Acta Scientific Veterinary Sciences (ISSN: 2582-3183)

Volume 5 Issue 1 January 2023

Mini Review

A Mini Review on the Significance and Virulence Factors of *Clostridium novyi*

Teferi Benti Moti*

Department of Veterinary Microbiology, Animal Health Institute, Ethiopia

***Corresponding Author:** Teferi Benti Moti, Department of Veterinary Microbiology, Animal Health Institute, Ethiopia.

Received: November 08, 2022

Published: December 23, 2022

Abstract

Clostridium novyi is one of the clostridia species, which are anaerobic, gram-positive, non-capsulated, motile, and have the ability to produce endospores. The spores of Clostridium species are highly resistant to environmental conditions and found in soil, on the faces of animals, and as endogenous sources in the intestinal tract and liver. The vegetative cells of Clostridium species are rod-shaped, frequently pleomorphic, and can be observed under a microscope in pairs, clusters, with rounded ends to their cells, and in short chains. The purpose of this review is to raise awareness of Clostridium novyi importance and its virulence factor. Clostridium novyi affects sheep, swine, cattle, and goats, but rarely equines. Black disease, or infectious necrotic hepatitis, is an acute, fatal disease characterized by edema, including swelling of the eyelids and nostrils, and noticeably elevated liver enzymes. Clostridium novyi is classified into four strains, designated A, B, C, and D, based on their soluble antigen, extent of their toxin production, and the diseases that they cause in humans and animals. Type A strains are of medical importance because they cause gas gangrene in humans, while type B is responsible for infectious necrotizing hepatitis, especially in sheep but occasionally in other animals. Clostridium novyi proliferates in the soft tissues of the head and neck, and the resultant clostridial toxin causes increased capillary permeability and the liberation of serous fluids in the tissues. Clinical history, routine necropsy, histopathology, and immunostaining of C. novyi in liver lesions are essential to giving a reasonable opinion of infectious necrotic hepatitis. Isolation isn't always successful, due to fastidious and strict anaerobic conditions of the microorganism; molecular identification by PCR is a precious tool to give an etiologic opinion and to separate C. novyi type B from other clostridial pathogens. Treatment is infrequently effective; however, control is achieved by disrupting the liver fluke life cycle by reducing populations of the intermediate snail host and actively immunizing with C. novyi toxoid.

Keywords: Bacterial Spores; *C. Novyi*; Diagnostic Technique; Infectious Necrotic Hepatitis; Toxin

Introduction

Dr. Frederick Novy discovered Clostridium novyi (C. novyi) from guinea pigs in1894, known as Bacillus oedematis maligni [1]. The endogenous source of C. novyi is the liver and gastrointestinal tract, and it is widely distributed in soil, water, and marine sediments [2]. It is Gram-positive, noncapsulated, motile, and is an obligate anaerobe that develops endospores to withstand unfavorable circumstances [1,3]. A wide group of bacteria that produce spores called "clostridia" usually cause disease by producing strong toxins [4]. In areas of liver necrosis brought on by the movement of liver flukes, the organism multiplies and creates a potent necrotizing toxin (alpha toxin). Wherever sheep and liver flukes are found, there is the disease, which is spreading and getting worse [5]. C.novyi is classified scientifically as belonging to the phylum formicates, class clostridia, order clostridiales, family clostridiaceae, and kingdom bacteria. Clostridium novyi type B causes infectious necrotic hepatitis (black disease) [5]. Since Clostridium novyi is pathogenic that secretes alpha toxins, a lethal exotoxin that induces edema, and cause a wide range of illnesses in both humans and animals [6,7].

