A new bacteriophage based luminescence assay for diagnosis of brucellosis

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Brucellosis is a highly contagious zoonotic disease of public health importance caused by the genus Brucella. It causes substantial economic losses in the livestock sector due to reproduction failure and decreased productivity of affected animals. Available pen side tests like Rose Bengal Plate Test (RBPT) are not accurate while advanced laboratory assays like ELISA and Complement Fixation Test (CFT) are expensive, cumbersome and require skilled personnel and sophisticated facilities. There is an urgent need for cost-effective and field applicable accurate diagnostic assays for Brucellosis to effectively control the disease. Bacteriophages lyse bacteria in a specific manner and can be employed in developing economic diagnostic kits for field use. In the present study, we explored the potential diagnostic application of a new lytic Brucellaphage isolated by us.

Materials and methods
Clinical samples
Twenty samples of uterine discharges from aborted (within 7 days of abortion) cattle and buffaloes from dairy farms in and around Ludhiana were collected in sterile containers and transported immediately on ice to the laboratory for further processing.

Isolation of Brucella organisms from stomach contents of aborted fetus
For primary isolation of Brucella organisms, Brucella selective medium (BSM) (Himedia, Mumbai) enriched with Brucella selective supplement (Himedia, Mumbai) was used. Samples of aborted fetal stomach contents were kept in microcentrifuge tubes and centrifuged at 6000 rpm for 10 min. The pellet was suspended in a small volume of BSM broth and inoculated on Brucella selective medium agar plate. After inoculation, the plates were incubated at 37°C under micro-aerophillic environment in a candle jar for 3-5 days and checked daily for the growth of Brucella organisms. Biochemical tests (Catalase, Oxidase, Nitrate Reduction, Urease, Indole and H₂S production tests) for confirmation of Brucella were performed on suspected colonies as per the standard methods.

The genomic DNA was extracted from Brucella abortus field isolates and the standard vaccine strain (S-19) by the method described earlier. Isolates were confirmed by PCR by using Brucella genus specific primers B4/B5 and F4/R2 obtained from Promega Biotech India Private Limited, Mumbai. The sequences of primers are given in Table 1. The DNA of microorganisms from samples was extracted as per the method described earlier.

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Table 1—Sequences of primers used for detection of B. abortus

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>Size of amplified product</th>
<th>Reference number</th>
</tr>
</thead>
<tbody>
<tr>
<td>F4</td>
<td>5'-TCG AGC GCC CGC AAG GGG-3'</td>
<td>905 bp</td>
<td>8</td>
</tr>
<tr>
<td>R2</td>
<td>5'-AAC CAT AGT GTC TCC ACT AA-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B4 F</td>
<td>5'-TGG CTC GGT TGC CAA TAT CAA-3'</td>
<td>223 bp</td>
<td>9</td>
</tr>
<tr>
<td>B5 R</td>
<td>5'-CGC GCT TGC CTT TCA GGT CTG-3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Isolation of brucellaphage

Sewage samples were collected from suspected Brucella affected (based on antibody titers of animals) sheds of the dairy farm and Veterinary Clinics of GADVASU, Ludhiana and were transported on ice to the laboratory for further processing. NZCYM+BSM agar plates were used for brucellaphage isolation.

Brucella abortus Strain 19 (isolated from live Brucella vaccine procured from Indian Veterinary Research Institute, Izatnagar) was used for isolation of brucellaphage from sewage samples. BSM agar plates were used for culturing and BSM broth was used for sub-culturing of Brucella abortus strain 19.

Ten milliliters of 24 h grown culture of Brucella abortus strain 19 in BSM broth was put into 25 mL of double strength NZCYM broth and 15 mL of sewage was poured into it and incubated at 37°C for 28 days. The processed sample was inoculated by agar overlay technique on alternate days (viz. 4, 6, 8 and so on up to 28 days) for bacteriophage isolation. The plates were checked out every day and those having plaque formation were preserved. The plaques were confirmed for the presence of phage by secondary streaking.

