Serological, Bacteriological, Molecular and Immunohistochemical Techniques for Diagnosis of Brucellosis in Buffaloes

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ABSTRACT

The present study was carried out on a total number of 102 Buffalo-cows. The obtained sera were investigated for detection of brucella-antibody titer using serological tests Rose Bengal plate Test (RBPT), Tube Agglutination Test (TAT), Mercaptoethanol Test (MET), Complement Fixation Test (CFT) and ELISA. A total number of 32 tissue samples were collected for bacteriological and Polymerase Chain Reaction (PCR) assays. The results of serological analysis revealed that the positive reactors were 17 (16.6 %), 12 (11.76 %), 13 (12.74 %) and 12 (11.76 %) and 14 (13.72 %) with RBPT, TAT, MET, CFT and ELISA, respectively. RBPT and ELISA tests showed the highest seropositivity. Meanwhile, the lowest ones were obtained by TAT and CFT tests. All seropositive animals gave positive PCR product (223bp) using of a Brucella species primer specific for 31 KD outer membrane protein gene and a positive PCR product (731bp) using primers specific for Brucella melitensis. The immunohistochemical examination of supramammary lymph node revealed moderate positive immuno-reactive Brucella antigen Intra cytoplasmic and extra cellular immunostaining. Electron microscopical finding revealed presence of moderate aggregations (clusters) of dark bodies of intact coco-bacilli within the cytoplasm of macrophages of medullary sinuses of supramammary lymph node. It could be concluded that ELISA is the most sensitive test for diagnosis of brucellosis in sera of infected buffalo cows. PCR assay is a sensitive, specific, rapid, relatively inexpensive method and diagnostic tool for detecting of brucella DNA. Also, immunoperoxidase and electron microscopical techniques could be used to enhance the diagnostic capabilities of brucellosis particularly in chronic infection.

Key words: Brucella-antibody titer, molecular, immunohistochemical, brucellosis, buffaloes

Introduction

Brucellosis is the most worldwide spread zoonotic disease and is of major public health and economic significance (Pappas et al., 2006). The disease is caused by brucella spp., which can infect several important livestock species, including cattle, water buffaloes, goats, sheep, and pigs (Di-Giannatale et al., 2008). The principal symptom of the infection in all animal species is abortion or premature expulsion of the fetus (Franco et al., 2007). The available strategies to control brucellosis are based on very strict management procedures including slaughter of all seropositive animals, and, where allowed, vaccination (Alvarez et al., 2011). Water buffalo (Bubalus bubalis) occupies an economically important place in the livestock industry in many parts of the world including Egypt. Brucella abortus causes reduced fertility and abortion in cattle (Enright, 1990) and water buffalo (Bubalus bubalis). However, many infected cows shed B. abortus in the milk.

Diagnosis of brucellosis can be established by laboratory methods such as serology and blood cultures. Prolonged incubation period, special growth media, and subcultures are required for the isolation of these fastidious, slow growing bacteria. However, cultures are not always positive when other tests are positive (Romero et al., 1995).

The TAT and the competitive ELISA have been validated as diagnostic screening and confirmatory tests, respectively, for brucellosis surveillance in water buffalo (Ghazi et al., 2006 and Ahmed et al., 2010). The sensitivities and specificities of serological tests reported differences in diagnosis of brucellosis between cattle and water buffalo (Fosgate et al., 2011).

The laboratory confirmation of brucellosis is based on microbiological, serological or molecular methods, each having its own advantages and disadvantages. Many serological tests such as Rose Bengal plate test (RBPT), complement fixation test (CFT), Coombs test, ELISA, and serum agglutination test (SAT) are used for the diagnosis of brucellosis (Islam et al., 2013). Each test has its own disadvantages, and the presence of antibodies doesn't always mean an active case of brucellosis.

As for other fastidious pathogens, amplification of DNA by PCR offers an alternative way for diagnosis of brucellosis (Alvarez et al., 2011).

