



Original Article

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Detection of Brucella organisms from Egyptian raw milk using cultural and molecular techniques

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Abstract

Aim: This study aimed to detect Brucella organisms in Egyptian raw milk using cultural and molecular techniques.

Methods: Two hundred bulk milk samples were collected from 2 cities at El-Gharbia and Kafer El-Sheikh Governorates during the summer and winter seasons. All samples were examined by the traditional cultural method and real-time polymerase chain reaction (qRT-PCR). All positive isolates were identified by biochemical tests and serotyping using monospecific antisera also examined by multiplex PCR primers for more identification.

Results: Samples collected in summer seasons from the two cities showed no colonies characteristic to Brucella organisms while in winter season five samples showed positive characteristic features of Brucella organisms after culturing on Brucella specific media. Using qRT-PCR, 43 samples (13 in summer and 30 in winter) were positive for Brucella organisms. All positive RT-PCR samples and isolated strains were examined for the detection of the virulence genes *Ure*, *bvfA* and *virB* using conventional PCR. *Ure*, *bvfA* and *virB* were detected in 88%, 79% and 74% of milk samples and 100%, 80% 100% of isolates, respectively.

Conclusion: These results suggested that the use of the specific PCR assay is a rapid, reliable, and accurate technique in comparison to traditional methods for the detection of Brucella species in milk samples.

Keywords: Milk, Brucella, Culture, PCR, Virulence genes.

1. Introduction

Raw milk is a natural biological fluid obtained from healthy udders of lactating cows. It provides compounds that play an important role in both nutrition and health protection for consumers of milk [1, 2]. Some epidemiological studies have clearly shown that raw milk can be infected with several pathogens, some of which are associated with human disease [3]. Brucellosis is a classic example of a zoonotic milk-borne disease caused by *Brucella* species that cause systemic disease and can also be found in mammary gland tissue and associated lymph nodes and shed in the milk in large numbers [4]. The disease is considered an occupational threat and is transmitted to humans through close contact with livestock, exposure to aborted materials and the ingestion of raw dairy products [5]. In Egypt, brucellosis is an endemic disease among animals and humans, *Brucella melitensis* biovar3 and *Brucella abortus* biovar1 are the main isolated species [6]. The infection in man occurs by two methods, one is wound infection and the other one is the ingestion [7]. The organism can survive in naturally contaminated unpasteurized milk for up to five days when kept at 4°C [8].

The routine identification and differentiation of Brucella species that based on culture and phenotypic traits are

associated with a high risk of laboratory-acquired infections and is very time consuming also require biosafety level 3 laboratories [9, 10]. So, nowadays diagnosis of Brucella is mostly based on PCR techniques [11]. The qRT-PCR was used for the detection and diagnosis of different Brucella species [12, 13]. Moreover, some virulence genes (such as *Ure*, *bvfA*, and *virB*) specific to Brucella species were detected by [14]. *BvfA* gene (*brucella* virulence factor antigen) plays a role in the intracellular establishment, *virB* gene (type IV secretion system) is responsible for the translocation of virulence factors into mammalian cells and *Ure* gene (urease) protects Brucella from low pH [14].

This study aimed to detect Brucella organisms in Egyptian raw milk using cultural and molecular techniques.

2. Materials and methods

This study was conducted after under the ethical approval from the Experimental Animals Care Committee in compliance with the guidelines of Kafrelsheikh University.

2.1. Sampling

A total of 200 samples of raw milk were collected from bulk milk tanks located in El-Santa city, El-Gharbia governorate, and Sidi Salem city, Kafer El-Sheikh governorate (100 samples from each city). Samples were collected from collection centers all over one year in sterile falcon tubes and were transferred as soon as possible in an icebox to the laboratory with a minimum of delay to be examined for Brucella species. Each sample (30 ml) was mixed by inverting the falcon tube three to four times. The first 20 ml was used for bacteriological examination and the second 10 ml was utilized for DNA extraction. Milk samples used for bacteriological examination were stored at 4°C while samples for PCR assays were stored at -20°C.

