"A Dried Blood Spot collection study for detection of Brucellosis in Bovine Population of Indian villages: an ELISA based system specific to Omp25 and Omp28 proteins of *Brucella abortus.*"

Final Project Report

Delhi University Innovation Project, 2015-2016

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Final Report

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PROJECT CODE: SHC-308

1. **PROJECT TITLE** :

"A Dried Blood Spot collection study for detection of Brucellosis in Bovine Population of Indian villages: an ELISA based system specific to Omp25 and Omp28 proteins of *Brucella abortus"*

2. **NAME OF COLLEGE/ INSTITUTION :** Shivaji College, University of Delhi

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Project Title : "**A Dried Blood Spot collection study for detection of Brucellosis in Bovine Population of Indian villages: an ELISA based system specific to Omp25 and Omp28 proteins of *Brucella abortus"***

Project Code : SHC 308

Abstract: We have done KAP [Knowledge, Attitudes and Practices] survey of cattle keepers. This survey is first of its kind for cattle keepers of Delhi. We surveyed 1200 cattle sheds in 11 districts of Delhi. We found that awareness about Brucellosis, vaccination and other precautions to be taken to prevent the spread of infectious diseases is very poor. We collected DBS of 100 buffalos and tested it for brucellosis infection. We used Rose Bengal Test[a WHO prescribed for detection of Brucellosis] on site of collection. We confirmed/ quantitated infection ve givby serum agglutination test in college lab. All these 100 samples were also tested by ELISA using Omp25 and Omp 28 as antigens. This study is the proof of the concept that Omp25 can be successfully utilized to diagnose infection with *Brucella abortus*.

Introduction: India is an agricultural country where livestock have a major contribution to the socioeconomic status of the rural households. Livestock contribute 32% to the Agricultural GDP of the country. India has largest buffalo population in the world. In villages each family rears buffalo whose number depends on the requirement of the family. But their health is generally ignored until a disease starts showing its symptoms and the buffalo is doomed to die. Although Govt. of India recommends regular vaccination of the cattle but a local survey done by the investigators of this project has revealed that villagers do not get their buffalos vaccinated. Their awareness about the need of vaccination is nil. In this scenario there are several cattle-prevalent diseases. One amongst them is **BRUCELLOSIS.**

What is Brucellosis? Brucellosis is a reproductive disease of livestock which induces infertility, delayed heat, interrupted lactation, abortion and loss of calves, loss of milk and meat production. Brucellosis is endemic in countries belonging to Mediterranean region, Asia, Middle East, Latin America and Africa. The disease causes economic loss and is endemic to all states of India [**Renukaradhya and Rajasekhar, 2002]**. The **causative agent** of this disease is a proteobacteria belonging to genus ***Brucella***. *Brucella* is gram negative, aerobic, facultative intracellular coccobacilli. There are several infective species of this genus; ***B.abortus*** infects cattle, ***B.melitensis*** infects sheep *and goats*, ***B.suis*** infects pigs, ***B.canis*** infects dogs. All the species of *Brucella* can infect humans but ***B.melitensis*** is the most common human pathogen. In humans it causes a severely debilitating disease that requires prolonged antibiotic treatment with a propensity of serial recurrence [**Mantur and Amarnath, 2008**].

Mode of transmission: *Brucellae* transmit from one animal to other through cuts, abrasions in skin and inhalation. The factors that facilitate transmission include poor farm hygiene, use of semen from infected bulls for artificial insemination, unrestricted trade and movement of animals from one district to another, use of local cattle yards and fairs for trading where mixing of cattle with sheep/goats occurs and chance of transmission rises. **Human transmissions are also common.** In rural area human beings come in regular contact with cattle and consumption of unpasteurized/ unboiled milk (rich in *Brucella*) is common. In urban areas *Brucella* transmits through consumption of infected meat and of dairy products prepared from unpasteurized milk like yoghurts, ice creams and soft cheese **[Smits and Kadari, 2005**].

Control and preventive measures include improved farm hygiene, mass vaccination of cattle and **diagnosis of Brucellosis** followed by removal of infected animal. Vaccination is crucial for eradication but due to lack of awareness amongst farmers and large number of buffalos, VACCINATION of each buffalo born in the country is an UNFATHOMABLE task. **Therefore, diagnosis of *Brucella* infection is of utmost priority for control of this disease.**

Basis of diagnosis: the diagnosis of brucellosis is based on detection of anti-*Brucella* antibodies in the blood of host because in 15-35% of cases (active disease) blood stays negative for *Brucella* bacterium by blood culture study [**Memish *et al,* 2002**].

Diagnostics available for Brucellosis: the classical methods include: 1.Isolation and identification of *Brucella* from blood of infected organism. **But this method is time consuming (45 days), not safe for laboratory personnel and impractical for large no. of animals**; 2. Rose Bengal Plate test (RBPT): serum from animal is added to the test reagent and agglutination on slide is observed; 3. Standard agglutination test (SAT): agglutination assay in tube [**Sadhu *et al,* 2015**]. The last two agglutination assays require that the reagents must be stored at 4 degrees. **But storage of reagents at 4ºC in field is very difficult in India. All these classical methods need to be replaced by ELISA-based diagnostics due to better sensitivity and economy associated with this tool.**

RESEARCH PROBLEM:

1. **Collection of sera of large no. of animals in field, their storage and their delivery to lab in safe time is very difficult.**
2. All the currently available diagnostic tests are based on detection of serum antibodies in infected animal to Lipopolysaccharide (LPS) portion of cell membrane of *Brucella*. This leads to false positives due to cross-reactivity of anti-LPS antibodies with other gram negative microbes like *Yersinia enterocolitica*, *Salmonella* and *E.coli*. **Therefore currently there is a search for non-LPS Antigens present on outer membrane of *Brucella* for use in ELISA as antigen** [**Chauhuri *et al,* 2010**].

OUR INNOVATIVE PROPOSAL:

1. **We proposed to collect Buffalo's blood by Dried Blood Spot technique.**
   Dried Blood Spot technique in nutshell: In this method blood is collected on filter paper and brought to lab where serum is eluted from the paper in phosphate buffered saline and can be used for diagnosis. This method is beneficial for large scale sera collection in field because Dried Blood Spots can be stored at ambient temperature in zip pouch for 14 days without deterioration of analytes. This method has been successfully used for detection of HIV, HCV and HBV infections [**Ross *et al,* 2015**].
2. **We proposed to test Omp25 protein as a novel molecule for diagnosis of Brucellosis.** Outer membranes of gram negative bacteria are rich in Outer membrane proteins/ porins. The major *Brucella* OMPs are identified and classified according to their apparent molecular masses. These include the 36- to 38-kDa OMPs (group 2 porin proteins), the 31- to 34-kDa and 25- to 27-kDa OMPs, which belong to the group 3 proteins. **Omp25 protein is highly conserved in *Brucella* species, biovars and strains** [**Cloeckaert *et al,* 1996**].**Therefore, it is a very promising protein candidate to be used for diagnosis of Brucellosis.** The protective efficacy of Omp25 as a vaccine candidate against *Brucella* has been established by the mentor of this project [**Goel and Bhatnagar, 2012**]**. Omp28** has been successfully used as a diagnostic antigen in indirect ELISA [**Chauhuri *et al,* 2010**], therefore, it was used as a **positive control** for our study.

OBJECTIVES:

1. Survey of the cattle keepers to assess their animal maintenance practices, their awareness about Brucellosis and their habits that unknowingly contribute to spread of infectious diseases.
2. Collection of Buffalo's blood.
3. Analysis of Seroprevalence of anti-*Brucella abortus* antibodies among the Buffalos
4. Indirect ELISA with Omp25 and Omp28 as test antigens

Methodology Techniques/Sampling /Tools/Materials

1. **KAP [Knowledge, Attitudes and Practices] Survey**: A cross-sectional study was carried out between August 2015 to August 2016 to estimate the awareness of cattle keepers about brucellosis and other infectious diseases. A standardized structured questionnaire including both open end and close end questions in Hindi was administered in Delhi. Delhi has 11 districts and 369 villages. Questionnaire was prepared to know the animal maintenance practices of cattle keepers. The common trends and Statistical Analysis was done. Copy of questionnaire has been attached.
2. **Blood Collection :** Animal handling was done by Animal Lab Technician trained at National Institute of Immunology for handling animals. To collect blood a small area of ear was cleaned with spirit swab, a prick was made using a 16 guage needle to collect blood in a 5 ml syringe from a visible ear vein from buffalos and saved by following ways:
    a) **Dried Blood Spot Collection :** ten drops of blood was dropped on Protein Saver cards made of Whatman filter paper [grade 903]. These cards were saved in a zip locked pouch.
    b) **Blood collection in tube:** blood [0.5 ml] was collected in microfuge tubes, these tubes were kept in vertical orientation in collection box. The tubes were brought to college within 3-4 hours of collection from village and centrifuged at 500xg to separate sera from blood clot. **All sera were treated at 60 Deg C for 30 mins for inactivating and stored at -20 Deg C.**

c) After blood collection the prick was pressed for some time to stop bleeding with a cotton swab and Neosporin powder was applied at the prick to avoid any future infection.

