Evaluation of strain-specific primer sequences from an abortifacient strain of ovine Chlamydophila abortus (Chlamydia psittaci) for the detection of EAE by PCR

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Received 17 March 2000; received in revised form 24 May 2000; accepted 24 May 2000

Abstract

Strain-specific primer sequences derived from the helicase gene of an ovine abortifacient strain (S26/3) of Chlamydophila abortus (Chlamydia psittaci) were evaluated for the diagnosis of enzootic abortion in ewes (EAE) by polymerase chain reaction (PCR). C. abortus DNA was amplified from tissues submitted from ovine abortion cases using genus-specific and strain-specific primers in a standard thermal cycler. Amplification was followed by Southern blotting and hybridisation with a strain-specific probe. Real-time PCR was also evaluated using strain-specific primers in a microvolume fluorimeter-based thermal cycler (LightCycler®). Detection using both PCR methods was compared with other diagnostic methods against the standard of McCoy cell culture isolation. In this paper we report the application of strain-specific PCR as a fast, sensitive, specific method for the detection of EAE. © 2000 Published by Elsevier Science B.V. on behalf of the Federation of European Microbiological Societies.

Keywords: Chlamydophila abortus; Chlamydia psittaci; Enzootic abortion; Polymerase chain reaction; Diagnosis; LightCycler®

1. Introduction

Chlamydiaceae are obligate intracellular Gram-negative bacteria, with nine species now recognised within the two genera of Chlamydia and Chlamydophila [1]. The species are widespread in the animal kingdom with the ovine species of Chlamydophila abortus (Chlamydia psittaci) being the causative agent of enzootic abortion in ewes (EAE) [2]. Not only is this disease of considerable economic importance, causing loss of lambs due to abortion and the birth of weak lambs, but it is also of importance due to its zoonotic potential, causing abortion in pregnant women.

To improve the efficiency of eradication schemes a simple, sensitive test for the specific identification of ovine C. abortus is required. At present, EAE diagnosis is achieved using either cell culture isolation, detection by immunofluorescence or by the use of genus-specific antigen detection commercial kits [3]. Although the commercial kit (Clearview Chlamydia, Unipath) used in this survey is fast and easy to use, it is designed for the detection of Chlamydia trachomatis in humans and based on antigen detection using a genus-specific lipopolysaccharide (LPS) monoclonal antibody. Detection of C. abortus by cell culture isolation is still regarded as the definitive test and the most sensitive [4], however, it is labour intensive and requires cell culture facilities.

Differentiation of chlamydial species can be achieved utilising differences in nucleic acid sequences. Primer sequences have been published from well conserved regions of the major outer membrane protein (MOMP). Following amplification using the genus-specific primers, the product can be analysed using restriction fragment length polymorphism (RFLP) to differentiate species [5,6]. This species differentiation can also be achieved using RFLP analysis of an amplified region between the 16S and 26S rRNA genes [7]. A two-step polymerase chain reaction (PCR) amplification test coupled with RFLP analysis [8] differentiates C. abortus, C. trachomatis and Chlamydophila pneumoniae. The primers are again situated in the MOMP gene with specific amplification carried out in a nested PCR amplification step. Chlamydial species differentiation using a multiplex PCR test with primers located in a cysteine-rich outer membrane protein (omp 2) has also been described [9].
Strain-specific sequences isolated using subtraction hybridisation have been reported [10]. The gene fragment isolated is reported to be a putative helicase that is repeated throughout the chlamydial genome. In this paper, we report the use of strain-specific primer sequences identified from the putative helicase gene of ovine Chlamydia abortus (S26/3) for the detection of EAE DNA in clinical samples.

2. Materials and methods

2.1. Preparation of clinical samples

The tissues used for the survey were ovine placenta, foetal lung and foetal abomasum which were submitted to the diagnostic laboratory from cases (n = 302) of ovine abortion where chlamydial infection was suspected. In some cases placental tissue was unavailable and only foetal lung and abomasum were submitted. From the placental tissues, homogenates were prepared from the cotyledon and intercotyledonary regions. This facilitates the identification of abortifacient organisms other than C. abortus and may also be necessary due to the necrotic condition of the tissue available. Twenty percent tissue homogenates were prepared in cell culture medium using previously described methods [11]. This homogenate was subsequently used for isolation of chlamydiae in McCoy cells and for DNA extraction.

