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OCCURRENCE OF BRUCELLA IN MILK AND CREAM

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ABSTRACT

A total of 200 samples (100 Farmer raw cow's milk, 60 market milk and 40 fresh cream) were collected from individual farmers, different dairy shops and milk separation centers distributed through Beni-Seuf and El-Minia cities, Egypt. The milk samples were subjected to serological test for detection of Brucella antibodies, using Milk ring test (MRT), Whey Buffered acidified plate antigen test (wBAPAT) and Whey Rose Bengal plate test (wRBPT). Out of 100 farmers milk samples examined by MRT. 30%, 7% and 63% were positive, suspicious and negative respectively. Also, 44% and 42% of these samples were positive for wBAPAT and wRBPT, respectively. On the other hand, Out of 60 market milk samples examined by MRT. 23.3%, 8.4% and 68.3% were positive, suspicious and negative, respectively, while 53.4% and 48.4% were positive for wBAPAT and wRBPT, respectively. All samples were examined bacteriologically for presence of Brucella organisms. The prevalence of Brucella species in farmers milk, market milk and cream samples were 28%, 60% and 32.5%, respectively. All isolates were typed as *Brucella melitensis* biovar 3. The public health significance and suggestive control measures were discussed.

Key words: *Brucella, Milk, Cream, Brucella antibodies.*

INTRODUCTION

In Egypt, direct consumption of raw milk is more frequent and more popular than the pasteurized one because it's believed, especially in rural areas, that the raw milk and its byproducts have nutritional advantages over the pasteurized one. Furthermore, milk is produced mainly by individual in small farms that lack proper sanitary measures and may be either consumed fresh, manufactured into dairy products or sell in retail markets that alarming as a major source of food borne brucellosis (El-Sayed *et al.,* 2011) and represent a serious human health problem.

Brucella is excreted in milk, it attains 10^4/ml at the beginning of the lactation and then it decline to 10/ml but may persist during successive lactation periods (Plommet *et al.,* 1988). The Food and Agriculture Organization (FAO), the World Health Organization (WHO) and the Office International des Epizooties (OIE) considered Brucellosis as one of the widest spread zoonotic diseases of domestic and wild animals throughout the world (Schelling *et al.,* 2003 and Thakur *et al.,* 2002). The World Health Organization (WHO) recently estimated that the median global number of cases of foodborne illness due to Brucella infection was 393,239 (Havelaar *et al.,* 2015). The main pathogenic species in both animals and human are *Brucella melitensis, Brucella abortus, Brucella suis,* and *Brucella canis* (Cloeckaert and Vizcaino, 2004 and Araj, 2010), while *Brucella melitensis* is currently the predominant species of Brucella present in Egypt (Holt *et al.,* 2011) precisely *Brucella melitensis* biovar 3 is the most common isolate of Brucella in Egypt (Refai, 2002 and Samaha *et al.,* 2007).

Brucellosis was first reported in Egypt in 1939 and is now considered endemic in most parts of the country (Refai, 2002 and Molina-Flores, 2010), it appear to be of particular risk in rural communities especially in Upper Egypt (Molina-Flores, 2010). Despite its economic and public health importance, the official Egyptian brucellosis control program does not appear to have been fully implemented (Refai, 2002 and Hegazy *et al.,* 2009).

The presence of Brucella organism in milk has conducted by several investigators (Abdel-Hakiem, 1999; Abd-Alla *et al.,* 2000; Meshref, 2000; Abdel-All, 2001; El- Sayed *et al.,* 2011; Abd Al-Azeem *et al.* (2012); Abosira 2015 and El-Diasty *et al.,* 2016).

Diagnosis of Brucellosis is the corner stone of proper eradication of the disease. Isolation of the causative agent is still the land mark for diagnosis of brucellosis (Alton *et al.,* 1988), however; it is

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difficult to recover from life infected animals, therefore diagnosis has been based mostly on the results of serological tests (Hamdy, 1997). It is easier for using milk and milk whey for diagnosing brucellosis as injuring animals for collecting blood samples is difficult (Farag, 1998).

