Middle East Journal of Applied Sciences, 4(3): 762-770, 2014

ISSN: 2077-4613

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 Nockler, 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.untill 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 -20C° 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 TGC TGG TCT GA).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 promide 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 glutraldehyde (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.

No. of examined animals	The different serological tests used for diagnosis of brucellosis									
	RBPT		TAT (positive at 80 IU/ml and higher)		MET (positive at 1:10 dilution and higher)		CFT (positive at 1:8 dilution)		ELIZA (positive	
	No.	%	No.	%	No.	%	No.	%	No.	%
102	17	16.6	12	11.76	13	12.74	12	11.76	14	13.72

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).

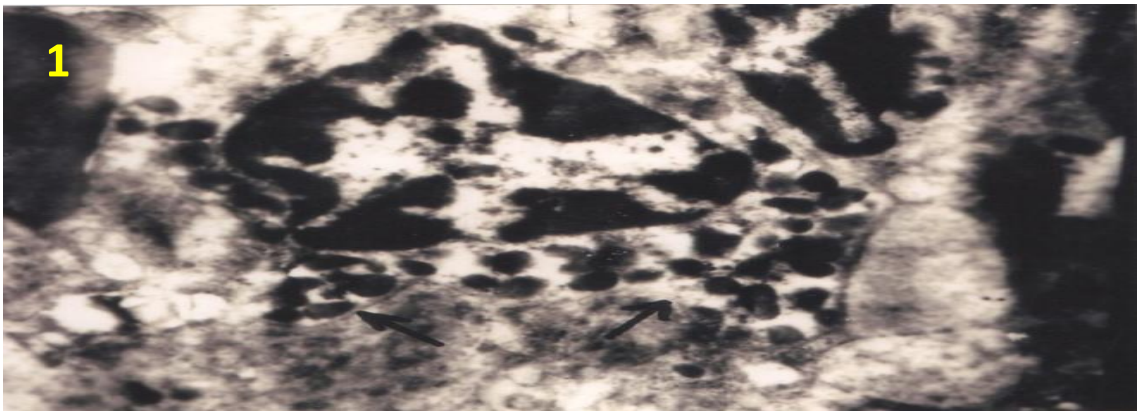

Fig. 1. Electron micrograph showing the presence of moderate aggregates of dark bodies of intact coco-bacilli within the cytoplasm of macrophage in the medullary sinuses of supra mammary lymph node (Uranyl acetate X 10000).

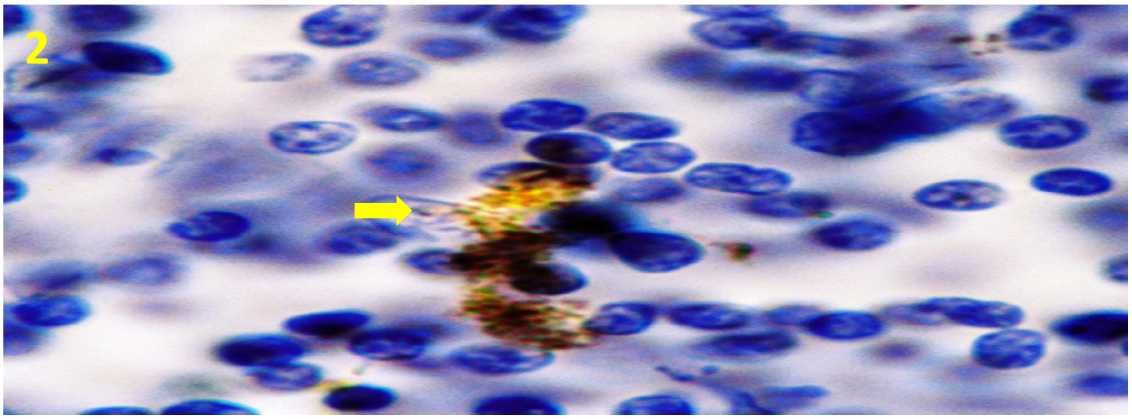

Fig.2. Supra mammary lymph node of Buffalo showing golden brown deposition of *Brucella melitensis* antigen in between lymphocytes and macrophages released from ruptured cells (yellow arrow) in the lymphoid follicles (moderate positive immunoperoxidase), indirect peroxidase technique.(DAB X 1000).

