A NEW BACTERIOPHAGE BASED LUMINESCENCE ASSAY FOR DIAGNOSIS OF BRUCELLOSIS

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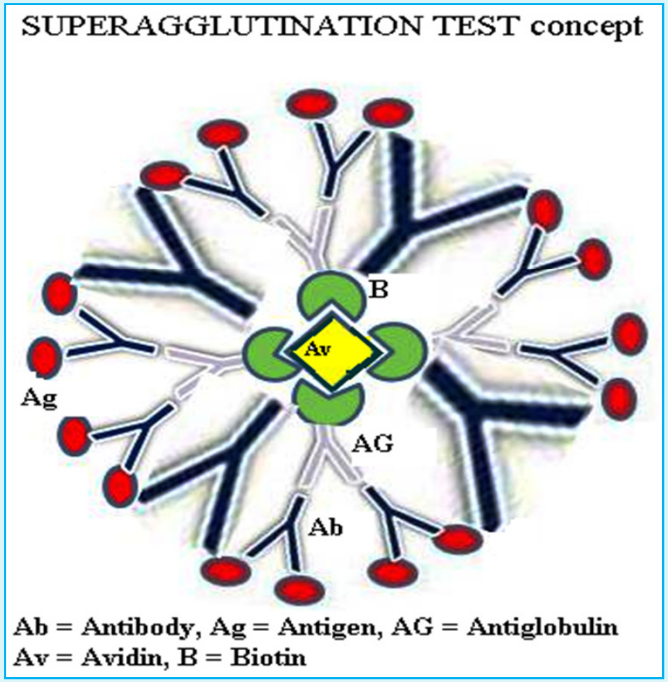
Brucellosis - a highly contagious zoonotic disease of public health importance caused by bacteria of the genus *Brucella.*

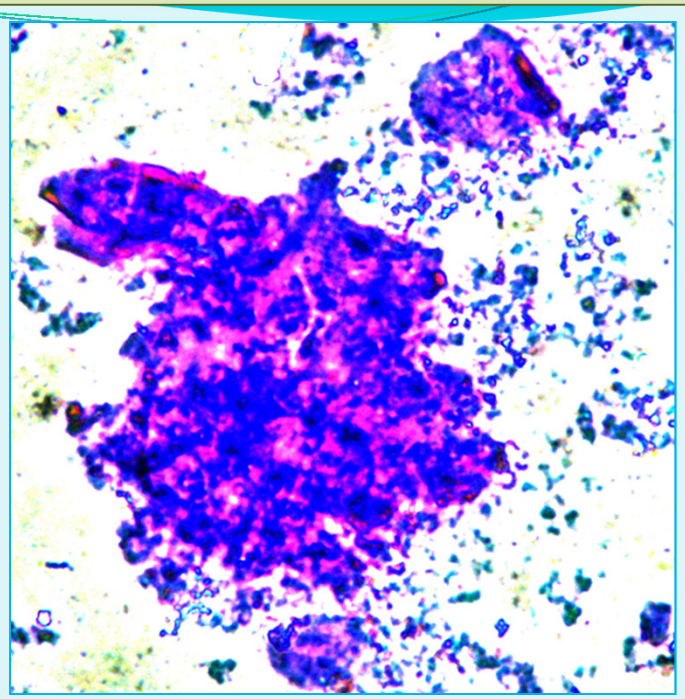
It causes substantial economic losses in the livestock sector due to reproduction failure and decreased productivity of affected animals.

Rose Bengal Plate Test (RBPT) & STAT- not very accurate.

ELISA & CFT -expensive, cumbersome, need skilled personnel, sophisticated facilities.

The new Superagglutination Test for serodiagnosis of Brucellosis





Test	Sensitivity in per cent	Specificity in per cent	PPV in per cent	NPV in per cent
Superagglutination	**95.88**	**89.32**	**89.42**	**95.83**
RBPT	93.33	88.18	86.60	94.17
STAT	94.25	68.14	69.49	93.90
iELISA	74.47	95.24	93.33	80.65
CFT	82.80	93.46	91.67	86.21

Antibody based tests may give cross – reactions with other organisms (RBPT for Brucellosis gives positive result with *Yersinia enterocolitica*).

Need cost-effective, field applicable, accurate diagnostic assays for brucellosis control.

Bacteriophages lyse bacteria in a very specific manner, and hence can be exploited for diagnostic application.

We isolated a new lytic Brucellaphage & explored its potential diagnostic application.

Materials and methods

Clinical samples

Samples of uterine discharges from aborted cattle and buffaloes from dairy farms

Isolation of Brucella organisms from stomach contents of aborted fetus

Brucella selective medium enriched with *Brucella* selective supplement (Himedia) used.

