The sensitivity of bacterial foodborne pathogens to *Croton blanchetianus* Baill essential oil

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Abstract

The aim of this study was to assess the activity of essential oil extracted from the leaves of *C. blanchetianus* Baill, popularly known as “marmeleiro”, in inhibiting the growth and survival of pathogenic microorganisms in food by determining their survival *in vitro* and by observing the behaviour of *Listeria monocytogenes* inoculated into a food model (meat cubes) that was stored at refrigeration temperature (7 ± 1 °C) for 4 days. The results indicated a bactericidal effect against *Aeromonas hydrophila* and *Listeria monocytogenes* and bacteriostatic action against *Salmonella Enteritidis*. A bacteriostatic effect on meat contaminated with *L. monocytogenes* was found for all concentrations of essential oils tested. These results showed that essential oil from the leaves of *C. blanchetianus* Baill represents an alternative source of potentially natural antimicrobial agents that may be used as a food preservative.

Key words: essential oil, *Croton blanchetianus* Baill, antimicrobial activity, foodborne microorganisms.

Introduction

Food preservation techniques are used to provide food with high nutritional quality and microbial stability, which is achieved by controlling the growth/survival of spoilage-associated and foodborne microorganisms (Baydar *et al.*, 2004; Benkbilia, 2004). Historically, several physical and/or chemical procedures have been used to ensure the microbiological safety of foods (Daferera *et al.*, 2003; Marino *et al.*, 2001). Recently, the use of chemical food preservatives has been questioned, particularly by consumers who demand more natural foods (Mendoza-Yepez *et al.*, 1997; Radhakrishnan-Sridhar *et al.*, 2003). More than 1,340 plants are known as potential sources of antimicrobial compounds, but very few of these have been studied scientifically (Seidil, 2000).

This scenario has encouraged research aimed at the possible development and use of vegetable products with antimicrobial properties (Souza *et al.*, 2005; Valero and Salmerón, 2003). Species from the genus *Croton* are frequently used in popular medicine (as infusions, teas and poultices) to relieve pain (Abreu *et al.*, 2001), constipation, diarrhea and other digestive symptoms, diabetes, wounds, inflammation, fever, and hypertension (Salatino *et al.*, 2007). Studies conducted in certain species have demonstrated several pharmacological properties of plants within this genus, such as antidiabetic (Barbosa-Filho *et al.*, 2005), anti-inflammatory, antiulcerogenic, analgesic and...
anti-hypertensive activities (Palmeira-Junior et al., 2006), among others. *C. blanchetianus* Baill (synonym *Croton sonderianus* Müll. Arg.) (Govaerts et al., 2000), known as “black marmeleiro”, is a widely disseminated shrub found in northeast Brazil. Its leaves and bark are used as popular medicines for treating gastrointestinal disturbances, rheumatism and cephalalgia (Chaves and Reinhard, 2003). It has a high essential oil content, and its yield may range from 0.5% to 1.5% (Chaves and Reinhard, 2003). Furthermore, this plant is rich in diterpenes with different biological activities, including anti-inflammatory, gastroprotective and antimicrobial properties (Marino et al., 2001).

The aim of this study was to evaluate the efficacy of essential oil from the leaves of *C. blanchetianus* Baill in inhibiting the growth of gram-positive and gram-negative pathogenic bacteria and to analyse the antibacterial effectiveness of *C. blanchetianus* Baill essential oil in meat cubes stored under refrigeration.

**Materials and Methods**

**Essential oil**

Fresh leaves of *C. blanchetianus* were collected in the rural municipality of Patos (7°05’10” S, 37°15’45” W), located in the semi-arid central region of Paraíba, Brazil, from February-March 2011. The species was identified by comparison with a herbarium specimen, and a dried sample (no. 1462) was deposited in the Brazil Herbarium, Federal University of Campina Grande, Patos, Paraíba.

Fresh leaves (150 g) were submitted to hydrodistillation for 3 h using a Clevenger type apparatus according to the method recommended in the European Pharmacopoeia (2004). The obtained oils were allowed to dry over anhydrous sodium sulphate. The oils were filtered and then stored at 4 °C prior to testing and analysis. The yield of the oil was 0.7%. The essential oil was tested at concentrations ranging from 80-0.6 µg.mL⁻¹. Essential oil solutions were prepared in Mueller Hinton broth (MH) (HiMed®, Mumbai, India) using bacteriological agar (0.15 g.100 g⁻¹) as a stabilising agent (Bennis et al., 2004; Mann and Markham, 1998).

**Bacterial strains**

Strains of *Aeromonas hydrophila* INCQS 7966, *Listeria monocytogenes* ATCC 7644 and *Salmonella* Enteritidis CDC 49812 were used as test microorganisms. Inocula used in antimicrobial assays were obtained from overnight cultures grown on MH agar slants at an optimum growth temperature (28 °C for *A. hydrophila* and 37 °C for the other bacteria). A loopful of the culture was diluted in sterile saline solution (0.85 g.100 mL⁻¹) to a final concentration of approximately 10⁶ colony-forming units per mL (cfu.mL⁻¹) adjusted according to the turbidity of 0.5 McFarland standards. The final concentration of the inoculum in the medium used for the antimicrobial assays was approximately 10⁷ cfu.mL⁻¹.

