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Polymerase Chain Reaction for Detection of *Chlamydia abortus* in Samples from Aborted Ruminants using Primers for *Chlamydia psittaci*

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Abstract

In this study, the CPsittF(R) and Or1(2) primers, targeting the *ompA* gene of *Chlamydia psittaci*, were used for PCR detection of *Chlamydia abortus* DNA in clinical samples from aborted animals. The sensitivity and specificity of the reaction were compared with those achieved by *C. abortus*-specific primers CpsiA(B). The comparative analysis of the results showed that all samples that were positive for *C. abortus* in PCR, performed with primers CpsiA(B) also gave a positive result in the PCR using the CPsittF(R) primers, generating a 1041 bp specific amplification product. PCR amplification using the Or1(2) primers was uncertain and produced an amplification product of 212 bp, which was different from the expected length of 245 bp. In an attempt to improve these primers, their sequence was modified at the 11th and the 21st nucleotide. Although the sensitivity of the reaction performed with the modified primers was improved, it was still lower compared to that achieved with the original *C. abortus*-specific primers CpsiA(B) and primers CPsittF(R). The results show that the primer pair CPsittF(R), developed for the detection of *C. psittaci* could be successfully used in the diagnosis of abortions, induced by *C. abortus,* while the primer set Or1(2) is less effective.

Key words: *Chlamydia abortus, Chlamydia psittaci*, primers, PCR

Резюме

Праймери CPsittF(R) и Or1(2), насочени към ompA гена на *Chlamydia psittaci* бяха изпитани в PCR за доказване наличието на ДНК на Chlamydia abortus в клинични проби от абортирали животни. Чувствителността и специфичността на реакцията бяха сравнени в PCR, проведена със специфичните за *C. abortus* праймери CpsiA(B). Сравнителния анализ показа, че всички изследвани проби с положителен резултат за *C. abortus* от PCR, проведена с праймери CpsiA(B), реагират положително и в PCR, проведена с праймери CPsittF(R), при което се генерира специфичен амплификационен продукт с големина 1041bp. Реакцията, проведена с праймери Or1(2) показа непостоянни резултати, като при това големината на получения продукт (212bp) не съответстваше на очакваната големина от 245bp. Тези праймери бяха модифицирани чрез замени в 11-та и 21-та база в нуклеотидната им последователност. Въпреки, че това повиши чувствителността на реакцията, тя остана по-ниска в сравнение с PCR, проведена с праймери CpsiA(B) и CPsittF(R). Резултатите показват, че праймери CPsittF(R), разработени за детекция на *C. psittaci* могат успешно да бъдат използвани в диагностиката на абортите, породени от *C. abortus*, докато праймерната двойка Or1(2) е по-малко ефективна.

Introduction

Chlamydia abortus is the main etiological agent of abortions and stillbirths in ruminants in Europe (Longbottom *et al*., 2013). Similar pathology, although with a lower incidence, it also causes in other animals (pigs, horses) (Bocklisch *et al.*,

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1991; Schautteet *et al*., 2011). This infectious agent has also proved to have a zoonotic potential, as it can cause abortions in risk-group pregnant women being in contact with infected animals, or flu-like conditions, characterized by fever, headache and muscle ache (Rodolakis and Mohamad, 2010).

Since a number of other viruses and bacteria also have an abortogenic potential, it is important to identify *C. abortus* etiology in view to develop an adequate strategy for treatment and prevention of such infections, respectively, abortions. In addition to conventional diagnostic assays, such as antigen detection by immunohistochemistry, microscopic observation of intracellular inclusions in stained impression smears and serological detection of antibodies by complement fixation test (CFT) and ELISA, the molecular methods, based on nucleic acid amplification are increasingly being used for detection of *C. abortus* and other *Chlamydiales*. Due to the close genetic relationship between the members of *Chlamydiaceae* family, they are commonly detected using conventional and real-time PCR as an initial screening step and, if necessary, differentiation at the species level is then done by sequencing (Kaltenboeck *et al*., 1993; Sheehy *et al*., 1996; Hartley *et al*., 2001) or restriction fragment length polymorphism (RFLP) analisys of the PCR products (Everettt and Andersen, 1999; Sait *et al*., 2011).

