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Comparisons of Polymerase Chain Reaction and Isolation in Cell Culture and Embryonated Hen's Eggs for the Detection of *Chlamydophila abortus* in Aborted Ovine/Caprine Placenta

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ABSTRACT

Enzootic abortion in ewes (EAE) is routinely diagnosed by the detection of elementary bodies of *Chlamydophila abortus* in placental smears stained with modified Ziehl-Neelsen's (MZN) stain. The *C. abortus* can also be isolated from infected material in McCoy cells or embryonated eggs. Currently, the polymerase chain reaction (PCR) is increasingly being used for the detection and differentiation of *Chlamydia* spp. and *Chlamydophila* spp. In the present study, placental samples from 65 aborting sheep and 2 aborting goats were used to compare the sensitivity and specificity of PCR using primers specific for the putative *helicase*, *omp2*, and *pmp* genes and for the 16S - 23S rRNA interspacer genes with bacterial isolation in cell cultures or embryonated eggs and detection of elementary bodies in impression smears stained with MZN. However, all the above mentioned strain specific primers have been used for detection of *Chlamydophila abortus.* We compared the diagnostic sensitivity of these primers and observed that primers specific to putative *helicase gene* and 16S-23S interspacer gene could be used for detection of *Chlamydophila abortus* from field samples of aborted ovine/caprine placenta. This study work is of significant importance for molecular epidemiology of *Chlamydophila abortus* in ovine and caprine population.

Key Words: Enzootic abortion in ewes, *Chlamdydophila abortus*, diagnosis, ovine, placenta, polymerase chain reaction

INTRODUCTION

The family *Chlamydiaceae* comprises of obligate intracellular Gram-negative bacteria characterized by a unique developmental cycle. The family consists of nine species belonging to two genera: *Chlamydia*, comprising *C. trachomatis, C. suis* and *C. muridarum*, and *Chlamydophila*, comprising *C. abortus, C. caviae, C. felis, C. pecorum, C. pneumoniae* and *C. psittaci* (Everett et al., 1999). *C. abortus* and *C. pecorum* are common pathogens of sheep (Aitken, 1993; Storz and Kaltenboeck, 1993; Everett et al., 1999). The former is associated with enzootic abortion in ewes (EAE), an endemic disease in most countries of the world (Aitken, 1993), and the later with polyarthritis, conjunctivitis, and pneumonia in sheep, cattle and other animals (Storz and Kaltenboeck, 1993).

Various antigen detection techniques can be used for the rapid diagnosis of EAE but these methods may not differentiate *C. pecorum*

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which is commonly present in the gastro-intestinal tract of sheep and cattle, and their differentiation by cultivation in culture and by serology is labour-intensive and time-consuming (Andersen, 1991). The demonstration of a rise of antibody titres against *C. abortus* can be used to establish retrospective serological diagnosis in animals which had aborted but not all animals readily seroconvert and *C. abortus* and *C. pecorum* share common antigens. Interpretation of serological tests is further complicated by antigenic cross-reactivity with other Gram negative bacteria. To improve the specificity of serological test, *C. abortus* specific recombinant antigens and monoclonal antibodies to some of the surface antigens have been developed for use in ELISA (Salti-Montesanto et al., 1997; Buendia et al., 2001; Longbottom et al., 2001). Recent advances in molecular biology have greatly helped to simplify the differentiation of species belonging to the genera *Chlamydia* and *Chlamydophila* (Everett et al., 1999).

Enzootic abortion in ewes (EAE) is routinely diagnosed by the detection of elementary bodies of *Chlamydophila abortus* in placental smears stained with modified Ziehl-Neelsen's (MZN) stain. *C. abortus* can also be isolated from infected material in McCoy cells or embryonated eggs; these techniques are considered the 'gold standard'. Currently, the polymerase chain reaction (PCR) is increasingly being used for the detection and differentiation of *Chlamydia* spp. and *Chlamydophila* spp. Primers targeting the genes encoding the interspacer regions of the 16S and 23S ribosomal rRNA genes (Medico et al., 2000), the conserved regions of the genes encoding the outer membrane protein (*omp*2) (Sheehy et al., 1996) the putative *helicase* gene and genes encoding the polymorphic outer membrane proteins (pmp) (Laurucao et al., 2001) have been used to improve sensitivity and specificity of detection. The aim of the present study was to compare staining with MZN and isolation in McCoy cells or embryonated eggs with the detection of nucleic acids using primers to amplify fragments of the 16S-23 gene interspacer region and the *omp2, pmp* and *helicase* genes for the detection of *C. abortus* in the placentae of ewes and goats that had aborted.

