Semina: Ciências Agrárias
ISSN: 1676-546X
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Universidade Estadual de Londrina
Brasil

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Semina: Ciências Agrárias, vol. 33, núm. 6, noviembre-diciembre, 2012, pp. 2337-2345
Universidade Estadual de Londrina
Londrina, Brasil

DOI: 10.5433/1679-0359.2012v33n6p2337

Comparative analysis of lesions caused by histotoxic clostridia in experimentally induced myonecrosis

Análise comparativa das lesões causadas por clostrídios histotóxicos em mionecrose induzida experimentalmente

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Abstract

The comparative anatomical and histopathological lesions caused by different histotoxic clostridia are considered an important step in the diagnosis of epidemiological mionecrosis. In the present study, guinea pigs (*Cavia porcellus*) were used to reproduce gas gangrene caused by *Clostridium septicum*, *C. chauvoei*, *C. novyi* type A, *C. perfringens* type A and *C. sordellii*. The clinical signs, gross and histopathological lesions were compared between inoculated groups and an immunohistochemistry (IHC) was standardized for detection of agents in tissues. On clinical evaluation, animals showed swelling at the injection point, discomfort and limited mobility. The intensity and extent of gross and microscopic lesions varied with the inoculated agent. IHC was able to identify each agents inoculated without cross reactions. All observed results demonstrate that the evaluation of lesions could be usefull in the presumptive etiologic diagnosis.

Key words: Blackleg, gas gangrene, myonecrosis, histopathology, immunohistochemistry

Resumo

O estudo comparativo das lesões anatomo-histopatológicas causadas pelos diferentes clostrídios histotóxicos é considerado uma etapa importante no diagnóstico epidemiológico das mionecroses. Para reproduzir experimentalmente a gangrena gasosa causada por *Clostridium septicum*, *C. chauvoei*, *C. novyi* tipo A, *C. perfringens* tipo A e *C. Sordellii* foram utilizados cobaios (*Cavia porcellus*). Os sinais clínicos, lesões macroscópicas e histopatológicas foram comparadas entre os grupos inoculados e uma imuno-istoquímica (IHQ) foi padronizada para detecção dos agentes nos tecidos das cobaias experimentalmente infectadas. Na avaliação clínica os animais apresentaram aumento de volume no ponto de inoculação, desconforto e dificuldade de locomoção. A intensidade e extensão das lesões macroscópicas e microscópicas variaram com o agente inoculado. Na IHQ foi possível identificar cada um dos agentes inoculados, sem a detecção de reações cruzadas. Todos os resultados observados demonstram que este tipo de avaliação das lesões é de extrema importância para o diagnóstico etiológico conclusivo, possibilitando assim a adoção de medidas preventivas acertivas.

Palavras-chave: Carbúnculo sintomático, gangrena gasosa, mionecrose, histopatologia, imuno-istoquímica

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Recebido para publicação 17/11/11 Aprovado em 21/11/12

Introduction

Clostridia are obligatory anaerobic Gram-positive spore forming bacilli, found ubiquitously in soil, water, food and intestinal contents of animals and humans. Among the clostridial diseases, the myonecrosis (gas gangrene and blackleg) are necrotizing soft tissue infections that affect cattle (ASSIS et al., 2010), sheep and goats (LIMA et al., 2006) leading to significant economic losses due to their high fatality rate (MIYASHIRO et al., 2007).

The detailed pathogenesis of blackleg has not been completely elucidated. The proposed pathogenesis indicates that the infection is acquired by the ingestion of *Clostridium chauvoei* spores. Either these spores, or spores produced following germinative cycles in the gut, are taken across the intestinal mucosa (maybe inside macrophages) and distributed to tissues. Gas-gangrene, caused by one or more pathogenic clostridia, including *C. septicum*, *C. chauvoei*, *C. novyi* type A, *C. perfringens* type A, and *C. sordellii*, is related to close contact between these agents and domestic ruminants, which allow the contamination of wounds in surgical interventions and/or in others procedures performed without adequate asepsis (VAN VLEET; VALENTINE, 2007).

