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Detection of Brucella among Voluntary Blood Donors in Turkey by Using a New Real Time PCR Method

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Abstract: Brucellosis is a worldwide zoonosis also the incidence of which has decreased in developed countries it is still effective in Asia, Africa, Central America and the Mediterranean basin (Turkey inside) that have insufficient public health and domestic animal health programs. There are many reports about Brucella endemics in Turkey. Although, human to human transmission is not frequently, it has been reported in association with blood transfusion, bone marrow transplantation and transplacental transmission in worldwide and Turkey. Human brucellosis is diagnosed based on clinical findings and laboratory studies that include bacteriological and serological tests. The conventional serological tests are insufficiently sensitive and specific to be used individually and often leading to difficulty in interpreting the results. In this study, researchers aimed the detection of possible Brucella carriers without manifest clinical finding among voluntary blood donors by using new real time PCR Method. Total 632 voluntary donor blood samples for four month were investigated for Brucella. The samples were subjected to both Wright and ELISA tests for Brucella. In the second step, leucocytes were separated from all samples and performed real time PCR based on BioRad I-Cycler. Although, the region is not high risk epidemiologic area for Brucellosis in Turkey 4 out of total 632 samples were detected by Wright agglutination and Brucella IgG, IgM ELISA while 2 positive samples were found by I-Cycler based real time PCR detection. All of positive samples were collected from rural area donors. Even it is not in high prevalence, this results and earlier studies were shown brucellosis could be a risk for blood transfusion. Researchers advice especially donors from coming rural area must be performed a questionnaire for brucellosis findings and Brucella screening tests must be performed for blood donors in endemic areas.

Key words: Brucella, blood transmission, real time PCR, donor, agglutination

INTRODUCTION

Brucella is a Gram-negative facultative intracellular bacterium, multiplies in macrophages of both human and animals and causes to chronic infections (Paranavitana *et al*., 2005). Brucellosis is one of the world's major zoonotic infections, transmitted from animals to humans animal products and is responsible for enormous economic losses as well as considerable human disease in endemic areas (Queipo-Ortuno *et al*., 2005).

Also, the incidence of which has decreased in developed countries it is still effective in Asia, Africa, Central America and the Mediterranean Basin (also Turkey inside) related to insufficient public health and domestic animal health programs. Four of known six *Brucella* species may cause human infections. *Brucella mellitensis* is the most common cause of human infection followed by *Brucella abortus*, *Brucella suis* and rarely *Brucella canis*. Brucella is able to invade, survive and even replicate in cells of the monocyte-macrophage system. This intracellular life cycle causes to unsuccessful antibiotics therapy and latent infections (Al Dahouk *et al*., 2005).

Disease is particularly acquired by consumption of contaminated dairy products, occupational contact with infected domestic animals or inhalation of contaminated

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dusts (Paranavitana *et al*., 2005; Schutze and Jacobs, 2000). Human to human transmission is rare but has been reported in association with blood transfusion and bone marrow transplantation from infected donors (Akcakus *et al*., 2005). In addition, neonatal infections have been reported which acquired transplacentally or during delivery. Nevertheless, sexually intercourse is a possible means of transmission (Palanduz *et al*., 2000).

Non-specific and irregular clinical symptoms mean that a diagnosis of human brucellosis always needs laboratory investigations (Queipo-Ortuno *et al*., 2005). Currently, diagnosis of brucellosis relies mainly on culture and serological tests. Although, culture is definitive it is a low yield procedure, time consuming and poses a serious threat to laboratory personnel. Serological tests, although valuable diagnostic and epidemiological aides can be presumptive and may have specificity problems due to their reliance on the detection of antibodies bacterial Lipopolysaccharide (LPS) (Elfaki *et al*., 2005). Furthermore, results of serological tests are difficult to interpret in endemic areas or in the context of permanently exposed individuals. The high sensitivity and additional advantages of PCR based assays for the diagnosis of human brucellosis recently were proven. Unlike conventional PCR, real time PCR Methods are easy to standardize and does not require extensive manipulations so that decrease to contamination risk (Queipo-Ortuno *et al*., 2005).

The aim of this study is the detection of possible Brucella carriers without manifest clinical finding among voluntary blood donors by using a new probe based real time PCR Method.

MATERIALS AND METHODS

Subjects: The study included total 632 samples collected from voluntary blood donors that appeal to Duzce University Medical School Research Hospital, Blood Transfusion Center. All donors were performed a questionnaire and compulsory serological tests according to American Association of Blood Banks (AABB) instructions.

Serological tests: For all samples, the Rose-Bengal agglutination test and Wright agglutination test were performed as described earlier (Alton *et al*., 1975). The samples were subjected to ELISA tests for anti Brucella antibodies of IgG and IgM (Vircell, Santa Fe, Spain).

DNA isolations: All samples were collected in CPT cell preparation tubes (Becton Dickinson, Sparks, USA) to separate white blood cells. Bacterial DNAs were isolated from samples using Minikit spin bacterial DNA isolation kit (Qiagen, Hilden, Germany). Aliquots of the final suspension were used for PCR analysis.

Real time PCR: Samples were performed probe based Real-Time PCR Method by using Flourion Brucella kit (Iontek Laboratories, Istanbul, Turkey) and IQ5-I-cycler equipment (BioRad, Hercules, CA, USA). Primers were designed from the 274 bp conserved region of the *alk B* gene of *Brucella abortus*. For signal detecting, FAM marked probes were used. Cy5 marked internal controls were included to all samples as the positive controls, comprised serial dilutions of *Brucella abortus* DNA from 10^3 to 5 fy. Reaction mixture without sample was used as a negative control.

