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Title: Quantitative real-time PCR improve conventional microbiological diagnosis in an outbreak of brucellosis due to ingestion of unpasteurized goat cheese.

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Abstract: Rapid diagnosis of individuals involved in brucellosis outbreaks can sometimes be difficult with conventional microbiological techniques. We analyzed, for the first time, the diagnostic yield of a real-time PCR assay in a family outbreak of brucellosis due to consumption of unpasteurized goat cheese. PCR correctly identified all symptomatic cases.

April 8, 2011.

"**Quantitative real-time PCR improve conventional microbiological diagnosis in an outbreak of brucellosis due to ingestion of unpasteurized goat cheese**"

Dear Sir

Please find enclosed the above mentioned paper for your consideration for publication in **Short Note** section of the ***Diagnostic Microbiology and Infectious Disease*.**

To our knowledge, this is the first study reported to date comparing the diagnostic yield of a real-time PCR assay with conventional microbiological methods in a Brucellosis outbreak.

The manuscript has been seen and approved by all the authors and they have contributed significantly to the work, it has not been published elsewhere and is not under consideration for publication elsewhere. All the authors have read and are familiar with the current "Instructions to Authors" and will comply with the instructions and stated conditions.

Trusting that the article will be of interest to ***Diagnostic Microbiology and Infectious Disease*** we remain.

Sincerely Yours

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REVISED VERSION

SHORT NOTE

TITLE

Quantitative real-time PCR improves conventional microbiological diagnosis in an outbreak of brucellosis due to ingestion of unpasteurized goat cheese.

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KEY WORD: Brucellosis, Outbreak, Epidemiology, Diagnosis, Real-time PCR.

RUNNING TITLE: Rapid diagnosis of brucellosis outbreak by PCR.

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ABSTRACT

Rapid diagnosis of individuals involved in brucellosis outbreaks can sometimes be difficult with conventional microbiological techniques. We analyzed, for the first time, the diagnostic yield of a real-time PCR assay in a family outbreak of brucellosis due to consumption of unpasteurized goat cheese. PCR correctly identified all symptomatic cases.

Since the discovery of *Brucella melitensis* in 1887, brucellosis has been an emerging and re-emerging disease [1-2]. Brucellosis is usually transmitted to humans by direct contact with infected animals, ingestion of unpasteurized dairy products or inhalation of aerosols generated when handling infected materials.

Many different brucellosis outbreaks have been described in farmers, abattoir workers, consumers of raw cheese or milk, veterinarians and microbiology laboratory workers [3-6]. Nevertheless, due to differences in the species of *Brucella* responsible for the infection, the scenarios in which it occurs and the methodology used, little is known about infection attack rates and effectiveness of the different diagnostic methods in brucellosis outbreaks. We describe a household outbreak of brucellosis due to the consumption of unpasteurized goat cheese and analyze the diagnostic yield of a real-time PCR method compared to conventional microbiological methods.

The index case was a 15-year-old male adolescent admitted to the Emergency Department of the Virgen de la Victoria University Hospital, Malaga, Spain in January 2007 with remittent fever, chills, profuse sweating, general malaise and arthralgia for the previous nine days. The liver and spleen were enlarged at physical examination and the patient reported having eaten unpausterized goat cheese two weeks before the onset of symptoms. A Rose Bengal test was positive, as were a standard agglutination test (SAT) and immunocapture-agglutination test, with titres of 1/5120 and 1/10240, respectively, and the blood cultures taken the day of admission grew *B. melitensis*. At this time a family epidemiological survey revealed that ten members of the same family had eaten the suspect cheese. No member had any known prior exposure to *Brucella* infection.

Over the following eight weeks another seven members of the family had clinical symptoms compatible with brucellosis, while the other two remained asymptomatic.

The mean age of the exposed subjects was 29.4±17.5 years (range 7-64 years). The duration of the incubation period was 42.1 days (range, 14-68 days). Seven (87.5%) of the eight subjects who became ill had fever with no apparent focus and the remaining patient, a 64-year-old woman with rheumatoid arthritis treated with infliximab, had fever and clinical data suggestive of subacute meningitis

Two blood culture sets, a quantitative real-time PCR assay in serum, Rose Bengal test, standard agglutination test (SAT) and immunocapture-agglutination test were performed for all the patients with suspected *Brucella* infection. In patients exposed but asymptomatic, only serology and real-time PCR were performed. The study protocol was approved by the IMABIS Committee on Human Research and written informed consent was obtained from all the patients.

