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DETECTION OF BRUCELLA IN MARKETABLE MILK SOLD IN ALEXANDERIA CITY

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ABSTRACT

The main object of this study is to detect the Brucella spp. in the marketable milk sold in Alexanderia city by using different methods such as MRT (milk ring test), ELISA, direct culture and PCR. A total of 170 milk samples were purchased as 70 cow's milk samples and 100 buffalo's milk samples. The obtained results indicated that the incidence of Brucella antibodies in milk samples were estimated by MRT in 16 samples (7 cow's milk and 9 buffalo's milk) out of the 170 milk samples ; and by ELISA in 35 samples (19 cow's milk and 16 buffalo's milk) out of the 170 milk samples. Moreover, Brucella spp. were detected in 4.3 % of the cow's milk samples and 5 % of the buffalo's milk samples by direct culture. Also the incidence of Brucella spp. gene were detected in 14 samples (8.2%) out of the 170 milk samples as 6 (8.6 %) for cow's milk and 8 (8%) for buffalo's milk samples by using PCR. In conclusion, PCR proved to be more suitable tools for Brucella detection than the culture techniques. A combination between molecular techniques and conventional techniques found to be a good reliable policy for controlling the disease. Achieved results set a warning for public health hazard due to habit of drinking of fresh raw milk.

***Key words**: Brucella spp., marketable milk, MRT, ELISA, PCR.*

INTRODUCTION

Brucellosis, also known as"undulant fever", "Mediterranean fever" or "Malta fever" is a highly contagious bacterial zoonotic disease that affect millions of people worldwide and a wide variety of farm animals (Mohsen, 2000; Bricker, 2002) and still remains a significant public health and economic problem in many developing countries (Hassan and Samaha, 2008). Six countries in the Middle East, report an annual total of more than 90000 cases of human brucellosis and the patient undergo long time of antibiotic treatment (FAO / WHO, 1995); The dairy animals, cattle, sheep, goat and camels are included within the reservoirs of the agent resulting in a decrease in reproductive efficiency and abortion (Adams and Moss, 1995), Moreover, it is a major reason for culling of animals due to the strategy of eradication program (Hopper *et al*., 1989). Man is often infected bydirect or indirect contact with the contaminated fetal membranes and infected animals or their products (Young, 1983; Wallach *et al.,* 1994) via consumption of contaminated foods, so it is also considered as food borne disease (Young, 1983).

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In the dairy animals, Brucella centralize in the supramammary lymph nodes which continue to excrete them in the milk (Cordes and Carter, 1979; Refai, 2003).

The genus of Brucella comprises of Gram-negative, non-motile and facultative intracellular pathogens and six species are recognized within the genus: *Brucella melitensis, B.abortus, B. ovis, B. canis, B. suis* and *B. neotomae* (Moreno *et al.,* 2002). All six Brucella spp. are considered to be potentially pathogenic to humans (Corbel and Brinley-Morgan, 1984). *Brucella melitensis* is the most virulent strain for humans. It is considered a level B biological weapon (Hoover *et al*., 2004). *Brucella melitens* biovar-3 considered as prevalent biovar in Egypt and reported an incidence of 61.0% in cattle and 24.0% in buffaloes (Ibrahim *et al.,* 2012). It is naturally infected raw milk and survived for 5 days at 4 °C and for 9 days at -20 °C (Hassan and Samaha, 2008).

The methods for the diagnosis of brucellosis firstly by serological tests via detection of antibodies specific for Brucella infection (Refai, 2003). Culture methods are well established for brucellosis but highly pathogenic for laboratory workers, difficult and lengthy processes that requires experienced technicians, finally, culture takes long time to growth (Kazemi *et al.,* 2008). Serological methods are not conclusive, because of the absence of the detectable level of antibodies by the infected animals. However,

milk ring test (MRT) is probably the most widely used test for screening and monitoring of brucellosis in dairy cattle (Alton *et al.,* 1988). PCR as amolecular techniquesh as the potential to meet the need for better diagnostic tools for several infectious diseases which caused by fastidious or slow growing bacteria (Romero *et al.,* 1995; Bricker, 2002).

MATERIALS AND METHODS

1-Collection of samples:
A total of 170 milk samples (70 cow's milk and 100 buffalo's milk) were collected from local markets in Alexanderia city. The samples were obtained as they sold and well mixed, then put in sterile poly ethylene bags and transported to the laboratory in an ice box, and freezed until analysis at Animal Reproduction Research Institute (Brucella Unit).

2-Milk Ring Test (MRT):
The test was performed in sterile was serman´s tubes. The milk samples were thoroughly shaken and 1 ml of the milk was transferred into a tube and a drop (30 µ1) of MRT antigen (stained brucella antigen) was added. The tubes were mixed thoroughly and incubated at 37 °C for 3 h. The positive results was indicated by the darker cream layer according to (Alton *et al.,* 1988).

3-Enzyme-Linked Immunosorbent Assay (ELISA):
The test was performed as described by the manufacturer from Synbiotic, France.

