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PREVALENCE OF *CLOSTRIDIUM PERFRINGENS* AND *CLOSTRIDIUM PERFRINGENS*-LIKE ORGANISMS IN FAECAL SAMPLES OF DOMESTIC ANIMALS

حدوث المطثيات الحاطمة والعضيات الشبيهه بها في عينات الروث في الحيوانات المستأنسة

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المستخلص

تهدف هذه الدراسة لتحديد معدل انتشار المطثية الحاطمة وشبيهات المطثية الحاطمة في المجترات الأليفة، الفصيلة الخيلية والدجاج، و لتوضيح الخلط بين هذه الميكروبات. جمعت عشرة ومئتا عينة من براز الأبقار، الضأن، الماعز، الإبل، الخيول، الحمير والدجاج، وقد أ شتملت على ثلاثين عينة من كل نوع . زرعت عينات البراز في وسط اللحم المطبوخ ثم تم التزريع من وسط اللحم المطبوخ في أجار الدم وتم التحضين تحت ظروف لاهوائية. تم تشخيص المستعمرات النقية على مستوى النوع بواسطة اختبارات كيموحيوية محددة، وإختبارإنتاج الليسيثين بالإضافة إلى إختبار التحلل الدموي التآزري. تم عزل المطثية الحاطمة وشبيهات المطثية الحاطمة من ثلاثين ومائة عينة برازمن أصل عشرة ومائتي عينة براز، خمسون عينة (38.5%) كانت عبارة عن المطثية الحاطمة بمعدل انتشار 23.8% من الحيوانات تحت الاختبار. ثمانون عينة (61.5%) كانت عبارة عن شبيهات المطثية الحاطمة والتي تضم المطثية الساردنينسية، المطثية الأبسونمية، المطثية البيجرنسكية، المطثية السيلاتمية والمطثية الباراتية بمعدل انتشار 27.6%، 4.8%، 3.3%، 1.9% و0.5% على التوالي. أعلى نسبة من المطثية الحاطمة سجلت في عينات الدجاج يتبعها الحمير ثم الإبل، بينما كانت أعلى نسبة من شبيهات المطثية الحاطمة في الحمير ثم الإبل. كما أوضحت النتائج أن المطثية الحاطمة وشبيهات المطثية الحاطمة وجدت في جميع أنواع الحيوانات التي تم إختبارها مع اختلاف في النسب المئوية لإنتشارها في هذه الحيوانات، بحيث يجب أن لا تغفل هذة النتائج أثناء عزل المطثية الحاطمة.

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Abstract

The aim of this study was to determine the prevalence of *Clostridium perfringens* and C. *perfringens* like organisms in domestic ruminants, equines and chicken and to clarify the confusion between these organisms.

In this study, two hundred and ten faecal samples were collected from seven animal species (cattle, sheep, goats, camels, horses, donkeys and chicken. Thirty samples were taken from each species. The faecal samples were cultured into cooked meat medium and then subcultured on blood agar under anaerobic condition. Pure colonies were identified to the species level using biochemical tests, lecithinase production as well as synergistic hemolysis.

Out of the 210 faecal samples, 130 isolates of *C. perfringens* and *C. perfringens*-like organisms were isolated. 50 isolates were *Clostridium perfringens* which constituted 38.5% of them i.e., with a prevalence rate of 23.8% in the animals tested. Eighty isolates (61.5%) were *C. perfringens*-like organisms which included *C. sardiniensis*, *C. absonum*, *C. beijerinckii*, *C. celatum* and *C. barati*, with prevalence rates of 27.6%, 4.8%, 3.3%, 1.9% and 0.5%, respectively. Chicken gave the greatest number of *C. perfringens* followed by donkeys and then camels, while the greatest number of *C. perfringens*- like organisms was detected in camels and then donkeys.

The results revealed that *C. perfringens* and *C. perfringens*-like organisms were detected in all animal species examined with varying percentages and not be overlooked during the process of isolation of *C. perfringens*.

Key words: Clostridium perfringens and Clostridium perfringens-like organisms.

