

Bacteria isolated from abscesses of small ruminants inspected in the semiarid region of Brazil

Bactérias isoladas de abscessos em pequenos ruminantes inspecionados na região semiárida do Brasil

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Abstract

Loss in the supply chain of small ruminants owing to condemnations of carcasses in the abattoirs and slaughterhouses is common in northeastern Brazil. This study aims to identify bacterial agents, including *Mycobacterium* spp., in the abscesses found in the postmortem analysis of the carcasses of sheep and goats bred in northeastern Brazil. Our analysis involved 679 goats and 1,838 sheep carcasses. Abscess samples were extracted and inoculated on blood agar and Lowenstein Jensen with pyruvate or glycerol for bacterial isolation. We then performed polymerase chain reaction of the *hps 65* gene; samples positive for *Mycobacterium* spp. were subjected to DNA sequencing. Relative frequencies of abscesses in goats and sheep were 5.44 and 3.26%, respectively. Microbiological analysis revealed 87.7% bacterial growth in the inoculated samples. Among these, *Corynebacterium pseudotuberculosis* represented 67.7% of the isolates. We observed 1.9% mycobacteria growth in the abscess samples inoculated on Lowenstein-Jensen medium. PCR of DNA extracted from abscesses samples showed amplification of 0.9% of samples. After sequencing, *Mycobacterium* spp. isolate was identified as *M. novocastrense*. *C. pseudotuberculosis* was the main agent responsible for the formation of abscesses in the examined animals, and we did not identify any species of the *M. tuberculosis* complex in the examined small ruminants.

Key words: Goat. Inspection. Isolation. PCR. Sheep.

Resumo

Perdas na cadeia produtiva dos pequenos ruminantes pela condenação de carcaças e vísceras são comuns nos matadouros/frigoríficos da região nordeste do Brasil. Este estudo objetivou identificar agentes bacterianos, inclusive *Mycobacterium* spp., no conteúdo de abscessos encontrados no exame post mortem de caprinos e ovinos criados em regiões do nordeste do Brasil. Foram inspecionados

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no exame post mortem 679 caprinos e 1.838 ovinos. Para o isolamento bacteriano as amostras foram cultivadas em meio Agar Sangue e Lowenstein Jensen contendo piruvato ou glicerol. Além do cultivo, foi realizada identificação molecular para *Mycobacterium* spp. pela técnica de Reação em Cadeia de Polimerase (PCR) em que foram utilizados primers que amplificam fragmentos de parte do gene hsp65. As amostras positivas para micobactérias foram submetidas ao sequenciamento de DNA. As frequências relativas de abscessos em caprinos e ovinos foram de 5,44% (37/679) e 3,26% (60/1.838), respectivamente. No exame microbiológico, utilizando o meio Ágar Sague, observou-se crescimento bacteriano em 87,7% (93/106) das amostras cultivadas. Destes, o *Corynebacterium pseudotuberculosis* representou 67,7% (63/93) dos isolados. Do cultivo em meio Lowenstein Jensen foram isolados micobactérias em 1,9% (2/106) das amostras de abscessos e, na PCR com DNA extraído direto das amostras de abscessos, observou-se amplificação em 0,9% (1/106) das amostras. No sequenciamento o isolado de *Mycobacterium* spp. foi confirmado como *M. Novocastrense*. *C. pseudotuberculosis* foi o principal agente responsável pela formação de abscessos nos animais avaliados e não foi identificada nenhuma espécie do complexo *M. tuberculosis* nos pequenos ruminantes inspecionados.

Palavras-chave: Caprino. Inspeção. Isolamento. Ovino. PCR.

Introduction

Breeding of sheep and goats is an important economic activity in the semiarid region of Brazil. This is possible because of the resistance of animals to the climate of this region, which is usually very warm, dry, and accompanied with long periods of drought (SARDI et al., 2012). Animals are usually bred in small or medium-sized rural properties, with simple stockyards, lacking sanitary handling of the animals, which makes prevention and control of infectious and parasitic diseases impossible (ALENCAR et al., 2010).

Infectious diseases cause losses in the small ruminants' production chain. Postmortem of carcasses and offal revealed the presence of abscesses, which are mainly caused by bacterial infection (RADOSTITS et al., 2002).