There is limited recent data on the prevalence of *Brucella* organisms in the Upper Egypt. Therefore, the present work was planned to investigate the incidence of Brucella organisms in milk and fresh cream and to throw the light upon the public health significance and preventive and control measures of brucellosis.

MATERIALS AND METHODS

1-Collection of samples:
A total of 200 random samples including Farmers milk (100 raw cow's milk samples were collected from individual farmers in Beni-Seuf and El-Minia cities), Market milk (60 samples were collected from different retail shops and vendors in Beni-Suef and El-Miniacities, Egypt) and Cream (40 samples from the separators in Beni-Seuf and El-Minia cities). All samples were collected in sterilized bottles and transported to the laboratory in an insulated ice box (4-6ºC) and kept in the refrigerator till be examined.

2- Serological examination:
Milk whey samples were prepared according to (Morgan *et al.,* 1978). The assigned tests were carried out on the samples as follow:

2.1. Milk Ring Test (Alton *et al.,* 1988).
2.2. Whey Buffered Acidified Plate Antigen Test (wBAPAT) (Alton *et al.,* 1988).
2.2. Whey Rose Bengal Plate Test (wRBPT) (Alton *et al.,* 1988).

3- Isolation and identification of *Brucella organisms*:

3.1. Direct culture method:
Briefly, the milk sample was centrifuged at 3000 rpm for 10 minutes to obtain the sediment–creammixture (Alton *et al.,* 1988) which then was cultured on duplicated plates of serum dextrose agar plates containing Brucella selective antibiotics (Oxoid code: SR0083, Hampshire, UK). The plates were incubated in presence of 5-10% CO_2 and aerobically at 37 ºC for up to 2 weeks. The plates examined every 2 days for any Brucella growth.

3.2. Indirect cultural method (Brodie and Sinton, 7. 1975):
Two ml of fresh cream were inoculated into bottles (50 ml volume) of serum dextrose broth containing Brucella selective supplements (Oxoid **code:** SR0083, Hampshire, UK), then incubated at 37◦C in carbon dioxide incubator (5-10% tensions) for 3-5 days. The broth was the sub-cultured onto selective serum dextrose agar plates and the plates as well as control ones were incubated at 37◦C in carbon dioxide incubator (5-10% tensions) for 3-5 days. The plates examined every 2 days for any Brucella growth.

All of the isolates were subjected to standard morphological and biochemical tests, including morphological characters of the colonies, microscopical appearance, CO2 requirement, growth in the presence thionin and basic fuschin dyes, and agglutination with *Brucella* anti-sera A and M.

4- Molecular examination of Brucella:

4.1. Extraction of DNA for PCR assay:
DNA was extracted from colonies by using QIA amp DNA Mini Kit Catalogue no.51304. It provides silica-membrane-based nucleic acid purification from different types of samples.

4.2 DNA amplification:
Conventional PCR (Bricker, 2002) was carried out for identification of the DNA extracts to confirm the presence of genetic material of genus Brucella. Amplification of target gene (Immunodominant antigen, gene bp26) was carried out in a final volume of 25µl in containing (12.5 µlBiomatik® master mix, 1 µl forward primer, 1 µl reverse primer, 7.5 µl nuclease free water and 3 µl DNA template. The amplification was performed in Labnet® Multigen Gradient thermal cycler, Catalog TC9600-G- 230V. (Labnet international, Inc. Edison, NJ, USA).

Cycling conditions of the different primers during cPCR

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
Immunodominant antigen, gene *bp26*	95˚C 4 min.	94˚C 45 sec.	60˚C 45 sec.	72˚C 60 sec.	35	72˚C 7 min.

Oligonucleotide primers sequences.

