Trial For Detection Of *Brucella* Microorganism From Milk And Milk Products Locally Produced Using PCR

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Abstract

A total of 57 samples, 31 milk (18 Cattle Milk and 13 Buffaloes Milk), 15 home made yoghurt (balady yoghurt) and 11 Kareesh cheese samples were collected from farmhouse and a street seller, wandering salesmen and some rural and urban markets, in addition 2 milk, 2 yoghurt and 2 soft cheese samples from known common companies in super markets, all sent to laboratory and were analyzed for direct culturing and polymerase chain reaction (PCR).

Three out of 18 (16.7%) cattle milk and 2 out of 13 (15.4%) buffaloes milk were detected positive by PCR, plus 2 out of 15 (13.3%) home made yoghurt (balady yoghurt) and 3 out of 11(27.3%) Kareesh Cheese were detected by PCR . Kareesh Cheese has the highest percentage of Brucella (27.3%) as compared with other locally produced dairy products. Direct culturing revealed no isolates obtained neither from locally produced dairy products samples nor from dairy products of commercial company in the super markets . The total *Brucella* DNA detected positive samples from home made Milk, Yoghurt and Kareesh cheese were 10/57(17.54%) which was identified as *Brucella melitensis* by PCR amplification of Brucella-DNA as molecular weight of bands was731 bp (*Brucella melitensis* biovar 3) which considered prevalent biovar in Egypt. PCR assay has several advantages over microbiological methods .A major advantage is the speed and the minimal sample preparation, live *Brucella* organisms are not necessary, less expensive,very amenable to automation and robotics, unaffected by pH or contamination by other microbes and biologically safe .

We concluded that PCR might be considered alternative to the traditional methods for *Brucella* diagnosis. Finally we advised to generalize using PCR as screening and confirmatory diagnostic tool for *Brucella* diagnosis in dairy products for saving cost and time. Results set a warning for public health hazard and to the fact that incorrect vaccination and / or inadequate management that occur in illegal dairy production should receive special attention from governmental agencies for the brucellosis control program .

Key words: *Brucella, detection, PCR, milk, yoghurt, Kareesh cheese .*

Introduction

Brucellosis is a specific contagious disease of animals and humans caused by bacteria of the *Brucella* group. The disease is considered by FAO, WHO and OIE as the most widespread zoonosis in the world.

Brucellosis is a significant public health problem in endemic areas such as the Mediterranean region, Western Asia, parts of Africa, **(OIE, 2009).**

Most of the produced milk is consumed mostly as fluid milk, cheese, ice cream, sweets and other products, does not meet the legal requirements (**Simone *et al.*,2007).**

Most infected cows also shed Brucella in their milk intermittently or continuously throughout lactation, and infected bulls may shed the bacteria in their semen**.** The route of human infection is mostly through consumption of unpasteurized dairy products. Dairy live stocks have an important position and are integral part of social life of rural people. Programmes for the control and eradication of bovine brucellosis markedly reduce the incidence of the disease in

humans. **(Tikare *et al.*, 2008).**

Dairy products prepared from unpasteurized milk such as soft cheeses, yoghurt, and ice-creams may contain high concentration of the bacteria and consumption of these is an important cause of brucellosis. 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**.**Soft cheese was a major vehicle of infection in the Mediterranean region, and the Middle East **(Mantur and Amarnath 2008** and **Abd EL –Razik *et al*., 2007).**

Brucella spp. were isolated from milk, cheese and vanilla flavored ice cream samples **(Kuplulu and Sarimehmetoglu 2004). I**n the Middle East, an annual total incidence of more than 90,000 cases and in each year half a million new human cases are reported worldwide **(WHO, 1997).**

Brucellosis is diagnosed using serological and microbiological methods. PCR is suitable diagnostic test for detection of *Brucella* in milk for the specificity and speed features,its sensitivity is extremely high as it could detect *Brucella* genome from 30 fg (femtogram) of total DNA (**Scholl *et al.*, 2010).** Rapid, sensitive, specific diagnostic tests are needed to identify infected animals and contaminated dairy products. Several PCR assays have been developed recently for the detection of genus-specific or species-specific se-quences of *Brucella* in milk and cheese samples **(Tantillo *et al.*,2003**) and **Abd El-Razik *et al.*,(2007)**who use PCR method for Brucella spp. for screening dairy products.