**Tests of cell viability**

Our previous experiments with *C. blanchetianus* Baill essential oil against the tested pathogens using disc diffusion revealed clearance zones of 10-12 mm and minimum inhibitory concentrations (MICs) of 20 µL.mL⁻¹, 1.25 µL.mL⁻¹ and 40 µL.mL⁻¹ for *Aeromonas hydrophila*, *Listeria monocytogenes* and *Salmonella* Enteritidis, respectively (unpublished data). Cell counts were performed to test cell viability at concentrations of MIC/2, MIC and 2x MIC. For this purpose, 5 mL of double concentrated MH broth was inoculated with 1 mL of bacterial suspension. Next, 4 mL of *C. blanchetianus* Baill essential oil solution was added to obtain an appropriate final concentration, and the system was incubated at 37 °C under aerobicism. At intervals of 0 min., 30 min, 1 h, 2 h, 4 h, 6 h, 8 h and 10 h after incubation, 1.0 mL aliquots of the suspension were diluted (10⁻¹-10⁻⁸) in sterile peptone water and uniformly seeded onto Petri plates containing MH agar using the plate spreading technique. After the incubation period, the viable cells were counted, and the resulting value was expressed in log cfu.mL⁻¹. Control samples without essential oil were tested in parallel (Barros et al., 2009).

**Antimicrobial activity in a food model**

Bovine meat knuckles were trimmed of all external fat and cut into pieces of uniform sizes (3x3x3 cm). The meat pieces were placed in glass flasks and sterilised using an autoclave (121 °C for 15 min, 121 atm). Then, meat pieces were inoculated with a microbial suspension by individual submersion in 50 mL of the bacterial inoculum (*L. monocytogenes* containing approximately 10⁸ cfu.mL⁻¹, prepared in sterile 0.85 g.100 mL⁻¹ saline solution) with rotation with a sterile glass rod for 1 min to ensure even inoculation. The inoculated pieces were air dried for 30 min in a bio-safety cabinet and finally washed with the essential oil (Oliveira et al., 2010). The inoculated pieces were randomly divided into five groups and treated for 30 s (1:4 w/v) as follows: (I) control, dipped in sterile distilled water; (II) dipped in 1x MIC essential oil solution; (III) dipped in 2x MIC essential oil solution; (IV) dipped in 5x MIC essential oil solution; and (V) dipped in 10x MIC essential oil solution. The pieces were then placed in sterile sealed polypropylene cups and stored under refrigeration (7 °C ± 1 °C). At 0, 24, 48, 72 and 96 h of storage, the meat samples were submitted to an *L. monocytogenes* count according to the procedure described by AOAC (1995). The results were presented in log cfu per gram of meat (log cfu.g⁻¹ of meat) (Oliveira et al., 2010). All antimicrobial assays were carried out in triplicate, and the results are expressed as an average of the three parallel assays.

**Statistical analysis**

Statistical analysis was performed to determine the significance of differences between groups (p < 0.05) using
the Tukey test to estimate the time of inactivation. Statistica software version 7.0 (StatSoft Inc., USA) was used for the data analysis.

**Results and Discussion**

Figures 1, 2 and 3 show the effects of MIC/2, MIC and 2xMIC of *C. blanchetianus* Bail essential oil on the viability of the bacteria of interest in food. The survival curve is presented as a way to measure the capacity of a compound to act on the viability of a microorganism. Furthermore, it may be inferred that the estimated mortality of a microbial population (determined by the number of viable cells on the plates) at a given concentration of an antimicrobial compound reflects the speed of the bactericidal effect or the duration of a bacteriostatic effect (Burt, 2004).

Three bacterial strains were selected for this test (*Aeromonas hydrophila*, *Listeria monocytogenes* and *Salmonella Enteritidis*), and the inhibition results of antimicrobial tests were used as the inclusion criteria. All of the growth curves obtained showed different activities for the different values of MIC. MIC and 2x MIC exhibited statistically significant inhibition of pathogen growth (p < 0.05) compared with the control sample.

Figure 1 shows that the microbial population size found at the last time interval analysed (10 hours) was approximately $10^3$ cfu.mL$^{-1}$, representing a reduction of 3-4 orders of magnitude in the number of viable cells relative to the initial inoculum. In turn, the microbial population size of the control sample was between $10^9$ and $10^{10}$ cfu.mL$^{-1}$ at time interval of 10 h, an approximately 100- to 1000-fold increase from the value of the initial inoculum. MIC was more effective in inhibiting the growth of bacterial strains after 8 hours of interaction, MIC/2 after at least 10 hours and 2x MIC after only 6 hours. A compound is recognised as having a strong bactericidal effect when it is capable of causing a 1000-fold reduction (3 orders of magnitude, or 99.9%) of the initial inoculum (LaPlante, 2007).

Figure 2 shows evidence of the bacteriostatic action (reduction of growth rate) of *C. blanchetianus* Bail essential oil against the bacterial strain studied. This bacteriostatic action was evident throughout the entire period of interaction (10 hours). The bacteriostatic activity is characterised by the ability of a substance to render a bacterium incapable of growing/multiplying in the broth but still capable of growth when removed from the broth and plated on appropriate agar (Smith-Palmer et al., 1998).

Some foodborne pathogens may show an initial exponential decrease in growth capacity followed by a subsequent increase in antimicrobial resistance upon exposure to compounds with antimicrobial properties (Rowan, 1999). This phenomenon occurs when the resistant microbial cells that represent only a small proportion of the initial microbial population are selected for and ultimately contribute a greater proportion of the total final population (Rowan, 1999).