Primer	Sequence (5'–3')	Amplicon size (bp)	DNA targets	Source of genetic Difference
BMEI0535f	GCG-CAT-TCT-TCG-GTT-ATG-AA	450	Immunodominant antigen, gene bp26	IS711 insertion in BMEI0535-BMEI0536 in Brucella strains isolated from marine mammals
BMEI0535r	CGC-AGG-CGA-AAA-CAG-CTA-TAA			

4.3 Detection and identification of PCR product.
The PCR products were detected through 1.5 % agarose gel stained with ethidium bromide solution (0.5 µg/ml) and visualized under an ultraviolet trans-illuminator and photographed.

RESULTS

Table 1: Incidence of Brucella organisms in the milk sample based on MRT.

Type of Samples	No. of Samples	MRT					
		+		±		-	
		No.	**%**	**No.**	**%**	**No.**	**%**
Farmers Milk	**100**	**30**	**30**	**7**	**7**	**63**	**63**
Market Milk	**60**	**14**	**23.3**	**5**	**8.4**	**41**	**68.3**
Total	**160**	**44**	**27.5**	**12**	**7.5**	**104**	**65**

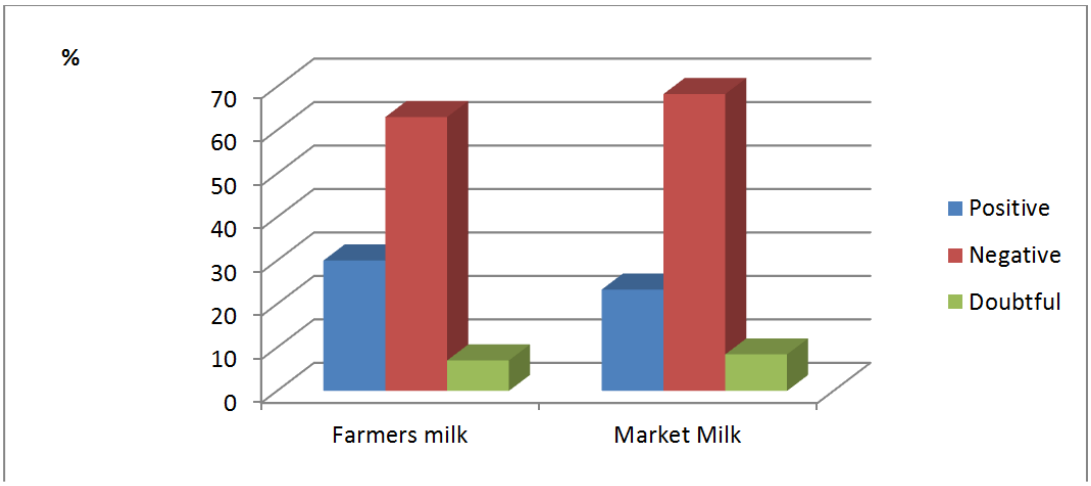


Figure 1: Incidence of Brucella organisms in the farmers and market milk based on the results of MRT.

Table 2: Incidence of Brucella organisms in the milk samples based on wBAPAT.

Type of Samples	No. of Samples	wBAPAT			
		+		-	
		No.	**%**	**No.**	**%**
Farmers Milk	**100**	**44**	**44**	**56**	**56**
Market Milk	**60**	**32**	**53.3**	**28**	**46.7**
Total	**160**	**76**	**47.5**	**84**	**52.5**