2.2. Reagents

Bacto Brucella agar (Catalogue no. DFO 96401-3, Difco Laboratories, Detroit, Mich, USA) and a freeze-dried vial of selective antibiotic supplement (Catalogue no. SR 83, Oxoid Limited, Basingstokes, Hampshire RG24 OPW, UK).were used for isolation of Brucella. Monospecific anti *Brucella abortus* (A) and anti *Brucella melitensis* (M) sera obtained from central veterinary laboratory, New HAW, Weybridge, England were used for serotyping. Lyophilized reference Brucella strains [*Brucella abortus* biovar1 (strain19) and *Brucella melitensis* biovar3] were obtained from Central Veterinary Laboratory New HAW, Weybridge, England. Power Prep™ DNA Extraction Kit was purchased (Reference no.E0101, Kogene Biotec). Gene JET Genomic DNA Purification Kit was purchased from Thermo Scientific™, USA, Cat. No. K0722. Primer design™Ltd Brucella genus (all species) L-glutamine: 2-deoxy- scylloinosose amino transferase genesig® Advanced Kit, Serial no. JN68610-21593 with Oasig™ Lyophilised or Precision PLUS™ 2x qPCR Master mix. 5x FIREPOL master mix ready to load with 12.5mM mgcl2, 1 ml (Solis BioDyNS Cat. No. 04-12-00125) was used.

2.3. Identification and serotyping of isolated cultures

Isolated cultures were identified and serotyped according to Alton et al. [15].

3.4. Detection of brucella by qRT-PCR

The extraction of bacterial DNA was performed according to the supplier manual using Kogene biotec kit for DNA extraction from milk samples and Gene JET Genomic DNA Purification Kit for DNA extraction from isolates. Brucella universal gene was amplified by qRT-PCR. using Primer design™ Ltd Brucella genus (all species) genesig® Advanced Kit. The primer had 100% homology with all Brucella reference sequences included in the NCBI database (CP001488.1, AE008917.1, P000887.1, CP000911.1, CP000872.1, CP000708.1, AE014291.4, AF047478.1, AM040264.1, AE017223.1) and therefore had a very broad detection profile. The qRT-PCR was carried out in 20 µl reaction mixture composed of 2x qPCR Mastermix (10µl), Brucella species primer/probe mix (1µl), RNase/DNAse free water (4µl), DNA template (5µl) and in case of negative control, 5µl of DNase/RNase free water was added instead of DNA sample and in case of positive control, 5µl of standard template of Brucella species was added for positive control. The PCR program was run on a real-time thermocycler (Stratagene MX 3005p) as follows: enzyme activation step at 95°C for 2 min, denaturation step at 95°C for 10 sec, then 50 cycles of hybridization, extension, data collection at 60°C for 1 min.

Fluorogenic data should be collected through the FAM channels. The interpretations of results were performed by detection of cycle threshold (CT). The positive control template is expected to amplify between CT 16 and 23 and amplification after CT 40 considered a negative result.

2.5. Conventional PCR for detection of virulence genes

Brucella melitensis virulence genes *Ure*, *BvfA* and *VirB* were amplified using specific oligonucleotide primers (Table1) as previously reported by Derakhshandeh et al. [14]. PCR was carried out in a 20µl reaction mixture composed of 5X Taq master mix (4µl), Primer 10 pmol (1µl), template DNA (3µl) then the reaction completed to 20µl with PCR grade water. The PCR was run on Thermocycler Bio-Rad (PTC 1148) as follows: initial denaturation step at 95°C for 5min then 32 cycles of denaturation 95 °C for 1min, annealing for 1 min (Table 1) and extension at 72 °C for 90 sec; then final extension at 72 °C for 5 min. PCR products were loaded and migrated on 1.5% agarose gel containing 0.5pg of ethidium bromide dye. The gel electrophoresis was run under constant voltage (85) for 45 min. The gel was visualized, photographed and analysed by Bio-Rad Gel documentation system (XR).