Evidence that this phenomenon occurred in our experiments may be observed in Figure 2, in which an exponential reduction of the microbial population is observed from 0-8 hours of interaction, followed by a smaller reduction effect and an ascending growth curve up to 10 hours of interaction.

Figure 3 demonstrates a strong bactericidal effect in that the microbial population found in the last time interval analysed (10 hours) present values of approximately $10^3$ cfu.mL$^{-1}$, a decrease of 3-5 orders of magnitude in the number of viable cells relative to the initial inoculum. In turn, the control sample showed a microbial population of between $10^9$ and $10^8$ cfu.mL$^{-1}$ at 10 h, that is, an increase of

![Figure 1](image1.png)  
**Figure 1** - Effect of the *Croton blanchetianus* Bail essential oil on the cell viability of *A. hydrophila*: (■) control (0 µg mL$^{-1}$); (●) MIC/2 (10 µg.mL$^{-1}$); (▲) MIC (20 µg.mL$^{-1}$); (▼) 2x MIC (40 µg.mL$^{-1}$) of essential oil.

![Figure 2](image2.png)  
**Figure 2** - Effect of the *Croton blanchetianus* Bail essential oil on the cell viability of *Salmonella Enteritidis*: (■) control (0 µg mL$^{-1}$); (●) MIC/2 (20 µg.mL$^{-1}$); (▲) MIC (40 µg.mL$^{-1}$); (▼) 2x MIC (80 µg.mL$^{-1}$) of essential oil.
approximately 10 to 100 times the value found in the initial inoculum. The greatest efficacy of essential oil in inhibiting the growth of bacterial strains was observed after 4 hours of exposure to the oil in 2.5 μg.mL⁻¹, and 6 hours in 0.6 and 1.25 μg.mL⁻¹ of essential oil. Thus, a significant reduction (p < 0.05) in bacteria count was promoted in comparison with the control sample. We also observed a prolonged lag phase (stationary phase) of the bacterial growth curve. The lag phase is typically considered an indicator of the time required to adapt to a new environment; therefore, the longer the lag phase, the more difficult it is for the microorganism to adapt to the new environment and establish exponential growth (Souza et al., 2005).

Even though a satisfactory antimicrobial efficacy was found for the MIC of C. blanchetianus Baill essential oil, particularly against L. monocytogenes. Figure 4 shows when applied to food model, it did not decrease the number of microbes relative to the initial inoculum, as observed in vitro; however, there was a bacteriostatic effect at all concentrations tested. Growth inhibition was more effective at MIC and 10x MIC, which maintained the initial contamination level of 10⁵ cfu.mL⁻¹ until the end of 4 days.

It must be noted that higher concentrations of essential oil are generally needed in food to achieve antimicrobial effectiveness similar to that obtained in laboratory experiments. In studies with food models, the required concentration values were two times higher in semi-skim milk (Karatzas et al., 2001), ten times in pork sausages (Pandit and Shelef, 1994), fifty times in a soup (Ultee and Smid, 2001) and twenty-five to one hundred times in cheese (Mendoza-Yepez et al., 1997). Our results showed that the effective inhibition of Listeria monocytogenes growth in meat required a concentration ten times higher than that used in the in vitro experiment.

The mechanism responsible for the lower antimicrobial effectiveness of essential oils when used in foods is still not well established (Gil et al., 2002). It has been suggested that the great variety of nutrients available in foods compared with culture medium could allow the microbial cell to recover more quickly from cell damage (Gil et al., 2002). It is also possible that the essential oil is dissolved in the lipid phase of the food, making it less capable of acting against the microorganisms present in the aqueous phase (Mjelholm and Dalgaard, 2002).

In food model systems, both the intrinsic properties of the food and extrinsic determinants may influence the antimicrobial effectiveness of essential oils. According to reports from Gutierrez, Barry-Ryan and Bourke (Gutierrez et al., 2008; Gutierrez et al., 2009), various essential oils applied alone or in combination were more effective against pathogenic bacteria when applied in a medium with high protein content and high acidity, low levels of fats and carbohydrates, and moderate levels of simple sugars. Therefore, the Figure 3 demonstrates the model meat food used in the present experiment should not have interfered substantially in the antimicrobial effectiveness of the essential oil. Parallel results suggested that a longer period of exposure of the microorganism to the essential oil might be necessary to obtain a better understanding of the antibacterial kinetics.

**Conclusion**

It was concluded that the essential oil of C. blanchetianus Baill is effective as an antimicrobial agent in vitro; however, in a model system using fresh meat, its efficacy was reduced, and consequently a higher concentration of essential oil was required to inhibit bacterial growth. A
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major challenge for the practical application of essential oils as food preservatives is to optimise the use of essential oil at low concentrations in conjunction with other conservation techniques that enable food security and longer shelf lives.

Acknowledgments

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Isolation of *Actinobacillus seminis* from a goat with clinical epididymo-orchitis in Brazil

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Abstract

The present study reports the first isolation of *Actinobacillus seminis* from a goat in Brazil. A four-year-old Moxotó breeding goat in a flock of 70 goats and 65 sheep reared together in the county of Patos, semiarid region of Northeastern Brazil, showed clinical signs of unilateral orchitis and epididymitis. Diagnosis of *A. seminis* infection was confirmed by association of clinical findings, bacterial isolation and 16S rRNA gene sequencing. This result suggests that *A. seminis* may be an additional cause of infertility in goats, and that sheep may be the source of infection because the mixed farming system allows the contact between sheep and goats in the semiarid region of Northeastern Brazil.