The reports about affected animals unfortunately only describe marked crepitation, swelling, and subcutaneous edema in affected tissues, toxemia, and sudden death. Most diagnoses are based only on clinical signs and poorly detailed gross lesions, with few reports having laboratory confirmation. The fluorescent antibody test (FAT) and immunohistochemistry (IHC) are the main diagnosis assays, but sometimes the etiological identification, by these techniques, is not enough for final diagnose (LOBATO; ASSIS; SALVARANI, 2007; RIBEIRO et al., 2012). Clostridia are frequent *post-mortem* invader, and laboratory identification could result in an inadequate diagnosis if there is a lack of tissue samples preservation (ASSIS et al., 2010). In veterinary medicine in Brazil, it is especially important, because most of the farms are far from research and diagnostic centers, which favors a corresponding increase in the multiplication of saprophytic clostridia.

Different lesions depending on the histotoxic clostridia specie could be expected (VAN VLEET; VALENTINE, 2007), because they produce toxins with specific action. Despite the major advance in the knowledge of the biological mode of toxins' action produced by these five species of histotoxic clostridia, there is a lack of studies comparing detailed gross and histological lesions among histotoxic clostridia in experimentally inoculated animals. For this propose, experimentally myonecrosis was reproduced in guinea pigs to evaluate anatomic-pathological findings which could be helpful to final etiological diagnosis. In addition, the present report gives access to a simple protocol for production and standardization of an IHC to the main histotoxic clostridial species.

Material and Methods

Reference strains

Strains of American Type Culture Collection (ATCC) of *C. septicum* (ATCC 12464), *C. chauvoei* (ATCC 10092), *C. novyi* type A (ATCC 19402), *C. perfringens* type A (ATCC 13124), and *C. sordellii* (ATCC 9714) were used to experimentally reproduce gas gangrene in guinea pigs (*Cavia porcellus*) and for primary antibodies production.

Gas gangrene model

The guinea pigs were divided into six groups with eight animals in each one. The negative control group was inoculated, intramuscularly, with 1 mL of sterile solution of 5% $CaCl_2$, euthanized and necropsied after 48 hours. The others groups were inoculated with 1 mL solution containing suspension of bacterial culture, corresponding to the tube eight of MacFarland Scale, with approximately 2.4 x 10^9

bacterial cells/mL, and of sterile solution of 10% $CaCl_2$ (1:1). These guinea pigs were observed until the death of the first animal, when the others were euthanized and necropsied. Muscle tissue, from the inoculation point, was fixed in buffered formalin for 72 hours, embedded in paraffin and stained with hematoxylin and eosin (HE) for histological evaluation with light microscopy.

The gross and histopathological lesions of each guinea pig were extensively examined for comparisons between the groups. On gross evaluation the increased of volume and gas bubble, hemorrhage and edema were classified on absence, mild, moderate and marked if the alterations were not observed, restrict to inoculation area or focal area in contralateral forelimb, extend to all inoculated member but do not affect extensively all muscles, extend and affect all muscle of inoculated member and adjacent areas, respectively. The color was characterized and gas bubbles associated with ulcerative epidermis were examined for presence and absence. On histological analyses the necrosis was classified on liquefative, hyaline and flocullar. On histological analyses the necrosis, gas, inflammatory infiltration and interstitial hemorrhage were also evaluated and classified on absence, mild, moderate and marked if the alterations were not observed, limited microscopic fields, multifocal microscopic fields and all examined microscopic fields of blade, respectively. The IHC were applied on tissue sections and the amount of bacillus were classified as in the histological evaluation.

Production and purification of primary antibody

The bacterins of each of five histotoxic clostridia, were obtained, as previous described for Assis et al. (2005), and emulsified (Sorvall mixer Omini-17106-Sorvall, USA) in a 1:1 ratio, with complete and incomplete Freund's adjuvant (Sigma-Aldrich, USA) for the first (day 0) and other inoculations (days 28, 49, 70 and 91), respectively. To obtain hyperimmune serum, three rabbits for each bacterin were used. Inoculations were done subcutaneously, with 1 mL per inoculum. Immunoglobulins G purification (IgG's) were performed by affinity chromatography (HiTrap Protein G HP, GE Healthcare, USA) according to manufacturer's instructions.

Streptavidin Biotin Peroxidase Technique (LSAB)

The unstained histological sections were deparaffinized, then treated with 3% hydrogen peroxide and 10% bovine serum albumin, and placed into a humid chamber at 37 °C for 30 and 15 minutes, respectively. Later, sections were covered and incubated with the primary antibody, previously, at 37 °C for 40 minutes. Then, they were incubated with biotinylated antibody (Dako, USA) and exposed to streptavidin peroxidase conjugate (Dako, USA). These steps were interspersed with three washes with phosphate buffer (1M PBS pH 7.4) for five minutes each. The reaction was revealed using DAB (Dako, USA). In all procedures tissue sections were counterstained with Harris's hematoxylin.