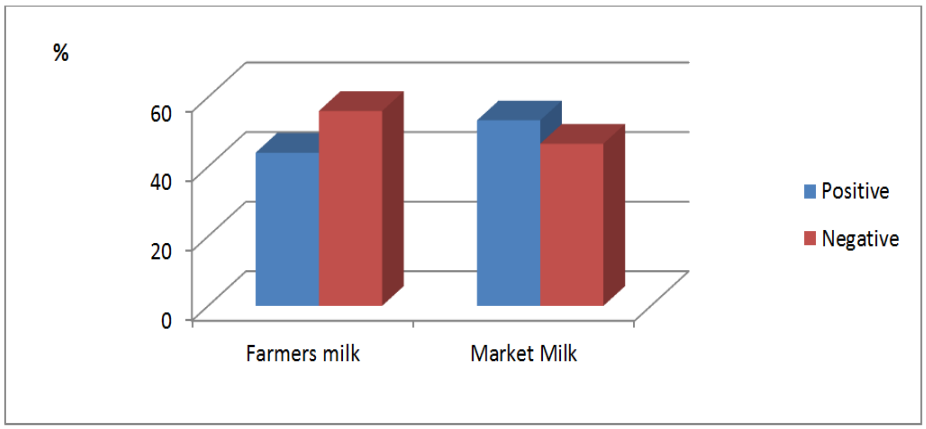


Figure 2: Incidence of Brucella organism in the farmers and market milk based on the results of wBAPAT.

Table 3: Incidence of Brucella organisms in the milk samples based on wRBPT.

Type of Samples	No. of Samples	wRBPT			
		+		-	
		No.	**%**	**No.**	**%**
Farmers Milk	**100**	**42**	**42**	**58**	**58**
Market Milk	**60**	**29**	**48.3**	**31**	**51.7**
Total	**160**	**71**	**44.4**	**89**	**55.6**

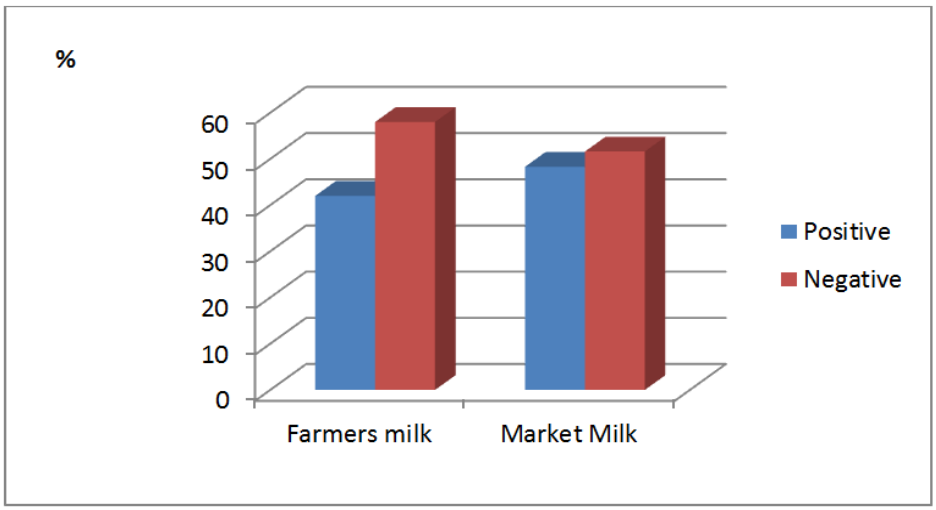


Figure 3: Incidence of Brucella organisms in the farmers and market milk based on the results of wRBPT.

Table 4: Isolation of Brucella organisms from the milk and fresh cream.

Type of samples	No. of Samples	Positive Samples	
		No.	**%**
Farmers milk	**100**	**28**	**28**
Market milk	**60**	**36**	**60**
Fresh Cream	**40**	**13**	**32.5**
Total	**200**	**77**	**38.5**