Key words: epididymitis, goats, *Actinobacillus seminis*, isolation.

Introduction

Goat breeding has great economic importance in many countries, including Brazil. However, several factors negatively affect goat breeding including nutritional and health issues, and infectious diseases (Pinheiro et al., 2000).

It is documented that ovine epididymitis due to *A. seminis* is one of the major cause of economic losses as it reduces and interferes with the fertility of infected ram. Its pathogenesis is uncertain, but it is suggested that *A. seminis* may ascend from the preputial cavity and colonizes the genital tract, causing the clinical signs, and that predisposing factors such as stress induced by hormone changes, as well as nutritional deficiencies can cause the development of orchitis and epididymitis, particularly in young sheep (Hajtós et al., 1987; Dibarrat et al., 2006).

The first isolation of *A. seminis* was reported by Baynes and Simmons (1960), in Australia, in semen of rams with epididymitis. Then, the bacterium was isolated in several occasions in different countries: United States of America (Livingston and Hardy, 1964), South Africa (Worthington and Bosman, 1968), New Zealand (Bruere et al., 1977), Hungary (Hajtós et al., 1987), Argentine (Robles et al., 1990), Brazil (Gomes et al., 2001; Gregory et al., 2009; Bezerra et al., 2012), United Kingdom (Heath et al., 1991), Spain (Puente-Redondo et al., 2000), Turkey (Diker et al., 1991), Kenya (Mbai et al., 1996) and Mexico (Narez et al., 1999). Interestingly, it was reported that *A. seminis* had been isolated from bulls and goat in South Africa (Van Tonder and Bolton, 1970; Van Tonder, 1973) and from cattle in New Zealand (Dixon et al., 1983).

This paper documents the first isolation of the microorganism from a goat with clinical signs of epididymitis and orchitis in Brazil.

Materials and Methods

Field observation and clinical signs

During a visit in a farm in the county of Patos, State of Paraíba, semiarid region of Brazil to investigate the reasons of reduced fertility, where there were 135 animals, 70 goats and 65 sheep reared, a four-year-old Moxotó breeding goat...
was found to have clinical signs of unilateral orchitis and epididymitis on the right side (Figure 1). Clinical examination of the area showed firm consistency of the epididymis and testis, with absence of ulceration or nodularity, sensitivity to palpation and adhesions of the scrotum.

Serological examination
Blood samples were collected from all animals by jugular vein puncture. Agar gel immunodiffusion (AGID) test was used for the detection of anti-B. ovis antibodies with commercial kits produced by the Institute of Technology of Paraná (TECPAR, Curitiba, Paraná, Brazil), using lipopolysaccharide antigen and proteins of B. ovis strain Reo 198. Buffered Acidified Plate Antigen Test (BAPA) was used for serodiagnosis of B. abortus using an inactivated B. abortus strain 1119-3 antigen, produced by the Institute of Technology of Paraná (TECPAR, Curitiba, Paraná, Brazil), at the cellular concentration of 4% and Rose Bengal stained (OIE, 2008).

Bacteriological examination
Weekly samples of semen were collected from the goat diagnosed with epididymitis and orchitis using electroejaculation for three successive weeks. Fine needle aspirates of a caseous and white to yellow material of the testis and the tail of the epididymis were also performed by aspiration with disposable syringes and needles. The samples were plated on blood agar and Brucella agar (Difco, Franklin Lakes, NJ, USA) enriched with defibrinated sheep blood at a concentration of 5% of the total volume. All samples were incubated at 37 °C in an atmosphere containing 10% of carbon dioxide (CO₂). Cultures were examined daily for five days for visible growth. The bacteria isolated were subjected to Gram staining and biochemical tests (catalase, oxidase, nitrate and esculin; and acid production from maltose, xylose, galactose and trehalose) according to Krieg and Holt (1984).

Molecular examination
Semen, aspirates and isolated bacteria samples were subjected to DNA extraction according to the manufacturer’s protocol using the commercial kit “Qiagen DNeasy® Blood and Tissue Kit” (Qiagen, Austin, TX, USA).

Polimerase Chain Reaction (PCR) technique for Brucella spp.
Specific primers directed to the 16S-23S ribosomal RNA (rRNA) interspace region of Brucella spp. (ITS66: ACATAGATCGAGGCCCAGTCA and ITS279: AGATACCGACGAAAACGCTAC) (Keid et al., 2007) were used, which amplify with 214 bp. The amplification reaction mixture was prepared in a volume of 12.5 μL containing 200 μM of each deoxynucleoside triphosphate, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5 mM MgCl₂, 0.5 mM of each primer, 1.5 U platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) and 5 μL of template DNA.

The reaction was performed in a DNA thermal cycler (MJ Research PTC 200 DNA engine, Watertown, MA, USA) without mineral oil. Ultrapure water was used as negative control while B. ovis strain 63/290 served as positive control. After an initial denaturation at 95 °C for 2 min, the PCR profile was set as follows: 30 s of template denaturation at 95 °C, 30 s of primer annealing at 62 °C and 30 s of primer extension at 72 °C, for a total of 40 cycles, with a final extension at 72 °C for 5 min.