Based on the toxins they produce, *C. novyi* is classified into four types: A, B, C, and D. *Clostridium novyi* Type A produces alpha, gamma, delta, and epsilon toxins. *C. novyi* Type B produces alpha, beta, and zeta toxins, while Type C produces gamma toxins [2,8]. C. novyi type D is thought to be a distinct species (*Clostridium haemolyticum)* from Clostridium types A, B, and C. [9] because it lacks alpha-toxin; it produces beta, eta, and theta toxins [10]. C. novyi type A

is frequently involved in gas gangrene infections in humans and animals, while type B is the etiological agent of infectious necrotic hepatitis (black disease), which is typically observed in sheep, swine, and cattle, although rarely in horses [11]. C. novyi type C is not known to cause illness in laboratory animals and is therefore considered non-pathogenic [8].

C. novyi type D, which only produces a beta toxin, is commonly known as *C. haemolyticum* and causes BH [12,13], while *C. novyi* types A and B (producing alpha toxin) cause sudden death in swine, and the carcasses exhibit gross distension and livers with gas bubble infiltration or sponge-like appearances [14,15]. Clostridium spp. is an obligate anaerobe and requires enriched media for isolation and identification, which are more time consuming and laborious [16]. In general, C. Novyi type B causes infectious necrotic hepatitis in domestic animals [17]. C. Novyi strains have peritrichous flagella; they are all motile and cause swarming on blood agar cultures [18]. The major goal of this review is to draw attention to the importance and virulence factors of C. novyi.

Review

Pathogenesis of *C. novyi* infections

Ingestion of environmental spores that seed Histocytes in the liver, bone marrow, spleen, and possibly other organs is the first step in the pathogenesis of the disorders. Clostridial spores that have already been swallowed, circulated, and deposited in tissues begin to germinate when anaerobic conditions are present, and this cause's tissue damage by way of toxin synthesis. INH, sometimes referred to as "black disease," is an acute toxemic disease of sheep produced by *C. novyi* type B [19,20]. Sporadic cases have been also reported in cattle, goats, pigs, [21] and horses [19,22].

In ruminants, the migration of the liver fluke is thought to be the main initiating event [5]. Any hepatic injury leading to the generation of anaerobic conditions may be a predisposing factor for these diseases. The liver and other organs enlarge with edema and tissue necrosis as a result of a toxin released by the vegetative bacteria. Both humans and animals can become infected by Clostridium novyi, which is widely found in the environment in soil and animal waste. Initial, vigorous, and spore formation are the three stages of infection for C. novyi. The bacteria are expanding throughout their first growth phase without releasing any poisons or pathogens. During strong growth, a lot of toxins are created. Spores and endospores are produced, and the level of toxin synthesis declines, during the final stage of infection. Endospores are robust structures that are inactive [19,23].

Rams with swollen heads are caused by Clostridium novyi type A, which is also one of the causes of gas gangrene in humans. In young rams in particular and is characterized by the onset of a prominent seditious oedema of the head and neck. This condition arises from head injuries that are frequently sustained during fighting and that become infected. The term "black diseases" refers to conditions when the corpse briefly turns black after death due to engorgement of subcutaneous blood veins [24,25]. Sheep, in particular, are susceptible to the condition known as infectious necrotic hepatitis, which is brought on by a Clostridium novyi infection. The common name "black disease" comes from the fact that the primary infection is intestinal and is spread by the faecal-oral rot spores of C. novyi that escape from the gut and lodge in the liver. There, they remain dormant until an injury creates anaerobic conditions for them to germinate. This causes local necrosis and widespread damage to the microvascular system, which leads to subcutaneous bleeding and a blackening of the skin [1].

Virulence factors of *C. novyi*

Toxins

The lethal and necrotizing toxins damage hepatic parenchyma, thereby permitting the bacteria to multiply and produce a lethal amount of toxin [26]. An area of necrosis is the distinctive lesion of contagious necrotic hepatitis in domestic animals or humans. An archipelago of symptoms, including refractory hypotension, leukocytosis, and multiple effusions, are produced by the alpha toxin of C. novyi strains [24]. A single peptide chain made up of roughly 250,000 MW makes up the massive clostridial cytotoxins collectively. Their activity is defined by fluid leakage into the interstitial space [27]. Particularly clostridial toxins disrupt normal cell contact and weaken vascular endothelial integrity by glycosylating GTPases involved in actin cytoskeleton function [28,29].