Introduction

Clostridium perfringens was reported to cause intestinal disturbance in man (Klein, 1895). It was also recovered from cases of gangrenous appendicitis in man (Veillon and Zuber, 1897) who called it *Bacillus perfringens*

Clostridium perfringens type A occurs in intestinal tracts of humans and other animals and in most soils. Types B, C, D, and E are found mostly in the intestinal tracts of animals, and their survival in soil is variable. Transmission is by ingestion and wound infection (Hirsh et al., 2004).

The major diseases associated with the various types of *C. perfringens* which include necrotic enteritis in chicken, necrotizing enterocolitis in piglets, canine hemorrhagic gastroenteritis, gas gangrene and food poisoning in humans were caused by type A. Lamb dysentery and hemorrhagic enteritis in calves and foals were caused by type B. Struck in adult sheep, sudden death in goats and feedlot cattle, necrotic enteritis in chickens, and hemorrhagic enteritis in neonatal piglets were caused by type C. Pulpy kidney in sheep and enterotoxaemia in calves and goats were caused by type D (Fox, 2007). Hemorrhagic enteritis in calves and enteritis in rabbits were caused by type E (Quinn et al., 2002), enterotoxaemia in humans were caused by type F (Stewart and Beswick, 1977).

Clostridium perfringens-like organisms are group of physiologically related strains. They have similar morphology and biochemical properties with *C. perfringens* (Abdel Salam and El Sanousi, 1991).

Although early workers described outbreaks of diarrhoea which they ascribed to *C. perfringens* (Klein, 1895; Andrewes, 1899; Simonds, 1915), little interest was given to the role of *C. perfringens* as a major cause of food poisoning until the report of Willis and Hobbs et al. (1959). They described a clearly defined form of food poisoning, with symptoms of diarrhoea and acute abdominal pain which usually begin 8-14 h after ingesting a meat meal containing large numbers of viable *C. perfringens* and persist for 2-24 h. The patients usually recover within 24 h.

Research in *C. perfringens*-like organisms is very meager. In Sudan only a few references were cited (Abdel Salam, 1986; Samia, 2003).

The need for public health laboratories to examine samples of food and faeces for C. perfringens during the routine investigation of food poisoning outbreaks should be apparent (Shapton and Board, 1975).

This study was intended to:

1. Isolate and identify *C. perfringens and C. perfringens*-like organisms from faecal samples of ruminants, equines and chicken.
2. Recognize the prevalence of these organisms in these animals to prevent the confusion between these organisms in isolation of *C. perfringens.*
3.

Materials and Methods

Sample Preparation and Isolation Cultivation

210 Faecal samples were collected from seven species of domestic animals (cattle, sheep, goats, camels, horses, donkeys, and chicken) from Khartoum State. Thirty samples were collected from each species by swabs taken directly from rectum (about 2 grams of faeces) then they were transported in ice box to the laboratory. On the same day of collection the samples were treated immediately as follows:

A portion of faeces (1 gram) was inoculated into preheated and quickly cooled cooked meat medium, and incubated in water bath at 42^{o} C for 2 hours, and was then subcultured on blood agar. This method enhanced growth of *C. perfringens* and *C. perfringens*-like organisms and suppressed the growth of other organisms (El Sanousi, personal communication). Blood agar plates were incubated anaerobically using a BTL jar (England) filled with hydrogen generated from a gas kit (Oxoid). After an incubation for 24 h, colonies suspected for *C. perfringens* and *C. perfringens*-like organisms (gray colonies which gave gram positive rod cells with blunted edges under microscope) were purified by further subculturing on blood agar and incubated anaerobically as described previously. Pure isolates were preserved in cooked meat medium.

Methods of the isolates

The scheme used was prepared by Abd Elsalam and El Sanousi (1991). It was designed to differentiate between *C. perfringens* and *C. perfringens*- like organisms. The scheme was used with some modifications. It included lecithinase production, synergistic hemolysis,

LGNM (lactose, gelatin, nitrate and motility) tests and sugar fermentation tests.