Among the microorganisms, which are responsible for the condemnation of carcasses at slaughterhouses, we focus on those of the *Mycobacterium tuberculosis* complex. These are especially relevant for the losses they cause to animal production and for the risk they cause to public health (CVETNIC et al., 2007; GUMI et al., 2012). Tuberculosis in sheep and goats is caused by *M. bovis*, and less frequently by *M. tuberculosis* and *M. caprae* (BEZOS et al., 2010; PIGNATA et al., 2009).

Animals affected by tuberculosis may not present visible clinical signs, but exhibit

progressive weight loss and peripheral lymph nodes infarction (necrosis) (RIET-CORREA et al., 2007). Macroscopic analysis revealed that a lymph node is a firm, white, grey, or yellow colored dry nodule, with a caseous and necrotic center. Moreover, calcification, which is commonly found in lesions, was detected by a gritty sound upon incision in the postmortem examination of affected animals (JONES et al., 2000). In addition, postmortem findings revealed similar results for other small ruminant diseases in slaughterhouses. For example, caseous lymphadenitis, caused by *Corynebacterium pseudotuberculosis*, is characterized by yellowish-green or whitish-yellow abscess formation, with the consistency varying from purulent to caseous (SOUZA et al., 2011).

Hence, the study aims to identify bacterial agents, including *Mycobacterium* spp., in the abscess of sheep and goats after postmortem examination.

Materials and Methods

Animals and abscesses sampling

In this study, we inspected 2,517 animals (679 goats, 1,838 sheep), slaughtered in abattoirs of rural regions of Petrolina (State of Pernambuco) and Juazeiro (State of Bahia), Brazil. Among these animals, 97 showed abscess formation in the entrails (internal organs) and/or in the whole carcasses

(among these, nine animals presented abscesses in several different organs). We collected 106 abscess samples from these animals for study. The samples were subjected to microbiological and molecular analyses.

The samples were collected during the process of evisceration of the animals. These samples were collected in two different sampling boxes: a sterile box, which had been refrigerated; and a bottle containing 10% buffered formalin. After collection, samples were taken to the laboratory for analysis.

Bacterial cultivation

For the cultivation of Mycobacteria, the samples were decontaminated according with Petrof method, as described by Quinn et al. (1994). After this, 0.1 mL of sample was grown in Lowenstein-Jensen medium with pyruvate and Lowenstein-Jensen medium with glycerol. The samples were then stored at 37°C for 120 days. Observations were recorded weekly.

For the differential identification of the bacteria, samples were grown in 5% sheep blood agar and stored for 48 h at 37 °C. Isolated bacterial colonies were identified by Gram staining and sugars fermentation tests (glucose, and maltose), esculine test, nitrate reduction, DNase and urease tests (QUINN et al., 1994).

DNA extraction

DNA was extracted from the tissues where the abscesses were present, using the following protocol: samples were macerated in liquid nitrogen. After this, 200 mg of sample was transferred to a microtube and 800 µL of extraction solution (50 mM Tris HCl pH 8; 25 mM EDTA; 400 mM NaCl; 100 µL SDS 10% and 0.2 mg K-proteinase) was added. The mixture was then homogenized and incubated at 65 °C for 5 h

After incubation, the sample was transferred into two microtubes, and 442 µL NaCl 2M was added to

each tube. The microtubes were then stored at 4 °C for 15 min. After this, 450 µL from each tube was transferred to a new microtube containing 50 µL sodium acetate (3M). Then DNA was precipitated using 1 mL of iced absolute ethanol. Microtubes were lightly shaken and centrifuged at 14,000 g for 15 min. Supernatant was discarded and 200 µL of 70% ethanol was added. The tubes were again centrifuged at 14,000 g for 5 min and the supernatant was discarded. The microtubes were left to dry and the material was suspended in 500 µL of TE (10 mM Tris/HCL pH 8 and 1 mM EDTA pH 8) and left at 4 °C overnight.

Next, 500 µL of chloroform-isoamyl alcohol (24:1) was added to the material and the tubes were shaken by inversion. After this, the tubes were centrifuged at 1700 g for 10 min, and the first phase of the material was collected in a new tube. DNA was precipitated using 1 mL frozen absolute ethanol. Tubes were lightly shaken and centrifuged at 14,000 g for 15 min. The supernatant was discarded and the tubes were left to dry for 15 min. In the end, DNA was re-suspended in 50 µL TE + 2.5 µL RNase and left to dry for 20 min at 37 °C.

