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Serological and molecular diagnosis of human brucellosis in Najran, Southwestern Saudi Arabia

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Summary This study aimed to investigate the prevalence of human brucellosis in Najran, southwestern Saudi Arabia, and to assess the performances of ELISA and PCR as diagnostic tools for brucellosis with respect to conventional methods. The study included 340 patients with clinical characteristics of brucellosis. Blood samples from cases and controls were subjected to culture, standard tube agglutination test (SAT), ELISA for IgM and IgG, and *brucella* PCR. The diagnosis of brucellosis was confirmed in 54 (15.9%) of the 340 provisionally diagnosed brucellosis patients. Blood culture identified only 14 (25.9%) cases. The SAT was positive for 50 (92.6%) cases, whereas ELISA IgM, IgG and PCR were found positive in 46, 52 and 38 cases respectively. The sensitivities of ELISA IgM and IgG were 85.2% and 96.3% respectively and the specificity was 100% for each. For PCR, the sensitivity and specificity were 70.4% and 100% respectively. In conclusion, ELISA offers a significant advantage over conventional serological methods in the diagnosis of brucellosis in endemic areas. The PCR test results can be particularly important in patients with clinical signs and symptoms, and negative serological results, allowing the early and rapid confirmation of the brucellosis.

Introduction

Brucellosis is a systemic disease caused by bacteria of the genus *Brucella* that affects humans and numerous animal species. The transmission to humans occurs by the ingestion of raw or unpasteurized milk and other dairy products, by direct contact with infected animal tissues, or by the accidental ingestion, inhalation, or injection of *Brucella* [1].

According to the World Health Organization, half a million of new human cases are reported each year, but these numbers greatly underestimate the true incidence of the disease in humans [2]. Although the disease incidence has decreased

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markedly in industrialized countries, brucellosis remains a major public health problem in many developing countries. In Saudi Arabia, brucellosis is hyper-endemic, with an incidence of 5.4 per 1000 per year. Its prevalence varies in different regions of the country, with values of 8.8—8% having been reported [3]. According to Memish and Mah [4], more than 8000 cases are reported per year to public health authorities.

In humans, brucellosis behaves as a systemic infection with a very heterogeneous clinical spectrum [5]. The infection is characterized by protean manifestations and prolonged recurrent febrile episodes. The features of acute disease are varied and may be insidious, whereas the features of chronic disease, which may persist or recur for years, are often vague [6]. The disease, therefore, cannot be diagnosed on a clinical basis alone, and microbiological confirmation is required through the isolation of *Brucella* spp. from blood cultures or the detection of specific antibodies through the use of serological tests. However, the established methods for laboratory diagnosis are often unreliable in several respects.

Culturing is a time-consuming procedure. Furthermore, failure to detect the pathogen is a frequent occurrence, and *Brucella* spp. are class III pathogens, posing considerable risk to laboratory personnel [7]. The common serological tests include the Rose Bengal plate agglutination test (RBPT), the standard tube agglutination test (SAT), the Coombs test and ELISA. Conventional serological methods also have important limitations: their sensitivity is poor during the early stage of the disease, and their specificity is reduced in areas where the disease is highly endemic and frequent relapses of the disease occur [8]. The development of specific PCR assays is a recent advance in the diagnosis of human brucellosis, but information concerning the use of this diagnostic tool is scarce.

This study aimed to investigate the prevalence of human brucellosis in Najran, southwestern Saudi Arabia, and to assess the performances of ELISA and PCR as diagnostic tools for brucellosis with respect to conventional methods.

