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Enterotoxemia in Albanian Zoo-Park Llama (*Lama glama*): *Clostridium perfringens*-type C was the Causative Agent

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Abstract: *Clostridium perfringens* causes enteric diseases in mammals, usually called enterotoxemia. *C. perfringens* was isolated from ileum and jejunum of dead llamas in one of zoo-parks in Tirana. Diagnosis of the disease was based on clinical sings, pathologically and also in isolation of the causes. Isolates colonies in agar blood were like dewdrops, smooth, gray, shiny and convex. In microscopic preparations colored and prepared with Gram technique were visible small bacillus, in sticks shape and Gram positive. Biochemical identification with API 20A kit valued isolates catalase and lecithinase positive, with hemolytic activity in agarblood from sheep with a dual zone of hemolysis. Isolates fermented producing gas and acid toward glucose, fructose lactose, sucrose and mannitol. Biological test of neutralization in white mice with antitoxins α and β, determined type C of *C. perfringens*as cause of llamas death, a result that was confirmed by ELISA kit. Besides treatment with drugs for pre-protection of llamas was used bivalent killed vaccine with types C and D of *Clostridium perfringens*.

Keywords: *Enterotoxemia, C.perfringens, API 20A, biological test of neutralization, ELISA, llamas.*

Introduction

Enterotoxemiacaused by *C. perfringens*is a fatal enteric disease and affects all agricultural animals, animals of zoo-parks and humans.This disease is one of the most frequent illness, widespread throughout the world, with a prevalence level ranging from 24,13 to 100% (El Idrissi, and Ward, 1992; Greco et.al. 2005). *C. perfringens*is a Gram positive bacillus, anaerobic bacteria that causes infections in animals and humans. Diseases caused by this microorganism in most cases are threatening the health of animals, which often end with their death. *C.perfringens*is widespread in the environment and also he is an integral part of ecological community of the intestinal tract of animals and humans. There are over 300 species but a small number of them, about 11 species, have interest in veterinary. Most of the pathogenic species produce one or more exotoxin with different potentials. Pathogenicity of this microorganism is closely related to exotoxins which he produces (Songer 1996; Uzal et.al. 2008).

Based on synthesis of four toxins, which have powerful lethal action (alpha, beta, epsilon and iota), *C. perfringens*consists of five types: A, B, C, D and E. However, as a result of different combinations *C. perfringens*can produce up to 15 toxins. *C. perfringens* type A produce only toxin alpha, type B produce toxins alpha, beta and epsilon, type C produce toxins alpha, beta, type D produce toxins alpha, and epsilon and type E produce toxins alpha and iota. Any type of toxin, in different animal species, causes specific enteric infection (Al-Humiany 2012; Fayez et al., 2013; McDonel 1986; Walker 1993). Diagnosis of enterotoxemia is usually based on clinical signs and anatomopathology, but to confirm the diagnoses is necessary identification of toxins in the content of intestine or in culture media developed in vitro. Classic identification of toxins is based on neutralization test in white mice and/or guniea pigs. However, this method it is tedious, expensive and takes time to be realized. In recent years for identification and characterization of toxins of *C. perfringens*is used successfully an ELISA kit with sensitivity 90.5% and specificity98,4% (Holt et al., 1994; Naylor et al., 1997; Sterne & Batty 1975).

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Material and Method

Samples from the intestinal content

Samples from the intestinal content were collected from dead llamas, which were suspected of having died from enterotoxemia. All samples were taken in aseptic form by ileum and jejunum. The sample was transferred to the laboratory in refrigerated conditions, after that were elaborated. Processed samples were conserved at –20 °C. Samples were divided in two parts. A part was used for bacterial isolation, and the rest after being diluted in the ratio 1/5 with PBS mediumwas centrifuged in 2000 g for 20 minutesat 4^0C. After centrifugation, the supernatant through membrane filter 0,45 μm and was preserved at -70^0C to satisfy the test for the identification of toxins (Fayez et.al 2013; McDonel 1986; Özcan & Gürçay 2000).

