

Molecular and Serological Studies on Detection of *Brucella Species* in Cattle and Buffaloes

¹Mohamed Wael Abd Al-Azeem, ²Laila Mustafa Elmalt, ³Alaa El Dein Zain El Abdein and ³Haitham Helmy Sayed

¹ Microbiology Department, Faculty of Veterinary Medicine, South Valley University

² Food Hygiene Department, Faculty of Veterinary Medicine, South Valley University

³Theriogenology Department, Faculty of Veterinary Medicine, Sohag University

ABSTRACT

Brucellosis is one of the most important diseases affecting both human and animal in most of the developing countries. It causes important economic losses. Rapid and accurate diagnosis is fundamental for control and eradication of brucellosis.

A total of 150 milk samples (three samples from each animal included in the study) and 50 blood samples were collected from 32 cows and 18 buffaloes suspected to be infected or had a history of brucellosis from villages and cities of Sohag, Beni-Suef and Giza governorates in Egypt. All tested animals gave a negative reactions to all serological test that included Buffered acidified plate antigen test (BAPAT), Rose bengal plate test (RBPT), Tube agglutination test (TAT) and Rivanol test (Riv.T).

Bacteriological and molecular examination of milk samples revealed detection of *Brucella* organisms in 40.6% and 53.1% from the cow's samples by the two examinations respectively and in 38.9% and 55.6% from the buffalo's samples by the two examinations respectively. All the isolated brucella organisms were *B. melitensis*.

Out of 32 cow 's serum samples examined by BAPAT, RBPT, TAT and Riv.T, antibodies against *Brucella* were found in 32 samples (100%), 30 samples (93.8%), 29 samples (90.6%) and 27 samples (84.3%) by these tests respectively. Out of 32 cow 's milk samples examined by MRT, antibodies against brucella were found in 28 samples (87.5%). On the other hand, Out of 32 whey samples examined by wBAPAT, wRBPT, wTAT and wRiv.T, antibodies against *Brucella* were found in 25 samples (78.1%), 24 samples (75.0%), 24 samples (75.0%) and 23 samples (71.9%) by these tests respectively.

Out of 18 buffalo 's serum samples examined by BAPAT, RBPT, TAT and Riv.T, antibodies against *Brucella* were found in 18 samples (100%) by BAPAT and in 16 samples (88.9%) by each from RBPT, TAT and Riv.T.

Out of 18 buffalo 's milk samples examined by MRT, antibodies against *Brucella* were found in 15 samples (83.3%). On the other hand, Out of 18 whey samples examined by wBAPAT, wRBPT, wTAT and wRiv.T, antibodies against *Brucella* were found in 14 samples (77.8%) by each of them.

Key words: - Brucellosis - Buffalo – PCR – Serology – Milk.

INTRODUCTION

Brucellosis particularly caused by *B. melitensis*, is endemic in Egypt, presumably affecting large numbers of animals as well as humans. It appears to be of particular risk in rural communities, especially in Upper Egypt (Molina-Flores, 2010). In many countries of the region, brucellosis continues to be reported in almost all domestic animals, particularly sheep, goats and cattle (FAO, 2010).

Brucellosis is caused by gram-negative coccobacilli of genus *Brucella* (Corbel, 1997). *Brucella* organisms are transmitted from the infected animals to man by ingestion of unpasteurized milk and milk products, by contact with infected animals or their discharges or by inhalation of aerosols containing *Brucella* organisms (Refai, 2003). Ingestion of unpasteurized animal milk (goat, sheep, cow or camel) or its products as soft cheese, account for most cases worldwide (Franco et al., 2007). Some specific occupational groups including farm workers, veterinarians, ranchers, and meat-packing employees are considered at higher risk (Tabak et al., 2008).

*Corresponding Author: Mohamed Wael Abd Al-Azeem, Microbiology Department, Faculty of Veterinary Medicine, South Valley University, Qena, Egypt. E-mail: Weal_2000uk@yahoo.co.uk

Diagnosis of brucellosis is the corner stone for its proper eradication and control (Alton *et al.*, 1988). Methods of diagnosis of brucellosis comprise tests for isolation and identification, tests for direct demonstration of the agent, its antigens or DNA, tests for detection and estimation of antibodies induced in response to the agent, as well as the allergic test, brucellin. Diagnosis of brucellosis is usually made by bacteriological and immunological tests (Refai, 2003).