The three basic characteristics of the alpha toxin (TcnA) are edematizing, deadly, and necrotizing [30]. Signal transduction pathways that are blocked because the disintegration of cytoskeleton structures, which results in morphological changes in all cell types, but especially in endothelial, cells [28]. The cells of the microvascular system undergo a spherical change, and the connec-

Type	Main	Alpha	Beta	Diseases
A		+++	-	Gas gangrene (humans and animals
B		++	+	Infectious necrotic hepatitis
C		-	-	No known disease association
D (or C. haemolyticum)		-	+++	Bacillary hemoglobinuria

Table 1: Shows C. novyi type and their main toxin.

– = no toxin produced; + to +++ = increasing amount of toxin produced.

Source: Navarro MA and Uzal FA. (2020).

tions between adjacent cells become weak. These outcomes cause fluid to leak from the capillaries, resulting in oedema in the connective tissue [19,27]. Gamma toxin is produced by Clostridium novyi type C characterized as hemolytic, lecithinase or phospholipid, is associated with gas gangrene in humans and animals. Delta-Toxin: is characterized as oxygen labile haemolysin. Clostridium type B produces alpha (TcnA) and beta- toxin in which necrotizing, hemolytic, and lethal) and is a phospholipase or lecithinase [19].

Clostridium novyi type B produce Alpha, Beta and Zeta toxin. The alpha toxin is similar to clostridium novyi type A and Beta and Zeta toxin cause hemolytic necrosis. Clostridium novyi type B toxin is considered the main virulence factor of this microorganism, and it's responsible for bacillary hemoglobinuria (BH), disease affecting substantially cattle that's clinically and pathologically veritably analogous to contagious necrotic hepatitis (INH). After ingestion, the spores of C. novyi type B are absorbed from the intestine and reach the liver via the portal rotation, after which they're spread to other organs [30]. The spores are phagocytized and survive in the liver, Kupffer cells, bone marrow, macrophages, and spleen'. Because both conditions are thought to develop after liver damage, necrosis and the ensuing anaerobic conditions necessary for the germination of latent spores and the production of toxins are believed to occur in both conditions. Necrotic hepatitis is typically thought of as the counterpart to bacillary hemoglobinuria (BH). The most important risk factor is the invasion of the liver by immature forms of liver fluke [31].

Clinical sign

Identification of diseased animals is challenging due to the disease's often peracute course and lack of clinical symptoms. The animals typically seem to be in good physical condition before they suddenly pass away, with unusually quick postmortem decomposition being a defining feature [32]. Rams can develop an illness called "Bighead" that causes non-gaseous, non-hemorrhagic head and neck edema. The edema could spread to ventral areas, like the throat. Eyelid and nostril swelling are additional clinical symptoms. The majority of animals die within 48 to 72 hours [33,34].

Clinical signs are rarely seen because of the per acute nature of the disease. Although the animals typically seemed to be in fair physical condition, several of them died unexpectedly from high rectal temperatures after trying to get well. Deaths were seen in flocks of pigs and lambs, and there was also a case of an equine documented [34,35]. The animal's body was well-fed and had a suitable amount of fat stores during necropsy. The left lobe of the liver was firm, dark red to black, and covered with copious fibrin, which had numerous subscapular and deep parenchymal gas bubbles. The liver was markedly enlarged and had rounded borders [20]. Gross distention of the body, grandiloquent skin abrasion, widespread edema, and subcutaneous infiltration with bubbles and foul-smelling bloody fluid in the pleural, pericardial, and abdominal depressions are patterns of pathological lesions. Generally, infected organs exhibit oppressive narcotization, gas filling, sponginess, and softening [19,20]. Samples of the brain, lung, spleen, heart, diaphragm, intestine, liver, kidney, stomach, pancreas, and mesentery were taken aseptically in order to isolate and identify the etiological agent [22,36].