Table 1. Oligonucleotide primers for *Brucella melitensis* virulence genes

Gene	Primer sequences (5' - 3')	Ta	Product (bp)
<i>bvfA</i>	F: ACCCTTCGTCGATGTGCTGA R: CCGCGCTGATTTCATCGCTG	65°C	1282
<i>virB</i>	F: CGCTGATCTATAATTAAAGGCTA R: TGCGACTGCCTCCTATCGTC	54°C	881
<i>Ure</i>	F:GCTTGCCCTTGAATTCCCTTGTTGG R:ATCTGCGAATTGCCGGACTCTAT	65°C	2100

Ta: Annealing temperature

2.6. Multiplex PCR for detection of Brucella species

IS711 gene for *Brucella abortus* and *Brucella melitensis* was amplified using specific oligonucleotide primers (Table 2) as previously described by Ali et al. [16]. PCR was carried out in a 20µl reaction mixture composed of 5X Taq master mix (4µl), Primer mix of *Brucella abortus* (1µl), primer mix of *Brucella melitensis* (1µl) template DNA (3µl) then the reaction completed to 20µl with PCR grade water. The PCR was run on Thermocycler Bio-Rad (PTC 1148) as follows: initial denaturation step at 95°C for 5min then 35 cycles of denaturation 95 °C for 1min, annealing at 60 °C for 1 min and extension at 72 °C for 1 min; then final extension at 72 °C for 5 min.

Table 2. Oligonucleotide primers for Brucella species

Species	Primer sequences (5'-3')	Ta	Product (bp)
Br. abortus	F:GACGAACGGAATTTTT CCAATCCC R:TGCCGATCACTTAAG GGCCTTCAT	60°C	498
Br.melitensis	F:AAATCGCGTCCTTGCT GGTCTGA R:TGCCGATCACTTAAG GGCCTTCAT		731

Ta: Annealing temperature

2.7. Statistical analysis

Data were statistically analyzed by using SPSS software, computed and represented in tables.

3. Results and discussion

Brucellosis is mainly a disease of animals, while humans get infected through contact with infected animals or consumption of raw animal products especially dairy products made from unpasteurized milk. It is one of the most widespread zoonosis worldwide [17]. The Nile Delta region in Egypt is reported to have the highest human and animal densities in the world [18]. In Egypt, 62.1% of brucellosis cases were reported to people consumed Kareish cheese made from raw milk [19]. In the present study, 5 (2 samples from El-Santa city and 3 samples from Sidi-Salem city) out of 200 (with an incidence rate of 2.5%) milk samples were positive for *Brucella* and grow on *Brucella* specific media (Table 3). These colonies appeared round with smooth margins, translucent of golden color (pale honey colored) (Figure 1). RT- PCR showed 43 positive samples with an incidence rate of 21.5% (10.5% and 11% from El-Santa city and Sidi-Salem city respectively).

Table 3. Incidence of *Brucella* in milk samples (n=200)

Source	Culture method		RT- PCR	
	No.	% *	No.	% *
El-Santa city	2	1	21	10.5
Sidi Salem city	3	1.5	22	11
Total positive	5	2.5	43	21.5

*Percent to total samples.

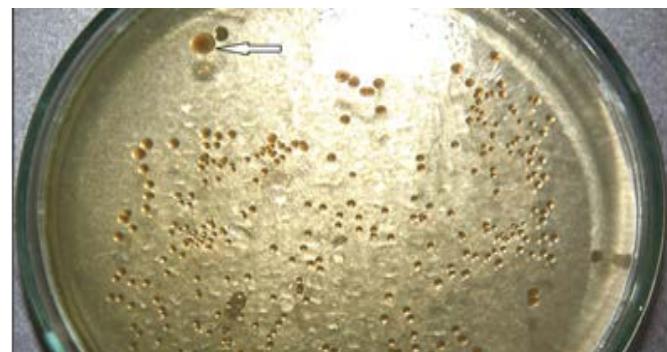


Figure 1. Colonies on *Brucella* agar media appeared round with smooth margins, round edges, translucent and of golden color (pale honey colored, arrow).

All colonies were identified biochemically, and with monospecific antisera as *Brucella melitensis*. The results were confirmed by using multiplex PCR with *Brucella abortus* and *Brucella melitensis* specific primers (Figure 2 and Table 4). All isolates were identified at product length 731 bp for *Brucella melitensis* (Figure 2).