PCR assay is able to detect as low as 1 CFU/ml of Brucella spp. in water and 3 CFU/ml of *Brucella* spp. in buffalo milk in less than 3 h without any enrichment step. The assay demonstrated a higher sensitivity in comparison to bacteriological analysis **Amoroso *et al.*, 2011; Ewalt and Bricker,(2000), Bricker and Halling (1994)** mentioned that AMOS PCR was described to identify and differentiate most of the *Brucella* species and biovars (*B. abortus* bv. 1, 2 and 4; *B. melitensis* bv. 1, 2 and 3; *B. ovis* and *B. suis* bv. 1). AMOS PCR assay is a multiplex primer assay that uses a five-primer cocktail. As designed, *B. abortus* (biovars 1,2, and 4) amplifies a 498-bp product, *B. melitensis* (bv. 1, 2, and 3) amplifies a 731-bp product, *B. ovis* amplifies 976-bp product, and *B. suis* (biovar 1) amplifies a 285-bp product. The assay described has several advantages over the current microbiological methods used to identify *Brucella* species. Major advantages are the speed, the minimal sample preparation as few as 10^4 bacteria can be added directly to the reaction mixture and live *Brucella* organisms are not necessary. This is significant because *Brucella* is a human pathogen. It is slightly less expensive to perform the PCR assay than the conventional methods in use. Because the assay is very amenable to automation and robotics, the costs could be reduced even further. Finally, the assay is unaffected by pH and contamination by other microbes that might be present in the tissue samples used for isolation. After collection, the bacteria can be killed and sent for identification.. It is both quick,less expensive, and additionally, PCR is biologically safe. PCR is a useful approach for diagnostics.

Leary *et al.*, (2006) and **Conde *et al.*, (2008)** reported that the isolation of *Brucella* spp. is attempted but it is time-consuming. In these cases, the use of PCR allows the detection, typing of Brucella sp. and differentiate it into four categories: field strains, vaccine strain 19 (S19),vaccine strain RB51 and the challenge strain 2308.The technique showed high sensitivity, specificity and accuracy. They recommend using PCR as an alternative to culture for diagnosis of brucellosis (**El Kholy *et al.*, 2009). Hamdy and Amin (2002) and Leal Klevezas *et al.*, (1995)** reported a reliable highly sensitive and specific PCR test for the detection of *Brucella* species from milk (detecting less than 10 cells in 1 ml of milk) and useful for a rapid screening of brucellosis in dairy cattle.

All *Brucella* isolates showed an ability to grow within the temperature ranges 18−44^{o}C and at pH 4–9. The optimum growth temperature is

36−38˚C, but most *Brucella* strains can grow between 20 ˚C and 40 ˚C. Brucella is inactivated by pasteurization or by prolonged boiling for 10 min (**Abbas and Aldeewan,2009)**
The aim of the current study was to assess the use of PCR in detection, identification and typing of *Brucella* from home made dairy products (milk, yoghurt and Kareesh cheese).

Material and Methods

Samples

A total of 57 samples, 31 milk (18 Cattle Milk and 13 Buffaloes Milk), 15 home made yoghurt (balady yoghurt) and 11 Kareesh cheese samples were collected from farmhouse and a street seller, wandering salesmen and some rural and urban markets, in addition 2 milk, 2 yoghurt and 2 soft cheese from known common companies in super markets,all sent to bacteriology and immunity Department, National Research Center.All samples were analyzed for direct culturing and polymerase chain reaction (PCR).

Isolation and identification of *Brucella* from milk and milk products was carried out as described by **Alton *et al.*, 1988.**

Cheese Sample processing was employed by using **Gene JET™** Genomic DNA Purification Kit,, **Fermintas** Company, www Fermintas.com, Quick Protocol QP14. This method was applied in **Biotechnology Department,Animal Health Research Institute (AHRI), Egypt.**

Polymerase chain reaction and gel electrophoresis

A primer cocktail consisting of sequence of oligonucleotide primers for PCR was used as

Primer	Sequence (5′-3′)
B. abortus-specific primer	GAC-GAA-CGG-AAT-TTT-TCC-AAT-CCC
B. melitensis-specific primer.	AAA-TCG-CGT-CCT-TGC-TGG-TCT-GA
IS711-specific primer	TGC-CGA-TCA-CTT-AAG-GGC-CTT-CAT

described by **Bricker and Halling (1994).**

The amplified products were detected by Ethidium bromide (0.5 g/mL) staining after electrophoresis at 60–70 V for 1 h in 1.5% agarose gels. PCR products with the molecular sizes of 498 and 731 bp were considered indicative for

Results and Discussion

Table (1). Results of PCR and direct culturing investigations for cow and buffaloes milk samples of the farmhouse and a street seller

Types of Samples	No. Of examined	Direct culturing	PCR		Biotyping of isolates by PCR
			No. of +ve	% of +ve	
Cattle milk	18	0	3	(16.7%)	*Br. melitens*is biovar
Buffaloes milk	13	0	2	(15.4%)	*Br. melitensis* biovar
Total	31	0	5	(16.1%)	

Table (2). Results of PCR and direct culturing investigations for home made yoghurt (balady yoghurt) and Kareesh cheese collected from the farmhouse and a street seller.