Blood samples were incubated in a non-radiometric semiautomatic BACTEC 9240 system (Becton Dickinson, Diagnostic Instrument Systems, Sparks, MD, USA). Blood culture incubation was maintained for 15 days, with blind subcultures on chocolate agar and *Brucella* agar performed after 7 and 15 days. The suspected colonies were identified by colonial morphology, Gram staining, oxidase, catalase and urease tests, and positive agglutination with specific antiserum. All isolated strains were sent to the Brucellosis Reference Laboratory (Valladolid, Spain).

The Rose Bengal card agglutination test (BioMérieux, Charbonieres les Banes, France) and SAT (Atom Biosystem, Barcelona, Spain) were performed according to previously described techniques [7]. The determination of total anti-*Brucella* antibodies was made by an immunocapture-agglutination test (Brucellacapt;

Vircell SL, Granada, Spain), following the manufacturer's instructions [8]. Significant titres were considered to be a SAT ≥1/160 and immunocapture-agglutination test ≥1/320.

For the detection of *Brucella* spp, a 223 bp fragment from the conserved region of the gene which encodes an immunogenic membrane protein of 31 kDa of *B. abortus* (BCSP31) specific to the *Brucella* genus was amplified.

Blood samples for PCR were taken at the same time as the blood cultures. DNA from serum and cerebrospinal fluid (CSF) were extracted using the UltraClean DNA-BloodSpin Kit and UltraClean Tissue DNA isolation Kit (Mo Bio Laboratories), respectively, according to the manufacturer's instructions.

PCR amplifications were performed in capillary tubes with a LightCycler instrument (Roche Diagnostic, SL, San Cugat del Valles, Spain) using the primers B_4 (5' TGG CTC GGT TGC CAA TAT CAA 3') and B_5 (5' CGC GCT TGC CTT TCA GGT CTG 3´) (Tib Molbiol, Berlin, Germany) according to the methodology previously described by our group [9]. Each assay was performed using a standard curve of genomic *B. mellitensis* DNA (2 $x10^5$ -2 copies/mL) and negative controls (water).

Blood cultures were positive in three (all of them identified as *B. melitensis* biovar 3) of the eight symptomatic cases (37.5%). The Rose Bengal test was positive in seven (87.5%) of the symptomatic subjects. SAT and the immunocapture-agglutination test had titres within the diagnostic range for six (75%) of these. SAT and immunocapture-agglutination test were positive but showed titres below the diagnostic range in the remaining two cases and in both asymptomatic subjects.

Real-time PCR was positive in all subjects who became ill and negative in those who did not develop symptoms. The demographic, clinical and microbiological characteristics of the subjects involved in the outbreak are summarized in Table 1.

In samples with positive real-time PCR results, the mean Cp (threshold cycle) was 33.3±4.6 cycles and all *Brucella*-specific amplicons of 223 bp could be distinguished by their characteristic melting temperature of 87.9ºC. The mean bacterial DNA load for household members with brucellosis was 2.18×10^3 copies/mL (range, 6.3×10^1-10.52×10^3 copies/mL). All three patients with positive blood cultures had a bacterial load equal to or higher than $1x10^3$copies/mL.

In the CSF sample from the patient with meningitis, cultures were negative, SAT and immunocapture-agglutination titres were 1/40 and 1/80, respectively, and real-time PCR assay was positive.

The total duration of the outbreak was two months. All subjects who became ill had a good response to treatment and there was no relapse during six months of follow-up.

Among the different *Brucella* species, *B. melitensis* is by far the most virulent to humans [10]. The high attack rate of the outbreak described here seems to confirm this point. Eighty percent of exposed subjects became ill, which is a similar figure to that reported by other authors when *B. melitensis* was the species responsible [11].

As the incubation period of brucellosis is extremely variable, the attack rate very high and the possibility of serious focal complications non-negligible, some authors have recommended treating all exposed subjects [12], However, following this strategy, between 20% and 40% of exposed subjects could be ordered to receive an unnecessary six-week period of treatment.

PCR methods are more sensitive than cultures and more specific than serological tests, both for the diagnosis of acute forms and for focal complications of the disease. Nevertheless, no study has yet examined the diagnostic yield of PCR in an outbreak of brucellosis.

Blood cultures should be performed whenever possible, but our findings confirm that they are of little practical use in the overall study of an outbreak due to their low sensitivity. On the other hand, confirmatory serological tests showed better sensitivity, but did not reach the diagnostic range in 25% of symptomatic subjects.