Table 4. Biochemical, serological tests and multiplex PCR for identification of *Brucella* isolates

Isolates NO	C ₂ O requirement	H ₂ S test	Urease test	Catalase test	Agglutination with mono-specific antisera		Multiplex PCR for IS711 gene	
					A	M	498bp	731bp
5	-	-	+	+	-	+	-	+

A: *Brucella abortus* antisera. M: *Brucella melitensis* antisera

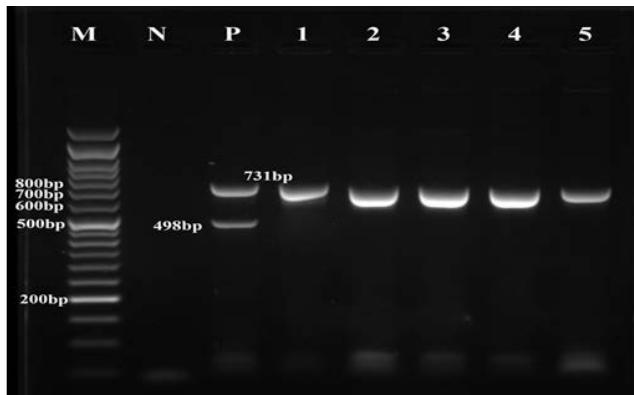


Figure 2. Agarose gel electrophoresis showed amplified fragments of 498bp for *Brucella abortus* and 731bp *Brucella melitensis* IS711 gene. Lane M: molecular marker (50bp), lane N: negative control, lane P: positive control and lane 1-5 *Br. melitensis* positive isolates. for *Brucella melitensis* IS711 gene

High prevalence rates of *Brucella melitensis* infections in sheep and goats was reported and *Brucella melitensis* infections of cattle and buffaloes have increased in Egypt [20]. Selim et al. [21] concluded that the predominant strain of *Brucella* species among ruminants in Kafr El-Sheikh governorate is *Brucella melitensis*. Montasser et al. [22] supported our results as they reported that *Brucella melitensis* was the most prevalent strain among cattle and the causative agent of brucellosis in Egypt due to Egypt has mixed populations of sheep, goats, cattle, and buffaloes which are reared together [23].

These results showed the higher sensitivity and specificity of RT-PCR for *Brucella* detection rather than culture method (Table 5).

Table 5. Incidence of *Brucella* in milk samples regarding seasons and sites (n=200)

	Positive samples by different tests			
	Culture method		RT-PCR	
	No.	% *	No.	% *
Summer	0	0	13	6.5
El-Santa city	0	0	6	3
Sidi_Salem city	0	0	7	3.5
Winter	5	2.5	30	15
El-Santa city	2	1	15	7.5
Sidi_Salem city	3	1.5	15	7.5

*Percent to total samples.

These variations were declared by Corbel [24] who reported that in countries with low temperature or cold climates there is a marked seasonal variation in the incidence of acute brucellosis, with most cases occurring in the spring and summer where the period for abortions and parturitions among farm animals while the peak period in the winter.

The positive samples in summer and winter seasons were 0 and 5 by culturing while 13 and 30 by RT-PCR, respectively (Table 6). The lower recovery by culture was explained by Aulakh et al. [25] who attributed this to inactive state of infected animals of shedding organisms in milk, or due to physical and chemical management of milk before reaching to the consumers. High positive milk samples by qRT-PCR when compared to bacteriological culture method could be attributed to the very low numbers of bacteria, which would be consistent with the small number of colony forming units detected in

milk samples by culture methods [26] and because PCR can detect living and dead organisms, while culture detects only living organisms [27]. In addition, the culture technique is time consuming and presents a great threat of infection for the laboratory personnel [28].

Table 6. Sensitivity and specificity of different tests in relation to RT-PCR as gold standard (n=200)

Test	Culture method		RT-PCR	
	TP-5	FP-0	TP-43	FP-0
	FN-38	TN-157	FN-0	TN-157
Sensitivity				
TP/(TP+FN)* 100		12%		100%
Specificity				
TN/(TN+FP)* 100		100%		100%

TP: true positive; FP: false positive; FN: false negative; TN: true negative

Of 200 milk samples examined 43 (21.5%) contained Brucella species as detected by qRT-PCR (Figure 3). Of them, 34 (79%), contained *bvFA* virulence gene (1282 bp), and 32 (74%) contained *virB* virulence gene (881bp) and 38 (88%) contained *Ure* virulence gene (2100bp) (Figures 4-6 and Table 7). Furthermore, all 5 Brucella isolates contained *Ure* and *virB* virulence genes and 4 of them contained *bvFA* gene (Table 7).