The aims of the present study are:

- 1) Applying a multiplex PCR technique as an accurate method for detection and identification of *Brucella spp.* and identification of isolated *Brucella spp.* from cow and buffaloes infected with brucellosis in some areas of Upper Egypt.
- 2) Evaluation of the used serological tests (BAPAT, RBPT, TAT and Riv.T) in addition to, evaluation of milk ring test and whey serological tests (wBAPAT, wRBPT, wTAT and wRiv.T) for detection of *Brucella spp.* from cow and buffaloes infected with brucellosis

MATERIALS AND METHODS

This study was carried out on 32 cows and 18 buffaloes suspected to be infected or had a history of brucellosis from villages and cities of Sohag, Beni-Suef and Giza governorates, also these animals had no history of vaccination against *Brucella*. Each animal gave positive reaction to one serological test at least from Buffered acidified plate antigen test (BAPAT), Rose bengal plate test (RBPT), Tube agglutination test (TAT) and Rivanol test (Riv.T).

1- Sampling:-

A) Collection of milk samples:-

Three milk samples were collected from each animal included in the study, one for bacteriological isolation, the second for PCR assay and the third for Milk ring test (MRT) and preparation of whey. The samples were collected in a sterile screw capped McCartney bottles.

B) Collection of blood samples

The blood samples were collected from Jugular vein after disinfection site of injection by ethyl alcohol 70%. About 8-10 ml of blood is collected from each animal in a plain vacutainer tube. The samples were coded and transferred to the laboratory with the minimal delay in an insulated ice box.

2- Serological examination:-

For serological examination of serum and milk, sera were prepared according to Alton *et al.*, 1988 and milk samples were also prepared according to Morgan *et al.*, 1978. The assigned tests were carried out on all samples as follow:

- A- Milk Ring Test (Alton *et al.*, 1988).
- B- Rose Bengal Plate Test (RBPT) (Alton *et al.*, 1988).
- C- Buffered Acidified Plate Antigen Test (BAPAT) (Alton *et al.*, 1988).
- D- Standard Tube Agglutination Test (STAT) (European method) (Alton *et al.*, 1975).
- E- Rivanol Test (Riv.T) (Alton *et al.*, 1988).

The test was performed on milk whey and serum samples

3- Isolation and identification of *Brucella* isolates:

A- Culture of milk samples (Alton *et al.*, 1988)

Brifely, the milk sample was centrifuged at 3000 rpm for 10 minutes to obtain the sediment - cream mixture (Alton *et al.*, 1988) which then was cultured on duplicated plates of *Brucella* selective medium. The plates were incubated in presence of 5-10% CO₂ and in normal air condition at 37 °C for up to 2 weeks. The *Brucella* isolates were identified through morphological characters of the colonies, microscopical appearance, CO₂ requirement, biochemical characteristics, growth in presence thionin and basic fuschin dyes, and agglutination with *Brucella* anti-sera A and M

B- Identification and typing of *Brucella* isolates:-

- I- Morphological characteristics of the colonies (Alton et al., 1975) this included the following techniques which involved direct examination of the colonies and acriflavine test .
- II - CO₂ requirement (Alton et al., 1988)
- III - Microscopical examination:- according to (Cruickshank et al.1982).
- IV – Biochemical identification:-

Catalase test (Alton et al., 1988), Oxidase test by Kovacs' method (Steel, 1961), Urease test (Alton et al., 1988), Production of H₂S (Alton et al., 1988), serological identification using reaction with *Brucella* control positive and negative sera and reaction with mono-specific sera A & M (Alton et al., 1988) and the growth in presence of different concentrations of Thionin and Basic fuchsin dyes (Alton et al., 1988)

4- Molecular examination (for milk samples):-

A- Extraction of DNA from milk samples for PCR assay:-

DNA was extracted from milk in a biohazard safety cabinet class II (N-biotek, model no. NB-602 wsl, China) by using QIAamp DNA Purification from body fluids protocol (Spin one) with application some essential modifications on it, according to (Vitale et al., 1998)

B- DNA amplification:-

PCR assay was made in 25 µl of a reaction mixture according to the instructions mastermix manufacturer containing 12.5 µl GoTaq® Hot Start Green Master Mix (Promega), 0.2 µl from each of *B. abortus*-specific primer, *B. melitensis*-specific primer and *B. suis*- specific primer, 0.6 µl IS711-specific primer, 1–5 µl extracted DNA and Nuclease-Free Water to complete the mixture 25 µl.

