Real time polymerase chain reaction: a new powerful tool for the diagnosis of neurobrucellosis

J D Colmenero, M I Queipo-Ortuño, J M Reguera, G Baeza, J A Salazar, P Morata

**Background/methods:** We compared the diagnostic yield of a real time polymerase chain reaction (PCR) assay in cerebrospinal fluid (CSF) samples with conventional microbiological techniques for the diagnosis of neurobrucellosis. Following amplification of a 223 bp sequence specific for *Brucella* genus, melting curve analysis was performed to verify the specificity of the PCR products.

**Results:** All six patients with neurobrucellosis (three meningitis and three meningoencephalitis) had a positive real time PCR assay, whereas CSF cultures and Wright seroagglutination tests were positive in only two and four cases, respectively. *Brucella* specific amplicons were easily demonstrated by their characteristic melting temperature in all the real time PCR assays.

**Conclusion:** LightCycler based real time PCR assay in CSF samples is more rapid and sensitive than conventional microbiological tests. This technique could be useful for the rapid diagnosis of neurobrucellosis.

**Patients and methods**

**Patients**

From January 1998 to December 2002, six patients with neurobrucellosis were diagnosed, treated, and followed at the Infectious Diseases Unit or Neurology Service of Carlos Haya University Hospital, Spain. The diagnosis of brucellosis was established according to one of the following criteria: first, isolation of *Brucella* spp in blood or any other body fluid or tissue sample, or, second, the presence of a compatible clinical picture together with the demonstration of specific antibodies at significant titres or seroconversion. Significant titres were considered to be a Wright’s seroagglutination ≥1/160 or a Coombs antibrucella test ≥1/320. Neurobrucellosis was diagnosed in patients with neurological symptoms and signs not otherwise explainable and at least one of the following criteria: (a) isolation of *Brucella* from CSF; (b) demonstration of antibodies to *Brucella*, agglutinating or non-agglutinating, in CSF (at any titre) in the presence of one or more abnormalities of the CSF (>10 cells/μl, protein levels >45 mg/dl, or glucose levels <40 mg/dl or 40% of the concomitant blood glucose level).

**Specimen collection and processing**

Samples of CSF were cultured onto blood and chocolate agar media, MacConkey agar, and *Brucella* agar (Biomedics, San Sebastian de los Reyes, Madrid, Spain). In order to rule out the possibility of mycobacterial and fungal infection all samples were also cultured onto Lowesteen and Sabouraud medium. The plates were incubated in a 10% CO₂ atmosphere at 37°C for at least 7 days. If growth appeared, the suspected colonies were identified by colonial morphology, Gram staining, oxidase, catalase and urease tests, and positive agglutination with specific antiserum.

**DNA extraction**

CSF samples were maintained at −20°C until processing. DNA was extracted, by boiling, from 200 μl to 1 ml of CSF placed in a 1.5 ml microcentrifuge tube and centrifuged for 15 min at 15 000 X g. The supernatant was eliminated and the pellet resuspended in 200 μl of sterile water and centrifuged for 10 min at 15 000 X g. The supernatant was again eliminated and the pellet resuspended in 40 μl of sterile water and subjected to boiling in a water bath for 10 min, cooled on ice and centrifuged for 10 s at 15 000 X g before storing at −20°C until use. A total of 2 μl of the suspension was used for PCR.

