The role of *Clostridium perfringens* in camel calf diarrhoea with special reference to the pathogenesis and pathology in the Sudan

By

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A thesis submitted in fulfillment of the requirement for the degree of Doctor of Philosophy (PhD.)

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December 2004
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Dedication

I dedicate this work to my mother, father, brothers, sisters and cousins.
Acknowledgement

I am grateful to the Sudan government for the scholarship which enable me to carryout this work.

It is pleasure to record my thanks and gratitude to my supervisor Prof. Suliman Mohamed ElSanousi for his guidance interest, encouragement, kindness and assistance throughout the execution and writing of this study.

I am indebted to the predecessor director of Central Vet. Res. Centre and the director of National Research council Prof. Ali Mohamed Abdel Majid for his encouragement, help and financial support during the field survey.

My thanks and gratitude goes to Prof. Mohammed Mohammed Salih the director of Cent. Vet. Res. Centre for his encouragement and help.

I wish to express my gratitude to Dr. A.V. Farzan, of the Department of Animal Anaerobic Bacteriology and Vaccine production, Razi vaccine and serum Research Institute for sending me C. perfringens antitoxin, and Prof. Mo.Salman of Colorado State University for sending me C. perfringens primers.

I am grateful to Dr. A/Malik Ibrahim Kalafalla and Prof. Imad ElDin Elamin Aradaib Arabia for the opportunity given to me to carryout the typing of the pathogen in their Departments of biotechnology.

Special gratitude to predecessor director of Kassala State Lab. Dr. Kamal Salih Hussein, successor director of Kassala State Lab. Dr. A/Hameed A/Razig, the director of Elgadarif State Lab. Dr. Ali Elgadal and Dr. Yahia Hassan Ali for their help during the field survey.

Special dept gratitude to my colleague Dr. Halima Mohammed Osman, Dr. Layla Ismail Mohammed Dr. Elzein Bahsir, Dr. Manal Hassan Salih, Dr. Dia El Din Ahmed Salih, Dr.Omer Mohamed Ahmed and Dr. Abuobieda for their value help and cooperation.
Thank are due to Mrs. Samira Amin Eisa and Mrs. Eltoma Mohamed Elamin for typing the manuscript.

Special gratitude to technical staff of Departments of Pathology and Diagnosis, in particular Babiker Ahmed Kalifa, A/Rahman Ahmed Saaid, Omima Hassan, Yousif A/Elwahab. The technical assistant of Sharf Eldin Mohamed Abdalla of the Department of Biological Product, Elfatih Hassan Abdalla of the Department of Biochemistry and Farog Idris of the Department of Radioisotopes are appreciated.

I gratefully acknowledge Dr. Gundi Suliman Gasmir for his value help and cooperation during field survey, experimental work and writing of the manuscript.
Summary

Diarrhoea and deaths in newly born camel calves, was noticed by veterinary investigators to be very high. Hence, it is thought necessary to investigate this problem from the bacteriological and pathological points of view.

Survey was conducted in diarrhoeic camel calves aged six months or younger. These animals were owned by the nomadic pastoralists of different tribes at Kassala, ElGedarif and Butana (Eastern region), Nahr ElNiel State (Northern region) and Kordofan Stae (Western region). These animals were local breeds including, Arab, Anafi, Bushari, Swahli and crosses between these breeds.

_C. perfringens_ was isolated from 97 (40.9%) out of 238 examined diarrhoeic faecal specimens. These isolates were analyzed by DNA amplification technique (PCR) in order to determine the prevalence of _α_, _B_, _B2_, _ε_, 1 and CPE toxin gene. All isolates were _C. perfringens_ evidenced by the demonstration of α-toxin. The most prevalent toxin type of the _C. perfringens_ isolates was found type A containing the α-toxin represented 97.9% (N=95), type D which was characterized by the production of α and E-toxin was isolates in 2.06% of all isolates (N=2). However, non of _C. perfringesn_ types B, C and E were identified. The enterotoxigenic _C. perfringens_ type A harbouring cpe gene represented 49.7% of type A (N=47). The recently discovered, not yet assigned, B2 toxigenic _C. perfringens_ type A was represented in 7.4% (N=7).

Experimental enterotoxaemia was successfully produced into camel calves following intraduodenal infusion of _C. perfringens_ type D strain 97, which produce α and ε-toxins and type A strain 95 which produce α-toxin, and B2 toxigenic type A strain 80 that screeat α and B2 toxins. Generally all three strains induced similar clinicopathological alteration with some minor differences in the intensity of
reaction. The necropsy lesion characterized by accumulation of oedematous fluid in pericardial, thoracic and peritoneal cavities. Haemorrhages, congestion and vasculitis with disrupted endothelial lining were hallmark in camel enterotoxaemia. These pathological changes were observed in brain, heart, lung, liver, kidney, lymph nodes, spleen, adrenal gland, pancreas, urinary bladder and gastrointestinal tract. The gross lesions were confirmed by histological findings. The most striking lesions in the brain were perivascular haemorrhages and oedema coexisting with focal to diffuse microglial proliferation, malacic lesions and neuronal degeneration. This lesion appeared to be more pronounced in animals inoculated with type D. Air bubbles in the encephalon were detected only in calves inoculated with type A strain 95. Accumulation of pink proteinaceous fluid in lung alveoli and dilated and Bowman’s space of kidney was initiated by the three strains, at variance, the proteinaceous maternal in the adrenal gland was produced by type D. Marked embarrassment to the circulation system was observed in animals inoculated with strain 80 particularly in the liver, where the outpouring of erythrocytes was detected in the dilated sinusoids. Odema of ruminal wall was more prominent in animals inoculated with type D. Gas gangrene was observed in cardiac and skeletal muscle of one animal inoculated with strain 95. Air bubbles in liver, spleen and adrenal glands were produced by type A.
CHAPTER 1

INTRODUCTION
Domesticated representative of family camelidae constitute an integrated part of national livestock population of many countries in arid and semi-arid zones of Africa and Asia as well as in the high mountain of Indies and South America.

The camel population in the world is estimated to be 18 millions camels. Of these about 16.5 million are the one-humped (*Camelus dromedarius*). Sudan and Somalia account for 70% of camels in Africa. In the Sudan, the last census estimate of camels’ population was 3 millions and is concentrated in two main regions. The Eastern state where camels are found in Butana plains and Red Sea hills and the Western regions, Darfour and Kordofan states, where camels are found in the northern parts.

The Butana plain, which occupied the area lying between Rivers Nile in the West and Atbara River in the East, is inhabited by different camels’ breeder tribes such as Shukriya, Lahawiyin, Kawahla and Rashaida. These tribes are ancient camels’ breeders who maintained a pastoralist life for centuries. Owning to fluctuation in rainfall and security of pasture, especially in rather long dry season (November to July) these tribes practice transhumance mode of range utilization.

Camels are well adapted to living in arid zone and had the ability to withstand thirst and survive on thorn and desert shrubs. These multipurpose animals represent an important source of food (milk and meat), clothing, shelter and transportation for the Bedwina.

Diarrhoea of newborn calves is one of the common diseases which the large animal clinician is faced with in practice (Blood, Henderson, Radostits, Arundel and Gay, 1979). Earlier neonatal calf diarrhea (NCD) was attributed to *Escherichia coli* (E.coli) and was designated calf scour. Subsequently, field and laboratory investigation confirmed that there may not be a single precise aetiology of NCD, but rather the cause is complex and usually involve the interplay between enteropathogenic bacteria, viruses, cryptosporidium parasite, immunity of host and effect of management and environment (Mebus, 1976; Blood et al., 1979; Tzipori, 1981 and Moore, 1989).
Clinically, NCD characterized by profuse watery diarrhea with consequent dehydration, electrolyte imbalance and death. Other clinical symptom such as septicaemia, enterotoxaemia and retarded growth were encountered (Radostits, Gray, Blood and Hinchcliff, 2000).

In the Eastern States of Sudan camel calf diarrhea (CCD) affect calves from seven days to five months of age with morbidity and mortality rate of 33% and 23% respectively (Abbas, 1993). The author attributed the aetiological agent to Salmonella spp. However, the epidemiological study of Agab and Abbas (1998) showed that diarrhea was the main cause of death among camel calves with the seasonal mortality rate of 80%, 39.1% and 20.6% during winter, summer and autumn respectively.

The entero-pathogen of CCD most commonly investigated or extensively studied were entero-toxigen E.coli (Romboli, 1942; Mohammed et al., 1998 and Salih, 1998); Rotavirus and coronavirus (Mohammed et al., 1998 and Ali, 2003). However, available reports in the association of C.perfringens with camel calves diarrhoea or enteric diseases were meager. Thus the current investigation was focused in the role and pathogenesis of C.perfringens in camel calves diarrhea. Furthermore, the study was conducted in response to the continuous complain of camel breeders to investigate the uncontrollable problem of CCD which affect the calf viability and herd growth.

The conventional method used for typing C. perfringens is the neutralization test either by intravenous inoculation of mice or intrademal inoculation of 9 pig skin. Though, this method is sensitive but have some limitation. Using living animals, which has inaccuracy of biological assay such as variation in individual sensitivity non specific toxicity from other substance, which may present in the intestinal content (Henderson, 1984), disfavour on humanitarian ground and animal welfare. Consequently the DNA amplification, polymearase chain Reaction (PCR), method was used in this study. Typing of C. perfringen was carried out using primer for α, B, E and I toxin gene (cpa-cpb, etx and AI). In this work primers of enterotoxin (cpe) and novel toxin B2 (cpb2) were incorporated because of the confirmed association between these toxin and animal enteritis (Sarker, Carman

The aim of this study is to define the important of *C. perfringens* as aetiological agent in camel calves diarrhoea, it prevalence and to recommend the measure need to be undertaken to minimize and control this syndromes.

I hope the information, which emerged in the current study, would be of value to ascertain and verify the importance of *C. perfringens* in camel calves diarrhoea.
CHAPTER II

LITERATURE REVIEW
2.1. Diarrhoea

Diarrhoea is the presence of water in faeces in relative excess in proportion to faecal dry matter. The consistency of faeces varies from soft to watery. Increase of frequency of defaecation is a common feature of diarrhea (Heywarth, 1985 and Radostitis, Gay et.al, 2000). Several enteric disease result in diarrhea such as colibacillosis and salmonellosis. Many of these diseases, tend to be clinically comparable in that profuse diarrhea, dehydration, electrolyte imbalance leading to acidosis and death or impaired growth are the major consequences. However specific enteric diseases causes diarrhea by varied characteristic mechanism included malabsorption, hypersecretion, hypermotility and increase motility (Moon, 1978).

2.1.1. Neonatal Calf Diarrhoea

Neonatal calf diarrhoea (NCD) is diarrhoea of newborn calf, calf scour, milk scour, dietetic diarrhea and calf dysentery are synonymous names for common enteric disorder which affect young animals such as cattle, lamb, foals, piglets and camels.

NCD is the most common disease complex that the large animals clinicians encountered in practice (Radostits, et.al, 2000). It has long been recognized as important cause of economic loss in calves of both dairy and beef herd industry worldwide. The economic loss is due not only to mortality, which vary from zero to eight, but also to medical cost and poor growth (Woode and Crouch, 1978).

Diarrhoea of young calf is a clinical entity with variable etiology involving the interaction between three components, host, microorganism and environment. Most investigators agreed that Diarrhoea can be attributed to infection with single or multiple agents (Morin, Lariviere, Lallier, Begin, Roy and Ethier, 1978; Tzipori, 1981 and Radostits et al., 2000). The enteropathogen incriminated in NCD including, rotavirus (Ares and Babiuk, 1978; Morin et al., 1978 and Abraham, Roeder and Zewdu, 1992); corona virus (Mebus, Stair, Rhodes and Twiehaus, 1973); reo-like virus (Mebus, Stair, Underdahl and Twiehaus, 1971; Acre and Radostits, 1976 and Lecce, King and Mock, 1976); Adenovirus (Bulmer, Tsai and Little, 1975), bovine
viral diarrhea (Lambert and Fernelius, 1968); paravovirus (Baker and Ames, 1987); Chlamydia (Doughri, Young and Storz, 1974); Escherichia coli (Acres, Saunders and Radostits, 1977 and Salih, Shigidi, Mohamed, McDough and Chang, 1998) Salmonella species (Tzipori, 1985 and Salih et al., 1998); Clostridium difficile (Jenes, Adney, Alexander, Shideler and Traub-Dargatz, 1988); Clostridium perfringens (Wernery, Ali, Wernery and Seifert, 1992; Elsanousi and Gameel, 1993) and Cryptosporidium species (Meuten, Van Kruiningen and Lein, 1974 and ElNour, 1994).

The most common clinical characteristic of acute enteritis, namely depression, anorexia, profuse watery diarrhea with or without dehydration are manifestation of infection by the majority of enteropathogen. Dehydration, emaciation and fluid filled intestinal tract with no obvious gross lesions are the most consistent necropsy findings. The exceptions are enteritis caused by Salmonella, C.perfringens and Eimeria species in which there are typical gross lesions (Radostits et al., 2000). Villous atrophy and replacement of enterocytes by immature cuboidal cells are typical histopathological changes shared by most of enteropathogen. Therefore the clinicopathological changes are inadequate criteria for diagnosis of specific pathogens. Differential diagnosis depends on laboratory results.

2.1.2. Camel Calf Diarrhoea

Literature on camel calf diarrhea is meager. However, epidemiological evidence suggested that in the Eastern State of Sudan camel calf diarrhea affect 33% of offspring causing 23% mortality (Abbas and Musa, 1988) and account for noticeable reduction in calf variability and herd growth (Abbas and Musa, 1988 and Rollelson, Mundy and Mathias, 2001).

The etiology of camel calf diarrhea is comparable to those of other animals namely cattle, lambs and piglets. It is a complex syndrome involving one or a combination of more than one specific agents. Previous investigation (Romboli, 1942) described E.coli infection in suckling camels, which resembled the neonatal coliform septicaemia in other animals. Furthermore, salmonella species was isolated from faeces collected from an outbreak of diarrhea in suckling camel calves in Somalia (Cheyne, Pegram and Catwright, 1977).
Epidemiological surveys of pathogens on faeces of scouring camel calves in Eastern Sudan revealed prevalence of 14.5%, 4.5% and 9% rotavirus, *E.coli* and *Salmonella species* respectively (Mohamed et al., 1998) and 66% *E.coli*, 13% *Salmonella typhi* and 10% untyped *Clostridium perfringens* (Salih et al., 1998). Later two outbreaks of *Clostridium perfringens* type A and D enteric disease in suckling camel calves had been reported from United Arab Emirates (Wernery, Ali et al., 1992) and Saudi Arabia (ElSanousi and Gameel, 1993) respectively. However, both outbreaks described severe disease which characterized by malaise, profuse diarrhea and the animal succumbed quickly. The most striking macro and microscopic changes were myocardial haemorrhages and degeneration. The field observation by Rolletson et al., 2001 showed that camel calf diarrhea complex is caused by either one or multiple etiological agents included rota and corona viruses, *E.coli*, *Salmonella* and suckling too much milk.

### 2.2. *Clostridium perfringens*

The term clostridium is a latin word means small spindle and perfringens was derived from latin word which means breaking through.

**Synonyms:** *Clostridium welchii, Bacillus aerogenous capsulatus, Bacillus phlegmonis emphsematosae, Welchii bacillus* and gas bacillus (Hagan and Brunes, 1961)

*C.perfringens* is the most widely occurring pathogenic bacterium (Smith and Willian, 1984) and certainly the most important cause of clostridial enteric and histotoxic diseases in both human and domestic animals (Senger, 1996 and 1997; Johnson and Gerding, 1997). This species produce a number of soluble substances that cause a variety of toxic effects in vitro and /or in vivo conditions. *C.Perfringens* has been divided into five types (A, B, C, D and E) on the basis of production of the major lethal toxin (table 1) (Sterne and Warrack, 1964). Some types of
Table 1: The neutralization reaction which occur between C. perfringens Antitoxins

<table>
<thead>
<tr>
<th>Type</th>
<th>Major toxins</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>α</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B</td>
<td>α, β, ε</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C</td>
<td>α, β</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D</td>
<td>α, ε</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>E</td>
<td>α, ι</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

+ Neutralization of toxin
- No neutralization of toxin
This organism, mainly type A, are consistently isolated from intestinal tract of animal and the environment, while other types (B, C, D and E) are less common in the intestinal tract of animal (Carter and Chengappa, 1991) and occasionally found in the environment where the disease produced by \textit{C. Perfringens} is enzootic (Niilo, 1980).

Historically, anaerobic infections have attracted the attention of observant physicians as early as 460 b.C. (McGowan and Gorbach, 1981). However, the recognition of pathogenic anaerobes occurred in 1891 when Achalme described \textit{C. perfringens} (cited by ElSanousi, 1975). It was first isolated and identified from decomposed human cadaver whose tissues were gaseous and was named \textit{Bacillus aerogenes capsulatus} (Welch and Nattal, 1892). Thereafter the organism was isolated from human cases with intestinal disturbances (Klein, 1895) and gangrenous appendicitis (Veillon and Zuber, 1897). The organism was named by the latter as \textit{Bacillus perfringens}. This strain of \textit{C. perfringens} which has been refered to as classical strain to distinguish it from other strains isolated from animals sources (Dalling and Ross, 1938).

Three organism closely resembling the classical type of \textit{C. perfringens} but different in the toxin production have been isolated from sheep. The first type was isolated from cases of entertoxaemia and was called lamb dysentery bacillus and \textit{Bacillus agni} (Gaiger and Dalling, 1923 and Dalling, 1926), the second type was isolated from a disease known in Britain as struck or strike and was termed \textit{C. paludis} (McEwen, 1929) and the third type was isolated from a case of pulpy kidney disease and referred to as \textit{C. ovitoxicum} (Bennetts, 1933). These subtypes were later designated as type B, C and D respectively.

2.2.1. \textbf{Characteristic features of \textit{C. perfringens}}

2.2.1.1. \textbf{Morphology and staining}

The organisms are rods shaped with blunt ends that occur singly or in pairs and occasionally in short changes. It measure 2-6\(\mu\)m length and .8-1.5 width. In smear prepared from infected tissue, distinct capsule may often be observed surrounding the
bacilli. Spores are rarely produced in culture media or pathological material, and do not bulge out of the cell. Young culture stain uniformly gram positive but in older cultures many organism may be granular and other stain gram negative (Buxton and Fraser, 1977).