The samples were cycled 35 times, each cycle consists of 1.15 minutes at 95 °C, 2.0 minutes at 55.5 °C and 2.0 minutes at 72 °C in a thermocycler (Biometra, TProfessional Basic, Germany). After the last cycle, the reaction mixtures were incubated for an additional 5 min at 72 °C before they were stored at 4 °C. It is worthy to mention that many trials were made to reach to the optimum conditions for PCR assay. *B. abortus*, *B. melitensis* and *B. suis* primers were used according to Bricker and Haling (1994) and were kindly offered from The Institute for Critical Technology and Applied Science, Virginia Polytechnic Institute and State University, Blacksburg, USA.

(Table 1) shows the sequences of the oligonucleotide primers used in PCR assay.

Table (1): Sequences of the oligonucleotide primers used in PCR assay are in the following.

primer	Sequence(5'-3')
<i>B. abortus</i> -specific	GAC-GAA-CGG-AAT-TTT-TCC-AAT-CCC
<i>B. melitensis</i> -specific	AAA-TCG-CGT-CCT-TGC-TGG-TCT-GA
<i>B. suis</i> -specific	GCG-CGG-TTT-TCT-GAA-GGT-TCA-GG
<i>IS711</i> -specific	TGC-CGA-TCA-CTT-AAG-GGC-CTT-CAT

C- Detection and identification of PCR product.

10 ul of PCR products was analyzed by electrophoresis (Biometra, P-30-BI 030090,Germany) through 1.5 % agarose gel stained with etidium bromide solution(0.5 mg/ml) and visualized under an ultraviolet transilluminator and photographed. Visible band of appropriate size of 498 bp for *B.abortus* and 731 bp for *B.melitensis* were considered positive.

RESULTS AND DISCUSSION

Brucellosis particularly caused by *B. melitensis*, is endemic in Egypt, presumably affecting large numbers of animals as well as humans. It appears to be of particular risk in rural communities especially in Upper Egypt (Molina-Flores, 2010).

Rapid and accurate diagnosis is fundamental for control and eradication of brucellosis (Alton et al., 1988; Refai, 2003). Culture provides the definitive diagnosis of brucellosis and it is considered the gold standard method for it (Alton et al., 1988). Because of difficulty of performing culture in the field, its consuming for the time, its health hazard and lack sensitivity of the most culture procedures, the serological

tests are the main tools used for detection of *Brucella* infection in animals (Refai, 2003). Although it is well known that there is no single serological test gave high sensitivity, and antigen combination showed 100% sensitivity and specificity simultaneously (Munoz et al., 2005). The limitations of both isolation and serological detection procedures have resulted in increasing the use of PCR-based methods for detection and identification of *Brucella* species due to their accuracy, sensitivity, speed and ability to work with DNA as opposed to highly infectious live cultures (Foster et al., 2008).

By comparing results of PCR assay with culture method for detection / isolation and identification of *Brucella* organisms from cow's and buffalo's milk samples as shown in Table 1 the study revealed that PCR assay detected and identified more *Brucella* positive samples than those by culture method from both cow's and buffalo's milk samples where PCR detected and identified *B. melitensis* from 17 (53.1%) and 10 (55.6%) samples out of the examined 32 cow's and 18 buffalo's milk samples, respectively while by conventional culture method, *B. melitensis biovar 3* was isolated and identified only from 13 (40.6%) and 7 (38.9%) samples from the same samples, respectively. The same finding was reached by Hamdy and Aminy (2002) that examined 52 milk samples by culture and PCR assay and detected *Brucella* organisms in 29 and 24 samples from them respectively and by Ibrahim et al. (2002) that examined 108 milk samples by culture and PCR assay and detected *Brucella* organisms in 59 and 30 samples respectively. Unlike finding of this study, Romero et al. (1995) and O'Leary et al. (2006) found that culture method detected more positive samples than PCR assay.

Table (1): Detection of *Brucella* organisms in milk samples by culture method and PCR assay.

