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Etiologic diagnosis of bovine infectious abortion by PCR

Diagnóstico etiológico de aborto infeccioso bovino por PCR

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ABSTRACT

*Infectious abortion is a significant cause of reproductive failure and economic losses in cattle. The goal of this study was to detect nucleic acids of several infectious agents known to cause abortion including **Arcanobacterium pyogenes**, Bovine Herpesvirus 1, **Brucella abortus**, **Campylobacter fetus** subsp. **venerealis**, **Chlamydophila abortus**, **Leptospira** sp., **Listeria monocytogenes**, Salmonella sp., **Mycoplasma bovis**, **Mycoplasma bovigenitalium**, **Neospora caninum**, and **Tritrichomonas foetus**. Tissue homogenates from 42 fetuses and paraffin-embedded tissues from 28 fetuses and 14 placentas/endometrium were included in this study. **Brucella abortus** was detected in 14.2% (12/84) of the samples. Salmonella sp. DNA was amplified from 2 fetuses, and there was one positive for **Neospora caninum**, and another for **Listeria monocytogenes**. This PCR-based approach resulted in identification of the etiology in 19% of samples, or 20% if considered fetal tissues only.*

Key words: *cattle, abortion, **Brucella abortus**, Salmonella sp., **Neospora caninum**, **Listeria monocytogenes**.*

RESUMO

*Aborto infeccioso é uma causa significativa de falhas reprodutivas e perdas econômicas na bovinocultura. O objetivo deste estudo foi detectar ácidos nucleicos de vários agentes infecciosos reconhecidos como causadores de aborto, incluindo-se **Arcanobacterium pyogenes**, Herpesvirus bovino tipo 1, **Brucella abortus**, **Campylobacter fetus** subsp. **venerealis**, **Chlamydophila abortus**, **Leptospira** sp., **Listeria monocytogenes**, Salmonella sp., **Mycoplasma bovis**, **Mycoplasma bovigenitalium**, **Neospora caninum** e **Tritrichomonas foetus**. Homogenados de tecidos de 42 fetos e tecidos incluídos em parafina de 28 fetos e 14 placentas/endométrio foram incluídos neste estudo. **Brucella abortus** foi detectada em 14,2% (12/84) das amostras. DNA de Salmonella sp. foi amplificado de dois fetos e houve um feto positivo para **Neospora caninum** e outro para **Listeria monocytogenes**. Essa metodologia baseada em PCR resultou na identificação da etiologia em 19% das amostras ou 20% se considerados somente os tecidos fetais.*

Palavras-chave: *bovino, aborto, **Brucella abortus**, Salmonella sp., **Neospora caninum**, **Listeria monocytogenes**.*

INTRODUCTION

Abortions have a highly negative impact on reproductive efficiency, resulting in significant economic losses for the cattle industry (DE VRIES, 2006). Under optimal laboratorial conditions, etiologic diagnosis is achieved in 23.3 to 45.5% of the cases (ANDERSON, 2007). Bovine abortion may be due to infectious, toxic, endocrine, physical or nutritional causes. Infectious agents associated with abortion in cattle include viruses, bacteria, protozoa, and fungus. The exact proportion of cases due to infectious agents is not known, but in 90% of cases in which an etiologic diagnosis is achieved the cause is infectious (NASCIMENTO & SANTOS, 2003).

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Efficient diagnosis requires a complete diagnostic protocol associated with submission of appropriate specimens and clinical history. Traditional diagnostic tools include serology, histopathology, bacterial and viral isolation, and for certain agents direct examination or immunohistochemistry (ANDERSON, 2007).