2.2.1.2. Culture features:

Although *C. perfringens* is anaerobic but does not require such strict anaerobic condition for growth. It grows in temperature range between 37-47 °C, the optimum begin at 43 °C (Buxton and Fraser, 1977). On surface of solid media two type of colonies are formed. Around 2-4 µm in diameter low convex, grayish yellow or opaque with smooth surface and entire edge, butyrous easily emulsifiable. The second type umbonate and is differentiated into an opaque brownish culture and lighter, translucent radialy straight periphery with crenated edge (Willson and Milles, 1975). On blood agar the colonies surrounded by narrow zone complete haemolysis and wider zone of partial haemolysis due to action of theta and alpha toxin respectively. The extend of haemolytic reaction depend on the amount of toxin and species of red blood cells incorporated in the media. The sheep and ox RBCs are most satisfactory (Cruickshank, Duguid, Marmion and Swain, 1975 and Buxton and Fraser, 1977).

2.2.1.3. The antigenic and toxin types

The capacity of *C. perfringens* to produce potent toxin when grown under suitable condition has long been recognized and specific antitoxins was prepared (Bull and Pritchett, 1917). The investigation of Henry (1923) showed that the toxin of the classical strain, type A, could be divided into two fractions, a haemotoxin capable of haemolysis of red blood cells and myotoxin that causes necrosis of muscle and skin. He further proved that both haemolytic and myotoxin were neutralized by the same antitoxin.

The first indication that different types of *C. perfringens* existed was reported by Dalling (1928), who worked with strains isolated from lamb dysentery. He observed that antitoxin of classical strain did not neutralize the potent toxin produced by lamb dysentery strain, whereas the antitoxin of lamb dysentery strain, neutralized its own toxin and that of the classical type of *C. perfringens*. Thereafter, attention was focused
on *B. paludis* which was isolated from struck (McEwen, 1929) and lamb dysentery bacilli. According to toxin antitoxin neutralization tests *B. paludis* was considered serologically identical to lamb dysentery type (Mason, Ross and Dalling, 1931). Subsequently, hitherto unrecognized variant of the organism was isolated from sheep suffering from pulpy kidney disease in Australia (Bennets, 19323), Tasmania (Oxer, 1932) and New Zealand (Gill, 1932). During that period, when different investigations on the antigenic relationship of *C. perfringens* in progress, Wilsdon (1931) established four toxin types, A, B, C and D by preparing toxin culture filtrates and their corresponding antitoxin sera, and observing the differential mouse protection test (table 1). He found that type A antisera neutralized only type A cultures, type B neutralized all four types, type C neutralized all except type D which neutralized both A and D toxin. He also found that type A comprised strain of classical *C. perfringens* of human gas gangrene, type B strain of lamb dysentery, type C and D strains responsible struck and pulpy kidney respectively. Two years latter Glenny, Barr, Liewellyn, Jones, Dallling and Ross (1933) confirm Wildson (1931) observation, they found a number of antigenic (lethal toxin) components in immunizing filtrates of type B, C and D. They distinguished between the different toxins by mean of Greek letters alpha (α), beta (β), Gamma (γ), delta (δ) and epsilon (ε). Hence, type A elaborates α toxin, type B produces α, β, ε, type C produces α and β and type D elaborates α and ε toxins. In addition, a lethal toxin designated γ (gamma) was detected in filtrates of type B and C, and haemolysin designated δ (delta) in type C filtrate. Though *C. perfringens* type A was first thought to contain α toxin (Prigge, 1937), Ipsen and Davoli (1939) detected two components α and zeta (ζ) and from one strain they extract a third component which was designated eta (η). Prigge’s ζ toxin was found equivalent to Glenny’s α (Dallling and Stephenson, 1942) who designated α and ζ of Prigge as theta (θ) and alpha (α) respectively. Subsequently four more serological identifiable substrate were detected and designated kappa (κ), lambda (λ), mu (μ) and nu (ν) (Oakley and Warrack, 1951).

In addition to the known types a fifth type (type E) was established by Boswarth (1943), who isolated from calf strain which produced a lethal toxin not neutralized by type A, B, C and D antitoxins or any combination of them. Whereas,
type E antitoxin neutralized type A toxin filtrate as well as those of homologous strains but not those of type B, C and D. He proposed that unique lethal factor in type E culture and he designated as iota (ι) toxin.

A six type, type F, had been isolated from an outbreak of necrotic enteritis in Germany (Zeissler and Rassfeld-Stenberg, 1949). This organism produced α and β and would have been classified with type C on that basis, but it was unusually heat resistant and lacked three minor toxin visa delta, theta and Kappa which were produced by type C. This distinction was subsequently deemed unjustified because discrepancies in ancillary characteristic among other strains were noted and establishing new types to denote such differences would become unmanageable (Sterne and Warrack, 1964). There were then sixteen toxin (table 2) by which C. perfringens have been typed and subtyped (McDonel, 1980 and Nillo, 1980). However, recently B₂ toxin, which is immunologically related to B toxin, has been identified from piglets died from necrotic enteritis. This strain was previously classified with type C, but it was found to be produced by some strains of type A (Hunter, Brown, Oyston, Sakurai and Titball, 1993 and Gibert, Jolivet-Renaud and Popoff, 1997), and D and E (Bueschel, Jost, Billington, Trinh and Songer,2003). Similarly the Iota toxin was found to consist of two independent proteins, an enzymatic component La and a binding component Lb (Perelle, Gibert, Boquet and Popoff, 1995)

2.2.1.4. Toxin and Virulence factors

C. perfringens produces a variety of virulence factors (table 3) which responsible for different pathological condition, varying from relatively mild food poisoning to life-threatening myonecrosis and enterotoxaemia. C. perfringens produces at least 16 potential virulence factors (Nillo, 1980 and Hatheway, 1990). The mechanism of action
### Table 2: Toxins produced by *C. perfringens*

<table>
<thead>
<tr>
<th>Types</th>
<th>Major toxins</th>
<th>Minor toxins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Toxins and enzyme substances</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Alpha</td>
<td>Beta</td>
</tr>
<tr>
<td>A</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>C</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>D</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>E</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

+ Produced by some strains of the type given. Quantities of toxin produced by different strains vary.
- not known to be produced by any strain of the type given. (+) Produced as prototoxin, activation require enzymes.

- Existence doubtful
- 0 not studied
<table>
<thead>
<tr>
<th>Type</th>
<th>Variety</th>
<th>Disease or occurrence</th>
<th>Variety antigenic difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>Gas gangrene of man and animals, Avian necrotic enteritis, necrotising colitis of equine, enterotoxaemia of lambs, cattle and camels, Intestinal commensals, soil Food poisoning of man and animals</td>
<td>Nonenterotoxaemia Produce enterotoxin</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>Lambs dysentry, enterotoxaemia of calves and foal</td>
<td>Produce lambda, mu, no. Kappa Produces Kappa, No Lambda, mu.</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Enterotoxaemia of sheep and goat (Iran)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>1</td>
<td>Enterotoxaemia of sheep (struck) Neonatal haemorrhagic enterotoxaemia of calves, lambs and foal. Enterotoxaemia of piglets Necrotic enteritis of man in Germany, and foals Necrotic enteritis of man in Papua New Guinea.</td>
<td>Produces delta No data combination theta, Kappa and Mu. Absence of the above antigens. As in variety 2 and 3 and produce enterotoxin</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Variety</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Unknown</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>1</td>
<td>Enterotoxaemia of sheep, goat and cattle Camels and Human (toxin not studied)</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>1</td>
<td>Enterotoxaemia of sheep and cattle and enteritis in rabbits.</td>
<td></td>
</tr>
</tbody>
</table>
these factors usually falls into one of three groups (Table 4). Some of these factors such as alpha toxin which is phospholipase C, and Kappa toxin, which is collagenase, are enzymes that hydrolyze substrate essential to integrity of membranes or other body structures. Other virulence factors such as beta, epislon and iota toxin act primarily on vascular endothelium causing increase capillary permeability especially in the brain (Gardner, 1974, and Worthington and Mulders, 1975). Still other factor, such as the delta and theta toxin, are essential haemolysin (Smith, 1985; Hatheway 1990 and Rood and Cole, 1991).

2. 2.1.5. The major toxin
2. 2.1.5.1. Alpha toxin:

The $\alpha$ toxin component, which produced by all C. perfringens types is thermostable, lethal for mice when inoculated intravenous or intraperitoneal and produce necrotic lesions following intradermal injection in guinea pigs. Nagler (1939) observed that toxin filtrate of type A-D produced opalescence in human sera proportional to their toxin content and that the reaction was specially inhibited by antisera to type A filtrate. Similar reaction was produced by $\alpha$ – toxin on extract of egg yolk (MacFarlane, Oakely and Anderson, 1941). They suggested that both phenomena were due to enzymatic splitting of lipoprotein complex in the sera and egg yolk. MacFarlane and Knight (1941) demonstrated quantitative splitting of lecithin by $\alpha$ toxin into phospharylcholine and diglyceride and necessity for $\text{ca}^{++}$ and $\text{mg}^{++}$ ions in the reaction. The $\alpha$ toxin is able to hydrolyze membrane phospholipids of erythrocyte result in haemoglobin release and haemolysis. It causes haemolysis of most animals erythrocytes except those of horse and goat. Erythrocytes of cattle and mice seem most susceptible, while those of rabbits, sheep, human are moderate in susceptibility (McDonal, 1980). In addition, to its effect in the erythrocytes $\alpha$ toxin lysis platelets and leucocytes (Michancea, Bittner, Toacsen, Ceacuruanu.
and Teodorescu, 1970 and Knoe and Inoue, 1990)), stimulate histamine release from mast cells (Habermann, 1960 and Strandberg, Mollby and Wadstrom, 1974); damage fibroblasts membrane (Mollby, Thelestam and Wadstrom, 1974); intact muscle cells (Boethius, Rydquist, Mollby and Wadstrom, 1973) and cause platelets aggregation (Sugahara, Takahashi, Yamaya, Ohsaka, 1976 and Ohsaka, Tsuchiya, Oshio, Miyara, Suzuki and Yamakwa, 1978). α toxin has been shown to affect myocardial function, causing hypotension and bradycardia, result in shock (Stevens, Troyer, Merrick, Mitten and Olson, 1988)

2. 2.1.5. 2. Beta toxin (β)

It is produced exclusively by C.perfringens type B and C. This toxin is not haemolytic, highly trpsin and heat sensitive protein (Sakurai and Duncan, 1977), which is responsible for mucosal necrosis and central nervous system sign (McDonel, 1986). It produces dermonecrosis following intradermal inoculation in guinea pig and rabbits, and lethal when given intravenous in mice (Sterne and Batty, 1975). Early studies in the pharmacologically active filtrates from type B and C indicate that beta toxin can cause contraction of smooth muscle, raise pulmonary arterial and veinous pressure and decrease systemic pressure which usually accompanied by heart block (Kellaway and Trethewie, 1942). Furthmore, the demonstration of β toxin to rabbit jejunum and ileum resulted in paralysis of motor activity of the intestine (Parnas, 1976).

2. 2.1.5.3. Beta2 toxin (β2)

A novel toxin β2 is produced by all type of C.perfringens ( A, B, C, D and E) (Bueschet, Jost, Billington, Trinho and Songer, 2003). It is lethal to mice and cytotoxic for cell line including cell rounding and lysis (Gibert et.al, 1997). In addition β2 toxin producing C.perfringens strains are associated with necrotic and haemorrhagic enteritis and diarrhea(Herholz, Miserez, Nicolet, Frey, Popoff, Gibert, Gerbes and Straub, 1999; Klaasen, Molkenboer, Bakker, Miserez, Hani, Frey, Popoff and Van den Bosch, 1999 and Manteca, Daube,
2. 2.1.5. 4. Epsilon toxin (ε):

The ε toxin is both lethal and necrotizing but not haemolytic. It is produced by type B and D C. *perfringens* in precursor form and is activated following the proteolytic breaking of the peptide bond between the 14\(^{th}\) and 15\(^{th}\) amino acids from amino acid termins (lys-14Ala-15) and releasing active peptide of 14 residues (Bhown and Habeeb, 1977) to give active epsilon toxin. Symptoms provoked by this toxin included increase intestinal permeability (Bullen and Batty, 1956 and Bullen, 1970); vascular endothelial leakage (Gardner, 1974; Morgan, Kelly and Buxton, 1975 and Buxton, Linklater and Dyson, 1978); leading to brain oedema (Gardner, 1974 and Morgan and Kelly, 1974) severe tissue oedema, lung, heart, brain and excess pericardial fluid (Griner 1961, and McDonal 1980). In addition ε toxin causes swollen hyperemic kidney few hours before death (Jansen, 1960, Gardner, 1973a and Rood and Colle, 1991); focal symmetrical encephalomalacia (FSE) in brain stem (Hartley, 1956; Griner, 1961 and Griner and Carlson, 1961). Clinically, ε toxin has been shown to cause hyperglycaemia and glycole urea (Gardner, 1973b) and increase blood pressure (Sakurai, Nagahama and Fujii, 1983).

2. 2.1.5. 5. Iota (ι) toxin:

Iota toxin is produced by type E only. It is thermostable, lethal and necrotizing but not haemolytic. It is elaborated as inert prototoxin and activated by proteolytic enzymes (Bosworth, 1943). Iota (ι) toxin comprise two distinct polypeptide chains which acted synergistically in mouse lethality and dermonecrosis (Stile and Wilkins, 1986) and increase capillary permeability (Craig and Miles, 1961).

2. 2.1.5. 6. Theta (θ) toxin:

All types of *C. perfringens* A-E produce a lethal necrotizing and haemolytic θ toxin, which is known as haemloysin perfringens O and perfringolysin O. It
is responsible for zone of clear haemolysis enclosing the colony on blood agar plate. \( \theta \) toxin plays a role in tissue necrosis associated with *C. perfringens* gas gangrene and responsible for depletion of polymorphnuclear leucocytes in affected zone. Low concentration of \( \theta \) toxin caused altered polymorphnuclear leucocytes morphology, metabolism and migration (Stevens, Mitten and Henry, 1987).

**2.2.1.5.7. Delta (\( \delta \)) toxin:**

Delta toxin is produced by young culture of type B and C. It is lethal, haemolytic for sheep, goats, pigs and cow erythrocytes, and necrotizing upon intradermal inoculation in guinea pig (Tixter and Alouf, 1976).

**2.2.1.5.8. Eta (\( \eta \)) and gamma (\( \gamma \)) toxins:**

Their existence is dubious (Rood and Cole, 1991)

**2.2.1.5.9. kappa (\( \kappa \)) toxin:**

It is produced by all types of *C. perfringens*. It is enzyme collagenase and gelatinase which attack the collagen and reticulin of muscle and gelatin. It is necrotizing and apparently lethal (Oakely, Warrack and Warren, 1948) and it digest dentine (Evans and Prophet, 1950).

**2.2.1.5.10. Lambda (\( \lambda \)) toxin:**

Lambda, which is protease and gelatinase, is produced by type B, E and some strain of type D. It was proved to be non lethal and non necrotizing (Oakely et al., 1948).

**2.2.1.5.11. Mu (\( \mu \)) toxin:**

It is hyaluronidase and produced in large amount by strains of type B but also produced by type A and D (McDonel, 1980) and type C (Smith, 1975 and Willis, 1969) \( \mu \) component has haemolytic, necrotic and lethal activities (Willis, 1969). It is heat labile (Robertson, Ropes and Bauer, 1940) and resistant to low and high pH. \( \mu \) toxin degrades hyaluronic acid, an important constituent of connective tissue, to release glucosamine (McDonel, 1980) and increase vascular permeability (Willis, 1969).
2.2.1.5.12. nu Dnase (ν) toxin:

It is deoxyribonuclease, produced by all type of C. perfringens, and most frequent by types A and C. It is believed to have lethal, haemolytic and necrotic activation. It is leucocidin that its ribonuclease activity result in destruction of nuclei of polymorphnuclear leucocytes and muscle cells (Robb-Smith, 1945).

2.2.1.5.13. Neuraminidase (sialidase)

It is produced by all type of C. perfringens A-E (McCrea, 1947; Collee, 1965; Fraser and Collee, 1975 and Fraser, 1978). It play a role in disease either by destroying the receptor molecules on cell surface and allow more direct contact of microorganism pathogen with the host cells or by disrupting the connective tissue (Cabexas, 1978). It may render the erythrocytes panagglutinable, resulting in increase of blood viscosity and promoting capillary thrombosis (Hatheway, 1990).