Types of Samples	No. Of examined	Direct culturing	PCR		Biotyping of isolates by PCR
			No. of +ve	% of +ve	
Home made yoghurt (balady yoghurt)	15	0	2	(13.33%)	*Br. melitensis* biovar
Kareesh Cheese	11	0	3	(27.27%)	*Br. melitensis* biovar
Total	26	0	5	(19.23%)	

Table (3). Results of PCR and direct culturing investigations for dairy products of some known commercial company in the super markets.

Types of Samples	No. Of examined	Direct culturing	PCR	
			No. of +ve	% of +ve
Pasteurized Milk (Juhynah)	2	0	0	0
Yoghurt(Juhynah and Nissla)	2	0	0	0
Cheese(Banda and Feta)	2	0	0	0
Total	6	0	0	0

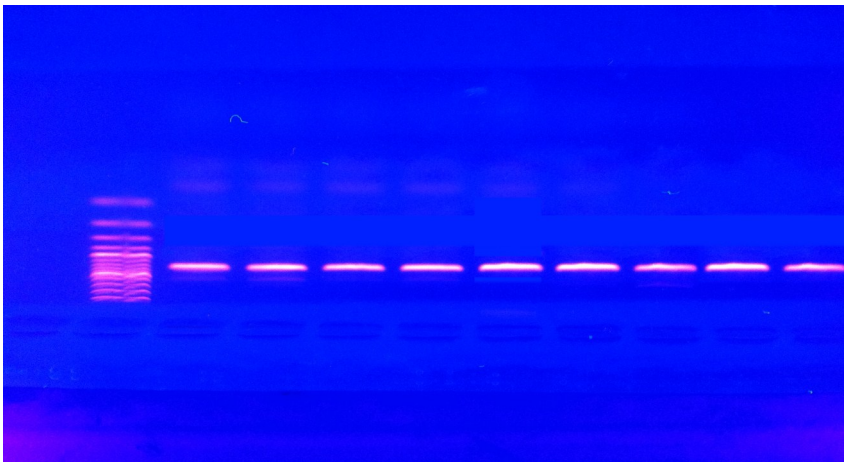

Fig. (1). Ethidium bromide–stained agarose gel electrophoresis of polymerase chain reaction (PCR) for dairy products.
Lane M: (Marker) 100-bp DNA ladder (Promega), Lane (1-3)cattle milk samples, Lane (4-5)buffaloes milk samples, Lane (6-7) Home made yoghurt samples, and Lane (8-9) Kareesh Cheese samples, *Brucella melitensis*-DNA amplified from samples (731-bp).

Three out of 18 (16.2%) cattle milk and 2 out of 13 (15.4%) buffaloes milk were detected positive by PCR,while direct culturing from 18 cattle milk and 13 buffaloes milk samples collected from farmhouse and a street seller, wandering salesmen revealed no isolates **(Table, 1)**

Two out of 15 (13.3%) home made yoghurt (balady yoghurt) and 3out of 11 (27.3%) Kareesh cheese samples were detected positive by PCR while direct culturing revealed no isolates from 15 Home made yoghurt (balady yoghurt) and 11 Kareesh cheese samples (**Table, 2).**
Neither direct culturing nor PCR revealed any isolates from milk, yoghurt and cheese samples from dairy products of some known commercial company in the super markets **(Table,3).**

Tables (1 and 2) revealed that out of 31 milk samples,(15 Home made yoghurt and 11 Kareesh cheese), 10 *Brucella* DNA were detected by PCR,while direct culturing from the same samples collected from farmhouse and a street seller, wandering salesmen revealed no isolates which explained that *Brucella* may descend intermittent in milk or may be dead by the action of acidity of milk products (affected by pH) or *Brucella* may be found in few number in milk so its undetectable by direct culturing but detected by PCR which revealed the highest specificity of PCR for detection of *Brucella* DNA indicating its ability to detect the higher number of positive milk samples to brucellosis. This result agreed with the results of **Ewalt**

and Bricker (2000), Leal-Klevezas *et al*., (2000) and **Patrick (2002)** who reported that this technique could be a potentially useful method for the diagnosis of brucellosis since it could detect the bacteria in paucibacillary samples and even in samples highly contaminated with other microorganisms.