The DNA of the bacterial colonies were extracted from the samples inoculated in Lowenstein-Jensen medium using the following method: the sample was inactivated by heat and left for 10 min at 80 °C; then 10 colony forming units (CFU) were added to 300 µL TE (Tris EDTA). After this, 70 µL of 10% SDS was added and the sample was quickly shaken using a vortex. Then, 100 µL of NaCl₂ 5M and 80 µL CTAB/NaCl were added and the sample was incubated at 65 °C for 10 min. Then, 700 µL of chloroform-isoamyl alcohol (24:1) was added and the sample was homogenized by inversion and centrifuged at 11,759 g for 5 min. The first phase was transferred to a new tube, and 450 µL of isopropanol was added and left overnight. The following day, the sample was centrifuged at 11,750 g for 15 min. The superficial layer was discarded and 500 µL of 70% ethanol was added. The sample was again centrifuged at 11,750 g for 10 min. The

superficial layer was again discarded and the sample was left to dry for 15 min. The pellet was suspended in 80 μ L TE (pH 8.0) and was left for 1 h at 65°C, and then stored at -20°C.

The sample was analyzed by 1% agarose-gel electrophoresis to determine the quality of the extracted DNA.

PCR for the detection of Mycobacterium spp.

DNA derived from the bacterial colonies of abscess samples seeded in Lowenstein-Jensen medium was analyzed for *Mycobacterium* spp. identification by PCR using Tb 11 (‘-ACCAACGATGGTGTGTCCAT) and Tb 12 (CTTGTCGAACCGCATACCCT) primers, as described in the literature (TELENTI et al., 1993). These primers amplified a 439 bp fragment of the *hsp65* gene. PCR was performed with a final volume of 20 μ L containing: enzyme buffer 1X, 1.25 mM of MgCl₂, 0.25 mM of each dNTP, 0.125 μ M of each primer, 2.5 units of *Taq* DNA polymerase, and 5 μ L genomic DNA. The amplification reaction was performed with an initial denaturation at 94°C for 10 min, followed by 45 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72 °C for 1 min. After these cycles, the amplified product was submitted for a final extension at 72 °C for 10 minutes. The products were then analyzed in 1.5% agarose gel and visualized under UV light.

DNA sequencing

The PCR products were purified using a purification kit (Quick Gel Extraction & PCR Purification COMBO Kit-Invitrogen) and were sequenced using the automatic sequencer *ABI-PRIM 3100 Genetic Analyser* by the Sanger method.

The electropherograms were analyzed using PHRED software to check the quality of the sequences. Sequences with PHRED quality scores ≥ 20 (EWING et al., 1998) were analyzed using the nucleotide blast option, nucleotide collection database, of the Basic Local Align Sequence Tool (<<http://blast.ncbi.nlm.nih.gov/Blast.cgi>>) to confirm the species.

Histopathologic examination

Fragments of the lesions were dehydrated in ethylic alcohol at progressively increasing concentration, diaphanized in xylene, and embedded in paraffin blocs. The blocs were later cut into 4 μ m thick blocs using a microtome. Slides were later stained using hematoxylin-eosin (PROPHET et al., 1992) and Ziehl-Neelsen staining.

Results

The distribution of the animals examined at the postmortem inspection showed greater number of abscesses in goats (Table 1). Bacteriologic analysis of abscesses from samples seeded on Lowenstein-Jensen medium revealed that 1.9% (2/106) of the isolated bacterial colony was characterized as acid-alcohol resistant bacillus (AARB) and confirmed as *Mycobacterium* spp. using PCR. The two isolates were from abscesses found in the liver and in the esophagus of a goat and a sheep, respectively. These isolates were observed after 4 weeks growth in Lowenstein-Jensen Medium with sodium pyruvate. The sequencing of these isolates indicated that they belong to *M. novocastrense* species. This analysis showed a total score of 654, e-value of 0.0, and identity of 97%.

Table 1. Distribution of sheep and goats examined at postmortem inspection and absolute and relative frequency of animals with abscesses.

