



Raw Milk as a Vehicle of Brucella Infection for Human in El-Behera Province

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ABSTRACT

Brucellosis is a worldwide zoonosis that has major public health concern in Egypt. The present work was conducted to investigate the seroprevalence of brucellosis in milk as well as in serum samples of humans on basis of the Milk Ring Test (MRT) and Rose Bengal plate test (RBPT), respectively and further confirmation by polymerase chain reaction (PCR). A total of 240 raw milk samples (98 from dairy markets, 128 samples from farm with history of mastitis and 14 samples from retailers) were collected during the period extended from November, 2017 to October, 2018 beside another 100 serum samples were collected from patients attending fever hospitals (60), individuals with occupational contact with investigated animals (20) and apparently healthy individuals attending private laboratories in Behera seeking for medical advice (20). It was found that the overall prevalence of brucellosis in milk was 10.8% by MRT. Also, it was observed that sero-prevalence of Brucella Abs in milk samples in relation to sources of milk revealed that the highest rate was recorded in milk samples collected from retailers (14.3%) followed by those collected from dairy farms (12.5%) then those of dairy markets (8.2%) with non-significant association between the rates of detection. Concerning season, there was highly significant relationship between season and percent of infection with Brucella, the high percent of infection found in winter season (15.4%) than in summer season (6.5%). On the other side, the seroprevalence of Brucella Abs in human serum samples by RBPT clarified that the highest detection rate occurred in individuals with occupational contact (25%) followed by patients with fever complain (18.3%) and finally apparently healthy individuals (10%) with significant association between them. Seasonal prevalence in human samples clarified higher summer prevalence (20.5%) compared to winter (16.4%). Moreover, there was significant relationship between age and infection rate in human ($P < 0.05$), with higher percentage of infection recorded in age group over than 40 years by 23.3% (10/43) followed by age group (15-40 years) (16.7%) (6/36) and lastly those less than 15 years (9.5%) (2/21). From our result, it is concluded that MRT and RBPT used as screening tests for detection the prevalence of species in milk and serum samples, Brucella infection is found with high percent in milk in Behera Province highlighting the hazardous role in milk in transmission of Brucella infection to consumers specially those used to drink raw milk in rural areas. In addition PCR is gold confirmatory technique for diagnosis of brucellosis in both milk and blood.

Keywords: *Brucella*; Milk; Serum; Human; Serology; PCR

1. Introduction

Billions of people consume milk and dairy products every day, in Egypt it ranks the second in contributing to the value of animal production (26.66% of the total animal production) (Mahrous 2016). Besides its beneficial effects on nutrition, it represents an ideal nutritive environment for numerous pathogens that might be a source of zoonotic infections as brucellosis, tuberculosis, salmonellosis and listeriosis. Despite of continuous effort for zoonotic brucellosis control, that represents a major public health threats, it remains endemic in the vast majority of Middle East countries, accused of tens of thousands of new cases yearly (Patel et al., 2017). There are about half a million new human cases of brucellosis occur every year worldwide making it the most common zoonosis (Seleem et al., 2010).

Transmission of brucellosis to human occurs through ingestion of the infected products, direct contact with infected animals and its materials and

through inhalation of the infected particles (Dieckhaus and Kyebambe 2017). Brucellosis is caused by Gram negative bacteria belonging to the genus *Brucella* (Cloeckeaert et al., 2003). Studies revealed that the national program under current regulations was not effective in control of brucellosis among animals in Egypt (El-tholth et al., 2016). Human brucellosis is mainly an occupational disease affecting animal caretakers, livestock farmers, artificial inseminators, abattoir workers, meat inspectors and veterinarians due to frequent exposure to infected animals. The common routes of transmission to humans are consumption of unpasteurized dairy products or through direct contact with infected animals, placentas or aborted fetuses.

The most common symptoms are fever, sweating, fatigue, weight loss, headache, and joint pain. Some cases may have neurological complications, endocarditis and testicular or bone abscess formation (Corbel, 2006). Close contact with animals may occur when humans assist animals during parturition or abortion or handling of stillbirth. It is also common for farmers to separate the placenta manually and they are likely exposed to tissues infected with *Brucella* (Agasthya et al., 2007).

The diagnosis of brucellosis is usually performed by a combination of serological and molecular methods. Definitive diagnosis is usually carried out through isolation and identification of the causative organism, but drawback is that it is time-consuming, must be performed by highly skilled personnel, and is hazardous. For these reasons, serological tests like Rose Bengal Plate Test (RBPT) and serum agglutination test are normally preferred (Poester et al., 2010). Diagnosis of brucellosis in humans and animals is mainly based on detection of *Brucella* LPS specific antibodies in milk and serum samples using serological tests (Jennings et al., 2007). The prevalence of human brucellosis in Egypt was estimated by Abou Eisha (2001), Habib et al. (2003), Afifi et al., (2005), Haggag and Samaha (2007) and El-Monir et al., (2016). A prospective study based on Ain Shams hospitals found that brucellosis was the most common infectious cause of fever of unknown origin among Egyptian adults (Ali-Eldin et al., 2011). The present study aimed to investigate the seroprevalence of Brucella Antibodies in raw milk samples and human in El-Behera province.