Real-time PCR was positive in all symptomatic subjects and negative in those exposed but who remained asymptomatic. In addition, there was a clear trend toward high bacterial loads in the three patients with positive blood cultures. Some reports suggest that small bacterial loads may persist for long periods in patients with recent brucellosis [13], though none of our patients had a past history of brucellosis. Moreover, multiple studies have demonstrated the high specificity of PCR-based methods even in endemic areas, provided subjects included as controls had not had a recent history of brucellosis

In conclusion, quantitative real-time PCR could be a useful tool for the rapid diagnosis of persons involved in outbreaks of brucellosis due to occasional consumption of unpasteurized milk or cheese. It is possible that this technology will have no practical use for handling outbreaks in occupationally exposed subjects and studies are needed to determine its usefulness in outbreaks affecting laboratory workers.

ACKNOWLEDGEMENTS

We thank Ian Johnstone for his help with the English language version of the text.

TRANSPARENCY DECLARATION

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Potential conflicts of interest. All authors read and approved the final manuscript

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Table

Table 1. Main demographic, clinical and microbiological data of subjects involved in the brucellosis outbreak.

Subject Number	Age (years) /Sex	Clinical Features	Incubation Period (Days)	Blood culture	Results of serological tests in serum samples			Real-time PCR	Bacterial load Copies/mL
					Rose Bengal test	SAT	Immunocapture agglutination test		
1	34/M	Fever without focus	51	+	+	1/320	1/640	+	$1.89x10^3$
2	37/W	Fever without focus	52	-	-	1/40	1/80	+	$3.35x10^1$
3	15/M	Fever without focus	14	+	+	1/5120	1/10250	+	$10.52x\ 10^3$
4	33/W	Fever without focus	54	+	+	1/2560	1/5120	+	$2.40x10^3$
5	64/W	Lymphocytic meningitis	52	-	+	1/160	1/1280	+	$6.30x10^1$
6	7/M	Fever without focus	18	-	+	1/640	1/2560	+	$2.45x10^2$
7	8/M	Fever without focus	28	-	+	1/640	1/2560	+	$2.30x10^3$
8	38/M	Fever without focus	68	-	+	1/40	1/80	+	$1.10x10^1$
9	40/W	Asymptomatic		ND	-	1/40	1/80	-	-
10	18/M	Asymptomatic		ND	-	1/40	1/80	-	-

ND, not done; PCR, polymerase chain reaction; SAT, standard agglutination test,

Dr Daryl J. Hoban
Associate Editor
Diagnostic Microbiology and Infectious Diseases

17 June 2011

Ref. No.: DMID-11-223
Title: Quantitative real-time PCR improves conventional microbiological diagnosis in an outbreak of brucellosis due to ingestion of unpasteurized goat cheese.

Dear Dr Hoban:

Further to your kind recommendations, we have undertaken a complete revision of the manuscript, retaining the original format, and incorporating as much of the additional information requested as possible, given the restraints in manuscript length, as well as other suggestions made by the reviewers. We regret to have slightly exceeded the number of words suggested for this type of manuscript, which was due to the large amount of additional information requested by Reviewer 3.

Please also find enclosed our item by item comments to the reviewers.

We hope that the revised manuscript will now be acceptable for publication in *Diagnostic Microbiology and Infectious Disease.*

Yours sincerely,

María Isabel Queipo Ortuño PhD.

Reviewer 1

We sincerely appreciate your comments about the quality of our work and its contribution to the diagnosis of brucellosis.

In the revised manuscript, information about the method of extracting DNA from samples of serum and CSF has been included
In addition, the revised version has been checked and the grammatical errors corrected:
p 3, lines 5, 10, 18, 19, 20, 22 and 24.
p 4 line 10.
p 5, lines 1, 10, 12, 17 and 23.
p 6, lines 1, 12, 13, 16, 17 and 19.
p 7, line 1.

Reviewer 2

We sincerely thank the reviewer for the comments.
1. Please note that the objective of this study was not to assess the specificity of our quantitative real-time PCR assay but to compare its diagnostic efficacy with conventional methods in an outbreak due to ingestion of unpasteurized goat cheese. Our group has already reported the diagnostic yield of this quantitative real-time PCR in a large group of patients with Brucellosis and controls, Reference 9.

2. The revised manuscript now includes primer sequences.

3. The revised version also includes standard agglutination and immunocapture-agglutination test titres considered to be significant.

4. There is some evidence that small bacterial loads may persist for long periods in patients with recent brucellosis (Ref 13). For this reason we wanted to be careful not to extrapolate the findings of this study to outbreaks among patients permanently exposed to Brucella infection.

