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Comparison of diagnostic tests for the detection of bovine brucellosis in the natural cases of abortion

Mahajan, V.[1*]; Banga, H. S.[2]; Filia, G.[1]; Gupta, M. P.[3] and Gupta, K.[2]

[1]Department of Animal Disease Research Centre, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana-141004, Punjab, India; [2]Department of Veterinary Pathology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana-141004, Punjab, India; [3]Department of Veterinary and Animal Husbandry Extension, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana-141004, Punjab, India

[*]***Correspondence:*** *V. Mahajan, Department of Animal Disease Research Centre, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana-141004, Punjab, India. E-mail: mahajanv17@gmail.com*

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Summary

Rapid and precise diagnosis in natural field cases of bovine abortion caused by *Brucella abortus* warrants the use of the most sensitive and reliable diagnostic methods. In the present study, bacterial isolation, serology, gross, histopathology, immunohistochemistry and polymerase chain reaction technique(s) were applied. Sero-prevalence studies showed the rate of 28.86% positive cases using the competitive ELISA. Histopathological changes were mainly seen in the placenta, fetal lungs, kidney, liver and spleen. Immunohistochemical (IHC) staining of *Brucella* spp. was evident as brown, finely granular intracytoplasmic staining in trophoblasts of placental sections and in section(s) of liver, lung, kidney and spleen. Twenty-eight out of the 103 samples (17 from stomach contents, 3 from placental cotyledons, 2 from vaginal discharges and 6 from pooled fetal tissues) produced 193 bp amplicon specific for *Brucella* genus. Moreover, the species-specific primers amplified a 498 bp amplicon which corresponded to *B. abortus*. Comparison of diagnostic tests revealed PCR and IHC provide a reliable test for the diagnosis of bovine brucellosis in aborted fetal tissue and placental cotyledons whereas serology is most important for detection of *Brucella* positive animals in a herd.

Key words: Abortion, Brucellosis, Immunohistochemistry, Polymerase chain reaction

Introduction

Brucellosis is a highly contagious, zoonotic and economically important bacterial disease of bovines that has been incriminated in causation of abortion(s), retention of placenta, stillbirth, infertility and increasing calving intervals (Singh *et al*., 2002). In India, brucellosis was first recognized in 1942 by Polding and is now endemic throughout the country. Rapid, definitive and accurate diagnosis of brucellosis is very important for a positive outcome of eradication programmes (Surucuoglu *et al*., 2009). There are various ancillary tests employed for the diagnosis of brucellosis including culture, serological, immunopathological and molecular methods. Of these, culture method has high specificity but is time consuming and requires laboratoryfacilities with an appropriate degree of bio-safety (Poester *et al*., 2005). Various serological tests are employed for diagnosis with varying degree of sensitivity and specificity. However, cross-reactions between *Brucella* species and other Gram-negative bacteria are a major problem of the serological assays (Muñoz *et al*., 2005). Immunohistochemical techniques have been used for detection of *Brucella* organisms in formalin-fixed, paraffin-embedded tissues of aborted fetuses (Pérez *et al*., 1998) and a complementary tool in suspected cases of brucellosis with negative bacteriologic culture or when serology is not possible or material fixed in formalin. PCR has been increasingly used as a diagnostic tool for etiologic diagnosis of abortion in cows either as a supplementary or as a replacement of time consuming traditional diagnostic methods (Anderson, 2007). There is no single test by which a bacterium can be identified as *Brucella*. A combination of growth characteristics, bacteriological, serological, IHC and molecular methods is usually required. The aim of the present study was to compare bacterial culture, cELISA, IHC and PCR as diagnostic techniques for brucellosis in natural field cases of bovine abortion.

Materials and Methods

Selection of farms and animals

In present study, animal farms (n=28) having history of abortion were selected from different agro climatic zones *viz*. sub-mountain region (annual rainfall 800-900 mm), central plain region (annual rainfall 500-800 mm) and arid-irrigated region (<400 mm) of Punjab (Fig. 1) by using 'Random Animal' program of the survey toolbox software (Cameron, 1999). Total bovine population of these selected farms was 1830, out of which 176 animals (approximately 10%) belonging to various livestock owners were randomly selected. None of the selected animals were vaccinated with *Brucella* calf hood cotton strain 19 vaccine.