3. **Analysis of Seroprevalence of anti-*Brucella abortus* antibodies among the Buffalos**

a) **RBT Assay :** RBT is a reagent approved by WHO used for detection of Brucellosis. It has pink colored dye called as Rose Bengal mixed with dead *Brucella abortus* in a solution of pH 3.5 to 3.7. This was done **'ON SITE'. It is a qualitative test.** Hundred microlitre of Rose Bengal test reagent was taken on a microslide with the help of a dropper. A drop of blood was added by syringe and mixed with RBT reagent with the help of a fresh all pin and reaction was observed for five minutes by tilting the slide.

b) **Agglutination Assay:** This assay was done by using the Brucella Serum Agglutination Test [SAT] antigen. SAT antigen is a suspension of a pure smooth culture of *Brucella abortus* in phenol saline obtained from Indian Veterinary Research Institute, Izatnagar. This was done in **'COLLEGE LAB'.** The assay was adapted from the original to reduce the volume of sera to be used. It was done in 96 –well ELISA plates. Here phenol saline was prepared by adding 0.5% phenol to normal saline. Phenol saline was aliquoted in volumes 80µl in first well of each row followed by 50µl in subsequent wells. Sera was added in a volume of 20µl to first well, mixed well and two fold serially diluted four more times by picking 50µl from first well. The sera and agglutination antigen was mixed well and incubated at 37 deg C for 20 ± 1 hours. All 100 sera samples were tested by SAT and

study was done 3 times. The result was observed after keeping plates for half an hour on the bench at the room temperature.

4. **Methodology of protein purification**: Due to lack of cold room or cold cabinet in Shivaji College, both the recombinant proteins, Omp25 and Omp28 of *Brucella abortus* were purified at the Mentor's lab. Expression vectors containing recombinant *Omp25* and *Omp28* genes were transformed in *E.coli* expression strain BL21.These BL21 cells were grown in 2 L cultures till A600 of 0.6 and induced with isopropyl-l-thio-β-D-galactopyranoside [IPTG]. The expression of induced proteins was checked on SDS PAGE.rOmp25 and rOmp28 containing inclusion bodies were solubilized by Urea lysis method. The His-tagged proteins were purified with help of Ni-nitrilotriacetic acid resin [Ni-NTA] and were eluted with imidazole. Purified proteins were dialyzed in Phosphate buffered saline. The purified protein has been saved in -80 Degree deep freezer at mentor's lab at School of Biotechnology, Jawaharlal Nehru University, Delhi. Just before conducting the experiment the proteins were shifted to -20 Deg deep freezer and thawed just before use.
5. Solid phase/ Indirect ELISA : The 96-well polypropylene ELISA plates were used for this assay. The antigen[omp25/ omp28] were coated at a 100 ng/ well in PBS concentration in a volume of 100µl overnight at 4 deg C. The antigen was thrown and coated wells were incubated with 2% Bovine Serum albumin [BSA] in PBS in a volume of 200 µl for two hours. Few uncoated wells were also incubated with BSA for negative control. BSA was discarded and all wells were washed with PBST [PBS+ 0.1% Tween 20/CWEEN detergent] five times to wash off any weakly interacting molecule. Sera was diluted serially and incubated in the wells in a volume of 100 µl for 2 hours.

The sera were discarded and well were washed with PBST five times by shaking on gyratory shaker. Secondary Antibody: The secondary antibody was a bovine specific antibody labelled with Horse Radish Peroxidase [HRP]. The range of dilution specified by manufacturer was 1:5000 to 1: 25,000. The dilution was standardized to be 1:20,000. Secondary antibody [1:20,000 in PBS] was incubated for 1 hour. Secondary Antibody was discarded and wells were washed five times with PBST. TMB substrate was added to each well and incubated till development of color and maintenance of gradient. The time was standardized and accordingly 2M H_2SO_4 was added to stop the reaction. The Optical Density was recorded at 450 nm in ELISA reader. The result was analyzed.

6. **Elution of sera from Dried Blood Spot:** From each Protein Saver Card multiple 6 mm circles were punched and these 6 mm circles were added in 96 well plate, 3 circles per well and 300 µl PBS was added to each well. These 96 well plates were incubated overnight on gyratory shaker. PBS containing sera was collected in microfuge tubes. These microfuge tubes were centrifuged at 500x g for 10 minutes to separate debris from sera. Sera were aliquoted in another microfuge tube and saved at -20 Deg C until further use. Reactivity of these sera with rOmp25 was analyzed by ELISA. The Comparison of ELISA OD values of card eluates with original serum.

RESULT AND DISCUSSION:

1. Analysis of the Survey: Survey has been done at 1200 cattle sheds with total no. of animals surveyed 5550 [including 4828 females and 722 males] in Delhi. This is first ever report of this kind from Delhi. **The trends were prepared as follows:**

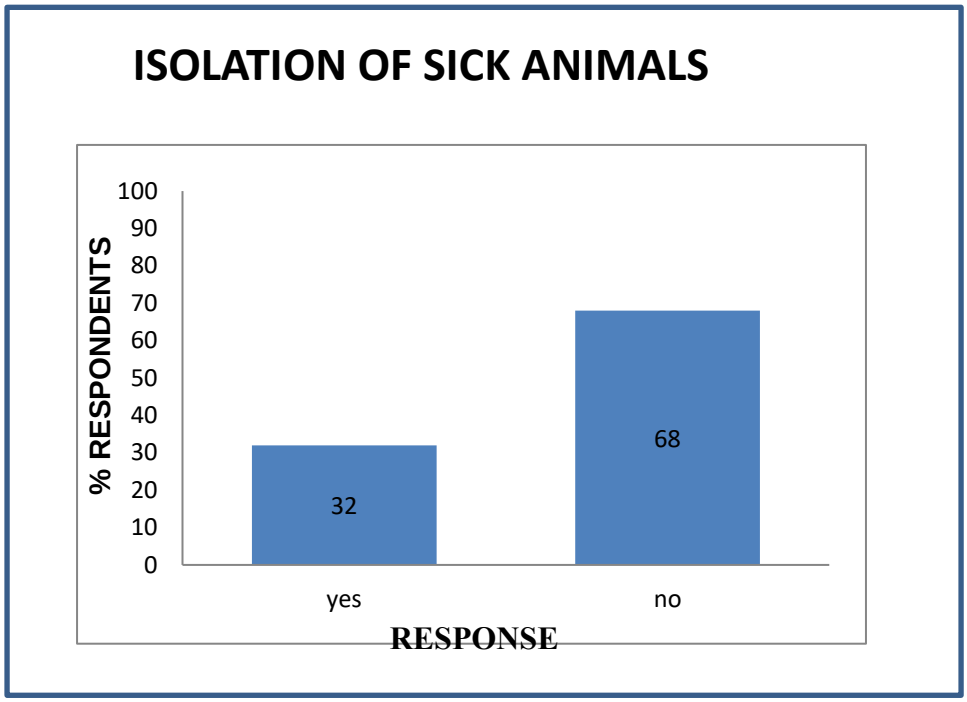


Figure 1a: percentage of respondents who separate their sick animals from healthy ones