MATERIALS AND METHODS

Source of Clinical Samples

Sixty-five placenta from ewes and 2 from goats that had aborted during late gestation were generously supplied by the Veterinary Laboratories Agency (VLA) at Carmarthen and Shrewsbury during the lambing seasons of 2004 and 2005. Ten control ovine placenta were obtained from the Animal Husbandry Farm, University of Liverpool, UK.

Detection and Isolation of *C. abortus*

The presence of *C. abortus* in placental tissues was investigated by staining smears from the cotyledons of aborted placenta with MZN, by isolation in McCoy cells or embryonated eggs and by PCR as described below.

***MZN staining*:** Impression smears of tissue from highly vascularized areas of cotyledonary and intercotyledonary placenta were made on glass microscope slides and fixed by gentle heating. The smears were stained for 10 minutes with carbol fuchsin. They were then washed with tap water and decolorized with 0.5% acetic acid, washed and counterstained with methylene blue for 10 seconds. The washed and dried slides were examined with microscope under 40X lens for the presence of inclusions of red elementary bodies against a blue background. The MZN staining was scored as ++ for smears showing multiple inclusions, + showing a few dispersed elementary bodies and – for no inclusion.

Preparation of samples for isolation of C. abortus and DNA extraction: The placental tissues from the cotyledon and inter-cotyledonary regions were homogenized by grinding in a sterile pestle mortar. These tissues were then suspended in transport medium containing streptomycin (1mg/mL), vancomycin (1mg/mL), gentamycin (50 µg/mL) to make a 10% suspension and treated with 3 cycles of sonication at maximum amplitude (Vibra Cell, Sonic Inc, Danbury, CT, USA), with each cycle lasting 15 seconds. After centrifugation at 200*g* for 10 minutes, the supernatants were collected and aliquoted in small volumes. These were either stored at -70^{o}C or used immediately for the isolation of *C. abortus* in McCoy cell and embryonated eggs or for the extraction of DNA.

Isolation of C. abortus: Monolayers of McCoy cells were seeded onto 24-well plates or 25 cm^2 flasks and once the cells had formed confluence, 500 µL of the inoculums was added before centrifuging the flasks for 30 minutes at 400 x *g*. The flasks were then pre-incubated for 2 hours at 37^{o}C under a humidified atmosphere and 5% CO_2. After removing the supernatant, fresh growth medium (Medium 199 with Earle's salt, Gibco, Paisley, UK), containing, vancomycin (100µg/mL), streptomycin (100 µg/mL), gentamycin (50 µg/mL) and amphotericin B (5 µg/mL), FCS (2%) and cycloheximide (0.5 µg/mL) was added and the plates incubated 24-96 hours. Cytospin smears were prepared on glass slides every 24 hours, air-dried, stained with a rapid Romanowsky staining kit (Diff-Quick, HD Supplies, Rabans Lane Industrial Estate, Aylesbury, UK) and the cells examined under a light microscope for the presence of typical inclusion bodies.

The same samples were also used for the isolation of *C. abortus* in the yolk sac membranes of 7-day-old embryo-nated hen's eggs. For this purpose, 7-8 day old embryonated eggs obtained from specific-pathogen-free (SPF) flock were inoculated through the yolk sac after cleaning the shell with 70% ethanol, marking air sac with a pencil, followed by drilling a small hole into the shell over the top of the air sac. After injecting about 300 µL of the inoculums, the hole was sealed with nail varnish.

The eggs were incubated at 37^{o}C for 9 days and candled daily to detect embryonic death; those eggs in which the embryo died within 24 hours after infection were discarded. Thereafter, eggs containing dead embryos were kept at 4^{o} C overnight and the yolk sac membranes were aseptically harvested in a safety cabinet after washing the eggshells with methanol. The yolk sac was washed twice with PBS (pH 7.2) and then cut into small pieces to be ground with a pestle and mortar to prepare a cell suspension as described earlier and the presence of *C. abortus* confirmed by staining impression smears with MZN, further isolation in McCoy cells or by the detection of specific DNA by PCR as described below.