Milk samples	No. of samples	Positive samples			
		Culture		PCR assay	
		No.	%	No.	%
Cow's samples	32	13	40.6	17	53.1
Buffaloe's samples	18	7	38.9	10	55.6

The higher sensitivity of PCR assay than the culture method in this study may be attributed to the high sensitivity of PCR to detect fewer number of *Brucella* organisms present in the milk sample than that can be detected by culture (Leal-Klevezas et al., 1995; Hamdy and Aminy, 2002). Also, PCR detects DNA which present in both living and dead *Brucella* organisms while culture detects only the living organisms (Hamdy and Aminy, 2002) and/or to occurrence of false-negative bacteriological results due to massive contamination of the milk samples or from inhibition of some *Brucella spp.* in the selective medium that are major factor that limit the use of conventional bacteriological methods. In these circumstances *Brucella* DNA can still be detected by PCR assay (Romero et al., 1995). Here, it must be mentioned that survival of *Brucella* organisms in milk is altered by the change in pH and storage temperature (Hamdy, 1992) and that *Brucella* content of milk depends on stage of the lactation (El- Berg, 1981) and infection (Romero et al., 1995).

Romero et al. (1995) and O'Leary et al. (2006) attributed the lower sensitivity of PCR assay than culture to presence of a number of *Brucella* organisms below the detection limit of the used PCR, degradation of target DNA in the sample (s) and/or to presence of polymerase inhibitors in the milk sample (s) and using inefficient *Brucella* DNA extraction protocol. Here, it is worthy to mention that in this study, essential modifications were made for the DNA extraction protocol provided by DNA extraction Kit manufactures according to Vitale et al. (1998) to make *Brucella* DNA extraction from milk samples more efficient and avoid polymerase inhibitors present in them. Romero and Lopez-gon (1999) proved that the use of high concentrations of proteinase K, and application of high temperatures of incubation was necessary for the efficient extraction of *Brucella* DNA from milk samples.

From our results the incidence of cows positive samples in both culture (40.6 -38.9) and PCR assay (53.1 -55.6) more than the incidence of buffaloes positive samples and this may be attributed to the susceptibility of cows to infection with the brucellosis more than buffaloes.

Table (2): PCR assay, culture method and different serological test results for milk and serum samples

Animal species	No. of Samples	PCR assay	Culture	BAPAT	RBPT	TAT	RIV.T
		P	P	P	P	P	P
Cows	32	17	13	17	17	17	17
Buffaloss	18	10	7	10	10	10	10

PCR = Polymerase Chain Reaction

BAPAT = Buffered Acidified Plate Antig

RBPT = Rose Bengal Plate Test.

Riv. T = Rivanol Test.

TAT = Tube Agglutination Test.

P = positive

From data illustrated in Table 2, the study revealed that all milk culture- positive samples either from cows or buffaloes were PCR positive. The same finding was reached by Hamdy and Aminy (2002) and Al-Mariri and Haj-Mahmoud (2010). This indicates that specificity of the PCR (100%) is highly satisfactorily to be used as confirmatory test for diagnosis of brucellosis.

By comparing results of PCR assay and culture method with those of serological tests for detection of cows and buffaloes infected with brucellosis as shown in Table 2, Our study revealed that while all positive milk samples by PCR and culture method were obtained from serologically positive animals, brucella organisms were not detected from all the serologically positive animals either by PCR or culture method. The same finding was reported by Hamdy and Aminy (2002) and Marianelli et al. (2008). This may be attributed to that excretion of brucella organisms in milk from the infected animals is intermittent (Morgan and Mackinon, 1979; Alton et al., 1988).

Concerning to *Brucella spp.* that were identified either by PCR or culture method, the study revealed that all the isolates from both cow's and buffalo's milk samples were *B. melitensis*. The same finding was found by Ali et al. (1993) in Assiut governorate, Abdel-wahab (2005) in Monofia governorate, Abdel-Hamid et al. (2008) in Assiut governorate and by Samaha et al. (2008) in Giza, Benisuef, Assiut, Alexandria, Behera, Monofia and Qalioubia governorates

Isolation of *B. melitensis* indicates that it is still the prevalent species in cattle and buffaloes. Increasing *B. melitensis* infections of cattle and buffaloes in Egypt may be attributed to presence of mixed populations of sheep, goats, cattle and buffaloes in the villages, high prevalence rates of *B. melitensis* infections in sheep and goats and the lack of control programs in such animals yet and subsequently its transmission to cattle and buffaloes (Refai, 2002), this in addition to that most sheep or goat flocks in Egypt are mobile and subsequently the infected flocks can contaminate pastures and spread the disease to the other animals in another herds or areas (Samaha et al., 2008). Transmission of *B. melitensis* from sheep and goats to cattle and buffaloes increases risk of causing human infections where milk and milk products of cows and buffaloes are more widely consumed by human than those of sheep and goat (El-Berg, 1981), in addition to that the major route of human infection with brucellosis is consumption of raw milk or its products (Christie, 1987).