Dilution of primary antibody and specificity test

To determine the sensitivity of primary antibodies in histological sections from inoculated animals, dilutions of 1:30, 1:100, 1:200, 1:400 1:1000, 1:2000, 1:3000 and 1:4000 were used and the optimum concentration was determined. Specificity was evaluated by applying the antibody in the same procedure, using the guinea pig affected muscle from animals inoculated with heterologous clostridia. The negative reactivity of the primary antibody was tested by replacing the incubation step of the histological sections with primary antibody by incubation with PBS.

Committee on animal experimentation

This study was approved by the Ethics Committee on Animal Experimentation of Universidade Federal de Minas Gerais (Protocol 189/09).

Results

Clinical course and gross and histological lesions in guinea pigs

The guinea pigs of the negative control group showed no significant clinical signs. On physical examination, there was only a slightly increased sensitivity in the inoculated leg through the 48 hours of observation. In experimentally infected guinea pigs, two hours after inoculation, increased volume and marked discomfort in the inoculated thigh were observed. Four hours after inoculation, it was also observed locomotor difficulty when forced to move. The period between inoculation and death of first guinea pig is presented in Table 1.

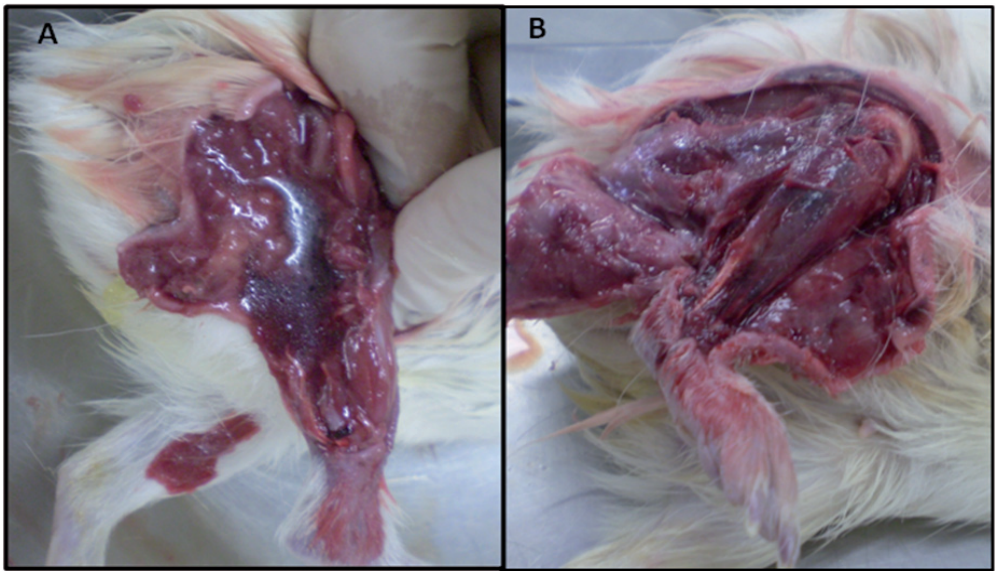
At necropsy, the muscles in the area of inoculation in the animals of the negative control group had mild localized subcutaneous edema. Focal petechiae and ecchymotic hemorrhages were also observed in the inoculated muscle. Comparing to control group, the gross lesions observed on infected guinea pig were noticeably more intense and extensive. The guinea pigs inoculated with the bacterial strains showed swelling of the entire leg, including the foot. At palpation, in general, especially in the inoculation area, the skin was tense and there was crepitation indicating presence of gas. On cut surface, muscle fibers were separated by the presence of gas and the muscles were dark-red (Figure 1A and 1B), sometimes with gray areas, indicative of necrosis. In the subcutaneous tissue, there was blood-stained edema with gas bubbles present over large areas of the medial side of the inoculated limb, often extending into inguinal and abdominal areas. In the guinea pigs infected with *C. septicum* and *C. sordellii,* large quantities of edema stained with blood extended to the subcutaneous tissue in the inner side of all four limbs and over the ventral surface of body. Particularly in guinea pigs infected with *C. perfringens* type A, the inoculated and adjacent areas showed marked liquefactive necrosis. Strong rancid odor also was detected and fat droplets were abundant (necrosis of adjacent adipose tissue) (Figure 1A). Details about intensity and extension of gross lesions varied with the different species of *Clostridium* inoculated and are presented in the Table 1.

