

Development of Polymerase Chain Reaction assays with host-specific internal controls for *Chlamydophila abortus*

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ABSTRACT: *Chlamydophila abortus* (*C. abortus*) is one of the most important infectious agents causing abortion in ruminants. The bacterium is obligately intracellular, cannot grow on agar, but it needs cell culture or embryonated eggs for growth. Therefore, culture-independent detection methods such as the polymerase chain reaction are increasingly important and needed. The aim of this study was to develop a polymerase chain reaction assay with an internal control for detection of *C. abortus* in clinical samples. Using newly-designed two primer sets specific for *C. abortus*, the polymerase chain reaction assay was first tested with positive and negative control DNA and its sensitivity and specificity were determined. A new polymerase chain reaction protocol was developed by combining the new primer pair sets with bovine (12SM-FW and 12SBT-REV2) and ruminant host-specific primer sets (12S-FW and 12S-REV). In conclusion, the developed polymerase chain reaction assays can potentially be used for direct detection of *Chlamydophila abortus* in bovine and ruminant samples.

Keywords: *Chlamydophila abortus*; internal control; PCR

Chlamydophila abortus (*C. abortus*) is an obligate intracellular bacterium and has the general characteristics of the Chlamydiaceae. This family consists of nine species under two genera (*Chlamydia* genus: *C. muridarum*, *C. suis* and *C. trachomatis*; and *Chlamydophila* genus: *C. abortus*, *C. caviae*, *C. felis*, *C. pecorum*, *C. pneumoniae* and *C. psittaci*). These species cause host-specific infections in humans and different animal species. Some strains are adapted to animals and have zoonotic potential (Everett et al. 1999; Sachse et al. 2009). *C. abortus* is responsible for ovine enzootic abortion (OEA) in sheep and goats (Longbottom and Coulter 2003), but is less common in cattle (Borel et al. 2006). Also, the organism can cause subclinical enteric disease in sheep without a history of abortion (Salti-Montesanto et al. 1997; Gut-Zangger et al. 1999). Differential diagnosis of the agent from other members of Chlamydiaceae, other abortion agents such as *Brucella* spp. and from abundant

microorganisms in the environment such as *E. coli* and *Staphylococcus* spp. is important. Isolation of the agent is considered as the gold standard for diagnosis of the disease. However, the agent can only be grown in the yolk sac of embryonated eggs or in tissue culture, and not on agar medium. Further, isolation of the agent is time-consuming and requires technical equipment and specialised staff. For these reasons, serological diagnosis methods have been used for many years. The complement fixation test was the first method for serological diagnosis. Due to the low sensitivity of this technique, however, the method has now been superseded by ELISA (Aitken and Longbottom 2004). PCR has been used widely in the diagnosis of infectious agents at the level of genus, species and strain (Sachse et al. 2009). It was shown that *pmp* gene-specific primers have a higher sensitivity and specificity for detecting *C. abortus* (Laroucau et al. 2001; Greco et al. 2005). Therefore, such an ap-

proach holds obvious advantages for the fast and reliable detection of the bacterium in clinical material. Using highly sensitive primers is important when samples are diluted to eliminate or diminish the levels of PCR inhibitors. Also, use of an internal amplification control is essential when performing PCR directly on clinical samples, which usually contain PCR inhibitors (Wilson 1997).

The aim of this study was to develop a PCR assay, with a host-specific internal amplification control, for detection of *C. abortus* in clinical samples. For this purpose, specific primers targeting the *pmp* genes of *C. abortus* were first designed and then these primers were combined with bovine and ruminant 12S rDNA-specific primers as host-specific internal controls.

MATERIAL AND METHODS

C. abortus DNA was used as a positive control. DNAs from *C. psittaci*, *C. felis*, *E. coli*, *Brucella abortus* S19, *Brucella melitensis* 16M and *S. aureus* were used to determine primers specificity. The DNA concentration of *C. abortus* was measured as 14.2 ng/μl (Nanodrop Spectrophotometer, ND 1000, USA). Ten-fold serial dilutions were prepared from positive control DNA. Twenty bovine and six caprine foetal abomasal contents were used in this study. The phenol-chloroform method was used for nucleic acid extraction (Sambrook and Russel 2001).

Capmp1a/Capmp1b and Capmp2a/Capmp2b are two specific primer sets for *C. abortus pmp* genes (GenBank accession no. CPU 65942) and were designed in this study using Primer3 (Yuryev 2007). Bovine- (12SM-FW and 12SBT-REV2) and rumi-

nant-specific primers (12S-FW and 12S-REV) were used as internal controls (Lopez-Calleja et al. 2005). Primer properties are shown in Table 1.

