Clinical, Serological, Hormonal, Bacteriological and Molecular Detection of Brucellosis in Aborted Cows and Buffalos

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Abstract

Abortion is the most obvious manifestation of *Brucella* infection. In this study, 59 aborted buffalos and 91 aborted cows were included. Diagnosis of *Brucella* infection in these abortions was based on clinical, serological, bacteriological, hormonal, and molecular assays. Serological studies included the use of RB and ELISA tests as screening tests for infection. Argumentative differences between RB and ELISA results have been shown. *Brucella* isolated and identified from aborted fetuses, vaginal discharge and milk samples were 7 isolates from aborted cows and 3 from aborted buffalos. *Brucella* isolates revealed amplification of a 223-bp fragment with B4 and B5 primers. Hormonal assessment in both, brucella infected cows and buffalos, registered significant decrease in progesterone and P/E ratio in comparison with that aborted due to other causes. Immunohistochemical study revealed down expression level of 3BHSD enzyme in placentas of *Brucella* positive animals. ELISA technique was the valuable serological test to confirm the diagnosis of brucellosis. In conclusion, both RB and ELISA are necessary to be performed together as screening tests in diagnosis of brucellosis, whereas serum hormonal, placental immunohistochemical, and molecular (PCR) assessments have an efficient diagnostic values which can be included for confirmation of brucellosis.

Keywords: *B. abortus*, *B. melitensis*, Brucellosis, ELISA, RB, PCR.

1. Introduction

Brucellosis, is a major infectious disease afflicting humans and a wide range of domesticated animals and wildlife. It is known to be a worldwide problem and one of the most important among zoonoses in the Mediterranean region, India, and Central and South America (1). Brucellosis results significant human morbidity (2). Reports from the areas where *Brucella melitensis* infection is endemic, suggest that there is an increased rate of abortion in asymptomatic pregnant women (3). Outbreaks of bovine brucellosis are associated with abortion during the last trimester of gestation, and produces weak newborn calves, and infertility in cows and bulls (4). The diagnostic method known to produce the best results in terms of specificity is the isolation of *Brucella* organisms from the suspected animal. However, this method has a limited sensitivity, is expensive and cumbersome and has the added difficulty of being unpractical to apply at a large scale in control

campaigns. Accordingly, the indirect diagnosis of disease based on serological tests is of choice in the eradication programmes. The standard Rose Bengal (RB) is the main serological test used to detect antibodies against *B. abortus* and *B. melitensis* infections. This test has been used for several decades, proving to be successful for eradicating bovine brucellosis in some countries. Nevertheless, there is evidence that RB test is significantly less effective for the diagnosis of brucellosis in sheep and goats than in cattle (5). During recent years, different ELISAs have been developed using more or less purified S-LPS as the antigen and have been reported to be at least as sensitive and specific for the diagnosis of brucellosis in ruminants (6, 7, 8). The aims of the present work were: (i) to compare the diagnostic performance of serological: standard RB and ELISA, hormonal; in the blood and placenta, and molecular; PCR studies in cattle with known clinical and bacteriological status, (ii) to determine the diagnostic performance of these tests in aborted cattle positive in the classical RB test, but in which *B. abortus* and *B. melitensis* could not be isolated.

2. Materials and Methods

Materials: Bovine brucellosis kit was provided by EUROPEAN VETRINARY LABORATORY; EVL, Netherland. Gram's stain solution and modified Ziehl-Neelsen stain solution was prepared and used as described by Alton *et al* .,(9). Rose Bengal antigen was provided by Omega company, UK. Monospecific antiserum; antibrucella abortus and antibrucella melitensis were supplied by Difico, USA. It was provided as a gift from FAO. Antibiotic supplements were provided by Himedia, India. Fetal calf serum was provided by Difico company, USA. Materials used in PCR; Wizard Genomic DNA purification kit GoTaq® Green Master Mix were provided by Promega company, USA. Primers used for diagnosis; the system used was B4/B5 (Baily) primers system; advised by Baily *et al*.,(10), and provided by Alpha DNA Company, as described below:

Primers name	Sequences	Predictive product
B4	5′-TGGCTCGGTTGCCAATATCAA-3′	223- bp region within a gene coding for 31-kDa membrane protein specific to the genus *Brucella*
B5	5′-CGCGCTTGCCTTTCAGGTCTG-3′	

Methods:

Samples collection.