Materials and methods

From April 2010 to September 2011, a total of 340 patients seen at the Infectious Diseases Clinic of King Khalid Hospital in Najran and presenting with clinical characteristics of brucellosis were included in this study. Based on the duration of their symptoms, the patients were classified into 3 groups: the acute group, with symptoms for <2 months ($N = 180$); the sub-acute group, with symptoms for 2—12 months ($N = 110$); and the chronic group, with symptoms for >1 year ($N = 50$). One hundred healthy personnel who were blood donors at the hospital's blood bank were enrolled as the controls; the median age of controls was 34.6 years (range, 18—57); and 74 were male, and 36 were female. Eight milliliters of blood (5 ml for culture and serology and 3 ml mixed with EDTA for PCR) was obtained from each patient and control. A diagnosis of brucellosis was made according to one of the following criteria: (i) the isolation of *Brucella* spp. in blood culture or (ii) the presence of a compatible clinical assessment together with a positive result by either SAT (at $\geq$1/160) or ELISA [8] or both.

Bacteriological and serological techniques

The blood cultures were processed inside a class III biological safety cabinet [7] using biphasic blood culture medium (BioMerieux, France) and were incubated at 37 °C in an atmosphere of 5—10% carbon dioxide for 30 days; sub-culturing was performed weekly. Suspected colonies were identified according to standard techniques [9].

For serology, all of the sera from the patients and controls were tested using SAT and ELISA for IgM and IgG antibodies against *Brucella* species. In the SAT, the serum samples were serially diluted in 0.5% saline, and equal volumes of the dilutions (from 1:10 to 1:1280) and of *B. abortus* and *B. melitensis* antigens (Omega Diagnostic Ltd, UK) were mixed in test tubes and incubated in an incubator at 37 °C for 24 h. Known negative and positive control sera were included. A titer of 1/160 was considered positive [8]. The ELISA testing for IgM and IgG against *Brucella* spp. was performed using commercial reagents (Genzyme Virotech, Germany). The absorbance values obtained were converted into Virotech units (VE) using the following formula, according to the manufacturer's instructions: patient sample (mean) absorbance $\times$ 10/mean absorbance value of cut-off controls (>11 VE was considered positive). Borderline results were re-tested and confirmed as either positive or negative.

Brucella PCR

The detection of a 223-bp target sequence within the gene coding for the production of a 31-kDa membrane protein specific to the genus *Brucella* was performed by PCR using specific primers (Qiagen, USA), as previously reported [10]. The sequences of these primers were forward

Table 1 Distribution of laboratory tests results according to the type of brucellosis.

Laboratory test	Brucellosis groups			
	Acute, *N* = 180 (%)	Subacute, *N* = 110 (%)	Chronic, *N* = 50 (%)	Total, *N* = 340 (%)
Culture	13 (7.2)	1 (0.9)	0 (0)	14 (4.1)
SAT	28 (15.6)	6 (5.5)	16 (32)	50 (14.7)
ELISA IgM	32 (17.8)	4 (3.6)	10 (20)	46 (13.1)
ELISA IgG	28 (15.6)	6 (5.5)	18 (36)	52 (15.3)
PCR	32 (17.8)	4 (3.6)	2 (4)	38 (11.2)

5′-TGGCTCGGTTGCCAATATCAA-3′ and reverse 5′-CGCGCTTGCCTTTCAGGTCTG-3′ [11].

Serum samples from the patients and controls were extracted for the isolation of *Brucella* DNA using the E.Z.N.A. commercial kit (Omega Biotech, USA), according to the manufacturer's instructions.

The PCR amplification mixture consisted of pure Taq ready-to-go PCR beads (Amersham Bioscience, UK), 10 pmol/μl of each primer and 50 pg of *Brucella* DNA extract in a total volume of 50 μl. The amplification was performed using a thermal cycler (Cyclogene, Techne, UK). The reaction mixtures were heated to 90 °C for 5 min, followed by 40 amplification cycles, each consisting of 60 s at 90 °C, 30 s at 60 °C and 60 s at 72 °C. A final extension cycle of 72 °C for 7 min was included. The amplified products were electrophoresed through 1.5% agarose gels, which were stained with ethidium bromide and visualized under an ultraviolet trans-illuminator (Cole-Parmer, USA). The presence of an obvious band of 223 bp was considered a positive result.