4-Isolation and identification of Brucella spp. from the milks amples:
The cream and sediment obtained after centrifugation (10 minutes at 5000 rpm) of 50 ml of milk were seeded on plates of Brucella agar medium (Oxoid), suspected colonies were characterized by biochemical tests such as oxidase, catalase, urease, CO_2 requirement, H_2S production, methyl red, in dole and sensitivity to thion in and basic fuchsin dyes according to (Maymona *et al.,* 2014).

5- Molecular characterization (Detection of Brucella spp. gene) by using polymerase chain reaction (PCR):
Primer set sequences used for Amplification of Brucella spp. was done according to (Baily *et al*., 1992). Amplification of 223 bpb and confirmed the isolate to be Brucella spp.

DNA amplification of Brucella spp. gene: A 500 µl of each milk sample was mixed with 100 µl of NET (50mM NaCl-125 mM EDTA-50 mMTris-HCl, pH 7.6). After incubation at 80°C for 15 minutes, sodium dodecyl sulfate (SDS) and proteinase K were added in a final concentration of 0.5% and 200 µg/ml, respectively. After incubation at 50°C for 3 hours, the cell debris was removed by precipitation with 5 M NaCl and a hexadecyl trimethyl ammonium bromide-NaCl solution at 65°C for 10 minutes (Wilson *et al*., 1990) After extraction of the DNA (Sambrook *et al*., 1989), the extracted DNA pellet from each milk sample was resuspended in 25 µl of sterile distilled water and one µg of this DNA suspension was added to the PCR mixture. Reaction mixture of 50 µl containing 10x PCR buffer (500mM KCl; 100 mMTrisHCl, pH 9.0; 1% Triton x -1001.5 mMMgCl, Promega, USA), 20 mMdNTPs (Boehringer Mannheim, Germany), 2.5 units of *Taq* DNA polymerase (Promega, USA), 1 µg of extracted DNA and 100 pmole of primer. Negative control consisted of sterile water instead of the DNA template was used. The thermal cycler (MJ research, USA) was programmed as first initial denaturation at 94°C for one minute followed by 39 cycles consisting of 94°C for one minute, 55°C for one minute and 72°C for one minute and 10 minutes at 74°C for final extension (Baily *et al.,* 1992). The amplified product was resolved using 1.5 % agarose gel electrophoresis that stained with ethidium bromide and photographed by photo-documentation system (UVP, USA) and analyzed by Gel-pro 3.1 Analyzer (MEDIA, USA) (Sambrook *et al*., 1989).

Table1: Oligonucleotide primers for Brucella spp. according to (Baily *et al.,* 1992).

Primer Code	Sequence (5` to 3`)	Product Size	Species Specificity
B4	TGGCTCGGTTGCCAATATCAA	223 bp	All
B5	CGCGCTTGCCTTTCAGGTCTG		*Brucella spp.*

RESULTS

Table 2: Incidence of Brucella spp. present in marketable milk samples collected from cow and buffaloes by using MRT, ELISA, direct culture and PCR methods.

Milk Samples	No. of the examined samples	MRT		ELISA		Directculture		PCR	
		Positive samples		Positive samples		Positive samples		Positive samples	
		No.	%	No.	%	No.	%	No.	%
Cow's milk	70	7	10%	19	27.1%	3	4.3 %	6	8.6 %
Buffalo's milk	100	9	9 %	16	16%	5	5 %	8	8%
Total	170	16	9.4%	35	20.6%	8	4.7%	14	8.2 %

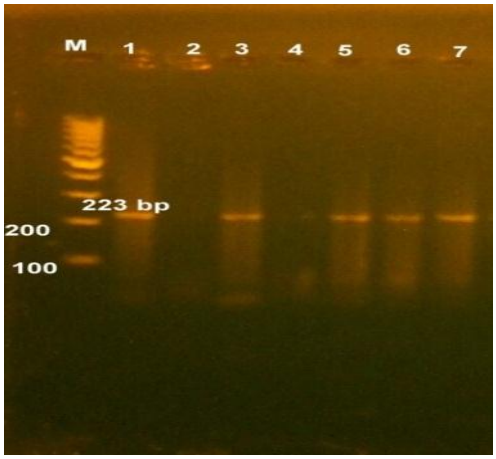

Figure (1): Agarose gel electrophoresis of multiplex PCR for detection of *Brucella spp.*
Lane M: Molecular weight marker (Gene Ruler 100 bp).
Lanes 1-3: Positive cow's milk samples DNA (223 bp).
Lane 4: Negative cow's milk samples.

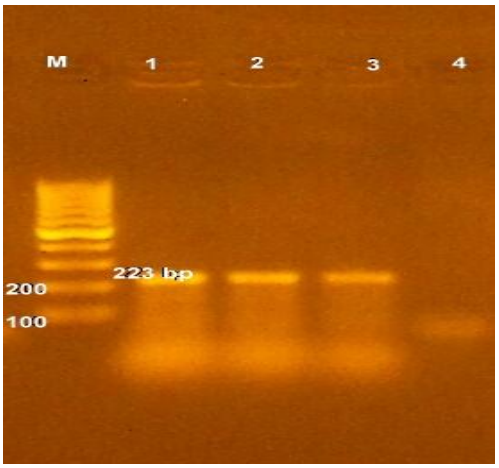

Figure (2): Agarose gel electrophoresis of multiplex PCR for detection of Brucella spp.
Lane M: Molecular weight marker (Gene Ruler 100 bp).
Lanes 1, 3, 5, 6,7: Positive buffalo's milk samples DNA (223 bp).
Lanes 2,4: Negative buffalo's milk samples.