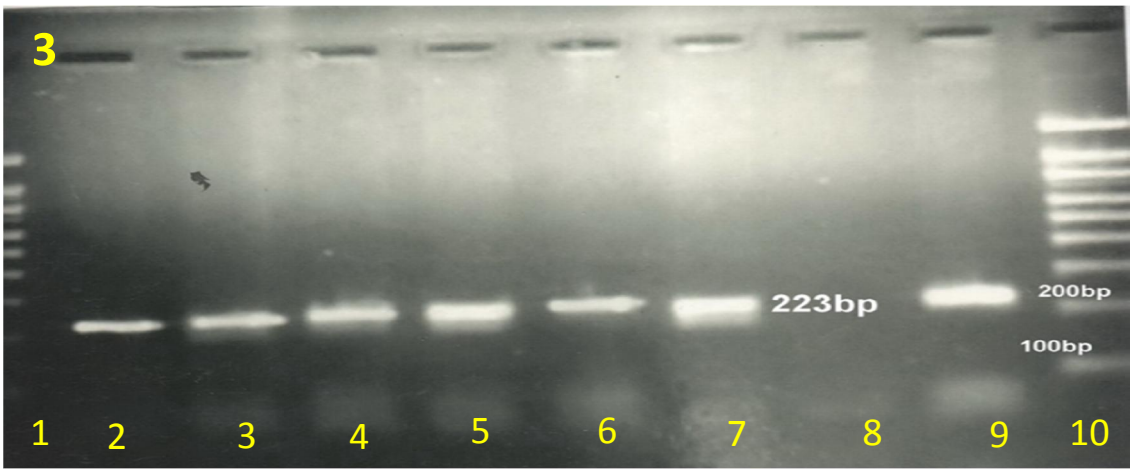

Fig.3. PCR amplification of *brucella* organism (using species primer) DNA Lane 1and10; Molecular weight marker (100) bp; Lane 2-7 tissue samples DNA PCR (223), Lane 8 control negative; Lane 9 control positive with *Br. melitensis*

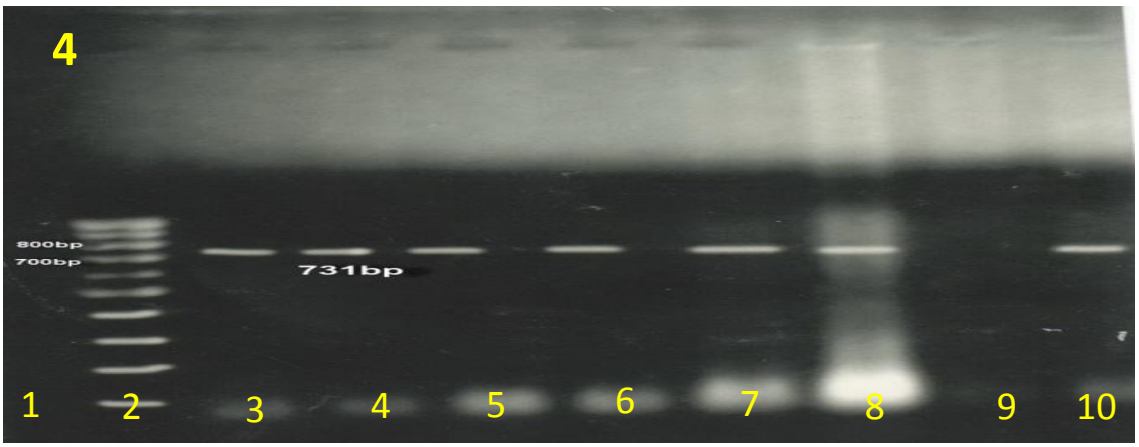

Fig 4: PCR amplification of *Br. melitensis* DNA from tissue samples. Lane 1: Molecular weight marker (100 bp). Lane 2-7 tissue samples DNA PCR (731). Lane 8 negative control; Lane 9 positive control with *Br. melitensis* DNA.

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 enterocolotica 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 buffaloes 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 truely 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 enterocolotica 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 cocco-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 *brucella*e (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

Abdel-Hafeez, M.M., H.A. Abd El-Kader, A.F. Bastawrows, M.M. Ali, and S.R. Sedik, 2001. Zoonotic importance of Brucellosis among farm animals and veterinary field employees at Assiut governorate. Assiut Vet. Med. J., 44: 119.

Adesiyun A.A., G.T. Fosgate, A. Persad, M. Campbell, R. Seebaransingh and A. Stewart-Johnson, 2010. Comparative study on responses of cattle and water buffalo (Bubalus bubalis) to experimental inoculation of *Brucella abortus biovar* 1 by the intraconjunctival route—a preliminary report. Trop. Anim .Health Prod ., 42:1685–1694.

Ahmed Y.F, S.M. Sokkar, H.M. Desouky, and A.A. Madboly, 2012. Pathological studies on buffalo-Cows naturally infected with *Brucella* melitensis. Global Vet., 9(6):663-668.