PCR technique for A. seminis
SRJAS1 (CTTATCTTTCTTAAGCCCTGAC) and SRJAS2 (AAGAAAGACGAGAGACATT) primers were used, which amplify the 16S rRNA gene with 436 bp (Appuhamy et al., 1998b). Amplification reactions were performed in a final volume of 25 μL containing 2.5 μL of PCR buffering solution (100 mM Tris-HCL, 15 mM MgCl₂, 500 mM KCl, pH 8.3), 2 μL (0.2 μM) of each dNTP, 1.0 μL (50 pM) of each primer, 0.125 μL (0.625 U) of Taq polymerase (Qiagen, Austin, TX, USA), 2.5 μL of genomic DNA and 16.87 μL of Milli-Q ultrapure water, according to the supplier’s protocol.

The thermal profile of the stages of reactions was measured on Thermal Cycler (Bioer Technology CO LTD, Hangzhou, China), consisting of an initial denaturation of DNA at 94 °C (1 min) and followed by 35 cycles at 94 °C for 30 s for denaturation, 55 °C for 30 s for annealing, 72 °C for 6 min for extension and final extension of 1 min at 72 °C, according to Appuhamy et al. (1998a).

Amplified products were analyzed by electrophoresis in a 2% agarose gel and then stained with ethidium bromide (0.5 mg/mL). The DNA bands were visualized under UV light.

DNA sequencing
Sequencing reactions were performed using the kit “The BigDye® Terminator v3.1 Cycle Sequencing” (Applied Biosystems, Foster City, CA, USA) and polymerization conditions were performed in 96 well plates according to manufacturer’s instructions. Samples were sequenced by the dideoxy terminator method in automatic in ABI PRISM.

Figure 1 - Four-year-old Moxotó breeding goat with clinical signs of unilateral orchitis and epididymitis.
Results

Serological findings

All animals were found to be negative (100%) for B. abortus by BAPA test, while five sheep (n = 5, 8%) and nine goats (n = 9, 13%) including the diseased goat were found to be positive for B. ovis by IDGA.

Bacteriological findings

Small smooth, shining and non-pigmented colonies (1 to 2 mm) were isolated from semen samples. They approved to be non-motile, Gram negative coccobacilli. Biochemically, isolated organism was catalase, oxidase, nitrate and esculin positive. It produces acid from maltose and xylose but not from galactose and trehalose.

PCR findings

PCR was negative for Brucella spp. However, A. seminis DNA was amplified from weekly-collected semen samples and isolated colonies (isolate SAAS01). Sequence of PCR products from semen samples and colonies that revealed a DNA fragment of a standard size showed 99% similarity with the region of the 16S rRNA gene of A. seminis.

Discussion

Positive sequencing following isolation of A. seminis in goats is the first report of this kind in Brazil. A fact that deserves attention concerns the mixed farming system of goats and sheep in Brazil. The isolation of A. seminis from a Boer goat, bull semen, and from an Angora goat, an Afrikaner and Friesland bull raised the wide range of host involvement and the role of both sheep and goat in the dissemination of actinobacillosis (Al-Katib and Dennis, 2005) and indicate that these species may serve as sources of infection for A. seminis and thus aid in the spread and transmission of infection when they are in contact with rams under intensive systems (Al-Katib and Dennis, 2009).

Clinical and pathological findings of the epididymitis and orchitis in the tested goat were properly similar to those described by others (Bezerra et al., 2012). The signs of infection by A. seminis deserve special consideration because the effects are not noticeable or measurable (Gomes et al., 2001), particularly in extensive breeding systems or when farmers are not aware of the economic importance of the disease (Bezerra et al., 2012). It should be stressed that the clinical changes observed are usually associated with low concentration, low motility and non viability of sperms, besides the presence of neutrophils in the semen, which affect the fertility rate of breeders, and in the regions where the disease has not been previously diagnosed, economic losses may be even greater (Baynes and Simmons, 1960; Van Tonder, 1973; Bezerra et al., 2012).

Our findings indicated that serological tests did not yield satisfactory results. In this study, positive AGID in the goat raises the possible simultaneous presence of B. ovis seropositive animal when infected with A. seminis, which should be subject of further investigations. This in contrast with those results reported by Gomes et al. (2001) and Gregory et al. (2009) that isolated A. seminis in sheep, which were AGID-negative for B. ovis.

In the present study, the isolation of A. seminis from a goat would suggest that infection be disseminated in adult breeding goats, which makes control difficult, because of the continued exchange of breeding goats between the farmers and their commercialization without proper healthcare.

The differential diagnosis of orchitis and epididymitis must be based on clinical and pathological diagnosis, the isolation and identification of the causative agent (Spönenberg et al., 1983). Our findings indicated that the morphological, and biochemical characteristics allowed its identification as A. seminis.

It is concluded that A. seminis should be considered as differential diagnosis in cases of epididymitis in goats particularly in the Northeastern Brazil where the mixed farming system is widely adopted, and the agent has already been isolated in sheep.

