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MICROBIOLOGY

Seroprevalence of brucellosis in buffaloes in North India

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Abstract

Brucellosis is an infectious disease characterized by abortion and infertility in several mammalian species including buffaloes, and is one of the most important zoonosis worldwide. The objective of present study was to establish the seroprevalence of brucellosis in buffaloes in North India and to know the exact causal species of *Brucella* organism. A total of 160 serum samples were analyzed. The seroprevalence of Brucellosis was 4.38% and 7.50% by Rose Bengal Plate Test (RBPT) and Standard Tube Agglutination Test (STAT), respectively. Seroprevalence was zero by both RBPT and STAT in organized dairy farm while in unorganized sector, 5.04 and 8.63% by RBPT and STAT, respectively. Age-wise seroprevalence was higher in 4.5 to 6 years old buffaloes than younger. Area-wise seroprevalence was higher in Satwari block of study area. On screening of 20 milk samples from serological positive and doubtful buffaloes by milk ring test, five samples were positive (25%). On isolation of from samples, only one sample yielded *Brucella abortus*. The results showed that younger buffaloes were less infected than adults. Prevalence of *Brucellosis* was higher in Satwari block of Jammu province compared to other study area. The presence of Brucellosis in bovine may pose a significant economic loss to the farmer and a public health hazard to the general population.

Highlights

- Brucellosis is an infectious disease characterized by abortion and infertility in several mammalian species including buffaloes.
- The seroprevalence of Brucellosis in buffaloes was 4.38 % by Rose Bengal Plate Test.
- Seroprevalence was zero in organized dairy farm while in unorganized sector occurrence of brucellosis was 8.63 % by STAT.

Keywords: Buffaloes, brucellosis, *Brucella abortus*, seroprevalence.

Brucellosis is contagious and common zoonotic bacterial disease of livestock (Munir *et al*., 2010) and continues to be of great health concern and economic importance in worldwide. Bovine brucellosis is usually caused by *Brucella abortus*, less frequently by *Brucella melitensis* and rarely by *Brucella suis*. *Brucella* is gram-negative, non-spore-forming, facultative, intracellular bacteria (Jagapur *et al*., 2013). The alternative methods involving caused by bacteria which affects both cattle and buffaloes serological tests are easy, safe, and less time and resource equally. This organism is also important causal agent of brucellosis in humans (Gul and Khan, 2007). Infection in animals frequently results in abortion and diminished milk production (Cutler *et al*., 2005). Its zoonotic importance and secretion of Brucella

organism in milk increase the interest of researchers to know the prevalence of this organism at local farms.

In India, the occurrence of brucellosis is to the extent of 10% in marginal herd and 50% in organized farms and the economic impact of this disease was estimated to be ₹ 500 Crores annually (Rajashekhar, 1995). Major economic loss due to this organism occurs because of abortion, loss of calves, reduced milk yield in females and infertility in males (WHO, 1971).

The control of bovine brucellosis is possible only by accurate diagnosis of the disease at the appropriate time. Diagnostic methods include direct tests, involving isolation of bacteria or DNA detection by polymerase chain reaction (PCR) or loop mediated isothermal amplification (LAMP) methods. Indirect test includes serological tests, which are applied *in vitro* using milk or plasma or serum and allergic test done *in vivo* in suspected animals. Serology can be used for a presumptive diagnosis of brucellosis, or to screen herds.

The isolation and characterization of Brucella organism is the gold standard confirmative diagnosis of brucellosis (Godfroid *et al*., 2010). *Brucella* spp. can be isolated on a variety of plain media, or selective media such as Farrell's medium or Thayer-Martin's modified medium. Enrichment techniques can also be used. *Brucella* colonies usually become visible after two days growth. Keeping the above facts in view, the present study was under taken to assess the status of brucellosis in buffaloes by examining serum and milk. Brucellosis causing organisms were isolated and characterized to find out the exact distribution of individual species of Brucella organism in the study area.