Ahmed Y.F, S.M. Sokkar, H.M. Desouky, Y.A. Ghazi, A.S. Amin and A.A. Madboly, 2010. Pathological and Molecular studies on mammary glands and supramammary lymph nodes of naturally Brucella infected buffalo-cows. J. of Reproduct and Infertility, 1(2):33-40.

Al Dahouk S. and K. Nockler, 2011. Implications of laboratory diagnosis on brucellosis therapy. Expert Rev Anti Infect Ther. 2011; 9(7):833_845.

Ali S, Q. Ali, F. Melzer, I. Khan, S. Akhter, H. Neubauer, S.M. Jamal, 2014. Isolation and identification of bovine Brucella isolates from Pakistan by biochemical tests and PCR. Trop. Anim. Health Prod., 46(1):73-8.

Alton, G. G., L.M. Jones, R.D. Angus, and J.M. Verger, 1988. Techniques for the Brucellosis laboratory. INRA publication, France.

Alvarez J, J.L. Sáez, N. García, C. Serrat, M. Pérez-Sancho, S. González, M.J. Ortega, J. Gou, L. Carbajo, F. Garrido, J. Goyache, L. Domínguez, 2011. Management of an outbreak of brucellosis due to *B. melitensis* in dairy cattle in Spain. Res. Vet. Sci., 90:208 –211.

Arenas, G.N., A.S. Staskevich, A. Aballay and L. S. Mayorga, 2000. Intracellular trafficking of *Brucella abortus* in J774 macrophages. Infect. Immun., 68: 4255–4263.

Baily, G.G., J.B. Krahn, B.S. Drasar, and N.G. Stocker, 1992. Detection of *Brucella melitensis* and *B. abortus* by DNA amplification. J. Trop. Med. Hyg., 95: 271-275.

Bancroft J.D. and A. Stenes, 1982. Electron microscopy 2: transmission (A) tissue preparation, (B) section and staining in: Theory and practice of histological technique, 2nd Ed., Robinson, G; Churchill Livingstone inc., New York pp: 482-518.

Borriello, G., R. Capparelli, M. Bianco, D. Fenizia, F. Alfano, F. Capuano, , D. Ercolini, , A. Parisi, S. Roperto, and D. Iannelli, 2006. Genetic Resistance to *Brucella abortus* in the Water Buffalo (*Bubalus bubalis*) Infection and Immunity, 74(4): 2115–2120.

Chand, P., H.V. Batra, and R.J. Sadana, 1988. Detection of Brucella specific protein –A reactive antibodies in buffalos by dot-enzyme-linked immunosorbent assay. Vet. Rec., 121: 162-163.

Corbel, M. J. 1997. Recent advances in Brucellosis. J. Med. Microbiol., 46: 101-103.

Corbel, M.J., and W.J. Brinley-Morgan, 1984 Genus *Brucella* Meyer and Shaw 1920, 173AL, p. 377–388. *In* N. R. Krieg and J. C. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. Williams and Wilkins Co., Baltimore, Md.

Diaz, R and I. Morityon, 1989. Laboratory techniques in the diagnosis of human Brucellosis. In:" Brucellosis: Clinical laboratory aspects of human infection". Young, E. J., and Corbel, J. M., (Eds). CRC Press, Boca Raton. 73-83.

Di-Giannatale E., F. De Massis, M. Ancora, K. Zilli, A. Alessiani, 2008.Typing of Brucella field strains isolated from livestock populations in Italy between 2001 and 2006. Vet. Ital., 44:383–388.

El-Taweel, A.H., 1999. Brucellosis Information workshop, Ram Allah, Oct. 19-20, Country reports.

Enright, F.M., 1990. The pathogenesis and pathobiology of Brucella infection on domestic animals. In: Nielsenand K. pp. 301–320. (CRC Press, Boca).

Essmail, M. E., I. G. Ibrahim and M. H. Yassen, 2002. Immunohistochemical detection of Brucella antigens in formalin-fixed, paraffin-embedded tissues of Buffaloes. J. Egypt. Vet. Med. Ass., 62: 127-136.

Fekete, A., J.A. Pantle, S.M. Halling and R.W. Stick, 1992. Amplification fragment length polymorphism in Brucella strains by use of polymerase Chain Reaction with arbitrary primers. J. Bacteriol., 174: 7778-7783.

Fink, S.L. and B.T. Cookson, 2005. Apoptosis, pyroptosis and necrosis mechanistic description of dead and dying eukaryotic cells.Infect. Immun., 73:1907-1916.