2.2.1.5.14. Enterotoxin:

It is responsible for food-borne and non food-borne human gastrointestinal illness. This toxin is elaborated mainly by type A but is found to be produced by other types B-E (McDonel, 1980; Rood and Cole, 1991; McClane; 1996 and Billington, Wieckowki, Sarker, Bueschel, Songer and McClane, 1998). Enterotoxin differ from other exotoxin and is only produced during sporulation (Duncan and Strong, 1969; Duncan, Strong and Sebald, 1972; and McClane, 1996). It is lethal to mice, increase capillary permeability and cause ertherma in guinea pig skin following intradermal inoculation (Strong and Duncan, 1971). It also increase permeability of intestine mainly jejunum and ileum causing profuse outpouring of water, sodium, chloride and inhibit glucose uptake (Hatheway, 1990).
2.2.2. *C. perfringens* gene structure and localization

The genes of *C. perfringens* toxins has been organised and the physical map of these genome has been established (Candard and Cole, 1989 and Road, 1998). The structural gene has been elucidated and located (table 4 and 5) (Petit, Gibert and Popoff, 1999). The genes encoding α-toxin, plcpla, θ-toxin or perfringolysinO, pfor, K-toxin or collagenase, Col A; μ-hyaluronidase, nag H and nan H and the nani

**Table 4:** The location, mode of action and biological activity of *C. perfringens* toxin encoding genes

<table>
<thead>
<tr>
<th>Toxin enzyme</th>
<th>Gene Structure</th>
<th>Genetic localization</th>
<th>Mode of action</th>
<th>Biological activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>PLc</td>
<td>Chromosome</td>
<td>Phospholipase c, Sphingomyelinase</td>
<td>Cytolytic, haemolytic, Necrotic, lethal</td>
</tr>
<tr>
<td>B1</td>
<td>Cpb1</td>
<td>Plasmid</td>
<td>Pore-forming activity, cell Membrane disruption</td>
<td>Cytolytic, dermonecrotic, Lethal, haemolytic, necrosis of intestinal mucosa.</td>
</tr>
<tr>
<td>ε</td>
<td>etx</td>
<td>plasmid</td>
<td>Sphingomyelinase</td>
<td>Liver, kidney and brain, Dermonecrotic, lethal.</td>
</tr>
<tr>
<td>t (la)</td>
<td>iap</td>
<td>plasmid</td>
<td>Pore-forming activity, cell Membrane disruption. Alteration of cell membrane Permeability (pore-forming activity).</td>
<td>Oedema in various organs</td>
</tr>
<tr>
<td>t (lb)</td>
<td>ibp</td>
<td>plasmid</td>
<td>Pore-forming activity.</td>
<td>Disruption act in cytoskeleton, disruption of cell barrier integrity, Dermonecrotic, lethal.</td>
</tr>
<tr>
<td>Enterotoxin</td>
<td>cpe</td>
<td>chromosome (Food Poisoning)</td>
<td>Pore-forming activity.</td>
<td>Cytotoxic erythematos, lethal, leakage of water and ions by enterocytes, diarrhoea.</td>
</tr>
<tr>
<td>(CPE)</td>
<td></td>
<td>plasmid (non food poisoning)</td>
<td></td>
<td>Additional virulence factor</td>
</tr>
<tr>
<td>θ (p of A)</td>
<td>PloA</td>
<td>Chromosome</td>
<td></td>
<td></td>
</tr>
<tr>
<td>κ</td>
<td>CoLA</td>
<td>Chromosome</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type</td>
<td>Major toxin</td>
<td>Genotype</td>
<td>Associated pathology</td>
<td></td>
</tr>
<tr>
<td>------</td>
<td>-------------</td>
<td>----------</td>
<td>----------------------</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>++</td>
<td>plc, cPe, plc, cpb₂, plc, cpe₂, cPB₁</td>
<td>Myonecrosis, fowl necrotic entritis, bovine enterotoxaemia, porcine necrotic enterocolitis, canine necrotic enterocolitis, gasgangrene, gastrointestinal disease, food poisoning, antibiotic associated diarrhoea, sporadic diarrhoea, cases of sudden infant death syndrome</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>+ + +</td>
<td>plc, cpb₁, etx, plc, cpb₁, etx, cpe</td>
<td>Lamb dysentery, chronic enteritis in lambs, ovine haemorrhagic enterotoxaemia, equine and bovine haemorrhagic enteritis.</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>+ +</td>
<td>plc, cpb₁, plc, Cpb₂, plc, Cpb₁, cpe₂, plc, cpe₂, cpb₁, cpe₂</td>
<td>Fowl necrotic enteritis, neonatal haemorrhagic or necrotic enterotoxaemia (ovine, bovine, caprine, Parambrand).</td>
<td></td>
</tr>
</tbody>
</table>

Table 5: showing *C. perfringens*, toxinotypes and associated diseases
genes encoding nuraminidase were located in the chromosomes. In contrast the
genes encoding β₁ and β₂ toxins, Cpb₁ and Cpb₂; ε-toxin, etx, la and lb, iap,
ibp; λ-protease; lam and urease were located in the plasmid (Katayamo,
toxin Cpe located either chromosomal or plasmid (Rood, 1998 and Sparks,
Carman, Sarker, 2001). Furthermore, the work by Sparks et.al., (2001) revealed
that C. perfringens enterotoxin associated with antibiotic association diarrhoea
(AAD) had plasmid cpe genes, while the food poisoning isolates of
C. perfringens carried chromosomal cpe genes.
The complete nucleotid sequence of C. perfringens toxins have been
determined for α-toxin (Titball, Hunter, Martin, Morris, Shuttleworth, Rubidge,
Anderson and Kelly, 1989); β-toxin (Hunter, Bown, et.al., 1993); ε-toxin
(Hunter, Clarka, Kelly and Titball, 1992), L-toxin (Perelle, Gibert, Boquet and
Popoff, 1993) and enterotoxin (Kokai, Kun, Songer, Czezulin, Chen and
McClane, 1994).

2.2.3. Diagnosis and typing of C. perfringens

Clostridium perfringens is an important agent of enteritis and fatal
enterotoxaemias in domestic animals and human (Niilo, 1993 and Songer, 1996),
causing diseases such as equine colitis, necrotic enteritis in poultry, food poisoning in
human, lamb dusentry, neonatal haemorrhagic or necrotic enterotoxaemia. The pathogenesis of these diseases is mediated by one or more of at least 18 toxins produced by *C. perfringens* (table 2 and 4). The elaboration of four major toxins (α, β, ε, ι) has become the basis of subspecies classification of the organism into five toxogenic phenotypes (table 1) (Buxton and Fraser, 1977, and Hatheway, 1990).

Routinely, typing of *C. perfringens* is performed by seroneutralization test either in guinea pigs skin or lethality by intravenous inoculation in mice (Sterne and Batty, 1975). This assay has become increasingly unsatisfactory because it is time consuming, expensive requires specific antisera for each toxin type, which are currently difficult to find commercially and need large amount of toxin. Using living animals which has the inaccuracy of biological assays such as variation in individual sensitivity, non specific toxicity from other substance that may be present in intestinal content (Henderson, 1984), disfavour on humanitarian ground and animal well fare. Also this method may not detect the non or poorly toxigenic variant found within the types of *C. perfringens* (Dalling and Ross, 1938 and McDonel, 1980). However, many diagnostic laboratory rely, instead, upon evaluation of clinical signs, macro. and microscopic pathology and direct or cultural examination of infected tissue. Immunoassay including gel diffusion (GD), florescent antibody (FAT), agglutination, immunodiffusion, reverse passive haemoagglutination (RPHA), counter immunoelectrophresis (CIE), latex agglutination (LA) and enzyme linked immunosorbent assay (ELISA) have been applied for detection enterotoxaemia associated toxin of *C. perfringens*. The detection of the four major toxins either from gut content, faecal samples, blood stream and serous exudate of affected animals, or from culture filtrates play a predominant role in the diagnosis of *C. perfringens* induced enteric diseases. All these methods may be useful in diagnosis of the organism. However, many of them have some limitations and are not practical for identification of *C. perfringens* into the serological types. Many investigators attempted to established the number of serological group within *C. perfringes* by the gel diffusion technique. Their results indicated a sharing of common antigens among the six types and strains variation in production of antigens within each type (Orlands and Jones, 1958 and Ellner and Bohan, 1961). The investigation of Yamagish,
Yoshizawa, Kawai, Seo and Nishida (1971) showed that fluorescent antibody and agglutination methods gave similar results in which the test antisera react fully with the homologous strains and with a few of heterologous strains. Both tests showed false positive and failed to identify non toxogenic variety of *C.perfringens*. FAT has been applied to the recognition of *C.perfringens* but owing to serological heterogenicity of the group of these organisms this methods becomes unreliable for routine diagnosis of these bacteria (Boxton and Fraser, 1977).

Reversed passive haemagglutination (RPHA) was used by Uemura, Sakagachi and Riemann (1973) who found it to be simple sensitive test for detecting as little as .5 ng of type A entotoxin. However, when this test applied to detect epsilon toxin in type D enterotoxaemia, Beh and Buttery (1978) found the test to be more sensitive than the mouse toxicity test in detecting spsilon antigen in normal intestinal content. In contrast, the latter authors found the single radial immunodiffusion (SRID) was insufficiently sensitive to detect epsilon toxin in the intestinal content of sheep that died from type O enterotoxaemia. Counterimmunoelectrophoresis (CIEP) was specific, quick and sensitive enough to detect 2 µg of *C.perfrinens* type A enterotoxin (Naik and Duncan, 1977). However, Henderson (1984) used CIEP to detect ε-toxin in the intestinal of affected sheep and reported 90.3% agreement with mouse neutralization test.

Enzyme linked immunosorbent assay (ELISA) was reported for the detection of *C.perfringen* ε when varying amounts of purified toxins were added to normal intestinal content (Weddell and Worthington, 1984). The authors obtained satisfactory results in samples containing 7.8-62.5 ng/ml of ε toxin. This method have been developed as an alternative to toxicity neutralization test in mice to detect *C.perfringens* type D ε toxin(Naylor, Martin and Sharpe, 1987 and Elidrissi and Ward, 1992); type C β toxin (Martin, Naylor and Sharpe, 1988) and type Aα toxin ( Naylor, Martin and Barker, 1997) in gut content of animal died in suspected of C.perfringens enterotoxaemia and from culture filterates. This method was described as sensitive, quantitative and gave excellent agreement with the conventional mice protection test. Hence, using ELISA α toxin in conjunction with t for β and ε toxins allows the differential diagnosis of C.perfringens type A, B, C and D enterotoxin.
from faecal, intestinal content and culture supernatant of C. perfringens (Naylor et al., 1997).

The latex agglutination test (LAT) readily detect the ε toxin quantitatively in the intestinal content of animal suspected or dying from enterotoxaemia. When it was compared with the ELISA, the LAT was proved easy to perform and had sensitivity and specificity slightly less than ELISA (Martin and Naylor, 1994).

2.2.4. Enterotoxin

Laboratory confirmation of C. perfringens type A enterotoxin outbreaks usually based on the following criteria, firstly large number (>10⁵ /g) of organisms on food, secondly, large number (>10⁵ /g) of C. perfringens in faeces of affected cases. Thirdly, the presence of the same serotype in most of affected cases, the contaminated food and in faeces (Berry, Rodhouse, Hughes, Bartholomew and Gibert, 1988). However, this confirmatory test may sometimes be inconclusive when any of the mention criteria is not obtained such as contaminated food was not available, or when the incident concerned carriage individual who carry large number of the same serotype without clinical symptoms (Sutton, 1966; Yamagishi, Serikawa, Morita, Nakamua and Nishida, 1976 and Stringer, Waston and Gilbert, 1985). Consequently several methods have been described for detection of C. perfringens type A enterotoxin, cytotoxicity assay (Berry et al., 1988 and Mohony Gilliatt, Dawtson, Sockdale and Lee, 1989) and immunoassays including CIEP (Naik and Duncan, 1977); ELISA (Olvik, Granum and Berdal, 1982; McClane and Stouse, 1984; Jackson, Yip-chuck and Broodsly, 1985, Berry et al., 1988 and Fach and Popoff, 1997).

Naik and Duncan (1977) reported a trial on the use of CIEP to detect enterotoxin of C. perfringens and they could detect as small as .2 µg/ml of enterotoxin. They described CIEP technique as rapid, sensitive, specific and easy.

A four-layer sandwich ELISA was established for the detection of C. perfringens enterotoxin produced by strains isolated from an outbreaks of food poisoning (Olvrik et al., 1982). They found that the ELISA technique was high sensitive to detect .1 ng/ml of purified toxin in trypicase soya broth and 1.0 ng/ml in heterogenous solution such as meat products and faeces. Other groups of
investigators believed that ELISA system has many advantages pertinent to the
diagnosis of food-borne diseases such as highly sensitivity, reagent stability,
flexibility in sample loading, ease manipulation and specificity essential for
Subsequently, Berry et al. (1988 compared ELISA, RPLA and vero cell assays in
the diagnosis of three hundred and ninety two faecal samples from different
seventy separate outbreaks of suspected C. perfringens food poisoning. They
concluded that the ELISA, though, was the most time consuming method, was the
specific and reproducible. RPLA was more sensitive than ELISA but showed
some nonspecific reaction. The vero cells were least sensitive and least
reproducible method being affected by some non specific cytotoxin and cytotoxic
reaction. In another comparative investigation McClane and Snyder (1987) found
strong correlation between SLA, ELISA, RPLA and CIEP, they concluded that
SLA offers several advantages over existing CPE assays, including rapidity 8
minutes, versus approximately 8-24 hours for ELISA and RPLA, adaptability for
handling large samples numbers while ELISA and RPLA are very tedious for
large outbreaks unless specialized equipment is available, economy and technical
simplicity where no sophisticated equipment is required; SLA test was also
described as simple rapid and sensitive method (Fach and Popoff, 1997).

Though ELISA assay appeared to be the most sensitive and specific
serological test for diagnosis and typing of C. perfringens. It is tedious is perform,
time consuming (McCland and Snyder, 1987) and have some limitation such as
elaboration of antibody, conjugate preparation protocol since ELISA kits for
diagnosis and typing of C. perfringens are not available commercially and the test
need to be performed separately for each toxin type.

During the last 10 years considerable research attention has been directed
or focused to established simple, fast, accurate, specific and reliable alternative in
vitro assay to in vivo sero-neutralization test for diagnosis and typing of
C. perfringens.
PCR technique has been applied in several areas since the late 1980s. This method has been highlighted as simple, rapid, specific and accurate assay for detection of low copy number of genes. Also the sensitivity and specificity of this method confirmed by amplification specific target DNA under unique conditions (Yoo, Lee, Park and Park, 1997). Nucleic acid base methods for detecting toxigenic *C. perfringens* have been reported. Oligonucleotide probes (Daube, Simon, Limbourg, Manteca, Mainil and Kaeckenbeeck, 1996) or PCR (Daube, China, Simon, Hvala and Mainil, 1994; Songer and Meer, 1996; Fach and Popoff, 1997 and Augustynowicz, Gzyl and Slusarczyk, 2000) have been used to detect *C. perfringens* genes, α, (Cpa); β, (Cpb); ε, (etx); i, (IA) and μ, (nagU) As well as enterotoxin (cpe) and sialidase θ (pfo). Both methods were described sensitive, specific, time saving and easy to perform.

Genes for α, β, ε, i, μ, θ and cpe were detected by DNA-DNA hybridization of more than 750 strains from cases of bovine enterotoxaemia in Belgium, all hybridized with probes of Cpa and nan H, most with probes for pfo and NagH, few with probes of cpe and non with probes for cpb, etx and iA (Daube, Simon, Limbourg, Renier and Kaeckenbeeck, 1994). Subsequently Daube et al. (1996) identified 11 pathogenic type base on hybridization with probes for the same genes. Though no strains for type B or E were identified, five strains of enterotoxigenic type D were found in sheep and deer. Primer derived from the sequences for cpa, cpb, etx, iA and cpe have been used successfully in PCR assays to type *C. perfringens* (Daube et al., 1994; Songer, 1995; Songer and Meer, 1996 and Meer and Songer, 1997). *C. perfringens* was detected by the amplification of cpa from faeces with a detection limit to 500 bacteria per gram of faeces (Fach and Guillou, 1993). Daube et al. (1994) examine five typed strains and ten field strains by PCR amplification fragment of cpa, cpb, etx and cpe in single reaction (multiplex). They were unable to amplify iA in the same reaction mixture or to consistently amplification cpa from enrichment cultures of intestinal content. In 1995 Songer examined by PCR 500 isolates of *C. perfringens*, which were readily placed into genotype A (86.74%); C (4%) and D (9.3%). PCR genotyping of 616 field isolates were assigned to
genotype A (92.7%), B (.1%), C (4.5%), D (2.1%) and E (0.6%). 8% (n=50) of total were PCR positive for Cpe with genotype A strains predominant, but one cpe positive type C, one type D and four type E (Songer and Meer, 1996). The PCR technique specifically differentiate *C. perfringens* type B and D from type A and C and from *Escherichia coli* in faeces and intestinal content of goats, it detected as few as 1.4 X 10^2 Cfu/g of sample (Uzal, Plumb, Blackall,Boyle and Kelly, 1996). Using PCFR assay Uzal, Plumb, Blackall and Kelly, (1997), identified the genes encoding the major toxin of *C. perfringens* in faeces of goat artificially spiked with these microorganisms. It detected as few as 1-1.5 X 10^2 Cfu/g the five types.

Another group of investigators successfully identified by PCR technique the genes encoding α, ε and β toxins of *C. perfringens* in 11 out of 13 formalin fixed paraffin-embedded specimens of intestinal tissues of goat and sheep known to infected with *C. perfringens* (Warren, Uzal, Blackall and Kelly, 1999). Two out of 13 samples gave negative results, this possibly because the bacteria were unevenly distributed in tissue. This result may be corrected if multiple or serial sections were used. Hence, the PCR technology could be valuable tool for analysis of archived material from pathology laboratory.

Recent investigation showed 100% correlation between genotype and toxinogenic phenotype (Moller and Ahrens, 1996). They developed PCR assay for individual major toxin gene including cpe in group of strains containing more than fifty isolates. Moller and Ahren found type A were common in specimens from pig, sheep and goat, type C and D isolates were obtained from piglets and sheep respectively; non were positive for enterotoxin gene cpe. Their results, also, showed that the PCR assay was superior to ELISA for detection of β toxin (gene) in necrotic intestinal mucosa without culturing. Therefore, the results of Moller and Ahren (1996) supported the results of successor researchers (Yamagish, Sugitani, Tanishima and Nakamura, 1997; Yoo et al., 1997; Miserez, Frey, Buogo, Capaul, Tontis, Burnens and Nicolet, 1998; Kadra et al., 1999 and Augustynowicz et.al, 2000) indicated that the PCR methods are an excellent alternative in vitro method to in vivo testing in laboratory animals for diagnosis and typing *C. perfringens*. 

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PCR methodology was, also found excellent alternative assay to the conventional seronutralization test for epidemiological surveys. Yoo et al. (1997) investigated the most prevalent type of *C.perfringens* in Korean calves, piglets and chicken with clinical symptoms such as diarrhea, enterotoxaemia and necrotic enteritis. Type A was the most prevalent type and was isolated from calves, chickens, while type C (2 out of 14 isolates) in addition to type A were isolated from piglets. They claimed that their results were comparable to those previously obtained by the classical method. In another epidemiological study Augustynowicz et al., (2000) found 97% prevalence of type A, 2% type C and 1% type E. The authors comment that their results documented the presence of rare toxin of genotype E of *C.perfringens* in a polish geographical region and indicated the suitability of multiplex PCR as a method supplementary to the classical technique and providing better insight into the prevalence of toxinogenic *C.perfringens* strains.