Bacterial isolation

Samples from intestinal contents were inoculated in medium: broth cooked meat (Oxoid), liquid broth (taroc)+ glucose +yeast extract than were incubated in Gas pack jar for 24 hours at 37^0C. From increased cultures through Drigalski spatulawere conducted passages in Petri dishes with medium agar blood from sheep 5%, which also contained neomycin sulphate supplement.Dishes were incubated in Gas pack jar for 48 hours at 37^0C. Identification of colonies was based on morphological characteristics and hue with Gram method. While biochemical identification was made by using commercial API 20A kit (bioMerieux SA, Marcy l'Etole, France). (Fayez et al., 2013; Songer 1996; Uzal et al., 2008; Walker 1993).

Biological tests for the presence of toxins from*C.perfringens*

Strains isolated and morphologically identified, with Gram stain and biochemical test were cultivated in broth cooked meat for 24 hours at 37^0C. After cultivation tubes were centrifuged at 3000 g for 15 minutes. Supernatant was taken and was divided in two parts. One part was treated with trypsin 1% to the quantity and then was incubatedfor 1 hourat 37^0C.

White mice, weighing 25-40 g, were injected intravenously with 0.3 ml supernatant and then were observed from 10 hours to 3 days after the injection, if there manifested death or clinical signs of enterotoxemia disease. In the same way, mice were injected with 0,3 ml of filtrate of intestinal contents. Control group of mice was injected with PBS medium (Naz et.al 2012; Sterne and Batty 1975; Uzal et.al 2003).

ELISA

For the detection and identification of toxins, from both, supernatant of cultures from isolated strains and filtrate of intestinal contents were examined with commercial ELISA kit (Bio-X Diagnostics). In rows, A, C, E and G are mounted specific antibodies monoclonal and polyclonal (antitoxins α, β, ε and a specific monoclonal antibody to a specific protein of *C. perfringens*), while in rows B, D, F and H are mounted non-specific antibodies, which serve as the negative control test. ELISA test was conducted and interpretation of results was under relevant instructions (El Idrissi & Ward, 1992; Naz *et al*., 2012; Uzal *et al*., 2003).

Results and Discussion

*C. perfringens*causes gastro- intestinal infections in almost all mammal species. These infections are usually nominated enterotoxemia because toxins produced in intestine can be absorbed and distributed through bloodstream. However, while this is true for many toxins of *C. perfringens*, some toxins produced in the intestine have only local action, while other toxins besides local action give systemic action. Clinical signs expressed more in llamas were: immediate loss of appetite, depression and in face they look disoriented, cramps and lying on one side of the body. High temperature and hemorrhagic diarrhea was observed (Greco et al., 2005; McDonel 1986; Songer 1996).

In post-mortem examinations noticed: the small intestine mucosa, mainly ileum and jejunum, presented with a hemorrhagic enteritis associated with necrosis in dark red color. Pathologically this one dedicated to toxin beta. Congestion and swelling were visible in the liver, kidney, lungs and spleen. Pericardial sacs when opened contained large quantities of pericardial fluid in the presence of fibrin. Lung presented with interstitial edema. Course of illness was fast and with a degree of almost 100% mortality (Greco et al., 2005; McDonel, 1986; Uzal et al., 2008).

Pathogenesis of infection caused by *C.perfringens*is directly related to the action of toxins and enzymes. Source of infection are usually spores that were taken through dirty food or found in the intestines of healthy animals, as saprophytes, which are virulent when the body of the animal is weakened, by bad condition of fertilization or by consumption of inappropriate foods and with high level of carbohydrates (McDonel 1986 ; Özcan & Gürçay 2000; Songer 1996).

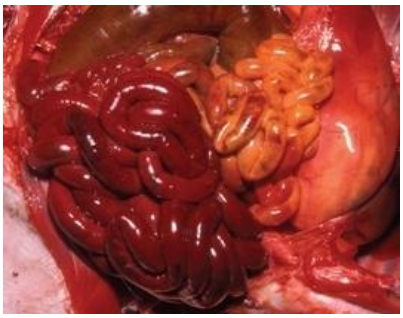

Figure 1. Hemorrhagic enteritiscaused by *C. perfringens*.