Table (3): Results of different serological tests for cow's and buffaloes serum.

Animal species	No. of sample	BAPAT		RBPT		TAT		Riv.T	
		No. of positive samples	%	No. of positive samples	%	No. of positive samples	%	No. of positive samples	%
Cows	32	32	100	30	93.8	29	90.6	27	84.3
Buffaloes	18	18	100	16	88.9	16	88.9	16	88.9

BAPAT = Buffered Acidified Plate Antigen Test.

RBPT = Rose Bengal Plate Test.

TAT = Tube Agglutination Test.

Riv. T = Rivanol T

From data illustrated in Table 3 for results of the different serological tests which were applied on cow's serum samples, the study revealed presence of the antibodies against *Brucella* organisms in 32 cows (100%), 30 cows (93.8%), 29 cows (90.6%) and 27 cows (84.3%) from the examined 32 cows by BAPAT, RBPT, TAT and Riv.T respectively. Very lower ratios of positive reactors by the same tests were reported by Samaha et al. (2008) that detected antibodies against *Brucella* organisms in 107 cows (5.44%), 98 cows (4.98%), 93 cows (4.73%) and 88 cows (4.48%) when they examined 1966 cows by BAPAT, RBPT, TAT and Riv.T, respectively. The very higher ratios of positive cows in this study can be attributed to sampling of that this study was carried out on cows suspected to be infected or had a history of brucellosis not on cows randomly.

From data illustrated in Table 3 results of different serological tests which were applied on buffalo's serum samples, the study revealed the presence of the antibodies against *Brucella* organisms in 18 buffaloes (100%) by BAPAT and 16 buffaloes (88.9%) by each from RBPT, TAT and Riv.T. Very lower ratios of positive reactors by the same tests were reported by Abdel-Hamid et al. (2008) that detected antibodies against *Brucella* organisms in 43 buffaloes (26.9%) by BAPAT and in 31 buffaloes (19.4%) by RBPT, TAT and Riv.T, the very higher ratios of positive buffaloes in this study can be attributed to that this study was carried out on buffaloes suspected to be infected or had a history of brucellosis not on buffaloes randomly.

From data illustrated in Table 4 for results of milk ring test and different whey serological tests which

were applied on cow's milk samples and its whey respectively, the study revealed the presence of the antibodies against *Brucella* organisms in 28 samples (87.5%), 25 samples (78.1%), 24 samples (75.0%), 24 samples (75.0%) and 23 samples (71.9%) from the examined 32 samples by MRT, wBAPAT, wRBPT, wTAT and wRiv.T respectively. Lower ratios of positive reactors by the same tests were reported by Hamdy (1997) that examined milk and whey of 51 serologically positive cows by MRT, wBAPAT, wRBPT, wSAT and wRT and detected antibodies against *Brucella* organisms in 66.6%, 54.9%, 39.2%, 50.9% and 49% of them respectively. While the result in buffalo's milk samples and its whey respectively, also the study revealed presence of the antibodies against brucella organisms in 15 samples (83.3%) by MRT and in 14 samples (77.8%) by each from wBAPAT, wRBPT, wTAT and wRiv.T respectively. This differs from the findings of Sedek *et al.* (2004) that could not detect any antibodies against *Brucella* organisms when they examined 50 buffalo's whey samples from Assiut governorate by wBAPAT, wRBPT, wTAT and wRiv.T.