1. Clinical observations: was performed on aborted cows and fetuses throughout the last few days of gestation period. At parturition, retained placenta, body temperature and bleeding status has been registered.

2. Blood samples: 150 blood samples (91 from cows and 59 from buffalos) were obtained. serum samples have been obtained for serological assessments using RB and ELISA tests.

3. Aborted fetuses: Specimens from 15 aborted fetuses, at the first and last stage of pregnancy (9 from aborted cows and 6 from aborted buffalos) were cultured in duplicated *Brucella* agar plates as described by Alton *et al* (9).

Serological assessments: were preformed according to OIE, (11).

Identification and isolation of *Brucella***:** *Brucella* growth was confirmed by bacteriological and biochemical tests as suggested by Alton *et al*., (12).

Molecular Identification by PCR-based assay:

A-DNA purification: was performed according to the manufacturer instructions.

B-The PCR amplification process: Enzymatic amplification of DNA was carried out in a final volume of 25 µl according to the recommendations of manufacture. The following reaction mixes were prepared on ice. For a 25µl reaction volume:

Component	Volume	Final Conc.
GoTaq® Green Master Mix, 2X	12.5µl	1X
upstream primer, 10µM	0.25–2.5µl	0.1–1.0µM
downstream primer, 10µM	0.25–2.5µl	0.1–1.0µM
DNA template	1–5µl	<250ng
Nuclease-Free Water to	25µl	N.A.

PCR consisted of a preheating at 95ºC for 5 min. After this initial denaturation step, the mixture was subjected to 40 amplification cycles as follow:

Loop's steps	Temperature	Time	Number of cycle
denaturation	94 ºC	1 min	40
annealing	55 ºC	1 min	
extension	72 ºC	1 min	
Final extension	72 ºC	7 min	1
Hold	4 ºC	Indefinite	1

C-Detection of PCR products by agarose electrophoresis: A horizontal slab gel electrophoresis apparatus was used. Ten µl of amplified products were mixed with 3 µl of loading buffer, analysed by electrophoresis in 2% agarose gel, and stained with 0.5 µg /ml ethidium bromide at 100 V for 1hr. in 1 X TBE buffer. Then visualized under UV light using ultraviolet transelumenater. DNA ladder (100-1000) was used and the gel was photographed when necessary by digital camera. A sample was considered positive for *Brucella* spp. when a specific fragment of 223 bp was detected in the gel (10).

Statistical Analysis: All data were analyzed using the statistical package for social science (SPSS) for Windows program on the computer. Chi-square was used to compare between the frequencies. Student *t* test was used to compare between means of groups. The significance was accepted as P value < 0.05 and <0.01.

3. Results

Clinical observation:

All positive cases for *Brucella* infection were found in late stage of pregnancy (5-9 month), some cases accompanied with abortion with retention of placenta, aborted buffalos were in herds not sporadically like cows (table 1). Pneumonia and pericarditis were the main complications shown in aborted fetuses positive for brucellosis. Placentas were, edematous and opaque with bleeding in some of cases.

Serological assessments:

ELISA, RB tests and clinical signs results are shown in tables (1 and 2) that RB test results were positive in 33 cases (22%) including 26 cases (28.5%) in cows and 7 cases (11.86%) in buffalo. ELISA results were positive in 38 cases (25.3%), including 21 cases (23%) in cows and 17 cases (28.8%) in buffalo. ELISA was positive in aborted cattle which had previous history of abortion, whereas RB test was positive in 2 cows that was negative for ELISA & bacterial isolation, also RB test was negative in 10 case of buffalo that aborted before several months, which was positive with ELISA test. According to the results of Pearson Chi-Square test, the difference in RB positive cases between groups was significant ($P<0.05$), whereas difference in RB negative cases between cows and buffalos was not significance ($P>0.05$). According to ELISA technique, statistical analysis showed insignificant difference ($P>0.05$) between groups.