Overnight grown Brucella S-19 culture (100 µL) was added to 3 mL molten cooled semisolid NZCYM agar, mixed well and poured on NZCYM+BSM agar plates. The plates were allowed to solidify at 37°C. The plaques suspected for brucellaphage were picked up by using platinum wire inoculation loop and streaked firstly as horizontal lines across the plates and later on as perpendicular lines dissecting the horizontal lines across the plate. The plates were then incubated at 37°C aerobically for 2-3 days. The clearing along the streak line indicated the presence of bacteriophage. The phage was eluted from the plates into sterile SM buffer.

Sterile SM buffer (5 mL) was poured over suspected bacteriophage plates showing clearance around the streak lines. The plaques along the line were disturbed using a platinum loop and the plates were kept in incubator at 37°C for 8 h. The elute was collected and centrifuged to discard any agar particle. The supernatant was filtered through 0.22 µm PVDF filter and preserved at 4°C.

The purified phage was serially diluted in SM buffer. Equal quantity of each dilution of the phage and overnight grown Brucella S-19 culture were added to 3 mL molten cooled semisolid NZCYM agar at 40°C, mixed well and poured on NZCYM+BSM agar plates. The plates were allowed to solidify at room temperature and then incubated at 37°C aerobically for 2-3 days. The plaques produced were counted and multiplied with the dilution factor to determine the phage titer. The titer was expressed in plaque forming units (pfu)/mL.

\[ \text{Pfu/mL} = \frac{\text{Number of plaques} \times \text{Dilution factor}}{\text{Volume of phage used (mL)}} \]

The lytic activity of the brucellaphage was tested against Brucella abortus field isolates, Brucella abortus strain 99 and Brucella melitensis (IVRI, Izatnagar) and several bacteria of heterologous species viz. Staphylococcus aureus, Salmonella species, Escherichia coli, and Pasteurella multocida.

Phage based luminescence assay

A rapid and sensitive method for detection of Brucella based on bacteriophage mediated lysis of Brucella was developed by suitably modifying the protocol described earlier and employing the ATP Determination Kit (Invitrogen Detection Technologies). The bacteriophage lytic to Brucella isolated by us was used for the development of the assay. The bacteriophage preparation was purified to remove any contamination by 2x centrifugation at 100000xg at 4°C for 2 h using an ultracentrifuge (Microoultracentrifuge CS 150 NX-Hitachi). Phage titers were ascertained by the plaque assay.

The reagents of the ATP determination kit were prepared as per the manufacturer’s instruction. The standard reaction solution was prepared by mixing the following: 8.9 mL deionised water; 0.5 mL 20X Reaction Buffer; 0.1 mL 0.1 M DTT; 0.5 mL of 10 mM D-luciferin; and 2.5 µL of firefly luciferase 5 mg/ml stock solution.

The standard curve for a series of ATP concentrations was generated using the standard ATP provided with the kit. One hundred microliters of the standard reaction solution was placed in sterile
96 well flat bottom plates (NUN 96 fw-LumiNunc FluoroNunc - Nunclon 96 flat white) and the background luminescence was measured at 460 nm in a Luminometer (Infinite F200 PRO-TECAN). The reaction was started by adding 10 µL of different dilutions of ATP standard solution. The volume of the dilute ATP standard solution added was not more than 10% of the total assay volume. One hundred micro liters of the standard reaction solution was placed in these ATP dilutions and the luminescence was measured by the Luminometer at 460 nm. A standard curve was generated for each series of ATP concentrations.

**ATP assay on clinical samples**

Samples of uterine secretions were concentrated by centrifugation at 10000 rpm for 10 min at 4°C. The pellet was dissolved in 200 µL of BSM broth before the assay.

One hundred micro liters of processed sample and 100 µL of phage were mixed and incubated at 37°C till the lysis of the bacteria by the phage. It was then used for ATP analysis using the kit. One hundred micro liters of the standard reaction solution was prepared and placed in two wells of sterile 96 well flat bottom plates (NUN 96 fw-LumiNunc FluoroNunc-Nunclon 96 flat white) and the background luminescence was measured at 460 nm. A standard curve was generated for each series of ATP concentrations.