After inoculation of contents, BSM agar plate incubated at 37^0C under microaerophillic environment in a candle jar for 3-5 days & checked daily for growth of *Brucella*

Biochemical tests for identification of bacteria

Catalase, Oxidase, Nitrate Reduction, Urease, Indole and H_2S production tests were performed on suspected colonies for confirmation of Brucella organisms.

Molecular characterization of Brucella

Genomic DNA extracted from *Brucella abortus* field isolates and vaccine strain (S-19).

DNA was confirmed by PCR using genus specific primers B4/B5 & F4/R2 (Promega).

Primers	Sequence	Size of amplified product	Reference
F4	5'-TCG AGC GCC CGC AAG GGG-3'	905bp	Romero et al (1995)
R2	5'-AAC CAT AGT GTC TCC ACT AA -3'		
B4 F	5'-TGG CTC GGT TGC CAA TAT CAA-3'	223bp	Bailey et al (1992)
B5 R	5'-CGC GCT TGC CTT TCA GGT CTG-3'		

Isolation of brucellaphage

Sewage from *Brucella* affected sheds of dairy farm & Vet Clinics of GADVASU were processed.

NZCYM+BSM agar plates were used for phage isolation.

B abortus S19 isolated from live *Brucella* vaccine (IVRI) was used for isolation of brucellaphage.

BSM agar plates were used for culturing & BSM broth for sub-culturing of strain 19.

10 ml of 24hr grown culture of strain 19 in BSM broth was put into 25ml of double strength NZCYM broth and 15ml of sewage was poured into it and incubated at 37^0C for 28 days.

The processed sample was inoculated by agar overlay technique on alternate days (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.

100µl overnight grown *Brucella* S-19 culture was added to 3ml molten cooled semisolid NZCYM agar, mixed well and poured on NZCYM+BSM agar plates.

The plates were allowed to solidify at room temperature.

The plaques suspected for brucellaphage were picked up by using platinum wire loop and streaked as horizontal lines across the plates and later on as perpendicular lines across the plate.

The plates were then incubated at 37^0C 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 (5ml) was poured over suspected plates showing clearance around streak lines.

The plaques along the line were disturbed using a platinum loop and plates kept at 37^0C for 8 hrs.

Elute was collected, centrifuged & supernatant filtered thru 0.22µm PVDF filter, preserved at 4^0C

Estimation of the titre of brucellaphage

The phage preparation was purified by 2x centrifugation at 100,000g at 4^0C for 2 hours using an ultracentrifuge. Phage titers were ascertained by the plaque assay.

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 3ml molten cooled semisolid NZCYM agar at 40^0C, mixed well and poured on NZCYM+BSM agar plates.

The plates were allowed to solidify at room temperature and then incubated at 37^0C aerobically for 2-3 days.

The plaques produced were counted and multiplied with the dilution factor to determine the phage titre.

Titre in Pfu/ml = Number of plaques × Dilution factor / Volume of phage used (ml)

Characterization of brucellaphage

The lytic activity of the brucellaphage was tested against:

Brucella abortus field isolates,
Brucella abortus strain 99
Brucella melitensis (IVRI, Izatnagar)

and several bacteria of heterologous species:

Staphylococcus aureus,
Salmonella species,
Escherichia coli, and
Pasteurella multocida.

Phage based Nitrate Reduction Assay for detection of Brucella

Live bacteria reduce nitrate to nitrite due to release of nitrate reductase by bacteria.

Presence of nitrite can be detected by nitrate reagents which produce change in colour.

100 µl of overnight grown culture of *Brucella abortus* S-19 was placed in microtitre plate.

To this equal volume of brucellaphage was added.

Control wells included *Brucella,* phage, and broth alone only.

20 µl nitrate broth was added in each well and incubated at 37^0C for $6^{1/2}$ hours.

After incubation, 20 µl each of reagent A and reagent B were added (Quinn *et al*., 1999).

Phage based luminescence assay

A rapid and sensitive assay was developed by suitably modifying the protocol developed by Blasco et al (1998) for adenylate kinase and employing the ATP Determination Kit (Invitrogen).

The reagents of the ATP determination kit were prepared as following:
8.9 ml deionized water
0.5 ml 20x Reaction Buffer
0.1 ml 0.1M DTT
0.5 ml of 10 mM D-Luciferin
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.

100µl 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 460nm in a Luminometer (Infinite F200 PRO-TECAN).

The reaction was started by adding 10 µl of different dilutions of ATP standard solution.

100µl of the standard reaction solution was placed in these ATP dilutions and the luminescence was measured by the Luminometer at 460nm.

Analysis of Clinical samples

Uterine secretion samples concentrated by centrifugation at 10000 rpm , 10 min at 4^0C. The pellet was dissolved in 200 µl of BSM broth.