**Real time PCR with SYBR Green I**

Primers were selected from the conserved region of the gene that encodes an immunogenic membrane protein of 31 kDa of *Brucella abortus* specific to the *Brucella* genus. The pair of 21 nucleotide primers B₄ (5’ TGG CTC GGT TGC CAA TAT CAA 3’) and B₅ (5’ CGC GCT TGC CTT TCA GGT CTG 3’) (Tib Molbiol, Berlin, Germany) were used in the amplification process. PCR amplifications were performed in capillary tubes with 20 μl of final volume in a LightCycler instrument (Roche Diagnostic, Mannheim, Germany). Reaction mixtures contained 2 μl of LightCycler FastStart DNA master mixture for SYBR Green I (Roche Diagnostic, Germany), 0.5 μM each primer, 4 mM MgCl₂, and 2 μl of DNA template. All capillaries were sealed and then centrifuged at 500 X g for 45 s, placed in a 10% CO₂ atmosphere, stored at −20°C until processing. PCR amplifications were performed in capillary tubes with 20 μl of final volume in a LightCycler instrument (Roche Diagnostic, Mannheim, Germany). Reaction mixtures contained 2 μl of LightCycler FastStart DNA master mixture for SYBR Green I (Roche Diagnostic, Germany), 0.5 μM each primer, 4 mM MgCl₂, and 2 μl of DNA template. All capillaries were sealed and then centrifuged at 500 X g for 45 s, placed in a 10% CO₂ atmosphere, stored at −20°C until processing.
stranded PCR product was measured during the 72 °C 
the binding of SYBR green to the product.

thermocycles were run with 10 s denaturation at 95 °C, 10 s 
(Table 2).

titres were higher than those of the agglutination test 
by Coombs antibrucella test. In fact, Coombs antibrucella 
the other two cases non-agglutinating antibodies were shown 
seroagglutination was positive in four cases (66.6%) and in 
meningitis in all cases (Table 2). Antibrucella antibodies 
were present fever and only three (50%) had clear signs of 

age was 37.6

RESULTS

Five of the patients were men and one a woman. Their mean 
was 37.6 ± 16.6 years (range: 21–69 years). Four patients 
had usual contact with sheep or goats, one habitually 
consumed unpasteurised dairy products, and the other had 
no known prior exposure to Brucella infection.

Three patients had meningococcal meningitis and three had 
meningitis. The duration of the symptoms prior to diagnosis 
was 90 ± 138 days (range: 5–365 days). Four patients (66.6%) 
presented fever and only three (50%) had clear signs of 
meningeal irritation. Other relevant clinical data are shown in 
Table 1.

Examination of CSF showed findings compatible with 
meningitis in all cases (Table 2). Antibruccella antibodies 
could also be demonstrated in CSF in all cases. Wright 
seroagglutination was positive in four cases (66.6%) and in 
the other two cases non-agglutinating antibodies were shown 
by Coombs antibruccella test. In fact, Coombs antibruccella 
titres were higher than those of the agglutination test 
(Table 2).

CSF IgG values were increased in all cases (range: 2.34– 
6.42 g/L), and CSF protein electrophoresis showed oligoclo-

DISCUSSION

The clinical spectrum of neurobrucellosis is very heteroge-

neous. Different clinical pictures have been described, 
including meningitis, meningoencephalitis, intracerebral 
haemorrhage, benign intracranial hypertension, optic neur-
itis, arachnoiditis, polyradiculoneuritis, myelitis, and various 
combinations of these. Accordingly, neurobrucellosis, like 
neurosphilis, is easily confused with many other neurolo-
gical, neurosurgical, or even psychiatric processes.

As seen in this and other studies, although the meninges 
are almost always involved in patients with neurobrucellos-
is, the clinical signs of meningeal irritation are often 
lacking or, if present, may well be discrete. Furthermore, 
the fact that CSF cultures are negative in over 50% of cases, that 
IgG oligoclonal bands are often present, and that these 
patients are not always seen by specialists in infectious 
diseases, explains why neurobrucellosis is not always 
considered in the differential diagnosis.

Adequate antibiotic treatment usually results in a good 
prognosis for patients with neurobrucellosis. However, 20– 
30% of patients still present neurological sequelae, which are 
clearly related to the delay in diagnosis and initiation of 
correct antibiotic treatment.

Our group has reported that PCR based methods provide 
better results than conventional microbiological techniques 
for the diagnosis of both primary infection and relapses of 
brucellosis. Real time PCR is a new automated amplification 
technique that quantitatively monitors PCR products as they 
accumulate during thermal cycling. LightCycler technology 
(Roche Diagnostic, Germany) combines rapid amplification 
and sequence-specific detection of amplicons in an auto-
mated and standardised format. Because this probe does 
not require post-amplification handling, much faster assays 
are possible.