In the PCR-based diagnostics of *C. abortus* infections, there is a wide range of primers targeting different regions of the chlamydial genome, e.g. the intergenic spacer region between the 16S and 23S ribosomal RNA (rRNA) genes (Madico *et al*., 2000), the conserved regions of the gene encoding the outer membrane protein (omp2) (Hartley et al., 2001), the helicase gene (Creelan *et al*., 2000), as well as the genes involved in the synthesis of the polymorphic outer membrane proteins (pmp) (Laurucao *et al*., 2001). However, the sensitivity of the reactions may vary depending on the primer set (Creelan *et al*., 2000; Greco *et al*., 2005; Soomro *et al*., 2012). Therefore, searching for new combinations of primers in order to optimize this molecular assay and expand the range of *C. abortus* strains which can be detected is needed.

Although both *C. abortus* and *C. psittaci* could infect ruminants (Cox *et al*., 1998; Lenzko *et al*., 2011), from practical point of view their species differentiation is not necessary since the therapeutic approaches in case of infection are one and the same. Presuming that, as well as the close genetic relationships between these two members of *Chlamydiaceae* family, we performed this study to examine whether *C. abortus* could be successfully detected by PCR in clinical samples from placentae and internal organs of aborted fetuses using primers, originally constructed for the diagnosis of *C. psittaci*.

Materials and methods.

Clinical samples

Between December of 2013 and February of 2015, fetuses and placentae from 21 sheep and 7 goats, originating from 23 flocks with a high percentage of abortion were delivered in the laboratory for diagnostic investigation for *C. abortus* and *Coxiella burnetii*. Samples were investigated by light microscope observation on impression smears from cotyledons, stained by Giemsa method for the presence of intracellular elementary bodies and PCR. The samples were tested in other laboratories both bacteriologicaly and virologicaly for the presence of other abortogenic agents. Where available, epizootiological data were collected for the period of the pregnancies to exclude any concomitant factors able to cause abortions (mycotoxicoses, feeding with frost-damaged feed and other technological errors).

DNA extraction

DNA was extracted from placental cotyledons or tissues (abomazum and lung) from aborted fetuses using Animal and Fungi DNA Preparation Kit (Jena Bioscience) or Tissue & Cell Genomic DNA Mini Kit (Guangzhou Geneshun Biotech., Ltd.) following manufacturer's instructions. Extraction efficiency was assessed by agarose gel electrophoresis and extracted DNA was stored at -20°C until use.

PCR

DNA was amplified by conventional PCR using the CpsiA and CpsiB primers, which were designed from the 4 available *C. abortus* pmp gene sequences (Larocaou *et al.,* 2001). An automatic FluoroCycler 12, HAIN, LKB, was used. The reaction mixtures were 25 µL and consisted of 12.5 µL of 2x PCR Taq Mix (Guangzhou Geneshun Biotech., Ltd), 2 µL of each primer at a concentration of 10pmol µL-1, 2 µL of target DNA and 6.5 µL ddH_2O. The amplification program was set as follows: initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 1 min and elongation at 72 °C for 2 min; and a final elongation step at 72 °C for 10 min. As a control, which was run in parallel, we used DNA, extracted from the placenta of an aborted goat, identified as *C. abortus* positive by

Table 1. Target genes and primer sequences used in the study:

Gene	Primer	Sequence	Amplicon size (bp)	Reference
pmp C. abortus	CpsiA CpsiB	5'-ATG AAA CAT CCA GTC TAC TGG-3' 5'- TTG TGT AGT AAT ATT ATC AAA-3'	300	Larocaou *et al*., 2001
ompA C. psittaci	CPsittF CPsittR	5'-GCT ACG GGT TCC GCT CT-3' 5'-TTT GTT GAT YTG AAT CGA AGC -3'	1041	Heddema *et al*., 2006
ompA C. psittaci	Or1 Or2	5'-TTT CGA TCG TGT ATT AAA AGT T-3' 5'-AGA AAA TGT CGA AGC GAT CCA-3'	245	Olsen *et al*., 1998
ompA C. psittaci	Or1m Or2m	5'-TTT CGA TCG TGT ATT AAA AGT T-3' 5'-AGA AAA TGT CRA AGC GAT CCG-3'	212	This study
*superoxide dismutase *Coxiella burnetii*	C.B.-1 C.B.-2	5'-ACT CAA CGT ACT GGA ACC GC-3' 5'-TAG CTG AAG CCA ATT CGC C-3'	257	Stein and Raoult, 1992