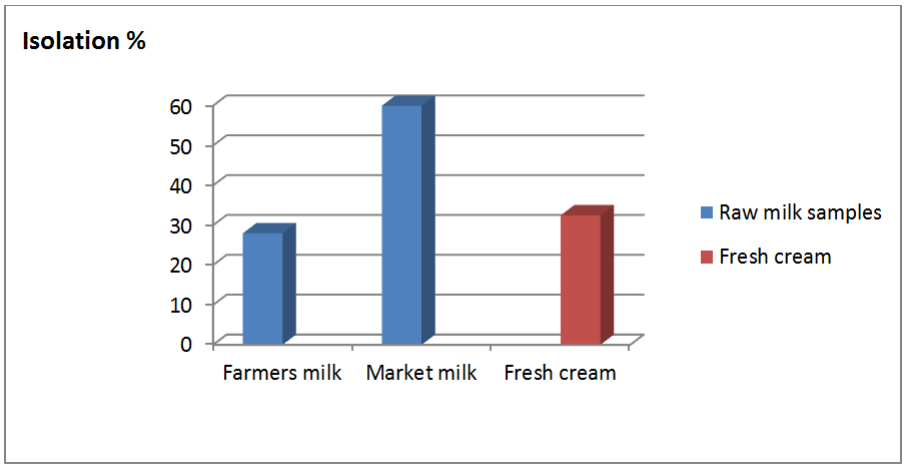
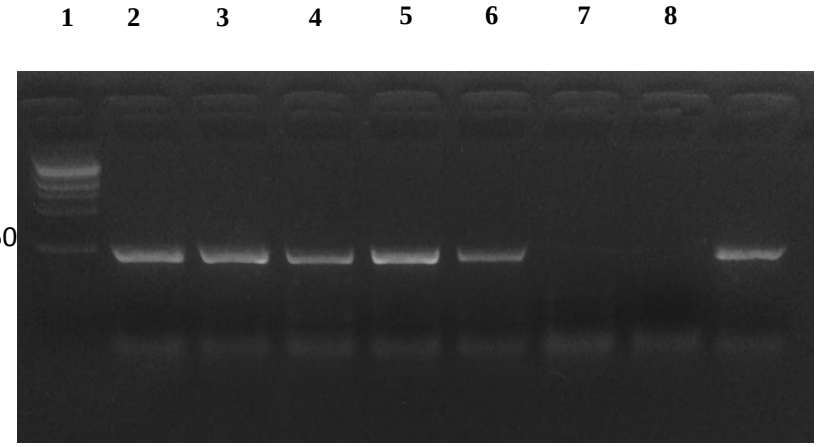


Figure 4: Isolation of Brucella organisms from the milk and fresh cream.

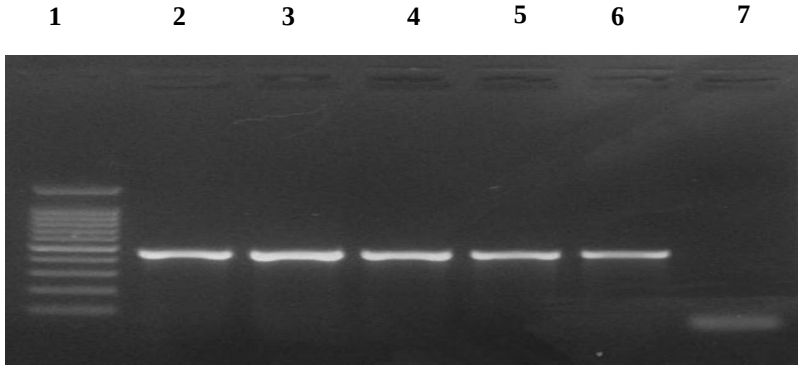
Table 5: Identification of isolated strains.

Strain	Farmers Milk (100)		Market milk (60)		Cream (40)	
	No.	**%**	**No.**	**%**	**No.**	**%**
Br. melitensis biovar 3	**28**	**28**	**36**	**60**	**13**	**32.5**
Br. abortus	-	-	-	-	-	-
Br. suis	-	-	-	-	-	-

Conventional PCR for detection of Brucella DNA on genus level



Lane 1:DNA ladder
Lane 2-6, 9: positive sample for Brucella
Lane 7-8: negative



Lane 1:100 bp DNA ladder
Lane 2-6: positive sample for Brucella
Lane 7: negative

Figure5: Gel electrophoresis of PCR products using universal primer set (with 450 bp expected product size).