Materials and Methods

Chemicals, media and glassware

In the present study, properly cleaned, neutral and standard glass wares and plastic wares were used for isolation and diagnostic protocols. *Brucella* agar base, *Brucella* selective supplement, MacConkey agar (MA), Motility test medium, broth base, Brain heart infusion broth and Gram's stain were purchased from Hi-media, Mumbai, India. Kovac's reagent was prepared by dissolved 2 gm dimethylaminobenzaldehede in 30 ml isoamyl alcohol, added 100 ml concentrated hydrochloric acid, mixed well and stored at 2 to 8 ^{o}C.

The RBPT antigen, *Brucella* STAT antigen, ABR Antigen and *Brucella abortus* positive serum were obtained from Division of Biological Products, Indian Veterinary Research Institute (I.V.R.I.), Izatnagar, Uttar Pradesh, India.

Sample collection

The study was conducted in the different parts of the Jammu region of North India. A total of 160 blood samples were collected randomly from organized (n = 21) and unorganized (n = 139) buffaloes farms to detect anti-*Brucella* antibodies. Brucella organism isolation study was done on 20 milk samples from sero-positive animals.

Serum

A total of 160 serum samples were collected from buffaloes for serological testing viz., Rose Bengal Plate Test (RBPT) and Standard Tube Agglutination Test (STAT). For serum sample collection, about 9 ml of blood was collected aseptically by jugular vein puncture of individual animal using sterilized disposable syringe and later transferred to serum separation vial (vacutainer). These vials were kept handled as described by manufacturer. The separated serum was collected in a screw capped plastic vials and transported to the laboratory. The sera were stored at -20^{o}C till further use. Collected serum samples were subjected to RBPT and STAT.

Milk

A total of 10 milk samples from buffaloes showing positive or doubtful reactions in RBPT or STAT, was subjected to Milk Ring Test (MRT). For the milk collection, the udder was thoroughly washed and cleaned with potassium permanganate solution (1:1000) and dried with clean cloth. Disinfection of the teat openings was done with 70% of ethyl alcohol.

After discarding few drops of milk, approximately 10 ml of milk from each quarter was collected in sterile screw capped plastic vial and transported on the ice to the laboratory. One fraction of milk was used for MRT and rest was kept at -20ºC for future use in cultural isolation.

Diagnostic tests

Rose Bengal Plate Test (RBPT)

Serum sample and RBPT antigen were brought to the room temperature and then one drop (0.03 ml) of serum was taken on a clean, dry and non greasy glass slide by micropipette. The antigen bottle was shaken well to ensure homogenous suspension and then one drop (0.03 ml) of the antigen was added. The antigen and serum were mixed thoroughly for four min along with continuous slide rotation. The result was noted immediately after four min. Definite clumping/agglutination was considered as positive reaction, where as no clumping / agglutination was considered as negative.

Standard Tube Agglutination Test (STAT)

For STAT, each serum sample was diluted using 0.5% phenol saline. In general, a minimum of eight dilutions were used and for high titer sera, higher dilutions were prepared in order to achieve end point titer. Eight agglutination tubes were placed in a rack. 0.8 ml of 0.5% phenol saline was taken in a first tube and 0.5 ml in rest of the tubes. 0.2 ml of serum was added in the first tube, mixed well and 0.5 ml of diluted serum was transferred to the next tube. Again, mixing and transfer of 0.5 ml of diluted fraction was followed. The process was repeated up to the eighth tube. 0.5 ml of diluted serum from the last tube was discarded. 0.5 ml of homogenous antigen was added to each tube and mixed thoroughly. This provided the final dilutions of serum as 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640 and 1:1280. All the tubes were incubated at 37ºC for 20 h before result was recorded. After incubation, the contents of tubes were compared with a control, prepared by mixing 0.5 ml of antigen with 1.5 ml of 0.5% phenol saline in an agglutination tube, simulating 50% agglutination. The tube showing ≥ 50% agglutination was selected for calculating end titer. The degree of agglutination was judged by opacity of the supernatant fluid. The highest serum dilution showing 50% or more agglutination (50% clearing) was considered for calculation of titer of serum. The results were expressed in terms of International Unit (I.U.) per ml of serum by multiplying the reciprocal of serum dilution of selected tube with two. 80 I.U. per ml or above was considered positive for brucellosis.