Several PCR and RT-PCR (reverse transcription-polymerase chain reaction) protocols have been recently developed for identification of infectious agents in aborted bovine fetuses, including ***Brucella abortus*** (LEAL-KLEVEZAS et al., 2000; CORTEZ et al., 2001; RICHTZENHAIN et al., 2002; BRICKER et al., 2003), ***Leptospira*** sp. (HEINEMANN et al., 1999; RICHTZENHAIN et al., 2002), ***Listeria monocytogenes*** (AZNAR & ALARCÓN, 2003), Bovine Herpesvirus 1 (KIBENGE et al., 1994; ROCHA et al., 1999), ***Campylobacter fetus*** subsp. ***venerealis*** (HUM et al., 1997; VARGAS et al., 2003), ***Neospora caninum*** (BASZLER et al., 1999; ELLIS et al., 1999; COLLANTES-FERNÁNDEZ et al., 2002; PEREIRA-BUENO et al., 2003), ***Mycoplasma bovis*** (AYLING et al., 1997; SUBRAMANIAM et al., 1998; HAYMAN & HIRST, 2003), ***Mycoplasma bovigenitalium*** (KOBAYASHI et al., 1998; HIROSE et al., 2001; CARDOSO et al., 2006), ***Chlamydophila abortus*** (MADICO et al., 2000; LAROUCAU et al., 2001; DEGRAVES et al., 2003), *Salmonella enterica* ser. Dublin or *Salmonella* sp. (KEROUANTON et al., 1996; WHYTE et al., 2002; AMAVISIT et al., 2001), and ***Arcanobacterium pyogenes*** (JOST et al., 2002). With the exception of ***Arcanobaterium pyogenes*** and *Salmonella enterica* ser. Dublin, these agents are primarily involved in reproductive diseases in cattle. Although ***A. pyogenes*** is not a specific cause of abortion, it may cause a suppurative placentitis and abortion (NASCIMENTO & SANTOS, 2003). Similarly, *Salmonella* Dublin is usually associated with enteric infection and diarrhea, particularly in calves, although infection of pregnant cows may result in abortion as the only clinical manifestation of infection in the herd (RINGS, 1985). Since the early 1990's, PCR has been increasingly used as a diagnostic tool for etiologic diagnosis of abortion in cattle either as a complement or replacement of time consuming traditional diagnostic methods such as bacterial or viral isolation (ANDERSON, 2007).

Considering the potential of PCR for etiological diagnosis of infectious bovine abortion, the objective of this study was to use several previously established PCR protocols as a comprehensive PCR panel for identification of etiologic agent in tissues from aborted bovine fetuses. This study included frozen tissues as well as formalin-fixed and paraffin-embedded tissues from aborted fetuses and placentas.

MATERIAL AND METHODS

Samples

Forty-two tissue samples from bovine aborted fetuses (n=38) and stillborn calves (n=4) were collected in several farms located in the States of Minas Gerais (n=11), São Paulo (n=26), Goiás (n=2), and Mato Grosso do Sul (n=3), from 1997 to 2004. These samples were homogenates of several fetal tissues pooled together, and were stored at -20°C until DNA extraction.

Formalin-fixed and paraffin-embedded tissue samples from 28 fetuses and 14 placentas and/or endometrium were obtained from archived tissues originally collected from Minas Gerais (n=6), Bahia (n=2), and São Paulo (n=1), although most of the samples did not have information regarding their origin (n=33). These samples were collected from 1954 to 2004, and were also used in this study. Five micrometer-thick sections were obtained from all paraffin-embedded tissues and stained with hematoxylin and eosin for histopathological evaluation.

DNA extraction

DNA extraction from frozen tissues samples was performed using a commercial kit (Wizard Genomic DNA purification Kit, Promega, USA) following the manufacturer's instructions. Briefly, 100µL of thawed homogenates of fetal tissues were mixed with 600µL of Nuclei Lysis Solution and homogenized for 10 seconds. Samples were incubated at 65°C for 30min, followed by addition of 17.5µL proteinase K (20mg mL^{-1}) and incubation at 55°C for 3 hours, vortexing every hour. Three microliters of RNase A (4mg mL^{-1}) were added, the samples were mixed and incubated at 37°C for 30min. After cooling, 200µL of Protein Precipitation Solution were added, followed by vortexing and centrifugation at 13,000 x g for 4min. The supernatant was transferred to a new microtube with 600µL of isopropanol, mixed, and centrifuged at 13,000x g for 3min. The supernatant was discarded and the pellet was washed with 600µL of 70% ethanol, followed by a final centrifugation at 13,000x g for 3min. Each pellet was dissolved in 100µL of DNA Rehydration Solution by incubating at 65°C for 1 hour.

DNA extraction from paraffin embedded tissue sections was performed as previously described (SHI et al., 2004). Briefly, 500µL of 0.1M NaOH were added to each microtube containing two 10µm-thick tissue sections, and heated at 100°C for 20min. Then, 500µL of phenol:chloroform:isopropanol alcohol (25:24:1) were added, followed by vortexing and centrifugation at 10,000 x g for 10min. The supernatant was transferred to another microtube and 1 volume of

chloroform was added, mixed by vortexing and centrifuged at 10,000x g for 5min. The upper phase of the supernatant was transferred to another microtube and mixed with 0.1 volumes of 3.0M sodium acetate, and 1 volume of isopropanol, followed by incubation at -20°C overnight. The suspension of precipitated DNA was then centrifuged at 10,000x g for 5 min at 4°C. The pellet was washed with 75% ethanol, dried, and diluted in 50µL of distilled water. DNA samples from different tissues of the same fetus were pooled prior to PCR amplification.