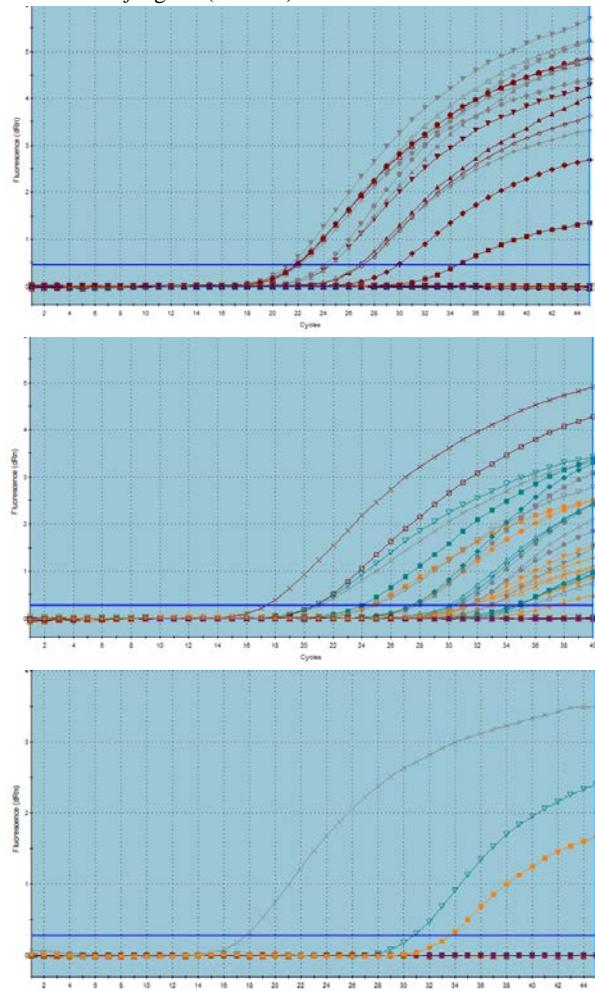


Figure 3. Real time PCR assay of Genus specific L-glutamine 2-deoxy scylloinosose amino transferase gene showing amplification curves, (upper): 94 samples (16 positive), (middle): 94 samples (25 positive), (lower): 12 samples (2 positive) referred to control positive (first curve on the left).

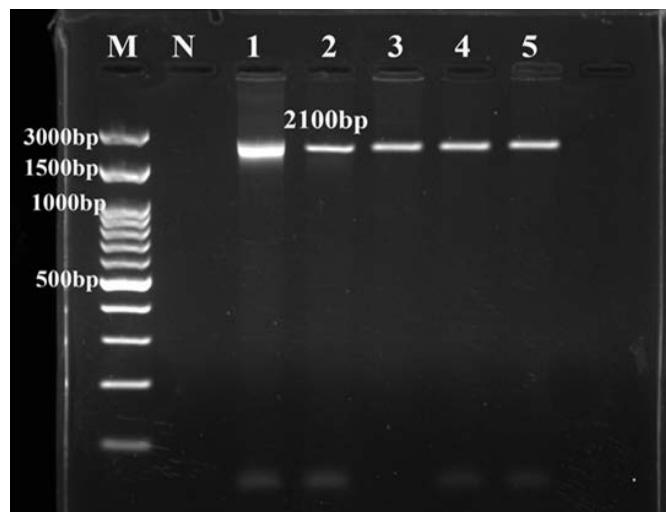


Figure 4. Agarose gel electrophoresis showed amplified fragment of 2100bp *Brucella melitensis ure* gene. Lane M: molecular marker (100bp), lane N: negative control and lane 1-5: positive *Brucella melitensis ure* gene.