Statistical methods

The data were analyzed using SPSS 10 for Windows (SPSS Inc, USA). The sensitivity, specificity, and positive and negative predictive values were calculated.

Results

We studied a total of 340 patients having presumptive diagnoses of brucellosis. The patients were between 19 and 82 years of age, with a mean age of 32.18 years and a standard deviation of ±11.73 years. Of these patients, 260 (76.5%) were male and 80 (23.5%) were female, for a male to female ratio of 3.3:1. There was no seasonal variation in the cases studied. The notable symptoms were fever, joint pain, lower backache, headache, and vomiting. The consumption of raw milk (205 patients) and direct contact with domestic animals (138 patients) were recognized as the major risk factors for the transmission of brucellosis in our study.

In this work, the diagnosis of brucellosis was confirmed in 54 (15.9%) of the 340 provisionally diagnosed brucellosis patients. Blood culture identified 14 (25.9%) cases. The SAT was positive for 50 (92.6%) cases, and ELISAs detecting IgM and IgG were positive in 46 (85.2%) and 52 (96.3%) cases, respectively, whereas PCR produced positive results in 38 (70.4%) cases. The distribution of the laboratory test results among the 3 brucellosis groups is listed in Table 1. The control blood samples were all negative by culture, SAT and PCR.

Twenty-one patients with SAT titers of 1/160 and 18 patients with SAT titers of 1/320 yielded positive results in the IgM and IgG ELISAs. The IgM and IgG ELISAs were positive for 4 patients for whom the SAT titer was 1/80. The PCR test was positive in 38 cases in which the SAT titers were 1/160 (11 cases), 1/320 (17 cases), 1/640 (6 cases) or 1/1280 (4 cases), as presented in Table 2. The sensitivity, specificity, and positive and negative predictive values of the ELISA-IgM, ELISA-IgG and PCR assays are presented in Table 3.

Discussion

Brucellosis is an important public health issue in many developing countries, including Mediterranean countries and countries on the Arabian Peninsula. The overall prevalence of human brucellosis in the present study was 16%; in an earlier report, the seroprevalence rate was 19% in the southern region of Saudi Arabia [12]. In a large-scale study investigating the seroprevalence of brucellosis in 24,000 subjects in different Saudi regions [13], the highest prevalence of brucellosis was found in the northern and southern regions, with prevalences of 20% and 18.3%, respectively, whereas the prevalences were 14.6%, 14% and 11.6% in the central, eastern and western regions, respectively. Many previous Saudi studies [14,15]

Table 2 Distribution of ELISA and PCR results according to SAT titers.

SAT titer	No of cases	ELISA IgM		ELISA IgG		PCR	
		Positive	Negative	Positive	Negative	Positive	Negative
1/40	96	0	96	0	96	0	96
1/80	194	4	190	4	190	0	194
1/160	18	18	0	18	0	11	7
1/320	21	21	0	21	0	17	4
1/640	7	3	4	7	0	6	1
1/1280	4	0	4	2	2	4	0

Table 3 Diagnostic yield of ELISA IgM, ELISA IgG and PCR.

Laboratory test	Sensitivity	Specificity	Positive predictive value	Negative predictive value
ELISA IgM	85.2%	100%	100%	97.3%
ELISA IgG	96.3%	100%	100%	99.3%
PCR	70.4%	100%	100%	94.7%

showed that the area of residence (northern or southern region) has a significant effect on the seroprevalence. Border locations in which there are uncontrolled movements of animals may have a high prevalence rate, especially in villages where Bedouins live in close contact with animals. However, in another recent study in the Najran region [16], the prevalence was 7.3% among 540 healthy subjects based on the IgG ELISA (the only diagnostic technique); thus, the authors might have underestimated the true prevalence of the disease. It has been reported that none of the serological techniques used in the diagnosis of brucellosis are 100% sensitive and specific [17,18]. Data from Middle Eastern countries revealed seroprevalence rates ranging from 8% in Jordan [19] to 12% in Kuwait [20].