Epizootology and Transmission: C. novyi toxins, which enter through wounds frequently connected to horn injuries sustained during combat, are the cause of bighead. Outbreaks are more frequent in the summer and fall and frequently occur after excavation or flooding. It is believed that the organism is ingested, enters the

gastrointestinal tract through the bloodstream, and then travels to the muscles. Black disease and bacillary hemoglobinuria are linked to concurrent liver disease, which is occasionally discovered as a result of liver biopsies and is frequently brought on by Fasciola infections [19,20].

Diagnostic techniques

For accurate diagnosis, it is essential to perform postmortem examination and collect samples as soon as possible after death. Clostridial disease is associated with advanced autolysis of organs because clostridial toxemia can mimic natural autolysis unless an outbreak of microflora is tampering with the results [19,32,37].

Necropsy: Postmortem lesions are typically used to make the diagnosis of black disease. Blood engorgement of subcutaneous arteries will give desiccated skin a black look. Dead bodies soon putrefy. In addition, endocardia hemorrhages and hepatic damage from flukes are frequent, and the latter can be so severe that a diagnosis is challenging. At necropsy, gross lesions were seen in the liver, heart and brain, and there was also significant ascites. The surface of the liver was dark red and merging white, with a faint necrotic patch. The developing white necrotic area resembled a honeycomb with gas bubble infiltration on the cut surface and a sponge-like appearance of the liver parenchyma [38].

It is essential to collect aseptic samples, transport them, use the appropriate preservatives, and understand the nature of the agent. After timely sample collection, microbial insulation, and application of PCR, a correct diagnosis of C. novyi infection was made. Sections of the liver, kidney, spleen, lung, heart, stomach, pancreas, brain, and small and large intestine served as samples for histological evaluation. These tissues were fixed in 10% formalin, reused often, and stained with hematoxylin and eosin (H and E) [31]. To isolate C.novyi, liver and brain tissue homogenates were added to cooking media containing 1% glucose and cultured at 37 °C in both anaerobic and aerobic environments. On blood agar, C. novyi colonies have a ground-glass appearance, are irregular in shape, spherical, and have a perimeter that measures 3 to 8 mm.Due of Clostridium novyi severe oxygen sensitivity and nutritional preferences, cultivation proved laborious and unreliable [1]. At necropsy, gross lesions were seen in the liver, heart and brain, and there was also significant ascites. The surface of the liver was dark red and merging white, with a faint necrotic patch. The developing white necrotic area resembled a honeycomb with gas bubble infiltration on the cut surface and a sponge-like appearance of the liver parenchyma [38].

Histological observation: The macroscopically visible hepatic lesion contained numerous vacuoles and necrotic hepatocytes that were causing the normal hepatic structure to deteriorate. The liver capsule was microscopically covered with copious fibrin mixed with cell fragments, and there were subcapsular and deep parenchymal emphysematous bullae of various diameters. The most dramatic result was a multifocal to focally expanding coagulative necrosis that was encircled by enormous numbers of live and degenerate neutrophils, fibrin, hemorrhage, cell debris, and significant gram-positive bacteria rod accumulations, some of which had sub-terminal spores [38,39].

The fluorescent antibody test (FAT) can be run on isolated cells or smears directly taken from clinical samples. The fluorescent antibody test is the most practical and quick discrimination assay for myonecrosis agents. It has been said that using immunofluorescence antibodies directly on liver smears is a highly sensitive individual method that is preferable to culturing. In order to prepare the liver tissues for immunohistochemistry, they were formalin-fixed, paraffin-embedded, and subjected to tissue section analysis [40]. A rabbit polyclonal antibody against Clostridia species was used, and detection was carried out using a commercial kit (N-Histofine) sample stain MAX PO(R), as directed by the kit's manufacturer [31,41,42].