100 µl of processed sample and 100µl of phage were mixed and incubated at 37^0C till the lysis of the bacteria by the phage.

ATP assay was performed using the kit.

100 µl of standard solution was placed in two wells of sterile 96 well flat bottom plates (NUN 96 fw-LumiNunc FluoroNunc- Nunclon 96 flat white) & background luminescence read at 460 nm.

20 µl of processed sample added to wells of sterile plates.

100 µl of fresh standard reaction solution was added to the test wells.

Luminescence was measured immediately at 460 nm.

Luminescence of test samples was calculated by subtracting the background luminescence from the reading of test sample.

Results

PCR Confirmation of Brucella

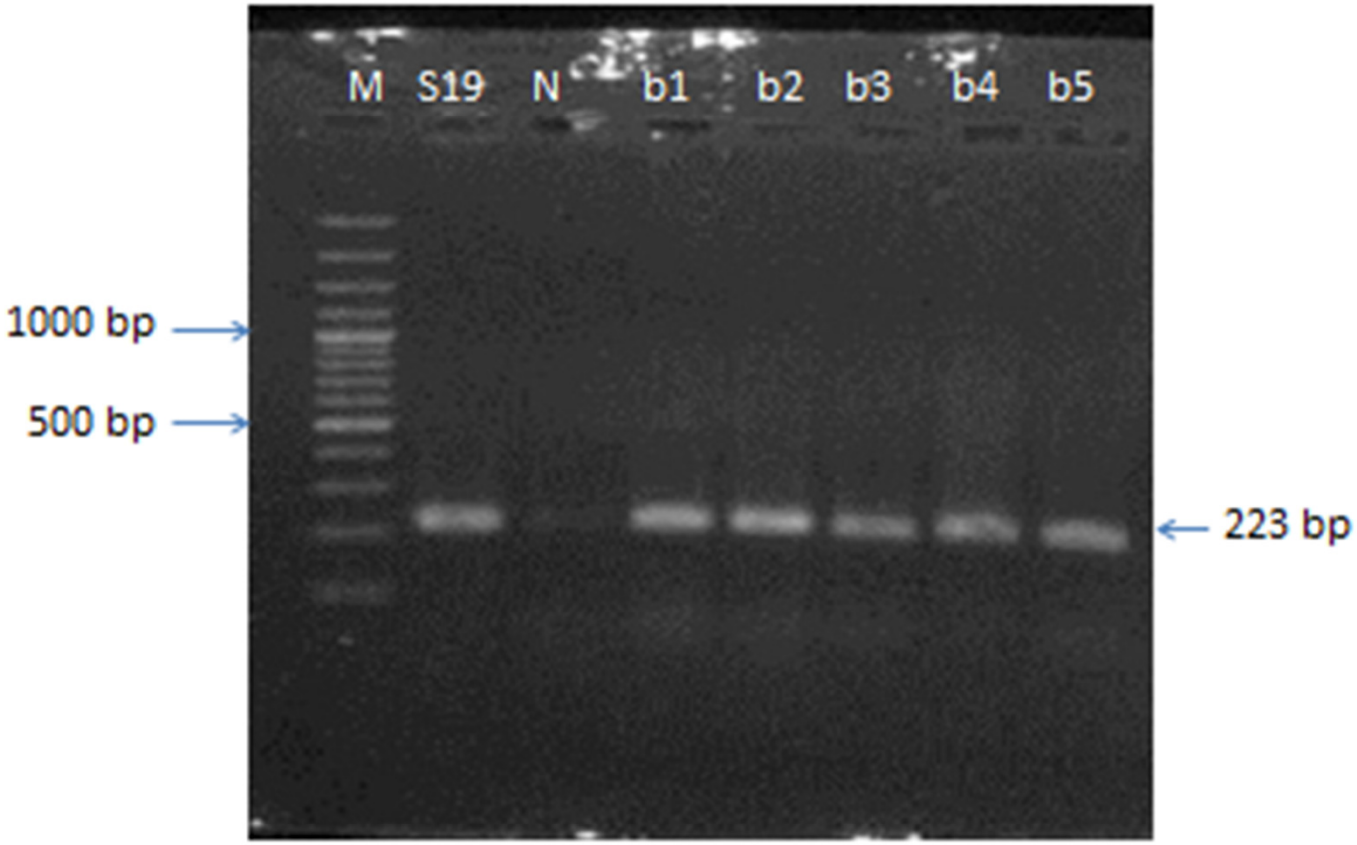
The DNA extracted from *Brucella abortus* isolates and S-19 revealed the desired amplicons at 223 bp and 905 bp, respectively by PCR using *Brucella* specific primers B4/B5 and F4/R2 (Fig 1, 2).

