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A N I N T E R N A T I O N A L J O U R N A L

Validation of a PCR Assay for *Chlamydophila abortus* rRNA Gene Detection in a Murine Model

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ABSTRACT

Chlamydophila abortus (C. abortus) is associated with reproductive problems in cattle, sheep, and goats. Diagnosis of C. abortus using embryonated chicken eggs or immortalized cell lines has a very low sensitivity. Polymerase chain reaction (PCR) assays have been used to detect C. abortus infection in clinical specimens and organ fragments, such as placenta, fetal organs, vaginal secretions, and semen. The aim of this study was to develop a PCR assay for the amplification of an 856-bp fragment of the rRNA gene of the Chlamydiaceae family. The PCR assay was evaluated using organs from 15 mice experimentally infected with the S26/3 reference strain of C. abortus. The results of the rRNA PCR were compared to the results from another PCR system (Omp2 PCR) that has been previously described for the Omp2 (outer major protein) gene from the Chlamydiaceae family. From the 15 C. abortus-inoculated mice, 13 (K=0.84, standard error =0.20) tested positive using the rRNA PCR assay and 9 (K=0.55, standard error=0.18) tested positive using the Omp2 PCR assay. The detection limit, measured using inclusion-forming units (IFU), for C. abortus with the rRNA PCR (1.05 IFU) was 100-fold lower than for the Omp2 PCR (105 IFU). The higher sensitivity of the rRNA PCR, as compared to the previously described PCR assay, and the specificity of the assay, demonstrated using different pathogenic microorganisms of the bovine reproductive system, suggest that the new PCR assay developed in this study can be used for the molecular diagnosis of C. abortus in abortion and other reproductive failures in bovines, caprines, and ovines.

Key words: bovine, ovine, caprine, reproductive failures, *Chlamydophila abortus*, PCR

INTRODUCTION

The *Chlamydiales* order includes obligatory intracellular Gram negative bacteria, which have a life cycle that alternates between the infectant (elementary bodies) and vegetative (reticulate bodies) forms. Species belonging to the *Chlamydiaceae* family cause infection in both humans and animals, and the *Chlamydophila* genus is considered one of the most important in veterinary medicine (Schachter and Caldwell, 1980; Corsaro et al., 2003).

Chlamydophila abortus (*C. abortus*) is associated with reproductive failures in cattle, sheep, and goats and occasionally in pigs and horses (Everett, 2000). Epizootic abortion of cattle and enzootic abortion of goats and sheep are the most important clinical manifestations and have been reported

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worldwide (Griffiths et al., 1995; Rodolakis et al., 1998; Rekiki et al., 2002; Szeredi and Bacsadi, 2002).

C. abortus infections have also been reported in abortion observed in laboratory animals (mice, guinea pigs, and rabbits), although the virulence of the particular strain is fundamental to the development of clinical signs (Buendía et al., 1999; Everett, 2000).

Diverse diagnosis methods can be used to identify *C. abortus* in biological samples such as placenta, fetal organs, vaginal discharge, and semen. These methods include culture in embryonated chicken eggs or in continuous cell lines (McCoy, VERO, and L929), as well as techniques for protein detection (direct immunofluorescence, immunohistochemistry, and ELISA) and nucleic acid detection (polymerase chain reaction, PCR) (Storz et al., 1968; Dagnal and Wilsmore, 1990; Buxton et al., 1996; Laroucau et al., 2001).

Identification of species belonging to the *Chlamydiaceae* family has been performed using PCR employing primers that amplify conserved regions of the Omp (outer major protein) gene or the intergenic space between the 16S and 23S rRNA genes (Everett, 2000; Madico et al., 2000). While the Omp gene sequence is extremely variable, making difficult to identify conserved regions (Watson et al., 1991; Kaltenboeck et al., 2005), the rRNA operon contains numerous regions conserved among all species of the *Chlamydiaceae* family. This feature has allowed the use of the PCR assay for proposing a new taxonomy, for identification of new species, and for diagnosis of infection (Everett et al., 1999; Kaltenboeck et al., 2005). Differentiation among species of the *Chlamydiaceae* family can be achieved by restriction fragment length polymorphism analysis of the generated amplicons (RFLP-PCR) or through direct sequencing of the PCR-amplified products (Sheehy et al., 1996; Everett, 2000; Hartley et al., 2001).