PCR procedures and protocols were optimised by adjusting the concentrations of PCR components such as MgCl₂, primers, Taq DNA polymerase and 10× PCR buffer concentrations in the mix and by testing different annealing temperatures and times according to the recommendations of Henegariu et al. (1997). The PCR amplification mixture was carried out in a final volume of 25 μl. The mixture consisted of 2 μl of extracted DNA template, 1.5 IU of TaqDNA polymerase (VivantisTechnologies, Malaysia), 3.5 μl of 10× PCR buffer (10× ViBuffer A, without MgCl₂), 3mM MgCl₂, 200μM each of dNTPs (Vivantis Technologies, Malaysia) and 20 pmol of each primer.

After an initial denaturation at 95 °C for 3 min, the PCR protocol was as follows: 60 s of template denaturation at 94 °C, 60 s of primer annealing at 54 °C, and 90 s of primer extension at 72 °C (total of 35 cycles), with a final extension at 72 °C for 5 min. The products were separated by electrophoresis in a 2% agarose gel, stained with ethidium bromide (0.5 mg/ml), and DNA bands were visualised under UV light.

RESULTS

In PCR assays, Capmp1a/Capmp1b and Capmp2a/Capmp2b primers amplified specific products in *C. abortus* and *C. psittaci* DNA, but no amplification products were detected for *C. felis* and other bacterial DNAs used for detection of specificity (data not shown). In analyses to compare the sensitiv-

Table 1. Properties of Primers used in the study

Target	Primer name	Primer sequences	Length of amplicons	Reference
<i>C. abortus pmp</i>	Capmp1a	5'-CGAGCTTAATCGTCTCGAACTCA-3'	180 bp	this study
	Capmp1b	5'-CTAACCCCGCATAGGCAATACA-3'		
<i>C. abortus pmp</i>	Capmp2a	5'-TCCGAGGAACCAGATCAAAA-3'	223 bp	this study
	Capmp2b	5'-TGTAACCATTTCCCAAGAAGGA-3'		
Ruminant 12S	12S-FW	5'-GGTAAATCTCGTGCCAGCCA-3'	720 bp	Lopez-Calleja et al. 2005
	12S-REV	5'-TCCAGTATGCTTACCTTGTTACGAC-3'		
Bovine 12S	12SM-FW	5'-CTAGAGGAGCCTGTTCTATAATCGATAA-3'	346 bp	Lopez-Calleja et al. 2005
	12SBT-REV2	5'-AAATAGGGTTAGATGCACTGAATCCAT-3'		

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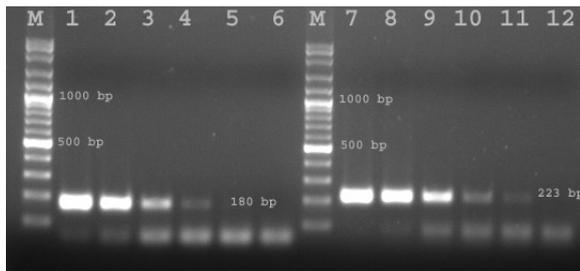


Figure 1. M; VC 100bp Plus DNA Ladder. Lanes 1–6: *C. abortus*-specific bands with Capmp1a/Capmp1b primers and ten-fold diluted *C. abortus* DNA (180 bp); lanes 7–12 *C. abortus*-specific bands with Capmp2a/Capmp2b primers and ten-fold diluted *C. abortus* DNA (223 bp)

ity of the two primer sets, the Capmp2a/Capmp2b primer pair (1.4 pg/μl) was found to be ten times more sensitive than the Capmp1a/Capmp1b primer pair (14 pg/μl) (Figure 1).

This newly developed PCR assay was tested on positive control DNAs and clinical samples. The primers effectively amplified products from DNA from clinical material and specific amplification products are shown in Figure 2.