DNA quality was assessed by spectrophotometry and PCR amplification of an internal control (a-actin). Samples that did not yield a a-actin amplicon or had DNA concentration lower than 100ng μL^{-1} as assessed by spectrophotometry were excluded from further analysis.

PCR

DNA samples were PCR tested for detection of 12 infectious agents known to cause abortion in cattle, including ***Arcanobacterium pyogenes,*** Bovine Herpesvirus 1, ***Brucella abortus***, ***Campylobacter fetus*** subsp. ***venerealis***, ***Chlamydophila abortus***, ***Leptospira*** sp., ***Listeria monocytogenes***, *Salmonella* sp., ***Mycoplasma bovis***, ***Mycoplasma bovigenitalium***, ***Neospora caninum***, and ***Tritrichomonas foetus***. PCR reactions were performed using 13µL of a commercial PCR mix (PCR Supermix, Invitrogen, USA), 0.75µL of a 25µM solution of each primer (Table 1), and 1µL of DNA (100 to 500ng per reaction). Amplification of Bovine Herpesvirus 1 sequence was achieved by nested PCR, using 1µL of the product from the first reaction for the second round of amplification. Parameters used were initial denaturation at 95°C for 5min, followed by denaturation at 95°C for 1min, annealing for 1min, extension at 72°C for 1min (except in the case of ***Mycoplasma bovis*** that had 2min extension), and a final extension at 72°C for 7min. The annealing temperatures and number of cycles for each agent are described in table 1. PCR products were resolved by electrophoresis in a 1% agarose gel stained with ethidium bromide. Positive controls included DNA from cultured organisms or infected tissues. Positive and negative controls (in which DNA template was replaced by PCR-grade water) were included in all reactions.

Statistical analysis

Frequencies of positive results were compared between tissue homogenates and formalin-fixed and paraffin-embedded samples by the Fisher's exact text, using Graphpad Instat software, version 3.05 (Graphpad Software, Inc., CA, USA).

RESULTS AND DISCUSSION

The PCR protocols employed in this study efficiently amplified sequences from the positive control samples, without any amplification of negative controls (Figure 1). Results obtained with DNA samples from tissue homogenates from aborted fetuses are described in table 2. Only DNA from ***Brucella abortus*** and ***Listeria monocytogenes*** were successfully amplified from formalin-fixed and paraffin-embedded tissue samples from aborted fetuses and placentas (Table 2).

Brucella abortus was the most frequent agent identified with an overall frequency of 14.28% (12/84), which was followed by two fetuses that were positive for *Salmonella* sp., one positive for ***Neospora caninum***, and another positive for ***Listeria monocytogenes***. These results support the notion that ***B. abortus*** is the most prevalent cause of infectious abortion in Southeastern and Central Western regions of Brazil, which is in good agreement with a recent PCR-based survey (CORTEZ et al., 2006). Importantly, there was no significant difference in the frequency of positivity for ***B. abortus*** between tissue homogenates collected from 1997 and 2004, and paraffin-embedded tissues collected from 1954 to 2004 ($P<0.05$, Fisher's exact test). Although the present study was not designed to study prevalence of infectious abortion, these results may indicate a relatively high frequency of bovine brucellosis during these two periods of time. Conversely, a recent study in Southern Brazil that included multiple diagnostic assays identified ***N. caninum*** as the most frequent cause of abortion in cattle (CORBELLINI et al., 2006).

Fetal epicarditis was observed in one of the cases of ***B. abortus*** infection, and was characterized by a lymphoplasmacytic and neutrophilic diffuse inflammatory infiltrate with accumulation of fibrin on the epicardium. Experimental infection of pregnant cows is often associated with fibrinous pericarditis (XAVIER et al., 2009), which is in good agreement with the finding of epicarditis in one of the aborted fetuses naturally infected with ***B. abortus*** in this study. The fetus that was positive for ***L. monocytogenes*** had a mild focal lympho-histiocytic endocarditis. Although ***L. monocytogenes*** can cause inflammatory changes in the heart (SCHLAFER & MILLER, 2007), in the present case the endocarditis can not be attributed to ***L. monocytogenes*** with certainty since no organisms were observed in association with the lesion, which is often the case in ***L. monocytogenes*** induced lesions. Multifocal miocardial necrosis and fibrosis, associated with centrilobular necrosis and fibrosis due to chronic passive congestion were observed in one of the fetuses.