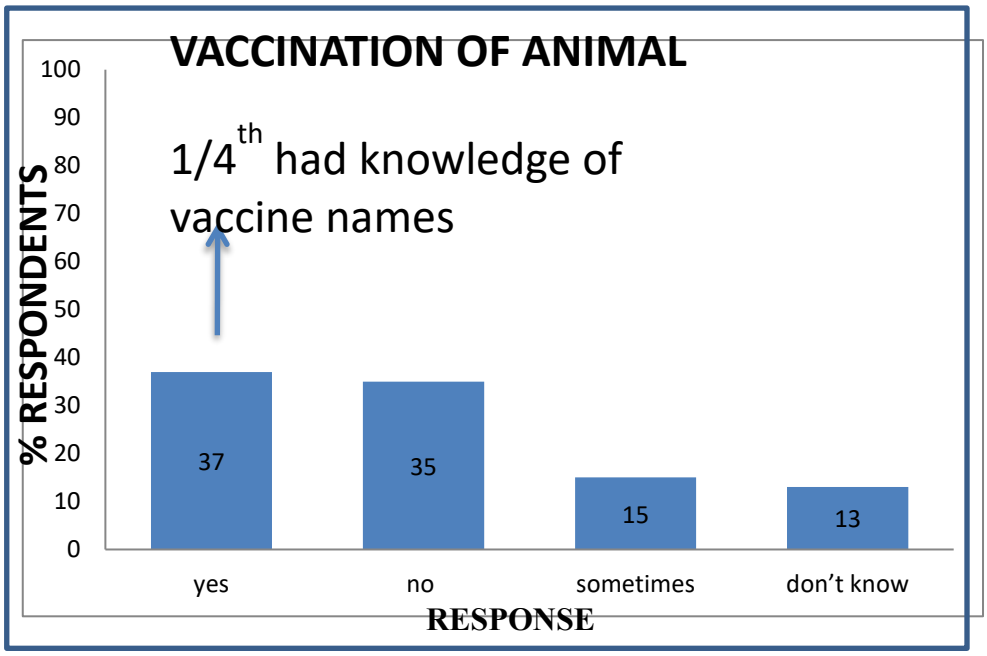


Figure 1b: Knowledge of cattle vaccines among respondents

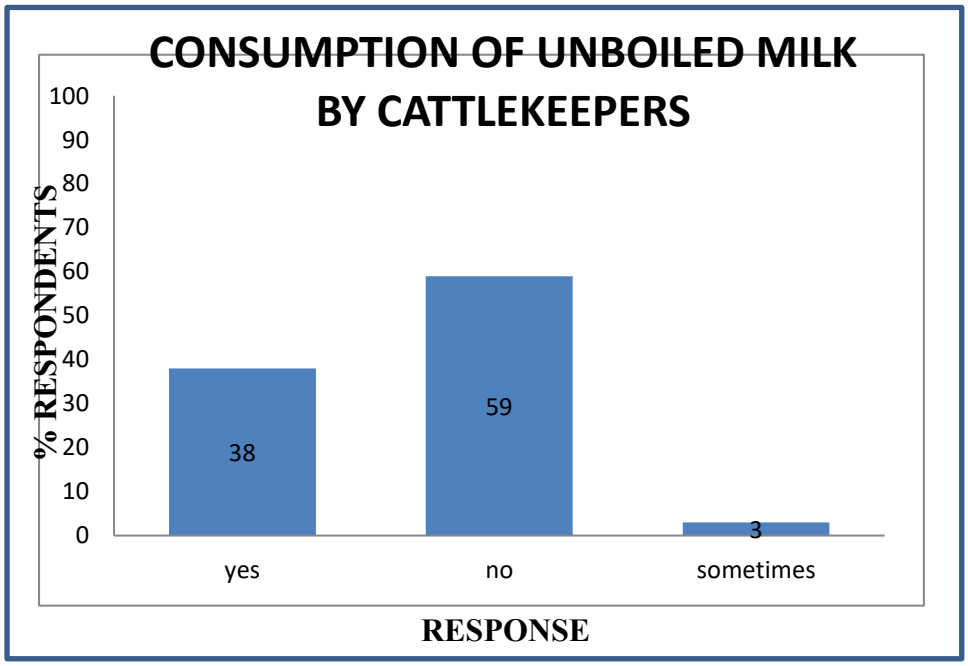


Figure 1c: Milk consumption habit of respondents

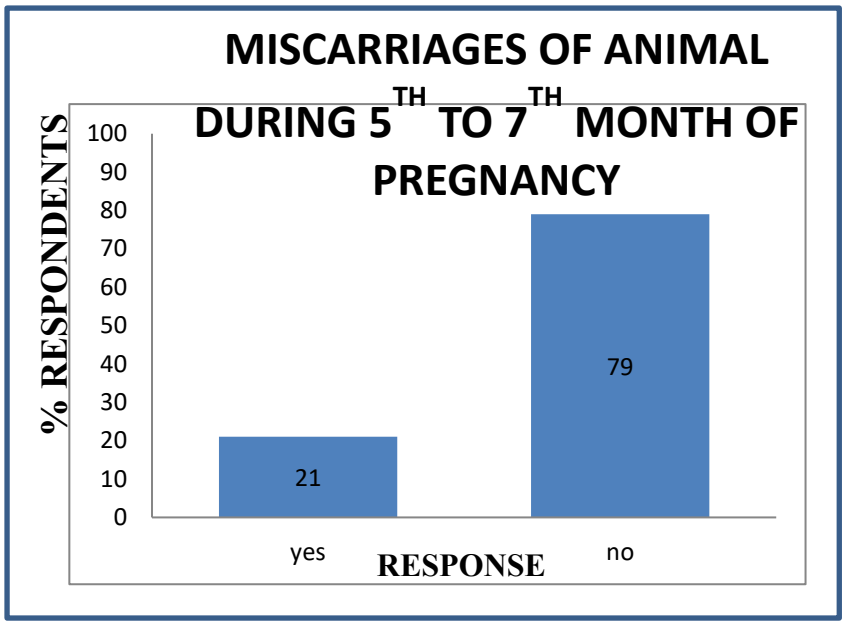


Figure 1d: Report on miscarriage of buffalos by respondents

As 68% of respondents don't separate their sick animals therefore the cattle under study are at high risk of infectious diseases (Fig.1a). Only one fourth out of 37% Vaccine aware respondents could name animal vaccines like anthrax spore vaccine, FMD vaccine, ET and BQ. But 0% had knowledge about *Brucella abortus* strain 19 vaccine (Fig.1b). Cattlekeepers consuming unboiled milk of their buffalos are at a risk of Brucellosis infection. Govt. support services for cattle keepers are inadequate (Fig.1c). Animals undergoing miscarriages are at high risk of *Brucella abortus* infection (Fig1d). Such a KAP survey has never been done in past. Last report about *Brucella* seroprevalence from Delhi was in year 1997 (Kumar et al.)

2. **Rose Bengal test:** Fifty seven samples out of hundred samples showed positive agglutination with RBT (fig.2A). The agglutination was correlated with blood samples that were negative for agglutination. Ten samples were doubtful cases. But the product insert instructs that any change in texture of the reagent within 4

minutes can be taken as a positive result. This test is a qualitative test for checking Brucellosis infection

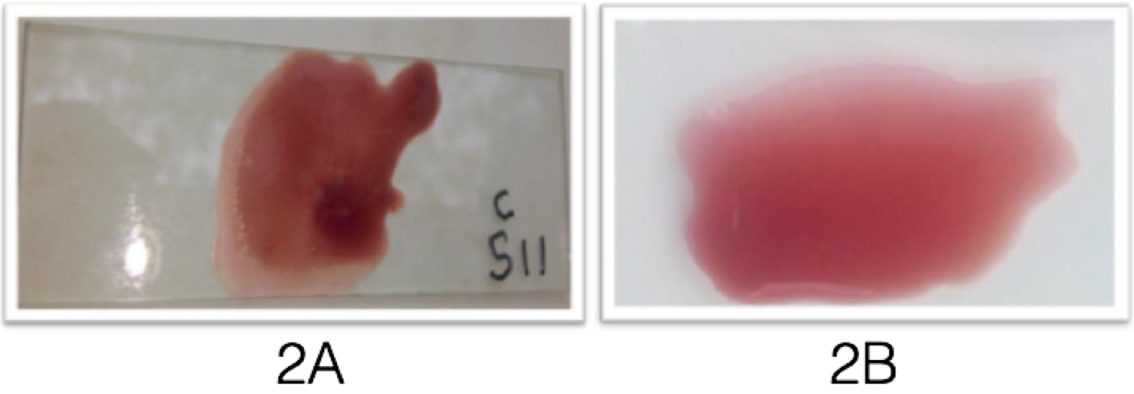


Figure 2, Result of Rose Bengal Test. A. sample showing agglutination with RBT. B. Sample negative for agglutination used as reference.

All the samples were tested by Serum Agglutination Test (SAT) also.

3. Serum Agglutination Test (SAT): with the help of this test the international unit of infection was calculated by following formula:

$$\text{I.U.} = \frac{\text{Titre of unknown serum X 1000}}{\text{The titre of international serum with the antigen to give 50\% agglutination}}$$

Here,

1. the titer of the international serum with the antigen specified on the reagent bottle was 1:500. This value was used to calculate IU of infection.

2. The titer of unknown was the last dilution showing agglutination with the antigen (tables)

3. IU equal or more than 80 IU indicates infection with *Brucella abortus.* 40 IU / above in cattle shows doubtful cases

SAMPLE → / Dilution ↓	1	2	3	5	6	9	10	11	12	15	16	17
1:5	+	+	+	+	+	+	+	+	+	+	+	+
1:10		+	+	+	+	+		+	+	+	+	+
1:20		+	+	+	+	+		+	+	+	+	+
1:40		+	+	+	+	+			+	+	+	+
1:80		+		+	+	+			+	+	+	+
International units of infection	10*	160	80	160	160	160	10*	40*	160	160	160	160

Table 1: Result of SAT. * indicates absence of infection. IU equal to or more than 80 IU for cattle shows *brucella* infection.

SAMPLE → / Dilution ↓	19	21	22	24	26	37	38	39	40	41	42	43
1:5	+	+	+	+	+	+	+	+	+	+	+	+
1:10	+	+	+	+	+	+	+	+	+	+	+	+
1:20	+	+	+	+	+	+	+	+	+	+	+	+
1:40	+	+	+		+	+	+	+	+	+	+	+
1:80	+	+	+		+	+	+	+	+	+	+	+
International units of infection	160	160	160	40*	160	160	160	160	160	160	160	160

Table 2: Result of SAT. * indicates doubtful infection. IU equal to or more than 80 IU for cattle shows *brucella* infection.

SAMPLE / Dilution	44	45	46	47	48	49	50	51	52	53	54	55
1:5	+	+		+	+	+	+	+	+		+	+
1:10	+	+		+	+	+	+	+		+	+	+
1:20	+	+		+	+	+	+	+		+	+	+
1:40	+	+	+	+	+	+	+	+		+	+	+
1:80	+	+	+	+	+	+	+	+		+	+	+
International units of infection	160	160	160	160	160	160	160	160	10*	160	160	160

Table 3: Result of SAT. * indicates absence of infection. IU equal to or more than 80 IU for cattle shows *brucella* infection.

SAMPLE / Dilution	56	57	58	60	62	63	64	65	66	68	69	70
1:5	+	+				+	+	+	+	+	+	+
1:10	+	+	+	+		+		+	+	+	+	+
1:20	+	+	+	+		+			+	+	+	+
1:40	+	+	+	+		+			+	+	+	+
1:80	+	+	+	+		+						
International units of infection	160	160	160	160		160	10*	20*	80	80	80	80

Table 4 : Result of SAT. * indicates doubtful infection. IU equal to or more than 80 IU for cattle shows *brucella* infection.

SAMPLE → / Dilution ↓	71	73	74	79	81	82	84	85	86	88	90	91
1:5	+	+	+	+		+	+	+	+	+	+	+
1:10	+	+					+	+	+	+		+
1:20	+	+					+	+	+	+		+
1:40	+	+					+	+	+	+		+
1:80							+	+	+	+		
International units of infection	80	80	10*	10*	0	10*	160	160	160	160	10*	80

Table 5: Result of SAT. * indicates doubtful infection. IU equal to or more than 80 IU for cattle shows *brucella* infection. Empty cells indicate no agglutination.

SAMPLE → / Dilution ↓	94	95	96	97	99	100
1:5	+	+	+	+	+	+
1:10		+	+	+	+	+
1:20		+	+	+	+	+
1:40		+	+	+	+	+
1:80		+	+	+		+
International units of infection	10*	160	160	160	80	160

Table 6: Result of SAT. * indicates doubtful infection. IU equal to or more than 80 IU for cattle shows *brucella* infection. Empty cells indicate no agglutination.