The molecular diagnosis of brucellosis can be performed using genus-specific Polymerase Chain Reaction (PCR) assays (Al Dahouk and Nockerl, 2011). Tissue samples from lymph glands appear as the most promising sample type for brucella detection by PCR (Leary et al., 2006).

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Immunohistochemical examination of paraffin wax-embedded tissues for Brucella 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 brucella infection (Meador et al., 1986 and Perez et al., 1998).

Therefore, the aim of present work is to diagnose brucella infection in buffaloes by using different serological, bacteriological and molecular methods, as well as, demonstrates the presence of Brucella spp. organism in tissues by using immunohistochemical and electron microscopical techniques.

Materials and Methods

Animals

A total number of 102 Buffalo cows from a governmental farm located at Ismailia governorate of Egypt, suffering from reproductive disorders as repeat breading, decrease milk yield and retained placenta were used in the present study. Also, 15 obligatory slaughtered cases from Ossim slaughter house in Giza governorate were examined for brucellosis by different serological tests.

Blood samples

A total numbers of 102 blood samples were collected from Ismailia buffalo farm. The blood samples were taken for separation of serum and then the sera were preserved at -20°C until used. These serum samples were used for serological examinations.

Tissue samples

Tissue samples (uterus, spleen, mammary gland and supramammary lymph node) were taken from a total number of 32 Brucella seropositive cases (17 cases from buffalo farm and 15 cases from slaughter house) for bacteriological, immunohistochemical, ultra structure examination and PCR assay.

Brucella antigens used for serological tests:

Antigens of Rose Bengal Plate Test (RBPT), Tube Agglutination Test (TAT) and Mercaptoethanol test (MET): were supplied by Vet. Serum and Vaccine Research Institute, Abbasia, Cairo, Egypt. Enzyme-Linked Immuno Sorbent Assay (ELISA) was applied: using crude Lipopolysaccharide (LPS) of Brucella abortus strain 19 with the optimized antigen concentration and serum dilution using checker Board titration (Narayanan et al., 1983) and modified by using protein G (Sigma Co).

Polymerase Chain Reaction assay (PCR):

Tissue specimens frozen at -20°C were thawed at room temperature and the extraction of genomic DNA was done according to Sambrook et al. (1989) and Fekete et al. (1992). Two oligonucleotide primers were used for amplification of Brucella DNA prepared according to the sequences of highly preserved region that coding for outer membrane protein (OMP2) (Baily et al., 1992). Primer sequences were:

Primer 1: Forward (B4 TGG CTC GGT TGC CAA TAT CAA). Reverse (B5 CGC GCT TGC CTT TCA GGT CTG). Specific for Brucella species whereas the expected PCR products are 223bp.

Primer 2: Forward (Bm- SP AAA TCG CGT CCT TGG TGT CAT). Reverse (IS711-SP TCG CGA TCA CTT AAG GGC CTT CAT). Specific for Brucella melitensis where the expected PCR products are 731bp.

The amplified products were visualized by electrophoresis using 2% agarose gel stained with Ethidium bromide according to Sambrook et al. (1989)

Isolation and identification

Tissue specimens from uterus, mammary gland, supramammary lymph node and spleen (the organs of predilection site for brucella organism) were taken as freshly as possible for isolation and identification of brucella organism. Culturing of brucella was done as referred by Alton et al. (1988).

Serological tests

Serum samples were investigated for the detection of brucella antibody titer using: RBPAT, TAT, MET and CFT according to Alton et al. (1988). Also, ELISA was carried out as described by Alton et al. (1988) and modified by using protein G according to Chand et al., (1988).

Tissue samples

Tissue samples were taken from supramammary lymph nodes for immunohistochemistry and ultrastructure examination.
Immunohistochemistry

Avidin-Biotin complex peroxidase technique was applied for detection of brucella in formalin-fixed, paraffin-embedded tissue sections from supramammary lymph nodes according to Haines and Clark, 1991, using peroxidase detection kit purchased from Novocastra co.UK.