Animals	Number of inspected animals	Animals with abscesses	
		Absolute frequency	Relative frequency (%)
Goats	679	37	5.44
Sheep	1.838	60	3.26
Total	2.517	97	3.85

Corynebacterium pseudotuberculosis was also found in the samples that were isolated and identified as *Mycobacterium* spp., as it was cultivated on blood agar. The microorganisms isolated from blood agar medium are presented in Table 2. A total of 93 out of 106 cultivated specimens were bacteria. Among the isolated species, *C. pseudotuberculosis* represented 59.4% (63/106) of the isolates, followed by *Bacillus* spp. (11.3%), *Pseudomonas* spp. (5.7%), *Staphylococcus* spp. (4.7%), *Micrococcus* spp. (2.8%), *Edwardsiella* spp. (2.8%), and *Shigella* spp. (0.9%).

Molecular analysis of DNA extracted from abscesses revealed that 0.9% (1/106) of the sample was characterized as *Mycobacterium* spp. (the positive sample was from abscess on the ribs region of a sheep). The sequencing of this isolate did not show high quality (Phred value < 20) product and hence could not help in the distinction of the species.

Histopathological analysis of positive specimens in cultivation for Mycobacteria showed areas with calcium salt deposition surrounded by areas of necrosis with large colonies, which is typical of caseous lymphadenitis. Besides this, we could not observe the presence of the alcohol-acid resistant bacillus on Ziehl Neelsen stain.

Discussion

The results show that the relative frequency of abscesses in goats was higher compared to sheep (Table 1). Andrade et al. (2012) found similar results in their study on external abscesses in sheep and goats from other regions of northeastern Brazil.

The identification of animals with abscesses at slaughtering houses causes significant economic loss because of the contamination of carcasses and the lack of sanitary barriers. Besides this, some of the bacteria, which are causative agents of these lesions may present high risk to the public health (COSTA, 2002; GUMI et al., 2012). The high frequency of abscesses in small ruminants bred in the semiarid region of Brazil may be explained by the lack of sanitary handling techniques and by local factors, such as the presence of many plants of the family Cactaceae in the pasturing areas, which may hurt the animal skin (LANGENEGGER et al., 1991; PINHEIRO et al., 2000).

Two abscess samples grown on selective medium revealed the presence of *Mycobacterium* spp., which was also confirmed by PCR. However, PCR of DNA extracted from abscesses showed the presence of another isolate. Previous studies reported low prevalence of *Mycobacterium* spp. on sheep and goat tissues slaughtered in Ethiopia (KASSA et al., 2012).

Bacteriologic analysis and PCR of DNA extracted from abscesses showed contradictory results, as samples that were positive for bacterial growth were later negative at PCR. PCR inhibitors in clinical samples may justify the false-negative results, which were shown using this technique (MICHEL et al., 2010).

Sequencing of *Mycobacterium* spp. positive samples isolated from abscesses showed microorganisms homologous to *M. novocastrense*. The effect of *M. novocastrense* on the health of small ruminants is still unknown. However, *M.*

novocastrense is isolated from granulomatous lesions of humans and confirmed by sequencing (SHOJAEI et al., 1997). The DNA from PCR-positive samples extracted from abscesses did not have PHRED quality and therefore was not used for BLAST alignment; thus, we can only say that it belongs to the genus of *Mycobacterium* spp.

Among other microorganisms identified in bacterial culture, *C. pseudotuberculosis* was found at a higher frequency. Several previous studies have shown the occurrence of caseous lymphadenitis in sheep and goats in Brazil (ANDRADE et al., 2012; PINHEIRO et al., 2000; SEYFFERT et al., 2010; SOUZA et al., 2011). Samples analyzed of infarcted lymph nodes presented prevalence of 74.5% of *C. pseudotuberculosis*, which is similar to our findings (SOUZA et al., 2011). These findings show the relevance of caseous lymphadenitis for the breeding of small ruminants in the semiarid region of Brazil, qualifying it as the main cause of contamination of carcasses and entrails in the region.