The outbreak of disease is conditioned by the presence of favorable conditions, that make possible to develop the cause of the disease, infestation of parasitosis, mainly fascioliasis.Regarding this problem among the most important factors that cause this pathology are: non-infectious factors and infectious factors et.al (Al-Humiany, 2012; Fayez *et al*., 2013; Sterne & Batty, 1975).

Isolated bacterial strains showed characteristics of *C. perfringens*. Colonies in agar blood were like dewdrops, smooth, gray, shiny and convex. In microscopic preparation colored and prepared with Gram techniques, were visible small bacillus, in sticks shape and Gram positive (Al-Humiany, 2012; Sterne & Batty 1975; Walker 1993).

Figure 2. Hemolisis caused by *C. Perfringens*in agar blood

Biochemical identification valued isolates as catalase and lecithinase positive, with a hemolytic activity in agar blood from sheep with a dual zone of hemolysin. They fermented, producing gas and acid from glucose, fructose, lactose, sucrose and mannitol. Also isolates were urease negative and gelatinase positive. Based on morphological characteristics, coloring,biochemicaltests and consulting with Bergey's Manual for bacteriological determination (Holt *et al*., 1994) isolates were identified as *C. perfringens*.

In connection with biological tests realized in white mice with broth supernatant, with trypsin supernatant and filtrate from intestinal content were obtained the following results: mice injected with broth filtrate and with the mixing broth filtrate +antitoxinαdied along, while mice injected with broth filtrate +antitoxinα+βand mice injected with broth trypsin filtrate +antitoxinαsurvived.

We emphasize that the mice died along within 10-12 hours of injection. Positive results in biological test of neutralization in white mice and clinic-pathologically in dead llamas prove that the cause of their death is *C. perfringens.*

Based on the results obtained, conclude that the cause of death in llamas is *C. perfringens* type C. Despite the fact that the mixture broth trypsin filtrate + antitoxinαwe have not included antitoxin βand come up to the conclusion that the cause of death in llamas is *C. perfringens*type C, toxin which is produced by this strain, but trypsin activates toxin ε and in the same time destroys toxin β. Note that toxin beta of *C. perfringens*is considered the major factor of virulence for type C of *C. perfringens* (Greco et al., 2005; McDonel 1986; Naz *et al*., 2012; Uzal., 2003).

ELISA kit identified toxins alpha and beta confirming that the cause of death in llamas is *C. perfringens* type C (El Idrissi & Ward 1992; Naylor et al., 1997; Uzal et al., 2003). Obtained results by ELISA kit comply with biological test of neutralization in white mice(El Idrissi & Ward 1992) state that ELISA kit compared with neutralization test in white mice and bacteriological isolation has a sensitivity and specificity on borders 90.5%-89.2%, respectively, for toxin β and for toxin α over98.4%.

Table 1. Neutralization test and biological characterization of *C. perfringens*

No	Mixture	Injected heads	Types of *C. perfringens*				Result
			A	B	C	D	
1	Broth filtrate	2	+	+	+	+	2 dead heads
2	Broth filtrate + antitoxinα	2	-	+	+	+	2 dead heads
3	Broth filtrate + antitoxinα+ antitoxinβ	2	-	-	-	-	2survived heads
4	Broth trypsin filtrate + antitoxinα	2	-	-	-	-	2 survived heads
5	PBS medium	2					Survived

Generally some commercial ELISA have higher sensitivity and can detect low levels of concentrations for toxins from intestinal contents and from other body fluids of animals, concentrations which are insufficient to cause disease. Exactly based on this finding, diagnosis of enterotoxemia should always be supported clinic- pathologically (El Idrissi & Ward 1992; Naylor et.al 1997; Uzal et.al 2003).

Treatment and prevention

Exotoxins produced by*C. perfringens*have almost all antigenic features and stimulate the body to produce specific antibodies, antitoxins. Exotoxins, as a result of their treatment with formalin, may be transferred to non-toxic agents called toxoid or anatoxin. Anatoxins have lost their toxic properties, while have preserved unchanged their antigenic properties, serving as an effective means for immunizing organism.