Synergistic Hemolysis Test (The CAMP Test)

The plate of blood agar was inoculated with a single diametric streak of ß-toxin-producing strain of *Staphylococcus aureus* (D.Z.1t). The test isolates were streaked (inoculated) at right angles to but not touching the staphylococcal inoculums and it was incubated anaerobically at 37^{o} C overnight.The plate was examined while still warm (Barrow and Felthman, 2003).

LGNM Tests and Sugar Fermentation Tests

Lecithinase Test (Opalescence on Egg-Yolk Agar)

Egg-yolk plate was inoculated with a single streak of organism and incubated anaerobically at 37^{o} C overnight.according to the method described by (Barrow and Felthman, 2003)

As stated by egg-yolk reactions depend on lecithinase C and lipase activity both types of enzymes produce quite distinct opacity changes in egg-yolk agar. Lecithinase C hydrolyzes lecithin into phosphorylcholine and an insoluble diglyceride, the reaction resulted

Motility Test

Tube of motility medium was Stab-inoculated to a depth of about 5 mm. It was incubated anaerobically at 37^{o} C for 5 days.

(Barrow and Felthman, 2003) reported that motile organisms which migrated throughout the motility medium, became turbid; growth of non-motile organisms were confined to the stab-inoculums .

Gelatinase Test

Tubes of gelatin of different concentrations (12%, 10% and 2%) were used in these tests. These were inoculated with the organism to be examined and it was incubated anaerobically at 37^{o} C for 7 days. The gelatin was placed in a refrigerator.

(Salle, 2007). reported that when the gelatin is hardened, the organism under examination does not produce gelatinase into the culture medium. When the gelatin is in the liquid form, the organism under examination produces gelatinase into the culture medium .

Sugar Fermentation Test

The organism was inoculated in the sugar media (Lactose, raffinose, galactose, cellobiose and sucrose) then it was incubated anaerobically at 37^{o} C. The media was examined daily for 7 days for acid (Barrow and Felthman, 2003 and(Baird *et al*., 2000)..

Nitrate Test

Nitrate Broth was inoculated lightly and it was incubated anaerobically at 37^{o} C for up to 5 days as described by (Barrow and Felthman, 2003). 1 ml of nitrite reagent A was added to nitrate medium followed by 1 ml of reagent B. A deep red color indicated the presence of nitrite and showed that nitrate had been reduced, this suggests a positive reaction. Powdered zinc was added (up to 5 mg/ml of culture) to tube that did not show a red color within 5 min.. Red color indicated the presence of nitrate in the medium i.e. not reduced by the organism. Absence of red colour indicated the absence of nitrate in the medium i.e. reduced by the organism to nitrite, (Barrow and Felthman, 2003).

Results

Isolation of *Clostridium perfringens* and *Clostridium perfringens*-like organisms

130 isolates (61.9%) out of the 210 faecal samples examined were found positive.

The Cell Morphology and Gram Reactions

All isolates were Gram-positive bacilli with blunt ends in smears prepared from fresh cultures.

Growth and Hemolysis on Blood Agar

All isolates developed grayish colonies, 2-4 mm in diameter, round in shape on sheep blood agar Some colonies were granular with irregular edges, glistening and with a low convex surface. All isolates were non mucoid except one isolate from a donkey. With regards to haemolysis, it was found that 122 (93.8%) isolates did not produce any hemolysis but 8 (6.2%) isolates developed double zone of hemolysis. Two of them were from sheep, one from camels and one from horses. All of these isolates were identified as *C. sardiniensis*. On the other hand one isolate from

horses was identified as *C. pefringens*, one isolate from goats was *C. barati* and one isolate from each chicken and donkeys was *C. celatum*. The colonies were surrounded by an inner zone of complete hemolysis followed by a zone of incomplete hemolysis.

Growth of pure isolates in Cooked Meat Medium

All isolates propagated in this medium for 24 h at 37^{o} C, produced a turbidity. The majority of isolates 79 (60.8%) were saccharolytic whereas 51 (39.2%) of them were non saccharolytic.