References


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PITIOSE CUTÂNEA CANINA – RELATO DE CASO

CANINE CUTANEOUS PYTHIOSIS - CASE REPORT

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RESUMO
A pitiose é uma enfermidade granulomatosa crônica, principalmente do tecido subcutâneo, causada pelo Oomiceto Pythium insidiosum que acomete humanos e animais. A espécie canina é a segunda dentre as espécies mais acometidas, sendo a manifestação clínica cutânea a forma menos comum. O sucesso da terapia é determinado pelo diagnóstico precoce da doença. A composição da parede celular torna os tradicionais fármacos antifúngicos ineficientes. Atualmente a imunoterapia se tornou uma alternativa terapêutica em potencial, entretanto, a excisão cirúrgica ainda é o principal meio de controle. Descreve-se um caso de pitiose em um cão da raça Pastor Alemão oriundo de área rural, enfocando características clínicas, aspectos histopatológicos e conduta terapêutica, onde pode-se ressaltar que a pitiose cutânea trata-se de um importante diagnóstico diferencial dentre as dermatopatias piogranulomatosas que acomete cães, principalmente naqueles oriundos de zona rural onde possuem acesso a açudes ou áreas alagadas. De modo geral, o conhecimento do ciclo epidemiológico do P. insidiosum aliados a exames complementares como a histopatologia e imunohistoquímica, são ferramentas imprescindíveis em sua definição diagnóstica.


SUMMARY
Pythiosis is a chronic granulomatous disease, especially in the subcutaneous tissue caused by the oomycete Pythium insidiosum that affects humans and animals. The canine species is the second among the most affected species, and the clinical cutaneous is the least common form. The success of therapy is determined by early diagnosis. The composition of the cell wall becomes ineffective traditional antifungal drugs. Currently immunotherapy has become a potential therapeutic alternative, however, surgical excision remains the primary mean of control. We describe a case of pythiosis in a German Shepherd dog come from rural area, focusing on clinical and pathologic features, as well as therapeutic management, where it might be noted that cutaneous pythiosis is an important differential diagnosis among pyogranulomatous skin diseases affecting dogs, especially those coming from rural areas where there might be free access to ponds or wetlands. In general, knowledge of the epidemiological cycle of P. insidiosum combined with tests such as histopathology and immunohistochemistry, are essential tools for its diagnosis.


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A pitiose é uma doença granulomatosa que atinge equinos, caninos, bovinos, felinos e humanos, ocorrente em áreas tropicais, subtropicais ou mesmo temperadas (MEIRELES et al., 1993; MENDOZA et al., 1993), causada pelo Oomiceto *Pythium insidiosum* (DE COCK et al., 1987). A espécie equina é a mais afetada, principalmente nas formas cutânea e subcutânea, seguida da canina (MENDOZA et al., 1996).

Não há predisposição por sexo, idade ou raça, obstante considera-se que caninos de maior porte são mais susceptíveis. A fonte de infecção são os zoósporos móveis aquáticos, inexistindo relatos de transmissão antropo ou zooantropozoonótica (MENDOZA et al., 1996). As condições ambientais são fundamentais para o desenvolvimento do organismo no meio ambiente. Para a produção de zoósporos são necessárias temperaturas entre 30 e 40°C e o acúmulo de água tépida ou quente em banhados e lagoas (MILLER & CAMPBELL et al., 1982).

Os caninos podem apresentar a forma cutânea e gastrointestinal, sendo esta última a mais comum, caracterizada por distúrbios digestivos como êmese, anorexia crônica, emaciação, diarréia, por vezes sanguinolentas e presença de massas nodulares, quando submetidos à palpação abdominal (FISCHER et al., 1994). A forma cutânea é caracterizada por lesões crônicas, ulceradas, nodulares, com várias extensões de drenagem, podendo acometer qualquer parte do corpo (DYKSTRA, 1999). A histopatologia, apresentam-se como dermatite piogranulomatosa ulcerativa, contendo áreas de necrose infiltrada por neutrófilos e macrófagos e granulomas eosinofílicos (FOIL et al., 1984; HOWERTH et al., 1989).

Tradicionalmente, o diagnóstico da pitiose baseava-se nas características clínicas, histopatológicas, no isolamento e na identificação do agente através de suas características culturais, morfológicas e reprodutivas. A identificação precoce da doença, no entanto, torna-se difícil por meio desses métodos. O diagnóstico diferencial deve incluir habronemose, neoplasia, quadros clínicos de drenagem, podendo acometer qualquer parte do corpo (DYKSTRA, 1999). À histopatologia, apresentam-se como dermatite piogranulomatosa ulcerativa, contendo áreas de necrose infiltrada por neutrófilos e macrófagos e granulomas eosinofílicos (FOIL et al., 1984; HOWERTH et al., 1989).

O tratamento de infecções pelo *P. insidiosum* em animais e humanos é complicado devido às características do agente, sobretudo de sua composição de parede celular, uma vez que os fungos verdadeiros possuem quitina em sua parede, enquanto o *P. insidiosum* contém célulose, β-gluconanos e não possuem ergosterol, que é o componente alvo de ação da maioria das drogas, tornando os fármacos antifúngicos tradicionais ineficientes contra o *P. insidiosum* (SATHAPATAYAVONGS et al., 1989; FOIL, 1996).

O sucesso das outras formas de tratamento é variável, sendo influenciado pelo tamanho e duração da lesão, idade e estado nutricional do animal (MILLER, 1981). Em geral, o tratamento cirúrgico apresenta bons resultados apenas em lesões pequenas e superficiais, nas quais seja possível a retirada de toda área afetada (LEAL et al., 2001). Uma alternativa para o tratamento da pitiose equina é o protocolo imunobiológico (imunoterápico) a partir de culturas do próprio agente (hifas sonificadas). O índice de eficiência obtido na imunoterapia varia de 53% a 75% quando associado à cirurgia (MILLER, 1981; MILLER & CAMPBELL et al., 1982). Portanto, descreve-se aqui caso de pitiose cutânea em um cão da raça Pastor Alemão originário de zona rural, enfocando características clínicas, aspectos histopatológicos e conduta terapêutica.

