Detection of *Brucella* Genome by Real Time PCR from the Milk of Small Ruminants in the West Bank, Palestine

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Abstract

Brucellosis is an endemic disease of the small ruminants in Palestine, posing a big threat to humans due to its zoonotic character. The rapid detection is the main goal in case of outbreak in order to implement the specific control policy. In the control of the brucellosis, the fast detection must offset the impairment of the “test and slaughter” policy caused by the financial burden. The aim of this study was to develop accurate and rapid molecular techniques to detect *Brucella* in sheep and goat milk. Recently, has been reported an outbreak in Qibbiya (small village of Ramallah district) counting 11 adult people and a 10 month old baby: subsequently, for serological testing, were collected serum samples from 350 animals from the 14 suspected flocks. The milk samples from 33 seropositive animals were checked using an in-house PCR protocol for the *Brucella* genome identification. The results revealed the presence of *Brucella* spp genome in 78.8% (26/33) of the milk samples, while only 27.2% (9/33) of milk samples were positive at isolation on *Brucella* agar. All suspected and confirmed animals were quarantined and necessary measures were taken. Two weeks later, all sero-negative and real time PCR negative animals, were retested using Rose Bengal test and were negatives. Identification of *Brucella* spp by classical methods delay the implementation of control measures. The development of such a rapid and sensitive assay as real time PCR give a reliable tool to be used by authorities, in order to control the spread of Brucellosis.

Keywords: brucellosis, milk samples, real-time PCR, zoonosis

INTRODUCTION

Brucellosis is zoonotic disease and important public health problem worldwide, especially in Mediterranean countries (Doganay and Aygen, 2003; Gul and Khan, 2007). The causative agent of Brucellosis is a intracellular gram-negative bacteria belonging to genus *Brucella*: at this time in this genus are 11 species. The most important pathogen species from this genera is *Brucella*

melitensis, due to his great prevalence in sheeps and goats bred in Mediterranean area and to zoonotic risk. (Scholz and Vergnaud, 2013; Mayer-Scholl *et al.*, 2010). Infections by Brucellosis are caused significant economic losses which include decrease productivity as a result of abortion, weak offspring, reduced milk production and losses of trade opportunities (FAO, 2010). The *B.melitensis* infections in small ruminants are still widespread

in West Bank area, causing human illness, primarily by consumption of contaminated dairy products or as professional diseases. (Saleem *et al.*, 2010; Kaoud *et al.*, 2010; Doganay and Aygen, 2003; Zvizdic *et al.*, 2006; Sanco, 2001). In human, if the disease is not diagnosed and treated promptly and effectively, it can become chronic and affect multiple body systems (FAO, 2010, Saleem *et al.*, 2010; Sanco, 2001; Zvizdic *et al.*, 2006; WHO, 1999).

Several Brucellosis eradication programs have been launched in many countries, but the success of these programs still has not reached the desired level. These programs are based on the vaccination of the animals, carrying out of studies to identify the infected farm animals, the slaughter of animals and limitation of movement from contaminated areas to free regions. (FAO, 2010).

Early detection by valid diagnostic tools is crucial for the control and eradication of Brucellosis (Al-Garadi *et al.*, 2011; Bricker, 2002; Surucuoglu *et al.*, 2009). Culture methods are not always successful as they are time-consuming and the handling of microorganism is hazardous. The sensitivity of the bacteriological culture methods depends on the viability and numbers of *Brucella* in the sample, and of the nature of the sample which is commonly contaminated with other bacteria (Refai, 2002). Milk is the foremost source of *Brucella* infection in humans and its testing by bacteriological culture has low sensitivity (Ning *et al.*, 2013; O'Leary *et al.*, 2006).

Serologic methods are rapid, but not conclusive, because not all infected animals produce detectable levels of antibodies and cross-reactivity with other antigens can give false-positive results (Gwida *et al.*, 2011).

Molecular diagnostic techniques represent an important breakthrough in the diagnostic practice. Most of the authors confirmed that real-time PCR is a very sensitive method for detection of *Brucella* from various samples (Doosti and Ghasemi-Dencodri, 2011; Safarpour-Denkordi, 2014; Newby *et al.*, 2003; Al-Garadi *et al.*, 2011; Foster *et al.*, 2008; Navarro-Martinez *et al.*, 2008). PCR also has the added advantage of being able to simultaneously detect all species of *Brucella* genome and differentiate them with high sensitivity and specificity (Sohrabi *et al.*, 2011; Garcia-Yoldi *et al.*, 2006; López Goñi *et al.*, 2009; Mirnejad *et al.*, 2012; Bounaadja *et al.*, 2009).