No.	History & clinical signs	Pregnancy period	Samples & isolation of agent	R.B	ELISA
1	Aborted before 2hrs with edema. of placenta	7 Months	Fetal stomach (+)	++	0.362
2	Pneumonia of Fetus.	8 Months	Fetal stomach (+)	++	0.409
3	Aborted before 6hrs with opaque placenta edema.	6 Months	Fetal stomach (+)	++	0.469
4	Aborted before 12hrs.	9 Months	Uterine fluid (+)	+++	0.772
5	Aborted before 24hrs with retained placenta.	9 Months	Uterine fluid (-)	+++	0.666
6	Aborted before 24hrs with Opaque placenta	8 Months	Uterine fluid (+)	++++	1.037
7	Aborted before 5hrs with pneumonia of Fetus	7 Months	Fetal stomach & Fetal organ (+)	+++	1.00
8	Aborted before 48hrs	7 Months	Uterine fluid (+)	++++	1.08
9	Aborted before 72hrs	6 Months	Uterine fluid (-)	+++	0.846
10	Aborted before 14 d.	5 Months	Milk (-)	+++	1.00
11	Aborted before 23 days.	8 Months	Milk (+)	++	0.676
12	Abortion pneumonia of Fetus with opaque of placenta	9 Months	Fetal stomach & Fetal organ (+)	+++	0.650
13	Retained of placenta	9 Months	Fetal stomach (+)	++	0.570
14	Opaque of placenta	8 Months	Placenta &uterine fluid(-)	+++	0.840

No.	History & clinical signs	Pregnancy period	Samples & isolation of agent	R.B	ELISA
15	Aborted before 40 d with retained placenta	6 Months	Milk (-)	++	1.026
16	Aborted before 60 d. (Abortion also occurred in all herds before 5 months at late pregnancy)	9 Months	Milk (-)	_	1.016

Table 1. History, clinical signs, pregnancy period, Samples and isolation, RB and ELISA results of aborted cattle and their fetuses.

	NO	R.B			ELISA			Bacterial isolation		
		+	-	%	+	-	%	+	-	%
Cow	91	26	65	28.5	21	70	23	7	79	13.18
Buffalo	59	7	52	11.86	17	42	28.8	3	55	6.77

***Only one** case had ELISA titer 10.09 (suspected grey zone)

Table 2. Results of RB test, ELISA and bacterial isolation for aborted cows and buffalos.

Bacterial isolation & identification:

Brucella organisms first recognized in smears obtained from fetal stomach stained with modified Ziehl Nielsen stain, which appeared red clumps against blue background. *Brucella* culture recognized on the basis of colonial morphology which appeared round translucent pale honey. Routine bacteriological examination has been carried out for identify the genus *Brucella* before they submitted for *Brucella* typing tests any isolate that was differ in even one test was excluded from further consideration as member of the genus *Brucella*. So the obtained isolates were Gram-negative, coccobacilli, arranged singly, in short chain pairs, with small groups, negative for haemolysis on blood agar, and it neither grow nor perform lactose fermentation on MacConkey agar, positive for nitrate reduction oxidase, catalase and urease, negative for MR-VP, gelatinase, Citrate utilization and indol production. Out of 91 aborted cows, 7 (13.18%) were positive by culture, whereas out of 59 aborted buffalos, 3 (6.77%) were positive for culture (table 1 and 2). Out of 15 aborted fetuses from cows and buffalos 4 from aborted cow's fetuses and 2 from buffalo's aborted fetus were positive by culture. Out of 12 uterine fluid swabs 2 from aborted cows and 1 from aborted buffalo were positive by culture. Out of 122 milk samples positive 1 from aborted cows, was positive by culture. Uterine swabs and milk samples where firstly cultured on the selective media, so *Brucella* colonies were recognized first by colonial morphology then subcultured to obtain pure culture before submitted to the bacteriological and biochemical test. According to the results of Pearson Chi- Square tests, isolates from cows specimens were significantly higher ($P<0.05$) than that isolated from buffalos (figure 2).

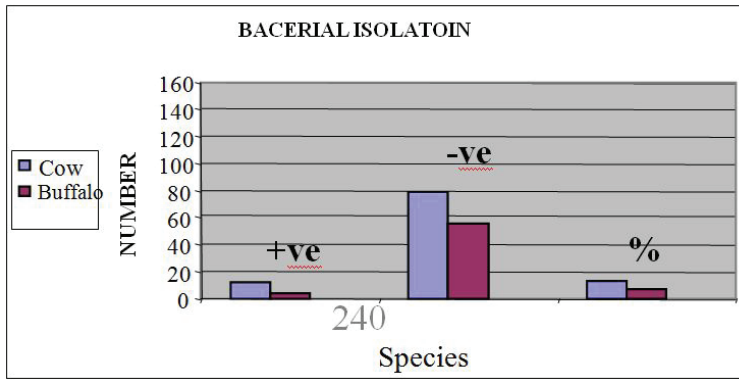

Fig 4. Illustrate the positive, negative and percentages of bacterial Isolation results for cattle.