The PCR was found to detect specifically α toxin gene of *C.perfringens* (Moller and Ahren, 1996). They carry out PCR protocol using α toxin primer sequences specifically designed for *C.perfringens* toxin genes. He used eleven reference strain belong to five types of *C.perfringens* A-E and four reference strains, which produce α toxin, including *C.bifermentans, C.sordelli, Bacillus cereus* and *Pseudomonas aeruginosa*. The PCR tests were 100% specific and sensitive when applied to type *C.perfringens* reference strains. Though α toxin is also elaborated by *C.sordelli, C.bifermenntans, B.cereus* and *P.aeruginosa* (Pritchard and Vasil, 1986; Johansen, Holm, Guddal, Sletten, Haugli and Little, 1988 and Tos and Siebel, 1989), the PCR for α toxin gene did not react with any of these strain. Similar results were obtained by Fach and Guillou (1993).
2.2.4. **C. Perfringens: Diseases and Pathogenesis**

*C. perfringens* in animal is a versatile pathogen that produces a variety of diseases in a variety of animals (table 3). The pathogenic important of this organism as causative agents of gas gangrene was discovered at the end of the nineteenth century and since then it has been object of intense study (Ispolatovskaya, 1971). Beside its involvement of gas gangrene the organism has been shown to cause food poisoning, necrotizing enterocolitis of infant and enteritis necroticans (pigbel) in man (Smith, 1979 and McDonel, 1980). It is also the causative agent of animal diseases such as lamb dysentery, ovine enterotoxaemia (Struck), pulpy kidney disease of sheep and other enterotoxaemia of animals (Niillo, 1980, Barker and Van-Dreumel, 1985 and Songer, 1996). Isolates of *C. perfringens* can be divided into five (A to E) based on particular extracellular toxins, which they produce. Each of these toxins types is responsible for specific disease syndromes.

2.2.4.1. **C. perfringens type A:**

Strains of type A are the most common and widespread of *C. perfringens* types in the intestinal tract and the environment (Mc Donel, 1980; Wierup and Dipietro, 1981 and Barker and Van-Dreumel, 1985). They were found in most soil that specifically culture for it (Ardehali, Moosawi and Pilehian, 1994). According to its toxigenic behavior type A can be subdivided into two varieties (table 3). The enterotoxigenic or food poisoning variety, characterized by enterotoxin production that is capable for causing diarrheic food poisoning in human and a similar enteritis in animals particularly pigs (Songer, 1996 and Radostits et al., 2000). The classical variety characterized by production of α-toxin, which associated with wound contamination, anaerobic cellulitis, gas gangrene and enteric disease and enterotoxaemias in animals (Hatheway, 1990).

Type A was first reported as cause of fatal haemorrhagic disease in sheep and cattle in Australia (Rose and Edager, 1936). Subsequently, similar disease
occurred in different localities and was designated as enterotoxaemia and yellow lamb disease in sheep (McGowan, Moulton and Rood, 1958; Greig, 1975 and Popoff, 1984); acute enteritis in calves (Macrae, Murray and Grant, 1943; Shirely, 1958 and Fleming, 1985) and cattle (Niilo, Moffatt and Avery, 1963; AL-Mashat and Taylor, 1983 and Worrall, Natalia, Rorohardjo and Tarmudji, 1987). In 1987 type A \textit{C. perfringens} was isolated from disease syndrome characterized by abomasitis, abomasal tympany and ulceration in calves (Roeder, Chergappa, Nagaraja, Avery and Kennedy, 1987). Year latter similar changes of abomasitis, abomasums tympany and abomasal ulceration was reproduced experimentally (Roeder et al., 1988). An acute haemolytic enterotoxaemia attributed to \textit{C. perfringens} type A was reported in foals (Dart, Pascoe, Gibson and Harrower, 1988 and Kanoe, Inoue, Abe, Anazi, Kamada, Imagawa and Kanemaru, 1990); horses (Wierup and Dipietro, 1981) and goats (Russel, 1970). Wernery et al. (1992) reported an outbreak of type A enteritis in camels calves suffering from constipation for a few days followed by severe diarrhoea and death. Similar syndrome was reported in adults camels (Chauhan, Kulshreshth and Kaushik, 1985; Gameel, EL-Sanousi, Musa and El-Owni, 1986 and Wernery et al., 1991).

Clinically, type A haemolytic disease characterized by acute onset of severe depression, collapse, mucosal pallor, jaundice, haemoglobin urea, dyspnea and presence of severe abdominal pain. Temperature range from normal to 41°C (106°C). The disease is highly fatal, most affected animals died within 12 hours of the onset of clinical signs although, occasionally, animals survive for several days. Large number of the organism and their toxins were detected in the intestine (Rose and Edgar, 1936; McGowan et al., 1958; Russel, 1970; Jansen and Swift, 1982; Fleming, 1985 and Radostits et al., 2000). Other clinical manifestations of type A toxaemia such as shivering, staggering gate, ataxia, opisthotonos and convulsion (Popoff, 1984, Chauchan et al., 1985 and
Wernery et al., 1991); protruding of the tongue and frothy from nostril and mouth (Worrall et al., 1987).

At necropsy, the cardinal features of type A enterotoxaemia included, pallor mucous membrane, Jaundice, petechial and ecchymotic haemorrhages in serosal surface and serosanguinous fluid (40-600mL) with fibrous flacks in the body cavities (Rose and Edgar, 1936; Macrae, 1943; McGowan et al., 1958; Popoff, 1984; Worrall et al., 1987 and Barker and Van-Dreumel, 1985).

2.2.4.2. C.perfringens type B:

Type B is the etiological agent of haemorrhagic and rapidly fatal disease of newborn lambs, known as lamb dysentery (Gaiger and Dalling, 1923 and Masson, Ross and Dalling, 1931). The disease occurred primarily in Great Britain, South Africa and Middle East (Dalling, 1926; Brook and Entessar, 1957; Jensen, 1961; Barker and Van-Dreumel, 1985 and Timoney, Gillespie, Scott and Barlough, 1988). Type B haemorrhagic enteritis was also reported in Montana (Tunnicliff, 1933). Older lambs may be involved in outbreaks but lamb dysentery typically developed during first few days of life (Dalling, 1926). Lamb dysentery organism was isolated from adult sheep and goats (Brook and Entessar, 1957); calves and cattle (Hepple, 1952; Frank, 1959; Buergelt, Chenoweth, Lopez and Aguila, 1999) and foals (Montgomerie and Rowlands, 1936; Mason and Robinson, 1938 and Stubbing, 1990).

In lambs the predominant clinical sign was sudden death without premonitory sign in peracute form. In acute cases cessation of feeding, severe abdominal pain, especially when the animal forced to raise, accompanied by passage of semi-fluid dark faeces mixed or coated with blood and abdomen may be tympanic. These clinical signs were followed by recumbency, coma and death less than 24 hours after the onset of the clinical disease. A more chronic form, which was called pine in England, occurred in older lambs and manifested by chronic abdominal pain without diarrhoea, unthriftness,
depression, reluctant to suckle and peculiar streaching when the animal rise (Sterne and Batty, 1975; Barker; Van-Dreumel, 1985 and Jensen and Swift, 1982).

In lambs gross lesions usually observed, though in exceptional some per acute cases they are indistinct. The most striking lesions were extensive haemorrhagic enteritis. Discrete and confluent ulceration developed if the course of the disease was long enough. The peritoneal cavity often contain small amount of serous and blood stained fluid. In advanced cases penetrating mucosal ulcers, which appeared through serosa as purplish areas limited to small intestine. The intestine contained blood stained fluid or pure blood. In severe disease there was peritonitis with red fibrous strands on the local mesentery and intestinal adhesion. In acute cases abomasal mucosa may be intensely congested and the mesenteric lymph nodes were oedematous and congested (Barker and Van-Dreumel, 1985).

The clinicopathological feature of the disease described in calves (Frank, 1959) and Foals (Stubbing, 1990) were comparable to those described for lambs. However, ulceration with extensive mucosal necrosis and patchy diphtheritic membrane specially in the ileum were described in calves (Barker and Van-Dreumel, 1985).

Little is known about the pathogenesis of enteric type B strain. There has been little experimental study of the pathogenesis of type B infection. It is not clear whether individual effect of α, β or ε toxins predominate or there are additive or synergistic effect. Recent advances in genetic manipulation of *C.perfringens* may allow the generation of appropriate mutants to experimentally answer these questions.

2.2.4.3. *C.perfringens* type C:

Type C is the aetiological agent of haemorrhagic or necrotic enterotoxaemia. Five antigenic pathogenic varieties of type C, differing in their
minor toxins from the classical type, have been implicated in various disease condition (table 3) and in different geographical location throughout the world (Brook et al., 1957; Niilo, 1980 and Hatheway, 1990). The classical type was first isolates from acute disease of young sheep called struck and designated as *Bacillus palludisi* (McEwen, 1929 and McEwen and Robert, 1931), which latter became type C in Wilsdon (1931) classification.

Struck is a disease of pastured animals that had mortality rate of 5-15% (Barker and Van Dreumel, 1985). In peracute form death occurred suddenly with terminal convulsive episodes, whereas in acute form the animal stand on a straining position, which indicates abdominal pain. Occasionally, in acute diseases animals manifested diarrhoea as cerebral disturbances (Montgomerie and Rowland, 1936 and Jubb and Kennedy, 1970).

At necropsy, the peritoneal cavity contained up to 3 liters of clear pale yellow fluid that clots on exposure to air. The peritoneal blood vessels specially those of omentum, small intestine and urinary bladder were intensely congested and multiple subserosal haemorrhages were present. The mucosa of small intestine was hyperaemic either in patches or along most of its length. Occasionally ulcers were present. The ulceration was more prominent in the Jejenum. In other organs lesions of severe toxaemia included copious pleural and pericardial transudate of Gelatinous fluid, subendocardial haemorrhages and haemorrhages in zona reticularis of adrenal glands (McEwen, 1929; McEwen and Robert, 1931; Montagomerie and Rowland, 1936 and Barker ad Van Dreumel, 1985). A degraded strain of type C that caused inflammation of abomasums and first portion of small intestine was reported in New Zealand (Buddle, 1954).

The clinicopathological features of type C infection in feedlot cattle were comparable those described for sheep (Lauerman, Jensen and Pierson, 1977).

The disease caused by type C in calves lambs and foal were similar. Affected animal were young suckling that contact the disease within first few
days of live often first hours. In peracute disease neonates animal died suddenly without forewaring symptoms. Often vigorous, healthy animal were affected. In subacute form calves showed abdominal pain, sudden onset of diarrhoea, unthriftness and death which preceded by spasmodic convulsion (Macrae et al., 1943 and Grinder and Bracker, 1953). Some times developed bloody diarrhoea (Niilo, Harries and Jones, 1974) nervous signs included frenzied bellowing, aimless running, tetany and opisthotonous (Songer, 1996). In subacute form affected lambs manifested abdominal pain and distension, dysentery, prostration followed by death within 12 hours (Grinder and Johnson, 1954 and Greig, 1975). Whereas, foals revealed depression, severe haemorrhagic diarrhoea, dehydration and occasionally colic (Dickie, Klinkerman and Petrie, 1978 and Sims, Tzipori, Hazard and Carrol, 1985).

At necropsy, calves, lambs and foals showed intestinal lesions vary from catarrhal to severe necrohaemorrhagic enteritis with mucosal necrosis and ulceration which were more prominent in the Jejunum and Ileum. The intestinal lumen contained free blood. Excess serous or blood stained fluid in the pericardial, pleural and peritoneal cavities. The mesenteric lymph nodes were enlarged, oedematous and congested. Ecchymosis of serous membrane and meninges of brain were frequently present (Macrae et al., 1943; Grinder and Bracker, 1953; Grinder and Johnson, 1954; Niilo et al., 1974; Howard-Martin, Marton, Qualls and MacAllister, 1986 and Pearson, Hedstrom, Sonn and Wedam, 1986).

2.2.4.3.1. Camel

Affected camels with type c kneeled away from herd and manifested profuse diarrhoea. Death occurred in 2-6 days and some died after 2-3 weeks after commence of clinical signs. Occasionally they developed clonic or tonic convulsion, salivation, and urine became brown. The most prominent gross lesions were ecchymotic and petechial haemorrhage in the wall of small intestine which covered with redish mucus. Serous exudates was observed in
peritoneal and pelvic cavities (Moebuu, Ayuurzand, Dashdava and Ipatenko, 1967 and Ipatenko, 1974).

### 2.2.4.3.2. Human

Type C was isolated from an outbreak of severe necrotic enteritis in human, called Darmbrand in Germany (Zeissler and Ranssfield-Sternberg, 1949) and Pigbel in New Guinea (Murrell and Roth, 1963; Egerton and Walker, 1964 and Murrell, Egerton, Rampling, Samuels and Walker, 1966). In human the onset of the disease was acute with severe pain and slight rigidity, mostly in the left lower abdomen, vomiting and profuse diarrhoea leading to general dehydration. This followed within few days by extreme circulatory collapse and general cyanosis. Grossly, there was necrotic inflammation of several areas of intestine specially the Jejunum and terminal duodenum.

### 2.2.4.4. C. perfringens type D

This type is the best known pathogenic *C. perfringens* types, being widely regarded as the aetiology of fatal enterotoxaemia, which also known as sudden death, pulpy kidney disease, overeating and braxy-like disease, in sheep of all ages except newborn (Niilo, 1993), and probably most prevalent in young lambs suckling heavily lactating ewes grazed on luxurient pasture. It is the predominant cause of death in weaned animals up to one year old (Barker and Van-Dreumen, 1985), often those fed rich ration in feedlots. The frequent association of enterotoxaemia with upset in the gut flora caused by the unmanaged change to rich diet give a rise to the common name overeating (Bull, 1970, Geering and Middleton, 1981 and Uzal and Kelly, 1996).

*C. perfringens* type D enterotoxaemia also occurred in calves (Griner, Aichelman and Brown, 1956; Mumford, 1961); goats (Oxer, 1956, Blackwell and Butler, 1992 and Uzal, Pasini, Olacchea, Robies and Elizondo, 1994); rarely in adult cattle (Itodo, Agba and Okwole, 1983 and Jeffery, 1992); camels (Ipatenko, 1974 and El sanousy and Gameel, 1993); other animals
Appropriate microenvironment lead to rapid multiplication of the organism and production of ε toxin which play a central role in the pathogenesis of type D (Bennet, 1932). Persistence of high level of high level of ε toxin in the intestine, increase the permeability of the intestine and facilitates its own absorption (Bullen, 1970). Absorption of ε toxin from intestine lead to toxaemia with little enteritis. ε toxin affect mainly central nervous system, circulatory and respiratory systems resulting in sudden death,(Kellaway, Trethewie and Turner, 1940; Griner and Carlosn, 1961; Gardner, 1973a and Buxton and Morgan, 1976), with some animal surviving long enough to display clinical signs such as dullness, yawning, facial movement, impaired vision, retraction of the head, opisthotonous and convulsion with agonal struggling (Hartley, 1956; Griner, 1961; Mumford, 1961; Barker and Van-Dreumen, 1985).

Focal symmetrical encephalomalacia (FSE) is a chronic neurological manifestation of type D infection. It occurs sporadically and characterized by haphazard roaming, inability to eat, blindness, ataxia, head pressing, posterior paresis, convulsion seizures, recumbency, paddling movement of limbs and death (Hartely, 1956; Griner, 1961).

Hyperglycemia and glucosurea are pathognomonic for type D enterotoxaemia (Gordon, Steward, Holman and Taylor, 1940; Gardner, 1973b; Pathak, 1990; Niilo, 1993 and Uzal and Kelly, 1996). This event apparently occur very rapidly in the first hours after ε toxin enter the circulation (Songer, 1996).

In goats diarrhoea is a prominent clinical sign of type D intoxication particularly those surviving for few days (Blackwell and Butler, 1992 and Uzal, Glastonbury, Kelly and Thomas, 1997). In peracute infection goats kids manifested convulsion, pyrexia (105°F), severe abdominal pain, dysentry and death within 4-36 hours. In peracute disease adult goats often found died...
whereas in acute form showed abdominal pain, diarrhoea followed by death or recovery within 2-4 days (Oxer, 1956; Blackwell, Buttler, Prescott and Wicock, 1991 and Blackwell and Butler 1992). In chronic disease the goats revealed anorexia, intermittent severe profuse watery diarrhoea and in some cases dysentery (Blackwell et al. 1991 and Blackwell and Buttler, 1992). Some animals manifested chronic wasting anaemia and emaciation (Oxer, 1956).

At necropsy, in some rapid fatal cases, there are no evidence of gross lesions. Often there is excess straw-colour pericardial fluid which clots when exposed to air. Congestion and oedema of lungs may be severe to form frothy in respiratory passages, haemorrhages on the subendocardial of left ventricle. In addition, there are haemorrhages on the serous membrane of epicardium, black haemorrhages in the parietal peritoneum, liver congestion, small intestine distended with gas and hyperaemic. Kidney congested, postmortem autolysis leads with few hours to interlobular haemorrhages which characteristicof the disease pulpy kidney (Jensen and Swift, 1982). Gross lesion of calves dying of type D enterotoxaemia closely resemble those of sheep, though rapid autolysis of the kidney is not prominent feature, subcapsular congestion, haemorrhages occur sometimes as black clots of blood up to 1.0 cm thick (Baker and Van-Dreumen, 1985). In neonatal and adult goats catarrhal, fibrinous or haemorrhagic enterocolitis are the consistent lesions (Blackwell et al., 1991; Blackwell and Butler, 1992 and Uzal and Kelly, 1996). Occasionally pseudomembranous colitis (Uzal and Kelly, 1998) and numerous ulcers on the intestinal mucosa (Uzal et al., 1994) were encountered.

It is widely accepted that the primary target of ε toxin is the C.N.S., where it produces foci of liquefactive necrosis, prevascular oedema and haemorrhages particularly in the meninges (Buxton et al., 1978). It has been shown that these changes were preceded by increase in vascular permeability using horse radish peroxidase (Gardner, 1974 and Morgan et al., 1975) and radioactive iodine (Grines and Carlos, 1961 and Worthington and Muldr, 1975) as traces of
vascular integrity. A receptor of ε toxin has been identified on the vascular endothelium lining certain blood vessels, luminal cells lining tubules of kidney and hepatic sinusoids (Buxton, 1978). Ultrastructural examination of brain tissue from animal inoculated with ε toxin revealed that tight junctions in the vascular endothelium degenerate (Gardner, 1974 and Buxton and Morgan, 1976). Subsequently the perivascular astrocytes processes swell and rupture, causing increase in vascular permeability (Finnie, 1986), and loss of plasma protein (Finnie and Hajduk, 1992), water and rapid extravasation of fluid in the brain forming brain oedema (Griner and Carlson, 1961; Gardner, 1974; Buxton and Morgan, 1976 and Finne and Hajduk, 1992). Brain oedema was succeeded by elevation of intracerebral pressure, focal to diffuse areas of degeneration and necrosis (Buxton and Morgan, 1976), and bilateral foci of malacic lesions (Hartely, 1956; Buxton et al., 1981 and Uzal et al., 1997). Focal symmetrical encephalomalacia was produced experimentally in lambs, mice and goats by intravenous administration of ε toxin (Griner, 1961; Finnie, 1984; Usal and Kelly, 1998). Lesions produced experimentally were comparable to those described in natural cases. The extent of clinical symptoms of C.N.S. derangement, including incoordination, convulsion is directly related to severity of lesions (Grinder, 1961).