Discussion : Cattle of rural and Urban area of our country in Delhi NCR region is at risk of infection with *Brucella abortus. Brucella abortus* is known to be endemic to South India, Punjab and Eastern states of India, but our study is first to report its incidence/ seroprevalence of Brucellosis in Delhi-NCR region. With the validation of indirect ELISA in the second half of this project by us we will be able to inform the cattle keepers about the *Brucella* infection of their cattle so that they will be able to get appropriate medical intervention**.**

3. **Protein Purification :** Recombinant Omp25 and Omp28 were purified to homogeneity as can be seen from fig.3.

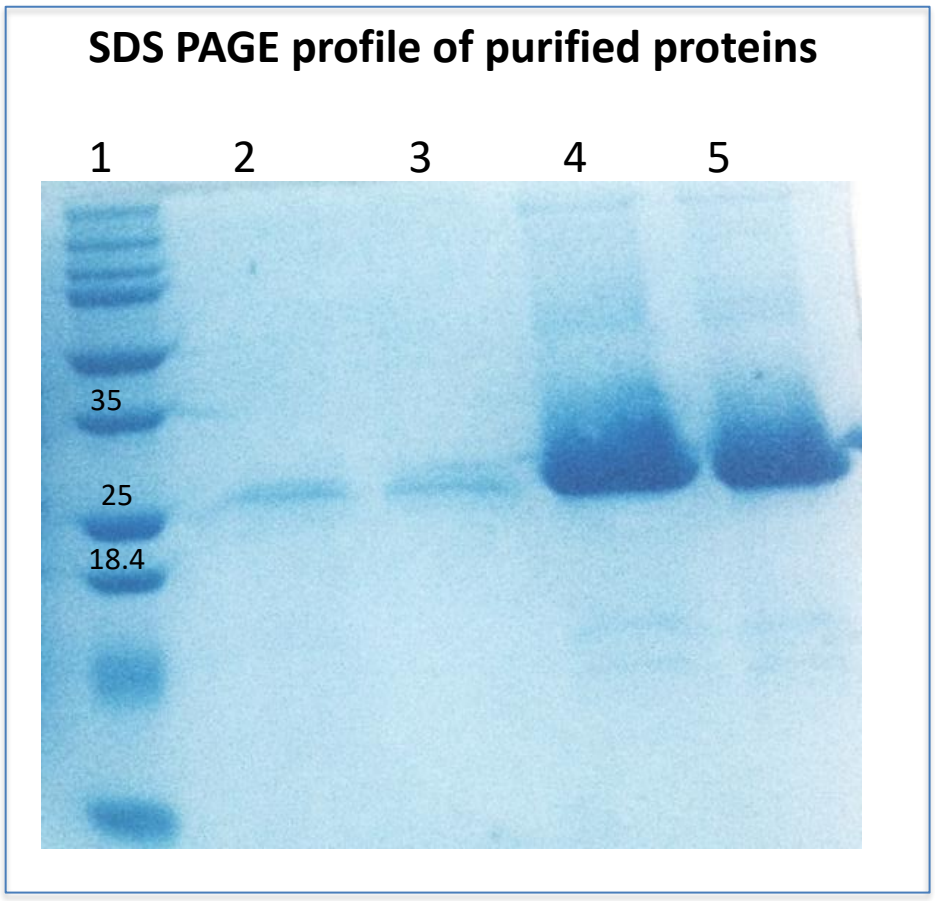
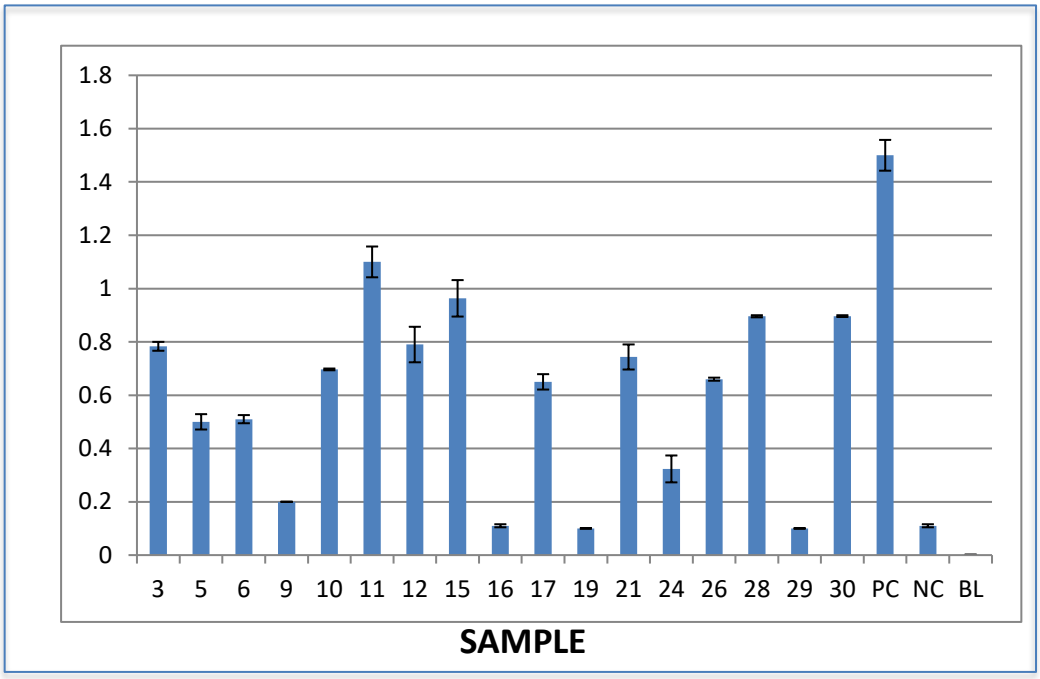


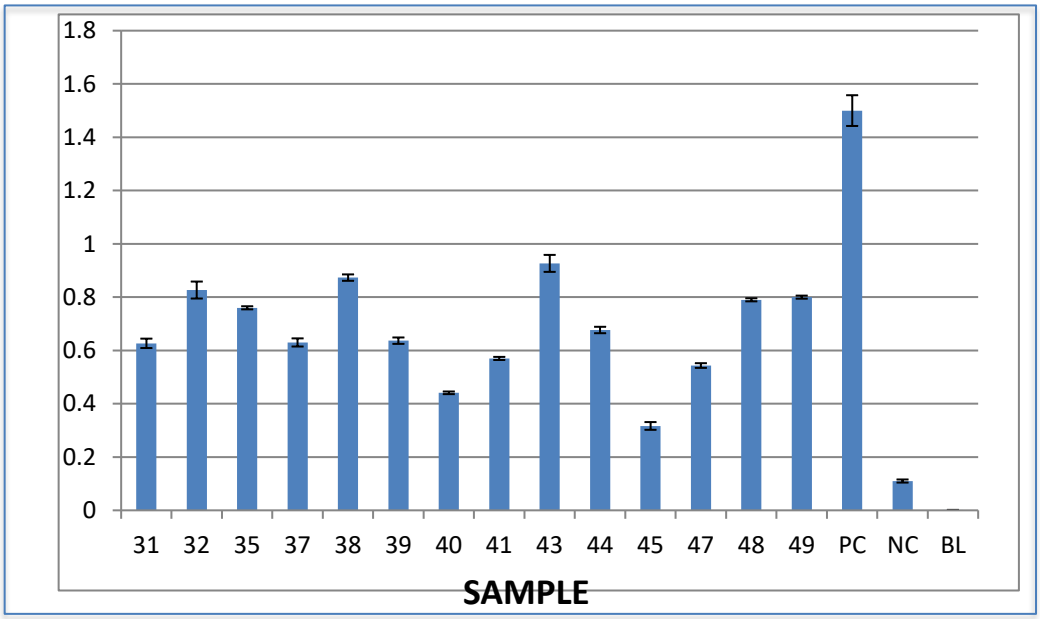
Figure 3: SDS PAGE profile of purified proteins. Here, 1: Molecular marker[kD], 2: Diluted Omp25, 3: Diluted Omp28, 4 : Omp 25 [300 mcg/ml] 5: Omp28[250 mcg/ml]

The protein concentration was determined by Lowry's reagent. The proteins were saved in deep freezer until use.

4. **Enzyme linked Immunosorbent Assay:** For all 100 samples titer was determined by pilot assay and rest of the assays were conducted by using 1:400 dilutions of sera samples. ELISA was carried out for both the antigens Omp25 and Omp28. Optical densities that were three times the OD of the negative control samples were considered positive for *brucella* infection. Figure 4A, 4B, 4C, 4D and 4E show ELISA result of 100 samples with Omp25 as antigen.