Table 1. Period between inoculation and dead, type and intensity of gross changes in guinea pigs experimentally inoculated with histotoxic clostridia.

Strain	Death (h)	Gross changes				
		Changes in the inoculated thigh and adjacent tissues				Contra-lateral forelimb
		Increased of volume and gas bubble	Color	Gas bubble and ulcerative epiderme	Hemorrhage and edema	Hemorrhage and edema
Negative control	Euthanized	+	Red	No	0	0
C. septicum	8	+++	Dark red	No	+++	+++
C. chauvoei	31	++	Dark red-to-black	No	++	+
C. novyi type A	17	++	Dark red to-black	Yes	++	+
C. perfringens type A	20	+++	Greenish to blackish green	Yes	+++	+
C. sordellii	21	+++	Dark red-to-black and grayish	No	+++	+++

Legend: 0 absence of specific lesion, (+) mild, (++) moderate, (+++) marked; Yes/No for alteration evaluated for presence or absence, but not quantified.
Source: Elaboration of the authors.

Figure 1. Skeletal muscles of guinea pig experimentally infected with *Clostridium perfringens type*(**A**) and *C. chauvoei* (**B**). On cut surface, there is marked liquefactive necrosis with hemorrhagic exudate and fat droplets (necrosis of adjacent adipose tissue) (**A**) and moderate subcutaneous blood-stained edema and the muscles of the femoral and tibia region are dark red (**B**).



Source: Elaboration of the authors.

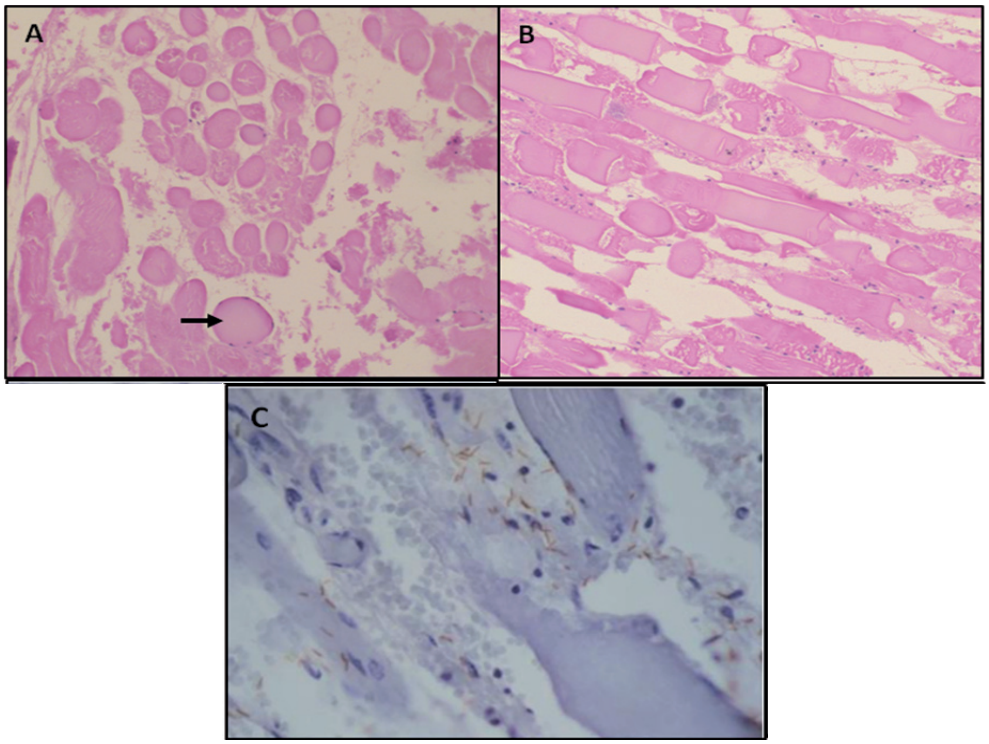
Likewise, the intensity and type of histological lesions produced by these clostridia were different (Table 2). The negative control group showed mild muscle fiber necrosis associated with mild neutrophilic infiltrates. The guinea pigs inoculated with strains of histotoxic clostridia showed typical lesions of clostridial myonecrosis. In general, there was acute neutrophilic myositis associated with variable degrees and types of necrosis. *C. septicum* presented predominantly hyaline necrosis. *C. chauvoei*, *C. novyi* type A and *C. sordellii* showed especially floccular necrosis (Figure 2B). However, *C. perfringens* type A presented particularly liquefactive necrosis (Figure 2A) associated with thrombosis and minimal inflammatory infiltrated. Additionally, vascular necrosis and thrombosis, gas bubbles and interstitial hemorrhages associated with variable amounts of bacilli were observed in all tissues inoculated with clostridia used in this study.