Table 1 - Primers sequence and PCR reaction parameters for detection of bovine infectious abortion agents.

Agent / gene	Primers	Annealing (°C)	Cycles	Product (bp)	Reference
Arcanobacterium pyogenes	ggcccgaatgtcaccgc aactccgcctctagcgc	55	35	270	JOST et al. (2002)
Bovine Herpesvirus 1 *	tcgaargccgagtacctgcg ccagtcccaggcraccgtcac	60	35	468	ROS & BELAK (1999)
	tggtggccttygaccgcgac gctccggcgagtagctggtgtg	62	35	344	
Brucella abortus	tggctcggttgccaatatcaa cgcgcttgcctttcaaggtctg	60	40	223	BAILY et al. (1992)
Campylobacter fetus subsp. ***venerealis***	cttagcagtttgcgatattgccatt gcttttgagataacaataagagctt	50	30	142	HUM et al. (1997)
Chlamydophila abortus	atgaaacatccagtctactgg ttgtgtagtaatattatcaaa	50	30	300	LAROUCAU et al. (2001)
Leptospira sp.	ggcggcgcgtcttaaacatg ttagaacgaagttacccccctt	63	29	331	MERIEN et al. (1992)
Listeria monocytogenes	cggaggttccgcaaaagatg cctccagagtgatcgatgtt	55	35	234	AZNAR & ALARCÓN (2003)
Mycoplasma bovigenitalium	ggtagatgccgcatggcatttacgg cattcaatatagtggcatttcctac	61	37	312	KOBAYASHI et al. (1998)
Mycoplasma bovis	ttacgcaagagaatgcttca taggaaagcaccctattgat	53	35	1626	SUBRAMANIAM et al. (1998)
Neospora caninum	cctcccaatgcgaacgaaa gggtgaaccgagggagttg	57	35	275	BASZLER et al. (1999)
Salmonella sp.	accgctaacgctcgcctgtat agaggtggacgggttgctgccgtt	56	35	159	AMAVISIT et al. (2001)
Tritrichomonas foetus	cgggtcttcctatatgagacagaacc cctgccgttggatcagtttcgttaa	67	40	347	FELLEISEN et al. (1998)
α-actin	tcaaggagaagctctgctacgtg ttgccgatggtgatgacctg	60	40	162	MÜLLER et al. (2003)

* Nested PCR.

A specific etiologic diagnosis in cases of bovine abortion is highly desirable since control measures vary according to the agent involved. The overall rate of successful diagnosis in the present study was 19.04% (16/84), which is a lower rate when compared to protocols that include several different diagnostic tests that usually result in success rates ranging from 23.3 to 45.5% (ANDERSON, 2007). Importantly, the success rate obtained in this study is similar to a recent PCR-based study that included four different agents, and resulted in an etiologic diagnosis in 22.6% of the cases (CORTEZ et al., 2006). The large proportion of negative results observed in this study (80.95%) may be attributed to non-infectious abortions, or infectious abortions caused by organisms that were not included in the panel or by abortions caused by