Ultra structure examination

Small tissue specimens were taken from supramammary lymph nodes for ultra structure examination. The specimens were fixed in 5% cold cocodylate buffer glutaraldehyde (4C 0.1N, pH 7.2) then kept at 4ºC until processing for electron microscopic examination (Bancroft and Stenes, 1982). This work was done at the electron microscope unit, National Research Centre, the model of apparatus is Zeiss E.M.10 – Germany.

Polymerase Chain Reaction (PCR)

Extraction of the Brucella genomic DNA

DNA extraction from the bacterial cultures and tissue samples was performed using enzymatic method (Fekete et al., 1992).

DNA amplification:

Different PCR assays were performed in this study, using 25 µl PCR reaction volumes. The amplification was performed in a programmable heating block, (Primus Thermal Cycler, MWG Biotech. Germany) according to Sambrook et al. (1989) and Fekete et al., (1992).

Results

Bacteriological findings:

Brucella was isolated from three (9.37%) out of a total number thirty two examined cases. Three isolates of Brucella culture growth were obtained mainly from supramammary lymph nodes. The cultures were smooth, glistening, transparent in appearance, pin point in size and distributed all over the selective brucella agar media. The suspected colonies were picked up for direct slide agglutination test where it gave positive results; also these colonies were picked up for differentiation of brucella species by using PCR assay. It was confirmed as B. melitensis which gave positive PCR product (223bp) and (731bp) using specific primers for brucella species and Br. melitensis respectively.

Serological examinations:

The results of serological tests applied on buffalo cows were demonstrated in Table (1). The results revealed that the highest positive reactors were obtained by RBPT and ELISA tests, while, the lowest ones were obtained by TAT and CFT tests.

Table 1. Incidence of brucellosis among buffalo cows with history of reproductive disorders using different serological tests.

<table>
<thead>
<tr>
<th>No. of examined animals</th>
<th>The different serological tests used for diagnosis of brucellosis</th>
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<tr>
<td></td>
<td>RBPT (positive at 80 IU/ml and higher)</td>
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<td></td>
<td>No.</td>
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<td>102</td>
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Electron microscopical findings:

The electron microscope revealed the presence of moderate aggregates (clusters) of dark bodies of intact coco-bacilli within the cytoplasm of macrophages in the medullary sinuses of supramammary lymph node (Fig. 1).

Immunohistochemical findings:

The supramammary lymph node showed strong positive immuno-reactive brucella antigen staining within the cytoplasm of macrophages in the germinal centers of lymphoid follicle. Moreover, extra cellular
multiple clusters of moderate to severe *brucella* antigen immuno-staining were observed in the cortex. Intra cytoplasmic and extra cellular immunostaining in some areas was intense and interfere with the visualization of the cellular and structural details. Also intra cytoplasmic an immunostaining reaction was found in the macrophages of the control positive sample. In all positive cases, deposition of golden brown chromogen pigment at the site of antigen-antibody reaction was seen within the cytoplasm of mononuclear cells and detected extracellularly among the lymphocytes in the germinal centers of lymphoid follicles (Fig. 2).

**Results of PCR:**

A six tissue samples out of a total number of a DNA successfully extracted nine tissue samples (gave high titer of antibodies by serological tests) of uterus and supramammary lymph node gave positive PCR product (223bp) as shown in (Fig.3) using *brucella* species primers specific for 31 kD outer membrane protein gene and a positive PCR product (731bp) using primers specific for *Brucella melitensis* as shown in (Fig.4).

![Fig. 1](image1.png)

**Fig. 1.** Electron micrograph showing the presence of moderate aggregates of dark bodies of intact coco-bacilli within the cytoplasm of macrophage in the medullary sinuses of supra mammary lymph node (Uranyl acetate X 10000).