The aim of this study was to develop a PCR assay to amplify the intergenic space in the 16S-23S rRNA sequence of the *Chlamydiaceae* family in order to detect and identify *C. abortus* in organs of experimentally infected mice.

MATERIALS AND METHODS

Chlamydophila abortus

The reference strain *C. abortus* S26/3 was propagated in the vitelline sac of embryonated chicken eggs and used as positive control in the PCR assay.

Mice

A total of 25 albino female mice (*Mus musculus*), age 4 weeks, were divided into two groups. The first group, consisting of 15 mice, was intraperitoneally (IP) inoculated with 0.2 mL of chicken embryo vitelline sac previously inoculated with 1000 inclusion-forming units (IFU) of *C. abortus* in 0.9% saline solution. The second group, which represented the negative control, included 10 mice inoculated (IP) with 0.2 mL of saline solution. Three animals from the first group and two from the second group were euthanized on days 5, 10, 15, 20, and 30 post-inoculation.

At necropsy, fragments of the liver, spleen, lung, and kidney were collected. The organ fragments were ground in phosphate-buffered saline solution (PBS, pH 7.2), and suspensions (10% w/v) were centrifuged at 1000 x *g* for 5 min at 4°C. A pool of supernatants was prepared, stored at 4°C, and used for nucleic acid extraction.

DNA extraction

For DNA extraction, aliquots (400 µL) from the pooled supernatants were prepared using the silica/guanidine isothiocyanate method (Boom et al., 1990). Negative controls consisting of aliquots of sterile ultrapure water were included in all DNA extraction procedures.

PCR assay

The DNA amplification was performed using two different systems:

PCR 1: The rRNA PCR was performed with the primers Ldn 2 (5'-CCCAAGGTGAGGCTGATGAC-3', positive sense, nt 1470-1489 in the 16S rRNA gene) and Ldn 4 (5'-GTTTCAGGTTCTATTTCACTCCC-3', negative sense, nt 2303-2325 in 23S rRNA gene), which amplify an 856-bp product from the intergenic space between the 16S and 23S rRNA genes. The Ldn2/Ldn4 primers were designed based on the genomic sequence of the *C. abortus* EBA strain (GenBank access number U76710). However, the *Chlamydophila psittaci* 6BC strain (U68447), the *Chlamydophila pecorum* UR 629 strain (U68434), the *Chlamydophila felis* UR 120 strain (U68457), the *Chlamydophila pneumoniae* N16 strain (U68426), the *Chlamydophila*

muridarum SFPD strain (U68437), the *Chlamydia caviae* CPIC U813 strain (U68451), the *Chlamydia suis* R22 strain (U68420), and the *Chlamydia trachomatis* L2434.BU strain (U68443) were also aligned to determine the most conserved region among the different species of the *Chlamydiaceae* family. Sequence alignment, primer design, and initial primer analysis were performed using the CLUSTAL W Multiple Alignment Program (http://www.ebi.ac.uk/clustalw), Gene Runner version 3.05 software (Hastings Software Inc., Hastings, USA) (http://www.generunner.com), and BLAST (http://www.ncbi.nlm.nih.gov/BLAST), respectively.

The PCR was carried out using 5 μL of extracted DNA and 45 μL of PCR-mix consisting of 20 pmol of each primer, 0.2 mM of each dNTP, 2.5 units of *Platinum Taq* DNA polymerase (Invitrogen Life Technologies, BR), 1.5 × PCR buffer (30 mM Tris-HCl, pH 8.4, and 75 mM KCl), 2 mM $MgCl_2$, and sterile ultrapure water to a final volume of 50 μL. Amplification was performed in a thermocycler (PTC 200, MJ Research Co., USA) with the following cycling profile: an initial step of 4 min at 94°C, followed by 40 cycles of 1 min/94°C, 1min/59°C, 1 min/72°C, and a final extension step of 7 min/72°C.

PCR 2: The Omp2 PCR was performed with the primers Ch1 (5'-ATGTCCAAACTCATCAGACGAG-3', positive sense, nt 529-550) and Ch2 (5'-CCTTCTTTAAGAGGTTTTACCCA -3', negative sense, nt 1093-1115), which amplify a 587-bp product from the Omp2 gene of *Chlamydiaceae* family representatives. The reaction was performed according to Hartley et al. (2001).