Table 2. Intensity and type of histologic changes in guinea pigs experimentally inoculated with histotoxic clostridia.

Strain	Histologic changes							IHC
	Necrosis			Vascular changes		Gas	Inflammatory infiltrated	Bacillus
	Hyalin	Floc-cular	Lique-factive	Intersticial hemorrhage	Vascular necrosis and trhombosis			
Negative control	0	+	0	+	No	0	0	0
C. septicum	+++	+++	0	+++	Yes	+	+++	+++
C. chauvoei	+	+++	0	++	Yes	++	+++	++
C. novyi type A	+	+++	++	++	Yes	++	+	+++
C. perfringens type A	++	++	+++	++	Yes	+++	+	+++
C. sordellii	++	+++	++	++	Yes	+++	++	+++

Legend: Immunohistochemistry (IHC), 0 absence of specific lesion, (+) mild, (++) moderate, (+++) marked; Yes/No for alteration evaluated for presence or absence, but not quantified.
Source: Elaboration of the authors.

Figure 2. Muscle tissue from guinea pig experimentally infected with *Clostridium perfringens* type A (**A**) and *C. novyi* type A (**B**). There are few cells roundness (arrow) and with loss of striations (hyaline necrosis), and other cells with fragmentation of the cytoplasm (floccular necrosis). The necrotic fiber bundles are separated by gas. H&E. 20X (**A**); and there are predominant floccular necrosis associated with gas separating the fibers. H&E. 60X (**B**); (**C**) Immunohistochemistry of muscle tissue from guinea pig infected with *C. perfringens* type A. Many bacilli *C. perfringens* type A positive can be observed associated with floccular necrosis.



Source: Elaboration of the authors.

Immunohistochemistry in experimental infected guinea pigs

The dilutions of primary antibodies for LSAB technique were 1:3000, 1:200, 1:30, 1:200 and 1:400 for *C. septicum*, *C. chauvoei*, *C. novyi* type A, *C. perfringens* type A and *C. sordellii*, respectively. The muscle sections labeled by the LSAB technique showed intense immunolabeling for the corresponding organism injected (Figure 2C). There was no cross-reactivity between antibodies. When the antibodies were tested on tissue sections from the negative control guinea pigs or when they were replaced by PBS no labeled rods were observed.

Discussion

Definitive diagnosis of clostridial myositis can be challenging, and requires association among the clinical, pathological and complementary laboratory tests in order to confirm the etiologic agent. The lesions observed in guinea pigs inoculated with different clostridia are compatible with the clostridial myositides affecting domestic animals (LIMA et al., 2006). Although some variations in gross and histological appearance of the lesion depending on the species of the principal pathogen were expected (VAN VLEET; VALENTINE, 2007), it is the first time that it was detailed in controlled inoculated groups.

Grossly, there were noticeable differences in the muscle color among the different groups of infected guinea pigs (Table 1), unusual reported on gas gangrene communications. The occurrence and intensity of color changes observed in infected guinea pigs are related to the intensity of vascular lesions and/or activity toxins produced by these clostridia (TITBALL; NAYLOR; BASAK, 1999). Especially in guinea pigs inoculated with *C. perfringens* type A, the change in the color of muscle and subcutaneous tissue was similar to pseudomelanosis and easily confused with *post-mortem* changes. But in these animals the change was definitely *in vivo* and can be attributed to the action of the bacteria's sulfated hydrogen which promoted rapid disintegration of hemoglobin (VAN VLEET; VALENTINE, 2007). This observation has not yet been reported in natural infection.