In particular, the supernatant portion of the liver samples as well as the isolated bacteria were used to harvest DNA. By using the polymerase chain reaction, a small portion of the 16S rRNA gene (rDNA) from the genomic DNA of the insulated bacteria was amplified (PCR). The thermocycler profiles were as follows: 94 °C for 5 min, 30 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1.5 min, and finally 72 °C for 7 min. The samples were kept at 4 °C while the PCR results were seen using ethidium bromide under ultraviolet light [31,43].

The uprooted DNA was used as a template to amplify portions of the flagellin genes (fliC) from C. novyi types A, and traditional PCR was used to amplify a member of the C. novyi type B toxin gene (TcnA), which is one of the key virulence factors of the patho-

gen. The oligonucleotide sequences used were as follows: Both the 5'TGATGTTGACCATCCTTGCTCT-3' (CNtBaFF) and the 5'-CCT-TATGCAAAGGGATGGCG-33' (CNt- BaR) amplified the target gene's 34222-bp DNA scrap [43,44].

According to [43] the legion PCR system, which is based on the gene sequence of the flic flagellin subunit, is capable of identifying and separating Clostridium novy type B and Clostridium haemolyticum, which have been removed from isolates and culture supernatants of liver and brain samples. The Clostridium-like organism with the accession number AB857215 was found to be phylogenetically most closely related to two strains of Clostridium novy type B, ATCC25758 (accession number AB035087), whose 16S rDNA sequence was the same. Based on analyses of 1468 base pairs of classification, the isolates and those two bacteria species shared an extraordinarily high degree of sequence similarity, both 99.9 [45]. The 427 bp-sized species-specific amplicons showed that the liver and brain contained Clostridium novyi type B [46].

Conclusions

This review indicates that Clostridium novyi strains are causes diseases in sheep, pigs, and large animals and that it is a rare disease that can harm equine. Despite being rare, Clostridium novyi is a very dangerous infection for humans. Molecular identification of Clostridium species and their strain by PCR is a valuable tool to provide an etiologic diagnosis and to differentiate Clostridium novyi type B from other Clostridia pathogens. Prevention is superior to treatment. A multivalent clostridial vaccine will protect an animal from various clostridial diseases, and it may even be useful during an outbreak. Management of fascioliasis is crucial for the treatment and prevention of black diseases.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Bibliography

1. Aronoff DM. "Clostridium novyi, sordellii, and tetani: mechanisms of disease". *Anaerobe* 24 (2013): 98-101.

2. Skarin H and Segerman B. "Plasmidome interchange between Clostridium botulinum, Clostridium novyi and Clostridium haemolyticum converts strains of independent lineages into distinctly different pathogens". *PLoS One* 9.9 (2014): e107777.

3. Guo P., *et al.* "Clostridium species as probiotics: potentials and challenges". *Journal of Animal Science and Biotechnology* 11.24 (2020).

4. P Bette., *et al.* "A comparative biochemical, pharmacological and immunological study of Clostridium novyi alpha-toxin, C. difficile toxin B and C". *Sordellii Lethal Toxin Toxicon* (1991).

5. Smith GW. "Black disease". In: Smith BP, ed. Large Animal Internal Medicine. 5th edition. St. Louis, MO: Mosby Elsevier (2015): 849-850.

6. Hatheway CL. "Toxigenic clostridia". *Clinical Microbiology Reviews* 3 (1990): 66-98.

7. Uzal Francisco A and J Glenn Songer. "Clostridial diseases". *Diseases of Swine* (2019): 792-806.

8. Sasaki Y., *et al.* "Phylogenetic analyisis and PCR detection of C. Novyi type A and B based on fillgellin". *Veterinary Microbiology* 86 (2002): 257-267.