Milk Ring Test (MRT)

The MRT was performed on milk samples of animals showing positive or doubtful reactions in RBPT or STAT. ABR Antigen and milk samples were brought to the room temperature prior to performing the test. About 30-50 µl of antigen was added to the 2 ml of milk in a narrow test tube and mixed thoroughly. The tubes were incubated at 37°C for 1 h together with positive and negative working standards. A strongly positive reaction was indicated by formation of dark pink ring above a white milk column. The test was considered to be negative if the pink colour of milk column underlying the ring exceeds that of the cream layer/ring. (Plate3.2)

Isolation and identification of *Brucella abortus* from bovine milk

For isolation and identification of *Brucella abortus* from buffaloes milk, the standard procedures (Alton *et al.,* 1988; Quinn *et al.,* 1994; OIE, 2004) were followed. Milk samples were centrifuged at 6000-7000 rpm for 15 min and about 100 µl of milk pellet and creamy layer were inoculated separately on *Brucella* agar medium (BAM) plates(Himedia). The plates were incubated at 37ºC for minimum 15 days under 10% CO_2 tension. The plates were observed at every 24 h for bacterial growth. The suspected colonies of *Brucella* were picked up and transferred to another BAM plates and incubated under 10% CO_2 tension to obtain pure culture. The isolates so obtained were streaked on plates of MacConkey agar. The isolates Non-lactose fermenting colonies on MA were suspected *Brucella* isolates. The isolates suspected for *Brucella* were subjected to Gram staining for checking the purity of cultures and morphological characters.

The isolates suspected to be of Brucella were subjected to agglutination and biochemical tests such as motility, acriflavin test, indole, oxidase, catalase, urease, nitrate reduction and H_2S production. Rapid slide agglutination test was performed using one drop (0.03 ml) of Brucella abortus positive serum, procured from Division of Biological Products, IVRI, Izatnagar, was taken on a glass slide by micropipette. The loopful culture from single suspected colony was mixed thoroughly with the spreader and the slide was rotated for four min. Definite clumping/ agglutination was considered as positive reaction indicating Brucella isolate, where as no clumping/ agglutination was considered as negative.

Seroprevalence study

Seroprevalence study was conducted on the basis of organized and unorganized farms, age-wise and region-wise. For age-wise prevalence, animals were classified into different age groups such as 1.5 to 3, 3 to 4.5 and 4.5 to 6 years. Different parts (Satwari, Akhnoor, Bhalwal and Dansal) of Jammu region were included in the present study.

Results and Discussion

In the present study, to determine the magnitude of occurrence of brucellosis in buffaloes, the serological test viz. RBPT and STAT were carried out. MRT to detect anti-Brucella antibodies in the milk, and Brucella organism was isolated from milk of animals showing serological positive or doubtful reactions was also done.

Seroprevalence of brucellosis

In the present study the seroprevalence in buffaloes of Jammu was assessed by RBPT and STAT. Out of the 160 serum samples tested, 7 (4.38%) samples were RBPT positive and 12 (7.50%) samples were STAT positive. The results of the present study showed that 7 (4.38%) samples were positive by both RBPT and STAT. For overall prevalence of brucellosis in buffaloes only true positive (positive for both RBPT and STAT) were considered and recorded overall seroprevalence as 4.38%.

Seroprevalence on the basis of organized and unorganized farms, our study recorded zero seroprevalence of brucellosis by both RBPT and STAT in organized dairy farm while in unorganized sector, 3.62% and 6.16% seroprevalence by RBPT and STAT, respectively, was recorded.