A



B

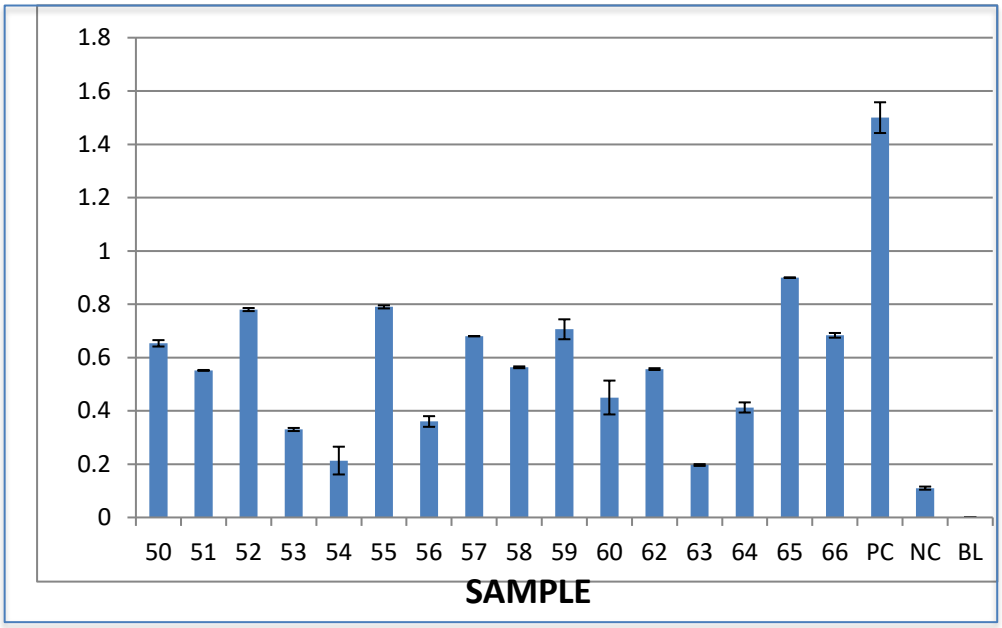


Figure 4 C

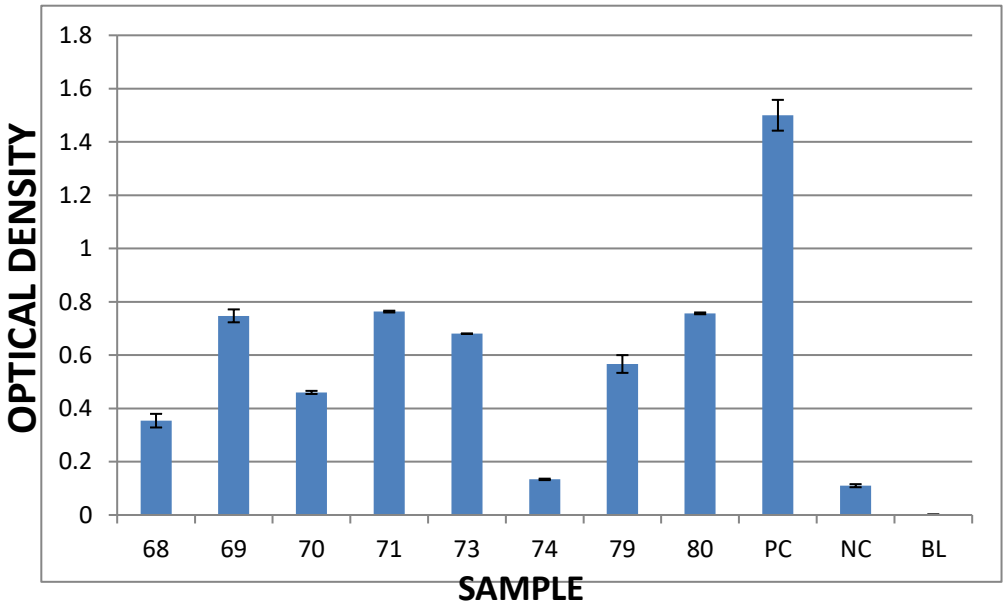


Figure 4D

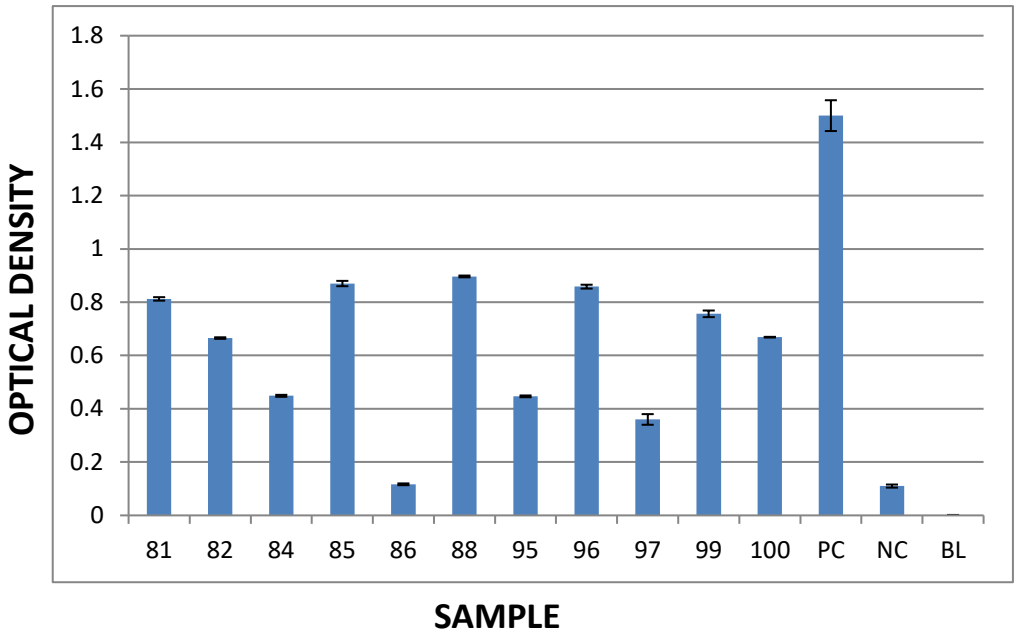


Figure 4E

Figure 4. A,B,C, D: ELISA of 100 samples of buffalo sera. Here, PC is positive control, NC is negative and BL is blank well.

A comparison of ODs of ELISA with Omp25 and Omp28: A comparison was drawn between the reaction of the same samples with rOmp25 and rOmp28. This was used to calculate accuracy, specificity and sensitivity of both the antigens as diagnostic markers. Here the samples showing OD three times the OD of Negative control were considered to be infected with *brucella*.