Brucellaphage

Brucellaphage (Fig 3) isolated from sewage gave clear zones along the streak lines on streaking the plaques on *Brucella* lawn (Fig 4).

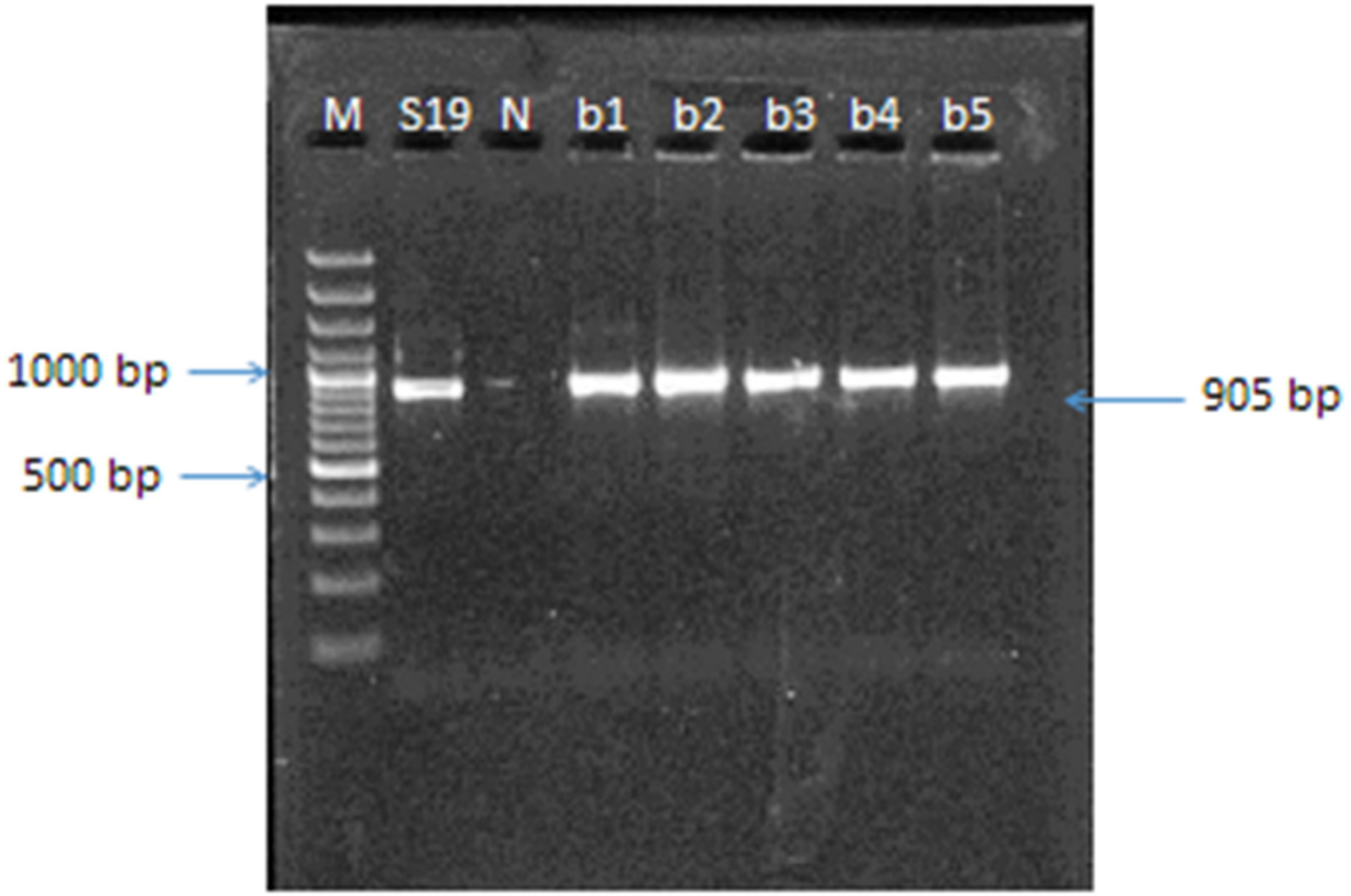
At a concentration of 10^{-4}, the phage count was 4.5×10^{6} plaques per ml.