Age-wise seroprevalence study found that buffaloes in age group of 4.5 to 6 years had highest (12.50% and 17.50%) seroprevalence of Brucellosis by RBPT and STAT respectively (Table 1).

Table 1. Age-wise prevalence in large ruminants as detected by RBPT and STAT.

Species	Age (Years)	No of samples collected	Samples Positive (%)	
			RBPT	STAT
Buffaloes	1.5 to 3	35	0 (0)	1 (2.86)
	3 to 4.5	85	2 (2.35)	4 (4.71)
	4.5 to 6.0	40	5 (12.50)	7 (17.50)

In our study area wise seroprevelance of brucellosis in cattle revealed more seroprevalence in Satwari block (6%) followed by Akhnoor, whereas no sample was positive from rest of 2 blocks by RBPT, while STAT showed 10% occurrence in Satwari block and followed by Akhnoor. The detail results are presented in Table 2.

Table 2. Area-wise prevalence in large ruminants as detected by RBPT and STAT.

Area	Samples collected	Samples positive (%)	
		RBPT	STAT
Satwari	100	6 (6.00)	10 (10.00)
Akhnoor	30	1 (3.33)	2 (6.66)
Bhalwal	18	0 (0.00)	0 (0.00)
Dansal	12	0 (0.00)	0 (0.00)

Milk ring test on bovine milk samples

Total twenty milk samples were collected from buffaloes which were positive or doubtful in serological studies were analyzed for detection of anti-*Brucella* antibodies using MRT. Out of 20 milk samples of buffaloes, 5 (25%) were positive.

Isolation and characterization of *Brucella* spp. from buffaloes milk

Total ten milk samples subjected to MRT were also processed for isolation of *Brucella abortus,* for which *Brucella* agar medium (BAM) was used as a primary culture medium. From the milk samples, milk and creamy layer separately, were inoculated on BAM under 10% CO_2 tension conditions for 15 days. The round, glistening and smooth colonies (Figure 1) on of BAM were further streaked on MacConkey agar (MA). The non-lactose fermenting isolates on MA plates were presumed to be that of *Brucella*. Of the 20 milk samples processed, only one (5%) presumptive isolate of *Brucella* was obtained.

The isolates presumptive to be of *Brucella* were subjected to Gram staining and were found to be Gram negative, coccobacillary rods (Figure 2A). All the isolates were found positive by Rapid Slide Agglutination Test (Figure 2B).

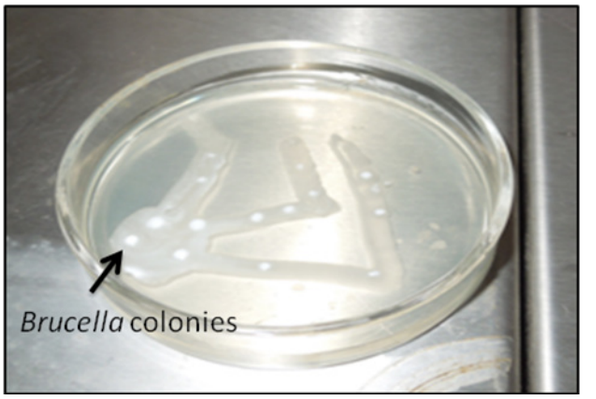

Figure 1. Brucella colonies on Brucella agar medium plate. Brucella colonies are smooth, round and glistening.