Table (4): Results of milk ring test and different serological tests applied on cow's and buffaloes milk samples

Animal species	No. of sample	MRT		W BAPAT		W RBPT		WTAT		W Riv.T	
		No. of positive samples	%	No. of positive samples	%	No. of positive samples	%	No. of positive samples	%	No. of positive samples	%
Cows	32	28	87.5	25	78.1	24	75	24	75	23	71.9
		15	83.3	14	77.8	14	77.8	14	77.8	14	77.8
Buffaloes	18	15	83.3	14	77.8	14	77.8	14	77.8	14	77.8

MRT = Milk Ring Test

wBAPAT = whey Buffered Acidified Plate Antigen Test.

wTAT = whey Tube Agglutination Test.

wRBPT = whey Rose Bengal Plate Test.

wRiv. T = whey Rivanol Test.

This study revealed that each serum serological test gave higher positive reactors than its corresponding whey serological test in addition to that there is no serum serological test negative sample gave positive whey serological test. Obtaining higher positive reactors by serum serological tests than its corresponding whey serological tests this could be attributed to the defatting process during whey preparation since most of the immunoglobulins are present on surface of the fat globules, also removal of solid parts by rennin during whey preparation, the change in the pH and the molecular weight of immunoglobulins could be another additional factors that lead to low sensitivity of whey agglutination tests (Hamdy, 1997; Abdel-Hamid *et al.*, 2008).

In Photo1 PCR products on an agarose gel stained by ethidium bromide following electrophoresis. Lane 1:molecular weight marker; lanes 2-6 :*B.melitensis* DNA were extracted from milk samples ; Lanes 7-9; negative milk samples ;lane 11:positive *B. abortus* DNA control(498) ;Lane 12: negative control. All the *Brucella* organisms detected and identified by PCR were identified as *B. melitensis*.

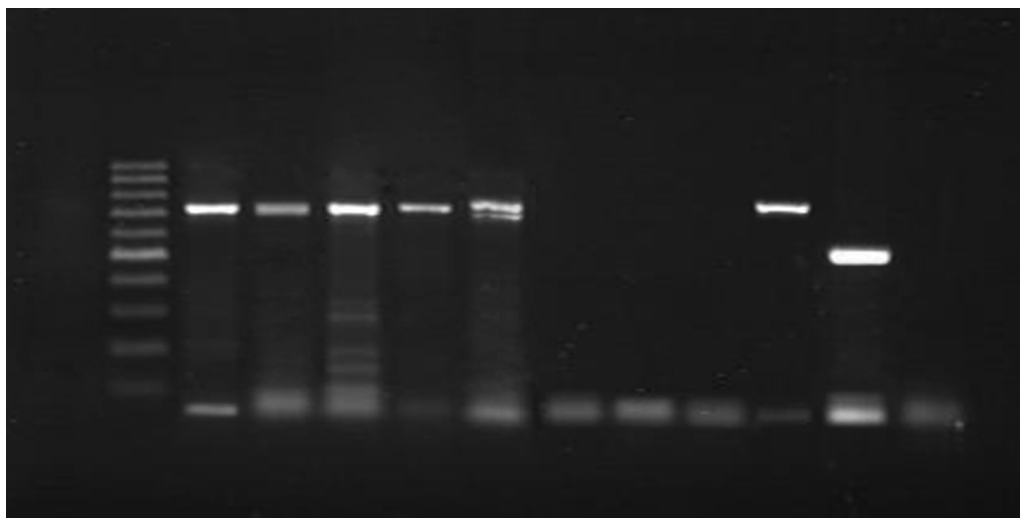


Photo (1): PCR products on an agarose gel stained by ethidium bromide following electrophoresis.

- Lane 1** : Molecular weight marker
Lanes 2 – 6 : *B. melitensis* DNA were extracted from milk sample
Lanes 7 – 9 : Negative milk samples
Lane 10 : Positive *B. melitensis* DNA control (731bp)
Lane 11 : Positive *B. abortus* DNA control (498bp)
Lane 12 : Negative control.

* All the *Brucella* organisms detected and identified by PCR were identified as *B. melitensis*.

Conclusions

According to data obtained, it was concluded that PCR assay is highly sensitive and specific method for detection and identification of *Brucella* organisms from milk samples. Isolation / detection of *Brucella* organisms from milk samples either by culture method or PCR is not recommended to be used alone for diagnosis of brucellosis from milk without the serological tests.

B. melitensis is still the prevalent species in cattle and buffaloes. There is no single serological test can identify all the infected cattle or buffaloes with brucellosis. MRT is more sensitive than whey serological tests in detection of antibodies against *Brucella* in milk samples, although it missed detection some infected animals detected by serum serological tests. The whey serological tests are less sensitive than serum serological tests.