2.2.4.5. C.perfringens type E

Type E organisms were originally isolated from calf with enterotoxaemia (Boswarth, 1943) and later from lambs (Ross, Warren and Barnes, 1949), case reports lining this organism to definite disease condition were rare that doubts were raised about the potential pathogenicity (Sterne and Batty, 1975). However, other reports described serious outbreaks of enteritis in a rabbit colony provided quite convincing evidence for the pathogenicity of type E iota toxin. Though, type E organisms were not isolated on these outbreaks, the presence of preformed iota toxin was confirmed by seroneutralization test in
rabbit caecal content (Patton, Holmes, Riggs and Cheeke, 1978 and Baskerville, Wood and Seamer, 1980). Clinically type E infection in rabbit is characterised by profused watery diarrhoea and death occurred within few hours of onset of disease. Lesions in all animals were confined to intestine and consisted of varying degree of inflammation of caecum which was distended with fluid content (Hart and Hooper, 1967 and Baker and Van-Dreumel, 1985) and manifested with haemorrhagic enteritis (Hart and Hooper, 1967 and Billington et al., 1998). The prominent gross lesion in calves were congestion and ulceration of abomasum and haemorrhagic enteritis, which occurred segmentally along the small intestine. The mesentric lymph nodes were enlarged and haemorrhagic and the pericardial effusion and serosal haemorrhages were present (Hart and Hoopes, 1967 and Barker and Van-Dreumel, 1985).

2.2.4.6. The enterotoxigenic variety

Some strains of type A and few strains of type B, C and D produced enterotoxin when undergoing sporulation. It is activated with tryps (Rood and Cole, 1996). This toxin is capable for causing diarrhoeic food poisoning in human and enteritis in animals (Rood and Cole, 1991). It was found implant in meat hygiene and food preparation (Genigeoogis, 1975).

In human type A enterotoxin infection manifested by diarrhoea vomition and general malaise (Niilo, 1993).

Affected horse and ponies with enterotoxin revealed abdominal pain, haemorrhagic gastroenteritis and celocolitis. Both small and large intestine were congested haemorrhagic and oedematous (Wierup, 1977 and Ochoa and Kern, 1980).

The disease in piglets is manifested with a watery yellow diarrhoea, occurring in piglets under 5 days of age and often in first 3 days of age, with high morbidity and low mortality. At necropsy, affected animal presented
enterocolitis and villous atrophy (Songer, 1996 and Radostits et al., 2000). Experimentally type A enterotoxin produced diarrhoea in lambs (Hauschild, Niilo and Dorward, 1970); monkeys (Hauschild, Walcroft and Cambell, 1971) and rabbits (Duncan and Strong, 1969). Inoculated lambs with enterotoxin developed transitory diarrhoea, lacrimation, salivation, nasal discharge, lassitude and dyspnea 1-5 hours post inoculation. Large inoculum caused rapid onset of clinical signs and subsequent death, grossly, the animal showed hyperaemia of small intestinal mucosa, some congestion of liver, lung, spleen and kidney (Niilo, 1971) who concluded that *C. perfringen* enterotoxigenic factor act on small intestine causing increase capillary permeability, vasodilation and increase intestinal motility. In other study, Niilo (1980) found that intravenous inoculation of this toxin produce vasodilation and profound decrease in blood pressure which lead to effects on many organs (Niilo, 1980).
CHAPTER III

MATERIAL AND METHODS

3.1. Equipment

Plastic bags, ice boxes; forceps, metal and plastic racks, slides, cover slides, wireloop (standard loop with 4 mm diameter), oil immersion lenses; test tubes different sizes (Pyrex England); culture tubes with caps (polystyrene, 17x10 mm). Pasteur pipettes; bijou, McCartney and universal bottles; disposable and glass
Petri dishes (Pyrex England); disposable syringes different sizes (1, 5, and 10 ml);
conical flat glass bottles (Pyrex England) round flask; measuring cylinders of
different sizes; graduated beakers (Pyrex, England), Millipore filter type HA (0.2
and 0.45 µm).

Microscope (Olympus BHC)
Cold centrifuge
Cold incubator thermostatically controlled from 10-50ºC (Gallenhamp).
Water bath, thermostatically controlled with temperature range from zero to
100ºC.
Autoclave (LS-B50L, China made).
Sensitive balance (Meter PN 323).
Scale balance.
Bench centrifuge (MSE).
Oven (Gallenhamp model OV 160).
PH meter
Anaerobic jar (a. BBL and Baird and Tatlock, England).
Gas generating kits (Oxoid).
Magnetic stirrer.
Hot plate (Gallenhamp)
Sucking pump
Dissection set

Equipment needed for PCR:-
1. Micropipettes (Eppendorf, 10µl, 100µl, 1000µl).
2. Micropipettes filter tips, (10µl, 100µl, 1000µl).
3. Microcentrifuge tubes (Eppendorf tube, 0.2ml, 0.5µl and 1.5ml).
4. Heating block 100ºC(Grant QBT1)
5. Microcentrifuge(Jouan)
6. Bench top microcentrifuge (Jouan Heraeus)
7. Mini-incubator 37ºC(Merk)
8. Microwave oven (Daewoo)
9. Gel apparatus (EC Electrophoresis)
10. Gel analyzer (Gene Genius)
11. Ice machine (Scotman)
12. Thermocycler (Perkin-Elmer 9700, 9600, 2400)
13. Vortex (2-geneie)
14. Polaroid gel camera
15. Spectrophotometer (Eppendorf-Biophotometer)

3.2. Cleaning and sterilization
Contaminated glassware was boiled immediately in pressure-cooker or autoclaved at 121°C for 15 minutes. It was washed with tap water to which detergent had been added, rinsed in tap water, followed by second rinse in de-ionized waters (DW), then finally dried and sterilized in oven at 160°C for 2 hours.

3.3. Reagents and media additive:
Normal saline
Normal saline was prepared according to Cruickshank, (1975).
Ingredients
Sodium chloride (Nacl) (BDH)  8.5 g  DW
1000 ml
The sodium chloride was dissolved in distilled water. The solution was dispersed in bottles of different size autoclaved at 121°C for 15 minutes and stored at 4°C.

Distilled water (DW)
Tap water was distilled using distiller (Elega, England).

Preparation of phosphate buffered saline (PBS)
Solution A
Sodium chloride (Nacl)  160 g
Potassium chloride (Kcl) (BDH) 0.4 g
Disodium hydrogen phosphate Na$_2$HOPO$_4$ (BDH) 2.3 g
Potassium dihydrogen phosphate KH$_2$PO$_4$ (BDH) 0.4
DDW completed to 1500 ml

**Solution B**

Hydrous Magnesium chloride, MgCl$_2$6H$_2$O (BDH) 0.426 g
(or unhydrous MgCl$_2$) 0.2 g
DDW 200 ml

**Solution C**

Hydrous CaCl$_2$ (BDH) 0.264 g
DDW 200 ml

Solution A, B and C were autoclaved and left to cool. Then solution A was added to solution B then solution C; the mixture was made up to two litres with DDW. The buffer was adjusted to appropriate pH by adding either NaOH or HCl.

**Dextrine (20% dextrine in normal saline)**

Dextrine 20 gm
Normal saline 100 ml
Autoclaved at 121°C for 15 minutes.

**Trypsin solution (10%)**

Ingredient

Trypsin 10 gm
D.W. 100 ml

The mixture was dissolved and sterilized by filtration through millipore seitz filter.

**Staining reagents**

**Crystal violet**

Crystal violet 1.0 g
D.W. 100.0 ml

**Lugol's iodine**
Iodine                                       1.0 g  
Potassium Iodide                      2.0 g  
DW                                          100.0 ml  

**Carbol fucsin**  
A. Basic fucsin                                   1g  
   Absolute ethanol                             10.0 ml  
B. phenol                                             5 g  
   D.W.                                               100.0 ml  
   Mixed     A and B

**Acetic acid solution**  
Acetic                                                  0.5 gm  
D.W.                                                   100.0 ml  

**Methylene blue**  
methylene blue                                     1 gm  
D.W.                                                   100.0 ml  

**3.4. Collection of a defibrinated blood.**  
**Equipment:**  
A five hundred (500) ml flask with arm jet was used. Clean glass beads were placed in the flask where the arm jet and mouth were plugged with cotton wool. Through the mouth plugged cotton wool glass tubing was inserted. The outer of the glass tubing was connected to the length of a rubber tubing to which a bleeding needle was connected and tied with a string. The needle was inserted into test tube and plugged with cotton wool. The arm jet, the mouth and tube containing the needle were then wrapped with aluminium foil and sterilized by autoclaving at 121ºC for 15 minutes.

**Collection of blood:**
A two-year old sheep was shaved at the jugular groove. The shaved area was disinfected with 70% alcohol. The needle was drawn from test tube and injected immediately in the jugular vein. The blood was drained into the flask with continuous shaking for defibrination. The needle was then withdrawn from the animal. The flask was transferred to the laboratory where the defibrinated blood was aseptically distributed in 20 ml aliquots into sterile McCartney bottles.

**Egg yolk emulsion**

Fresh eggs were washed thoroughly with soap and tap water, scrubbed several times with ethyl alcohol and left to dry in clean metal tray. The egg shell was opened and the egg white was aseptically discarded. The egg yolks were then collected aseptically into sterile beaker. An equal volume of sterile normal saline was added to egg yolk, mixed with shaking or stirring with sterile glass-rod. The egg yolk emulsion was then distributed in sterile 100 ml bottles and kept at 20°C for further use.

**Skim milk**

**Ingredient**

- Bacto-skim milk (Difco) 100 gm
- DW 100 µl

The ingredient was mixed and dissolved by stirring using magnetic stirrer and sterilized in autoclave for 15 minutes at 121°C.

**3.5. Reagent in PCR**

**Bromophenol Blue saturated**

- Bromophenol Blue 10%
- Bromophenol Blue 5 g
- DW 50 ml

Stored at room temperature.

**Ethidium bromide solution**

10 mg/ml used to visualize DNA

- Ethidium bromide 0.2 g
Gel loading buffer. Used to load DNA samples in gels.

- Glycerol 5 ml
- TAE buffer (40x) 250µl
- Bromophenol blue, saturated 1 ml
- Xylene cyanol, 10% suspension 1 ml
- DW 2.75 ml
- Total 10 ml

Divided to 1 ml aliquots and stored at -20°C.

Preparation of TAE buffer

Solution A: Tris Hcl 1M
- Tris 60.55 gm
- Hcl 18.75 ml
- DDW complete to 500 ml

Solution B: EDTA 1 mM
- EDTA 292.25 gm
- DDW complete to 1000 ml

Solution C
- Na acetate 136.0 gm
- DDW 1000 ml

Solution A was added to solution B then solution C as follow:-
- Solution A (Tris Hcl) 40 ml
- EDTA 0.5845 gm
- Solution C (Na acetate) 20 ml
- DDW complete to 1000 ml

The mixture was made to up to one litre with DDW. The buffer was adjusted to appropriate pH by adding either NaoH or Hcl.
**TE buffer (tris/EDTA)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris 10 mM pH7.4</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>0.1 mM EDTA, pH 8</td>
<td>20 µl</td>
</tr>
<tr>
<td>H₂O to make</td>
<td>99.3 ml</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>100 ml</td>
</tr>
</tbody>
</table>

Store at room temperature.

**Agarose gel:**

1.5% agarose containing 5mg/ml ethidium bromide

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose</td>
<td>0.75 g</td>
</tr>
<tr>
<td>TAE buffer (1x)</td>
<td>50 ml</td>
</tr>
</tbody>
</table>

The mixture was melted in a microwave oven, cooled down, 2.5 µl of ethidium bromide was added to final dilution of 5 µg/ml, and swirled poured in loading chamber. The comb (gel cutter) was placed and the gel was left to solidify for 15 minutes.

### 3.6. Media used:

#### 3.6.1. Reinforced Clostridial Medium (RCM):

Thirty-eight grams of RCM (Oxoid, England) was suspended in 1 litre of distilled water (DW), and heated in thermostatically controlled water bath until dissolved completely. The media was then sterilized by autoclaving at 121°C for 15 minutes.

#### 3.6.2. Cooked Meat Media

Ten grams of granules of cooked meat medium (Oxoid, England) was transferred into McCartney bottles, and 10 ml of DW was added. The media was then autoclaved at 121°C for 15 minutes for sterilization.

#### 3.6.3. Blood Agar

**Ingredient**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood agar base (Oxoid, England)</td>
<td>40 g</td>
</tr>
<tr>
<td>DW</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>
Preparation of the media:

Forty grams of blood agar base was suspended in a litre of DW and boiled in water bath until completely dissolved. Then the media was autoclaved at 121°C for 15 minutes for sterilization. The media was cooled to 45-50°C and the defibrinated blood was added to concentration of 7%. The media was distributed into sterilized Petri dishes and left to solidify.

3.6.4. Shahidi and Ferguson Perfringens Agar (S.F.P.A.).

This media was prepared as described by Shahidi and Ferguson (1971):

Ingredient (gram/litre, W/V).

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptose (Difco)</td>
<td>15g</td>
</tr>
<tr>
<td>Yeast extract (Difco)</td>
<td>5 g</td>
</tr>
<tr>
<td>Ferric ammonium citrate (BDH)</td>
<td>1g</td>
</tr>
<tr>
<td>Sodium metabisulphate (BDH)</td>
<td>1g</td>
</tr>
<tr>
<td>Polymixin B sulphate (Wellcome, England)</td>
<td>300IU</td>
</tr>
<tr>
<td>Kanamycin sulphate.</td>
<td>0.012 g</td>
</tr>
<tr>
<td>Bacteriological agar (Difco)</td>
<td>20g</td>
</tr>
<tr>
<td>D.W.</td>
<td>900ml</td>
</tr>
<tr>
<td>Egg yolk emulsion (50% in normal saline)</td>
<td>100ml</td>
</tr>
<tr>
<td>pH</td>
<td>7.5</td>
</tr>
</tbody>
</table>

All ingredients with exception of egg yolk emulsion were dissolved in water. After thorough mixing the pH was adjusted to 7.6. The media was autoclaved at 121°C for 15 minutes. The media cooled to about 50°C and 100 ml of 50% egg yolk in normal saline was added, thoroughly mixed and aseptically dispensed in 10 to 12 ml amounts into sterile Petri dishes. The media was allowed
to solidify, incubated over night at 37°C, and examined for dryness and sterility on the following day.

3.6.5. SFP overlay agar:
The medium was prepared according to Shahidi and Ferguson (1971). The medium consisted from the same ingredient described for SFPA except that the egg yolk emulsion was omitted and the ingredients were dissolved in 1000 ml of distilled water. The media was dispensed into McCartney bottles in 20 ml amount and autoclaved at 121°C for 15 minutes.

Lactose Motility (LM)

Ingredients

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptose</td>
<td>15 g</td>
</tr>
<tr>
<td>Yeast extracts</td>
<td>10 g</td>
</tr>
<tr>
<td>Lactose</td>
<td>10 g</td>
</tr>
<tr>
<td>Sodium phosphate (dibasic)</td>
<td>5 g</td>
</tr>
<tr>
<td>Phenol red</td>
<td>0.05 g</td>
</tr>
<tr>
<td>Bacteriological agar</td>
<td>3 g</td>
</tr>
<tr>
<td>D.W.</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

The ingredient except phenol and lactose was dissolved in distilled water by heating, the pH was adjusted to 7.5 and lactose and phenol were added. The media was dispensed in 10 ml amount into test tubes plugged with cotton wool and autoclaved at 121°C for 15 minutes.

3.6.6. Lactose Egg yolk milk agar
The media was prepared as described by Buuxton and Fraser (1977).

Ingredients

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteriological agar</td>
<td>4.8 g</td>
</tr>
<tr>
<td>Lactose</td>
<td>4.9 g</td>
</tr>
<tr>
<td>Egg yolk emulsion</td>
<td>15 ml</td>
</tr>
</tbody>
</table>
Skimmed milk 60 ml
Meat infusion broth 100 ml
Sodium thioglycollate (0.1 percent)

The ingredient except the egg yolk emulsion and skimmed milk, were mixed dissolved by heating in water bath and the pH was adjusted to 7.6. The media was then autoclaved at 121ºC for 15 minutes, and cooled to 50ºC. The egg yolk emulsion and skimmed milk were added and media was dispensed into sterile Petri-dishes. The plate were allowed to solidify and kept at 4ºC until used.

**3.6.7. Medium for preparation of Clostridium perfringens epsilon toxin.**

The medium was prepared as described by Beh and Buttery (1978).

**Ingredient**

Proteose peptone 15 g
Liver digest (Oxoid) 2.5 g
Yeast extract 15 g
Na$_2$HPO$_4$ 2.0 g
Dextrin 20 g
KHPO$_4$ 1.0 g
D.W. 1000 ml

The ingredient was dissolved in distilled water by heating in water bath and the pH was adjusted to 7.5. The media was then distributed in McCartney bottles, 15 ml each and autoclaved at 121ºC Lb for 15 minutes.

**3.6.8. Medium for preparation of Clostridium perfringens alpha toxin (α).**

This was prepared according to Moosawi, Ardehali and Pilehchian (1949) as follow

**Ingredients**

Protease peptone 3%
Na$_2$HPO$_4$ 1%
NaCl .......................... 0.25%
Glucose ........................................ 1%

The ingredients were mixed, dissolved by heating in water bath. The pH was adjusted to 7.5 and the medium was distributed in McCartney and autoclaved at 121°C Lb for 15 minutes.

3.6.9. Sporulation medium:
This medium was prepared as described by Dunkan and Strong (D&S) (1968).

Ingredients
Yeast extracts ................................ 2.0 g
Proteose peptone .......................... 7.5 g
Soluble starch (Oxoid, England) ... 2.0 g
Na₂HPO₄ ......................................... 2.65 g
D.W. ............................................. 500 ml

Ingredient was dissolved by heating in water bath. The pH was adjusted to 7.5 and the media was distributed in McCartney bottles and sterilized by autoclaving at 121°C for 15 minutes.

Animals and animal origin.

3.7. Field survey
Camel-calves aged six months or younger owned by nomadic pastoralists of different tribes at Kassala, El-Gedarif and Butana (Eastern State), Nahr El-Niel State (Northern State) and Kordofan State (Western States) were surveyed for camel calves diarrhea. These animals included local breeds vizä Arab, Anafî, Bushari, Sawahli and crosses between these breeds.