The main purpose of this study was detection *Brucella* in milk samples from sero-positive infected animals by using *Brucella* group RT-qPCR protocol developed in-house. This technique could be really useful for rapid quarantine of infected animals, implementation preventive measures and management of outbreaks. Up to now no article has documented about RT-qPCR investigation of *Brucella* genome in Palestine. This will enable the veterinary services control and eradicate Brucellosis in Palestine.

MATERIALS AND METHODS

Samples collection Sheep and goat blood samples were collected from the jugular veins in plain vacutainer tubes without anticoagulant by using aseptic techniques. The samples were kept on ice at about 4°C and transported immediately to the laboratory. Tubes were centrifuged at 3000 rpm for 3 min to separate the serum. The milk samples from sero-positive animals were collected and transported to the laboratory. All milk samples were obtained from animals during their routine milking time, from all 4 quarters of the mammary gland and 200 µl sediment of milk was used for detection of *Brucella* genome by Real-Time PCR assay and culture purpose.

The serological Rose-Bengal test. According to OIE and FAO recommendation the Rose-Bengal test was carried out by the addition of 30µl of serum and 30 µl of Rose-Bengal reagent (Jovac, Jordan) and was well mixing it was shaken for 4 minutes. Sera were considered positive when showing any degree of agglutination detected as score (+, ++, +++, +++) depending on strength of agglutination.

Bacterial isolation from milk samples. The cream and sediment mixtures from each 33 milk samples of sero-positive small ruminants were assessed after centrifugation of milk samples (10 mL) at 2 000g, 4°C for 20 minutes. Cream and sediment were then spread onto *Brucella* agar plates (Oxoid, UK), incubated at 37°C for 2 to 7 days and plates were daily examined for the colony presence and identification according to standard methods.

DNA extraction. Milk samples were centrifuged at 8000 rpm for 15 min to sediment the bacteria (Romero and Lopez-Goni, 1999). The fatty top layer and supernatant were discarded and 200µl pellet used for the extraction procedure by a commercial

kit QIAamp RNA Mini Kit (Qiagen, France) according to the manufacturer's instructions. Also was extracted Rev.1 vaccine (Ovejero, Spain) as positive control in this study.

The Real Time PCR Test. In order to perform RT-qPCR in-house protocol to detect of the *Brucella* spp, DNA of 33 sero-positive milk samples, vaccine strain Rev 1 (Avejero, Spain) as positive control and *Brucella* negative milk samples were used. Real time PCR was performed with set of primers *Brucella* group of the region *bcsp31* forward 5'-GCTCGGTTGCCAATATCAATGC-3', reverse 5'-GGGTAAAGCGTCGCCAGAAG-3' and probe 5'-6FAM AAATCTTCCACCTTGCCCTTGCCATCA-BHQ1-3' (Probert, 2004). DNA amplification mixture was composed of 7.12 µl of nuclease free water, 5 µl Qiagen 1 step RT PCR buffer x5 (Qiagen, Cat. No: 210212), 0.8 µl dNTPs (200 µM) (Qiagen, Ct. No: 210112), 0.5 µl forward and reverse primer (20 µM/µl), 0.5 µl (5 µM/µl) probe (Syntheza, Israel), 1 µl Taq polymerase enzyme (Promega, Cat. No: D1806) and 5 µl of DNA product. Amplification was performed by using SmartCycler, France. Reaction was initiated with denaturation at 95°C for 3 minutes followed by 50 cycles: 95°C for 15 sec, annealing and extension at 60°C for 35 minutes.

RESULTS AND DISCUSSIONS

In this study Rose-Bengal test was used to screen serum of 350 suspected animals for *Brucella* species. Results showed that 9.4% (33/350) of serum were positive for *Brucella*. The milk from sero-positive animals was tested by developed RT-qPCR protocol in-house and the results showed that 78.8% (26/33) were positive. Only 9 samples were positive for *Brucella* when isolation was done (Table 2). By testing milk samples obtained from 33 serologically positive animals, all the 9 (27.2%) positive ruminants detected by bacterial isolation

were also detected as positive by the RT-qPCR in-house assay. Furthermore, 18 (69.2%) additional milk samples that were negative by bacterial isolation, but positive by RBT gave positive results by RT-qPCR assay. Consequently, the agreement score between the bacterial isolation and the RT-qPCR assay was low.

