil, monocyte and eosinophil counts in the *B. abortus* positive cattle than the standard values.

He also found Hb, PCV, TEC, TLC, lymphocytes and basophils values of infected cattle within the reference range. Lymphoid depletion in the thymic cortex in natural and experimental could give lymphopenic condition.\textsuperscript{35} Biochemical parameters such as serum glucose, serum total protein, serum albumin, and creatinine, revealed higher value in infected cattle but, other parameter such as AST, ALT and SD were lower than the reference value in the present study. El-Boshy et al.,\textsuperscript{36} reported significant increases (P<0.05) in serum SD, AST and ALT levels and non-significant-variations in creatinine in *Brucella* infected animals. Forbes et al.,\textsuperscript{37} confirmed stable and similar haemato-biochemical parameters in moose experimentally infected with *B. abortus* biovar 1. Several treatment trials for brucellosis have been previously attempted, but none was entirely successful. The *Brucella* bacterium is protected from antibiotics since it survives within phagoecytic cells of the reticuloendothelial system. Successful treatment needs permeability of drug across the cell wall of bacterium. Present therapeutic trial on *Brucella* infected cattle showed; long acting oxytetracycline and streptomycin combination gave better results than the drugs given alone. However, they did not cure infection completely.

Animals either stopped shedding of pathogen or reduced its level to such an extent to diagnose by test implied. Similarly, antibody level in some of animals reduced to such a level as it was undiagnosed by different diagnostics used both on serum and milk. Oxytetracycline and streptomycin are capable of penetrating the bacterial cell wall, inhibiting protein synthesis and providing long lasting concentrations in the plasma and hence considered most effective in the treatment of brucellosis.\textsuperscript{34} Combination of both has demonstrated synergistic effect in *vitro*.\textsuperscript{38} Long term treatment with high doses of oxytetracycline and streptomycin combination in *Brucella* infected Neumann’s gazelles resulted in eradication of the infection.\textsuperscript{35} But, relapse and abortion were noticed frequently in animals which were received antibiotic treatments.\textsuperscript{36,37} Streptomycin-tetracycline could be the choice of therapy for brucellosis, particularly in severe cases.\textsuperscript{38} Combination therapy of long acting oxytetracycline and streptomycin revealed a significant increase (p<0.05) in Hb, TEC, TLC, lymphocytes count, ALT, AST and SD and significant decrease (p<0.05) in the values of glucose, total protein and albumin and non-significant alteration (p>0.05) in other parameters. Omer et al.,\textsuperscript{39} found significant changes in Hb, ALT and glucose, after combination therapy in *Brucella* infected gazelles. But changes in other haemato-biochemical parameters following the treatment were non-significant.

**Conclusion**

Long acting oxytetracycline and streptomycin in combination could be used for treatment of *Brucella* infected cattle, but it needs further validation. Shedding of pathogen in milk or other body secretions of animal should be monitored for longer duration, not only by PCR but also by isolation of bacterium on suitable media. Relapse of infection or abortion in subsequent pregnancy should also be monitor to validate the treatments. Use of haemato-biochemical parameters as indicator of *Brucella* infection is not warranted, as alterations in these parameters are also evidenced in other bacterial infections. Therefore, these alterations should be carefully interpreted to give a final decision. It is better to complement a serological or molecular test to attain a final conclusion.

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**Conflicts of interest**

The authors declared there is no conflict of interest.

**References**

29. RAR. University of Minnesota, United States; 2011.