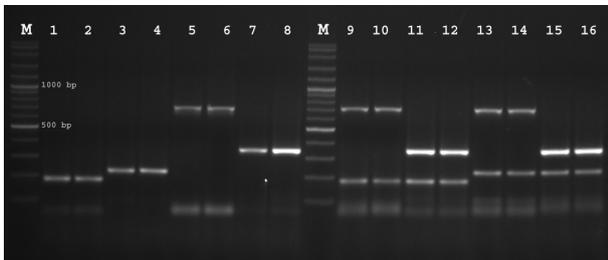


Figure 2. M; VC 100bp Plus DNA Ladder. Lanes 1–2 *C. abortus*-specific bands with Capmp1a/Capmp1b primers (180 bp). lanes 3–4 *C. abortus*-specific bands with Capmp2a/Capmp2b primers (223 bp); lanes 5–6 Ruminant 12S rDNA-specific bands with 12S-FW/12S-REV (720 bp); lanes 7–8 Bovine 12S rDNA-specific bands with 12SM-FW/12SBT-REV2 primers (346 bp); lanes 9–10 *C. abortus*-specific bands with Capmp1a/Capmp1b primers (180 bp) and Ruminant 12S rDNA-specific bands with 12S-FW/12S-REV primers (720 bp); lanes 11–12 *C. abortus*-specific bands with Capmp1a/Capmp1b primers (180 bp) and bovine-specific bands with 12SM-FW/12SBT-REV2 primers (346 bp); lanes 13–14 *C. abortus*-specific bands with Capmp2a/Capmp2b primers (223 bp) and ruminant-specific bands with 12S-FW /12S-REV primers (720 bp); lanes 15–16 *C. abortus*-specific bands with Capmp2a/Capmp2b primers (223 bp) and bovine-specific bands with 12SM-FW/12SBT-REV2 primers (346 bp)

DISCUSSION

Abortion causes significant economic losses in ruminant farming. The cost of an abortion case is between 480 and 700 US dollars (Thurmond and Picanso 1990), and can reach 1200 dollars with treatment and milk discharge costs (Kossaibati and Esslemont 1997). *C. abortus* is an important septic abortion agent in ruminants, especially in sheep and goats. Reliable and fast diagnosis of the agent in abortion cases is important in preventing the spread of disease in the herd, and can reduce economic losses.

In this study a PCR assay with an internal amplification control for detection of *C. abortus* was developed. For this purpose, specific primers targeting the *pmp* genes of *C. abortus* were designed and then these primers were combined with bovine and ruminant host-specific 12S rDNA primers.

It was previously shown that CpsiA/CpsiB primers, specific for *pmp* genes, can detect *C. abortus* with high sensitivity and specificity (Laroucau et al. 2001). Also, in another study, four different primer sets for detection of *C. abortus* from tissue samples were compared and it was reported that the CpsiA/CpsiB primer set was the most sensitive among the tested sets (Greco et al. 2005). In this study *pmp* gene-specific primers were designed and compared for their sensitivity and specificity. These primers amplified products from *C. abortus* and *C. psittaci*. Other bacterial DNAs did not yield any amplification products. It is important to note that the primers designed in this study can potentially detect *C. psittaci* in avian samples. Similarly, it was reported that CpsiA/CpsiB primers were effective for detecting avian chlamydiosis (Laroucau et al. 2007; Sareyyupoglu et al. 2007).

In PCR analyses, especially from clinical material, the presence of inhibitory substances can lead to the generation of false negative results (Wilson 1997). It was suggested that the dilution of clinical samples, checking with host specific primers or adding known amounts of target bacterial DNA to the mix in negative samples can be effective in detecting false negative results or in decreasing the effect of inhibitory substances (Navarro et al. 2004). The use of internal amplification controls in PCR analyses has also been suggested by the European Standardisation Committee and International Standard Organisation (Anonymous 2004).

In this study, Capmp1a/Capmp1b and Capmp2a/Capmp2b primers specific for *C. abortus pmp* genes were combined with bovine and ruminant host-spe-

cific primers for mitochondrial 12S rDNA reported previously (Lopez-Calleja et al. 2005). There are different strategies for using internal controls in PCR. The detection of host DNA simultaneously in the same PCR protocol can be especially useful for identifying false negative results from the sampling to amplification stage. In this method, however, the sample has to contain sufficient numbers of host cells. Therefore, the detection of host DNA can be an important marker for the quality of nucleic acid extraction and PCR amplification steps for this type of samples (Helps et al. 2003).

In conclusion, in this study, PCR procedures were developed for the direct detection of *C. abortus* in clinical ruminant samples using target bacterium and host-specific primer sets. The newly developed PCR assay has the potential to detect *C. abortus* in cases of abortion in bovines and other ruminants. Also, the assay can be useful set for the detection of *C. psittaci* in avian samples.

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