DISCUSSION

Brucellosis is common in rural areas because farmers live in close contact with their animals and often consume fresh unpasteurized dairy products. However, the vending of dairy products may also bring the disease to urban areas (Abd EL –Razik *et al.,* 2007).

The obtained results in Table 2 are not agree with the Egyptian Organization for standardization and Quality Control (E.O.S.Q.C.) (2005) which recorded that milk must be free from the pathogenic bacteria. Higher and lower results for detection of Brucellaein raw milk by using MRT and ELISA were recorded by many researchers [Farag (1998), Hamdy and Amin (2002), Abdalla and Hamid (2012), Ibrahim *et al.* (2012) and Abo-shama, (2013)]. Serological tests are faster but antigen–antibody interactions can be faulted by non-specific interactions. (Mohsen, 2000).

Higher numbers of the positive milk samples for Brucella spp. was obtained by PCR in comparison to the direct culture applied in the present study. And that may be attributed to the ability of PCR to detect the specific gene of the bacteria regardless living or dead organism (Amin *et al.,* 1995); (Brodie and Sinton, 1975). Microbiological culture depends on organism viability, quality of the sample, contamination of the sample with other microorganisms and time between collection and analysis, while DNA detection by PCR does not depend on these factors.

The 223 bp RNA gene was amplified by PCR indicating 14 milk samples with Brucella spp. (Fig.1) and (Fig.2) which, confirmed to be Brucella spp. strains. This assay offers a very specific, quick and reliable technique. Sequence analysis of 16S rRNA gene is extensively used for molecular detection of different bacterial species; 16SrRNA gene sequence among Brucella species is significantly conserved and it has been reported that 16S rRNA gene sequencing is a reliable tool for rapid genus level identification of Brucella (Fitch *et al.,* 1990). PCR was done mostly on isolated colonies in order to confirm the routine diagnostic procedure and it was concluded that PCR is a good diagnostic tool to evaluate presence or absence of *Brucella* species in the grown culture. Finally, Brucella is inactivated by pasteurization or by prolonged boiling for 10 min (Abbas and Aldeewan, 2009); So, consumption of un-pasteurized milk or milk products prepared under unsuitable conditions exhibit the level of potential risk for public health (Kasimoglu, 2002).

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الكشف عن البروسيلا في الألبان المباعة فى مدينة الإسكندرية

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هدفت هذه الدراسة الى الكشف عن ميكروب البروسيلا في الألبان المباعه فى مدينه الإسكندرية باستخدام طرق مختلفة مثل اختبار اللبن الحلقى والايليزا (المقاسه الامتصاصيه المناعيه للانزيم المرتبط) والزرع مباشرة على المستنبتات واختبار تفاعل البلمرة المتسلسل حيث تم تجميع ١٧٠ عينه من اللبن تم شراؤها ممثله فى ٧٠ عينه لبن بقرى و ١٠٠ عينه لبن جاموسى، وقد أشارت النتائج التي تم الحصول عليها إلى وجود الأجسام المضادة للبروسيلا في العينات بواسطه الاختبار اللبن الحلقى فى عدد ١٦ عينه (٧ عينات لبن بقرى و ٩ عينات لبن جاموسى) من اصل ١٧٠ عينة وبواسطة الايليزا ٣٥ عينه (١٩ عينه لبن بقرى و ١٦ عينه لبن جاموسى) . وعلاوة على ذلك، تم الكشف عن البروسيلا في ٤.٣٪ من اللبن البقرى و ٥٪ من اللبن الجاموسى عن طريق الزرع المباشر وأيضا تم تحديد جينات البروسيلا باستخدام اختبار تفاعل البلمرة المتسلسل فى ١٤ عينة (٨.٢٪) من أصل عدد ١٧٠ عينة لبن حيث فى ٦ (٨.٦٪) عينات لبن بقرى و 8(8٪) عينات لبن جاموسى. ونستخلص ان اختبار تفاعل البلمرة المتسلسل أكثر ملاءمة لتشخيص البروسيلا عن طريقه الزرع المباشر ووجد أن الجمع بين التقنيات الجزيئية والتقنيات التقليدية هو سياسة جيدة يمكن الاعتماد عليها للسيطرة على المرض. وقد حققت نتائج الدراسه الحاليه تحذيرا لمخاطر الصحة العامة خاصة بسبب عادة شرب اللبن الخام الطازج.

الكلمات الداله : البروسيلا ، الالبان المباعه ، اختبار اللبن الحلقى ، الايليزا ، اختبار تفاعل البلمرة المتسلسل.