2.2. DNA extraction

The ovine abortifacient strains of C. abortus (S26/3 and C94-1) and the Chlamydophila pecorum strains (W73, C95-38 and P787) were propagated in embryonated hen eggs using previously described methods [12]. DNA was extracted from these strains using a standard phenol/chloroform technique. Negative control DNA was extracted from uninoculated eggs. DNA was extracted from clinical tissue using the homogenate prepared for cell culture isolation. The extraction was carried out from the homogenate using the Qiamp DNA mini kit (Qiagen) according to the manufacturer’s instructions.

2.3. DNA amplification using genus-specific chlamydial primers

Amplification of a 1-kb fragment from the MOMP gene was carried out using the primer sequences previously published [5] which will subsequently be referred to as MOMP primers. Placental DNA samples were amplified using these primers, however in the absence of placental tissue, foetal lung and abomasum were used from a number of selected cases. Amplification conditions were as follows; 1 cycle at 94°C for 5 min, 25 cycles of 94°C for 1 min, 57°C for 1 min and 72°C for 2 min, followed by 1 cycle at 72°C for 7 min. Amplification products were separated by electrophoresis on a 1% (w/v) agarose gel and visualised by ethidium bromide fluorescence. All PCR products containing a band at 1 kb and had their specificity confirmed by Southern blotting, followed by hybridisation with a strain-specific, ovine, abortifacient, C. abortus S26/3 DNA probe which was derived from the 1-kb fragment amplified using MOMP genus-specific primers. This probe was peroxidase-labelled using the enhanced chemiluminescence (ECL) direct nucleic acid-labeling system as described previously [10]. Selected PCR products were confirmed as C. abortus by restriction endonuclease analysis. This was carried out by purification of the amplified product using Wizard PCR Preps DNA purification system (Promega), followed by restriction digestion using the restriction endonuclease AluI. The restricted fragments were electrophoresed and the chlamydial species identified using restriction endonuclease profiles previously published [5].

2.4. DNA amplification using strain-specific primers

The sequence for the EAE strain-specific primers was derived from a 479-bp strain-specific C. abortus fragment (accession number AF051935) isolated by subtraction hybridisation [10]. Henceforth, strain-specific primers will be referred to as clone 8 primers and their sequence is as follows: clone 8 forward primer, 5’ TGG TAT TCT TGC CGA TGA C 3’; and clone 8 reverse primer, 5’ GAT CGT AAC TGC TTA ATA AAC CG 3’. DNA samples used for amplification were as described for genus-specific primers. In each reaction, the final concentration of reagents was as follows; 1×PCR Supermix (Life Technologies (20 mM Tris–HCl pH 8.4, 5 mM KCl, 1.5 mM MgCl₂, 200 μM deoxynucleoside triphosphates, 2 U recombinant Taq DNA polymerase)) and 1 μM of each clone 8 primer. Amplification conditions were 1 cycle at 95°C for 5 min, followed by 40 cycles of 94°C for 1 min, 45°C for 1 min and 72°C for 2 min and a final extension cycle of 72°C for 7 min.

Amplification products were separated by electrophoresis on a 1% (w/v) agarose gel and visualised by ethidium bromide fluorescence. All samples containing a band at 479 bp were Southern-blotted using standard techniques and the specificity confirmed using a strain-specific probe derived from the 479-bp fragment amplified using clone 8 strain-specific primers. This probe was peroxidase-labelled using the ECL direct nucleic acid-labelling system as described previously [10].

2.5. Fluorimetric detection of amplified DNA using clone 8 primers

Only placental DNA samples were used for amplification using clone 8 strain-specific primer sequences described previously. To enable ‘hot start’ PCR using Plat-
inium Taq antibody (Life technologies), 40 U Taq antibody was added to 500 µl 2×PCR Master Mix (DNAmp). After incubation at room temperature for 20 min the antibody-master mix was ready for use. In each 20-µl PCR reaction, the final concentration of reagents was 1×PCR Master Mix containing 2 mM MgCl₂ (DNAmp). Sybr green (Idaho Technology) was used at 1:20 000 dilution of stock according to manufacturer's instructions. The reaction mix including target DNA was placed in 5-µl volumes in reaction cuvettes (Idaho Technology). A low-speed centrifugation step (285 ×g for 30 s) was carried out to place the reaction mix at the tip of the cuvette and then the tube was sealed with a plug. Amplification was carried out in LightCycler® LC32. The amplification conditions were as follows: 35 cycles of 94°C for 0 s, 47°C for 0 s, and 72°C for 10 s. The melting temperature (Tₘ) of the PCR product was determined by plotting the negative derivative of fluorescence with respect to temperature (−dF/dT versus T). Confirmation of results from clinical samples with reduced melting peak signals was carried out by Southern blotting using a peroxidase-labelled clone 8 fragment positive control and detection was carried out as previously described.