In addition, PCR technique could detect more infected animals compared to culturing and serological methods in agreement with the results by **Ongor *et al.*,(2006), Leal Klevezas *et al.*, (1995),Romero *et al.*,(1995) and Bricker and Halling (1994)** who reported that PCR assay detect as little as 30 fg of total DNA and the culture delay of about one week before results were issued and PCR a reliable highly sensitive, specific and single step for the detection of *Brucella* species from milk (detecting fewer than 10 cells in 1 ml of milk) obtained from infected livestock and the several advantages of PCR over the bacteriological methods for the diagnosis of brucellosis, such as speed, safety, high sensitivity and specificity, and application could be more useful for a rapid screening of Brucellosis in dairy cattle,and serological methods used for diagnosis of *Brucella* which were not sensitive or specific, moreover, they had repeatedly been reported to cross-react with antigens other than from *Brucella* species and represent a great risk of laboratory techniques.

In addition **Tantillo *et al.*, (2001**) also failed in cultivating *Brucella* despite reporting a high prevalence rate (46%) in the PCR examination of the cheese samples. The reason for failure in culture may be massive contamination of the cheese samples with other organisms, like *Lactobacillus, Escherichia coli,* or the absence of viable organisms.

PCR might be considered alternative to the traditional diagnostic methods which agree with **Simone *et al.,*(2007)** who reported that 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 means of PCR does not depend on these factors. It can detect few number as 10^4 bacteria.

Live *Brucella* organisms are not necessary for the assay, this is significant because *Brucella* is a human pathogen, speed with which the assay can be performed, i.e., less than a single working day and the minimal sample preparation. It is slightly less expensive to perform the PCR assay than the conventional methods in use. Because the assay is very amenable to automation and robotics, the costs could be reduced even further. Finally, the assay is unaffected by pH and contamination by other microbes that might be present in the tissue samples used for isolation. It is both quick and inexpensive. Additionally, PCR can use specimens in which the pathogenic organisms have been rendered biologically safe as described by **Hamdy and Amin (2002) and Gupta *et al.,* (2006)** who stated that PCR has low detection limit (detect few numbers of *Brucella*) than direct culturing and evaluated the use of a PCR assay on the detection of *Brucella* spp., from cheese. PCR procedure has been considered more sensitive and specific in comparison to bacteriological analysis and serological methods and reported that the detection limit of their assay was 100 colony forming unit (CFU)/ml and agree with **Amoroso *et al.*,2011** who mentioned that PCR is able to detect as low as 1 CFU/ml of *Brucella* in water and 3 CFU/ml in buffalo milk in less than 3 h without any enrichment step.

Also agree with that of **Radostits *et al.*,2002** who reported that most infected cows shed *Brucella* in their milk intermittently or continuously throughout lactation. Also agree with **Khoudair (2004)** who reported that for the last few years, there is no single test satisfies all criteria of diagnosis, until PCR had appeared such test proved to be the best possible use of those available tests including direct culturing.

There are number of specific screening tests for detecting antibodies of *brucella* in milk are very useful in the presumptive diagnosis of infection **(Chand *et al.*,2005).** False-negative reactions may occur in a significant number of serum samples from chronically- or latently-infected animals. Animals in the early stages of

infection may also fail to react (**Timoney *et al.*, 1988)**

Fig (1). PCR amplification of *Brucella*-DNA obtained from dairy samples revealed that 10/57 (17.54%) from Milk, Home made yoghurt (balady yoghurt) and Kareesh Cheese were detected by PCR and molecular weight of bands were 731 bp which identified as *B. melitensis* biovar 3.Our results agree with that of **Bricker and Halling (1994), Tantillo *et al.*,2003, Leary *et al.*, (2006),Conde *et al.*, (2008)** and **Labmedica International Staff Writers (2011**) who used PCR method for *Brucella* spp. for screening dairy products (milk and cheese samples). The assay demonstrated a higher sensitivity in comparison to bacteriological analysis and reported that field strains of *B. abortus* amplified a 498-bp DNA fragment. While

B. melitensis were identified by amplification of a 731-bp fragment..These results were similar to **Khoudair (2004)** who detected *Brucella* from milk samples by PCR and identified as *B. melitensis* biovar 3, which is most prevalent strain in Egypt.