**Results**

In the present study, a total of 20 samples of uterine fluid from suspected bovines (cattle and buffaloes) were analyzed for brucellosis (Table 2).

**Isolation of Brucella abortus from clinical samples**

*Brucella abortus* isolated from samples of uterine secretions and stomach contents of aborted fetuses were confirmed to be *Brucella* by PCR. The genomic DNA of *Brucella abortus* field isolates and the standard vaccine strain (S-19) were extracted. The OD260/280 value for isolated DNA was ~1.8 - 1.9 for all the samples indicating the purity of DNA.

The DNA extracted from *Brucella abortus* isolates and S-19 when subjected to PCR using *Brucella* specific primers B4/B5 and F4/R2 revealed the desired amplicons at 223 bp and 905 bp, respectively (Fig. 1 A & B).

**Isolation of brucellaphage**

Brucellaphage was isolated from a sewage sample. Streaking the plaques on *Brucella* lawn gave clear zones along the streak lines (Fig. 2). At a concentration of $10^4$, the phage count was $4.5 \times 10^6$ plaques per mL.

The isolated brucellaphage lysed all the 12 *Brucella abortus* field isolates, *B. abortus* strain 99 and *B. melitensis* (IVRI, Izatnagar) but did not lyse any of the heterologous species tested viz. *Staphylococcus aureus*, *Salmonella* sp., *Escherichia coli* and *Pasteurella multocida*.

**Phage based luminescence assay**

The amount of luminescence measured with different concentrations of standard ATP provided in the kit is given in Table 3. There was a positive correlation between the concentration of ATP and the amount of luminescence measured. It was observed that a minimum of 0.001 µM concentration of ATP

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Age of animal (years)</th>
<th>Month of abortion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cows</td>
<td></td>
<td></td>
</tr>
<tr>
<td>44b</td>
<td>6</td>
<td>5.5</td>
</tr>
<tr>
<td>48b</td>
<td>5</td>
<td>6.5</td>
</tr>
<tr>
<td>50b</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>55b</td>
<td>5</td>
<td>6.5</td>
</tr>
<tr>
<td>57b</td>
<td>4</td>
<td>H/O prolapse</td>
</tr>
<tr>
<td>58b</td>
<td>5</td>
<td>H/O ROP</td>
</tr>
<tr>
<td>59b</td>
<td>6</td>
<td>H/O Dystocia</td>
</tr>
<tr>
<td>60b</td>
<td>6</td>
<td>H/O ROP</td>
</tr>
<tr>
<td>72b</td>
<td>4.5</td>
<td>H/O Dystocia</td>
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</tr>
<tr>
<td>88b</td>
<td>4</td>
<td>H/O Dystocia</td>
</tr>
<tr>
<td>95b</td>
<td>5</td>
<td>H/O ROP</td>
</tr>
<tr>
<td>100b</td>
<td>7</td>
<td>H/O Prolapse</td>
</tr>
<tr>
<td>8b</td>
<td>6</td>
<td>NA</td>
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<td>17b</td>
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<td>NA</td>
</tr>
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<td>18b</td>
<td>4</td>
<td>NA</td>
</tr>
<tr>
<td>Buffaloes</td>
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</tr>
<tr>
<td>82b</td>
<td>5</td>
<td>H/O Dystocia</td>
</tr>
<tr>
<td>83b</td>
<td>4</td>
<td>H/O Dystocia</td>
</tr>
<tr>
<td>87b</td>
<td>6</td>
<td>H/O ROP</td>
</tr>
<tr>
<td>96b</td>
<td>5</td>
<td>H/O Dystocia</td>
</tr>
</tbody>
</table>

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Table 2—Uterine fluid samples processed for isolation of *Brucella* organisms
**ATP release by Brucella abortus positive samples after phage mediated lysis**

In the present study, the release of ATP after phage mediated specific lysis of bacteria in samples was estimated for Brucella using a phage lytic for the bacteria. Employing the new assay, field samples (Table 4 and Fig. 4) from animals suspected for brucellosis were analyzed by luminescence assay for the presence/absence of bacteria followed by reconfirmation with standard laboratory diagnostic procedures. It was observed that after 5 h of incubation of phage-bacteria suspension, there was significant increase in luminescence in the sample with phage lysed bacteria compared to the sample alone as determined by the Luminometer reading.