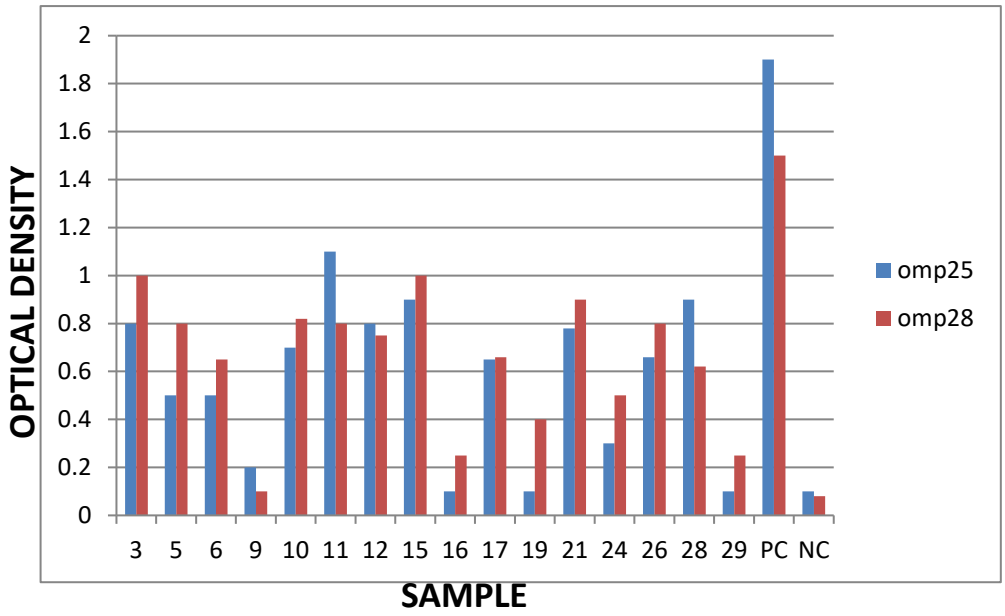


Figure 4F

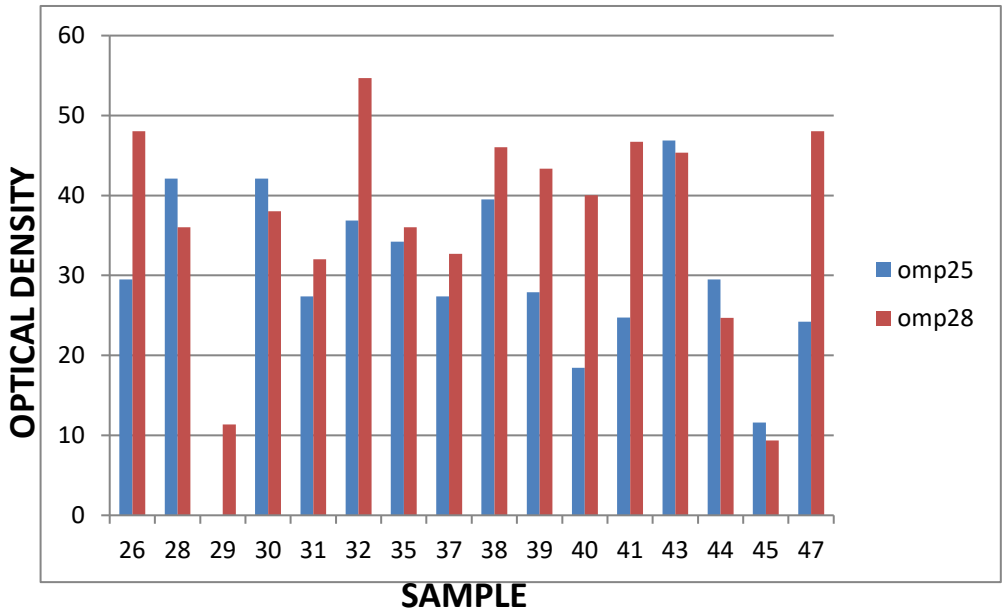


Figure 4G

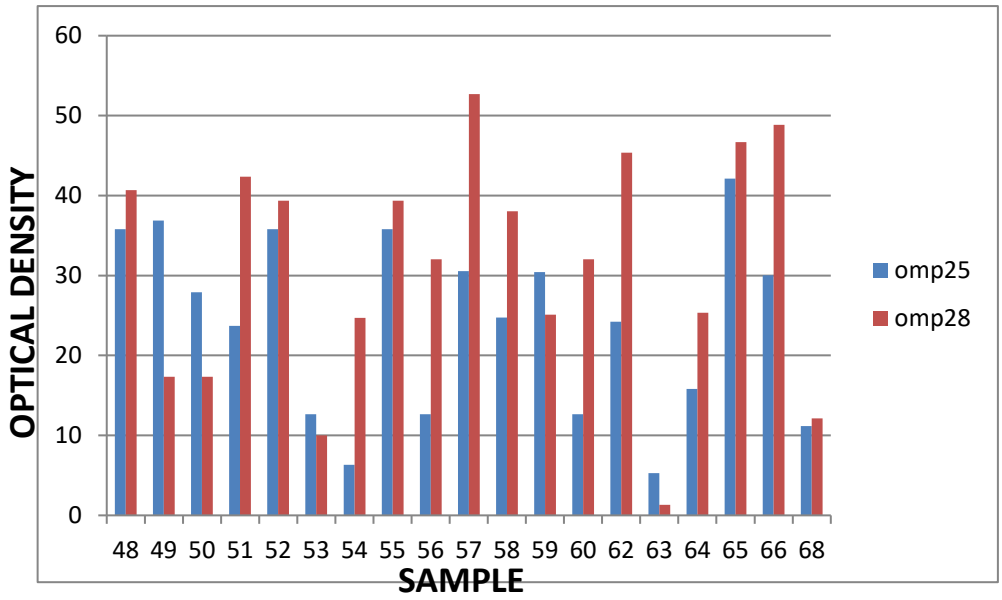


Figure 4H

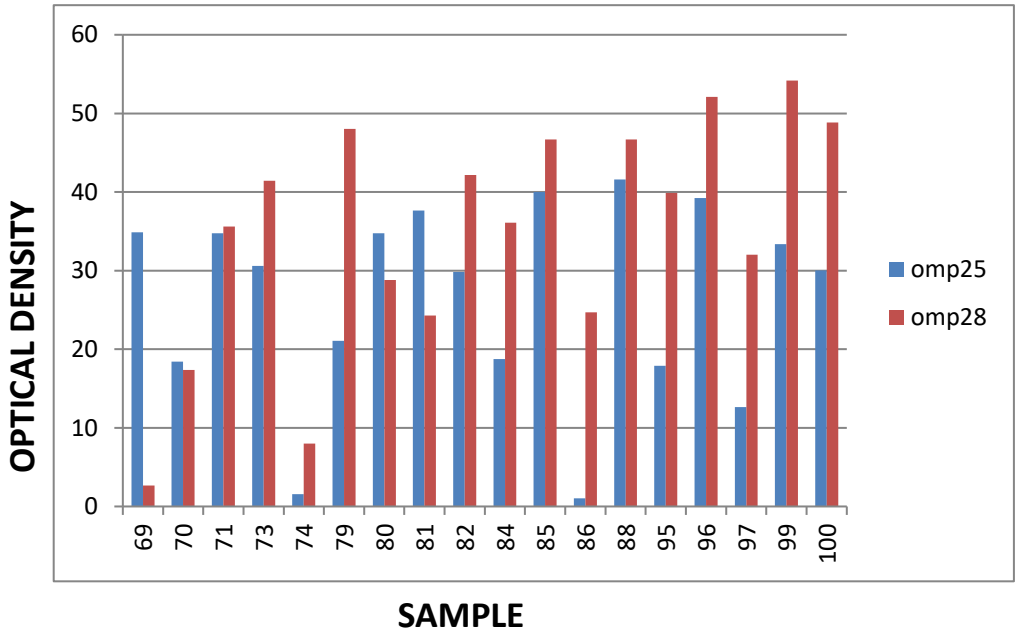


Figure 4I

Figure 4: F,G,H,I show result of ELISA of 100 samples using Omp25 or Omp28 as antigen.

Evaluation of Diagnostic Values of omp25 and omp28 in comparison with RBT: On the basis of the ODs obtained from ELISA the diagnostic potential of these 2 proteins was gauged. Table 8 summarize the results.

	omp 25	*omp 28*
Relative SENSITIVITY	**89.2%**	**93.6%**
SPECIFICITY	**10.76%**	**6.34%**
Relative ACCURACY	**50%**	**50%**

In previous report, In previous report (Gangaplara et al., Cellular and Molecular Probes, 2010) the Sensitivity of Omp 28 was reported to be 88.7%, Accuracy= 92.9% . There the samples used were of Sheep and goat. We have reported for domesticated Buffalos which have not been vaccinated with S19 *Brucella abortus* vaccine.

Elution of sera from Dried Blood Spot: sera eluted from card were titrated the same way as described and ELISA ODs were compared to calculate the Pearson's coefficient.ELISA was done with card eluates by using Omp25 and Omp 28 both. Figure 5 shows the comparison of ODs.

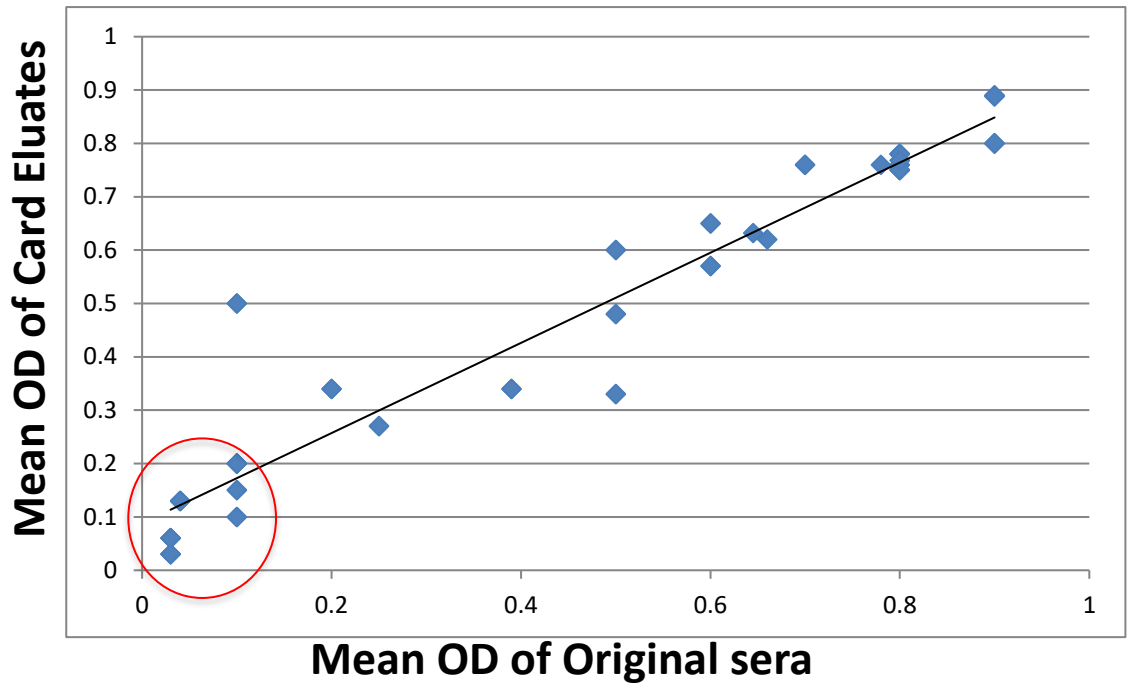


Figure 5: **Scatter Plot to compare the serum samples as card eluates at after 4 months of collection as original sera. ODs of samples negative for infection have been encircled.**

As can be seen from figure 5 Significant positive correlation can be seen between the card eluates and original serum of 60 samples. The calculated Pearson's Correlation Coefficient = 0.96.Statistical analysis has been done with Excel

SCIENTIFIC INNOVATION OF THE PROJECT

1 : Collection of bovine blood by Dried Blood Spot technique: We have collected very small volume of blood (ONLY 10 BLOOD DROPS) from buffalos on filter paper cards(Grade 903 filter cards) in field and brought to college for diagnosis.

- We have been able to show that Dried Blood Spot keeps sera stable for 4 months if stored at 4 °C after drying the blood spot. All the reports in NCBI have tested performance of DBS eluted sera with in 10 days of DBS collection.

2 : All the currently available diagnostic tests including Rose Bengal Test are based on detection of serum antibodies specific to Lipopolysaccharide (LPS) portion of cell membrane of *Brucella* in infected animal. This leads to false positives due to cross-reactivity of anti-LPS antibodies with other gram negative microbes like *Yersinia enterocolitica, Salmonella* and *E.coli.* TECHNOLOGICAL INNOVATION : We have shown that omp25 protein is as good a molecular marker for diagnosis of brucellosis as omp28. Omp25 protein is highly conserved in *Brucella* species, biovars and strains [Cloeckaert *et al*, 1996].

CONCLUSION: Omp25 holds the potential to be used as a molecular marker for diagnosis of Brucellosis. DBS of blood samples collected in field can be saved for 4 months.

- **FUTURE DIRECTIONS:** We plan to use the sera of these domesticated buffalos for epitope mapping project to identify peptides that could form promising vaccine candidates.

- This will help in formulation of a safe and efficacious vaccine as currently *brucella* strain 19 is being used for vaccination. This strain can be used for animal vaccination but it is completely unsuitable for human use. A well defined subunit vaccine could be tested for human beings as well.