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



M= Marker, N = Negative control, b = *Brucella* isolates

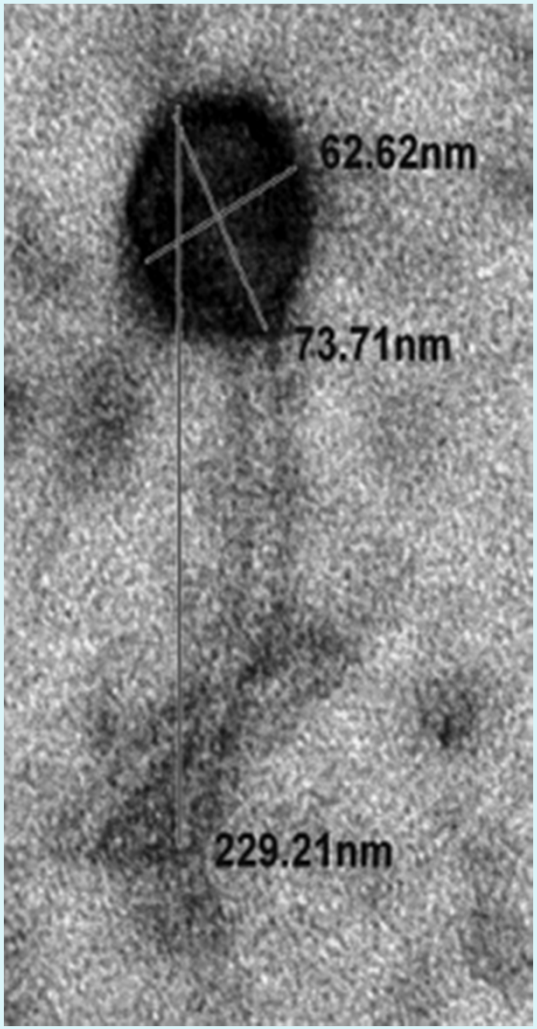
Confirmation by PCR with B4/ B5 *Brucella* primers