9. Nakamura S., *et al.* "Taxonomic relationships among Clostridium novyi types a and B, Clostridium haemolyticum and Clostridium botulinum type C". *The Journal of General Microbiology* 129 (1983): 1473-1474.

10. Farias L., *et al.* "Acute myonecrosis in horse caused by Clostridium novyi type A". *Brazilian Journal of Microbiology* 45 (2014): 221-224.

11. Brazier JS., *et al.* "Isolation and identification of Clostridium spp. from infections associated with the injection of drugs: experiences of a microbiological investigation team". *Journal of Medical Microbiology* 51.11 (2002): 985-989.

12. Navarro M., *et al.* "Bacillary hemoglobinuria. In: Uzal FA, *et al*., eds (2016). Clostridial Diseases of Animals. Hoboken, NJ: Wiley (2016): 265-274.

13. Navarro MA., *et al.* "Pathology of naturally occurring bacillary hemoglobinuria in cattle". *Veterinary Pathology* 54 (2017): 457-466.

14. Akiyama N., *et al.* "Fatal Clostridium novyi type B infection in a sow". *Japan Agricultural Research Quarterly* 51.1 (2017): 85-89.

15. Friendship CR and Bilkei G. "Concurrent swine erysipelas and Clostridium novyi infections associated with sow mortality in outdoor sows in Kenya". *The Veterinary Journal* 173.3 (2007): 694-696.

16. Busch C., *et al.* "Characterization of the catalytic domain of Clostridium novyi alpha-toxin". *Infection and Immunity* 68.11 (2000): 6378-6383.

17. Sweeny HJ. "Infectious necrotic hepatitis in a horse". *Equine Veterinary Journal* 18 (1986): 150-151.

18. Wiwanitkit V. "Clostridium novyi infection". *Journal of Swine Health and Production* 18.1 (2010): 10-10.

19. Cullen JM and Stalker MJ. "Liver and biliary system". In: Maxie MG, ed. Jubb, Kennedy, and Palmer's (2016). Pathology of Domestic Animals. 6th ed. St. Louis, MO: Elsevier (2016): 258-352.

20. Ahmed., *et al.* "Factors Affecting on Production of Clostridium novyi type (B) Alpha Toxin". *Journal of Applied Veterinary Sciences* 7.2 (2022): 53-57.

21. Abdolmohammadi Khiav L and Zahmatkesh A. "Vaccination against pathogenic clostridia in animals: a review". *Tropical Animal Health and Production* 53.2 (2021): 1-12.

22. Nyaoke AC. "Infectious necrotic hepatitis caused by Clostridium novyi type B in a horse: case report and review of the literature". *Journal of Veterinary Diagnostic Investigation* 30 (2018): 294-299.

23. Emadi A., *et al.* "Combined Molecular and Biochemical Identification of Alpha Toxin in Local Isolated Clostridium novyi from the Sheep Liver". *Archives of Razi Institute* 77.5 (2022): 1769-1777.

24. Schranner I., *et al.* "Isolation of immunogenic and lethal peptides of alpha-toxin from Clostridium novyi type B". *Toxicon* 30 (1992): 653-666.

25. Maxie MG., *et al.* "Necrotic hepatitis (black disease) (2016): In: Pathology of Domestic Animals. 6th edition., Vol. 2. Philadelphia, PA: Elsevier (2016): 316.

26. Ryan JM., *et al.* "Clostridium novyi infection: a fatal association with injecting drug users". *Emergency Medicine Journal* 18 (2001): 138-139.

27. Popoff MR and Bouvet P. "Clostridial toxins". *Future Microbiology* 4.8 (2009): 1021-1064.

28. Pires PS., *et al.* "Comparative analysis of lesions caused by histologic clostridia in experimentally induced myonecrosis". *Semina: Ciencias Agrarias (Londrina).* 33 (2012): 2337.