3.8. Experimental animals

3.8.1. Camels-calves (Camelus dromedaries):
Eight male camel-calves, 4-6 months of age, and their dams were purchased from the market. They were kept in a shed at Central Vet. Res. Laboratory Centre and fed dry sorghum valgaris (Abu Sabbæein) and green lucerne, (Berseem) and
water ad libitum. These animals were examined for ecto-ento and blood parasites. They were then treated with long acting oxytetracycline HCL 200 mg/ml. Each calves received 1 ml/10kg body weight intramuscularly and repeated at 48 hours intraval and sulphamethazein 33.3% containing 333 mg sulfadimidin was injected in rate of 6 ml/10 kg body weight as initial dose, then 3 ml/10 kg body weight as maintenance dose for three consecutive days.

The calves were kept under observation for two weeks before the experiment commence.

Bacterial strains used as inocula: organisms isolated and typied in chapter four were used.

3.8.2. Laboratory animals:
White swiss mice of 20-30 grams weight were obtained from the department of Small Laboratory Animals at Central Veterinary Laboratory, Khartoum, Sudan.

3.9. Sample collection:
Two hundreds and thirty eight faecal samples were collected from camel calves with clinical sign of diarrhea. These animals were not treated with any antibiotics during the diarrhea episode. The specimens were collected in sterile plastic bags which were held against the anal opening. These samples were kept immediately after collection in ice boxes where they had been transferred to the laboratory. A single sample was obtained from each animal during the study course.

3.10. Laboratory Examination:
In the laboratory the faecal specimens were examined for consistency, color, odour and presence of parasites. From each sample thin smear was prepared and stained with gram's stain.
3.10.1. Culturing and isolation procedures.

A loop full of faecal specimen was streaked on a dried SFP plate, left to dry and overlayed with Shahidi and Ferguson overlay agar. The inoculated plates were incubated anaerobically using gas bags generating kits at 37°C for 24 hours. When black colonies developed in this media, a part of single pure colony was picked with needle and stab was made three to fourth distance into lactose motility (Lm) agar which has been boiled for 10 minutes and cooled just before use. A second part of the same colony was subcultured in blood agar. These subcultures were incubated as before and results were recorded after 24 hours incubation. A third piece of the colony was used for preparation of thin smear, which was stained with gram. Colonies developed in blood agar surrounded by dural haemolysis, phenomenon of \textit{C. perfringens} were subcultured in RCM for propagation. Gram's stained smears were made from the RCM cultures. Pure cultures were inoculated in cooked meat media and kept as stock culture in -20°C.

3.10.2. Preparation of smears:

A drop of sterile normal saline or buffer saline (PBS) was placed on clean slide. Small portion of single pure colony was transferred from the solid media to the slide, emulsified with a drop of normal saline or PBS and spread on the slide. The smears were allowed to dry and fixed by passing through flame three times.

From liquid culture a loop full of the inoculum was transferred to clean slide, spreaded in the slide, air dried and fixed by gentle heating as before.

3.10.3. Staining methods

\textbf{Gram stain:}

This was performed according to Buxton and Fraser (1977). Briefly, thin air dried smears were fixed by gentle heating and crystal violet was applied for 2-3 minutes. The slides were then washed with tap water and stained with Lugol's iodine for 1 minute. The slides were washed again with tap water and decolorized with absolute ethanol for 15 seconds or acetone for few seconds. The slides were
immediately rinsed in tap water counterstained with diluted carbol fuchsin for 15 seconds and washed again in tap water, blotted dry and examined microscopically under oil immersion lense.

**Modified Ziel-Nelsen (Zn) staining methods.**

The method described by Buxton and Fraser (1977) was used. Thin air dried smears were prepared and fixed by passing through flame. Flood slides with dilute carbol fuchsin solution, allowed to react for 15 minutes and washed in tap water. Decolorized smears with acetic acid solution for 10 seconds. The slides were then washed in tap water counterstained with methylene blue for 15 minutes blotted dry and examined under oil immersion lense.

**Lactose fermentation and Motility test:**

Lactose fermentation and motility were both examined in lactose motility agar (LMA) medium as described by Shahidi and Ferguson (1997). The inoculum was stabbed deeply to one third of the depth in the medium which was heated and allowed to solidify just before use. Growth was observed after 24 hours anaerobic incubation at 37°C. Hanging-drop technique was also used to test for motility. A loopful of liquid culture was transferred to coverslides. The coverslide was hang to glass slide using petroleum fasline and examined microscopically.

**Nagler’s reaction**

**Lecithinase production**

The isolated organism were streaked in lactose milk egg-yolk medium and incubated anaerobically overnight at 37°C. Positive reaction was indicated by the presence of opacity around the growing colonies.

**Half plate neytralization test:**

Standard *Clostridium perfringens* alpha, beta, and epsilon antitoxin was donated by Dr. Farazan, A.V. of the Department of Veterinary Anaerobic Bacteria Vaccine, Razi Vaccine and Serum Research Institute, Iran.
Standard *C. perfringens* antisera type A antisera was spreaded to cover half of the egg-yolk plate, tilted and allowed dry. Pure colonies of the isolates were subcultured on the prepared half plate antitoxin milk egg-yolk agar starting from the neat untreated half of the plate. The plates were then incubated anaerobically at 37°C. for 24 hours.

Standard *Clostridium perfringens* alpha, beta, and epsilon antitoxin was donated by Dr.Farazan, A.V. of the Department of Veterinary Anaerobic Bacteria Vaccine, Razi Vaccine and Serum Research Institute, Iran.

**Haemolysis test**

7% sheep blood agar plates were inoculated with isolated organism. The plates were incubated at 37°C. anaerobically for 24 hours.

### 3.11. Toxogenicity

**Preparation of *Clostridium perfringens* toxins**

The toxins were prepared using the method of Sterne and Batty (1975). 18 to 24 hours culture of *Clostridium perfringens* were centrifuged in cold centrifuge, at 6000 revolution per minute for 20 minutes. The supernatant was aseptically collected in sterile McCartney bottles. **Activity of epsilon toxin:**

The concentration of trypsin needed was 1:250 therefore, 0.1 ml of 10% trypsin solution was added to 2.5 ml of the toxin filtrate and was incubated at 37°C for 45 minutes for activation.

**3.11.1. Mice inoculation:**

This test was conducted according to the method of Sterne and Batty (1975) to detect the lethal effect of *Clostridium perfringens* toxin on mice. Two mice were used for each strain. Two sets of toxins under test were prepared one part was treated with trypsin as described early and the second part was left neat. The mice were marked with boric acid solution to indicate the stain under test and whether the injected toxin was trypsinized or not.

The tail of the mouse was rubbed with a piece of cotton soaked in xylene to expose the lateral caudal tail vein. 1 ml disposable syringes were used to inoculate
0.5 ml of trypsin activated and unactivated toxin in tail vein of two mice. The mice were kept in labeled cages in the laboratory animal house with food and water ad libitum.

3.11.1.1. Animal inoculation

Preparation of inocula:

*C. perfringens* type A strain 95, 80 and type D strain 97 stock cultures maintained in cooked meat media were used for preparation of the inoculum. Then strains were subcultured in blood agar plates and incubated anaerobically at 37°C for 24 hours. Pure colonies from each plate were cultured in 50 ml toxin medium.

Type 1 strain 97 was cultured in Beh and Buttery (1978) media for epsilon toxin production. Type A strain 95 and 80 were cultured in Moosau et al. (1994) media to enhance alpha (α) toxin production. They were then incubated anaerobically for 8 hours at 37°C. Each inocula was further seeded in 500 ml bottles of the same media and incubated for 8 hours at 37°C.

Colony forming units (CFU):

1. Serial to fold dilutions were prepared from bacterial cultures in nutrient broth, 0.05 ml drop from each dilution was pipetted in plate of blood agar and incubated anaerobically at 37°C for 24 hours. The titre was calculated from the number of colonies obtained in highest dilution.

Method of administration of inoculums:

The surgical techniques were employed for intraduodenal inoculation of whole culture. The experimental animals were fastened for 24 hours before surgery. They were tranquilized with acpromazine maleate (Alvetra- Germany) /25-0.5 ml/50 kg body weight. Then the anaesthesia was maintained with Alfacaine 2% and adrenaline (20 mg Lindocane hydrochloride and 10 mg adrenaline) paravertebral lumbar anaesthesia 3 ml were injected between last thoracic and first lumber vertebra.

Experimental procedure
The skin at the site of incision was cleaned and prepared for surgery. Laboratory was performed in the right side flank ventral to the third or fourth lumbar vertebral transverse process. The pyloric area of the abomasums and the first portion of the duodenum were exposed using a drip 300 millitre (ml) of 20% dextrine solution was infused into the abomasums. Subsequently the animals were dosed (table 6) with whole culture which was infused intraduodenally using the same drip over period of approximately ten minutes. Then the abdominal incision was closed by separate muscle and skin suture. Necropsy was performed immediately after the animal died or sacrificed. Fresh samples were collected from different areas of intestine, liver, lung, kidney in sterile plastic bags and kept in refrigerator for bacteriological examination. Smears were prepared from intestinal content and stained with Gram stain. Brain, heart, lung, liver, kidney, spleen, lymph nodes, pancreas, adrenal gland, rumen, abomasums, urinary bladder and different parts of small and large intestine were immediately fixed in 10% formalin.

3.12. Histopathology Method

Selected portions of tissue were fixed in 10% formalin and process for paraffin wax embedding section using the convertial methods. 5µ sections were cut and stained with haematoxylin and eosin (H & E).

3.13. Performance of PCR assay

3.13.1. DNA Extraction:

5-10 colonies of *C.perfringens* were collected from blood agar plate and resuspended in 200µl of DDW in 1.5 ml Eppendorf tube.300 µl of liquid culture of Clostridium perfringens was transferred in microtubes (1.5ml) using micropipettes. The samples were boiled at 100°C. for 20 minutes, snapped cooled on ice for 5 minutes and centrifuged at 13000 revolution per minute (rpm) for 3 minutes. The supernatant was transferred to clean tubes and stored at -20°C.

3.13.2. Oligonucleotides:
PCR primers of Qiagen, 1000 Atlantic A ve, suite 108, Alameda, CA94501, were kindly supplied by Dr. Songer, J.G. of the Department of Veterinary Science, University of Arizona, Tucson.

The Tag polymerase, dNTPs, buffers and magnesium chloride (Mgcl$_2$) were supplied by Promega corporation, 2800 Woods Hollow Road, Madison, W1 53711-5399, USA.

**Table. 6.** Demonstrate type of inocula, dose in ml and time (in hours) elapsed between dosing and death and clinical symptoms.

<table>
<thead>
<tr>
<th>Group</th>
<th>Animal No.</th>
<th>Type of inocula</th>
<th>Dose in ml</th>
<th>Time between dosing and death</th>
<th>CFU</th>
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<p>| | | | | | | |</p>
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<tr>
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<td>1</td>
<td>2944</td>
<td>D, 97</td>
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<td>18</td>
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<tr>
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<td>5</td>
<td>D, 97</td>
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<td>24</td>
<td>14X10^{-5}</td>
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<tr>
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<td>2943</td>
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</tr>
<tr>
<td></td>
<td>7</td>
<td>A, 95</td>
<td>300</td>
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<tr>
<td>3</td>
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<tr>
<td></td>
<td></td>
<td>A, 80</td>
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<td></td>
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<tr>
<td>4</td>
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<td>No death</td>
<td></td>
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<td>6</td>
<td></td>
<td>500</td>
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**Primers used:**

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<td>C. <em>perfringen</em> α toxin</td>
<td>cpa-1+cpa-2</td>
<td>324</td>
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<tr>
<td>C. <em>perfringen</em> β toxin</td>
<td>Cpb-1+cpb-2</td>
<td>196</td>
</tr>
<tr>
<td>C. <em>perfringen</em> ε toxin</td>
<td>E1x-1+e1x-2</td>
<td>655</td>
</tr>
<tr>
<td>C. <em>perfringen</em> 1 toxin</td>
<td>1a-1+1a-2</td>
<td>446</td>
</tr>
<tr>
<td>C. <em>perfringen</em> ε toxin</td>
<td>Cpe-1+ cpe-2</td>
<td>233</td>
</tr>
<tr>
<td>C. <em>perfringen</em>-B toxin</td>
<td>Cpb2-1+cpb2-2</td>
<td>567</td>
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</tbody>
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### Primer sequences:

<table>
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<tr>
<th>Sequence</th>
<th>Name</th>
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</thead>
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<td>Cpa</td>
<td>Forward cpa-1</td>
<td>GCTAATGTTACTGCGTTGA</td>
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<tr>
<td></td>
<td>reverse cpa-2</td>
<td>CCTCTGATACATCGTGTAAG</td>
</tr>
<tr>
<td>Cpb</td>
<td>Forward cpb-1</td>
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</tr>
<tr>
<td></td>
<td>reverse cpb-2</td>
<td>GCAGGAACATTAGTATATCTTTC</td>
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<tr>
<td>EtX</td>
<td>Forward etx-1</td>
<td>GCGGTGATATCCATCTATTAC</td>
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<td></td>
<td>reverse etx-2</td>
<td>CCACCTACTTGGTCCTACTAAC</td>
</tr>
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<td>1a</td>
<td>Forward 1a-1</td>
<td>ACTACTCTCAGACAAGACAG</td>
</tr>
<tr>
<td></td>
<td>reverse 1a-2</td>
<td>CTTCCTTTCTATTACTATACG</td>
</tr>
<tr>
<td>Cpe</td>
<td>Forward cpe-1</td>
<td>GGAGATGGTGGATATTAGG</td>
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<td></td>
<td>reverse cpe-2</td>
<td>GGACCAGCAGTTGTGATA</td>
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<tr>
<td>cpb2</td>
<td>Forward cpb 2-1</td>
<td>AGATTTTAATATGATCCTAACC</td>
</tr>
<tr>
<td></td>
<td>reverse cpb 2-2</td>
<td>CAATACCCCTTCACCAAATACTC</td>
</tr>
</tbody>
</table>

### 3.13.3. PCR reaction:

The reagent were prepared and mixed as follow fro PCR reaction of 50 µl master mix for all toxin (multiplex PCR)

Volume (µl) per 50 µl reaction

<table>
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<th>Component</th>
<th>Volume (µl)</th>
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<tr>
<td>PCR buffer (x10)</td>
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<tr>
<td>dNTPs</td>
<td>1.2</td>
</tr>
<tr>
<td>Magnesium chloride (MgCl₂)</td>
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</tr>
<tr>
<td>1a</td>
<td>1.3</td>
</tr>
<tr>
<td>cpa</td>
<td>1.2</td>
</tr>
<tr>
<td>etx</td>
<td>1.1</td>
</tr>
<tr>
<td>cpb</td>
<td>0.9</td>
</tr>
<tr>
<td>cpe</td>
<td>0.9</td>
</tr>
</tbody>
</table>
The volume of each primer set listed referred to a mixed of the forward and reverse primers. Each primer was resuspended to 40 µm and then equal volumes of the forward and reverse primers were mixed together.

3.13.4. PCR Method:
1. A cocktail containing all PCR reagent was prepared for number of sample to be tested, vortex a, centrifuge briefly.
2. Aliquot 40µl of the cocktail to each .5 ml PCR tube which were placed back on ice.
3. 10µl of the supernatant of boiled cells were added to each 50 µl PCR reaction.
4. The tubes were then placed in a Biometra DNA thermal cycler (for PCR).

**Amplification of C. perfringens:**

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>94°C</td>
<td>2 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>1 min</td>
</tr>
<tr>
<td>Annealing</td>
<td>55 °C</td>
<td>1 min</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>1 min</td>
</tr>
<tr>
<td>Number of cycles</td>
<td>35</td>
<td></td>
</tr>
</tbody>
</table>

3.13.5. Detection of PCR product.

The gel electrophoresis separation method was used. A 1.5% agarose gel stained with 2.5 µl ethidium bromide was prepared. 10µl of the PCR product is mixed with 2µl bromophenol blue dye (loading buffer) 2µl of a 100 pb DNA molecular weight marker is loaded with 2µl loading buffer and transferred into the first well. In the following wells samples DNA were transferred followed by positive template and last by negative template. Gel electrophoresis is performed
at 75 9V) for 45 minutes and the analysis was done by using an automated photo documentation system (Bio. Doc. Analyza, digital).
4. Results

Unsatisfactory methods were previously used for isolation and enumeration of *C. perfringens* (Shahidi and Ferguson, 1971 and Hauschild and Hillsheimer, 1974). The egg-yolk free tryptose, sulphite, cycloserine
and neomycin blood agar was useful but expensive (Hauschild, Desmarchelier, Gilbert, Harman and Vahlefeld, 1979). Others morphological and biochemical tests were reported for the identification of pathogen (Carter, 1997 and Barrow and Felthman, 1999). These methods were labrous and time consuming. Alternatively, Shahidi and Ferguson (1971) successfully used SFP agar with conjunction with LM agar as confirmatory. This method save considerable time, effort and material towards the final identification in 48 hours. The SFP agar consisted of ferric ammonia citrate to demonstrate H₂S production and egg-yolk to demonstrate lecithinase production. The LM agar to demonstrate lactose fermentation and motility of *C. perfringens*. Various confirmatory tests performed such colony morphology, Gram stain, Neglar and Neglar half plate inhibition test (Buxon and Frazer, 1977).

Different methods are available for typing *C. perfringens* including the conventional serum neutralization in mice (Sterne and Batty, 1975), other immunoassay tests (Beh and Buttery, 1978; Henderson, 1984 and Naylor *et al*., 1997), biotechnology (Daube *et al*., 1996 and Yoo *et al*., 1997). The later is super to the serological methods because it is time saving, sensitive, more specific and reliable.

*C. perfringens* was isolated from 97 out of 238 faecal samples (40.7%). The prevalence of this organisms in differed location was illustrated in table 6.

**4.1. The cell morphology and Gram reactions:**

Cells of all isolated (100%) were Gram positive polymorphic bacilli with blunt ends in smears prepared from faecal specimens or fresh cultures. In old culture the organism possessed less affinity to stain with crystal violet and stained Gram negative. The organisms occurred singly,
in pairs and in 3 isolates were in chains. Distinct capsules were seen surrounding the bacilli Gram stained smears prepared from faecal samples (Fig. 1) and cultures of two isolates.

4.2. Growth in SFP medium:
On SFP media all isolates (100%) developed black colonies, 2-4 mm in diameter encircled by distinct zone of opalescence (Fig. 2) indicating the lecithinase enzyme production.