Despite the important advances made in the diagnostics of human brucellosis following the introduction of automated blood culture techniques, the diagnosis of this disease is still based primarily on serological and molecular methods. Among the newer serological tests, ELISA appears to be the most sensitive [5,6]. In this work, concordant results between the IgM and IgG ELISA titers and the SAT titers were found for 88.5% of the patients within the three groups. Discrepant results were obtained for 6 patients: 4 acute brucellosis patients with negative SAT titers were positive by ELISA for IgM and IgG, and 2 chronic brucellosis patients tested positive only for the IgG ELISA. This scenario for serological results is very similar to that reported elsewhere in the world [17,21,22]. Mantur et al. [17] also reported positive ELISA results for 36 brucellosis patients, in addition to positive IgM and IgG ELISA results for 16 acute brucellosis patients and positive IgG ELISA results for 18 chronic brucellosis patients with negative IgM ELISA and SAT titers. Irmak et al. [21] found that 9% of 26 acute brucellosis patients tested positive by the IgM assay and tested negative by the IgG assay, 56% tested positive by both tests, 26% tested positive by only the IgG assay, and 9% tested negative by both of the tests. It is known that the IgM antibody may be detected after the first week following the start of the infection; the peak level is reached 4 weeks later [22]. Although the IgG antibody has a delayed appearance, it is found mixed with IgM 4 weeks after the initial antigenic stimulus; the IgM antibody level always exceeds the IgG antibody level during the acute stage of the disease. Our findings agree with the principle that the IgM test is more indicative of acute infection, whereas IgG is more useful for the diagnosis of sub-acute and chronic infections.

Different studies have obtained different results regarding the sensitivity and specificity of ELISA, but most of these studies have identified ELISA as the best diagnostic test because of its high sensitivity. The sensitivity and specificity of ELISA in the present study are in agreement with those reported in other studies [23,24]. Araj et al. [23] reported sensitivities of 100% and 91% for IgM and IgG ELISAs, respectively, and a specificity of 100% for both assays. Mantur et al. [17] reported an ELISA sensitivity of 71.3% and a specificity of 100%. Memish et al. [24] found that the respective sensitivity and specificity of the IgM ELISA were 79.1% and 100%, whereas they were 45.6% and 97.1% for the IgG ELISA; combining the IgM and IgG ELISA positivities in their study increased the sensitivity to 94.1% and the specificity to 97.1%.

PCR-based assays are promising alternatives for the diagnosis of brucellosis. In this work, PCR correctly diagnosed the 32 acute brucellosis patients. In accordance, Mitka et al. [25] found that the percentage of PCR-positive results among 200 acute brucellosis patients was 99%, whereas this percentage was 91.2% (31 of 36 patients) in the study of Surucuoglo et al. [26]. In contrast, our PCR test results were only positive for 11.1% of the patients with chronic disease. This lower rate might be due to the low bacterial load in the blood of patients with chronic brucellosis. One of the primary characteristics of the PCR assay that enhances its value, as the results of the present study confirm, is its ability to establish the diagnosis of acute brucellosis.

Although the PCR specificity in this work was 100%, its sensitivity was 70.4%; in previous studies, the PCR sensitivities varied from 66% to 94% [25—28]. The disparate results may be related to a lack of uniformity and standardization among studies with regard to the PCR protocols, such as the optimal clinical specimen, sample volume, extraction method, primer and target sequences, storage conditions of the samples or experimental design [25,26].

Conclusion

The results of this study showed that ELISA offers a significant advantage over conventional serological methods in the diagnosis of brucellosis in endemic areas: its ability to measure 2 specific antibodies makes ELISA an effective diagnostic tool for brucellosis. This specificity is especially important, as it may be possible to use ELISA to confirm the clinical stage of the disease. The PCR test results can be particularly important in patients with clinical signs and symptoms and negative serological results, allowing the early and rapid confirmation of the brucellosis.

Conflict of interest statement

Funding: No funding sources.
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