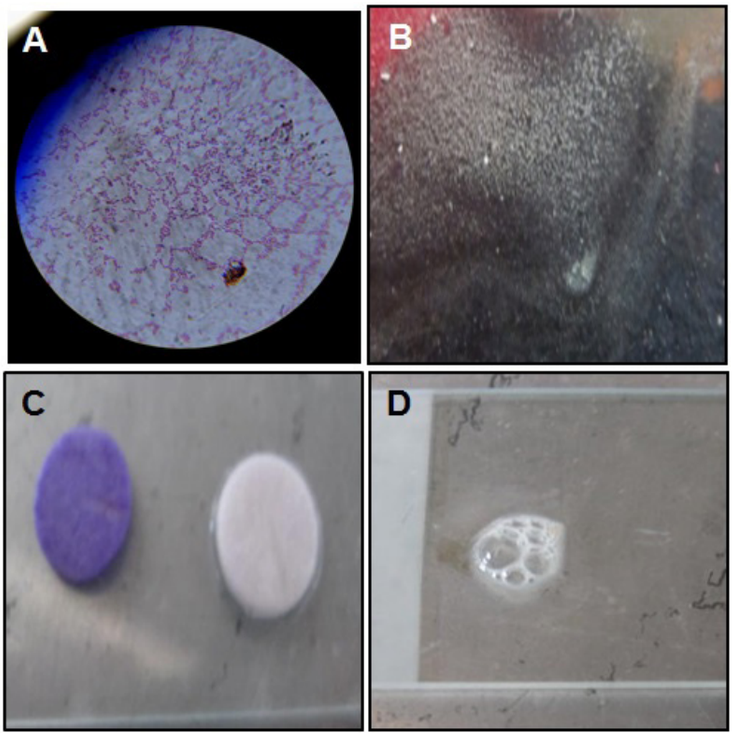

Figure 2. Characterization of isolated Brucella from suspected buffaloes milk samples. [A] Colonies subjected for Grams stain and smear showed Gram negative coccobacillary rods; [B] Rapid slide agglutination test showed strong clumping or agglutination reaction positive for Brucella; [C] Oxidase test is clearly showing the positive reaction in the left as blue color spot and white as negative reaction; [D] Showing positive catalase test with production of bubbles on addition of H2O2.

The isolates were further identified by biochemical tests and it was observed that the isolates were positive for Oxidase (Figure 2D), catalase (Figure 2D) and urease whereas negative for indole test. Further, the isolates produced H_2S in the MSM and reduced nitrate to nitrite. Acriflavine test revealed that both the isolates were smooth forms of the organism On the basis of morphological, biochemical tests and Rapid Slide Agglutination Test , the isolates were confirmed to be that of *Brucella abortus.* .

The present study was undertaken in the Jammu region to study the occurrence of brucellosis in buffaloes of Jammu region of North India. The present study revealed high seroprevalence of Brucellosis by both RBPT and STAT in the study area. Similar higher seroprevalence was shown by Prahlad *et al*. (1999) found 7.09%, 2.70%, 11.14% and 8.10% seropositivity by RBPT, STAT, CFT and dot-ELISA, respectively, in buffaloes. Ali *et al*. (2013) have also been reports the similar results of seroprevalence (6.6%) in buffaloeses. Rahman *et al*. (2011) have been reported low prevalence (2.87%) of Brucellosis in buffaloes by RBPT. The higher seroprevalence in the present study shown by STAT might be due to the possibility of cross-reactions of *Brucella* antibodies with those against other gram-negative bacteria, such as *Vibrio cholerae*, *Yersinia enterocolitica* serotype 09, *Francisella tularensis* and *Vibrio cholera*. Although STAT is the most commonly used test which detects antibodies and is considered as the quantitative test for brucellosis. But it shows high false positive, results due to cross reactions, and is not a prescribed or alternate test by OIE (OIE, 2004). RBPT is a simple spot agglutination test, using antigen stained with Rose Bengal and buffered to low pH, usually 3.65. RBPT is very sensitive, but like all other serological tests, it can give false positive result because of S19 vaccination or either cross – reactions. However cross-reactions are lower as compared to STAT amid acidic pH.