![Fig. 2](image2.png)

**Fig.2.** Supra mammary lymph node of Buffalo showing golden brown deposition of *Brucella melitensis* antigen in between lymphocytes and macrophages released from ruptured cells (yellow arrow) in the lymphoid follicles (moderate positive immunoperoxidase), indirect peroxidase technique.(DAB X 1000).
Although brucellosis is a notifiable disease in many countries, official figures do not fully reflect the number of cases reported annually, and the true incidence has been estimated to be between 10 and 25 times higher than what the reported figures indicate (Corbel, 1997). In Egypt subcontinent, brucellosis is prevalent among all domesticated animals and humans (Siftuentes-Rincon et al., 1997).

Diagnosis of brucellosis is the cornerstone of any control program and is based on bacteriological examination, serological and molecular findings. Serological methods are not always sensitive or specific due to cross reactivity with other antigens (Ali et al., 2014). In addition, the presence of antibodies does not always mean an active case of brucellosis, since vaccinated animals tend to yield persistent post-vaccinal immune responses, and other gram-negative bacteria such as Yersinia enterocolitica may cross-react with smooth *brucella* species (Diaz and Morityon, 1989).

Serological results in the present study, revealed that the incidence of *brucella* infection in the buffalo cows suffered from reproductive disorders in Ismailia governorate were 17 (16.6 %), 12 (11.76 %), 13 (12.74 %) and 12 (11.76 %) and 14 (13.72 %) by using RBPT, TAT, MET, CFT and ELISA respectively. The
incidence of brucellosis in buffalo cows was always very low during the last 50 years (Refai, 2003). However, high incidences of positive reactors in buffalo were 10.2 % using TAT and 7.6 % using RT (Refai et al., 1989), 23% in South Africa (Refai, 2003). Moreover, positive rates of 31 % in buffaloes in Italy (Borriello et al., 2006), 4.8% in Brazil (Silva et al., 2013) and 4.0% in Bangladesh (Islam et al., 2013) were recorded.

On the other hand, low incidence of brucellosis in buffaloes ranged between 0.24 % and 0.48 % in Egypt were recorded by (El-Taweel, 1999 and Abdel-Hafeez et al., 2001). Variation in the incidence of infection is related to the course of the diseases, locality, rate of exposure, reproductive status, sex, improvements in the diagnostic techniques and vaccination strategies (Ghazi et al., 2006). It has been reported that the genetic variation within the host may play a part in the resistance to brucellosis (Silva et al., 2013). Resistance of water buffaloes to B. abortus infection is associated with the gene Nramp1 which control the replication of B. abortus inside the macrophages (Borriello et al., 2006).

RBPT showed high incidence of infection whereas it is still an efficient screening test for detection of both early and chronic brucella infection. However the acidic pH of RBPT permits lesser amounts of IgM to share in the reaction (Mikolon et al., 1998), so decrease its sensitivity. The high positive reaction of buffaloes to RBPT as compared with positive TAT can be explained on the basis that; some of the suspicious cases in TAT (1/20 titer) may be truly infected but did not reach the positive titer (Waghela et al., 1980).

Incidence of infection by using MET (12.74 %) was higher than that of TAT (11.76 %) which indicates the chronicity of infection (Rust, et al 2004). This finding comes in accordance with histopathological results which characterized by occurrence of granulomatous endometritis, granulomatous mastitis and lymphoid depletion in spleen and lymph nodes (Ahmed et al., 2010 and Ahmed et al., 2012).

In the present work, sero-positivity of CFT and TAT were the lowest in serological tests. This condition could be explained on the basis of that exposed animals to field (natural) infection, develop TAT and CFT reactions at approximately the same time although the CFT may detect diagnostically significant levels before the TAT (Sting and Ortmann, 2000). Moreover, it was reported that CFT is considered as the best confirmatory test for the diagnosis of brucellosis in buffaloes due to the good balance of sensitivity and specificity. This may be attributed to its high ability to detect low concentration of IgG1 characteristic for brucella infection (Montasser et al., 2002).

In the current work, the results of sero-positive ELISA were higher than those of TAT, MET and CFT. This ind icates that ELISA was the most sensitive test (Saz et al., 1987). The test is rapid; easy to perform and can be automated (Osoba et al., 2001). Moreover, ELISA is a valuable and reliable addition of brucellosis sero-tests (Sayour, 1995).