Sequencing and speciation of Brucella DNA: In order to identification of bacterial DNA templates, primers were designed from the conserved region of the gene encoding an immunogenic membrane protein of 31 kDa (BSCP31) of *Brucella abortus* that is specific to the *Brucella* genus and in all known biovars (Mayfield *et al*., 1988). All positive PCR products were sequenced by using ABI PRISM Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, Madrid, Spain) and ABI 3100 automated sequencer (Applied Biosystems, Foster City, CA, USA). DNA sequences were aligned by using Bionumerics Software (Applied Maths, Sint-Martens-Latem, Belgium) and comparisons with Genbank database were searched for species or genus assignment using BLAST (NCBI, NLM, Bethesda, USA).

Statistical analysis: Data were analyzed using SPSS Ver., 15.0 for windows (SPSS Inc., Chicago, IL USA). Student's t-test, Chi-square and Fisher's exact test were used for comparisons. The $p<0.05$ were accepted significant.

RESULTS AND DISCUSSION

All donors were appropriate to donor acceptation criteria according to universal questionnaire and negative for compulsory screened known infection agents.

Socio-demographic specifications of blood donors were shown in Table 1. Comparison of real time PCR and serological tests were shown in Table 2. Four out of total 632 samples were detected by Wright agglutination and Brucella IgG, IgM ELISA while 2 positive samples were found by real time PCR detection. All of positive samples were collected from rural area donors.

Table 1: Socio-demographic characteristics of blood donors

Characteristics	PCR positive (%) (n = 2)	PCR negative (%) (n = 630)	Total (%) (n = 632)	p-value
Age (median±SEM)	33.7±11.3	31.3±8.6	32.5±10.9	>0.05
Sex (Male/Female)	2/0 (100/0)	489/141 (77.6/22.4)	491/141 (77.7/22.3)	>0.05
Rural/City dweller	2/0 (100/0)	337/293 (53.4/46.6)	339/293 (53.6/46.4)	>0.05
Continuous/First donation	1/1 (50/50)	411/219 (65.2/34.8)	412/220 (65.2/34.8)	>0.05

Table 2: Comparison of the 632 donors PCR results with the other serological tests

Tests	PCR positive (%) (n = 2)	PCR negative (%) (n = 630)	Total (%) (n = 632)	p-value
Rose Bengal agglutination test	2 (100)	5 (0.8)	7 (1.1)	>0.05
Wright agglutination test (>1/160)	2 (100)	3 (0.5)	5 (0.8)	>0.05
Anti Brucella IgG ELISA	2 (100)	2 (0.3)	4 (0.6)	>0.05
Anti Brucella IgM ELISA	1 (50)	0	1 (0.1)	>0.05

The identity of real time PCR results and definition of *Brucella* species were performed by nucleic acid sequencing and the both of two PCR positive samples were described as *Brucella abortus* Biovar 1.

Brucellosis is one of the major zoonotic diseases that widely distributed in both human and animals. In contrast to many Northern and Central European countries where brucellosis has been gradually eliminated its prevalence is still high or even increasing in countries of Mediterranean basin representing a significant public health problem in Northern Africa, Spain, Portugal, Southern France, Italy, Greece and Turkey (Godfroid and Kasbohrer, 2002; Taleski *et al*., 2002). During the last decade, about 9000 cases of human brucellosis have been officially reported in Turkey (Mert *et al*., 2002).

The most likely transmission source of brucellosis is the consumption of unpasteurized milk or fresh cheese which are important components of the diet of rural Turkish individuals. This route of infection leads non-selective affects to both genders in contrast to the much higher male to female ratio in endemic countries where brucellosis is recognized to be an occupational disease (Colmenero *et al*., 1996; Lulu *et al*., 1988). The demographic results were correlated with these values.

Transfusion of blood and blood products exposes recipient patients to both non-infectious and infectious adverse effects. Approximately, 11-12 million units of blood are transfused annually with an average of 3.5 units per patient (Schleupner, 1999). Human to human transmission of Brucella is rare but since, 1976 a lot of researchers reported the cases in association with blood transfusion, bone marrow transplantation, transplacental or perinatal exposure (Palanduz *et al*., 2000; Economidou *et al*., 1976; Giannacopoulos *et al*., 2002). Most of these cases were informed from Turkey (Palanduz *et al*., 2000; Chheda *et al*., 1997; Doganay *et al*., 2001). Although, the region of Turkey where performed the present study is semi endemic for Brucella infection, the results obtained from healthy blood donors support these case reports. Insufficient numbers of positive results obstructed to get statistically significant results.

Conventional serological tests may cause to false results related to specificity and sensitivity. The PCR offers an alternative over the conventionally available methods for an accurate diagnosis of brucellosis. Ensuring high standards of performance with the PCR technique is complex and technically demanding and it is reasonable to consider what its place is when serology using either the traditional SAT or more recent ELISA techniques yield satisfactory results. The analytical sensitivity of the I-cycler based real time PCR assay was higher than these conventional PCR Methods (Queipo-Ortuno *et al*., 2005; Al-Nakkas *et al*., 2005).

After sequencing, the both of positive samples were described as *Brucella abortus* can be related to low virulence ratio of this bacterium.

CONCLUSION

Even it is not in high prevalence, this results and earlier studies were shown brucellosis could be a risk for blood transfusion. Researchers advice especially donors from coming rural area must be performed a questionnaire for clinical findings and etiological factors of brucellosis and Brucella screening tests must be performed for blood donors in endemic areas. Consumption of animal products such as milk, cheese, yogurt and people should be asked and the necessary steps should be taken to prevent contamination.

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