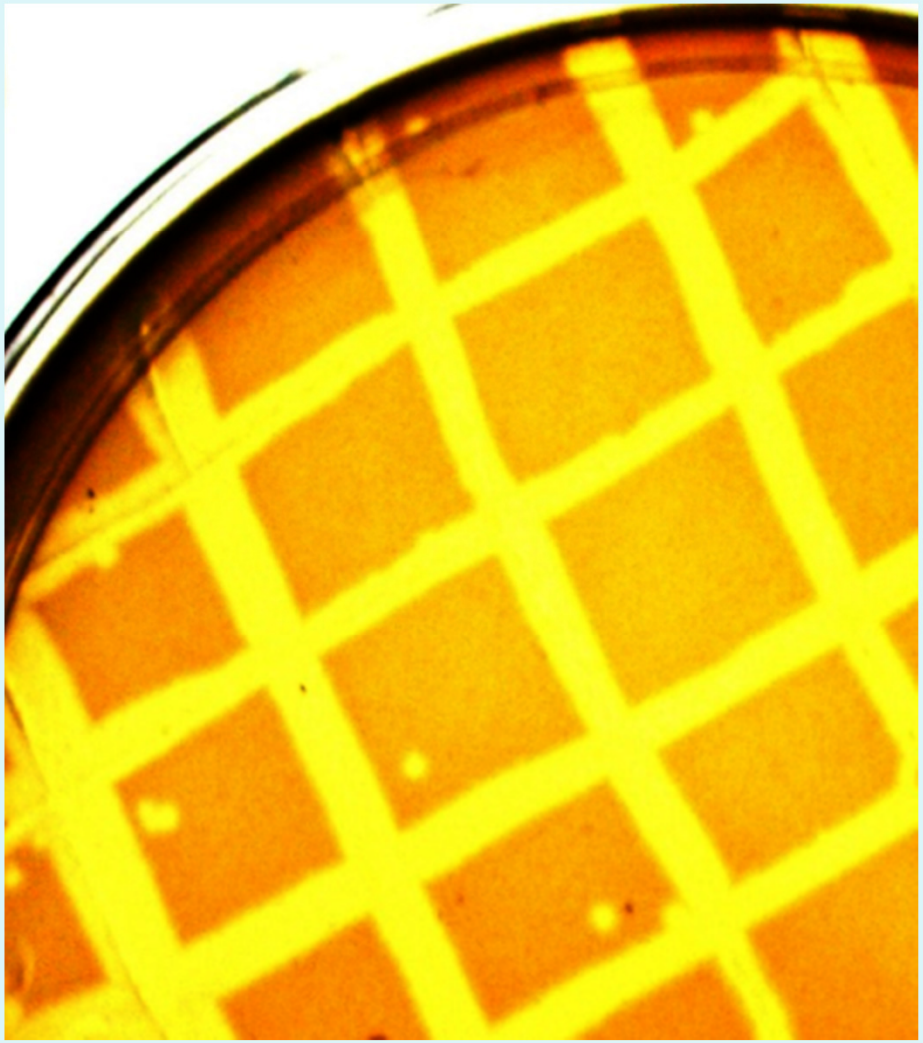


M= Marker, N = Negative control, b = *Brucella* isolates

Confirmation by PCR with F4/R2 Brucella primers

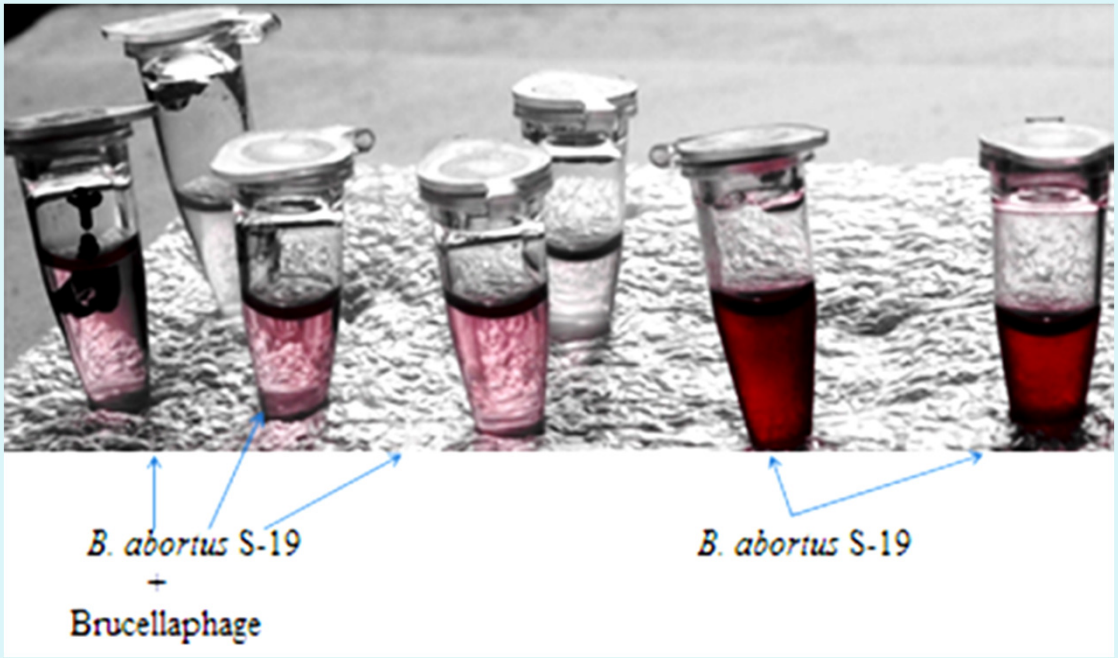
Brucellaphage isolated at GADVASU, Ludhiana





Lysis of Brucella organisms by the phage

We have adapted Nitrate Reduction assay for use with phage in detection of Brucella organisms.



Nitrate Reductase test for *Brucella abortus*

In Phage based Nitrate Reduction Test, red colour was noticed in the case of live Brucella isolates alone, but in case of Brucella incubated with phage for 8 hours, it remained colourless.

Phage based luminescence assay

The amount of luminescence measured with different concentrations of standard ATP provided in the kit is given in Table 4.