Sero-prevalence studies

Blood samples (5 ml) were collected from the

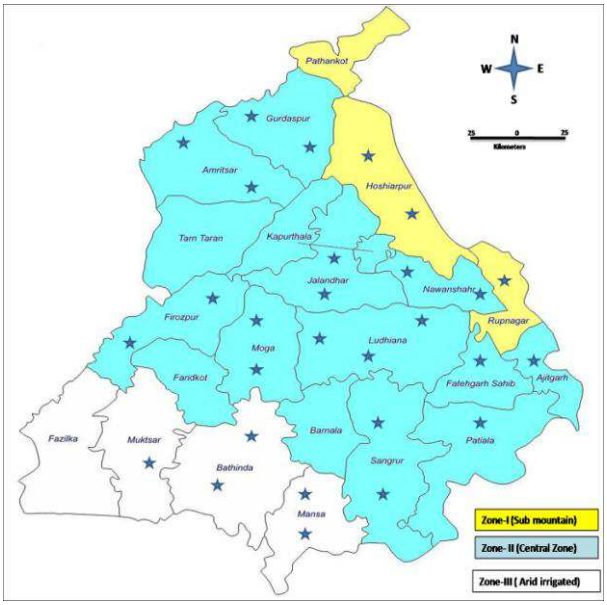

Fig. 1: Agro-ecological zones of Punjab

jugular vein; the sera were separated and stored at -20°C until they were tested for antibodies to *Brucella* by Rose Bengal Plate Test (RBPT) and Competitive ELISA kit (Svanovir, Uppsala, Sweden) as per the manufacturers guidelines. The optical density (OD) of the controls and the test samples at 450 nm was measured in an ELISA reader.

The percent inhibition (PI) of sample was calculated as given below:

$$PI = \frac{100 - (OD\ value\ of\ the\ sample/control \times 100)}{OD\ of\ conjugate\ control}$$

Test samples with≥30 PI were considered as positive and samples with PI <30 were considered as negative.

The data was analyzed using survey toolbox and SPSS (statistical package for social sciences) for Window version 11.0.1 (SPSS Inc., Chicago, Illinois, USA).

Bacterial isolation

For bacterial isolation, seventy-six samples comprised of stomach contents (n=46), vaginal discharge(s) (n=14) and placental cotyledons (n=16) samples from aborted bovines were streaked on selective medium consisting of *Brucella* agar base containing 5% horse serum, *Brucella* selective supplement (1%). The inoculated plates were incubated at 37°C under 5% CO_2 tension in the dessicator for 10-12 days. The isolates suspected to be of *Brucella* were subjected to rapid slide agglutination and biochemical tests (Oxidase test, Catalase test, Nitrate reduction).

Pathological studies

For pathological studies, tissue sample from aborted fetuses (n=27) and placental cotyledons (n=16) were collected in 10% neutral buffered formalin for histopathology and immunohistochemistry for fixation. The paraffin sections were stained with routine hematoxylin and eosin technique (Luna, 1968).

For immune-histochemical studies 4-5 µ thick paraffin-embedded tissue sections were cut and mounted on Superfrost Plus, positively charged microscopic slides (Fisher Scientific, USA). Immunohistochemical staining was performed by using advanced SS^{TM} One-Step Polymer-HRP IHC Detection System (BioGenex Laboratories Inc., San Ramon, California, USA) as per manufacturer's instructions. A commercially available monoclonal antibody against *Brucella abortus* (ABD serotac) was used in a dilution of 1:50. As negative control, sections were incubated with PBS instead of the primary antibody.

