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

Journal:	*Clinical Microbiology and Infection*
Manuscript ID:	Draft
Manuscript Type:	Research Note
Date Submitted by the Author:	n/a

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Key Words:	Brucellosis, Outbreak , Diagnosis, Real-time PCR, Epidemiology

Abstract:	Rapid diagnosis of individuals involved in brucellosis outbreaks can be sometimes difficult with conventional microbiological methods. In the present study we analyzed for the first time, the diagnostic yield of a real-time PCR assay in an outbreak due to consumption of unpasteurized goat cheese. PCR correctly identified all symptomatic cases and was negative in exposed subjects who did not develop symptoms. Despite the small number of subjects involved in this outbreak, our results suggest that real-time PCR could be a useful tool in diagnosis of brucellosis outbreaks due to occasional consumption of unpasteurized milk or cheese.

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RESEARCH NOTE

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

RUNNING TITLE: Rapid diagnosis of Brucellosis Outbreak by PCR.

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22 **ABSTRACT**

23 Rapid diagnosis of individuals involved in brucellosis outbreaks can be sometimes
24 difficult with conventional microbiological methods. In the present study we analyzed
25 for the first time, the diagnostic yield of a real-time PCR assay in an outbreak due to
26 consumption of unpasteurized goat cheese. PCR correctly identified all symptomatic
27 cases and was negative in exposed subjects who did not develop symptoms. Despite the
28 small number of subjects involved in this outbreak, our results suggest that real-time
29 PCR could be a useful tool in diagnosis of brucellosis outbreaks due to occasional
30 consumption of unpasteurized milk or cheese.

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

39 Many different brucellosis outbreaks has been described in farmers, abattoir
40 workers, consumers of raw cheese or milk, veterinarians and microbiology laboratory
41 workers [3-6]. Nevertheless, due to the differences in the species of *Brucella*
42 responsible for the infection, scenario in which it occurs and methodology carried out,
43 little is known about infection attack rate and effectiveness of the different diagnostic
44 methods in brucellosis outbreaks. In present study we describe a household outbreak of
45 brucellosis due to the consumption of unpasteurized goat cheese and analyze the
46 diagnostic yield of a real-time PCR method compared to conventional microbiological
47 methods.

48 The index case was a 15-year-old male adolescent admitted to the Emergency
49 Department in January 2007 with remittent fever, chills, profuse sweating, general
50 malaise and arthralgia during the last nine days. Liver and spleen were enlarged at
51 physical examination and the patient reported having eaten an unpausterised goat cheese
52 two weeks before symptoms begin. A Rose Bengal test was positive, standard
53 agglutination test (SAT) and immunocapture-agglutination test were positives with a
54 titres of 1/5120 and 1/10240 respectively and in the blood cultures taken the day of
55 admission grew *B. melitensis*. At this time a family epidemiological survey revealed
56 that the ten members of the same had eaten the suspect cheese. No member had any
57 known prior exposure to *Brucella* infection.

58 Over the next eight weeks others seven members of the family had clinical
59 symptoms compatible with brucellosis, while the other two remained asymptomatic.

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60 Mean age was 29.2±16.65 years (range 7-64 years). The duration of the incubation
61 period was 42.1 days (range, 14-68 days). Seven (87.5%) of the eight subjects who
62 became ill had a fever without apparent focus and the remaining, a 64 year-old female
63 with rheumatoid arthritis treated with infliximab, had fever and clinical data suggestive
64 of subacute meningitis

65 Two blood cultures, a real-time PCR assay, Rose Bengal test, SAT and
66 immunocapture-agglutination test, were performed for all patients with suspicion of
67 *Brucella* infection. In patients exposed but asymptomatic, only serology and real-time
68 PCR were performed. The study protocol was approved by the IMABIS Committee on
69 Human Research and a written informed consent will be obtained for all patients.

70 Blood samples were incubated in a non-radiometric semiautomatic BACTEC
71 9240 system (Becton Dickinson, Diagnostic Instrument Systems, Sparks, MD, USA).
72 Blood cultures incubation was maintained for 15 days, with blind subcultures on
73 chocolate agar and *Brucella* agar being performed after 7 and 15 days. The Rose Bengal
74 card agglutination test and SAT were performed according to previously described
75 techniques [7]. The determination of total anti-*Brucella* antibodies was made by an
76 immunocapture-agglutination test (Brucellacapt; Vircell SL, Granada, Spain), following
77 the manufacturer's instructions [8].