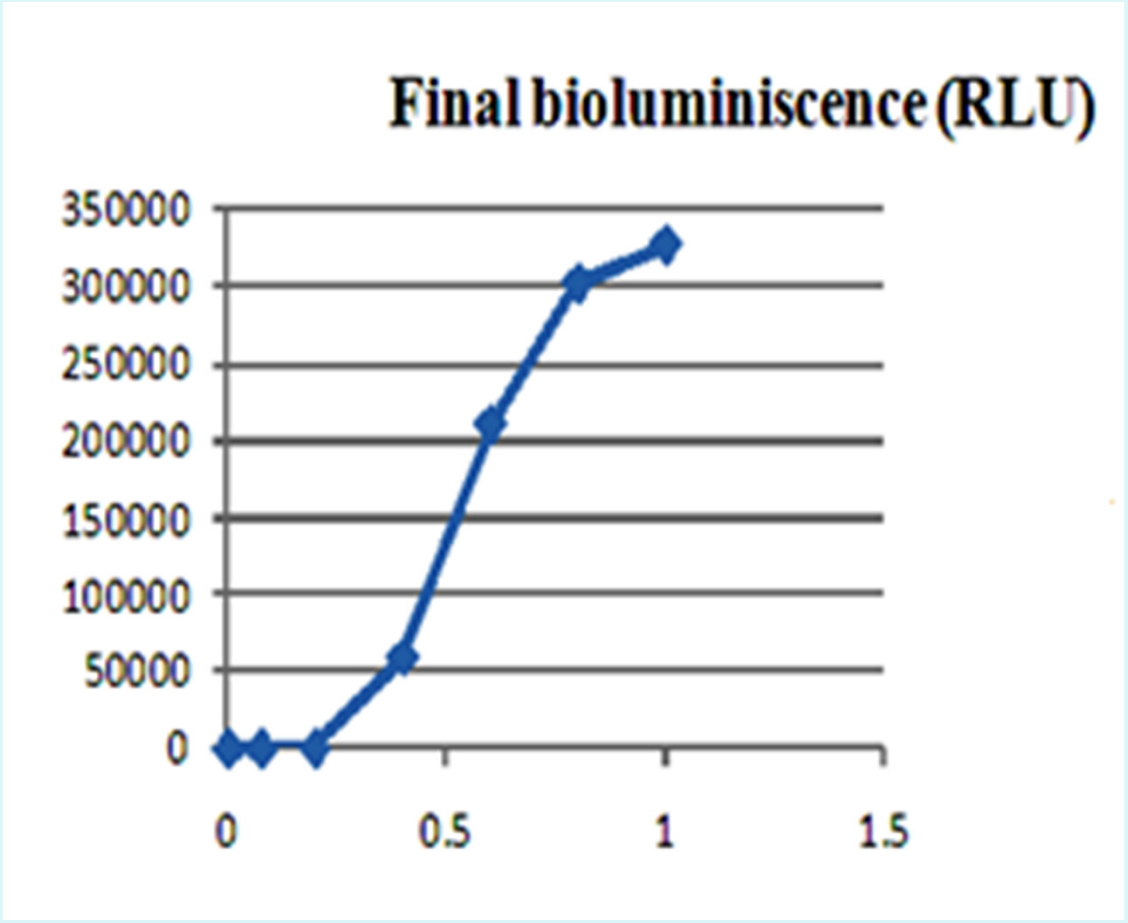
There was a positive correlation between the concentration of ATP and the amount of luminescence measured.

It was observed that a minimum of 0.001µM concentration of ATP was necessary to produce detectable luminescence (Table 3, fig 5).

Final bioluminescence = Luminescence measured – Background luminescence (BGL).

Table 3: Luminescence values for generating the standard curve for ATP assay

S. No.	Concentration of ATP (µM)	Final luminescence (RLU)
1	1.0	324660
2	0.8	300056
3	0.6	209939
4	0.4	60270
5	0.2	180
6	0.075	66
7	0.001	25



Standard ATP curve

Uterine fluid samples processed for isolation of *Brucella* organisms

	Sample No.	Age of animal (years)	Month of abortion
Cows	44b	6	5.5
	48b	5	6.5
	50b	6	7
	55b	5	6.5
	57b	4	H/O prolapse
	58b	5	H/O ROP
	59b	6	H/O Dystocia
	60b	6	H/O ROP
	72b	4.5	H/O Dystocia
	73b	6.5	H/O Dystocia
	88b	4	H/O Dystocia
	95b	5	H/O ROP
	100b	7	H/O Prolapse
	8b	6	NA
	17b	6	NA
	18b	4	NA
Buffaloes	82b	5	H/O Dystocia
	83b	4	H/O Dystocia
	87b	6	H/O ROP
	96b	5	H/O Dystocia

Analysis of isolates and samples by ATP assay

Field samples (Table 4, Fig. 6) from animals suspected for brucellosis were analyzed by luminescence assay for the presence/absence of live bacteria.

Reconfirmation was done with standard laboratory diagnostic procedures.

Uterine secretion samples from animals confirmed for *Brucella* infection, showed an increase in luminescence from **3.4 to 17.2 folds** after 5 hours of incubation with brucellaphage comparable to the luminescence observed after incubation of brucellaphage with the standard culture of *Brucella abortus* S-19.

The negative samples showed a decreased bioluminescence after 5 hours of incubation.

Animals were confirmed to be Brucellosis positive/negative by RBPT & ELISA.

Luminescence of Uterine fluid samples after incubation with the phage

S. no.	Positive Sample alone (A)	Positive sample + Phage (B)	Negative sample + Phage (C)
1	1517	22256	984
2	2323	15906	828
3	1009	17359	289
Mean ± SD	**1616.333 ± 662.608**	**18507.000 ± 3327.018**	**700.333 ± 364.664**

In case of Brucellosis positive animals, the mean luminescence was 1616.33 for uterine secretions alone and 18507 for phage treated samples