Um cão da raça Pastor Alemão, macho com um ano de idade foi atendido com queixa principal de lesões pruriginosas na cauda, de tal intensidade ao ponto de desenvolver automutilação, há cerca de quatro meses. Foi reportado que o animal era oriundo de uma propriedade rural, com regime de criação semi-domiciliar e periodicamente tinha acesso a um açude de vegetação aquática não bem caracterizada.

Ao exame físico, o animal apresentava-se em bom estado geral, parâmetros fisiológicos (temperatura retal e frequências cardiorrespiratória) nos limites de referência para a espécie (FEITOSO, 2008), mucosas visíveis normais e linfonodos poplíteos aumentados. Na cauda, foi observada alopecia que se estendia da base da cauda até aproximadamente a 8ª vértebra coxigea, com lesões cutâneas nodulares ulceradas e exsudativas de bordos circulares e irregulares com diâmetros que variaram entre 0,5cm a 3,5cm de diâmetro (exsudato sanguinolento e mucopurulento), tumefação intensa e focos de necrose (Figura 1).

O hemograma revelou apenas leucocitose (20.750 leuc/mm³) com neutrófilia (14.300 neu/mm³) e eosinofilia (1.452 eos/mm³), relativo aos parâmetros sanguíneos para a espécie canina (GARCIA-NAVARRO & PACHALLY, 1994). A citologia das lesões corando-se pelo método de Panótico revelou a presença de neutrófilos segmentados e eosinófilos. Alguns neutrófilos encontravam-se degenerados, havia presença de hemácias e de alguns macrófagos em pleno processo de fagocitose com restos celulares. Na histopatologia cutânea foi evidenciado pela coloração de hematoxilina e eosina, intenso infiltrado inflamatório polimorfonuclear representado por neutrófilos integrados e degenerados, plasmócitos e histiócitos permeando a derme superficial e profunda, alcançando o subcutâneo, coexistindo granulomas multifocais, que era compatível com dermatite piogranulomatosa. Havia áreas de necrose com infiltrado de neutrófilos e macrófagos e granulomas eosinofílicos. A coloração convencional foi auxiliada por colorações imunohistoquímicas e imunofluorescentes específicas, que permitiram identificação de anticorpos específicos para *P. insidiosum*.

Antes mesmo do resultado histopatológico, ainda sob suspeita clínica inicial de pitiose cutânea, foi prescrito itraconazol (5mg/kg/BID/VO), cefalexina (30mg/kg/BID/VO) e meloxicam (0,1mg/kg/SID/VO) até o estabelecimento diagnóstico (aproximadamente 10 dias), entretanto, não foi observada resposta...

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satisfatória. Logo, mediante a definição histopatológica do quadro de pitiose, foi proposta a excisão cirúrgica, sendo o animal submetido a uma caudectomia total. Além da terapia pós-operatória antimicrobiana, antiinflamatória e analgésica, a conduta terapêutica antifúngica foi mantida após procedimento durante 60 dias, sendo o animal reavaliado clinicamente e por histopatologia aos 30 e 60 dias, onde não apresentava mais sinais de infecção por *P. insidiosum* nas proximidades do sítio cirúrgico.

A pitiose canina é uma enfermidade com distribuição mundial, de caráter crônico/progressivo, potencialmente fatal na maioria dos casos e está diretamente relacionada à exposição do cão ao ciclo de vida do *P. insidiosum*, onde os zoósporos livres encistam e emitem o tubo germinativo, dando origem a um novo micélio e, assim, completando o seu ciclo no animal (MILLER, 1983).

Os aspectos ambientais parcialmente descritos neste relato que incluíam o cão no ciclo de colonização e desenvolvimento do *P. insidiosum* se enquadram nas condições descritas por outros autores, onde os cães afetados são, normalmente, oriundos de regiões rurais ou tiveram eventual acesso a lugares alagados (FOIL et al., 1984). A grande maioria dos casos de pitiose é observada durante ou após a estação chuvosa. Baseado em dados clínico-epidemiológicos, acredita-se na existência de um período de incubação de várias semanas (LEAL et al., 2001).

Cães infectados por *P. insidiosum*, na maioria das ocasiões, manifestam lesões gastrointestinais ou cutâneas, sendo a forma gastrointestinal a mais comum e pouco frequente a forma cutânea isoladamente (MILLER, 1983; SMITH et al., 1989; FISCHER et al., 1994). É rara a ocorrência associada das apresentações clínicas em um mesmo animal (GROOTERS et al., 2003). Entretanto, em dois casos descritos no Brasil, as duas manifestações clínicas em um mesmo cão foram relatadas (NONNEMACHER et al., 2009; RECH et al., 2004).