4.3. growth in blood agar and haemolysis test:
Grow the in blood agar:
On sheep blood agar all isolated developed colonies which vary in shape and size. Colonies of many isolated were grayish 3-5 mm in diameter flat, umbonate with radilly straited surface and dentate edges. Opaque or glisting colonies 2-3 mm diameter rounder, low convex with entire edges were demonstrated by same isolator.

Haemolysis test:
On sheep blood plates colonies of all isolates (100%) developed double zone of haemolysis. The colonies (Figure 3) were surrounded by inner narrow zone of complete haemolysis followed by wide zone of incomplete haemolysis.

4.4. Growth in cooked meat medium:
All isolated propagate in this media with production of turbidity and gas which in many isolates produces stromy growth with the meat particles pushed to the surface. 98 isolates were saccharolytic whereas 5 of them were non saccharolytic.
Figure 1: Smear prepared from faecal sample No. 74.  
Note capsulated pleomorphic Bacilli gram stain XX 40

Figure 2: Growth of C. perfringens in SFA agar.  
Note, black colonies surrounded by zone opalacence
4.5. Growth in RCM and DS media:

Following 24 hours anaerobic incubation C. perfringes isolates probates in both media with production of turbidity and gas.

growth aerobically:

There were no growth in all cultured blood agar incubated at 37°C for 24 hours.

Lactose fermentation:

All isolates changed the colour of LM agar from red to yellow (figures 4) and produced gas which indicative for lactose fermentation.

Motility:

All isolates (100%) were non motile (Figure 4) whether examined in LM agar or hanging drop.

Modified Zn stain – from – isolates ( %) of sporulate in DS medium. Spoorulated organism stain blue with central or subterminal but as bulging spores stained red.

Naylar’s reaction:

All isolates demonstrated the opalescence in egg yolk agar and in shahidi and Ferguson perfringers agar.
Figure 3: Growth of C. perfringens in blood agar medium. 
Note, colonies encircled by double zone of haemolysis.

Figure 4: Demonstrated Motility and lactose fermentation. 
Note change of colour from red to yellowish.
4.6. Nagler’s half-plate antitoxin:
This test was conducted to show the inhibitor of lecithinase production by \textit{C. perfringe} type A antitoxin. Out of 97 \textit{C. perfringes} isolates examined 87(89.7\%) were completely inhibited by \textit{C. perfringen} Type A antisera whereas 10(10.3\%) showed incomplete inhibition (Figure 5 a &b).

4.7. Result of PCR product
The amplified target sequence of \textit{C. perfringens} specific toxin gene by M PCR was observed on the agarose gel as bands at approximately the 324 bp level for cpa; 655 bp for etx; 233 for cpe and 567 for cpb2(Fig. 6 and 7). All isolates 100%(N=95) produced fragment of cpa(\(\alpha\) toxin gene) confirmed that all isolates were \textit{C. perfringens}. The vast majority of these isolates were type A (N=95) and two isolates were type D(Table 7). The prevalence of enterotoxigenic \textit{C. perfringens} type A(cpe) and/or type A with cpb2 fragment (\(\beta_2\) toxin gene) were illustrated in table 8.
Figure 5a: Demonstrated complete inhibition of lecithinase by type A antitoxin ($\alpha$–antitoxin)

Figure 5b: Demonstrated incomplete inhibition of ecithinase by type A antitoxin ($\alpha$ – antitoxin)
Figure 6: Agarose gel-electrophoresis of the PCR product (stain with ethidium bromide) obtained after MPCR amplification of C. perfringens specific toxin gene. Lane 1, DNA Marker; Lane 2, negative control; Lane 4, No. 64, Cpa + cpe (toxigenic type A); Lane 5 and 6, No. 97 and 27, Cpa + etx gene (type d); Lane 7, No. 29 cpa + cpe; lane 8, No. 80, Cpa + Cpb2 toxin gene (type A + β2 toxin).

Figure 7: Lane 1, DNA Marker; Lane 2, No. 179, cpa + cpe + cpb2; Lane 2, Cpa, Lane 3 and 4, Cpa + cpe + cpb2; Lane 5 and 8, cpa
Table 7: The prevalence of *C. perfringens* in different locations

<table>
<thead>
<tr>
<th>Location</th>
<th>Samples examined</th>
<th>Position No.</th>
<th>C. perfringens types</th>
<th>Entero toxin</th>
<th>B₂ toxin</th>
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<tr>
<td></td>
<td></td>
<td>%</td>
<td>A</td>
<td>B</td>
<td>C</td>
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<tr>
<td>Kassala</td>
<td>2</td>
<td>1</td>
<td>1</td>
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<td>0</td>
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<tr>
<td>Gedarif</td>
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<td>21</td>
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<td><strong>Total</strong></td>
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<td><strong>97</strong></td>
<td><strong>95</strong></td>
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</table>
Table 8: Prevalence of enterotoxin in $\beta_2$ toxin in different locations

<table>
<thead>
<tr>
<th>Location</th>
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4.8. Result of toxigenicity of mice

4.8.1. Toxicity examination
It was found that 78 (92.9%) of examined isolates were toxic and killed the mice, whereas 6 (7.1%) of the isolates resulted in an unthriftness followed complete recovery of the inoculated mice within 24 hours. The degree of toxigenicity was summarized in table 9.

4.8.2. Clinical signs
Affected mice demonstrated a wide variety of neurological manifestation. The predominant clinical symptoms included dullness, reluctant to move, followed by incoordination of movement, paralysis or sometimes tremor of hind quarters, abdominal respiration and death at various time post inoculation, just prior to death many of the mice stretched their body having tendency to flaccid paralysis. Generally, affected mice died within 30 minutes to 72 hours post infection. Ten mice showed shaking of head and/or tail. Three mice revealed rough coat and two mice manifested jerky movement jumping from one side to the other side of cage. However, one of the inoculated mice did not show any clinical abnormalities.

4.9. Macroscopical findings
No characteristic gross lesion apart of congestion of blood vessels of the brain. Two mice revealed subcutaneous oedema and haemorrhage at the site of inoculations. Hydrothorax and hydrometroonium were observed in the other two mice.
<table>
<thead>
<tr>
<th>Total</th>
<th>ND</th>
<th>No. examined</th>
<th>Degree of toxigenicity</th>
<th>Animal No.</th>
<th>%</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>97</td>
<td>13</td>
<td>84</td>
<td>++++</td>
<td>5</td>
<td>6.0</td>
<td>7.1 of the inoculated survived after showing an initial unthritness within the first 24 hours followed by complete recovery</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+++</td>
<td>43</td>
<td>51.2</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>++</td>
<td>28</td>
<td>33.3</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>2</td>
<td>2.4</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>0</td>
<td>7.1</td>
<td></td>
</tr>
</tbody>
</table>

++++ Highly toxic, death within 30 minute to one hour.
+++ Toxic, death within 1-6 hours.
++ Moderately toxic, death within 6-24 hours.
+ Mild toxic, death within 48-72 hours.
- Non toxic, no death after 72 hour.
ND Not done.
4.10. Clinicopathological aspect experimental C. perfringens enterotoxaemia in camel (Camelus dromedaries)

Relevant literature of C. perfringens infection in camels elucidate that only fragmentary inconclusive result of observation of some natural sporadic cases or outbreaks. Although the clinicopathological alteration subsequent to type D C. perfringens have been produced experimentally and well described in sheep, goats and cattle (Niilo, et al., 1963; Gardner, 1973; Blackwell et al., 1991 and Uzal and Kelly, 1998), to my knowledge, there is till a paucity of information regarding the pathology and pathogenesis of this disease in camels. In addition, clinically type A and B C. perfringens induced nervous sign (Jensen and Swift, 1982; Chauchan et al., 1985, Barkes and Van Dreumel, 1985 and Werney et al, 1991). However, there was lack of record of neuropathological lesions of the A & B. Furthermore, the role of alph (α) and Beta (B) toxins in these nervous manifestation need to be verified. Consequently, this work was designed to establish experimentally type D and A C. perfringen enterotoxaemia in camels and to study the pathology and pathogenesis of these organism in camels.

4.10.1. Experimental design:

The camel calves were allocated into four groups. Each group consisted of two animals (Table.6.). These animals were clinically examined and blood in EDTA and sera were collected before and periodically after infusion. All groups were inoculated with dextrine solution into the abomasums in addition, group one was inoculated with type D stain 97, group 2 and 3 received type A strain 95 and 80 respectively. Each animal of group four was inoculated with one of the sterile toxin media. No. 2894 was
infused with Beh and Butlery 1978 media and No. 6 was inoculated with Moosaw et al (1994) media.

Results

1. Pathological findings following intraduodenal infusion of type D strain 97 in camels

4.10.2. Clinical symptoms

Infected animals with type D revealed normal body temperature (39.0), weakness followed by lateral recumbency, prostration, stiffness of the neck and leg and death 18 and 21 hours post inoculation (HPI) One of the animals (No 2944) developed diarrhoea 6HPT.

4.10.3. Macroscopic findings:

At necropsy, the carcases were in good body condition. The most striking change was massive pericardial, pleural and peritoneal effusion of exudates. The pericardial and pleural cavities contained clear straw colour fluid, whereas the abdominal cavity was overfilled with approximately 5 liters of serosangunous fluid containing fibrous flacks. Encephalic lesions, apart of congestion of all blood vessels, were not observable. However, congestion, petechial and ecchymotic haemorrhages were more marked in subendo-and subepicardial (Figure 8), liver, lung, kidney (Figure 9), lymph nodes, larynx and throughout the mucosa and serosa of gastrointestinal tract (Figure 10). Paint brush haemorrhages were encountered in ileum which was
purplish red and contained dark purplish red ingesta. All mesentery was hyperaemic. The ruminal and abomasal wall enormously distended with oedema fluid. The mucosa of both was tinted with ecchymotic haemorrhages and congestion (Figure 11).

Figure 8: Heart, Note, heamorrhage
Figure 9: Kidney. Note haemorrhage

Figure 10: Intestine. Note haemorrhages and hyperaemia through the serosa and mucosa of small and large intestine.
Figure 11: Rumen and abomasums. Note mucosal and serosal haemorrhage and swelling of the wall

4.10.4. Microscopic findings:
Generally, the microscopic changes were inco-ordinate with gross lesion observed in all organs examined including brain, heart, lung, liver, kidney, spleen lymph nodes, pancreas, adrenal gland, rumen, abomasums, intestine, and urinary bladder. The most striking histopathological alteration was severe vasculitis with dilatation of numerous small and large blood vessels. The endothelial lining of many of these vessels was severely damaged, discontinued and with subsequent haemorrhages.

4.10.4.1. Brain:
Microscopically the brain of infected calves displayed congestion of most of blood vessels, pronounced vasculitis associated with perivascular haemorrhages (Figure 12) and/or perivascular and intercellular oedema
(Figure 13 and 14). There were marked focal to diffuse microglial proliferation in the basal ganglia, thalamus, corpus callosum, internal capsule particularly in subventricular area (Figure 15 and 16) where the adjacent ependymal cell layer was intensely infiltrated with microglial cells (Figure 16). In addition, the subventricular area presented focal and diffuse areas of early liquefactive necrosis. The ependymal cells in the vicinity of these necrotic areas showed vasculature necrosis and denudation (Figure 17). There was varying degree of neuronal degeneration including chromatolysis, distortion and collapsed (Figure 14). Examination of cerebellum disclosed necrosis and dissolution of the molecular layer and the adjacent purkinje cell stratum. Furthermore, there were fragmentation of more fibre and vacuolation of cerebellar peduncle ground substance (Figure 18).

4. 10. 3. 2. Heart:

There were severe subendo-and-epicardial haemorrhages which in many parts penetrated between the myocardial muscle (Figure 119). The myocardial muscle and purkinje fibre or the vicinity of haemorrhages revealed degeneration vacuolation and necrosis.
Figure 12: Section of brain. Note perivasular haemorrhage (arrow). H&EX10
Figure 13: Brain. Note, congestion and perivascular edema (arrow). H&E X10.

Figure 14: Brain. Note intercellular oedema and necrotized, collapsed neuron (arrow) H&E X40
Figure 15: Section of brain. Note glial nodules (arrow), proliferation of microglial in subepidermal area (large arrow). H&EX4

Figure 16: Brain. Note diffuse gliosis H&EX10
Figure 17: Brain, Note early liquefactive necrosis, necrosis and denudation of epyndiamal layer (arrow). H&E X10.

Figure 18: Brain cerebellar peduncle. Note fragmentation of nerve fibre, vacuolation of ground substance. &EX10.
4. 10. 3. 3. Lung:

The lungs of intoxicated calves showed severe congestion and haemorrhage. Many of alveoli were distended with oedematous fluid and aggregates of lymphoid cells (Figure 20).
4. 10. 3. 4. Liver:
The most prominent hepatic changes were a tropic collapsed hepatocytes with dilalated and congested sinusoids (Figure 21), In severely affected areas the hepatocytes were vacuolated and some disintegrated and replaced by tissue debris and extravasated erythrocytes. Multiple cyst like structures were observed mainly in the periportal area (Figure 22).

4. 10. 3. 5. Kidney:
Changes in the renal tissue included collapsed segmented glomerular tufts with dilatation at the Bowman’s space which in many filled with pink staining proteinaceous material (Figure 23). Severe haemorrhages was detected particularly in medularly rays. Some renal tubule lining epithelium showed dissociation and disintegration.

4. 10. 3. 6. Spleen and lymph nodes:
Changes in these organs included atrophic lymphoid follicles with depletion of cells of the germinal center and haemosiderin pigment deposition. These follicles were separated by lakes of extravasated erythrocytes (Figure 24).

4. 10. 3. 7. Pancreas
The most marked histological findings were subcapsular and interlobular haemorrhage, and oedema (Figure 25).
Figure 20: Lung. Note, oedema, and haemorrhages. H&EX40

Figure 21: Liver. Note, haemorrhages dissociated hepatocytes. H&EX 10
Figure 22: Liver. Note, large cavities. H&EX10

Figure 23: Section of kidney. Note, collapsed glomerula with pink proteinaceous material, haemorrhages H&EX40.
Figure 24: Spleen. Note atrophic lymphoid follicle, haemorrhage. H&EX10

Figure 25: Pancreas. Note, subcapsular haemorrhages congestion and oedema. H&EX10
4. 10. 3. 8. Adrenal gland:
The cells of the adrenal, cortex zona glomerulosa were dissociated and some were ruptured and disintegrated. Similarly the cells of the zona fasciculate and zona reticularis were swollen and or ruptured. Numerous large cysts, many of which were distended with homogenous pink material were scattered in the cortex and medulla. The majority of these cysts were present in zona reticularis and medulla intermixed with extravasated erythrocytes and mild mononuclear cells reaction (Fig.26).

4. 10. 3. 9. Stomach:
Both rumen and abomasum revealed degeneration and denudation of lining epithelium, marked oedema in the lamina propria, submucosa and between the separated muscle fibre of muscularis mucosa. The serosa showed oedema, congestion of all blood vessels and haemorrhage which also detected between adjacent muscle bundle (Figure 20).

4. 10. 3. 10. Intestine:
Intestine showed congestion, severe mucosal haemorrhages associated with epithelial degeneration and denudation. The mucosal crypts were separated from each other and some were filled with pink homogenous material. In many areas the epithelial cells of the submucosal Brunner’s glands were detached and some were fragmented. The intact gland were engorged with pink staining material (Figure 28). Oedema of the submucosa, mucosa and serosa where haemorrhage and congestion of all blood vessels were prominent.
4. 10. 3. 11. Urinary bladder:
There were oedema, haemorrhages and congestion of blood vessels in the connective tissue concomitant with necrosis desquamation of the lining epithelium (Figure 29).

4. 10. 4. Bacteriological Results:
*C. perfringens* was reisolated from intestinal content mixed with few colonies of *E. coli*.

No bacterial growth after 24 hours incubation of cultures of lung, livers and kidney.
Figure 26: Adrenal gland. Note, large cysts like stone filled with pink material and some inflammatory cell, haemorrhage, and oedema. H&EX10

Figure 27: Rumen. Note, serosal haemorrhage and oedema, separation of muscle bundles by oedema fluid. H&EX10
Figure 28: Intestine. Note several mucosal haemorrhages. Necrosis and denudation of lining epithelium, separated Brunner glands which were filled with haemorrhage material, detachment of acini from basement membrane, dissociation of lining cells of some gland. H&EX10.

Figure 29: Urinary bladder. Note, desquamation of mucosal epithelium, superficial haemorrhage, oedema and congestion in connective tissue H&EX10.
4.11. The pathological findings following intraduodenal infusion with type A strain 95 in camels

4.10. 11.1. Clinical symptoms:
Intoxicated calves, with strain 95 culture revealed normal body temperature (38.9 – 40.1) dul, dejected, lathery, lateral recumbency, prostration and death of both No 2943 and 7 after 18 and 30 HPI respectively. In addition, animal No. 2943 developed diarrhea 6 HPI.

4.11. 2. Macroscopical findings:
At necropsy, both carcases were in fairly good body condition. The pericardial and thoracic cavitier contained clear straw colour fluid with fibrous flacks. Large amount of approximately 5 liters of serosanguineous fluid containing strands of fibrous were present in the abdominal cavity. Both animals showed congestion of most blood vessels of brain whereas No. 7 showed cysts like cavities (Fig.30), scattered in most of brain tissue. Endocardial, pericardial epicardial sac and ribs ecchymosis was encountered in both animals (Figure 31). In constrast No 7 presented subendocardial paint brush haemorrhage with myocardial muscle became swollen redish black with offensive odour, sanguineous exudates and gas bubbles (Figure 32). Typical lesions to those of myocardial muscle were detected in subcutaneous muscle of fore and hind limbs. In addition, congestion, petechial and ecchymoric haemorrhages were evident in liver, lung (Fig.33), kidney and urinary bladder (Figure 34) and adrenal gland of both animals. Patchy ecchymosis and hyperaemia were detected through the serosa and mucosa of gastrointestinal tract, but were more marked in the item which was dark purplish and containing purplish to black ingesta.
Figure 30: Brain of animal No. 7. Note cyst-like structure.

Figure 31: Ribs, heart and pericardial sac. Note haemorrhges
Figure 32: Heart animal No. 7. Note black muscles with gas bubbles.

Figure 33: Lung note.
Figure 34: Kidney and urinary bladder: note haemorrhges
4.11.3. **Microscopical Findings:**

Congestion, haemorrhages and vasculitis were the predominant histopathological changes in all organs examined.