From the results we revealed that pH is a great factor on Brucella growth and survival in direct culturing but PCR is not affected by pH which agree with **Abbas and Aldeewan (2009)** and **El-Kholy *et al.*, (2009)** who stated that all *Brucella* isolates showed an ability to grow within the temperature range of 18-44 ^{o}C and at pH 4–9 and using PCR as an alternative to culture for diagnosis of brucellosis.

Consumption of un-pasteurized milk or milk products prepared under unsuitable conditions exhibit the level of potential risk for public health **Kasimoglu (2002**) and responsible for human brucellosis cases. The fact that the consumption of cheese, prepared from non pasteurized milk, without proper ripening is rather common in some parts of the country makes the problem more dramatic. Urgent attempts should therefore be made to make consumers aware of the importance of ripening cheese for at least 60 days before consumption.

Conclusion: PCR assay could be recommended as a screening and confirmatory method and an alternative to culture for diagnosis of brucellosis. as its speed, safety, high sensitivity, specificity and saving cost and time. The present trial was efficient in the standardization of sensitive and specific molecular techniques that may be applied to dairy products for the detection of *Brucella* spp., as well as aiding brucellosis control programs. Results set a warning for public health and to the fact that incorrect vaccination and / or inadequate management that occur in illegal dairy production should receive special attention from governmental agencies for the brucellosis control program effectively to reach its objectives.

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WWW Fermintas.com,Quick Protocol QP14, GeneJET™ Genomic DNA Purification Kit, # K0721,

محاولة اكتشاف وجود ميكروب البروسيلا في اللبن ومنتجاته المصنعة محليا باستخدام تفاعل إنزيم البلمرة المتسلسل

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الملخص العربي

أجريت هذه الدراسة على 57عينة عبارة عن 31عينة لبن (18 لبن أبقار+13لبن جاموس) و15 عينة زبادي بلدي ،11 عينة جبن قريش جميعها مصنعة فى المنازل و مجمعة من الأسواق والباعة الجائلين فى القرى والمدن بالإضافة لعدد 2 عينة لبن و2 عينة زبادي و2 عينة جبن من شركات معروفة.

باستخدام تفاعل البلمرة المتسلسل تم اكتشاف 3عينة لبن أبقار إيجابي من مجموع 18 عينة (16.7%) و2عينة لبن جاموس إيجابي من مجموع 13 عينة (15.4%) وكذلك 2 عينة زبادي بلدي مصنعة فى المنازل إيجابي من مجموع 15 عينة (13.3%) وكذلك 3 عينة جبن قريش إيجابي من مجموع 11 عينة (27.3%) كلها مجمعة من الأسواق والباعة الجائلين في القرى والمدن ،ولم يكتشف أي عينات إيجابية سواء أكانت لبن أو زبادي أو جبن من شركات معروفة. بينما لم يسفر العزل البكتيريولوجى لجميع العينات عن أي عزلات. وجد أن نسبة البروسيلا في الجبن قريش اكبر عنها في منتجات الألبان الأخرى مثل الألبان والزبادي المصنعة في المنازل. كان الوزن الجزيئى للعينات الإيجابي باستخدام تفاعل البلمرة المتسلسل(731 bp) مما يعنى أنها بروسيلا مليتنيسسز النوع الثالث وهى العترة السائدة والمنتشرة في مصر.

يستنتج من التجربة أن تفاعل البلمرة المتسلسل يتميز عن العزل البكتيريولوجى انه لايتاثر بان الميكروب حي أو ميت وله القدرة على اكتشاف عدد قليل من الميكروب في العينة ولايتاثر بدرجة الحموضة والقاعدية pHولايتاثر بوجود ميكروبات أخرى في العينة كما انه اختبار دقيق وسريع لذا يوصى باستخدامه كاختبار ماسح سريع وتأكيدي لاكتشاف البروسيلا في جميع منتجات الألبان واستخدامه كطريقة بديلة لطرق تشخيص البروسيلا المعروفة وكذلك استخدامه في جميع برامج مكافحة البروسيلا وكذلك يوصى بتشديد الرقابة الحكومية على جميع منتجات الألبان المنتجة في المنازل والتي تباع في الأسواق ومع الباعة الجائلين في القرى والمدن حفاظا على الصحة العامة.