In the current investigation, brucella organisms were isolated from uterus and supramammary lymph node of three cases (9.37%) out of total number thirty two cases of sero-positive ELISA test. These findings are come in accordance with (Essmail et al., 2002) who isolated B. melitensis from supramammary lymph nodes of 3 out of 16 cases (19 %) of naturally infected buffalo cows. In this respect, isolation of brucella organism is often difficult from the tissue samples (Corbel et al., 1984). On the other hand, a high rate of isolation of brucella organism from the udder and supra mammary lymph node was reported by Laing et al., (1988). Tissue samples which gave negative results may be due to the longtime required for culturing field specimens can be and/or tissues are contaminated with a low number or nonviable brucella organism. Despite of brucella isolation and identification are a direct and reliable methods for diagnosis, they are time consuming, cumbersome and represent human health hazard (Leyla et al., 2003).

The present work revealed the presence of positive immunostaining brucella antigen in formalin-fixed, paraffin imbedded tissue sections of uterus and spleen by using avidin–biotin complex peroxidase technique. Similar results were reported in cows, goats and mice inoculated with Br. abortus (Xavier et al., 2009), in tissues of naturally aborted bovine fetuses infected with Br.abortus (Perez et al., 1998) and in adult female buffaloes naturally infected with Br. melitensis (Essmail et al., 2002). They concluded that this technique is sufficiently sensitive for detecting brucella antigens in formalin fixed, paraffin embedded tissues and could be a complementary tool to serological and bacteriological examination for diagnosis of brucellosis.

Moreover, Staak et al. (2000) reported that immunoperoxidase technique may enhance diagnosis capabilities of brucellosis particularly in chronic infection and is an efficient mean for detecting brucella organisms when are inherently slow or difficult to diagnose by isolation or culture from tissues obtained from field cases due to contamination. In addition, this technique is relatively rapid and enables detection of dead and/or low numbers of bacteria (Haines and West, 2005) although, cross reaction of the polyclonal antibodies with other microorganisms such as Yersinia enterocolitica and E.coli cannot rule out.

Also, immunohistochemical staining has been used to study and assist in the understanding of the pathogenesis of infectious agents as the quantity, tissue and cellular locations of agent can be visualized (Meador et al., 1989). This technique could be used as a complementary tool to serology and bacteriology for the diagnosis of brucellosis (Perez et al., 1998).The ultrastructural finding of the current study, showed the presence of aggregates of intact electron dense cocci or coco-cocci-bacilli within the macrophages which means the ability of the organism to survive intracellularly (Pei et al., 2006). It has been suggested that lymph nodes
draining areas of infections or lesions have the highest chance of being positive for *brucellae* (Adesiyun et al., 2010). Since, it was found that *Br. abortus* is incorporated into phagosomes and remains in membrane-bound compartment until the host cell dies. The ability of *brucella* to survive in the intracellular environment is apparently due to inhibition of phagosome-lysosome fusion (Arenas et al., 2000).

Transmission electron microscope (TEM) studies of rough strain *brucella* infection showed that in addition to necrosis, *brucella*–infected macrophages underwent oncosis, which is a prelethal pathway leading to cell death characterized by cell organelle swelling, cell blebbing and increased membrane permeability (Fink, and Cookson, 2005). These findings were confirmed by previous reports indicating that infected cells were not killed via apoptosis (Pei and Ficht, 2004). The outcomes of infection can be explained as the organism can reach replication niches and survive and the host cells will be killed. Otherwise the bacteria will be cleared by the host cells (Majno and Joris, 1995).

Finally, it could be concluded from the present study that ELISA is the most sensitive test for diagnosis of brucellosis in sera of infected buffalo cows. PCR assay is a sensitive, specific, rapid, relatively inexpensive method and diagnostic tool for detecting *brucella* DNA. Moreover, immunoperoxidase and electron microscopical technique enhance the diagnostic capabilities of brucellosis particularly in chronic infection in buffalo cows.

**References**