Histological lesions show a marked difference between the species of clostridia (Table 2). Clostridial myonecrosis is commonly characterized by degenerating muscle fiber (VAN VLEET; VALENTINE, 2007), but on histological observations at least three presentations could be observed: hyaline necrosis, liquefative necrosis, and flocular necrosis. Hyaline necrosis of muscle fibers characterized by increased volume and roundness of the fiber, eosinophilia and loss of striations, was observed in all infected groups, but these changes were more intense and multifocality distributed in the muscle tissues of guinea pigs infected with *C. septicum*. The alpha toxin produced by this bacterium determines the formation of pores in cell membranes, leading to abnormal fluid influx and rapid intracellular accumulation of fluid (KNAPP et al., 2010) in muscle fibers (KENNEDY et al., 2009). It is presumably this mechanism that is responsible for the hyaline necrosis seen prominently in *C. septicum* infections. The direct action of alpha toxin on endothelial cells of the microcirculation also causes ischemia, which may be responsible for the additional floccular necrosis and extensive hemorrhages, moreover favoring the multiplication of *C. septicum* (KENNEDY et al., 2009).

The liquefactive necrosis observed in guinea pigs infected with *C. perfringens* type A (Figure 1A) was more intense in comparison with other agents (Table 1). The alpha toxin produced by *C. perfringens* type A hydrolyzes the phospholipid membrane of eukaryotic cells including erythrocytes, fibroblasts, endothelial cells and muscle, culminating in lysis. Furthermore, the toxin promotes platelet aggregation and vasoconstriction which leads to thrombosis and subsequent tissue anoxia (HICKEY et al., 2008). Possibly, in addition to its direct necrotizing action, the alpha toxin induced rapid and irreversible reduction of blood flow with greater intensity producing marked muscle ischemia, which probably led to liquefactive necrosis in guinea pigs inoculated with this agent (BRYANT et al., 2006).

Floccular necrosis (Figure 2B) was the predominant lesion in guinea pigs inoculated with *C. sordellii* and *C. novyi* type A. *C. sordellii* produces several virulence factors, but the major are the lethal toxin, and a hemorrhagic toxin. The *C. novyi* type A produces alpha toxin, which has a similar mode of action of letal toxin. Both of these disrupt actin filaments resulting in cell rounding, loss of intercellular junctions and increased endothelial permeability that is compatible with edema in the infections caused by this bacterium (JUST; GERHARD, 2004). The same alterations were observed lesion in guinea pigs inoculated with *C. chauvoei*, but there are a lack of information about the action its toxin.

Generally, neutrophils are never numerous because collected samples are taken at the advancing margins of the lesion. In addition, inflammatory infiltration is effectively immobilized and destroyed by the toxins (VAN VLEET; VALENTINE, 2007). The paucity of phagocytic cells in guinea pigs inoculated with *C. perfringens* type A in the area of the infection could be mediated by the cytotoxic effects of perfringolisin O on leukocytes and by the ability of alpha toxin to activate synthesis of cell adhesion molecules, causing neutrophils to adhere and migration at remote locations from the

site of infection that impede neutrophil diapedesis (BRYANT et al., 2006). The low inflammatory infiltrated observed in guinea pigs inoculated with *C. novyi* type A, and *C. sordellii* could be explained by apoptotic process induced by major toxins (JUST; GERHARD, 2004). Nevertheless, on guinea pigs experimentally infected with *C. septicum* and *C. chauvoei* it was observed a large number of neutrophils.

In the present study, there was strong and specific positivity in the IHC, with each antibody binding only to the tissues from the guinea pigs infected with the particular strain, supporting that the gross lesions were caused only by the inoculated agents and their toxins. Although the inoculation of guinea pigs with histotoxic clostridia has already been described (ASSIS et al., 2005), this is the first time this model is scrutinized. Detailed lesion reports will not avoid laboratorial etiological diagnosis, but it will permit an accurate diagnosis.

Conclusion

The guinea pigs model reproduced the gross and histological alterations observed in natural clostridial myonecrosis infection and it was variable among histotoxic clostridia species. The results presented demonstrated the importance of a detailed study of cases to obtain a presumptive etiologic diagnosis, which generally interfere directly with preventive measures. Also, the procedures described for antibody production and purification and standardization of IHC revels simple protocols for etiological diagnosis.

Acknowledgments

This work was supported by CNPq, FAPEMIG, INCT/IGSPB – CNPq and FEPMVZ. FCFL and RE were supported by fellowship from Conselho de Desenvolvimento Tecnológico e Científico (CNPq, Brasília, Brazil).

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