For the epidemiological studies samples were collected age wise and area wise. Serum samples from buffaloes were taken from three different age groups 1.5 to 3 years, 3 to 4.5 years and 5 to 6.5 years. The higher prevalence of the brucellosis in aged animals has been reported by various workers (Chakarborty *et al*., 2000; Kubuafor, 2000). A recent study has been reported the significant association between age group and the prevalence of brucellosis in buffaloes (Rahman *et al.*, 2011). Nevertheless, the susceptibility to brucellosis appears to be more commonly associated with sexual maturity than age which is known to be due to presence of sugar named 'erthyritol' in genital organs of adults (Radostits *et al*., 2000).

Further the epidemiological study on the basis of organized and unorganized farms, recorded zero seroprevalence of brucellosis by, both, RBPT and STAT in organized sector while in unorganized sector, the values were 8.63 and 5.04% by STAT and RBPT. The higher prevalence dairy farms was recorded by Nasir *et al.* (2004), who performed seroprevalence of brucellosis using RBPT and STAT in 481 and 223 buffaloes, respectively and found 15.38% prevalence by RBPT. The zero seroprevalence in organized dairy farm in present study might be due to regular screening of the herd on regular basis, hygienic sheds, proper disposal of animals waste and semen used in artificial insemination from brucellosis screened bulls, as the organized farm was the government owned.

From the seropositive animals and few doubtful reactors milk was collected. These milk samples were used for detecting antibodies in milk by using MRT and for isolation of the *Brucella abortus.* In the present study, a total 20 milk samples were tested by MRT and recorded 5 (25%) positive samples. The results were found almost similar as in case of work done by Mahato *et al.* (2004) to detect *Brucella* antibody in individual milk samples of 67 cows by using MRT and found 24 (35.82%) samples as positive. Thus in their study, slight higher positivity could be due to false-positive reactions which may occur in cattle vaccinated less than 4 months prior to testing, recent parturition, end of lactation and due to sub-clinical mastitis (Alton *et al.*, 1988) or in samples containing abnormal milk (such as colostrum). Therefore due to some of these reasons, it is not recommended to use

this test in very small farms where these problems may have a greater impact on the test results.

The milk samples were further used for the isolation of the *Brucella abortus*, which was done as per the standard procedure (OIE, 2004). In the present study out of 20 milk samples only 1 (5%) samples yielded recovery of *Brucella* in milk. It has been reported that animals showing antibodies both in serum as well as in milk may not reveal the presence of *Brucella* in milk by cultural isolation. This might be due to the chronic disease or intermittent shedding of *Brucella* in milk (Corbel, 1988). Similar results were shown by Chatterjee *et al.* (1995), who revealed 6.2% of isolation rate from milk, vaginal swab, hygroma fluid and semen samples of 177 cows and bulls having *Brucella* agglutinins at positive diagnostic level (80 IU/ml).

Whereas, Kaur *et al.* (2006) isolated *Brucella* from vaginal mucus, foetal membranes and foetal stomach content of aborted cattle and buffaloes which were RBPT and STAT positive. They were also able to isolate *B. abortus* from RBPT and STAT negative animals and concluded that the isolation method was most sensitive in comparison to RBPT and STAT.

References

Ali, S., Ali, Q., and Akhter, S. 2013. Sero-prevalence of *Brucella abortus* Among Dairy Cattle and Buffaloes in Pothohar Plateau, Pakistan. *Pakistan Journal of Zoology* **45(4)**: 1041-1046.

Alton, G., Jones, L. M., Angus, R. D., and Verger, J. M. 1988. *Techniques for the Brucellosis Laboratory*. INRA, Paris.

Chakraborty, M., Patgiri, G. P. and Sarma, D. K. 2000. Use of Rose Bengal Plate Test, Serum Agglutination Test and Indirect-ELISA for detecting brucellosis in bovines. *Indian Journal of Comparative Microbiology Immunology and Infectious Diseases* **21**: 24-25.