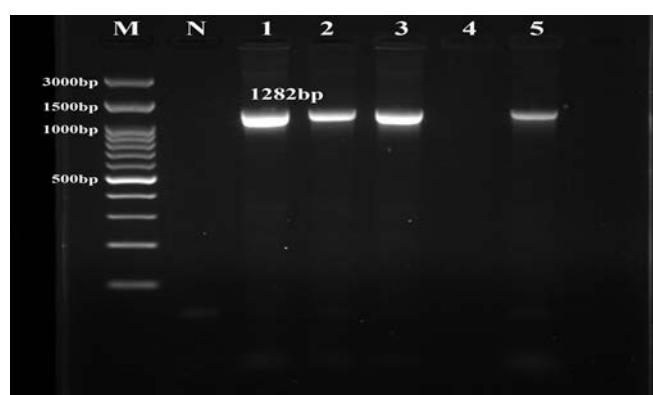


Figure 5. Agarose gel electrophoresis showed amplified fragment of 1282bp of *Brucella melitensis bvfa* gene. Lane M: molecular marker (100bp), lane N: negative control and lane 1, 2, 3 and 5: positive *Brucella melitensis bvfa* gene and lane 4 is negative for *Brucella melitensis bvfa* gene.

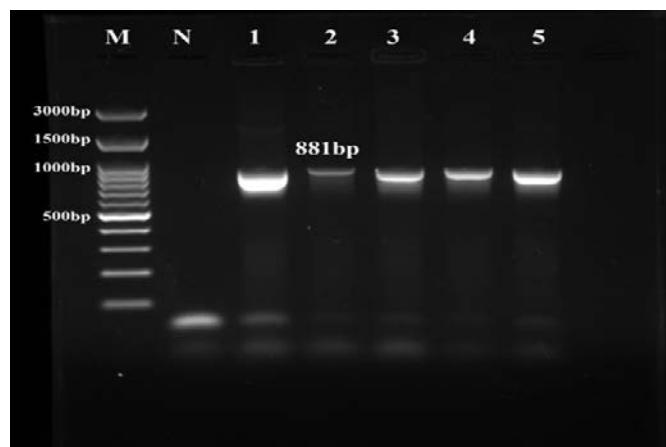


Figure 6. Agarose gel electrophoresis showed amplified fragment of 881bp of *Brucella melitensis virB* gene. Lane M: molecular marker (100bp), lane N: negative control and lane 1-5: positive *Brucella melitensis virB* gene.

Table 7. Incidence of *Brucella melitensis* virulence genes in positive milk samples and isolates by conventional PCR (n=43 for milk samples and n=5 for isolates)

NO. of positive samples by	Virulence genes					
	<i>Ure</i>		<i>bvfa</i>		<i>virB</i>	
	No.	%	No.	%	No.	%
RT-PCR	Positive milk samples					
	38	88	34	79	32	74
43	Positive isolates					
5	5	100	4	80	5	100

The *Ure* gene is the most virulence factor for *Brucella melitensis* as it responsible for urease activity which plays a role in the resistance of *Brucella melitensis* to low pH conditions consequently protect *Brucella melitensis* through the passage from the stomach [14]. Furthermore, some isolates, such as *Brucella abortus* are urease negative, while they seem to retain most of their pathogenic potential [29]. Whole-genome sequencing revealed the presence of a second urease operon (*ure2*) with all the genes potentially active in *Brucella suis* and *Brucella melitensis*. On the other hand, *Brucella abortus* had two frame shift deletions in *ureE2*, and *Brucella ovis* had deletions in *ureG2* and *ureT* [29]. Mirnejad et al. [30] showed that *Ure* gene more frequently existed in *Brucella melitensis* strains. Because of the critical role of urease in pathogenesis, it has been hypothesized that *Brucella melitensis* is more virulent than *Brucella abortus*. So, each step in the Brucella infection process can be mediated by a number of virulence factors and each strain may have a unique combination of these factors that affected the rate of bacterial pathogenesis.