The highest frequency of abscesses found at postmortem examination of carcasses was in the lungs (Table 2). Granulomatous lesions in the respiratory tract of small ruminants are common and may be an important source of contamination of the flock through the production of infected aerosol (BAIRD; FONTAINE, 2007; FONTAINE; BAIRD, 2008; MELO et al., 2012). These lesions may also be found in mesenteric lymph nodes, sub mandibular lymph nodes, spleen, tunicas serosas, and utero (BENESI et al., 2008; NEILL et al., 1994).

Histopathologic analyses showed typical features of caseous lymphadenitis. Besides this, we could not observe the presence of the alcohol-acid resistant bacillus on Ziehl Neelsen stain. This confirmed the results of the cultivation in blood

agar, where isolates of *C. pseudotuberculosis* were found as responsive for the macroscopic lesions seen in the postmortem examination of the animals. Histologic lesions with necrotic central area made of concentric layers, with large bacterial colonies and points of mineralization, surrounded by a band of inflammatory infiltrate with macrophage are common in *C. pseudotuberculosis* lesions, but they cannot be considered pathognomonic as they are common in lesions caused by other pyogenic organisms and with tuberculosis (SOUZA et al., 2011).

Conclusion

No species of the *M. tuberculosis* complex were found in abscesses of sheep and goats examined in the northeastern region of Brazil. *M. novocastrense* was detected, but this species does not represent a risk for the health of small ruminants.

The results of the postmortem examination revealed that goats are quite susceptible to infections, causing abscesses in carcasses and entrails, mainly liver and lungs. *C. pseudotuberculosis*, responsible for caseous lymphadenitis in small ruminants, was the main causative agent of the abscesses found in the examined sheep and goats carcasses, stressing out the relevance of the organization of the sanitary inspection system, considering the relevance of these bacteria for public health.

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Table 2. Distribution of microorganisms isolated from abscesses of small ruminants bred in Petrolina (Pernambuco State) and Juazeiro (Bahia State), 2013.

Organs	C. pseudotuberculosis		Bacillus spp.		Staphylococcus spp.		Pseudomonas spp.		Micrococcus spp.		Edwardsiella spp.		Shigella spp.		No isolation		Total	
	N°	%	N°	%	N°	%	N°	%	N°	%	N°	%	N°	%	N°	%	N°	%
Lung	29	74.4	4	10.2	-	0.0	1	2.6	1	2.6	-	0.0	-	0.0	4	10.2	39	36.8
Liver	16	43.2	4	10.8	3	8.1	2	5.4	2	5.4	3	8.1	1	2.7	6	16.2	37	34.9
Trachea	4	66.7	2	33.3	-	0.0	-	0.0	-	0.0	-	0.0	-	0.0	-	0.0	6	5.7
Esophageal lymph node	4	80.0	1	20.0	-	0.0	-	0.0	-	0.0	-	0.0	-	0.0	-	0.0	5	4.7
Mesenteric lymph node	1	20.0	1	20.0	2	20.0	-	0.0	-	0.0	-	0.0	-	0.0	1	20.0	5	4.7
Spleen	3	100	-	0.0	-	0.0	-	0.0	-	0.0	-	0.0	-	0.0	-	0.0	3	2.9
Retropharyngeal lymph node	1	50.0	-	0.0	-	0.0	1	50.0	-	0.0	-	0.0	-	0.0	-	0.0	2	1.9
Diaphragm	1	50.0	-	0.0	-	0.0	-	0.0	-	0.0	-	0.0	-	0.0	1	50.0	2	1.9
Mediastinal lymph node	1	50.0	-	0.0	-	0.0	-	0.0	-	0.0	-	0.0	-	0.0	1	50.0	2	1.9
Ribs	2	100	-	0.0	-	0.0	-	0.0	-	0.0	-	0.0	-	0.0	-	0.0	2	1.9
Uterus	-	0.0	-	0.0	-	0.0	1	100	-	0.0	-	0.0	-	0.0	-	0.0	1	0.94
Kidney	-	0.0	-	0.0	-	0.0	1	100	-	0.0	-	0.0	-	0.0	-	0.0	1	0.94
Marrow	1	100	-	0.0	-	0.0	-	0.0	-	0.0	-	0.0	-	0.0	-	0.0	1	0.94
Total (%)	63	59.4%	12	11.3	5	4.7	6	5.7	3	2.8	3	2.8	1	0.9	13	12.3	106	100

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