Aliquots of 10 μL of the PCR products were analyzed by electrophoresis on a 2% agarose gel. The gel was run at a constant voltage (90 V) for approximately 45 min, stained with ethidium bromide 0.5 (μg/mL) in TBE buffer pH 8.4 (89 mM Tris, 89 mM boric acid, 2 mM EDTA), and visualized under UV light.

RFLP-PCR

The specificity of the amplified products from the two PCR assays was confirmed by RFLP analysis with *Alu*I enzyme (Invitrogen Life Technologies, USA). The reaction was performed following the manufacturer's instructions. The obtained fragments were also analyzed by electrophoresis on a 2% agarose gel stained with ethidium bromide.

For a comparative analysis, the restriction profiles of nine *Chlamydophila* and *Chlamydia* species were obtained through *in silico* analyses using Gene Runner version 3.05 software.

Sensitivity

The lowest limit of detection of *C. abortus* DNA for both PCR assays was defined using suspensions of aborted calf organs that had been artificially contaminated. The bovine abortion was previously known to be negative for *C. abortus* infection by inoculation in an embryonated chicken egg and by rRNA and Omp2 PCR assays. A pool of suspensions was filtered using a 45 μm membrane and was submitted to counting of inclusion-forming units (IFU) according to Hartley et al. (2001). After determination of the IFU/mL, DNA was extracted from 10-fold serial dilutions using the silica/guanidine isothiocyanate method (Boom et al., 1990).

Specificity

The specificity of both PCR systems was evaluated using extracted DNA from: i) two species of *Chlamydophila* (*Chlamydophila psittaci*, Dade Behring strain, Germany; and *Chlamydophila felis*, Fel-o-vax IV Fort Dodge® vaccine, USA); ii) microorganisms commonly associated with reproductive failures in cattle [*Leptospira interrogans* serovar Hardjo (Hardjoprajitno); *Leptospira interrogans* serovar Icterohaemorrhagiae (RGA); *Leptospira interrogans* serovar Canicola (Hond Utrecht); *Brucella abortus*, antigen TECPAR-PR; *Mycoplasma bovigenitalium*; *Mycoplasma bovis*; *Ureaplasma* sp.; bovine herpesvirus 1; and bovine viral diarrhea virus]; iii) environmental microorganisms (*Escherichia coli*; *Salmonella* sp.; *Staphylococcus aureus*; and *Streptococcus* sp.).

Statistical analysis

The Kappa (*K*) indicator (Cohen, 1960) was used in order to establish an agreement between the results obtained from the two PCR assays evaluated in this study and the experimental inoculation in mice (gold standard), according to the following criteria: K=0 (poor agreement); K=0.1-0.2 (low agreement); K=0.21-0.4 (medium agreement); K=0.41-0.6 (moderate agreement);

K=0.61-0.8 (substantial agreement), and *K*>0.81 (almost perfect agreement) (Landis and Koch, 1977).

RESULTS

Of 15 inoculated mice, 13 (*K*=0.84, standard error=0.20) tested positive using the rRNA PCR and 9 (*K*=0.55, standard error=0.18) tested positive using the Omp2 PCR assay (Table 1). All 10 non-inoculated mice (negative control group) were negative in both PCR systems. The specificity of the assays for *C. abortus* was confirmed by RFLP with *Alu*I enzyme (Fig. 1). No background or nonspecific amplifications were visualized using an agarose gel. None of *C. abortus*-inoculated mice died during the experiment.

Table 1 - *Chlamydophila abortus* rRNA or Omp2 gene detection by PCR assays performed in suspensions of organs from mice experimentally infected.