DISCUSSION

Data presented in Table (1) and Figure (1) showed the incidence of Brucella antibodies in the examined raw milk based on the milk ring test (MRT).Out of 100 Farmers milk samples, 30 (30%) were positive, 7 (7%) were suspicious and 63 (63%) were negative.

This positive result agreed with those recorded by Abosria (2015) and nearly simulated to the finding recorded by El-Gibaly *et al.* (1995) and Miller *et al.* (2015) which were 28.2% and 28.7% respectively, while the gained result was higher than those obtained by Mohamed (1989), Zowghi *et al.* (1990), Hosein and El-Kholy (1993), Kadry (1996), Abdel-Hakiem (1999), El-Sherbini *et al.* (2002), Shehata (2004), Abd El Hamid (2008), El-Kholy *et al.* (2008), Shafee *et al.* (2011) and Ali *et al.* (2014), whom reported incidences of 22.25%, 25.2%, 4.1%, 0.99%, 8%, 10%, 12.38%, 14.7%, 8.2%, 4.6%, and 6.7% respectively.

The relatively high results obtained by using MRT could be attributed to the fact that MRT is highly sensitive, rapid screening test (Ferguson and Rebortson, 1954; El Gibaly, 1969 and Salem *et al.*, 1987).

On the other hand, it was relatively lower than those reported by Meshref (2000), Abd El-All (2001), Hamdy and Amin (2002), Ibrahim *et al.* (2002), Hashim *et al.* (2007), El-Diasty (2009), Abdalla and Hamid (2011), El-Sayed *et al.* (2011), Ibrahim *et al.* (2012) and Al-Mariri (2015) whom found incidence of 73.68%, 38.33%, 48.1%, 48%, 80%, 74%, 36.7%, 60.2%, 47.8% and 57%, respectively.

Despite MRT was recommended by (FAO/WHO, 1986) as surveillance test for detection of Brucella in milk due to its economical and practical advantages, the major limitations of the test are mastitis milk, skimmed milk, colostral, the dilution factors as well as milk agglutinations are locally produced in the udder of the infected cases and clustering of fat globules or low level of IgA and IgM tend to yield false negative results (Corbel *et al.*, 1984).

On the other hand the result illustrated in Table (1) and Figure (1) revealed that out of 60 market milk samples, 14 (23.3%) were positive, 5(8.4%) were suspicious and 41 (68.3%) were negative.

Our result was higher than those recorded by Kang'ethe *et al.* (2004), Bertu *et al.* (2010) and Ior and Chukwu (2015), whom reported that 3.9%, 12% and 12.5% of their samples, were positive respectively. In the contrary, Abd El Hamid (2008) reported that all market milk samples were negative to MRT.

Concerning whey buffered acidified plate antigen test, the recorded results in Table (2) and Figure (2) showed that the incidence of Brucella antibodies in the examined whey. Out of 100 Farmers milk samples, 44 (44%) were positive and 56 (56%) were negative. Also, out of 60 market milk samples, 32 (53.3%) were positive and 28 (46.7%) were negative.

Many reports dealing with prevalence of Brucella in milk have been accumulated. In those studies, various rates of prevalence were reported as 38.9%, 54.9%, 50%, 4.29%, 6.67%, 2.4%, 20.6%, 14.1%, 56% and 78.1% were obtained by Abdel-Rahman (1991), Hamdy (1997), Meshref (2000), El-Bassiony *et al.* (2007), Omran (2007); Oraby *et al.* (2007), Abdel-Hamid (2008) and El-Kholy *et al.* (2008), El-Diasty (2009) and Abd Al-Azeem *et al.* (2012) respectively.

The obtained results in Table (3) and Figure (3) showed that the incidence of Brucella antibodies in the examined whey samples based on the result of whey Rose Bengal plate agglutination test. Out of 100 Farmers milk samples, 42 (42%) were positive and 58 (58%) were negative. On the other hand, in case of market milk, out of 60 examined samples, 29 (48.4%) were positive and 31 (51.6%) were negative.