**C. burnetii* amplification was performed as described by Stein and Raoult, 1992 with modifications.

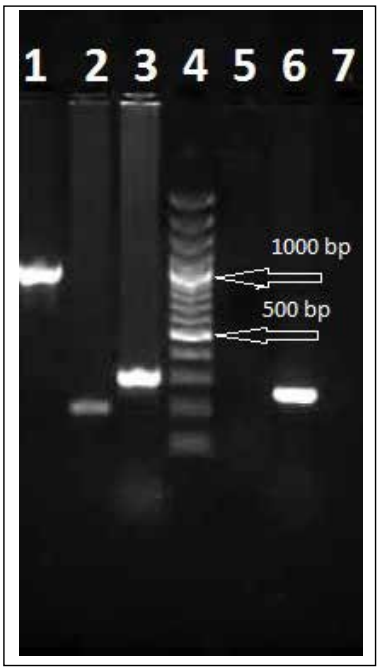

Fig.1. Results of PCR analysis of *C. abortus* DNA and *Coxiella burnetii* DNA, using *C. abortus*- and *C. psittaci*-specific, and *Coxiella burnetii*-specific primers, respectively. Lane 1, primers CpsittF(R), 1041bp; lane 2, primers Or1(2), 212bp; lane 3, primers CpsiA(B), 300 bp; lane 4, DNA molecular size marker 100bp; lane 5, negative control (DNA, tested negative with CpsiA(B) and re-tested with Cpsitt(F)R; lane 6, PCR product of placenta form goat, tested with C.B.-1(2) primers (*Coxiella burnetii*), 257bp; lane 7, ddH_2O and C.B.-1(2) primers (*Coxiella burnetii*)-negative control.

microscopy of stained impression smears, PCR and serology (ELISA).

To check for a possibility to detect *C. abortus* using *C. psittaci*-specific primers, three CpsiA(B) PCR positive and three negative samples were tested twofold by PCR using the CPsittF(R) and Or1(2) primers. The same amplification program as for CpsiA(B) PCR was used. The target genes, primer sequences, amplification product lengths and the corresponding references are given in Table 1.

All amplified fragments were analyzed using 2% agarose gel electrophoresis and GeneRuler 100bp Plus DNA Ladder.

Software

Comparison and modification of primers was done using the Basic Local Alignment Search Tool (BLAST) (National Center for Biotechnology Information-NCBI, http://www.ncbi.nlm.nih.gov/), software MEGA 6.06 (Tamura *et al.*, 2013) and its integrated alignment tool (Edgar, 2004).

Results

In the CpsiA(B) PCR 5 of 21 sheep and one of seven goat resulted positive. One goat was positive for *Coxiella burnetii* infection (Q fever), however coinfection with *C. abortus* was not detected. BLAST analysis of the CpsiA(B) primers showed that they are targeted to more than one region of the *C. abortus* genome as the forward primer (A) displays complementarities to other 3 regions and the reverse primer (B) to another 2 regions of the se-

quenced *C. abortus* genome (GenBank, accession number (LN554882.1, CR848038.1). In addition, difference was found in the sequence complementary to CpsiA in position 10, where C→T.

In the comparative study with different primers in all three samples PCR, performed with CPsittF(R) generated products with the expected size of 1041bp. (Fig.1). In contrast, on the first run of the reaction carried out with Or1(2) one of the samples resulted negative but, after being repeated, all samples gave positive results. Nevertheless, at electrophoresis the generated amplicons were with a size of about 200bp, which differed significantly from the theoretically expected size of 245bp, indicated in the original report (Olsen *et al.,* 1998). In addition, visualized bands showed low intensity staining. In an attempt to find a possible explanation for the unsatisfactory results, the homology of the reported Or1(2) primer pair with their nucleic acid sequences was analyzed by comparison to GenBank (NCBI) data, using MEGA 6.06 and an alignment tool (Edgar, 2004). The alignment revealed the presence of two discrepancies as follow: G→A(R) in position 11 (in two sequences, KM609418.1 and KM609419.1) and A→G in position 21 (in all sequences). Moreover, BLAST analysis revealed a difference of 34 bases in the size of the fragments encompassed by Or1(2) primers as compared to that, reported by Olsen *et al.* (1998).