Polymerase chain reaction (PCR): DNA was extracted from homogenized placental tissue supernatant, yolk sac membrane suspension and McCoy cell cultures by the ammonia digestion method as described previously. Briefly, 50µL of the sample was placed in a 1.5mL DNA/RNA free 'sure lock' microcentrifuge tube, and 500 µL of 1.25% NH_3 solution added. After mixing by vortex and centrifugation, the tubes were placed in a preheated heating block at 100^{o}C for 30 minutes. The tubes were removed and the process repeated until the evaporation has reduced the volume to about half of the original volume. Ten-fold dilution of the DNA extract were then prepared in RNase-DNase-free DEPC water and stored at -20^{o}C as 100 µL aliquots in RNase-DNase- free 0.5mL micro tubes. The DNA extracts prepared from yolk sacs infected with the reference strain S26/3 of *C. abortus* and T22 of *C. pecorum* were used as positive controls and extracts prepared from

Table 1 Primers used for the detection of *Chlamydophila abortus* in aborted ovine placenta

Target gene	Primer sequence	Reference
Helicase	F: 5' TGG TAT TCT TGC CGA TGA C 3', R: 5' GAT CGT AAC TGC TTA ATA AAC CG 3'	Creelan and McCllough, (2000)
omp2 gene specific to *C. abortus*	F: 5' TCA GTG CCA ATC CGT CGA TA 3', R: PCR-D2 5' CCT TCT TTA AGA GGT TTT AAA 3'	Sheehy et al. (1996)
pmp gene	F: 5' ATG AAA CAT CCA GTC TAC TCG-3', R: 5' TTG TGT AGT AAT ATT ATC AAA-3'	Laroucau et al. (2001)
16S-23S rRNA inter spacer genes	F: 5' CCC AAG GTG AGG CTG ATG AC 3', R: 5' CAA ACC GTC CTA AGA CAG TTA 3'	Madico et al. (2000)

normal ovine placenta, embryonated eggs and McCoy cells as negative controls.

DNA from other bacteria: To test for the specificity of the PCR assays DNA extracts were also prepared from *Chlamydophila pecorum, Escherichia coli*, *Staphylococcus aureus*, *Campylobacter fetus* and *Anaplasma phagocytophilum*.

Amplification of DNA: DNA samples were amplified by polymerase chain reaction. For each reaction 50 µL of a reaction mix was prepared by adding 45µL of super mix (Invitrogen) with each forward and reverse primer and 3 µL template DNA. Samples were prepared in 0.5 mL DNase-RNase free micro centrifuge tubes, placed in the sample block of a PCR machine (Thermohybid PX2, Thermo Fisher Scientific, UK), that was pre programmed for primers specific for amplification of DNA encoding the putative *helicase, omp2, pmp* and *16S-23S* inter spacer genes as previously described (Sheehy et al., 1996; Creelan and McCllough, 2000; Madico et al., 2000; Laroucau et al., 2001). The cyclic conditions for the helicase gene were as described by Creelan and McCllough, (2000) and those for *omp2*, *pmp* and *16S-23S* inter spacer genes were as described by Sheehy et al (1996), Laroucau et al (2001) and Madico et al (2000) respectively. All PCR products underwent electrophoresis in 2% gel. Ten µL samples were prepared by adding 4µL loading buffer to 6 µL of PCR product; 2 µL of diluted DNA marker was added to 4µL loading buffer. The samples were then added to each column using 10µL pipette tip, the gel tank covered and power adjusted to 500mAMP and 100V for 20 minutes. The gel was then removed from the tank placed in ethidium bromide for 5-10 minutes, and the gel visualized under UV light.

RESULTS

Elementary bodies were detected in 66/67 placental samples (98.5%) obtained from aborting sheep and goats after staining with MZN. However, 30 (44.7%) of the positive samples were weakly positive. *C. abortus* was isolated from 63 of 64 (98.4%) placental samples by culture in McCoy Cells and in 28 of 30 (93.3 %) samples inoculated into embryonated eggs.

Using DNA extracted from McCoy cells infected with the reference strain S26/3, PCR products 479bp, 330bp, 315bp and 111bp in size were generated using primers for the putative *helicase, omp2, pmp* and *16S –23S rRNA* interspacer genes respectively. Similar bands were detected using DNA extracted from placental samples obtained from aborting ewes and goats. Highest percent of positive samples were recorded through PCR by amplifying DNA with primers specific to putative *helicase* gene followed by primers specific to the *16S –23S rRNA* interspacer genes, omp2 and *pmp* gene. These results

show that PCR using the putative *helicase* gene was as sensitive as isolation in McCoy cell culture.