In our study, the positive samples showed increased luminescence after 5 h of incubation with brucellaphage which was comparable to the luminescence observed after incubation of brucellaphage with the standard culture of Brucella abortus S-19. We investigated the possibility of using bacteriophage based ATP - catalyzed luminescence assay for the detection of Brucella organisms in samples of uterine secretions from animals suspected for brucellosis.

**Analysis of clinical samples by ATP assay**

Uterine secretion samples from animals confirmed for Brucella infection, showed an increased luminescence by the ATP assay ranging from 3.4
Table 4—Luminescence of Uterine fluid samples after incubation with the phage

<table>
<thead>
<tr>
<th>S. no.</th>
<th>Sample alone (A)</th>
<th>Positive sample + Phage (B)</th>
<th>Negative sample + Phage (C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1517</td>
<td>22256</td>
<td>984</td>
</tr>
<tr>
<td>2</td>
<td>2323</td>
<td>15906</td>
<td>828</td>
</tr>
<tr>
<td>3</td>
<td>1009</td>
<td>17359</td>
<td>289</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>1616.333 ± 662.608</td>
<td>18507.000 ± 3327.018</td>
<td>700.333 ± 364.664</td>
</tr>
</tbody>
</table>

[Difference between A and B, B and C significant (\(P < 0.01\)). Difference between A and C is not significant]

Discussion

Although several serological tests are available for diagnosis of Brucellosis, including the recent Superagglutination test\(^6\), the antibody based tests suffer from the problem of cross reactions with other organisms (e.g., RBPT gives positive result with both Brucella abortus and Yersinia enterocolitica). Phage offers a means of accurate identification of bacteria due to highly specific lysis and hence can be exploited for diagnostic application.

In the present study, phage mediated lysis of Brucella abortus was detected by monitoring the release of ATP. There was an appreciable increase in the amount of ATP generated on lysis along with corresponding luminescence in samples with phage treated bacteria in comparison to the untreated samples. Bacterial intracellular ATP released by phage was available to luciferin-luciferase enzyme-substrate system for generation of light by luminescence. Low amount of ATP released from the samples uninfected by phage could have been due to spontaneous death and autolysis of a few Brucella or other contaminating bacteria. The observed background luminescence in our study could be due to the spontaneous low level release of adenylate kinase by bacteria.

The decrease in the luminescence of phage treated samples compared to the untreated samples in case of Brucellosis negative animals could possibly be due to the utilization of ATP released from the unrelated contaminant microorganisms by the phage itself leaving little ATP for the luciferin – luciferase reaction.

Our results indicate that phage based ATP Luminescence assay is a useful diagnostic assay and has the potential for field application with suitable modification for visual detection of luminescence (e.g., visualizing the reaction in a dark box).

It has been suggested\(^7\) that the bacteriophage can be used as a targeted reagent for the release of intracellular ATP from Listeria and observed that bacteriophage lysis combined with ATP detection enabled the identification of 2.5 x 10\(^5\) cells. Bacteriophages have been used earlier\(^5\) also to provide specific lysis of the bacteria and the release of the cell contents was measured by luminescence. They, however, focused on the bacteria’s adenylate kinase as the marker instead of ATP. They have also reported decreased signal (luminescence) in the presence of nonspecific contaminant, but the assay otherwise remains unaffected.
Serological methods of diagnosis\textsuperscript{8,9} have their limitations in diagnosis of Brucellosis. Vaccination with \textit{B. abortus} S19 induces antibodies to lipopolysaccharide which interfere with the serological testing programs as they are indistinguishable from antibodies arising due to infection\textsuperscript{10}.