Molecular studies

One hundred and three samples comprised of stomach contents (n=46), placental cotyledons (n=16), pooled tissue samples (n=27) including lungs, heart, liver, kidneys, spleen, brain of aborted fetuses and the vaginal mucus/uterine discharges (n=14) of aborted animals were collected and stored at -20°C until further use. DNA extraction from frozen tissues samples was performed using a commercial kit (HiPura mammalian genomic miniprep purification spin kit Himedia) following the manufacturer's instructions with slight modifications. For extraction of DNA from pure culture boiling and snap chilling method was used. Samples were processed for genus specific PCR for *Brucella* and those found positive were then processed for species-specific PCR for *B. abortus*. All the reactions were performed in a master cycle gradient thermocycler (Hybaid, Touch Down) with a preheated lid. *Brucella abortus* strain obtained from the Division of Standardization, IVRI, Izatnagar was used as the standard in the molecular diagnosis. For genus specific PCR the primers JPF-GCG CTC AGG CTG CCG ACG CAA and JPR-CCA GCC ATT GCG GTC GGT A encoding for an outer membrane protein (*omp2*) designed previously by Leal-Klevezas *et al*. (1995) were used in the study. For species differentiation of positive samples the method described by Bricker and Halling (1994) was used. The three primer cocktail (*B. abortus* specific-GAC GAA CGG AAT TTT TCC AAT CCC, *B. meltensis* specific-ACC AGC CAT TGC GGT CGG TA, IS711-TGC CGA TCA CTT AAG GGC CTT CAT) were used to differentiate *Brucella* species DNA into *B. abortus* and *B. melitensis*. The amplified product was analysed on 1.5% agarose gel (50 min at 70 V) by electrophoresis and visualized by using gel documentation system (Bio Rad, USA).

Results

Sero-prevalence studies

Serum samples from the randomly selected animals were analyzed by RBPT and cELISA which revealed an overall apparent prevalence of brucellosis to be 31.81 and 29.00%, respectively. Given the sensitivity and specificity of the cELISA at 98 and 99%, respectively,

true prevalence was calculated to be 28.86%. Of the total 176 animals tested, percentage of sero-prevalence in sub-mountain zone (zone I), central zone (zone II) and arid-irrigated zone (zone III) was found to be 36.84, 32.11 and 24.14%, respectively when tested by RBPT and 23.68, 33.94 and 17.24%, respectively by cELISA.

Isolation studies

Of the 76 samples processed, only five samples (3 from stomach contents, 1 from vaginal discharge(s) and 1 from placental cotyledon(s) yielded the round, glistening and smooth or mucoid colonies on plates presumed to be of *Brucella* spp. All the isolates were found positive by rapid slide agglutination test. The isolates were further identified by biochemical tests. Oxidase, catalase, urease and hydrogen sulphide (H_2S) were produced by all the isolates but none of the isolates produced indole and all were found non-motile. All the isolates were found to reduce nitrate.

Pathological and immunohistochemical studies

In the present study, most of the abortion(s) occurred between the seventh and ninth months of gestation with average gestational age of eight months. Gross lesions in aborted fetuses varied and the most important gross lesion recorded were: focal to diffuse pneumonic areas on lungs (Fig. 2), edematous subcutaneous tissue(s), excess of red tinged fluid in the thoracic cavity, serosanguinous fluid in abdominal cavities, spleenomegaly and hemorrhagic areas on spleen. The normal abomasal content of a fetus was clear, translucent, thick and viscid whereas the abomasum content of fetus aborted due to brucellosis suspected cases contained a whitish cloudy yellow fluid with suspended flecks of fibrin. Examination of placenta revealed hemorrhagic necrotic cotyledons with diffused hemorrhagic inter-cotyledonary areas (Fig. 3).

Histopathological changes were mainly seen in the placenta, fetal lungs, kidney, liver and spleen. Lesions in the placentas were characterized by edema of the chorionic stroma and loss of trophoblastic epithelial cells accompanied by infiltration of lympho-mononuclear cell and neutrophils besides presence of bacterial colonies in trophoblastic epithelial cells. The lesion in lungs composed of multifocal area of bronchitis and bronchopneumonia with predominantly lympho-mononuclear cell infiltration and occasional neutrophils (Fig. 4). Alveolar septa was thickened, edematous and infiltrated with lympho-mononuclear cells. In kidneys: focal interstitial lympho-mononuclear accumulations were present in the renal cortex and cortico-medullar junction along with enlarged degenerated tubules and widespread hemorrhage(s). In spleen there was depletion of the white pulp (lymphoid) and focal mononuclear infiltration of the red pulp. In some of the aborted fetuse(s), lesion in cornea due to brucellosis was characterized by loss of epithelium and subepithelial infiltration of lympho-mononuclear cell in connective tissue stroma of eye. Immunohistochemical staining of placental sections demonstrated numerous labelled bacteria in trophoblasts (Fig. 5) and positive immunostaining was present in section(s) of liver, lung (Fig. 6), kidney, spleen and appeared as brown, finely granular intracytoplasmic staining.