Results comparing the PCR with isolation from McCoy Cell Culture and embryonated hen's egg and other antigen detection methods are shown in Table 2.

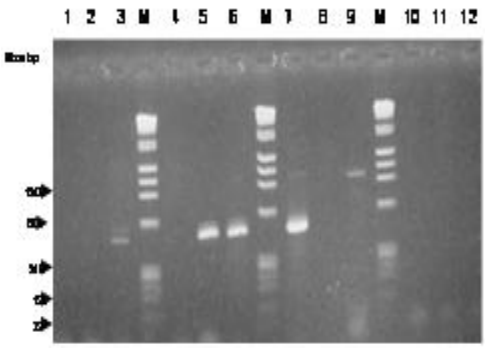

Figure 1 Lane 1-2T34, T22 (*C. pecorum* strains): Lane 3-4, 2WB, 2W7 (field isolates of caprine *C. abortus* Lane 5, S 26/3 (reference *C. abortus* strain): Lane 6-7 (field isolates ovine *C. abortus* strains Lane 8-11, *Staph. aureus*, *E. Coli*, Anaplasma, *Camp fetalis* respectively. Lane 12, Negative control, Lane M. Marker

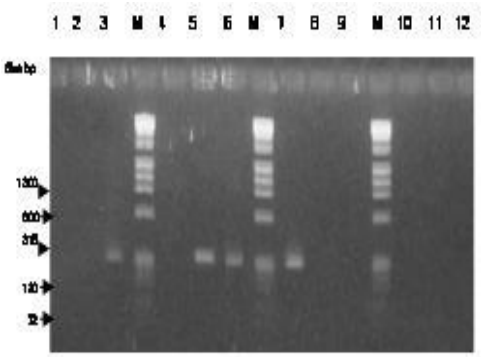

Figure 2 Lane 1-2T 34, T22 (*C. pecorum* strains): Lane 3-4, ZW6, ZW7 (field isolates of *Caprine caborlus*: Lane 5, S26/3 (reference *C. abortus* strain): Lane 6-7 (field isolates ovine *C. abortus* strains: Lane 8-11, *Staph. aureus*, *E. coli*, Anaplasma, *Camp fetalis* respectively. Lane 12, Negative control; Lane M. Marker

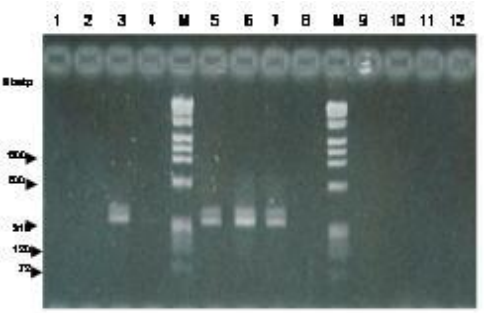

Figure 3 Lane 1-2T 34, T22 (*C. pecorum* strains): Lane 3-4, ZW6, 2W7 (field isolates of *Caprine caborlus*: Lane 5, S 26/3 (reference *C. abortus* strain): Lane 6-7 (field isolates ovine *C. abortus* strains: Lane 8-11, *Staph. aureus*, *E. coli*, Anaplasma, *Camp fetalis* respectively. Lane 12, Negative control; Lane M. Marker

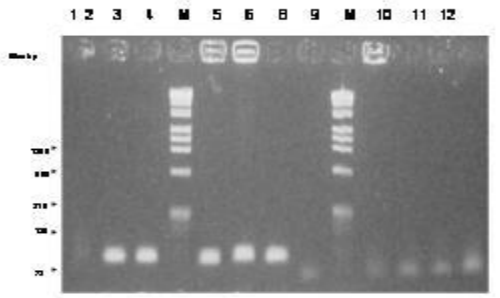

Figure 4 Lane 1-2 T34, T22 (*C. pecorum* strains): Lane 3-4, ZW6, ZW7 (field isolates of caprine *C. abortus*: Lane 5, S26/3 (reference *C. abortus* strain): Lane 6-7 (field isolates ovine *C. abortus* strains: Lane 8-11, *Staph. aureus*, *E. coli*, Anaplasma, *Camp fetalis* respectively. Lane 12, Negative control; Lane M. Marker.