2.6. Additional diagnostic methods used

Ovine tissues were also tested by cell culture isolation, Clearview Chlamydia (Unipath), modified Ziehl-Neelson (MZN) staining and the fluorescent antibody test (FAT) on frozen cryostat sections.

The Clearview Chlamydia antigen detection kit was used on all placental tissues available. A sterile cotton swab was rubbed against a freshly cut section of placental tissue before the test was carried out according to the manufacturer’s instructions.

Cell culture isolation of chlamydiae from placenta, lung and abomasum was carried out in McCoy cells. All placenta tissue was tested in this way, however in the absence of placenta, both lung and abomasum were tested. Forty-eight h post-inoculation the cells were fixed and stained using a fluorescein isothiocyanate (FITC)-labelled chlamydia LPS-specific monoclonal antibody as previously described [11]. Selected tissue homogenates were passaged into embryonated hen eggs [12] following passage in McCoy cell culture to optimise the growth of chlamydiae.

MZN staining of chlamydiae in placental smears was carried out using standard techniques, as previously described [2]. Staining of chlamydia in frozen placenta, lung and abomasum cryostat sections using FITC-labelled chlamydia LPS-specific monoclonal antibody was carried out using previously described methods [11].

The sensitivity and specificity of the diagnostic tests used was calculated with the isolation of chlamydiae in McCoy cell culture as the standard test and using methods described in Table 2.

3. Results

3.1. Specificity of chlamydial primers

The results obtained when clone 8 and MOMP primers were used to amplify five strains of chlamydial DNA are shown in Fig. 1. The clone 8 primers amplified only the two abortifacient strains of C. abortus with no amplification of the three strains of C. pecorum taking place (Fig. 1A). When MOMP primers are used against the same strains of chlamydiae, a 1-kb fragment was amplified from all C. abortus S26/3 DNA; lane 2, C. abortus Haden DNA; lane 3, negative control DNA; lane 4, C. psittaci C94-1 DNA; lane 5, C. pecorum W73 DNA; lane 6, C. pecorum P787 DNA; lane 7, C. pecorum C95-38 DNA.

3.2. Detection of EAE DNA using fluorimetric detection of amplified product

An example of melting peaks obtained following amplification using clone 8 primers and fluorimetric detection
using the LightCycler® is shown in Fig. 2. The melting temperature of the ovine abortifacient C. abortus S26/3 control was approximately 85°C and any primer complexes in the negative control were easily distinguished from the S26/3 by melting peak analysis. Although the melting temperature is affected by changes in sequence, and slight variations in sequence are expected from field isolates, positive field samples were readily identified by their melting peaks. A small number of placental samples gave positive results but with a reduced level of fluorescence, resulting in a lower melting peak. These samples were confirmed as positive by Southern blotting of the amplified product and hybridisation with a relevant C. abortus-labelled probe.

3.3. Southern blot analysis of PCR-amplified EAE DNA field samples using genus-specific and strain-specific primers

All placental samples which gave bands at 1 kb and 479 bp following amplification with MOMP and clone 8 primers, respectively, were confirmed as positive by Southern blotting and hybridisation with the relevant C. abortus probe as described previously.

3.4. Comparison with other diagnostic methods used

Placental tissues were tested by McCoy cell culture isolation, FAT, PCR (using MOMP and clone 8 primers), LightCycler® (using clone 8 primers), MZN and Clearview Chlamydia. McCoy cell culture isolation was also carried out on a proportion of lung and abomasum tissues submitted. The sensitivity and specificity of the tests carried out on placental tissues and the isolations carried out on lung and abomasum were calculated by comparison with McCoy cell culture isolation in placental tissue as this is regarded as the definitive standard for diagnosis of chlamydia infection [4]. The results of specificity and sensitivity and the number of samples positive and negative by each test are shown in Tables 1 and 2, respectively.