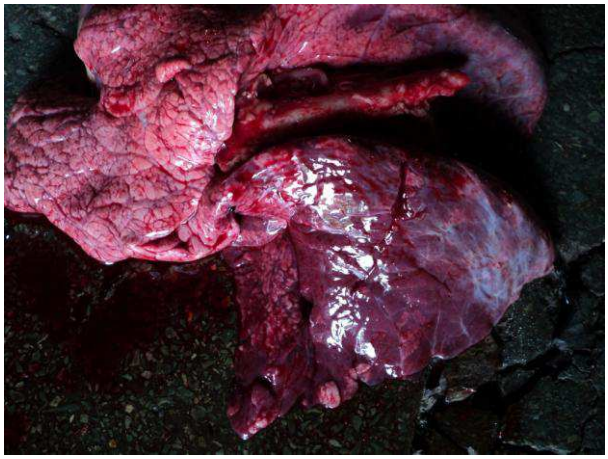

Fig. 2: Diffused pneumonic area on lung

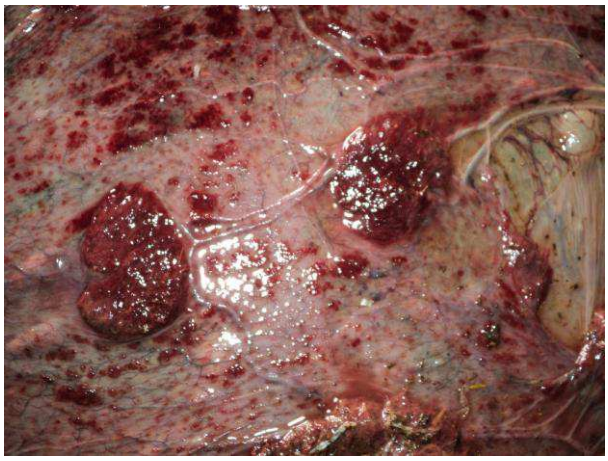

Fig. 3: Necrotic and hemorrhagic cotyledons with diffused hemorrhagic inter-cotyledonary area

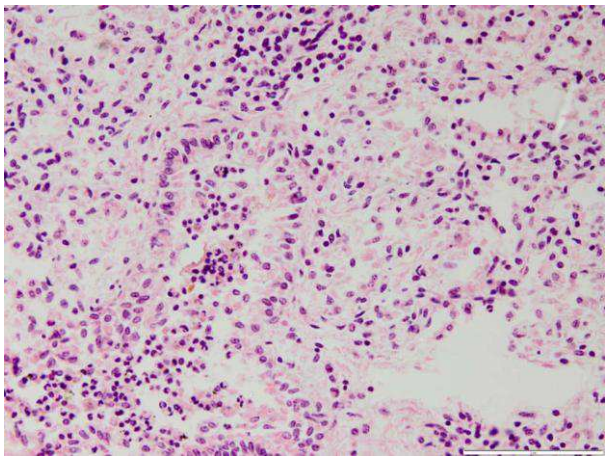

Fig. 4: Lung-bronchopneumonia with mixed cell infiltration (H&E, ×40)

Molecular studies

JPF/JPR primer pair amplified a 193 bp region of the

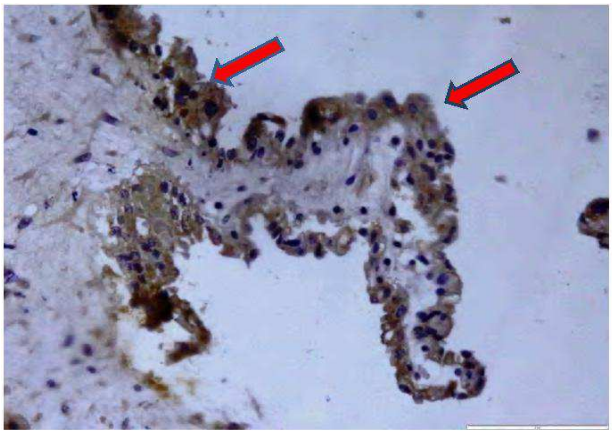

Fig. 5: Placenta- Immunohistochemical staining showing numerous labelled bacteria evident as brown, finely granular intracytoplasmic staining in trophoblasts, (×100)