2. Material and methods

2.1. Samples

2.1.1. Milk samples

A total of 240 raw milk samples (98 from dairy markets, 128 samples from farm with history of mastitis and 14 samples from retailers) were collected during the period extended from November, 2017 to October, 2018. Udder especially teats were cleaned and dried then each teat end was scrubbed with a piece of cotton moistened with betadine. Milk samples were obtained in sterilized jars (500 ml capacity) then placed in an ice box and transported directly to the laboratory of department of Animal Hygiene and Zoonoses, Faculty of veterinary Medicine, Damanhur University to be examined for presence of Brucella antibodies by Milk ring test (MRT).

2.1.2. Human serum samples

A total of 100 serum samples were collected from patients attending fever hospitals (60), individuals with occupational contact with investigated animals (20) and apparently healthy individuals attending private laboratories in Behera seeking for medical advice (20).

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Collection of samples was carried out according to Alton et al., (1988) by allowing 5 ml of blood to flow freely from radial vein of human beings by using sterile dry special double ended needle into a sterilized vacutainer tube in which blood samples were left at room temperature for 30 minutes, then centrifuged at 3000 rpm for 10 minutes and placed in the refrigerator for 24 hours and when the clot retracted, clear serum was obtained by using sterile Pasteur pipette, then kept in Eppendorf tubes and labeled. They were stored at -20° C in the deep freezer till examined serologically by RBPT. Full history from each patient was taken including; age, sex, residence, type of animal contact, manner of milk consumption and health condition

2.2. Serological detection of *Brucella*

2.2.1. Milk Ring Test (OIE, 2008)

The test was performed by adding 30 µl (0.03 ml) of *B. abortus* Bang Ring Antigen (hematoxylin-stained antigen manufactured by VSVRI, Abbassia, Cairo, Egypt). The height of the milk column in the tube was kept up to 25 mm. The milk (antigen) mixtures were incubated at 37°C for 1 h, together with positive and negative control samples. Agglutinated *Brucella* cells were picked up by fat globules as they rose, forming a dark cream layer on the top of the sample. A strongly positive reaction was indicated by formation of a dark blue ring above a white milk column. The test was considered negative if the color of the underlying milk exceeded that of the cream layer and when the cream layer was normal. Samples were read as negative, 1+, 2+, 3+ and 4+ depending on the intensity of color in the cream layer.

2.2.2. Rose Bengal plate test (RBPT)

The test was carried out according to OIE, (2012). Rose Bengal stained *B. abortus* strain 99 cells in lactate buffer (pH 3.65) was obtained from Veterinary Serum and Vaccine Research Institute (RSVRI), Abbassia, Cairo, Egypt. Serum samples to be tested and antigen were brought at room temperature before testing. Using antigen dropper delivering 0.03 ml per drop, one drop of antigen was placed on a dry white enamel plate, then 0.03 ml of the tested serum was placed alongside the antigen. The antigen and serum were thoroughly mixed, and reading was done within 4 minutes rocking period. Results were recorded as follows; no agglutination was negative; any degree of agglutination was positive.

2.3. Molecular detection of *Brucella* by PCR

Application of PCR for detection of the gene derived from insertion sequence 711 (IS711) specific for identification and characterization of *Brucella abortus* at 498 bp and *Brucella melitensis* at 731 bp was performed essentially by using primer sequence according to Bricker, (2002) as shown in Table 1.

Table (1): IS711 gene Primers of *Brucella* used in the PCR reactions

Primer	Oligonucleotide sequence (5' → 3')	Product size (bp)
F*	5' TGCCGATCACTTAAGGGCCTTCAT '3	498
R*	5' GAC GAACGGAATTTTCCAATCCC '3	
F [§]	5' TGCCGATCACTTAAGGGCCTTCAT '3	731
R [§]	5' AAA TCGCGTCTTGCTGGTCTGA'3	