Lower results were detected by Abdel-Rahman (1991), Hamdy (1997), Abd-Alla *et al.* (2000), El-Bassiony *et al.* (2007), Omran (2007), Oraby *et al.* (2007), Abdel Hamid (2008) and El- Kholy *et al.* (2008) where as they reported 22.1%, 39.2, 3.53, 4.29%, 6.67%, 2.4%, 15.7% and 11.8% respectively, but a nearly similar result (44%) was detected by Meshref (2000) and El- Diasty (2009), while higher value (75%) was recorded byAbd Al-Azeem *et al.* (2012).

The difference in the incidence rate might be due to the variation of the degree of infection, also the appearance of antibody is related to many factors such as size and method of exposure, virulence of organism, stage of pregnancy and previous exposure. The antibody titer usually reach diagnostic level by four weeks after exposure during fourth to sixth month of gestation and at 10 weeks after exposure in non-pregnant or in the first trimester gestation (Nicoletti, 1990). The high results may be correlated with high prevalence in unknown history of animals which might be due to lack of appropriate diagnostic facility at field level and screening of animals for brucellosis prior to purchase.

The result obtained in Table (4) and Figure (4) revealed that 28 (28%) out of 100 Farmers milk samples were found to be contaminated with Brucella organisms. This finding was nearly similar to the result (28.57%) that was reported by Abosria (2015), while higher prevalence (40.6%) was recorded by Abd Al-Azeem *et al.* (2012) and (33.8%)

was recorded by Ibrahim *et al.* (2012), but lower prevalence (7.5%) was recorded by Abd-Alla and Hamid (2011) and (4%) by Abdel-Kareem *et al.* (2011).

On the other hand the summarized result in Table (4) and Figure (4) revealed that 36 (60%) out of 60 market milk samples were found to be contaminated with Brucella organisms which was much higher than that reported by Meshref (2000) and Abdel-Hamid (2008) who failed to isolate any Brucella organisms from market milk.

The higher percent in farmers milk may be attributed to the lack of proper sanitary measures under which milk is produced as well as, the high incidence of Brucella infection in the dairy cows. Moreover, higher prevalence in market milk may attributed to the fact that the milk collected from different sources blended before the selling, and explained by the fact that the majority of milk sales are in the hands of farmers who are known to harbor beliefs that milk is inherently hygienic and less likely to get concerned about the hygiene and conservation of milk before sale. (All the collected samples were from bulk milk from various herds and milk sellers each represent). The infected animals serve as sources of infection to healthy animals within the herds as well as other neighboring herds as the animals graze around unrestricted area, making contact between different herds possible, it was understood that most of the milk sold do not undergo any form of heat treatment such as pasteurization or boiling. The recovery of Brucella from milk samples is of great public health significance and presents a particularly serious hazard as previously reported by El-Sayed *et al.* (2011).

MRT showed lower incidence rate (23.3%) in market milk when compared to that recorded by culture method (60%) and that may attributed to the dilution factor as the milk from different sources may be blended before selling. On the other hand, MRT showed higher incidence rate in farmers milk (30%) when compared to that recorded by culture method (28%) and that may attributed to the intermittent secretion of Brucella in milk (El-Berg, 1981).

As presented in Table (4) and Figure (4) Brucella organisms were detected in 13 (32.5%) out of 40 fresh cream samples. The obtained result was nearly similar to those reported by Meshref (2000) who found that (33.3%) of the cream samples were contaminated. The higher incidence could be attributed to the fact that the cream is usually more heavily infected than whole milk as the Brucella organisms tend to adhere to the surface of fat globules forming a complex which rise to top of milk by means of specific gravity (Champneyz, 1953). In addition, the samples were collected randomly from general separators, which separate the milk of at least 50 small herds/day. Small amounts of milk remains in the separator after each herd are considered as a good mechanical transmitter for pathogenic organisms including Brucella organisms to the following milk. Moreover, poor sanitary measures of the separator whereas washing and sanitation of the separator was done only once/day at the end of day work without using any sterilizer acts as main predisposing factor in mechanical transmission of Brucella organisms from infected to non-infected milk through the separators (Meshref, 2000).