Steroid hormones assessments:

Cows and buffalos positive for *Brucella* infection showed insignificant differences (P>0.05) of serum estradiol concentration compared with that of non infected (463.92±176.57 pg/ml for infected versus 589.66±235.67 pg/ml for non infected). Serum cortisol in infected animals revealed insignificant higher concentration (P>0.05) than non infected animals (78.36±12.14 nmol/l versus 65.48±13.38 nmol/l, respectively). on the other hand, serum progesterone concentration revealed significant decrease (P<0.05) in infected animals (0.495±0.13 ng/ml) compared with (18.468±6.26 ng/ml) in non infected group. This decrement of progesterone concentration reflected on the progesterone/ estradiol balance (P/E Ratio) which reach (0.107%) in infected group compared with (3.132%) in non infected group (figure 2).

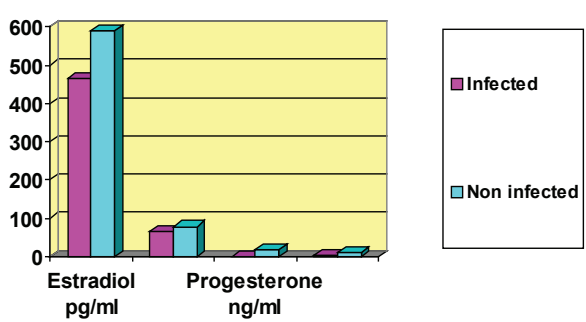

Fig 2. Mean ±SE of steroid hormones levels in aborted cattle.

Immunohistochemical assay:

Results of IHC analysis demonstrated positive staining for 3β-HSD in placentas of cattle, in which the localization was found in both chorionic villi and chorionic plate (fig. 3a and b). It has

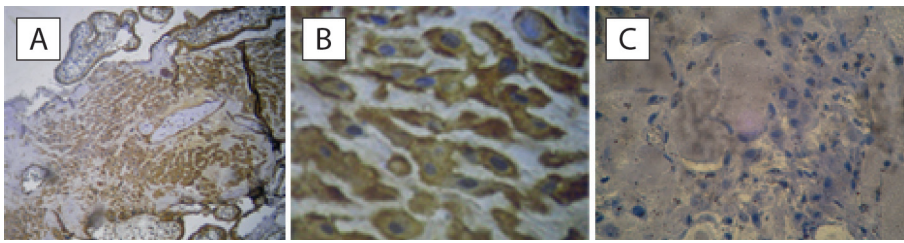

Figure 6. IHC staining results. A & B: cow placenta negative for brucellosis, specific staining of chorionic plate with DAB chromogen (brown) and counterstained with Hematoxylin (blue) (A:100x, B:400x). C: cow placenta positive for brucellosis; stained by DAB chromogen (brown) and counterstained with Hematoxylin (blue) notice, non IHC reaction for 3BHSD enzyme .100x.

Molecular detection of Brucella spp by PCR technique:

The primer pair used in this study succeeded in the amplification of a 223-bp fragment from *Brucella* isolates cultures that were studied, meanwhile, the DNA extracted from culture harboring *Brucella*'s DNA, so that they yielded predicted 223-bp fragment. All *Brucella* strains that studied with PCR have same 223-bp fragment. (figure 8).

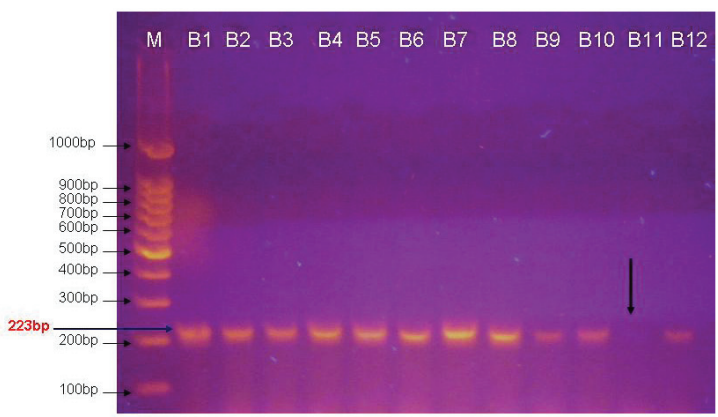

Figure 4-23. Agarose gel electrophoresis for PCR products of Brucella isolates, where 223bp PCR products appear as positive results.