Fosgate, G.T., M.D. Diptee, A. Ramnanan, A.A. Adesiyun, 2011. Brucellosis in domestic water buffalo (*Bubalus Bubalis*) of Trinidad and Tobago with comparative epidemiology to cattle. Tropical Animal Health and Production, 43: 1479–1486.

Franco M.P., M. Mulder, R.H. Gilman, H.L. Smits, 2007. Human brucellosis. Lancet Infect. Dis., 7:775–786.

Ghazi Y.A., K.A. Abd El-Razik and M.B. Kadry, 2006. Evaluation of Brucella diagnostic techniques in the Egyptian buffaloes. Proc. 3rd Inter.Conf.Vet.Res.Div., NRC, Cairo, Egypt, pp.23-34.

Haines, D.M. and E.G. Clark, 1991. Enzyme immunohistochemical staining of formalin-fixed tissues for diagnosis in veterinary pathology. Can. Vet. J., 32: 295-302.

Haines, D.M. and K.H. West, 2005. Immunohistochemistry: Forging the links between immunology and pathology. Vet. Imm. and Immunopath., 108: 151–156.

Islam M.A., M.N. Khatun, S.R. Were, N. Sriranganthan, S.M. Boyle, 2013. A review of Brucella seroprevalence among humans and animals in Bangladesh with special emphasis on epidemiology, risk factor. Vet. Microbiology, 166(3-4):317-326.

Laing, J. A., W. J. Morgan, and W. C. Wagner, 1988. Brucellosis In: Fertility and infertility in veterinary practice. Corbel, M. J., Edited by English book language book society, Bailliere, Tindall., pp. 189-220.

Leary, O. S., M.B. Sheahan, T.A. Sweeney, 2006. *Brucella abortus* detection by PCR assay in blood, milk and lymph tissue of serologically positive cows. Research in Veterinary Science, 81: 170–176.

Leyla, G., G. Kadri, O.K. Umran, 2003. Comparison of Polymerase Chain Reaction and bacteriological culture for diagnosis of sheep Brucellosis using aborted fetus samples. Vet. Microbiol., 93: 53-61.

Majno, G. and I. Joris, 1995. Apoptosis, oncosis and necrosis .An overview of cell death. Am.J.Path.,146: 3 - 5.

Meador, V.P., B.L. Deyoe and N.F. Cheville, 1989. Pathogenesis of *Brucella abortus* infection of the mammary gland and supramammary lymph node of the goats. Vet. Pathol., 26: 357-368.

Meador, V.P., L.B. Tabatabaia, W.A. Hagermoser and B. Deyoe, 1986. Identification of *Brucella abortus* in formalin fixed, paraffin embedded tissues of does, goats, and mice with Avidin-Biotin-peroxidase complex immuno enzymatic staining technique. Am. J. Vet. Res., 47: 2147-2150.

Mikolon, A.B., I.A. Gardner, S.K. Hietala, J.H. Anda, E.G. Pestana, R.S.G. Hennage & A.J. Edmondson, 1998. Evaluation of North America antibody test for diagnosis of brucellosis in goats. J Clin Microbiol., 36(6):1716-1722.

Montasser, A.M., M.E. Hamdy, S.I. Ibrahim, and H.M. Ghobashy, 2002. The impact of low serological antibody titers on diagnosis & control of brucellosis in infected farm animals. J.Egypt.Vet.Med.Ass.,62: 167-170.

Narayanan, P.R., G.S. Acharyulu, P.V. Krishnamurthy, Abdul, Rauoof and S.P. Tripathy, 1983. Evaluation of ELISA as a diagnostic test in pulmonary tuberculosis: Indian J. T.B., 30(1): 29-32.

Osoba, A.O., H. Balkhy, Z. Memish, M.Y. Khan, A. Al-Thagafi, B. Al-Shareef, A. Al-Mowallad, G.A. Oni, 2001. Diagnostic value of Brucella ELISA IgG and IgM in bactereamic and non-bactereamic patients with brucellosis. J Chemother. 13 Suppl., 1:54-9.

Pappas G, P. Papadimitriou, N. Akritidis, L. Christou, E.V. Tsianos, 2006. The new global map of human brucellosis. Lancet Infect. Dis., 6:91–99.

Pei, J. and T.A. Ficht, 2004. *Brucella abortus* rough mutants are cytopathic for macrophages in culture. Infect. Immun., 72:440 - 450.

Pei, J., J.E. Turse, Q. Wu, and T.A. Ficht, 2006. *Brucella abortus* rough mutants induce macrophage oncosis that requires protein synthesis and direct interaction with macrophage. Infec. Immun., 74:2667-2675.