All the obtained isolates from milk and cream were typed as *Brucella melitensis* biovar 3 as illustrated in Table (5). Isolation of *Brucella melitensis* biovar 3 from milk was reported by Meshref (2000), Abd El-all *et al.* (2001), Montasser *et al.* (2002), Shalaby *et al.* (2003), Zahran (2004), Abdel Wahab (2005), El-Diasty (2009), Moawad *et al.* (2013) and Abosria (2015). It is evident that *Brucella melitensis* biovar 3 is still the prevalent one among cattle in Egypt, also *Brucella melitensis* is considered the indigenous strain prevalent in sheep and goats in Egypt (El Gibaly *et al.*, 1993 and El- Sheery, 1993). Cattle are readily infected with brucellosis when they are in close contact with infected goats and sheep on communal grazing or at watering (Hellstrom, 1991).

CONCLUSION

Results of the study clearly indicate that the milk and fresh cream in both examined cities play a dangerous role in transmitting infection to man, so efficient boiling or pasteurization of milk before consumption or processing especially in infected areas to safeguard the consumers should be done. Urgent need for effective program for the control of this disease in reservoir animals in Egypt and educational programs to those sharing in milk production and handling as well as processing of dairy products and at risk population should be encouraged. Further studies on brucellosis should be conducted in other areas for setting up priorities for control measures.

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تم تجميع ٢٠٠عينة (١٠٠عينة لبن بقري خام من صغار المربيين و ٦٠ عينة من اللبن الخام المعروض في السوبر ماركت ومحلات بيع الالبان و٤٠ عينة قشدة طازجة) من مدينتي بني سويف والمنيا وفحصها باختبارات (اختبار اللبن الحلقي، اختبار الانتيجن الشريحي المحمض علي الشرش واختبار الانتيجن الشريحي المحمض وقد أسفرت نتائج اختبار اللبن الحلقي عن تواجد الاجسام المضادة لميكروب البروسيلا في اللبن الخام من صغار المربين في ٣٠% في حين ان ٧% عينة مشكوك فيها و ٦٣% عينة سلبية؛ اما عن العينات من السوبر ماركت ومحلات بيع الالبان فأظهرت ان ٢٣.٣% كانت ايجابية ، ٨.٤% مشكوك فيها و ٦٨.٣% عينة كانت سلبية. في حين أظهرت نتائج كل من اختبار الانتيجن الشريحي المحمض علي الشرش واختبار الروز بنجال علي الشرش علي اللبن الخام من صغار المربين ٤٤% و ٤٢% عينة ايجابية ع التوالي. في حين ان نتائج كل من اختبار الانتيجن الشريحي المحمض علي الشرش اختبار الانتيجن الشريحي المحمض علي اللبن الخام المعروض في السوبر ماركت ومحلات بيع الالبان أظهرت ان ٥٣.٤% و ٤٨.٤% عينة كانت ايجابية ع التوالي. وبالفحص البكتريولوجي ٢٨%، ٦٠% و ٣٢.٥% من عينات اللبن البقري الخام من صغار المربيين ، اللبن الخام المعروض في السوبر ماركت ومحلات بيع الالبان وعينة قشدة طازجةعلي التوالي ملوثة بميكروب البروسيلا وتم تصنيف جميع العترات المعزولة من عينات اللبن الخام والقشدة وجد أنها تنتمي الي عترة البروسيلا مليتنسيس النوع الثالث والتي تعتبر من أخطر العترات علي صحة المستهلك واكثرها شيوعا في مصر.