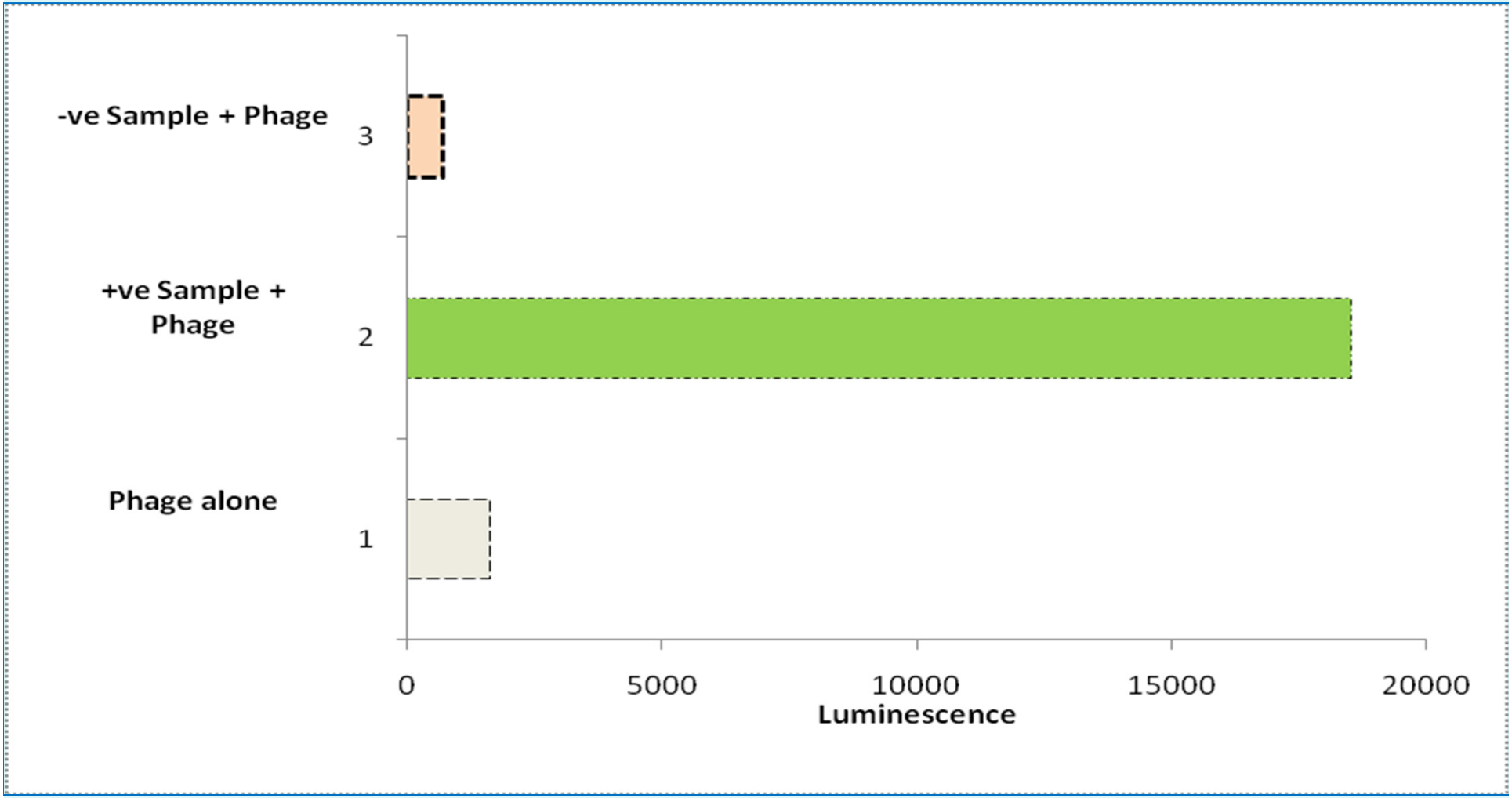
The difference was very significant ($P < 0.01$).

The average increase in luminescence was **10.03 folds**.

In case of Brucellosis negative animals, the mean luminescence of uterine secretions incubated with phage was 700.33.

The difference between the luminescence of sample alone and sample incubated with the phage was not significant.

However, the difference between the luminescence of positive and negative samples incubated with phage was very significant ($P < 0.01$).



Luminescence of uterine fluid samples with or without incubation with the phage

Low amount of ATP released from the samples untreated by phage could have been due to spontaneous death and autolysis of a few Brucella or other contaminating bacteria.

The observed background luminescence may possibly also 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.

Phages have been used earlier in the detection of several organisms like:

Salmonella (Favrin et al., 2001),
Mycobacterium (McNerney *et al., 2004),*
Cyanobacterium (Hennes et al., 1995),
Listeria (Loessner *et al., 1996),*
E. coli (Goodridge et al., 1999) etc.

However, we did not come across any report on phage based assays for *Brucella.*

This may possibly be the first report on adaptation of ATP assay along with a new Brucellaphage for detection of *Brucella.*

Conclusion

Phage based ATP assay is a simple, easy and cost effective penside test for accurate diagnosis of Brucellosis .


Thanks!