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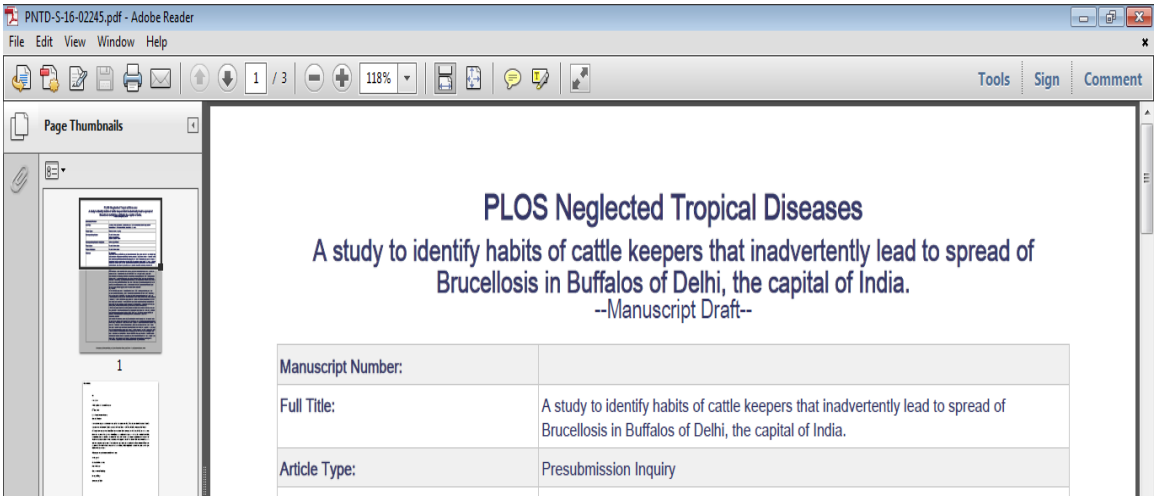
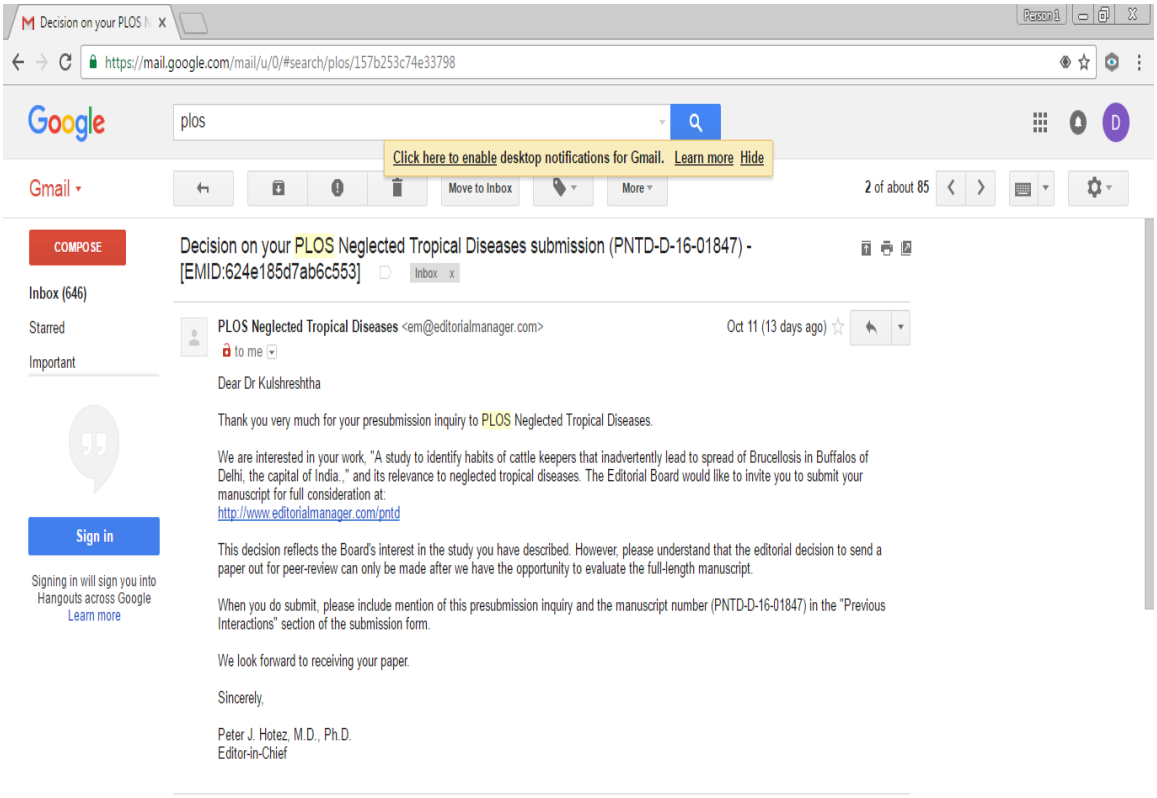
9. Goel D and Bhatnagar R (2012) Intradermal immunization with outer membrane protein 25 protects Balb/c mice from virulent B. abortus 544. Molecular Immunology 51, 159– 168.

PUBLICATIONS :

We submitted our abstract as pre-submission Inquiry to a prestigious International Journal



The Editor has asked us to submit the complete article, we are working to finalize the manuscript and will try to submit it as soon as possible



PRESENTATIONS AT THE CONFERENCES:

- Poster presentation at Annual Symposium, "BIOEPOCH 2016", at Jawaharlal Nehru University , "Seroprevalence analysis of Brucellosis in local bovine population of Delhi-NCR and Haryana region."
- Poster presentation at NATIONAL SYMPOSIUM, "Trends in research and Innovations in Life Sciences at Undergraduate level", organized by Deen Dayal Upadhyaya College, University of Delhi. "Seroprevalence analysis of Brucellosis in local bovine population of Delhi-NCR and Haryana region."
- Abstract has been selected for an International Conference, "International Brucellosis Research Conference",organised by Department of Biotechnolgy at Indian Agricultural Research Institute, Delhi to be held between 17th Nov. to 19 Nov. 2016. "A study to identify habits of cattle keepers that lead to spread of infectious diseases and prevalence of brucella –specific antibodies among buffalo population of Delhi"

SEROPREVALENCE SURVEY OF BRUCELLOSIS IN BUFFALO POPULATION OF DELHI-NCR REGION

Authors: Nimita Kant, Rashmi Singh, Anuradha Mal, Aas Mohammed, Amita Dwivedi, Rinkle Mehra, Riya Ahuja, Shashikant, Shilpa Kaushik, Shipra, Shrikrishn and Parul Kulshreshtha#

Author affiliation: Department of Zoology, Shivaji College
 # Corresponding author

ABSTRACT

A survey was done to analyze the maintenance practices and hygiene observed by cattlekeepers. A questionnaire was prepared in Hindi language to assess the habits of the cattlekeepers which unknowingly contribute to the spread of infectious diseases. Our survey presents data of **100 cattlesheds** with a total of **766 buffalos** [678 female and 88 males] from Delhi, Uttar Pradesh, Haryana and Rajasthan. Analysis of survey revealed that only 37% of the cattlekeepers got their buffalos vaccinated, out of which 1/4th could name any vaccine but 0% had knowledge about *Brucella abortus* strain 19 vaccine. At the same time Seroprevalence of Brucella abortus, the causative agent of Brucellosis, was assessed by Rose Bengal test, a test approved by WHO for detection of *Brucella* infection. It was found that 50% of buffalos were infected with *Brucella abortus.*

INTRODUCTION

India has the largest population of Buffalos in the world. Buffalo milk protein is now considered to be superior than the milk protein of *Bos taurus*, the western Cow. The semen of Murrah breed from Haryana is being sold in USA for superior offsprings. Buffalos are thus very important for the socioeconomic status of our rural and urban cattlekeepers. Still, the health of the cattle is generally ignored until a disease starts showing its symptoms and the cattle is doomed to die. In this scenario there are several cattle-specific diseases. One amongst them is BRUCELLOSIS Causative Agent: *Brucella* is a gram negative, aerobic, facultative intracellular coccobacilli. There are several infective species of this genus. *B. abortus* infects cattle. Disease spreads due to Poor Farm hygeine, Use of semen from infected bulls for artificial insemination, Unrestricted trade and movement of animals from one district to another. Use of local cattle yards and fairs for trading where mixing of cattle with sheep/goats occurs and chance of transmission rises. Human transmissions of this disease are also common. In rural area: population comes in regular contact with cattle and consumption of unpasteurized/ unboiled milk is common. In urban area: consumption of dairy products prepared from unpasteurized milk like yoghurts, ice creams and soft cheese.

OBJECTIVE -1

Survey of the cattle keepers to assess their animal maintenance practices, their awareness about Brucellosis and their habits that unknowingly contribute to spread of infectious diseases.

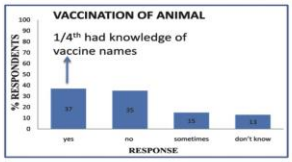
Methodology: Questionnaire was prepared to know the animal maintenance practices of cattle keepers. Common trends were drawn. Survey has been done at **100 cattle sheds with total no. of animals surveyed 766 [including 678 female and 88 males]** from following places:

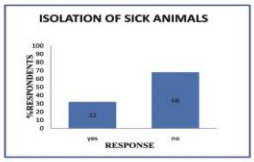
Delhi[Ujjwa, Palam, Prahladpur, Kirbi Palace, Nangalrai, Sadar Bazar, Burari, Nangli Dairy, Gola Gaon, Barwala, Shahadra]

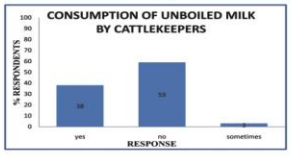
Uttar Pradesh[NOIDA,, Jaunpur, Rajgarh, Bareilly, Meerut, Mujjafarnagar , Kaser]

Haryana [Sonepat,Tajpur and Chitrakoot]

Rajasthan [Jaipur, Bijwad Chauhan, Alwar]







INTERPRETATION OF SURVEY

Only one fourth out of 37% Vaccine aware respondents could name animal vaccines like anthrax spore vaccine, FMD vaccine, ET and BQ , But 0% had knowledge about *Brucella abortus* strain 19 vaccine

As 68% of respondents don't separate their sick animals therefore the cattle under study are at high risk of infectious diseases

Animals undergoing miscarriages are at high risk of *Brucella abortus* infection

Cattlekeepers consuming unboiled milk of their buffalos are at a risk of Brucellosis infection

OBJECTIVE -2

ANALYSIS OF SEROPREVALENCE OF ANTI-BRUCELLA ABORTUS ANTIBODIES AMONG THE BUFFALOS

METHODOLOGY

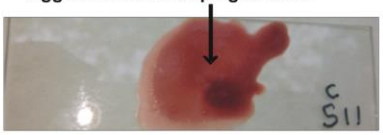
Blood has been collected from 50 buffalos. Our target is 100 buffalos

Animal handling was done by **Animal Lab Technician** from Zoology Dept. of University of Delhi. To collect blood a small area of ear was cleaned with spirit swab, a prick was made using a 16 guage needle to collect blood in a 5 ml syringe from a visible ear vein from buffalos.