PCR assay	Pooled organs / Days post-inoculation						Organs (*n*=15)			
	5th	10th	15th	20th	30th	Total	Lung	Spleen	Kidney	Liver
	Positives samples / Total samples									
rRNA (*K*=0.84)	3/3	3/3	3/3	2/3	2/3	13/15	13/15	13/15	12/15	13/15
Omp2 (*K*=0.55)	3/3	3/3	3/3	0/3	0/3	9/15	9/15	9/15	9/15	9/15

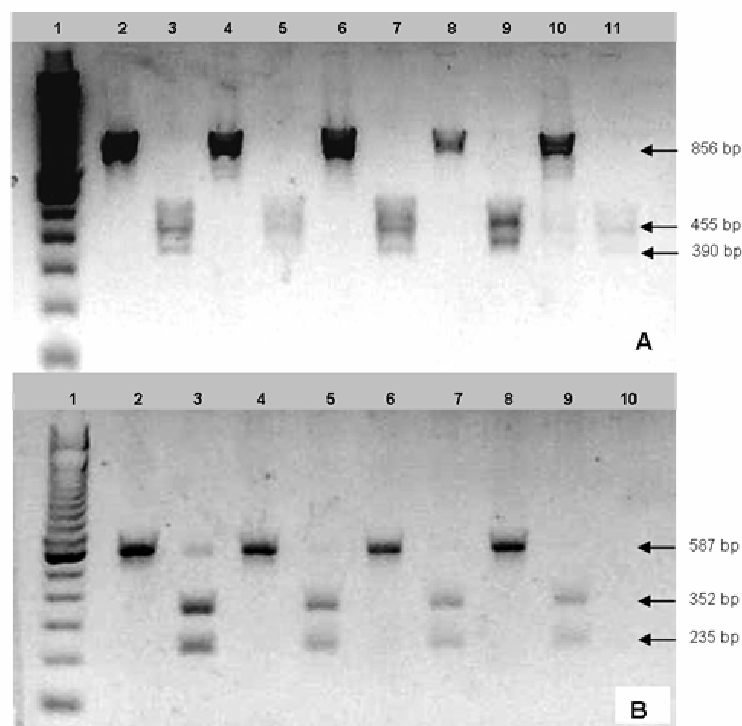

Figure 1 – Agarose gel electrophoresis of PCR products and *Alu*I restriction profiles obtained using organs of mice experimentally infected with Chlamydophila abortus. Panel A) rRNA PCR (856 bp). Lane 1: 100 bp molecular size marker (Invitrogen, USA); Lanes 2, 4, 6, 8 and 10: PCR products from mouse organs (pooled) at days 5, 10, 15, 20 and 30 after experimental inoculation, respectively; Lanes 3, 5, 7, 9, and 11: Restriction profiles (*Alu*I enzyme – Invitrogen , USA) of the PCR products from lanes 2, 4, 6, 8, and 10, respectively. Panel B) Omp 2 PCR (587 bp). Lane 1:100 bp molecular size marker; Lane 2: positive control (embryonated chicken egg inoculated with *C. abortus*); Lane 3: Restriction profile of the PCR products from lane 2; Lanes 4, 6, and 8: PCR products from mouse organs (pooled) at days 5, 10, and 15 after experimental inoculation, respectively; Lanes 5, 7 and 9: Restriction profiles of the PCR products from 4, 6, and 8, respectively: Lane 10: negative control.

Regarding the sensitivity of the PCR assays, DNA extracted from aborted calf organs that had been experimentally contaminated with *C. abortus* strain S26/3 could be amplified by PCR with primers Ldn 2/Lnd 4 (for the rRNA PCR) up to a dilution of 10^{-3} (1.05 IFU) and up to a dilution of 10^{-1} (105 UFI) with primers Ch1/Ch2 (for the Omp2 PCR) (Fig. 2).

In the evaluation of the specificity of the rRNA and Omp2 PCRs, products of 856 and 587 bp in size, for *C. psittaci* and *C. felis* respectively, were observed. Other bacterial and viral strains gave negative results in both PCR assays. Additionally, no false-negative or false-positive results were observed in PCR reactions with sterile ultrapure water or with *C. abortus* strain S26/3 (positive control).