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

81 Blood samples for PCR were taken at the same time as the blood cultures. PCR
82 amplifications were performed in capillary tubes with a LightCycler instrument (Roche
83 Diagnostic, SL, San Cugat del Valles, Spain) using the primers B_4 and B_5 (Tib Molbiol,
84 Berlin, Germany) according to the methodology previously described by our group [9].

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85 Blood cultures were positives in three of the eight symptomatic cases (37.5%).
86 All isolated strains were identified as *B. melitensis*, biovar 3 (Brucellosis Reference
87 Laboratory (Valladolid, Spain). The Rose Bengal test was positive in seven (87.5%) of
88 the symptomatic subjects. SAT and the immunocapture-agglutination test had titres
89 within the diagnostic range for six (75%) of these. Both confirmatory tests were positive
90 but showed titres below the diagnostic range in the remaining two cases and in both
91 asymptomatic subjects.

92 Real-time PCR was positive in all subjects who became ill and negative in those
93 who did not develop symptoms. Demographic, clinical and microbiological features of
94 subjects involved in the outbreak are summarized in the Table 1.

95 In samples with positive real-time PCR results, the mean Cp (threshold cycle)
96 was 33.3±4.6 cycles and all of them *Brucella*-specific amplicons of 223 bp could be
97 distinguished by their characteristic melting temperature of 87.9ºC. The mean bacterial
98 DNA load for household members with brucellosis was 2.1×10^{3} copies/mL (range,
99 6.3×10^{1}-10.52×10^{4} copies/mL). All three patients with positive blood cultures had a
100 bacterial load equal or higher than $1\text{x}10^{3}$ copies/mL.

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

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

107 Among the different *Brucella* species, *B. melitensis* is by far the most virulent to
108 humans [10]. The high attack rate of the outbreak described here seem to confirm this

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109 point. Eighty percent of exposed subjects became ill, similar figure to that reported by
110 other authors when *B. melitensis* was the species responsible [11].

111 As the incubation period of brucellosis is extremely variable, the attack rate very
112 high and the possibility of serious focal complications non-negligible, some authors
113 have recommended treating all exposed subjects [12], However, with this strategy,
114 between 20 and 40% of subjects exposed, could be sentenced to receive unnecessarily a
115 six-weeks treatment.

116 PCR methods are more sensitive than cultures and more specific than serological
117 tests, both for the diagnosis of acute forms and for focal complications of the disease,
118 nevertheless, so far, there is no study that has examined the diagnostic yield of PCR in
119 an outbreak of brucellosis.

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

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

132 In conclusion, quantitative real-time PCR could be a useful tool in the rapid
133 diagnosis of persons involved in outbreaks of brucellosis due to occasional consumption

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134 of unpasteurized milk or cheese. It is possible that this technology had not a practical
135 use for handling outbreaks of occupationally exposed subjects and studies are needed to
136 determine its usefulness in outbreaks affecting laboratory workers.

137 **ACKNOWLEDGEMENTS**

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

139

140 **TRANSPARENCY DECLARATION**

141 This work received financial support from the Instituto de Salud Carlos III (ISCIII),
142 F.I.S (grant PI06/0495), and the Consejería de Innovación Ciencia y Empresa (grant
143 CTS-276 and P-08 CTS 3969), both of the Junta de Andalucía (Spain).

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

145 **AUTHORSHIP CONTRIBUTION**

146 Colmenero JD, participated in the design of the study, analysis and interpretation of data
147 and helped to draft the manuscript.
148 Clavijo E, have made substantial contributions to conception and the design of the
149 study, carried out the microbiological studies and helped to draft the manuscript
150 Morata P, participated in the design of the study and helped to draft the manuscript.
151 Bravo MJ, participated in the design of the study and helped to draft the manuscript.
152 Queipo-Ortuño MI, conceived and designed the study, carried out the real-time PCR
153 assays and helped to draft the 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			Real-time PCR
					Rose Bengal test	SAT	Immunocapture agglutination test	
1	34/M	Fever without focus	51	+	+	1/320	1/640	+
2	37/W	Fever without focus	52	-	-	1/40	1/80	+
3	15/M	Fever without focus	14	+	+	1/5120	1/10250	+
4	33/W	Fever without focus	54	+	+	1/2560	1/5120	+
5	64/W	Lymphocytic meningitis	52	-	+	1/160	1/1280	+
6	7/M	Fever without focus	18	-	+	1/640	1/2560	+
7	8/M	Fever without t focus	28	-	+	1/640	1/2560	+
8	38/M	Fever without focus	68	-	+	1/40	1/80	+
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

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