Animals that show negative result do not mean that they are not infected with *Brucella* spp. Several possible reasons have been taken into account to explain the relatively inconsistent performance of *Brucella* detection. The *Brucella* could be located only in the lymph nodes and did not yet reach the milk at the sampling time.

The stage of the infection may influence the antibody level and the number of the bacteria (Alton *et al.*, 1988); the presence of large amounts of host genomic DNA may inhibit the PCR reaction (Navarro *et al.*, 2002); the DNA extraction method used may be crucial for the ability of the PCR assay to detect the bacterium (Romero and Lopez-Goni, 1999). Factors that may compromise DNA recovery from milk include difficulties in disrupting bacterial cell walls, loss of DNA template through extraction procedures, or the presence of potential polymerase inhibitors.

In addition, the amount of milk used for PCR is significant smaller than that required for bacteriological methods, so the number of organisms contained in a sample may thus not reach PCR detection limits (Yousef-Beingi *et al.*, 2005). Therefore, the milk samples were centrifuged and only concentrated bacteria (pellet) was used for extraction. Two weeks later, all serological and RT-qPCR negative animals, were retested using Rose Bengal test and gave negative result. In case of the high risk of contamination and spread of the agent, the shortest period of time for diagnosis of brucellosis in milk are to take into consideration, so the use of RT-qPCR is advantageous.

Tab. 1. Detection of *Brucella* spp. in serum and milk samples by RT-qPCR and bacterial isolation recovered from 33 serologically positive animals.

		Rose-Bengal test			RT-qPCR assay		Bacterial isolation	
		positive	negative		positive	negative	positive	negative
No of samples	Serum 350	33	317	Milk 33	26	7	6	24
% Percentage		18.2%	90.5%		78.8%	21.2%	18.2%	72.7%

This was the first study carried out in practice using the developed in-house protocol of RT-qPCR and it proved to be successfully.

Despite the intensive vaccination program by *Brucella melitensis* Rev 1 vaccine, the Brucellosis remains in Palestine a significant problem. Identification of *Brucella* spp by classical methods is time-consuming, delay the implementation of control measures, and it increase the risk of occurrence of more human cases.

Therefore, to dispose of a rapid, accurate and highly sensitive assay as RT-q PCR gives a reliable tool to be used by authorities, in order to implement measures and control of outbreak for the prevention and spread of the disease among human and animal population. In addition to the molecular assays, the serology methods must be performed and, if possible, confirmed by bacteriological isolation, in accordance with the epidemiological purpose.

The implementation of "test-and-slaughter" policy for infected animals is hindered by lack of financial resources to compensate affected owners.

Some farmers agree to slaughter their animals, but, due to the high level of errors in serology methods, the culling of all sero-positive animals in the flock leads to high economic losses. It was pointed by our study: instead of slaughtering 33 sero-positive animals, only 26 were slaughtered according to the result of real-time PCR assay and moreover, the necessary preventive measures were sooner implemented.

To reduce the prevalence of human infections, in the endemic areas it is extremely important to carry out awareness campaigns to emphasize to farmers to use only boiled milk for all purposes, because most cases occur after consumption of unpasteurized dairy product.

In addition, measures should be adopted to deal with animal health in general and with infected animals in particular. Finally, eradication of brucellosis can only be achieved by test-and-slaughter policy combined with animal movement control. Implementing these preventive measures and compensating owners may be effective in controlling brucellosis in Palestine.

Fig. 1. Performance of RT-q PCR. No significant fluorescence was observed in negative milk samples and negative control.

CONCLUSION

The main purpose of this study was to detect *Brucella* in milk samples from sero-positive animals using *Brucella* group RT-qPCR protocol developed in-house. This technique proved to be really useful for rapid quarantine of infected animals, for the implementation of preventive measures and for the outbreaks management. Up to now no article has documented about RT-qPCR investigation of *Brucella* genome in milk samples from Palestine. This will enable the veterinary services control and eradicate Brucellosis in Palestine.

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