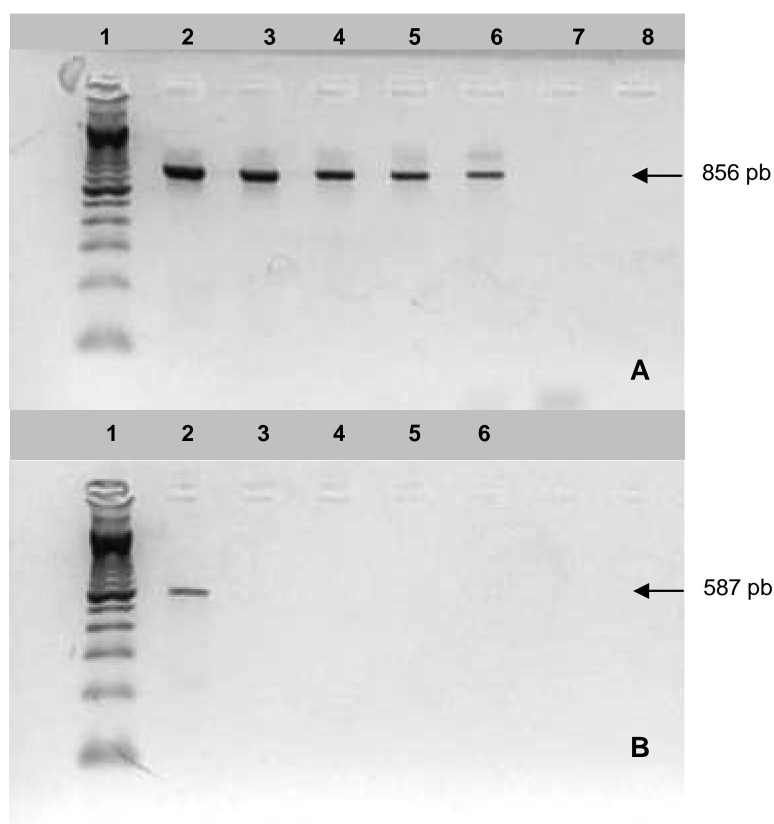

Figure 2 - *Chlamydophila abortus* detection limit using the rRNA and Omp2 PCR assays to evaluate the filtrate of aborted calf organs (pooled) that had been experimentally contaminated. Panel A) rRNA PCR (856 bp). Lane 1: 100 bp molecular size marker (Invitrogen, USA); Lane 2: positive control (embryonated chicken egg inoculated with *C. abortus*); Lane 3: filtrate of aborted calf organs (pooled) that had been experimentally contaminated with *C. abortus*; Lanes: 4, 5, 6, and 7: 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} dilutions of the filtrate from lane 3, respectively; Lane 8: negative control. Panel B) Omp2 PCR (587 bp). Lane 1: 100 bp molecular size marker; Lanes 2, 3, 4, and 5: 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} dilutions of the filtrate of aborted calf organs (pooled) that had been experimentally contaminated with *C. abortus*, respectively; Lane 6: negative control.

DISCUSSION

C. abortus is considered one of main microorganisms associated with reproductive failures in sheep, goats, and cattle (De Graves et al., 2003). Standard techniques for diagnosis of *C. abortus*, such as bacterial isolation in cell culture and Gimenez or Stamp staining, have limited sensitivity. The recent development and validation of different PCR assays has been done with the aim of reducing these gaps (Pelletier et al., 2006).

PCR has been considered the most adequate technique for the detection of *C. abortus* in samples from aborted calves (Reitt et al., 2007). Although they allow the identification of all species of the *Chlamydiaceae* family, PCR assays that amplify segments of the 16S and 23S rRNA genes present high sensitivity and specificity (Meijer et al., 1997; Madico et al., 2000).

In this study, the development and validation of a PCR assay for the detection of the intergenic space in the 16S-23S rRNA region of the DNA of the *Chlamydiacea* family was achieved. When compared to the Omp2 PCR system developed by Hartley et al. (2001), our rRNA PCR assay presented a detection limit 100-fold lower when using aborted calf organs that had been experimentally contaminated and when using experimentally infected mice.

Agreement between the rRNA PCR and the experimental inoculation of mice (*K*= almost perfect agreement) was also better than for the Omp2 PCR (*K*= moderate agreement). De Graves et al. (2003) have also found significant differences in sensitivity among PCR assays for the Omp gene and 23S rRNA, both of which were tested using vaginal cytology samples collected from heifers. In the aforementioned study, prevalences of 53% and 22% were obtained, respectively, using the 23S rRNA and Omp PCRs when analyzing the same clinical samples.