Seven samples found negative by McCoy cell culture isolation but positive by nucleic acid amplification using MOMP primers, were restriction endonuclease digested by AluI. The species of chlamydiae was identified according to previously published methods [5]. In all seven cases, the PCR product was identified as C. abortus. In an attempt to optimise chlamydial growth in these seven cases, McCoy cell culture isolation was followed by a passage in embryonated hen eggs. Five days post-inoculation, the yolk stalks were examined for C. abortus infection using immunofluorescence antibody test, according to previously published methods [11]. All seven cases were found to be negative after this additional passage in eggs. In these seven specific cases that were negative by cell culture but positive by MOMP PCR, three were also positive by clone 8 PCR. This result may be due to the presence of EAE DNA but the lack of viable elementary bodies (EB) required for cell culture isolation.

### Table 1

<table>
<thead>
<tr>
<th>Diagnostic test used</th>
<th>Number of samples positive by each test (%)</th>
<th>Total number of samples tested by each method (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>McCoy cell culture isolation (placenta)</td>
<td>86 (37.2%)</td>
<td>231 (100%)</td>
</tr>
<tr>
<td>McCoy cell culture isolation (lung)</td>
<td>28 (13.9%)</td>
<td>201 (100%)</td>
</tr>
<tr>
<td>McCoy cell culture isolation (abomasum)</td>
<td>18 (9.5%)</td>
<td>190 (100%)</td>
</tr>
<tr>
<td>Clone 8 PCR</td>
<td>73 (31.6%)</td>
<td>231 (100%)</td>
</tr>
<tr>
<td>MOMP PCR</td>
<td>71 (30.7%)</td>
<td>231 (100%)</td>
</tr>
<tr>
<td>LightCycler®</td>
<td>74 (32.0%)</td>
<td>231 (100%)</td>
</tr>
<tr>
<td>FAT</td>
<td>90 (50.6%)</td>
<td>178 (100%)</td>
</tr>
<tr>
<td>MZN</td>
<td>34 (27.4%)</td>
<td>124 (100%)</td>
</tr>
<tr>
<td>Clearview Chlamydia</td>
<td>107 (46.5%)</td>
<td>230 (100%)</td>
</tr>
</tbody>
</table>

Results are based on tests carried out on placental tissue unless otherwise stated.
Table 2
Sensitivity and specificity of diagnostic tests compared with McCoy cell culture isolation in placenta tissue [16]

<table>
<thead>
<tr>
<th>Diagnostic test used</th>
<th>Sensitivitya (%)</th>
<th>Specificityb (%)</th>
<th>Number of false negatives</th>
<th>Number of false positives</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clone 8 PCR</td>
<td>81.40</td>
<td>97.93</td>
<td>16/159</td>
<td>3/73</td>
</tr>
<tr>
<td>MOMP PCR</td>
<td>74.42</td>
<td>95.17</td>
<td>22/160</td>
<td>7/71</td>
</tr>
<tr>
<td>LightCycler® (using clone 8 primers)</td>
<td>80.23</td>
<td>96.55</td>
<td>17/158</td>
<td>5/74</td>
</tr>
<tr>
<td>Clearview Chlamydia</td>
<td>88.34</td>
<td>77.46</td>
<td>10/123</td>
<td>32/107</td>
</tr>
<tr>
<td>FAT</td>
<td>80.28</td>
<td>69.61</td>
<td>14/88</td>
<td>31/90</td>
</tr>
<tr>
<td>MZN</td>
<td>68.89</td>
<td>97.26</td>
<td>14/90</td>
<td>2/34</td>
</tr>
<tr>
<td>McCoy cell culture from lung</td>
<td>37.20</td>
<td>97.50</td>
<td>27/173</td>
<td>2/28</td>
</tr>
<tr>
<td>McCoy cell culture from abomasum</td>
<td>26.83</td>
<td>98.73</td>
<td>30/172</td>
<td>1/18</td>
</tr>
</tbody>
</table>

All diagnostic tests were carried out using placental tissues unless otherwise indicated.

aUsing the isolation of chlamydiae from placenta by McCoy cell culture as the standard for diagnosis, the sensitivity was calculated as follows: the sensitivity is the percentage of samples deemed positive by McCoy cell culture isolation that were also found positive by the diagnostic method in question.

bUsing the isolation of chlamydiae from placenta by McCoy cell culture as the standard for diagnosis, the specificity was calculated as follows: the specificity is the percentage of samples deemed negative by McCoy cell culture isolation that were also found negative by the diagnostic test in question.