**B. abortus*, §*B. melitensis*

Brucella DNA was obtained from serologically positive milk and whole blood human samples using QIA amp kit according to O'Leary et al. (2006). Amplification reaction for IS711 gene was performed according to Garshasbi et al., (2014) using a Thermal Cycler (Master cycler, Eppendorf, Hamburg, Germany). The PCR reaction was set up in 25µl volume. The PCR reaction was initially optimized by using varying concentrations of molecular biological chemicals and varying cycling conditions: 4.5 µl nucleic acid free water (Fermentas), 2.5 µl 10x PCR buffer, 1.5 µl 25 mM magnesium chloride, 1 µl 10 mM dNTPs, 0.5µl of Taq polymerase (5 U/µl) (Fermentas), 5 µl of 10 µMol forward and reverse primer. The reaction mixture was mixed gently by vortexing and spinning. In the end, 5 µl of DNA extracted from a different sample was added. The amplification programs for *B. abortus* and *B. melitensis* consisted of initial DNA denaturation at 95 °C for 3 min followed by 35 cycles at 95 °C for 90 sec, annealing at 65 °C for 1 min, extension at 72 °C and final extension step of 5 min at 72 °C. The PCR products were electrophoresed in 1.5% of agarose gel electrophoresis (Applichem, Germany, GmbH) in 1X TBE buffer stained with ethidium bromide (2 mg/ml) and captured as well as visualized on UV transilluminator. A 100 bp plus DNA Ladder was used to determine the fragment sizes

3. Results

Table (2): Sero-prevalence of *Brucella* Abs in milk samples in relation to sources of milk

Sources of milk	No. of milk samples	Positive		Chi ² value
		No.	%	
Dairy markets	98	8	8.2	1.55 NS
Farms	128	16	12.5	
Retailers	14	2	14.3	
Total	240	26	10.8	

NS= Non-significant at (P<0.05)

Table (3): Sero-prevalence of *Brucella* Abs in milk samples in relation to seasons of the year by MRT

Seasons	No. of milk samples	Positive		Chi ² value
		No.	%	
Winter	117	18	15.4	6.25**
Summer	123	8	6.5	
Total	240	26	10.8	

** = Significant at (P < 0.01)

Table (4): Seroprevalence of *Brucella* Abs in human serum samples by RBPT

Source of samples	No. of serum samples	Positive		Chi ² value
		No.	%	
Patients attending fever hospitals	60	11	18.3	7.89 **
Apparently healthy individuals	20	2	10.0	
Occupational contact individuals	20	5	25.0	
Total	100	18	18.0	

Table (5): Seroprevalence of *Brucella* Abs in human serum samples in relation to seasons of the year

Seasons	No. of serum samples	Positive		Chi ² value
		No.	%	
Winter	61	10	16.39	7.89 **
Summer	39	8	20.51	
Total	100	18	18.0	

** = Significant at (P < 0.01)

Table (6): Seroprevalence of *Brucella* Abs in human serum samples in relation to age groups

Age groups	No. of serum samples	Positive		Chi ² value
		No.	%	
< 15	21	2	9.5	5.56*
15 - 40	36	6	16.7	
> 40	43	10	23.3	
Total	100	18	18.0	

* = Significant at (P < 0.05)

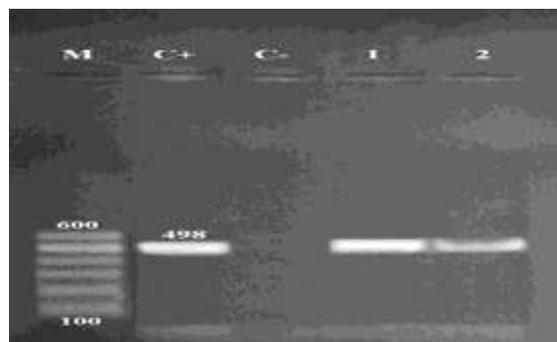


Figure (1): PCR products of IS711 (498 bp) gene specific for identification of *Brucella abortus* isolated from the milk. Lane M: 100 bp ladder as molecular size DNA marker. Lane C+: Control positive *Brucella abortus* for IS711 gene. Lane C-: Control negative. Lanes 1 and 2: Positive *Brucella abortus* strains for IS711 gene.



Figure (2): Identification of amplified fragments of IS711 (731 bp) specific for identification of *Brucella melitensis* detected in human blood. Lane M: 100 bp ladder as molecular size DNA marker. Lane C+: Control positive *Brucella melitensis* for IS711 gene. Lane C-: Control negative. Lanes 1 and 2: Positive *Brucella melitensis* strains for IS711 gene.