Since eradication of brucellosis from cattle and buffaloes depends mainly on the rapid and accurate diagnosis of the infected animals, also eradication of brucellosis from human depends mainly on its eradication from the animals.

REFERENCES

- Abdel-Hamid, R.F., Mohammed, I.S., Ahmed, S.A., Hassan, E.A. and El-Bauomy E.E.M. (2008): Studies on *Brucella* in milk and some milk products in Assiut city. M.V.Sc. Thesis, Fac. of Vet. Med., Assiut Uni., Egypt.
- Abdel-Wahab, M.A. (2005): Public health importance of brucellosis in El- Menofiya governorate. Ph.D. Thesis, Fac. of Vet. Med. Zagazig Uni., Egypt.
- Al-Mariri, A. and Haj-Mahmoud N. (2010): Detection of *Brucella abortus* in bovine milk by polymerase chain reaction. ACTA Vet. BRNO., (79): 277-280.
- Ali, H.S., Ibrahim, S.L. and Thabet, A. (1993): Some studies on brucellosis in water buffaloes during time

- of abortion at Assiut governorate. *Assiut Vet. Med. J.*, 29 (57): 143-150.
- Alton, G.G., Jones, L.M., Rogerson, B.A. and Mcpherson G.G. (1975): Serological diagnosis of bovine brucellosis an evaluation of complement fixation, serum agglutination and rose bengal test. *Aust. Vet. J.*, (51):57-63.
 - Alton, G.G., Jones, L.M., Verger, J.M. and Angus, R.D. (1988): Techniques for the brucellosis laboratory. *Institute National De Le Recherché Agronomique. (INRA) Publications, Paris, France.* pp. 190.
 - Al-Mariri, A. and Haj-Mahmoud N. (2010): Detection of *Brucella abortus* in bovine milk by polymerase chain reaction. *ACTA Vet. BRNO.*, (79): 277-280.
 - Braun, W. and Bonestell, A.E. (1947): Independent variations of characteristics in *Brucella abortus* variants and their detection. *Am. J. Vet. Res.*, (8): 386:390.
 - Christie, A.B. (1987): Brucellosis (Undulant fever, Malta fever or Mediterranean fever). In: Christie. A. *Infectious Diseases. Vo1. (2), 4th Ed. Churchill Livingstone, Edinburg, London, Melbourne and New Yo*
 - Corbel, M.J. (1997): Brucellosis, an overview. *Emerg. Inf. Dis.*, (3): 213- 221.
 - Cruickshank, R., Duguid, J.P., and Swain, R.H.A. (1982): *Medical Microbiology. 12th Ed. Vol. 2: The practice of medical microbiology. Churchill Livingstone, Edinburgh, UK.*
 - El-Berg, S.S. (1981): A guide to diagnosis, treatment and prevention of human brucellosis. WHO, VPH/81, 31.
 - FAO (2010): An update on transboundary animal diseases in the Near East. The 30th FAO regional conference report, Sudan, (8): 3-20.
 - Foster, J.T., Okinaka, R.T., Svensson, R., Shaw, K., De, B.K., Robison, R.A., Probert, W.S., Kenefic, L.J., Brown, W.D. and Keim, P. (2008): Real-time PCR assays of single-nucleotide polymorphisms defining the major *Brucella* clades. *J. Clin. Microbiol.*, (46): 296–301.
 - Franco, M.P., Mulder, M., Gilman, R.H. and Smits, H.L (2007): Human brucellosis. *Lancet Infect. Dis.*, (7):775–786.
 - Hamdy, M.E. (1997): Correlation between humoral and cellular immune response in dairy cows naturally infected with *Brucella abortus*, *J. Egypt Vet. Med. Assoc.*, 57 (1): 331-349.
 - Hamdy, M.E. and Aminy, A.S. (2002): Detection of *Brucella* species in the milk of infected cattle, sheep, goats and camels by PCR. *Vet. J.*, (163): 299-305.
 - Henry, B.S. (1933): Dissociation in the genus *Brucella*. *J. Infect. Dis.*, (52):374-402.
 - Ibrahim, A.K., Ibrahim, I.G., Ghoneim, M.A. and Awad, W.S. (2002): Evaluation of Polymerase Chain Reaction (PCR) and conventional brucellosis diagnostic techniques in milk samples from different animal species. *J. Egypt. Vet. Med. Assoc.*, 62 (2): 119-131.
 - in New Zealand. *J. Clin. Microbiol.*, (44): 4363–4370.
 - in New Zealand. *J. Clin. Microbiol.*, (44): 4363–4370.
 - Leal-Klevezas, D.S., Martinez-Vazquez, I.O., Lopez-Merino, A. and Martinez-Soriano, J.P. (1995): Single-step PCR for detection of *Brucella spp.* from blood and milk of infected animals. *J. of Clin. Microbiol.*, (33): 3087–3090