RBT Assay : RBT is a reagent approved by WHO used for detection of Brucellosis. It has pink colored dye called as Rose Bengal mixed with dead *Brucella abortus* in a solution of pH 3.5 to 3.7. We took100 µl of Rose Bengal test reagent on a microslide with the help of a dropper. A drop of blood was added by syringe and mixed with RBT reagent with the help of a fresh all pin and reaction was observed for five minutes by tilting the slide.

OBSERVATION OF ROSE BENGAL TEST FOR AGGLUTINATION WITH BLOOD SAMPLES

Agglutination/ clumping of RBCs



One of the slides showing agglutination of blood sample S11 with RBT

Control slide : A sample of blood (S2) lacking agglutination with RBT

RESULT

50% blood samples gave positive agglutination test done in field. 10% samples were doubtful cases, where agglutination was not very clear. But the product insert instructs that any change in texture of the reagent within 4 minutes can be taken as a positive result.

Interpretation : Cattle of rural and Urban area of our country in Delhi NCR region is at risk of infection with *Brucella abortus.*

Discussion: *Brucella abortus* is known to be endemic to South India, Punjab and Eastern states of India, but our study is first to report its incidence/ seroprevalence of Brucellosis in Delhi-NCR region.

Urban Cattle sheds were found to be not as well maintained as rural ones, therefore risk of infecton is higher in Cities. Thus, our project is acting as a Surveillance program where cattle keepers are being informed about vaccinations and hygienic as well as preventive practices to enhance biosecurity of the cattle for better reproductive health and milk production.

ACKNOWLEDGEMENT

This work has been funded by University of Delhi under its Innovation project program.

Aas Mohammed, Amita Dwivedi, Mohit Tehlan, Paritosh Ahmed, Rinkle Mehra, Riya Ahuja, Shashikant,

Shilpa Kaushik, Shipra, Shrikrishn Shukla get fellowship from University of Delhi to carry out this project, SHC 308, entitled.

"A Dried Blood Spot collection study for detection of Brucellosis in Bovine Population of Indian villages: an ELISA based system specific to Omp25 and Omp28 proteins of *Brucella abortus.*"

NATIONAL SYMPOSIUM

on

"Trends in Research and Innovations in Life Sciences at Undergraduate level"

organized by

Department of Zoology
Deen Dayal Upadhyaya College (University of Delhi)
Karampura, New Delhi-110015

CERTIFICATE

Dr. /Mr. /Ms. Rashmi Singh from Shivaji College

has participated/presented poster entitled Seroprevalence survey of Brucellosis in buffalo population of Delhi-NCR region in the symposium.

Dr. S.K. GARG
(Patron)

30th March, 2016

Dr. ANITA GULATI
(Convenor)

Brucellosis 2016
International Research Conference
17th -19th November, 2016 , New Delhi, India

Department of Biotechnology
Ministry of Science and Technology
Government of India

ICAR
Indian Council of Agricultural Research

To,

5th September, 2016

Dr. Parul Kulshreshtha
Shivaji College,
University of Delhi,
Delhi, India
E-mail: parulkuls26@gmail.com

Subject: Invitation to participate in "International Brucellosis Research Conference including the 69th Annual Brucellosis Research Meeting", New Delhi, India, November 17th to 19th, 2016.

Dear Dr. Parul Kulshreshtha,

Greetings!

On behalf of the organizing committee of "**Brucellosis 2016,International Brucellosis Research Conference including the 69th Annual Brucellosis Research Meeting"** jointly being organized by Department of Biotechnology, Ministry of Science and Technology and Indian Council of Agricultural Research**,** Department of Agricultural Research and Education, we are pleased to inform you that your abstract entitled 'A study to identify habits of cattle keepers that lead to spread of infection and the prevalence of *Brucella*-specific antibodies among the Buffalo population of Delhi' has been accepted for POSTER presentation in the conference to be held from 17-19th November, 2016 at National Agricultural Science Centre (NASC) Complex, New Delhi, India.

We have the great pleasure in inviting you to participate in the conference. The exact time and session of your presentation will be specified in the Brucellosis Conference Program online at http://www.dbtbrucellosis.in/brucellosis2016/scientific_program by second week of October, 2016.

Please consider this letter to be an official invitation to facilitate the processing of documents needed to attend the Conference. Kindly note that you will be responsible for the payment of all fees and expenses related to your participation in the Conference, as well as any necessary insurance for health, travel, etc. This invitation letter does not constitute any financial or legal obligation on behalf of the organizing committee.

Further, you are requested to kindly complete your registration. For registration, hotel booking and other information please visit our website http://www.dbtbrucellosis.in/brucellosis2016/.

We anticipate that we all will learn much from the dynamic sharing and partnering that will occur before, during and after the Conference. Please do not hesitate to contact us, if you have any queries.

Looking forward to meeting you at Brucellosis 2016 and sharing a wonderful experience together.

Best Regards,

(Dr. Padma Singh)
Office Secretary

Conference Secretariat:
Contact Details: Dr. Padma Singh,
Scientist 'D', Department of Biotechnology, Block-2,
8th Floor, CGO Complex, Lodhi Road, New Delhi, India-110 003
Ph: 011-24364447, Fax 011-24360295
Email: padma.singh@nic.in

Conference Manager:
CIMGLOBAL
Suite No. 309 - 311, DLF City Court,
MG Road Gurgaon 122 004, Haryana, India
Ph: +91 124 461 7700
Email: hitanshu@cimglobal.net

Project related photos

Performing ON SITE Rose Bengal Test

Collection of Blood, saving the blood drops on protein saver cards, student noting down survey

Distributing “pashudhan se sambandhit karya” the cattle management manual by Indian Agricultural Institute among the cattle keepers.

Final Utilization Certificate
Innovation Project 2015-16

Project Title - "A Dried Blood Spot collection study for detection of Brucellosis in Bovine Population of Indian villages: an ELISA based system specific to Omp25 and Omp28 proteins of *Brucella abortus.*"

Audited Financial Statement under Innovation Project scheme

College: Shivaji College, University of Delhi

Project Investigators: Ms. Nimita Kant, Dr. Anuradha Mal(Botany), Dr.Parul Kulshreshtha, Dr. Rashmi Singh(Zoology)

Grant Sanctioned Rs	(In figures) 5,00,000 Five lakh only	Amount utilized	Balance
Equipments/Consumables	2,25,000 +55000+20000	2,99,083/-	917/-
Travel	55,000/- t/f to E/c		
Stationery	20,000/ t/f to E/c		
Honorarium	25,000/-	25,000/-	-
Stipend	1,20,000/- (12months)	1,20,000/-	-
Contingency	55,000/-	54,292/-	708/-
Total amount utilized Rs. (In figures and words)	Rs. 4,98,375/- (Four lakh ninety eight thousand three hundred & seventy five only)		
Amount remaining Rs. (In figures and words)	Rs. 1625/- (One thousand six hundred and twenty five only)		

Certified that out of Rs. **5,00,000** (Five Lakh only) sanctioned to Innovation Project Code **SHC-308** ,**Rs. 4,98,375/-** has been utilized during the period of the project. The remaining amount Rs. **1625/-** (One thousand six hundred and twenty five only) sis being returned back to the University.

Signature of Project Investigators

Signature of Principal

04.11.16.

Financial Audit Clearance
and Stamp of Charted Accountant

VIMAL DHAWAN & ASSOCIATES
CA
M. No. 518146
NEW DELHI
Chartered Accountants

University of Delhi

RC/2015/9435 31 August, 2015

The Principal,
Shivaji College
Ring Road, Raja Garden,
New Delhi-27

Subject: - **Innovation Projects 2015-16**

Dear Principal,

The University of Delhi is pleased to announce the third round of the undergraduate research initiative in colleges, Innovation Projects 2015-16. You will be glad to know that the following project submitted by your college has been selected for award

Project Code: SHC 308
Project Title: A Dried Blood Spot Collection Study For Detection Of Brucellosis In Bovine Population Of Indian Villages: An ELISA Based System Specific To Omp 25 And Omp28 Proteins Of Brucella Abortus

The distribution of grant under different budget heads as below:

Sr. No.	Budget Head	Amount
1.	Equipment/Consumables	Rs 2,25,000/-
2.	Stipends	Rs. 1,20,000/- (1000x10x12)
3.	Travel	Rs 55,000/-
4.	Honorarium	Rs 25,000/-
5.	Stationery/Printing	Rs 20,000/
6.	Contingency	Rs 55,000/-
	Total	Rs 500,000/-
Rs 5 lakhs (Rupees five lakhs only)		
Amount to be released in first phase by Finance Branch- Rs 3,50,000/		

Budget head No. 1 and half of the remaining grant will be released as the first instalment. The second and final instalment will be released after submission of half-yearly report (by 15 February 2016), satisfactory review and recommendation of release of the second instalment.
Please refer to the detailed guidelines for implementation of the project. Any queries may be addressed to- innovationprojects1516@gmail.com.

With best wishes,

Yours sincerely,

Prof. Malashri Lal