Specificity of the Primers for *C. abortus*

All four primers amplified DNA obtained from aborted placental samples and reference strain S26/3 of *C. abortus.* None of primers amplified strain T22, T34 of *Chlamydophila pecorum*, *Escherichia coli*, *Staphylococcus aureus*, *Campylo-bacter fetus*, *Anaplasma phago-cytophilum* showing their specificity to *C. abortus* (Figures 1, 2, 3 and 4).

Table 2 Comparison of PCR, McCoy cell culture, embryonated egg isolation and other diagnostic test used for detection of *C. abortus*

Diagnostic test use	Number of samples positive (%)	Total number of samples tested
McCoy cell culture isolation	63 (98.4)	64
Embryonated egg isolation	28 (93.3)	30
Clone8 PCR	63 (94.0)	67
omp2 PCR	50 (74.6)	67
pmp PCR	41 (61.1)	67
16S-23S interspacer PCR	61 (91.0)	67
MZN	66 (98.5)	67
Immunostaining	66 (98.5)	67

DISCUSSION

Most diagnostic veterinary laboratories use the detection of EBs in placental smears stained with MZN for the presumptive diagnosis of EAE, but laboratory confirmation requires isolation in cell culture systems or in embryonated hen's eggs. However, these methods are useful in confirmation but expensive and time-consuming. Staining with MZN and the isolation in McCoy cell culture and embryonated egg depends on the viable *C. abortus* elementary bodies in the infected material. In the present study, we observed that *C. abortus* was detected in McCoy cells 48 hours after inoculation from those samples which were recorded as ++ positive after microscopic examination of MZN stained smears. The other samples were positive 96 hours post-inoclation and from some samples isolation of *C.abortus* in culture was only possible after second passage in McCoy cells. This suggests that speed of growth of *C. abortus* may be related to the number of bacteria present in the inoculum or stage of their developmental cycle. Many other factors such as contamination, storage conditions, and homgenization of tissue may affect on the viability *C.abortus* elementary bodies and cause slow growth and appearance in culture. Furthermore as cultural methods can not differentiate *C. abortus* from *C. pecorum*, the definitive diagnosis of EAE requires further antigenic tests preferentially using monoclonal antibodies (Andersen, 1991). We use immunostaining method for detection of *C. abortus* antigen using anti chlamydophila abortus antibodies; results were as sensitive as of culture method.

In the present study, we compared the sensitivity and specificity of detection of *C. abortus* in ovine placenta by PCR using primers for the *omp*2, putative *helicase, pmp* and *16S-23S* interspacer genes. *C. abortus* was detected by PCR using primers specific to the *16S-23S* interspacer gene in 61 of 67 (91.0%) of the culture-positive samples compared to 63 of 67 (94%) using primers specific to the putative *helicase, 50* of 67 (74.6%) using primers specific for the *omp2* gene and in 41 of 67 (61.1 %) samples using primers specific to the *pmp* gene. The lowest detection rates were observed using primers specific to the *pmp* gene. Another weakness of using primers for the *pmp* gene is that the amplicons are not specific to *C. abortus*. These primers were reported to generate 300

bp amplicons from *C. abortus*, *C. psittaci* and *C. caviae* strains but not from *C. pecorum*, *C. felis*, *C. pneumoniae* and *C. trachomatis* (Laroucau et al., 2001). Differentiation of *C. abortus* from *C. psittaci* and *C. caviae* was reported to be possible by looking at the RFLP patterns after digestion with *Alu*I. However, this is likely to be complicated by the possible amplification of four known pmp genes (*pmp*91A, *pmp*91B and *pmp90A*/B), which may have different RFLP patterns (Laroucau et al., 2001). The results showed the putative *helicase* gene and the *16S-23S* interspacer genes were good targets for detecting *C. abortus* by PCR. Our results confirm previous observations by other workers that the *16S-23S rRNA* interspacer genes and the putative *helicase* gene are appropriate targets for the identification of *Chlamydia* spp. and *Chlamydophila* spp. (Madico et al., 2000; Creelan and McCllough., 2000). The results of this study indicate that isolation in cell culture or embryonated eggs is marginally more sensitive than PCR for the detection of *C. abortus* in field samples but it is more time-consuming and labour-intensive. Of the primers tested, those specific for the putative helicase gene were more sensitive. Primers derived from putative *helicase* provided good results on amplification of crude DNA extracted from placental tissues. Therefore, it could be used for detection of *C. abortus* from field samples. Studies could be carried out to improve sensitivity of primers using real time PCR or in Situ Hybridization on placental tissue for detecting *C. abortus*.

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