4. Discussion

The specificity of clone 8 primers to ovine abortifacient C. abortus DNA, shown in Fig. 1, avoids the possibility of false positives in the presence of C. pecorum. Although it is unlikely that C. pecorum would be found in the ovine placental tissue, it is possible that faecal contamination of samples could occur in the field or in the post-mortem room when sampling is taking place.

A higher sensitivity of clone 8 primers in the detection of EAE DNA compared to MOMP primers (81.40 and 74.42%, respectively) has been shown (see Table 2). This can possibly be explained by the multiple copies of the helicase gene throughout the chlamydial genome [10]. MOMP primers were selected for this study because of their ability to detect a wide range of Chlamydia species and also because typing by MOMP-gene restriction mapping has been well documented.

An advantage in the use of PCR amplification for the detection of EAE is that viable chlamydiae EB do not have to be present, unlike the requirement for cell culture isolation. Tissue from field abortion cases may undergo degradation due to environmental conditions. This may explain results from seven placenta samples which are positive by PCR but negative by repeated cell culture isolation (in these cases attempted cell culture isolation was also followed by passage in embryonated hen eggs). It is therefore probable that these correlating PCR results are genuine EAE positive results and are not false positives as first suspected.

Detection of EAE DNA using the LightCycler® shows a slight reduction in sensitivity compared to that of the standard PCR but sensitivity is still higher compared to MOMP PCR. The greatest advantage of fluorimetric detection of the amplified product using the LightCycler®, is the speed at which results are produced. For nucleic acid amplification in a standard thermal cycler, amplification may take 4–5 h, followed by visualisation of the PCR product on an agarose gel. If confirmation by Southern blotting is required the result may not be confirmed for another 24–36 h. Monitoring the fluorescence of the double strand-specific dye used, Sybr green, allows the amplification of DNA to be detected cycle by cycle throughout the amplification cycle and may be used for quantification using appropriate internal standards. Melting curve analysis continuously monitors the level of fluorescence of the Sybr green throughout an increasing temperature cycle. The fluorescent signal falls rapidly as the double-stranded DNA product reaches its denaturation temperature. As the melting temperature of the product is dependent on GC/AT ratio, product length and sequence, specific amplified products can be distinguished from artefact bands and primer complexes [13,14]. Using the LightCycler® strain-specific EAE amplicons can be detected and confirmed in less than 30 min.

The sensitivity of McCoy cell culture isolation from foetal abomasum and lung is 26.83 and 37.20%, respectively (see Table 2), when compared to isolation from placental tissue. The results from these tissues are disappointingly low especially as placental tissue is not always available from field abortion cases. A small proportion of lung and abomasum tissues was also tested by PCR using clone 8 and MOMP primers. However, they detected such a small percentage of McCoy cell culture positives that amplification from these tissues was discontinued. These results stress the importance of obtaining placental samples from ovine abortion cases where EAE is suspected. In the absence of placental tissue, diagnosis may have been possible from vaginal swabs, however, these were not submitted from field abortion cases.

The use of the Clearview Chlamydia test kit provides a fast, easy to use method for the detection of chlamydia. It has a higher sensitivity than PCR but a specificity of only 77% when compared to McCoy cell culture isolation. These results show that it is a useful screening tool but it is not a species-specific test for C. abortus.

The MZN test shows a high specificity but a low sensitivity for the detection of EAE. The test is also difficult to
read with a high degree of technical expertise required to obtain accurate results. False positives may also occur when reading this test as *Coxiella* and *Brucella* may be confused with *Chlamyphila* [3].

The use of ovine abortifacient strain-specific primers for the detection of EAE DNA from placental tissue has been demonstrated to be a diagnostic method that shows both high sensitivity and specificity when compared to other diagnostic methods available. The specificity of the clone 8 primers for the abortifacient strain of *C. abortus* avoids the potential detection of *C. pecorum* infection, which is known to infect sheep [15].

The use of EAE-specific primers coupled with fluorimetric detection of amplified PCR product using the LightCycler® has potential for a sensitive, fast diagnostic test for EAE.

Acknowledgements

The authors thank Paul McFarland for excellent technical assistance and Dr. Martha Spagnuolo-Weaver for assistance with the LightCycler®. Chlamydial strains S26/3, W73 and P787 were kindly supplied by Dr. G.E. Jones, Moredun Research Institute, Edinburgh, UK. Strains C95-38 and C94-1 were kindly supplied by Dr. B.M. Markey, University College, Dublin, Ireland.

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