Perez, J., M. Quezada, J. Lo´pez , O .Casquet, M.A. Sierra and J. Martin de lasMulas, 1998. Immunohistochemical detection of *Brucella abortus* antigens in tissues from aborted bovine fetuses using a commercially available polyclonal antibody. J .Vet .Diagn. Invest., 10:17–21 .

Refai, M., S. El-Gibaly, and S. Salem, 1989. Brucellosis in cow and Buffalos in Egypt. In: Advances in Brucellosis Research. L. G. Adams. Ed.) Texas A&M Univ. Texas. USA. ISBN 0-89096-447.

Refai, M.K., 2003. Brucellosis in animals and man in Egypt. Egypt. J. Vet. Sci., 1-31.

Romero C., C. Gamazo, M. Pardo and I. Lo'pez-gon, 1995. Specific detection of Brucella DNA by PCR J. Clin. Microbiol., 33:615-617.

Rust, R.S., K.S. Aashit, T.P. Francisco, P.T. Florian, 2004. Brucellosis E. medicine Instant Access to the mind of medicine.

Sambrook, J., E.F. Fritsch, T. Maniatis, 1989. Molecular Coloning: A laboratory Manual, 2nd Edition. Cold Spring Harbor Laboratory Press, New York, pp. 2100.

Sayour, A.E. 1995. An approach towards the use of some unconventional Serological tests for the diagnosis of brucellosis. M.V.Sc. Thesis, Microbiol. Fac.Vet.Med.Cairo Univ.

Saz, J.V., M. Beltrán, A. Díaz, A. Agulla, F.J. Merino, P.A. Villasante, A.C.Velasco, 1987. Enzyme-linked Immunosorbent Assay for diagnosis of brucellosis. Eur. J. Clin. Microbiol., 6 (1):71-74.

Siftuentes-Rincon A.M., A. Revol and H.A. Barrera-Saldana, 1997. Detection and differentiation of the six Brucella species by polymerase chain reaction. Mol. Med., 3:734-739.

Silva J.B. C.P. Rangel, A.H. Fonseca, E. Morais, W.M.S. Vinhote, D.H.S. Lima, N.S. Silva and J.D. Barbosa, 2013. Serological survey and risk factors for brucellosis in water buffaloes in the state of Pará, Brazil. Tropical Animal Health and Production 46(2):385-9.

Staak, C., A. Drager, P. Bahn, and K. Nockler, 2000. Reacting antibodies in Brucellosis serology. 1. Reaction with various Yersinia serotypes and antibody avidity. Berl. Munch Tierazil Wochenschr., 113 : 361-367.

Sting, R. and G. Ortmann, 2000. Experience with simple ELISA test system for Brucella serology in cattle sheep & goats. Berl Munch Tierarztl Wochenschr.,113(1):22-28.

Waghela, S., J.G. Wandra, and G. Wagner, 1980. Comparison of four serological tests in the diagnosis of Caprine brucellosis. Res. Vet. Sci., 28:168-171.

Xavier, M.N., T.A. Paixao, F.P. Poester, A.P. Lage and R.L. Santos 2009. Pathological, immunohistochemical and bacteriological study of tissues and milk of cows and fetuses experimentally infected with *Brucella abortus*. J. Com. Path., 140:149-157.

Presumptive diagnosis of brucellosis can be made by demonstrating high or rising antibody titers to Brucella antigens. Diagnosis is confirmed with the isolation of the organism from blood, bone marrow, or tissue cultures. Evidence in support of the diagnosis of Brucellosis includes: A history of recent exposure to a known or probable source of Brucella spp.Â Prevention of brucellosis in humans: Key points. The prevention of human brucellosis is based on occupational hygiene and food hygiene. The best way to prevent brucellosis infection is to be sure you do not consume undercooked meat and unpasteurized dairy products, including milk, cheese and ice cream. All dairy products should be prepared from heat-treated milk. Bacteriological and serological techniques The blood cultures were processed inside a class III biological safety cabinet [7] using biphasic blood culture medium (BioMerieux, France) and were incubated at 37 â—¦ C in an atmosphere of 5â€”10% carbon dioxide for 30 days; sub-culturing was performed weekly. Suspected colonies were identiï¬ed according to standard techniques [9]. For serology, all of the sera from the patients and controls were tested using SAT and ELISA for IgM and IgG antibodies against Brucella species.Â The sequences of these primers were forward. Serological and molecular diagnosis of human brucellosis in Najran, Southwestern Saudi Arabia Table 1. Distribution of laboratory tests results according to the type of brucellosis. Laboratory test.