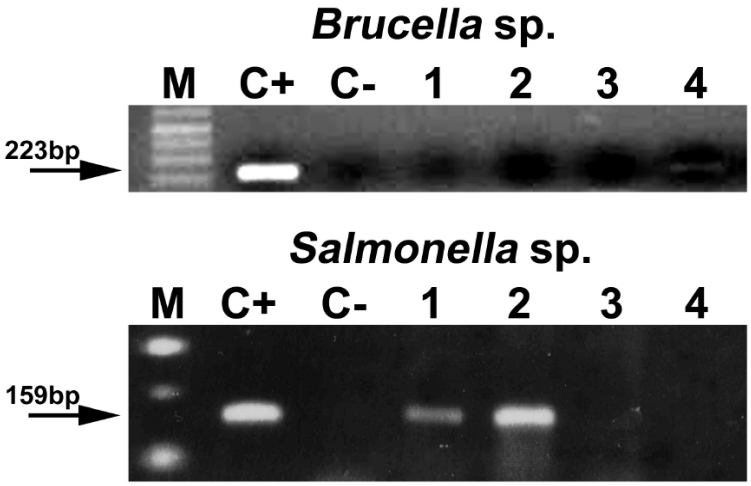

Figure 1 - Representative results of PCR amplification of genomic DNA of infectious agents of abortion in cattle. Upper panel: ***Brucella*** sp.-specific PCR (amplicon of 223 base pairs as indicated by the arrow); M: molecular weight marker, C+: positive control, C-: negative control, 1-4 samples from aborted fetuses – number 4 is weakly positive. Lower panel: ***Salmonella*** sp.-specific PCR (amplicon of 159 base pairs as indicated by the arrow); M: molecular weight marker, C+: positive control, C-: negative control, 1-4 samples from aborted fetuses –number 1 is weakly positive and number 2 is strongly positive.

one of the organisms included in the panel, but with concentrations of genomic DNA in the sample that was below the limit of detection of the method. Therefore, these results indicate the potential of PCR as an additional diagnostic tool for identification of infectious agents causing abortion in cattle. Importantly, when several diagnostic approaches are employed together, the rate of success apparently is increased in comparison to PCR alone (ANDERSON, 2007). Each one of the agents may be identified by other specific techniques such as bacteriologic or viral isolation, immunohistochemistry or immunofluorescence, serology, among others (ANDERSON, 2007). However, availability of these methods for all major infectious causes of abortion in cattle requires optimal laboratorial conditions. It is noteworthy that extremely high sensitivity of PCR methods under laboratorial conditions may not be reproducible under field conditions since suboptimal sampling and storage may have a major impact on sensitivity as recently reported for the application of a diagnostic PCR-based method under field conditions in Brazil (PAIXÃO et al., 2008).

Table 2 - PCR results for etiologic diagnosis of abortion in bovine tissue homogenates from aborted fetuses (n=42) from the states of Minas Gerais, São Paulo, Goiás, and Mato Grosso do Sul; and formalin-fixed and parafin-embedded bovine tissues from aborted fetuses (n=28) and placentas (n=14)*.

Agent	Tissue Homogenates (n=42)	----------Parafin-embedded tissues----------		
		Fetuses (n=28)	Placenta (n=14)	Histopathology
Brucella abortus	5 (11.9%)	5 (17.8%)	2 (14.3%)	Epicarditis (1/1)
Salmonella sp.	2 (4.7%)	0 (0%)	0 (0%)	N/A
Neospora caninum	1 (2.4%)	0 (0%)	0 (0%)	N/A
Listeria monocytogenes	0 (0%)	1 (3.6%)	0 (0%)	Endocarditis (1/1)

* PCR reactions for detection of Bovine Herpesvirus 1 (nested PCR), ***Leptospira*** sp., ***Campylobacter fetus venerealis***, ***Arcanobacterium pyogenes***, ***Chlamydophila abortus***, ***Mycoplasma bovis***, ***Mycoplasma bovigenitalium***, ***Tritrichomonas foetus***, yelded negative results with all samples.
** N/A = not applicable.

Although infectious agents are recognized as the most important cause of abortion in cattle, since 90% of the cases in which the cause is identified are infectious, abortion in cattle may be due to non infectious causes. These include toxic (e.g. toxic plants such as ***Tetrapterys multiglandulosa*** or ***Ataleia glazioviana***), endocrine dysfunction (either endogenous or exogenous/iatrogenic such as administration of prostaglandin F2α or corticosteroids), traumatic (manipulation during diagnosis of pregnancy or placement of intrauterine pipettes), or nutritional deficiency (NASCIMENTO & SANTOS, 2003).

Although there are several recent reports of development and application of PCR for diagnosis of specific causes of abortion (HUM et al., 1997; KOBAYASHI et al., 1998; SUBRAMANIAM et al., 1998; BASZLER et al., 1999; AMAVISIT et al., 2001; LAROUCAU et al., 2001; JOST et al., 2002; RICHTZENHAIN et al., 2002; AZNAR & ALARCÓN, 2003), in this study a comprehensive panel of PCR was applied to fetal tissue homogenates stored at -20ºC as well as paraffin-embedded tissues with satisfactory results. These data will be useful for developing a four-channel real time multiplex PCR protocol targeting the most significant causes of abortion in Brazil.

CONCLUSION

The use of a comprehensive PCR panel including 12 infectious agents associated with abortion in cattle resulted in the establishment of an etiologic diagnosis in 19% of the cases, which included paraffin-embbedded tissues and frozen tissue homogenates. ***Brucella abortus*** was the most frequent infections agent identified by this PCR panel.

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