4. Discussion

Clinical observation: Clinical findings observed in aborted cattle were in agreement with other researchers (13, 14). Abortion is a frequent complication of brucellosis in animals, where placental localization is believed to be associated with erythritol, a growth stimulant for *B. abortus*. Whether the rate of abortions from brucellosis exceeds rates associated with bacteremia from other bacterial causes is unclear. In any event, prompt diagnosis and treatment of brucellosis dur-

ing pregnancy can be lifesaving for the fetus (1). It has been postulated that generalized suppression of adaptive immune response mainly occurred during pregnancy. This immune suppressed state prevent maternal rejection of the fetus but has unfortunate consequence of increasing maternal susceptibility to certain infectious agents (15).

Bacterial isolation and identification: *Brucella* strains isolated from cattle were obtained from aborted fetuses and vaginal discharge and were compared to that isolated from milk samples. The number of *Brucella* organisms in milk and colostrums samples was lower than that in abortion material, fetus stomach, fetal fluids and membranes, also milk samples is highly contaminated with other organisms. These results were in agreement with that mentioned by OIE (11). On the other hand, bacteriological and biochemical test of *Brucella* isolates were same as that advised by Alton *et al*. (12) and Qunin *et al*. (13).

Serological results: Serological examination performed by RB test in the present study gave positive result in some aborted animals but were negative for ELISA and bacterial isolation, also it gave negative result in buffalo aborted before 2 month or more. This diversity may attributed to the infection with microorganisms other than *brucella* spp. This infection are likely to cause cross-reactions in serological tests with smooth *Brucella* antigens and give false positive for RB. These results indicate that RB test is not confirmative test for diagnosis of brucellosis. Although RB test is known to have many false positive or negative results, but generally it is simple, rapid and can be used as screening method for infection (16). For confirmation of brucellosis, ELISA technique is the suitable and precise detectable test. It has been recorded that antibodies to *Brucella* appear in the serum within 1-2 weeks of infection. The initial response is the appearance of IgM isotype (which can be easily detected by RB) followed by a switch to IgG, after a while titers of both immunoglobulins classes increase. Distinct most of the usual serological tests, ELISA is effective in detecting all immunoglobulins (antibodies) classes and sub-classes important in diagnosis and appears to be the most sensitive serological test (17). IgM is produced soon after infection but declines quickly when production of IgG increases. IgM reacts non specifically in many serological tests and can cause high rates of false positive reactions. IgG1 is consistently produced at high levels in *Brucella*-exposed cattle sera and has a high affinity and specificity for *Brucella* antigens particularly the O-chain.

Steroid hormones interference: It has been registered, in the present study, that the results of reproductive hormones (E and P) concentrations in aborted cows and buffalos positive for brucellosis were in orchestration with that registered in immunohistochemical assays. Although estradiol concentration slightly decreased in brucella-infected aborted animals, but progesterone concentration sharply decreased. The important significant point was that related to the decrement of P/E ratio. On the other hand, immunohistochemical results revealed that 3β-HSD enzyme expression on trophoblast registered significant decrease, where 3β-HSD considered as main enzyme in progesterone biosynthesis that play important role in maintenance of pregnancy (18, 19, 20). Gorvel and Moreno (21) showed that in trophoblasts, *B. abortus* induces steroid biosynthesis and modulates the metabolism of prostaglandin precursors, favoring bacterial growth. These changes mimic to some extent what happens during parturition and are likely to contribute to the abortion.

Immunohistochemistery assay: The decrement in the expression of 3β-HSD enzyme in brucella infected animals may attribute to utilization of this enzyme by *Brucella* itself, which may results in the decrement of progesterone biosynthesis. Or due to infection of placenta with *Brucella* that may caused damage in placental tissue. This results were in agreement with that recorded by Samartino *et al*. (22). From the present findings, it can be demonstrated that *B.* abortus may traffics from a phagosome compartment towards the endoplasmic reticulum of the host cell; where the organism has an optimal environment for replication (23).

Brucella detection by PCR: All *Brucella* strains that were studied with PCR have the same 223-bp fragment. Results of PCR were the same as that obtained by Baily *et al.* (10) whom used PCR amplification contained a single pair of oligonucleotide primers designed to amplify a 223 bp product. Fekete *et al*. (24) used PCR in the diagnosis of brucellosis and described it as specific, sensitive and simple and could become a routine diagnostic test for brucellosis.

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