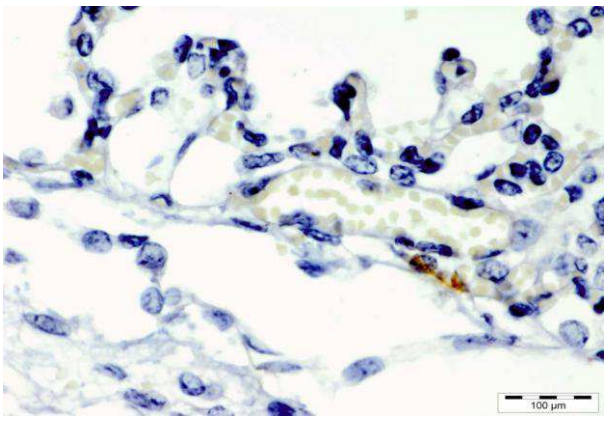

Fig. 6: Lung-immunoreactivity to the anti-*Brucella abortus* monoclonal antibody (×100)

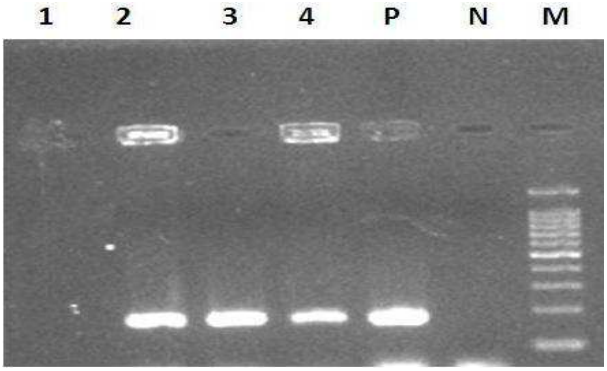

Fig. 7: *Brucella* genus species PCR from aborted material. Lane M: Molecular weight marker (100 bp). Lanes P and N: Positive and negative controls, and Lanes 1-4: Samples (2, 3 and 4 tested positive for *Brucella* spp. at 193 bp)

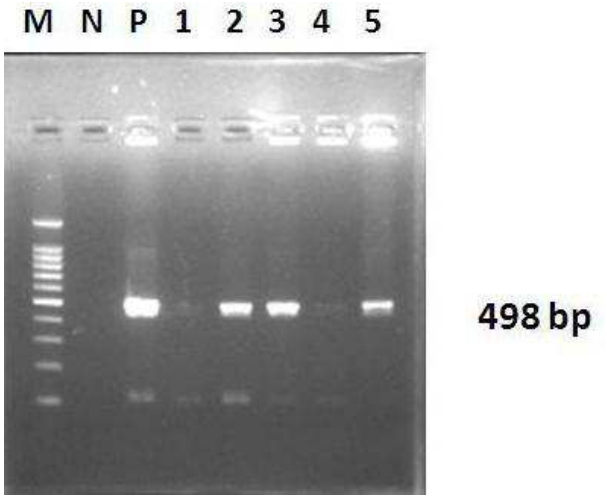

Fig. 8: *Brucella abortus* PCR from aborted material. Lane M: Molecular weight marker (100 bp). Lanes N and P: Negative control and positive control, and Lanes 1-5: Samples (2, 3 and 5 tested positive for *B. abortus* at 498 bp)

sequence encoding an outer membrane protein (*omp2*). Reference strains as well as 28 of the 103 samples (17 from stomach contents, 3 from placental cotyledons, 2 from vaginal discharges, and 6 from pooled tissue(s)) produced 193 bp amplicon (Fig. 7). The species-specific PCR was performed on these *Brucella* genus specific amplified samples. The species-specific primers amplified a 498 bp amplicon (Fig. 8) which corresponded to *B. abortus*.