T4SS of Brucella encoded by the *virB* operon is a major virulence factor for Brucella [31]. Gorvel [32] and Celli et al. [33] reported that A mutant of the VirB type IV secretion system, which is necessary for intracellular survival, was unable to sustain interactions and fuse with the endoplasmic reticulum, and was killed via eventual fusion with lysosomes. Delrue et al. [34] stated that the type IV secretion system has been described in both *Brucella melitensis* and *Brucella abortus*. *Brucella* virulence factor A (*bvfa*) is a small 11 kDa periplasmic protein unique to the genus Brucella with no homologs in GenBank. Although *bvfa* was essential for *brucella* virulence, it may play a role in the establishment of the intracellular replication [35]. Derakhshandeh et al. [14] was in agreement with our study as, of 42 *Brucella melitensis* isolates; 33 (78.5%) isolates had *bvfa* genes, 31(73.8%) isolates had *virB* genes and 37 (88.09%) isolates had *ure* genes.

References

- Ceballos LS, Morales ER, de la Torre Adarve G, Castro JD, Martínez LP, Sampelayo MRS. Composition of goat and cow milk produced under similar conditions and analyzed by identical methodology. Journal of Food Composition and Analysis 2009; 22:322–329.
- Afzal A, Mahmood MS, Hussain I, Akhtar M. Adulteration and microbiological quality of milk. Pakistan Journal of Nutrition 2011; 10: 1195–1202.
- Oliver SP, Boor KJ, Murphy SC, Murinda SE. Food safety hazards associated with consumption of raw milk. Foodborne Pathogens and Disease 2009; 6:793–806.
- Jeffrey T, Päivi JR. Unpasteurized Milk: A continued public health threat. Food Safty 2009 ;48: 93-100.
- Holt HR, Eltholth MM, Hegazy YM, El-Tras WF, Tayel AA, Javier G. *Brucella* spp. infection in large ruminants in an endemic area of Egypt: cross sectional study investigating seroprevalence, risk factors and livestock owner's knowledge, attitudes and practices (KAPs). BMC Public Health 2011; 11:page341.
- Wareth G, Melzer F, Elschner MC, Neubauer H, Roesler U. Detection of *brucella melitensis* in bovine milk and milk products from apparently healthy animals in Egypt by real-time PCR. The Journal of Infection in Developing Countries 2014; 8: 1339-1343.
- Yasmin B, Lone S. Brucellosis: An Economically important infection. Journal of Medical Microbiology and Diagnosis 2015; 4: 208-216.
- Samaha H, Al-Rowailly M, Khoudair RM, Ashour HM. Multicenter study of brucellosis in Egypt. Emerging Infectious Diseases 2008; 14:1916–1918.
- Navarro E, Casao MA, Solrea MA. Diagnosis of human brucellosis using PCR. Expert Review of Molecular Diagnosis 2004; 4: 115-123.
- Carver TJ, Rutherford KM, Berriman M, Rajandream MA, Barrell BG, Parkhill J. The Artemis comparison tool. Bioinformatics 2005; 21: 3422–3423.
- Navarro E, Segura JC, Castano MJ. Use of real-time quantitative polymerase chain reaction to monitor the evolution of *brucella melitensis* DNA load during therapy and post therapy follow up in patients with brucellosis. Clinical Infectious Diseases 2006; 42: 1266- 1273.
- Foster T, Okinaka R, Svensson R, Shaw K, Robison R, Probert W, Kenefic L, Brown W, Keim P. Real time PCR assays of single nucleotide polymorphisms defining the major *brucella* clades. Journal of Clinical Microbiology 2008;46:296-301.
- Doosti A, Dehkordi PG. Application of real time PCR for identification and differentiation of *brucella abortus* and *brucella melitensis* in cattle. Bulgarian Journal of Veterinary Medicine 2011; 14: 109–115.
- Derakhshandeh A, Firouzi R, Goudarztaejerd A. Detection of virulence genes (*bvfa*, *virB* and *ure*) in *brucella melitensis* isolated from aborted fetuses of sheep and goats. Iranian Journal Microbiology 2013; 5: 402–405.
- Alton GG, Jones LM, Anges RD, Verger JM. Techniques for the brucellosis laboratory. Institute National de la Recherche Agronomique (INRA) Paris 1988.
- Ali S, Ali Q, Melzer F, Khan I, Akhter S, Neubauer H, Jamal SM. Isolation and identification of bovine *brucella* isolates from Pakistan by biochemical tests and PCR. Tropical Animal Health and Production 2013;46: 73–78.
- Makita K, Fevre EM, Waiswa C, Eisler MC, Welburn SC. How human brucellosis incidence in urban Kampala can be reduced most efficiently? A stochastic risk assessment of informally marketed milk. PLoS ONE 2010; 5:e14188.
- Hegazy YM, Moawad A, Osman S, Ridler A, Guitian J. Ruminant brucellosis in the Kafr El Sheikh Governorate of the Nile Delta, Egypt: prevalence of a neglected zoonosis. PLoS neglected tropical diseases 2011; 5(1): e944.
- El-Ghitany EM, Omar SR, Abaza AM, Hassan EM, El-Wahab EWA. Trends in the Epidemiology of Brucellosis in a Highly Afflicted Region in Egypt: A 16 Year Experience (1997-2012). International journal of tropical disease and health 2014; 4: 250-271.
- Center for disease control and prevention (CDC) 24/7. Risks from Unpasteurized Dairy Products 2017.
- Selim A, Gaber A, Moustafa A. Diagnosis of brucellosis in ruminants in Kafr El-Sheikh governorate, Egypt. International Journal of Advanced Research 2015; 3: 345–350.
- Montasser AM Hamdy ME, El-Biomy EM, Khoudier RM. Bacteriological profile of *brucella* isolated from cattle in Egypt. 6th Sci. Cong., Egyptian Society for Cattle Diseases Assiut, Egypt 2001; 4:163-170.
- Refa M. Incidence and control of brucellosis in the Near East region. Veterinary Microbiology 2002; 90: 81-110.