29. Müller H., *et al.* "Morphological changes of cultured endothelial cells after microinjection of toxins that act on the cytoskeleton". *Infection and Immunity* 60.7 (1992): 3007-3010.

30. Navarro MA and Uzal FA. "Pathobiology and diagnosis of clostridial hepatitis in animals". *Journal of Veterinary Diagnostic Investigation* 32.2 (2020): 192-202.

31. Noshahri NG., *et al.* "Identification and cloning of highly epitopic regions of Clostridium novyi alpha toxin". *Turkish Journal of Biology* 40.6 (2016): 1219-1226.

32. García A. "Sanidad del ganado porcino ibérico". Principales enfermedades infecciosas. Librería Técnica Figueroa-2 (eds) PUBLICEP-Producción Humanes de Madrid, Madrid (2013): 127-130.

33. Tony Redford., *et al.* "Myocardial Necrosis Associated with Clostridium Novyi Infection In a Bighorn Sheep (ovis Canadensis)". *Journal of Wildlife Diseases* 53.3 (2013): 695-698.

34. Li J., *et al.* "The Agr-like quorum-sensing system regulates sporulation and production of enterotoxin and beta2 toxin by Clostridium perfringens type A non-food-borne human gastrointestinal disease strain F5603". *Infection and immunity* 79.6 (2011): 2451-2459.

35. Jandowsky A., *et al.* "Sudden death of outdoor housed pigs caused by Clostridium novyi. A case report". *Tierarztliche Praxis. Ausgabe G, Grosstiere/Nutztiere* 41.6 (2013): 392-395.

36. Sasaki Y., *et al.* "Phylogenetic positions of Clostridium novyi and Clostridium haemolyticum based on 16S rDNA sequences". *International Journal of Systematic and Evolutionary Microbiology* 51.Pt 3 (2001): 901-904.

37. Garcia A., *et al.* "Clostridium novyi infection causing sow mortality in Iberian pig herd". *Journal of Swine Health and Production* (2009).

38. Kulikova VV., *et al.* "Gene cloning, characterization, and cytotoxic activity of methionine γ-lyase from Clostridium novyi". *IUBMB Life* 69.9 (2017): 668-676.

39. Widulin N., *et al.* "Sponge-like appearance of the liver parenchyma due to fatal intrauterine gas gangrene following mechanical abortion". *Forensic Science, Medicine and Pathology* 9 (2013): 274-276.

40. Assis RA., *et al.* "Immunohistochemical detection of Clostridia species in paraffin-embedded tissues of experimentally inoculated guinea pigs". *Pesquisa Veterinária Brasileira* 25 (2005): 4-8.

41. Almond PH and Bilkei G. "Clostridium novyi caused outdoor sow mortality in croatia". *Berliner Und Munchener Tierarztliche Wochenschrift* 118.7-8 (2005): 296-299.

42. Assis RA., *et al.* "Detection of several clostridia by a direct fluorescent antibody test in formalin-fixed, paraffin-embedded tissues". *Arquivo Brasileiro de Medicina Veterinária e Zootecnia* 59 (2007): 1319-1322.

43. Hauer Paul J and Ricardo F Rosenbusch. "Appendix a. multiplex polymerase chain reaction for identifying alpha and beta toxin genes in clostridium novyi and clostridium hemolyticum". Phospholipase C of Clostridium hemolyticum: gene characterization and proof of its role as a protective immunogen in guinea pigs (2013): 73.

44. Kojima A., *et al.* "Rapid detection and identification of Clostridium chauvoei by PCR based on flagellin gene sequence". *Veterinary Microbiology* 78.4 (2001): 363-371.

45. Heffron A and Poxton IR. "A PCR approach to determine the distribution of toxin genes in closely related Clostridium species: Clostridium botulinum type C and D neurotoxins and C2 toxin, and Clostridium novyiα toxin". *Journal of Medical Microbiology* 56.2 (2007): 196-201.