5. On p. 6, "sentenced" has been replaced with "ordered" and "more high" with "higher".

Reviewer 3

We welcome the comments and apologize for the grammatical errors in the original version. The errors have been corrected in the revised manuscript.

We feel we have to disagree with your comment that our paper has a "lack of many controls, explanations and pertinent background information". Our group has been working on the molecular diagnosis of human brucellosis for over 15 years and, since one of our first international publications in 1997 (*Rapid diagnosis of human brucellosis by peripheral-blood PCR assay. Queipo-Ortuño MI et al. J Clin Microbiol. 1997; 35: 2927-3.)* we have published 17 articles reporting the design and methodology of various PCR-based techniques and their diagnostic yield in different clinical settings. One of these articles (**Reference 9:** *Usefulness of a quantitative real-time PCR assay using serum samples to discriminate between inactive, serologically positive and active human brucellosis. Clin Microbiol Infect. 2008;14:1128-34*) describes and evaluates in a large sample of patients with brucellosis and controls the diagnostic efficacy of the technique used in this study.

This time our interest has focused exclusively on assessing the usefulness of our quantitative real-time PCR assay in the diagnosis of a brucellosis outbreak, a scenario in which the diagnostic usefulness of molecular methods has never been assessed. Furthermore, it is impossible to provide detailed information in a paper with a space limitation of 1000 words.

As I am sure you are aware, in Spain and other Mediterranean countries the incidence of brucellosis is still high. The Rose Bengal Agglutination test is a rapid test used all over the world for the screening of Brucellosis (*Rose Bengal test: diagnostic yield and use for the rapid diagnosis of human brucellosis in emergency departments in endemic areas. Clin Microbiol Infect. 2005; 11:221-5)* and, obviously, it is commercially available.

Regarding the serological tests for confirmation; the standard agglutination test (SAT) and the immunocapture-agglutination test have both been widely referenced in the literature and their diagnostic titres are well known. Nevertheless, in the revised version of the manuscript we now include information about the companies supplying the serological tests and the titres considered positive.
Regarding the diagnostic yield of the quantitative real-time PCR, this, too, has been well studied and reported recently (see Reference 9).

The revised manuscript now includes the full name of the hospital where the patients were treated.

The sample used in the real-time PCR was serum. We thought this was clear on page 5 (PCR amplifications were performed in capillary tubes with a LightCycler instrument according to the methodology previously described by our group [9]). However, to avoid any doubt, this point has been clarified in the revised version.

As is clearly indicated in paragraph 2 of page 4, "Two blood cultures were performed for all patients with a suspected *Brucella* infection". Following the usual protocols of good clinical practice, each blood culture set included aerobic and anaerobic bottles.

As I am sure you well know, the isolation of Brucella spp in blood cultures "can never be considered a contaminant". Therefore, to specify whether Brucella melitensis was isolated in one or more blood culture bottles is redundant.

After isolation, Brucella colonies were identified following the usual methods; colonial morphology, Gram staining, oxidase, catalase and urease tests, and positive agglutination with specific antiserum. All strains of Brucella were sent to the Brucellosis Reference Laboratory (Valladolid, Spain) for definitive identification and typing. We again emphasize that it is impossible to provide all the information requested and still remain within the journal requirements (*Papers for the Notes category, which is intended for the presentation of brief observations should not contain any section heading and should not exceed 1,000 words).* Nevertheless, the revised manuscript includes this information. We hope the Editor understands our dilemma and await his opinion.

In reference 9 we described with absolute precision the methodology and rationale for use of a quantitative real-time PCR. As with any outbreak, this study (Ref 9) included individuals who were serologically positive as well as those who were either asymptomatic or oligosymptomatic

The two children younger than nine years of age were treated with the combination of rifampicin and TMP-SMZ for 6 weeks. The five uncomplicated symptomatic adults received doxycycline 100 mg PO twice daily for 45 days plus streptomycin sulphate 1 g IM daily for the first 14 days, and the patient with meningitis was treated with streptomycin sulphate 1 g IM daily for 14 days, doxycycline 100 mg PO b.i.d. and rifampicin 15 mg/kg/d q.d., both for three months.
We hope you understand that space limitations prevent us including this information in the manuscript.

Reviewer 4

We thank the reviewer for the suggestions.
The revised manuscript now includes the results of the quantification in Table 1.
We believe that paragraph 2, page 6 of the original version makes clear that the results of serological tests refer to the CSF sample. However, in order to rule out any possible doubt, we have amended Table 1 to specify that the serological results are serum samples.