Growth in Air

There was no growth of all isolates on blood agar incubated aerobically at 37^{o} C for 24 hours.

Opalescence on Egg-Yolk Agar

One isolate from horses (0.8%) produced opalescence (lecithinase positive) on egg-yolk agar as judged by an intense diffuse opaque haloes in the medium around colonies and zones of growth.

Synergistic Hemolysis

One isolate from goats (0.8%) produced synergistic hemolysis with *Staphylococcus aureus*. It was diagnosed as *C. barati*. Diffusion of ß-toxin produced a zone of discolouration of the sheep erythrocytes. Production and diffusion of the CAMP factor from organisms under test yielded a completely clear area having a bullet-like shape in the zone of discolouration caused by the ß-toxin .

LGNM Tests and Sugar Fermentation Tests

All isolates were tested on LGNM media (Lactose, Gelatin, Nitrate and Motility). Only 57 (43.8%) isolates were diagnosed to the species level by these tests, 50 of them were *C. perfringens* and 7 (5.4%) of them were *C. beijerinckii* (Table 1). All isolates were lactose fermenters, 123 (94.6%) of them were nitrate reducers and non motile and 7 (5.4%) of them did not reduce nitrate and were motile. The majority of the isolates 78 (60%) did not liquefy gelatin, 47 (36.2%) of them were strong gelatin liquefiers and 5 (3.8%) of them which were isolated from poultry samples, were weak gelatin liquefiers

Table (1): Lactose, Gelatin, Nitrate and Motility tests for identification and differentiation of *Clostridium perfringens* and *Clostridium perfringens*-like organisms.

Identification	Lactose	Gelatin	Nitrate	Motility
C. perfringens	+	+	+	-
C. beijerinckii	+	±	-	+

Isolates were not identified by the LGNM tests to the species level (72 isolates) were tested by raffinose medium. Only 58 (44.6%) of isolates did ferment raffinose and diagnosed as *C.* sardiniensis. The rest isolates (14, 10.8%) were diagnosed by Galactose, Cellobiose, Succrose and gelatin (gelatin 2% and gelatin 10%) media. Ten of them were *C. absonum* and 4 were *C. celatum* (Tables 2).

Table (2): Galactose, Sucrose, Cellobiose and Gelatin (2% - 10%) tests for identification and differentiation of *Clostridium perfringens and Clostridium perfringens-like organisms.*

Identification	Galactose	Sucrose	Cellobiose	Gelatin 2%	Gelatin 10%
C. absonum	+	+	-	+	-
	-				
C. celatum	-	+	Not done	+	+

Fifty Clostridium perfringens were isolated out of 130 isolates (38.5%) with a prevalence rate of 23.8%. Clostridium perfringens-like organisms were counted in 80 isolates with a prevalence rate of 61.5%. Fifty eight isolates were identified as Clostridium perfringens-like organisms

including C. sardiniensis, 10 C. absonum, 7 C. beijerinckii, 4 C. celatum, and 1 C. barati. The prevalence of these organisms in different domestic animals is illustrated in Tables 3 and 4.

Table (3): Total Prevalence of C. perfringens and C. perfringens-Like Organisms in Faecal Samples of Some Domestic Animals.

Animal Species	*C. perfringens*	percentage	*C. perfringens*-Like Organisms	percentage
Cattle	5	**16.7**	12	**40**
Sheep	1	**3.3**	10	**33.3**
Goats	4	**13.3**	11	**36.7**
Camels	8	**26.7**	18	**60**
Horses	3	**10**	6	**20**
Donkeys	11	**36.7**	14	**46.7**
Chicken	18	**60**	9	**30**
Total	50	**38.5**	80	**61.5**

Table (4): Prevalence of C. *perfringens*, C. *beijerinckii*, C. *barati* C. *sardiniensis*, C. *absonum* C. *celatum* in Faecal Samples of Some Domestic Animals.