**Figura 1**: 1A – Cão da Raça Pastor Alemão apresentando lesão em cauda; 1B – Lesões com inflamação e ulceração cutânea com necrose multifocal (superfície direita); 1C – Lesões ulcerativas com presença de exsudato serosanguinolento e/ou muco-sanguinolento; superfície cutânea com aspecto friável (superfície esquerda).
Figura 2 - Fragmento de pele de cão com Pitiose cutânea: 2A – Padrão granulomatoso, Hematoxilina e eosina (100x); 2B - Hifas evidenciadas pela coloração prata pela técnica de Grocott (400x); 2C – Presença de estruturas de coloração marrom amarelado (imunomarcados), indicando hifas e fragmentos fúngicos de P. insidiosum. Técnica de estreptoavidina-biotina-peroxidase (400x).

O primeiro caso de pitiose canina relatado no Brasil data de 1997, no Estado de São Paulo envolvendo fêmea com lesões cutâneas no membro pélvico esquerdo (LARSSON et al., 1997).

Os casos de pitiose cutânea geralmente acometem cães de grande porte, de um a três anos de idade (FOIL et al., 1984; DYKSTRA et al., 1999). As lesões em geral são variavelmente pruriginosas (DYKSTRA et al., 1999) e progredem rapidamente mesmo quando sob tratamento com fármacos dotados de ação antifúngica, antibiótica ou excisão cirúrgica (FOIL et al., 1984). O quadro prurítico e de automutilação observado neste caso é uma manifestação rara segundo Foil et al. (1984), podendo essa condição estar relacionada ao quadro de piodermite profunda secundária instalado, conduzindo ao estímulo periférico do prurido (GNIRS E PRÉLAUD, 2005). Os achados clínicos-patológicos do cão objeto deste relato foram similares àqueles descritos por outros autores, onde as lesões frequentemente têm evolução de um a três meses e consistem de nódulos, únicos ou múltiplos, ulcerados, de um a oito cm de diâmetro (DYKSTRA et al., 1999), ou ainda em massas ulceradas de até 30 cm de extensão, que envolvem tanto a pele como o pânico (FOIL et al., 1984; HOWERTH et al., 1989). Independentemente do tamanho, as lesões contêm tratos fistulosos que drenam exsudato serosanguinolento ou purulento (BENTINCK-SMITH et al., 1989; DYKSTRA et al., 1999; HENSEL et al., 2003). Em casos mais avançados, como o deste relato, a massa pode envolver, além da derme, a lipoderme. Parte desse envolvimento se deve à proliferação de tecido de granulação junto com áreas de necrose (BENTINCK-SMITH et al., 1989). Na histopatologia, o padrão de inlação mais comum pela coloração de HE, é de dermatite ulcerativa e piogranulomatosa (RIVIERRE et al., 2005), composta por áreas de inflamação e necrose da derme, com presença de neutrófilos e eosinófilos (HENSEL et al., 2003), às vezes combinadas a granulomas conspicuos formados unicamente por macrófagos epitelióides e células gigantes.
multinucleadas ou repletos de detritos celulares eosinofílicos (FOIL et al., 1984; HOWERTH et al., 1989).

No que se refere ao tratamento, a terapia proposta neste caso foi a de excisão cirúrgica associada à terapia antifúngica. A remoção cirúrgica das lesões continua sendo o procedimento de eleição no controle da pitiose cutânea, entretanto, bons resultados são observados apenas em lesões pequenas e superficiais, nas quais é possível a remoção de toda área afetada (LEAL et al., 2001).

Estudos avaliando a eficiência de conduta associada, excisão cirúrgica e terapia antifúngica com Anfotericina B, demonstraram sucesso de 50% (MCMULLAN et al., 1977). Ímportantes relatos revelaram que o uso isolado de drogas antifúngicas apresentam resultados variáveis in vitro e in vivo (LEAL et al., 2001), sendo que a Itraconazol apresentou efeito sinérgico resultando no cura de 53% a 83,3% e de 100% em bovinos (MONTEIRO, 1999). Por outro lado, ensaios inibitória do crescimento de Pythium insidiosum (SHENEP et al., 1998). Naquele estudo, a associação de Terbinafina e Itraconazol apresentou efeito sinérgico no controle da pitiose cutânea, entretanto, bons resultados são observados apenas em lesões pequenas e superficiais, nas quais é possível a remoção de toda área afetada (LEAL et al., 2001).

Um dos objetivos deste relato foi o de ressaltar a pitiose cutânea como um importante diagnóstico diferencial dentre as dermatopatias piogranulomatosas que acometem cães, tais como leishmaniose, granuloma leproide, piogranuloma estéril idiopático, celulite juvenil e micoses sistêmicas que cursam com granuloma leproide, piogranuloma estéril idiopático, celulite juvenil e micoses sistêmicas que cursam com infeção facial. A imunoterapia é uma alternativa em potencial para o tratamento da pitiose, demonstrando em equinos considerado com atividade moderada e a Terbinafina como a de maior atividade inibitória do crescimento de P. insidiosum (MENDOZA & NEWTON, 2005).

Respostas ao tratamento (DYKSTRA et al., 1999; FOIL et al., 1984; HOWERTH et al., 1989). No que se refere ao tratamento, a terapia antifúngica a base de Itraconazol. A remoção de áreas alagadas.

Para o tratamento da pitiose, demonstrando em equinos, a proposta neste caso foi a de excisão cirúrgica associada a terapia antifúngica. A remoção cirúrgica das lesões continua sendo o procedimento de eleição no controle da pitiose cutânea, entretanto, bons resultados são observados apenas em lesões pequenas e superficiais, nas quais é possível a remoção de toda área afetada (LEAL et al., 2001).

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