Presuming this we modified Or1(2) primers, in which these mismatches were corrected. The modified primers Or1(2)m showed greater sensitivity in PCR amplification (two positive from two runs) and generated a 212 bp product, which corresponded well to the length of the targeted region of the *ompA* gene. Nevertheless, the electrophoretic bands in most cases showed lower intensity as compared to those generated by CpsiA(B) and CPsittF(R) PCR. PCR of samples that tested negative with CpsiA(B) gave the same results when performed with primers CPsittF(R), Or1(2) and Or1(2) m.

Discussion

The results obtained by PCR for diagnosis of C. *abortus* (21.43% positives) confirmed the important role this agent plays as an etiological agent of the abortions in the sheep and goats from tested flocks. These values exceeded the percent of positive reagents detected by Giemsa-staining of impression smears (unpublished data) and showed the high diagnostic potential of PCR for diagnosis of chlamydial infections.

Considering the close genetic relationship within the *Chlamydiaceae*, we examined the availability of primers CPsittF(R) and Or1(2), designed for the detection of *C. psittaci* to diagnose *C. abortus* in clinical samples by PCR. In this we consider also studies demonstrating that primers targeting *C. abortus* genes can be successfully used for amplification of DNA extracted from *C. psittaci* (Creelan and McCullough, 2000; Larocaou *et al.*, 2001), *C. caviae* (Larocaou *et al.*, 2001), as well as primers targeting sequences common for all members of order *Chlamydiales* (Lienard *et al*., 2011). In our study, the results from CpsiA(B)-PCR coincided entirely with those of PCR, conducted with primers CPsittF(R), although these primers target different genes of the chlamydial genome, *pmp* and *ompA*, respectively. This indicates that the two primer pairs amplify sequences in these genes, which are identical in both *C. abortus* and *C. psittaci.*

Some differences in terms of repeatability of the results and the size of the amplification products were observed when the same samples were tested in PCR, using primers Or1(2). We suggest that the mismatches we identified between the primer sequences and their complementary regions in the *ompA* gene of *C. psittaci* could serve as a possible explanation, since such mismatches are known to reduce the specificity of PCR amplification.

Although some authors report differences in the detection rates of the PCR reactions performed with different primers (Greco *et al*., 2005; Soomro *et al*., 2012), in our study we did not observe such variation in the sensitivity and all three samples that proved positive by CpsiA(B) PCR also resulted positive in CPsittF(R) and Or1(2) PCR. It is known that *C. abortus* is characterized by low genetic heterogeneity (Denamur *et al*., 1991) and inter-strain differences can only be detected using monoclonal antibodies, sequencing or restriction fragment length polymorphism (RFLP) analysis of the PCR amplification products. Recent advancements in modern techniques for analysis of tandem repeats in DNA (multiple loci VNTR analysis (MLVA) and multi locus sequence typing (MLST)) have even made possible to divide *C. abortus* strains in different genotypes (Laroucau *et al*., 2009; Pannekoek *et al*., 2010; Siarkou *et al*., 2015), partly dependent on the geographical origin as well (Laroucau *et al*., 2009). In this study, only samples collected from Bulgaria were analyzed and no comparative analysis with reference strains or isolates from other countries was carried out. Moreover, only a limited number of samples were tested. Thus, the existence

of strains within *C. abortus* which cannot be detected by the PCR primer pairs used for detection of *C. psittaci* cannot be excluded completely.

Although different annealing temperatures have been reported in the original papers as optimal for the primers, compared to this study, 50°C for CpsiA(B), 62°C for CPsittF(R) and 48°C for Or1(2), respectively, we performed reactions following one and the same regime of amplification at annealing temperature of 50°C and obtained positive results with all of the primers tested. This allows simultaneous amplification of different genome segments of one and the same sample, when further sequencing or RFLP analyses are planned. In addition, the primers used by us for detection of *C. abortus* also have the advantage to generate amplicons of different size, thus facilitating their electrophoretic differentiation when other abortogenic agents (most commonly *Coxiella burnetii*) have been examined simultaneously in multiplex PCR.

Due to the uneven results observed with Or1(2), we do not recommend these primers for routine PCR diagnosis of *C. abortus* in small ruminants. In contrast, *C. psittaci* specific primer pair CpsittF(R) could be efficiently used for this purpose. However, additional techniques such as sequencing or RELF analysis are needed to specifically discriminate *C. abortus* from *C. psittaci* or other *Chlamydia* that may be involved in inducement of abortions.

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