Comparison of diagnostic tests

The comparisons between diagnostic tests are presented in Tables 1 and 2. Out of 46 samples of stomach content, 16 samples of placental cotyledon, 27 pooled fetal tissue samples and 14 samples of vaginal discharges, bacterial isolation was possible in 5 cases and PCR detected 28 positive cases. In the same 16 placental cotyledons and 27 pooled fetal tissue samples, IHC detected eight and PCR detected nine positive cases. Serum samples were collected from 40 animals from which either stomach content, vaginal discharge, placental cotyledons or fetal tissues were collected. For these 40 animals, 16 animals were positive for brucellosis by cELISA, bacterial isolation was successful in two cases (one sample of stomach content and one of vaginal discharge), IHC was positive in five cases (two samples of placental cotyledons and three fetal tissue samples) and PCR was positive in nine cases (four samples of stomach content, one sample of vaginal discharge, two placental cotyledons and two fetal tissue samples).

Table 1: Comparison of diagnostic tests

Samples	Bacterial isolation	Immunohistochemistry	PCR
Stomach contents (46)	3	Not performed	17
Vaginal discharges (14)	1	Not performed	2
Placental cotyledons (16)	1	3	3
Fetal tissue (27)	Not performed	5	6
Total (103)	5/76 (6.56%)	8/43 (18.6%)	28/103 (27.1%)

Table 2: Comparison of serological tests (RBPT and cELISA)

Serological tests		RBPT		Total
		+ve	-ve	
cELISA	+ve	45	6	51
	-ve	11	114	125
Total		56	120	176

Kappa= 0.772 (95% CI= 0.637-0.858), relative sensitivity= 80%, and relative specificity= 95%

Discussion

Serology is a standard method for the epidemiological surveillance of brucellosis (Leuenberger *et al*., 2007). However, many authors have emphasized the importance of using more than one type of diagnostic technique(s) for the detection of *Brucella* positive animals, especially for epidemiological purposes (Nielsen, 2002; Gall and Nielsen, 2004; Ilhan *et al*., 2008; Geresu and Kassa, 2016; Zamri-Saad and Kamarudin, 2016). In the present study, RBPT and cELISA were used for serological diagnosis of brucellosis which revealed overall prevalence of brucellosis to be 31.81 and 29.00%, respectively. Substantial degree of agreement (kappa= 0.772, 95% CI= 0.637-0.858) was found between two tests (Table 2) and relative sensitivity and specificity of RBPT to cELISA was 80 and 95%, respectively. Even though, the two tests showed degree of agreement; however the variation in prevalence by the two tests could be due to false positive. Rose Bengal Plate Test has been described as a highly sensitive but not specific test, while the cELISA is both specific and sensitive test (Paweska *et al*., 2002; Chand and Sharma, 2004; Agrawal *et al*., 2007) and can eliminate cross-reaction due to heterogeneous bacteria and can minimize false positive animals. Earlier studies have also estimated the disease status in the state which varied from as low as 7.54% to as high as 18.07% (Sandhu *et al*., 2001; Sharma *et al*., 2007). In the present study, animals were selected from farms having history of abortion which might be responsible for higher sero-prevalence of brucellosis. The percent prevalence of brucellosis in central zone was non-significantly higher (Chi-square= 0.850, P=0.654) as compared to the other zones. The central zone is the most fertile and large-sized dairy farms are reported here, resulting in maximum sales and purchase of animals in this zone of the state. Moreover, cow slaughter is banned in the state, hence the dairy farmers mostly sell *Brucella* positive animals in cow markets and the disease gets transmitted to another farm(s). Furthermore, facilities of screening the animals against brucellosis prior to purchase are not available at doorsteps. Consequently the introduction of a single infected animal in the herd can cause havoc by causing abortion storm in the herd. Earlier studies have also reported higher prevalence of the brucellosis in central zone (Aulakh *et al*., 2008). Maximum percentage of abortion was found in 6-9 months (22/31) (70.97%) of gestation. There was a significant association (Chi-square= 32.560, P=0.000) of occurrence of brucellosis with stage of abortion in third trimester. The higher incidence of abortion in third trimester may be due to increased vulnerability of uterus due to conducive uterine environment and the presence of erythritol for the multiplication of the bacteria (Anderson, 2007). The bacteria during the third trimester causes fetal death and abortion by invading placental cotyledons and destroying villi.