24. Corbel MJ. Brucellosis in humans and animals. World Health Organization 2006.
25. Aulakh HK, Patil PK, Sharma S, Kumar H, Mahajan V, Sandhu KS. A Study on the epidemiology of bovine brucellosis in Punjab (India) Using Milk-ELISA. Acta Veterinaria Brno 2008;77:393-399.
26. O'Leary S, Sheahan M, Sweeney T. *Brucella abortus* detection by PCR assay in blood, milk and lymph tissue of serologically positive cows. Research in veterinary science 2006; 81: 170-176.
27. Miranda KL, Poester FP, Dorneles EMS, Resende TM, Vaz AD, Ferraz SM, Lage AP. *Brucella abortus* RB51 in of vaccinated adult cattle . Acta Tropica 2016;160:58-61.
28. Muhammad A, Mehwish M, Muhammad JA. Bovine Brucellosis: Old and New Concepts with Pakistan Perspective. Pakistan Veterinary Journal 2012; 32: 147-155.
29. Sangari FJ, Seoane A, Rodriguez MC, Aguero J, Garcia Lobo JM. Characterization of the urease operon of *brucella abortus* and assessment of its role in virulence of the bacterium. Infection and Immunity 2007; 75: 774-780.
30. Mirnejad R, Jazi F M, Mostafaei S, Sedighi M. Molecular investigation of virulence factors of *brucella melitensis* and *brucella abortus* strains isolated from clinical and non-clinical samples. Microbial Pathogenesis 2017; 109:8-14.
31. Delrue RM, Lestrate L, Tibor A, Letesson JJ, Bolle XD. *Brucella* pathogenesis, genes identified from random large scale screens. FEMS Microbiology Letters 2004; 231: 1-12.
32. Gorvel JP. *Brucella* intracellular life from invasion to intracellular replication. Veterinary Microbiology 2002;90: 281–297.
33. Celli J, de Chastellier C, Franchini DM, Pizarro-Cerda J, Moreno E, Gorvel J. *Brucella* evades macrophage killing via VirB dependent sustained interactions with the endoplasmic reticulum . Journal of experimental medicine 2003; 198: 545–556.
34. Delrue RM, Deschamps C, Leonard S, Danese I, Schaus JM, Bonnot S, Ferooz J, De Bolle XD. A quorum sensing regulator controls expression of both the type IV secretion system and the flagellar apparatus of *brucella melitensis*. Cellular Microbiology 2005; 7: 1151–1161.
35. Lavigne JP, patey G, Sangari FT, bourg G, O'callaghan D. Identification of a new virulence factor, bvfA, in *brucella suis*. Infection and Immunity 2005; 73: 5524-5529.