4. Discussion

Each year half a million cases of brucellosis are reported worldwide but according to WHO, these numbers are greatly underestimated. Even so, brucellosis is distributed throughout the world wherever livestock are being raised. Likewise, in many less developed countries and in developing countries brucellosis continues to cause major losses in livestock and poses a serious threat to people. As shown in Table (1), it was found that the overall prevalence of brucellosis in milk was 10.8% by MRT. This result was lower than recorded by Cadmus et al., (2008) who found that overall, 18.61% of the milk samples were positive according to the MRT in India while it was higher than that recorded by Kumar et al., (2016) who examined 483 milk samples and found that 4.35% of samples were positive by MRT. Also, it was recorded in Table (1) that seroprevalence of *Brucella* Abs in milk samples in relation to sources of milk revealed that the highest rate was recorded in milk samples collected from retailers (14.3%) followed by those collected from dairy farms (12.5%) then those of dairy markets (8.2%) with non-significant association between the rates of detection. It was concluded that MRT can be used as a spot screening test and the presence of antibodies with their prevalence in milk warrants a systematic preventive strategy that is used to control brucellosis. Data recorded in Table (2) showed that there was highly significant relationship between season and percent of infection with *Brucella*, the high percent of infection found in winter season (15.4%) than in summer season (6.5%). This result agreed with Haggag and Samaha (2007) who found that the highest prevalence was in winter season. On contrary, it disagreed with Nossair (2005) who recorded that most cases occurred in spring season because of moderate atmospheric temperature that permits the survival of *Brucella* organisms in the environment. On the other side, tabulated data in Table (3) showed that the overall prevalence of brucellosis in human by RBPT was 18%. RBPT is a very useful test for the diagnosis of human brucellosis. It needs no complicated infrastructure or sophisticated training; it is exceedingly cheap, highly sensitive and easily adaptable to test serum dilutions (Díaz et al., 2011). The recorded prevalence was higher than recorded by Abou Eisha (2001) (5.1%), Afifi et al., (2005) (11%), Haggag and Samaha (2007) (14 %) and Tumwine et al., (2015) (17.0 %). On the other hand, it was extremely higher than that recorded by Assenga et al., (2015) (0.6 %) and El-Monir et al., (2016) (1.25%). On contrary, it was lower than that recorded by Habib et al. (2003) (37.6%), Chegeni et al., (2014) (29.5%) and Zolzaya et al., (2014) (27.3%) and Diab et al., (2018) (24.3%). This variation in prevalence of brucellosis in humans in the current work and others may be attributed to different geographic locations, variation in occupational contact and the type of used tests.

Data in Table (3) also showed that the highest detection rate of *Brucella* Abs in human serum samples occurred in individuals with occupational contact (25%) followed by patients with fever complain (18.3%) and finally apparently healthy individuals (10%) with significant association between them. The obtained result in the current study was supported by that of Meko et al., (2007) who proved that working with animals and eating ice-cream bought from street vendors were significantly associated with the affections and they concluded that contact with infected animals and their products was the most important method of transmission. Moreover, Tumwine et al. (2015) noticed that prevalence of human brucellosis was parallel with animal prevalence.

Seasonal prevalence in human samples was presented in Table (4) and clarified higher summer prevalence (20.5%) compared to winter (16.4%). This result agreed with De Massis et al. (2005), Nossair (2005) and

Haggag and Samaha (2007) who confirmed that the seasonal occurrence of human cases of brucellosis showed a peak in summer. On contrary, it disagreed with Diab et al., (2018) who noticed that the highest infection rate occurred during winter season. As shown in Table (5), there was significant relationship between age and infection rate in human ($P < 0.05$), with higher percentage of infection recorded in age group over than 40 years by 23.3% (10/43) followed by age group (15-40 years) (16.7%) (6/36) and lastly those less than 15 years (9.5%) (2/21). The increased prevalence in the age group (20 - > 40 years) may be attributed to this age group represents the most active age of work. It was in agreement with Abou Eisha, (2001) who reported that the prevalence of *Brucella* infection increased among the age group 30-39 years and El Mabrouk, (2013) who found that the highest prevalence was found in the age group (30 - < 45 years) (7.5 %) On contrast, it disagreed with the results obtained by Nasinyama et al. (2014) who found that there was no association between sero-positivity with age.

The diagnosis of brucellosis is challenging as culturing of *Brucella* and sero-conversion are time consuming (Al Dahouk and Nockler, 2011). Therefore, molecular techniques like as PCR are promising alternatives for the diagnosis of infectious diseases caused by fastidious or slowly growth microorganisms such as *Brucella*. Application of PCR for detection of the gene derived from insertion sequence 711 (IS711) specific for identification and characterization of *Brucella abortus* at 498 bp and *Brucella melitensis* at 731 bp was performed successfully (Figures 1 and 2). It was clear that PCR assay was a highly sensitive and specific diagnostic method for the detection of *Brucella* in milk and human blood samples.

From our result, it is concluded that MRT and RBPT used as screening tests for detection the prevalence of species in milk and serum samples, *Brucella* infection is found with high percent in milk in Behera Province highlighting the hazardous role in milk in transmission of *Brucella* infection to consumers specially those used to drink raw milk in rural areas. In addition, PCR is gold confirmatory technique for diagnosis of brucellosis in both milk and blood.

Conflict of interests

The authors have not declared any conflict of interests.

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