The gold standard diagnostic technique(s) continues to be based on isolation of suspicious bacterial colonies from aborted fetal stomach contents, fetal tissues and vaginal exudates, followed by bacteriological characterization (Alton *et al*., 1975). Moreover, isolation of organisms is tedious, cumbersome, time consuming and also health hazardous to the laboratory workers and the results are not always definitive (Bricker, 2002), thus it is generally not being followed as a routine diagnostic procedure.

Immunohistochemical techniques have been utilized to detect the location of *Brucella* organisms in formalin-fixed, paraffin-embedded tissues of cows (Meador *et al*., 1989). Pneumonia has been described as the most common lesion in fetuses aborted due to *B. abortus* infection (López *et al*., 1984). In the present study, *B. abortus* positive aborted bovine fetuses developed histopathologic changes similar to those in experimental and natural infections in cows (López *et al*., 1984; Pérez *et al*., 1998). The intracellular localization of *Brucella* in the placental trophoblast has been reported in the placenta of bovine (Meador and Deyoe, 1989) similar to the present study. Trophoblasts are thought to be the primary target cell for invasion and multiplication of *B. abortus* in the placenta. This tropism may be due to the presence of erythritol, or due to hormone synthesis by trophoblastic cells (Samartino and Enright, 1993). In the present study, the fetal kidney, lung and spleen contained cells with low numbers of organisms immunoreactive for *B. abortus* as compared to placenta which was consistent with other natural infections (Pérez *et al*., 1998). Thus, IHC examination of paraffin wax-embedded tissues for *B. abortus* antigens is not only both sensitive and specific, but also clearly shows tissue morphology; it is, therefore, capable of demonstrating the distribution of organisms in the tissues, a valuable attribute for the study of pathogenesis of *B. abortus* infection (Meador *et al*., 1986; Pérez *et al*., 1998).

Several PCR protocols have been developed for identification of infectious agents in aborted bovine fetuses, including *B. abortus* (Cortez *et al*., 2001; Richtzenhain *et al*., 2002). PCR based assays have been proved to be an important alternative rapid technique that overcomes problems and disadvantages of currently used traditional methods. The specificity and high sensitivity of PCR in the present study provides a valuable tool for the rapid diagnosis of brucellosis.

When investigating natural abortion cases, use of the most sensitive and reliable methods is important for rapid and precise diagnosis. Comparison of immunohistochemistry and bacterial culture in the present study for *Brucella* diagnosis was in agreement

with the observations of various studies (López *et al*., 1984; Pérez *et al*., 1998) that reported higher sensitivity of imminohistochemistry in comparison to culture which might be due to degenerated microorganisms, deficient isolation technique, or cross-reaction of antibody with another antigen. Diagnostic test agreement revealed slight degree of agreement (kappa= 0.249, CI= 0.079-0.295) between bacterial isolation and PCR and showed PCR to be more a sensitive and specific technique for the diagnosis of brucellosis. In the present study, PCR and IHC provide a reliable test for the diagnosis of brucellosis in bovine abortion cases on aborted fetal tissue and placental cotyledons as revealed by kappa value of 0.927 (CI= 0.548-0.927) which reflect an almost perfect degree of agreement between PCR and IHC. Comparison of PCR with other ancillary diagnostic techniques was in agreement with several previous studies that reported on the usefulness of PCR and advocated its use for the disease diagnosis (Leal-Klevezas *et al*., 1995; Keid *et al*., 2007). However in autolyzed fetuses, fetus stomach content and in aborted animals vaginal mucus/uterine discharges were found positive for brucellosis by PCR indicating higher sensitivity of PCR over IHC. In the present study, although 16 cows out of 40 cows with a history of abortion tested seropositive for *Brucella* by cELISA. However, IHC, bacterial culture and PCR detected fewer *B. abortus* positive cases than did the serological test(s). The differences amongst serological and culture techniques, and those of IHC and PCR might be due to the development of an immune response following an infection and the consequent clearance of the bacteria from the tissues.

Results of the present study revealed PCR and IHC were most specific tests for quick diagnosis of brucellosis in aborted fetuses and placenta, whereas cELISA is the most important serological test for detection of *Brucella* positive animal in a herd.

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