- Marianelli, C., Martucciello, A., Tarantino, M., Vecchio, R., Iovane, G. and Galiero, G. (2008): Evaluation of molecular methods for the detection of *Brucella* species in water buffalo milk. Dairy Sci. J., (91): 3779–3786.
- Molina-Flores, B. (2010): Field experience with the control of *Brucella melitensis* from selected countries. In: *Brucella melitensis* in Eurasia and The Middle East, FAO 2010, (10): 9.
- Morgan, W.J.B. and Mackinon, D.J. (1979): Brucellosis. In Laing J.A. (Ed). Fertility and infertility in domestic animals, 3rd edition. ELBS, Bailliere Tindall, chapter 9, pp. 171-198.
- Morgan, W.J.B., Mackinon, D.J., Gill, K.P., Gower, S.G.M. and Norris, P.I.W. (1978): Brucellosis diagnosis standard laboratory techniques. Ministry of Agriculture, Fisheries and Food. 2nd Ed. Central Vet. Laboratory, New Haw, Weybridge, Surrey, England.
- Munoz, P.M., Marin, C.M., Monreal, D., Gonzalez, D., Garin-Bastuji, B., Diaz, R., Mainar-Jaime, R.C., Moriyon, I. and Blasco, J.M. (2005): Efficacy of several serological tests and antigens for diagnosis of bovine brucellosis in the presence of false-positive serological results due to *Yersinia enterocolitica* O:9. Clin. and diag. laboratory immunol., (12): 141–151.
- O’Leary S., Sheahan, M. and Sweeney, T. (2006): *Brucella abortus* detection by PCR assay in blood, milk and lymph tissue of serologically positive cows. Res. in Vet. Sci., (81): 170-176
- Refai, M. (2002): Incidence and control of brucellosis in the Near East Region. Vet. Microbiol., (90): 81–110.
- Refai, M. (2003): Application of biotechnology in the diagnosis and control of brucellosis in the Near East Region. World J. of Microbiol. and Biotechnology (19):443 - 449.
- Romero, C. and Lopez-gon, I. (1999): Improved method for purification of bacterial DNA from bovine milk for detection of *Brucella spp.* by PCR. Appl. and environ. Microbiol., (65): 3735–3737.
- Romero, C., Pardo, M., Grillo, M.J., Diaz, R., Blasco, J.M., and Lopez-gon, I. (1995): Evaluation of PCR and indirect Enzyme Linked Immunosorbent Assay on milk samples for diagnosis of brucellosis in dairy cattle. J. of clin. Microbiol., (33): 3198–3200.
- Samaha, H., Al-Rowaily, M., Khoudair, R.M. and Ashour H.M. (2008): Multicenter study of brucellosis in Egypt. Emerg. Infect. Dis. J., (14):1916:1918.
- Sedek, O.A., El-Basiony, T.A. and El-Brince, E.E. (2004): Serological studies on milk for diagnosis of *Brucella* infection in Assiut governorate M.V.Sc. Thesis Fac. of Vet. Med., Assiut Uni., Egypt.
- Steel, K. J. (1961): The oxidase reaction as a taxonomic tool J. Microbiol., (25): 297-306
- Tabak, F., Hakko, E., Mete, B., Ozaras, R., Mert, A. and Ozturk, R. (2008): Is family screening necessary in brucellosis? Infection, (36): 575–577.
- Vitale, F., Capra, G., Maxia, L., Reale, S., Vesco, G., and Caracappa, S. (1998): Detection of *Mycobacterium tuberculosis* complex in cattle by PCR using milk, lymph node aspirates, and nasal swabs. J. Clin. Microbiol., (36):1050–1055.