Chatterjee, A., Mondal, P., De, B. N., and Sen, G. P. 1995. Cultural isolation of *Brucella* in relation to serum agglutination level. *Indian Veterinary Journal* **72:** 211-215.

Corbel, M. J. 1988. Brucellosis. In: Laing, J. A. (ed). *Fertility and Infertility in Veterinary Practice* 4th Edition. ELBS, Bailliere Tindall, pp. 189-221.

Cutler S. J., Whatmore, A. M., and Commander, N.J. 2005. A review: Brucellosis - new aspects of an old disease. *Journal of Applied Microbiology* **98**:1270-1281.

Godfroid, J., Nielsen, K., and Saegerman, C. 2010. Diagnosis of Brucellosis in Livestock and Wildlife. *Croatian Medical Journal* **51**: 296-305.

Gul, S. T., and Khan, A. 2007. Epidemiology and epizootology of brucellosis: A review. *Pakistan Veterinary Journal* **27**: 145-151.

Jagapur R. V., Rathore R., Karthik K., and Somavanshi R. 2013. Seroprevalence studies of bovine brucellosis using indirect-enzyme-linked immunosorbent assay (i-ELISA) at organized and unorganized farms in three different states of India. *Veterinary World* **6(8)**: 550-553.

Kaur, P., Sharma, N. S., Jand, S. K., and Oberoi, M. S. 2006. Isolation and identification of *Brucella abortus* from aborted cattle and buffaloes and evaluation of their antibiogram. *Indian Journal of Animal Research* **76:** 105-108.

Kubuafor,D.K., Awumbila, B., Akanmori,B.D., Williams,D.J.L. (ed) Akanmori, B.D. (ed); and Wasling; J.M.(2000). Seroprevalence of brucellosis in cattle and lumanin the Akwapim south district of Ghana: Public health implication. Special issue: Tropical Zoonoses in West Africa. *Acta Tropica* **76(1**): 45-48.

Mahato, G., Sharma, K., and Mahanta, P. N. 2004. Comparative evaluation of serological tests for detection of Brucellosis in bovine. *Indian Journal of Veterinary Medicine* **24:** 46.

Munir, R. M., Afzal, M., Hussain, S.M.S., Naqvi, and A Khanum, 2010. Outer membrane proteins of B. abortus vaccinal and field strains and their immune response in buffaloes. *Pakistan Veterinary Journal* **30**: 110-114.

Nasir, A. A., Parveen, Z., Shah, M. A. and Rashid, M. 2004. Seroprevalence of brucellosis in animals at Government and private livestock farms in Punjab. *Pakistan Veterinary Journal* **24:** 144-146.

OIE (World Organisation for Animal Health) 2004. Bovine brucellosis, Section 2.3. In: OIE Manual of standards for diagnostic tests and vaccines, 5th Ed. OIE, Paris.

Prahlad, K., Singh, D. K. and Barbuddhe, S. B. 1999. Sero-prevalence of brucellosis and comparison of serological tests to diagnose in buffaloes. *Buffalo Journal* **15**: 361-370.

Quinn, P. J., Carter, M. E., Markey, B., and Carter, G. R. 1994. Clinical Veterinary Microbiology Wolfe publisher, England.

Radostitis, D. M., Blood, D. C. and Gay, C. C. 2000. Veterinary Medicine: A text book of the diseases of cattle, sheep, pigs, goats and horses, 10th ed. Bailliere Tindall, Great Britian.

Rahman M. S., Faruk M. O., Her M., Kim J. Y., Kang S. I., and Jung S. C. 2011. Prevalence of brucellosis in ruminants in Bangladesh. *Veterinarni Medicina* **56(8)**: 379–385.

Rajasekhar, M. 1995. Bovine brucellosis progressive control programme concept paper. *Animal disease monitoring and surveillance project Indian.* Council of Agriculture Research. Banglore.

WHO, 1971. Technical Report Series, Joint FAO/WHO Expert Committee in Brucellosis, 5th report: No. 464.