To prevent enterotoxemia, vaccination against *C. perfringens*is the only important bioproduct for protection of animals (Bernáth et al., 2004; Naz et al., 2012). Typesidentification of *C. perfringens*in certain areas are necessary because on this identification defined types of *C. perfringens* that should be included in the vaccine.

In this zoo-park besides the use of antibacterial agents, which made possible reducing the severity of clinical signs of the disease, symptomatic therapy was used. To prevent recurrence of infection was used killed vaccine with types C and D of*C. perfringens*according to a well-defined scheme.

Conclusions

The cause of death in llamas is *C. perfringens*. Biochemical confirmation of C. perfringens were carried out with commercial API 20A kit.Neutralization test and ELISA test identified as the cause of death of llamas type C of *C. perfringens*. For specific pre-protection against enterotoxemiain the zoo-park was used bivalent killed vaccine with types C and D of *C. perfringens*.

References

Al-Humiany A., (2012). Microbiological Studies On Enteritis Caused By Clostridium perfringens Type A, In Sheep In Saudi Arabia. *J. Appl. Sci. Res*., **8**, 836-844.

Bernáth S, Fábián K, Kádár I, Szita G, Barna T, (2004) Optimum time interval between the first vaccination and the booster of sheep for Clostridium perfringens type D. Acta Vet. Brno. **73**, 473- 475.

El Idrissi AH, Ward GE, (1992). Evaluation of enzyme-linked immunosorbent assay for diagnosis of Clostridium perfringens enterotoxemias. *Vet. Microbiol*. **31**, 389-396

Greco G, Madio A, Buonavolia D, Totaro M, (2005). Clostridium perfringens toxin-types in lambs and kids affected with gastroenteric pathologies in Italy. *Vet. J.* **170**, 364-350.

Fayez MM, Al Musallam A, Al Marzoog A, (2013) Prevalence and toxinotyping of the toxigenic C.perfringens in sheep with suspected enterotoxemia. *Nature & Science*, **11**, 15-21.

Holt GH, Krieg NH, Sneath PHA, Stanley JT, Williams ST, (1994) Bergey's manual of Determinative Bacteriology. 9th Edition. Williams and Wilkins publications Baltimore.

McDonel J. L. (2013). Toxins of Clostridium perfringens type A, B, C, D, and E. In: Dorner F., Drews, H. Eds., Pharmacology of Bacterial Toxins. Pergamon Press, Oxford1986. *Nature & Science,* **11**(8) 21.

Naylor RD, Martin PK, Barker LT, (1997) Detection of Clostridium perfringens toxin by enzyme-linked immunosorbent assay. *Res. Vet. Sci*. **63**:, 101-102.

Naz S, Ghuman MA, Anjum AA, (2012) Comparison of Immune Responses Following the Administration of *Enterotoxaemia* Vaccine in Sheep and Goats. *J. Vet. Anim. Sci*. **2**, 89-94.

Özcan C, Gürçay M, (2000) Enterotoxaemia incidence in small ruminants in Elazıg and surrounding provinces in 1994-1998. *Turk. J. Vet. Anim. Sci*. **24**, 283-286.

Songer JG. (1996). Clostridial enteric disease of domestic animals. Clin. Microbiol. 9: 216-234

Sterne M, Batty I, (1975). Criteria for diagnosing clostridial infection, p79–122. In. Pathogenic clostridia, Butterworths, London,United Kingdom

Uzal FA, Kelly WR, Thomas R, Hornitzky M, Galea F, (2003) Comparison of four techniques for the detection of C.perfringens type D epsilon toxin in intestinal contents and other body fluids of sheep and goats. *J. Vet. Diagn. Invest*. **15**, 94-99.

Uzal FA, Songer JG, (2008) Diagnosis of *C.perfringens* intestinal infections in sheep and goats. *J. Vet. Diagn. Invest*. **20**, 253-265

Walker PD, (1993). Clostridium. Diagnostic procedures in veterinary bacteriology and mycology. 229-251.