Animal Species	*C. perfringens*	*C. beijerinckii*	*C. barati*	*C. sardiniensis*	*C. absonum*	*C. celatum*
Cattles	5	4	0	6	2	0
Sheep	1	1	0	9	0	0
Goats	4	0	1	9	1	1
Camels	8	0	0	13	3	1
Horses	3	1	0	5	0	0
Donkeys	11	1	0	9	3	1
Chicken	18	0	0	7	1	1
Total	**50**	**7**	**1**	**58**	**10**	**4**
Percentage	**23.8**	**3.3**	**0.5**	**27.6**	**4.8**	**1.9**

Discussion

The study was designed to isolate and identify *Clostridium perfringens* and *Clostridium perfringens*-like organisms.

The scheme used in this study was made by Abdel Salam and El Sanousi (1991) and it was based on the findings mentioned by Willis and Hobbs (1959); Nakamura *et al.* (1973); Buchanan *et al.* (1974); Smart *et al.* (1979); Gubash (1980); Narayan (1982); De Boer and Boot (1983) and Krieg and Holt (1986).

On sheep blood agar, 122 (93.8%) isolates of *C. perfringens* and *C. perfringens*-like organisms didn't cause any haemolysis but 8 (6.2%) isolates developed double zone of hemolysis indicating that *C. perfringens* and *C. perfringens*-like organisms may be found in isolates that didn't cause a double zone of haemolysis . These findings disagree with the results obtained by Sterne and Batty (1975) and Samia (2003), who reported that, all isolates showed hemolysis for sheep erythrocytes and produced double zone of hemolysis. On the other hand these findings agree with Abdel Salam and El Sanousi (1991), who reported that some isolates of *C. perfringens* and *C. perfringens*-like organisms were non hemolytic on sheep blood agar due to weak production of hemolysin.

One isolate from goats (0.8%) produced synergistic haemolysis with *Staphylococcus aureus* and gave bullet shaped synergistic hemolysis. It was diagnosed as *C. barati.* This finding agrees with Abdel Salam and El Sanousi (1991).

In cooked meat medium all isolates produced turbidity. Some isolates, 79 (60.8%) were saccharolytic whereas 51(39.2%) of them were non saccharolytic. This finding is similar to the findings of Abdel Salam and El Sanousi (1991) and Samia (2003).

All isolates did not produce opalescence on egg-yolk agar i.e they did not produce lecithinase enzyme except one isolate from horse which was identified later as *C.* perfringens. This finding agrees with Abdel Salam and El Sanousi (1991).

The majority of isolates 123 (94.6%) were non motile, 7 (5.4%) were reported to be motile. *C. beijerinckii* was the only isolate that showed motility. This finding agrees with Haroun (1989), Abdel Salam and El Sanousi (1991) and Samia (2003).

Many isolates, 78 (60%) did not liquefy gelatin which were *C. perfringens*-like organisms. Forty seven (36.2%) of isolates were strong gelatin liquefiers and 5 (3.8%) of them were weak gelatin liquefiers. The gelatin liquefiers were *C.* perfringens and C. perfringens-like organisms. These findings agree with Abdel Salam and El Sanousi (1991).

Only 72 out of 130 isolates were tested by raffinose media. Fifty eight of them were raffinose fermenters which were diagnosed as *C. sardiniensis*. This finding agrees with Abdel Salam and El Sanousi (1991).

The remaining 14 isolates, which did not ferment raffinose, were tested by galactose and cellobiose. Only 6 of them were galactose fermenters and they didn't ferment cellobiose.

The previous fourteen isolates were tested by sucrose and gelatin (gelatin 2% and gelatin 10%) media. Four of them were sucrose fermenters and gelatin 2%, 10% liquefiers which were diagnosed as *C. celatum,* 10 of them were sucrose fermenters and gelatin 2% liquefiers but did not liquefy gelatin 10% and were identified as *C. absonum.* These findings agree with Abdel Salam and El Sanousi (1991).

Chicken showed the greatest number of *C. perfringens,* this result may be due to the high temperature of chicken. Camels showed the greatest number of *C. sardiniensis*.

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