A rapid diagnostic technique was described\textsuperscript{11} for detection of significant bacterial pathogens (\(\geq 10^4\) cfu/mL) in bronchoalveolar lavage (BAL) samples. This assay utilized a kit based on luciferin-luciferase reaction to detect bacterial adenosine triphosphate (ATP) used for screening bacteriuria in urine specimens. A combined immunomagnetic separation (IMS)/ATP assay described\textsuperscript{12} for the detection of \textit{Mycobacterium tuberculosis} in urine using labeled immunomagnetic beads for capture of MTB. MTB was distinguished from common bacteriuria isolates and other nontarget bacteria by its ATP results. IMS/ATP detected 19 of 28 samples of MTB in synthetic urine with a limit of detection of \(10^3\) CFU/ml. Sensitivity and specificity were 67.9\% and 82.1\%, respectively. Phage-based platforms have been used for the detection of \textit{Mycobacterium tuberculosis}, \textit{Yersinia pestis}, \textit{Bacillus anthracis} and \textit{Staphylococcus aureus} in the clinical field\textsuperscript{13}.

In another study\textsuperscript{14}, three consecutive sputum samples collected from HIV patients for phage based diagnosis of pulmonary tuberculosis. After decontamination and concentration techniques, samples were used for phage assay, cultured on Lowenstein-Jensen medium and smears were prepared. The overall sensitivity, specificity, PPV and NPV of phage assay when compared to LJ culture were 83.3\%, 100\%, 1.0 and 0.92, respectively. With respect to smear-negative specimens, the sensitivity was 50\%.

We adapted MTT assay and nitrate reduction assay\textsuperscript{1} for use along with phage in detection of \textit{Brucella} organisms. In phage based MTT assay, all the standard isolates of \textit{Brucella} changed colour from yellow to purple upon addition of MTT after 6.5 h of incubation of bacteria alone. However, in case of wells containing bacteria incubated with the specific brucellaphage, no colour change was observed after 6.5 h of incubation because of the specific lysis of bacteria by the phage. In phage based Nitrate Reduction Test, red colour was noticed in the case of live \textit{Brucella} isolates alone, but in case of \textit{Brucella} incubated with phage for 8 h, it remained colourless.

Phage based diagnostic methods are cheap, simple and highly specific for bacterial diseases and are of bed side and pen side applicability. Phage based diagnostics have employed phage amplification and phage plaque method, reporter genes for luminescence, Fluorescence assays, MTT assay, Adenylate Kinase assay and ATP assay\textsuperscript{15}.

We isolated a broad acting bacteriophage lytic to \textit{Salmonella typhimurium}, \textit{S. pullorum}, \textit{S. gallinarum} and \textit{S. dublin} and investigated its application in diagnosis employing MTT assay and nitrate test\textsuperscript{16}. On addition of MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to live bacteria alone of isolates of \textit{Salmonella}, its colour changed from yellow to purple. However, after 4 h of incubation of bacteria with bacteriophage, no colour change was observed because of lysis of bacteria by the phage. After incubation with salmonellaphage, twelve out of forty clinical samples showed no colour change indicating lysis of \textit{Salmonella} by phage, whereas samples without \textit{Salmonella} showed colour change due to other bacteria. In nitrate test, live bacteria reduce nitrate to nitrite which can be detected by change in colour. No colour developed on prior incubation of \textit{Salmonella} with the phage.

Phage based ATP assay for detection of \textit{Brucella} organisms in clinical samples as demonstrated in this study offers a new approach for a highly accurate diagnosis of Brucellosis in 5 h compared to the serodiagnostic assays which often give false results due to cross reactions of the antibody, and the molecular assays which are expensive and require sophisticated lab facilities and skilled personnel. The phage based assay is cost effective and can be adapted for point of care application.

**Acknowledgement**

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**Conflict of interests**

The authors declare that there are no conflicts of interests.

**References**