This study showed real time PCR assay to be positive in all 
six cases of neurobrucellosis, whereas the sensitivity of

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Summary of clinical finding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patient 1</td>
</tr>
<tr>
<td>Duration of the symptoms</td>
<td>5 days</td>
</tr>
<tr>
<td>Fever</td>
<td>+</td>
</tr>
<tr>
<td>Vomiting</td>
<td>-</td>
</tr>
<tr>
<td>Headache</td>
<td>-</td>
</tr>
<tr>
<td>Stiff neck</td>
<td>-</td>
</tr>
<tr>
<td>Obnudation</td>
<td>+</td>
</tr>
<tr>
<td>Papilloedema</td>
<td>-</td>
</tr>
<tr>
<td>Impaired vision</td>
<td>-</td>
</tr>
<tr>
<td>Hemiparesis</td>
<td>-</td>
</tr>
<tr>
<td>Deafness</td>
<td>-</td>
</tr>
<tr>
<td>Seizures</td>
<td>+</td>
</tr>
<tr>
<td>Ataxia</td>
<td>+</td>
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seroagglutination and cultures of CSF samples was 66.6% and 33.3%, respectively. Real time PCR was negative in all the control samples, which is in keeping with the specificity of the primers used. Previous studies have demonstrated the high specificity of the B4 and B5 primers with a wide panel of microorganisms.13

The LightCycler assay used is rapid, easy, and objective, allowing sensitive and specific identification of PCR products in less than 2 hours. Moreover, the technique is a closed-tube system; this considerably reduces the risk of contamination by product carryover and allows simultaneous handling of a large number of samples, making it very suitable for use in a clinical laboratory. Finally, it should not be forgotten that \textit{Brucella} spp are class III pathogens requiring special protection measures. PCR assays completely obviate the need for direct handling of the pathogen, thus greatly reducing the risk of infection of laboratory personnel.

In conclusion, although the results of this study are preliminary, the high sensitivity and specificity of this real time PCR assay, together with its speed and versatility, make it a very useful tool and could lead to it being considered the new gold standard for the diagnosis of neurobrucellosis.

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Competing interests: none declared

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REFERENCES

**Table 2** Biological and microbiological finding in CSF samples of patients with neurobrucellosis

<table>
<thead>
<tr>
<th>Glucose mmol/L</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
<th>Patient 4</th>
<th>Patient 5</th>
<th>Patient 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF/plasma glucose ratio (%)</td>
<td>4.49</td>
<td>2.55</td>
<td>1.05</td>
<td>0.61</td>
<td>1.66</td>
<td>1.27</td>
</tr>
<tr>
<td>CSF WBC/ml (% lymphocytes)</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Adenosine deaminase U/L</td>
<td>85 (75%)</td>
<td>825 (70%)</td>
<td>579 (75%)</td>
<td>540 (70%)</td>
<td>45 (95%)</td>
<td></td>
</tr>
<tr>
<td>Protein g/L</td>
<td>0.85</td>
<td>3.03</td>
<td>5.35</td>
<td>0.84</td>
<td>0.80</td>
<td>1.80</td>
</tr>
<tr>
<td>Real time PCR</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Culture</td>
<td>–</td>
<td>B melitensis</td>
<td>–</td>
<td>–</td>
<td>B melitensis</td>
<td>–</td>
</tr>
<tr>
<td>Agglutination*</td>
<td>20</td>
<td>320</td>
<td>–</td>
<td>–</td>
<td>40</td>
<td>80</td>
</tr>
<tr>
<td>Coombs test*</td>
<td>160</td>
<td>5120</td>
<td>1280</td>
<td>640</td>
<td>160</td>
<td>5120</td>
</tr>
</tbody>
</table>

*Expressed as reciprocal of titres.

CSF, cerebrospinal fluid; ND, not done; PCR, polymerase chain reaction; WBC, white blood count.
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