Adequate primer design, the genomic region to be amplified, and the optimization of the PCR reaction all have direct impacts on the sensitivity and specificity of a technique (Everett et al., 1999; Butler et al., 2001). In this study, the primers developed for the rRNA PCR had similar annealing temperatures and did not form primer-dimers, primer-hairpin loops, primer-bulge loops, or primer-internal loops. Moreover, the region amplified by the Ldn2/Ldn4 primers is highly conserved and is specific for all species belonging to the *Chlamydiaceae* family. Additionally, rRNA PCR also amplifies heterogenic segments that allow differentiation among different species by cleavage with restriction enzymes or by direct sequencing of amplicons. The differentiation between *C. abortus* and other species of the *Chlamydiaceae* family can be performed using *Alu*I enzyme.

The choice of an adequate method for nucleic acid extraction is also important in the optimization of PCR systems (Innis and Gelfand, 1990). In this study, extraction methods using phenol/chloroform/isoamyl alcohol (Sambrook et al., 2001), silica/guanidine isothiocyanate (Boom et al., 1990), and a combination of both techniques (Alfieri et al., 2006) were comparatively evaluated for *C. abortus* detection in aborted calf organs that had been experimentally contaminated (data not shown). Since both methods employing silica presented similar results, the silica/guanidine isothiocyanate method was chosen to avoid additional steps in the DNA extraction procedure. In addition, this method is known to efficiently remove PCR inhibitors from clinical samples and it provides greater stability to the extracted nucleic acid (Romero, 1999; Rassol et al., 2002; Paula et al., 2003).

Although mice are susceptible to infection with C. *abortus* strain S26/3, no infected mice died or presented any clinical sign during this experiment. The fact that clinical manifestations can be affected by the particular *Chlamydiaceae* species, the virulence of the involved strain, the infective dose, and the host immunologic factors could explain this observation (Everett, 2000; Bouakane et al., 2003).

Since negative results were obtained for samples collected from mice euthanized on days 20 and 30 p.i. using the rRNA PCR assay, the elimination of *C. abortus* infection by the development of a specific immune response must be considered (Mestecky et al., 2005).

A PCR assay that amplifies the intergenic space of the 16S-23S rRNA genomic region of *Chlamydiaceae*, including the *C. abortus* species, was developed and evaluated in this study. This new PCR assay presented greater sensitivity and specificity than the previously described Omp2 PCR assay. The validation of this rRNA PCR assay opens the possibility of its use in epidemiological studies of the incidence of *C. abortus* in Brazilian cattle and small ruminant herds.

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RESUMO

Chlamydophila abortus (*C. abortus*) é frequentemente associada a distúrbios reprodutivos em bovinos, ovinos e caprinos. Para o diagnóstico, os métodos de cultivo em ovo embrionado de galinha e em células de linhagem contínua apresentam baixa sensibilidade. A reação em cadeia da polimerase (PCR) tem sido utilizada em placenta, órgãos fetais, secreção vaginal e sêmen para o diagnóstico da *C. abortus*. O objetivo deste trabalho foi desenvolver um sistema de PCR para a amplificação de um fragmento de 856-pb do gene rRNA da família *Chlamydiaceae*. A PCR foi avaliada em órgãos de 15 camundongos infectados experimentalmente com a estirpe de referência S26/3 da *C. abortus*. Os resultados foram comparados com os obtidos em outro sistema de PCR, previamente descrito para o gene Omp2 (*outer major protein*) da família *Chlamydiaceae*. Dos 15 camundongos inoculados com *C. abortus*, 13 (*K*=0,84, erro padrão=0,20) foram positivos na rRNA PCR e nove (*K*=0,55, erro padrão=0,18) na Omp2 PCR. O limite de detecção da *C. abortus* na rRNA PCR (1,05 UFI) foi 100 vezes inferior à Omp2 PCR (105 UFI). A maior sensibilidade em comparação ao sistema de PCR anteriormente descrito, bem como a especificidade demonstrada frente a diferentes microrganismos patogênicos do sistema reprodutivo, abrem a perspectiva da utilização da PCR desenvolvida nesse estudo para o diagnóstico molecular da *C. abortus* em casos de abortamentos e outros distúrbios reprodutivos em bovinos, ovinos e caprinos.

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