4.11.4. **Brain:**

The most striking encephalic lesions were vasculitis with necrosed discontinued endothelial lining, perivascular and intercellular oedema and haemorrhages (Figure 35 and 36). Though the majority of blood vessels were engorged with erythrocytes, some of them contained erythrocytes mixed with inflammatory cells or contained pink homogenous material. Early malaci lesions associated with microglial microglial proliferation were frequently seen particularly in subepyndemal area, where the epyndemal cells were eroded (Figure 37). There were vacuolation of while matter mainly in the cerebral hemisphere, where the neuron presented chromatolysis distortion and some of them devoid of nucleus (Figure 38). Severe reaction was demonstrated in cerebellum which included softening and vacuolation of ground substance, detachment of pia matter, oedema and necrosis of Purkinje stratum. Many of Purkinje cells were distorted while some collapsed and/or devoid of nucleus. Small areas of absence or disintegration of Purkinje cells were encountered (Fig.39). Cysts like cavities were scattered in different parts of the brain of No. 7. Many area of brain stem revealed effusion of mononuclear cells intermixed with proliferated microglial cells and vacuolation of ground substance (Figure 40). The choroids plexus presented oedema, congestion and sloughing of lining epyndemal cells (Figure 40).
Figure 35: Section of brain. Note perivascular oedema. H & E X10

Figure 36: Section of brain. Note the cellular oedema. H & E X40
4.11.5. Heart:
There were massive accumulation of extravasated erythrocytes in subendocardial, subepicardial and between the myoccardial muscle. In addition animal No.7 showed varying degree of degenerative change in cardiac muscle (Fig.42), manifested by eosin staining of muscle fibre with disappearance of sarcolemal nuclei which appeared to be due to haryolyis. Some pyknotic nuclei and fragmented chromatin masses were also seen scattered in muscle fibre. In severely affected area the necrosed fragmented muscle fibre were separated from one another and from endomysial connective tissue by oedema containing same inflammatory cells mainly neutrophil and lymphocytes particularly a rounded the blood vessels. Two distinct forms of myonecrosis which included coagulative necrosis and fragmentation and dissolution of muscle fibre were encountered coexisting with cyst like structure or air bubbles, hemorrhages and inflammatory exudates. Some of the cysts like structure contained pink amorphous material.

4.11.6. Lung:
Oedema, congestion were the common findings coexisting with subpleura and intra-alveolar haemorrhages. Focal collection of aggregates of lymphoid cell were seen in perivascular, peribronchial and intraalveolar areas.

4.11.7. Liver:
Changes of the liver included enlarged vacuolated, hepatocytes, collapsed sinuslodes, congestion and hemosiderin pigment deposition. In addition, liver of animal No. 7 presented cysts like structure of various size.
Figure 37: Brain infiltration of subepidermal layer

Figure 38: Section of brain. Note, neuron degeneration and vacuolation of ground substance.
Figure 39: Section of brain. Note, vaculated cerebellum, ground substance, detachment of dura matter, necrosis of Perkinje stratum and adjacent granular layer, dissolution and absence of some Perkinje cells. H&E X 10.

Figure 40: Brain. Note vaculation of ground substance and effusion of mononuclear cells and macroglial. H&E X40.
Figure 41: Chroid plexus. Note oedema and sloughing of lining epyndermal cells layer. H&E X10.

Figure 42: Muscle. Note severe myonecrosis with dissolution muscle fibre and accumulation of oedema fluid between the necrosed muscle bundles and in the intersitium.
4.11.8. Kidney:
There were glomerulitis with collapsed segmented glomerular tuft, dilatation of Bowman’s space where many of them filled with pink proteinous material. There were marked haemorrhages particularly in renal rays.

4.11.9. Spleen and lymph node:
The most prominent lesions were atrophic follicular germinal center, oedema and haemorrhges. Numerous cysts-like structures were scattered in lymph node of animal No. 7(Fig.43).

4.11.9. Adrenal gland:
This organ presented degeneration and dissociation of zona glomerulosa cells. Many of these cells clumped in the Lumina of the acini. The bounder of some of these acini ruptured forming large cavities (Fig.44). There were severe haemorrhages and congestion in the medulla.

4.11.10. Pancrease:
There were marked subcapsular and interlobular haemorrhage and oedema.

4.11.11. Stomach:
Both obomasum and rument presented muosal haemorrhages, necrosis and denudation of lining epithelium. The mucosal changes were accompanied with oedema, congestion and haemorrhages of connective tissue lamina propria, submucosa, muscularis mucosa and serosa.

4.11.12. Intestine:
There were necrosis and desquamation of the lining epithelium, oedema and aggregate of cellular exudates in connective tissue lamina propria, submucosa and muscularis mucosa where the muscle fibre. necrosis and disintegration was marked. The muscle fibre was separated by
Figure 43: Spleen. Note, Haemorrhages and cyst-like structure. H&E X10

Figure 44: Adrenal gland. Note, detachment of cell of Zona granulose, large cavities. H&X1
Figure 45: Intestine. Note oedema, haemorrhage in subserosa and between necrosed disintegrated muscle fibres. H&E X10.
oedematous fluid and haemorrhages. There were also oedema and haemorrhages in subserosa Figure 45).

4.11.13. Urinary bladders:
There were haemorrhage and erosion of the lining epithelium, oedema of connective tissue and congestion and extravasated erythrocytes in the serosa.

4.11.14. Bacteriology
*C.perfringens* was isolated from intestinal content of both animals and from liver, lung, kidney and muscle of animal No.7 only.

4.12. The pathological findings following intraduodenal infusion with type A strain 80 in camels

4.12.1. Clinical symptoms:
Intoxicated animals revealed normal body temperature, weakness, dull and depression, followed by lateral recumbency, prostration and death after 24 HPI.

4.12.2. Macroscopic findings:
At necropsy, the animals appeared to be in good body condition. The pericardial sac contained clear straw colour fluid with strands of fibrine, whereas the abdominal cavity contained approximately 5 liters of serosanguinous fluid and small amount, about 15 ml, of serous fluid was detected in the thoracic cavity. All brain blood vessels were congested and prominent. Congestion, petechial and ecchymotic haemorrhages were the common findings in the epi-endo and myocardial muscle (Fig.46), lung, liver (Figure 47) and kidney. Patchy haemorrhages was detected in mucosa and serosa off gastrointestinal tract particularly the ileum which was dark redish and contained dark red injesta. The rest of intestine was filled with yellowish ingesta.

4.12.3. Microscopical findings:
Generally the most stricking lesions were haemorrhages, congestion and vasculitis with disrupted endothelial cells involving all organs.
4.12.4. Brain:
Histologically, the predominant encephalic lesions were congestion of most blood vessels, vaculitis with endothelial lining of many vessels were discontinued resulted in perivascular haemorrhages and perivascular and intercellular oedema. Focal and diffuse microglial proliferation were frequently observed particularly in subventricular area associated necrosis.
Figure 46: Heart. Note, endocardial haemorrhages.

Figure 47: Liver. Note, haemorrhages and congestion
and detachment of ependymal cell lining the ventricle and early malacic lesions (Figure 48 and 49). More advance malacic lesions were recognized in hypocampus which showed necrosis and dissolution of neurons and liquefaction of ground substance (Figure 48). Sections of cerebellum showed degeneration necrosis of Purkinje cells stratum where the ghost Purkinje cells were distorted, devoid of nuclei and focal absence of some Purkinje cells. There were rarefaction of the ground substance of the molecular layer, dilatation of subarachnoid space and detachment of pia mater from the cortex (Figure 51). Other encephalon changes included necrosis of nerve fibres of internal capsule with discontinued cell membrane, oedema, congestion of choroids plexus and sloughing of ependymal cells (Fig.52).

4.12.5. Heart:

The heart presented severe subendo – and subepicardial haemorrhages which pentrated between the myocardial muscle fibres. In severely affected areas the normal heart tissue was replaced by accumulation of extravasated erythrocytes (Figure 53). The Purkinje cells fibre were vacuolated and necrosed whereas many myocardial muscle fibres revealed necrosis dissolution and were replaced by extravasated erythrocytes.

4.12.6. Lung:

This organ demonstrated oedema, congestion and haemorrhages in the alveoli and subpuleral tissue.

4.12.7. Liver:

There were marked damaged to the blood vessels endothelium resulted in outpouring of erythrocytes in the dilated sinusoids and between the dissociated hepatocytes (Figure 54). Focal areas of hepatic cells necrosis dissolution and haemosiderin pigment deposition were present.
Figure 48: Brain. Note, focal gliosis, detachment of the degenerated ependymal layer, early malacic lesions. H&E X10.

Figure 49: Brain. Note, diffuse glial cells proliferation. H&E X10.
Figure 50: Brain. Note, necrosis, dissolution of hippocampus granular layer. H&E X10.

Figure 51: Brain, cerebellum. Note, necrosis of purkinje cell stratum, dilatation of subarchnoid space, discontinued pia matter. H&E X10.
Figure 52: Brain, Choroid plexus. Note, oedema, congestion and detachment of ependymal Cell. H&E X10.

Figure 53: Heart. Note endocardial and myocardial harmorrhages. H& EX1
4.12.8. Kidney:

The glomeruli showed collapsed segmented glomerular tuft, dilalation of Bowman’s space which in many of them contained pink amorphous fluid. The epithelium lining many of the renal tubules was detached from basement membrane and disrupted. Some of these degenerated tubules contained pink amorphous fluid. Severe haemorrhages was observed particulary in renal rays coexited with haemosidern pigment deposition.

4.12.9. Spleen and lymph nodes:

Both spleen and lymph nodes showed atrophic lymphoid follicles with marked depletion of lymphoid cells of their germental center, oedema, haemorrhages and haemosiderin pigment deposition.

4.12.10. Adrenal glands:

The zona glomerulosa presented dissociation and disintegration of most of the secretory cells resulted in formation of large cavities ensheathing few cells (Figure 55). The cells of zona fascicularis and reticularis were enormously distended and some ruptured. Large cysts like structure were scattered in both cortex and medulla which displayed haemorrhages and congestion.

4.12.11. Stomach:

The pathological changes encountered in rumen and abomasums included mucosal haemorrhages and denudation of superficial lagers, oedema, haemorrhages, congestion and effusion of some lymphoid cells in lamina propria, submucosa, smuscularis mucosa and serosa.
Figure 54: Liver. Note, hepatocytes dissociation necrosis of vascular endothelium, accumulation of erythrocytes in the sinusoids. H&E X10.

Figure 55. Adrenal gland. Note, cyst-like structure. H&EX10
4.12.12. Intestine:

The intestine showed similar lesions to those described in stomach.

4.12.13. Urinary bladder:

This organ showed swelling vacuolation and erosion of the superficial lining epithelium (Fig. 56). The epithelial reaction was accompanied by oedema, congestion and extravasation of erythrocytes in the connective tissue.

4.12.14. Bacteriology:

*C. perfringens* was isolated from the intestinal content, but not from lung, liver and kidney.
Figure 56: Urinary bladder. Note erosion of lining epithelia, congestion and haemorrhages. H&E X10
CHAPTER V
DISCUSSION
The data of the current investigation recorded the first genotyping of the C. perfringens isolates in the Sudan. This isolates were from foecal specimens collected from diarrhoeic camel calves at different location in the Sudan (Map 1). The toxin types of these isolates were determined with six toxingene specific multiplex PCR (MPCR) assay (CPA, ETX, CPB, LA, CPE and CPB2). The distribution of these toxin type in different location were shown in tables 7 and 8. It appear that type A C. perfringens was the most prevalent and consisted of 95 out 97 isolates (97.9%), whereas, the other 2 isolates were type D. The percent distribution of subtype A were 44 (46.3%) type A producing α toxin; 44 (46.3%), type A producing α and CPE toxins; 4(4.3%) type A producing α and B2 toxins and 3(3.2%) type A producing α, CPE and B2.

From this results it was evident that type A was the predominant type in Sudan. This is in line with the previous findings (Abdelsalam, 1986) that all isolates (100%) from broiler chickens were type A C. perfringens. Similarly, previous molecular epidemiological surveys revealed that type A. C. perfringes is the predominate type. Only type A (100%) was isolated from calves and chickens and 87% were identified from piglets (Yoo et al., 1997); all 616 field isolates were found type A (Songer and Meer, 1996) and 95% of 361 isolates from human and animal were typed A (Meer and Songer, 1997). Furthermore, 97% and 92.4% type A were recorded by Augustynowicz et al. (2000) and Bueschel et al. (2003) respectively.

The highest percentage of the present isolates were type A suggesting the possible association between this type and neonatal camel calves diarrhoea. Type A has been linked with acute haemorrhagic enteritis in calves (MacRae et al., 1943) and adult cattle (Shirely, 1958); necrotic enteritis and/or diarrhoea of chicken (Nair, Bamford, 1967 and Bain, 1968); intestinal clostridiosis with profuse watery diarrhoea in horses (Wierup, 1977 and Wierup and Dipietro, 1981) and enteric diseases of racing camels (Wernery et al., 1992). Furthermore, type A C. perfringens known to cause haemolytic diseases in sheep and cattle in Australia (Rose and Edger, 1936) and lambs in California (McGowan et al., 1958) A similar syndrome was reported in goat (Russel, 1979).
Despite the reports of the type A enterotoxaemia, little was known about the absorption of alpha-toxin from the intestine, and its contribution as virulence factor in pathogenesis of classical type A enterotoxaemia (Niilo, 1993, Daube, 1994 and Petit et al., 1999). However, the investigation of Ginter, Williamson, Dessy, Coppe, Bullifent and Howells (1996) showed that some variant of alpha-toxin produced by type A isolate, have been implicated in some gastrointestinal disease of domestic animals. Lambs and calves inoculated intravenously with alpha-toxin developed transient diarrhoea and mucosal hyperaemia (Niilo, 1971 and 1973). In feeder pigs produces necrotizing enteritis and villous atrophy (Popoff and Jestin, 1985). Similar syndrome was produced by oral infusion of Gontobiotic colostrum deprived pigs as well as conventional weaner pigs (Jestin Popoff and Mahae, 1985).

Enterotoxigenic type A C. perfringens, which frequently isolated in present study, is primary of human health important from view of food-borne infection (Genigeogis, 1975). It is considered as leading cause of bacterial food borne illness in countries where consumption of meat and poultry is high for example, each year in USA, this organism is the second or third most common cause of confirmed cases of food borne, illness (Cliver, 1987 and Lin and Labbe, 2003).

The percentage distribution of CPE-positives C. perfringens isolates were 65.1%, 60%, 40% and 9% from Gadarif, Butana, Nahr Elneel and Kurdofan respectively. C. perfringens type A enterotoxin CPE hold considerable pathogenic important because of their established role as major virulence factor in food poisoning and non food poisoning gastrointestinal (G1) disease including antibiotic associated diarrhoea and sporadic diarrhoea (Brett, Rodhouse, Donovan, Febbutt and Hutchinson, 1992; Collie, Kokai-Kun and McClane, 1998 and Sarker et al., 1999). Previous study McDonel and Duncan, 1977 and Sherman, Klen and McClane, 1994) have shown that rabbit ileal loops treated with highly purified CPE accumulated significant amount of luminal fluid and developed extensive intestinal histopathological damage. Furthermore, human volunteer feeding experiment (Skjelkvale and Uemura, 1977) have demonstrated that injection of highly purified CPE resulted in abdominal cramping and diarrhoea that characterize the GI illness associated with CPE positive C. perfringens type A isolates. Nevertheless, evidence increasingly suggested that enterotoxigenic isolates played important role in the

CPE associated with enteropathy in dogs displayed soft to watery diarrhoeas with or without haemorrhages (Carman and Lewis, 1983 and Kruth, Prescott, Welch and Brodsky, 1989). Acute colic and haemorrhagic gastroenteritis could be induced experimentally in shetland ponies after intravenous infusion of enterotoxin (CPE) obtained from enterotoxigenic C. perfringens type A (Ochoa and Kern, 1980).

The pathogenesis of enterotoxigenic C. perfringes type A was described (Niilo, 1993) as multisteps process involving, brushborder, membrane of villous epithelium of mucosa. It acted rapidly in intestine mucosa mainly in jejunum and ileum causing profuse fluid outpouring and diarrhoea. Furthermore, recent studies fulfilling the molecular koch’s postulate have proof that CPE expression in important, if not essential, for the GI virulence of CPE positive C. perfringens type A food poisoning human GI disease isolates in animal model (Sarker et al., 1999). Some estimate suggested that this bacteria account for 5 to 20 % of all cases of antibiotic-associate diarrhoea (AAD) and sporadic non food-borne diarrhoea (Carman, 1997).

Recent studies have, also, established that CPE gene can have either a chromosomal or plasmid (Cornillot, Sant-Joanis, Daube, Kalayama, Graum, Carnard and Cole, 1995 and Colliex and McClane, 1998). However, initial studies have shown that CPE gene has a chromosomal location in food poisoning isolates (Cornillot et al, 1995, Kalayama et al., 1996 and Collie and McClane, 1998) but is located in a plasmid in non-food-borne human GI disease isolate (Collie and McClane, 1998, Myamoto, Iven and McClane, 2004). However, even if atypical, the recently identified (Tannka, Isobe, Hosorogi, Kimala, Shirmizu, Kalori, Gyobu, Nagai, Yamagishi, Karasaura and Nakamura, 2003) of Japanese food poisoning outbreaks involving isolates with a plasmid CPE remain interesting.

The present work recorded the presence of low incidence of a new yet, unsigned type of C. perfringesn containing α–toxin gene Cpa and noval B2–toxin gene Cpbe in faecal samples of diarrhoeic camel calves. Only 7.4% of 97 examined isolates
revealed the gene of cpa and cpb2. Several investigators have noticed an association of B2-toxin (Cpb2) positive isolates C. perfringens type A and occurrence of enteric disease in several animals particularly piglets (Klaasen et al., 1999; Garmory, Charter, Frensh, Bueschel, Songer and Titball, 2000 and Waters, Savoie, Garmony Bueschel, Popoff, Songer, Titball, McClane and Sarker, 2003); horse (Herholz et al., 1999), dogs (Thiede, Goethe, Amrsberg, 2001); elephant (Bacciarni Pagan, Frey and Grone, 2001); lambs (Gkiourtzidis, Frey, Bourtzi-Hatzopoulour, Iliadis and Sarris, 2001). The presence of CPb2 gene in isolates of piglets with enteritis and lack of these gene in isolates from healthy piglets indicate strong association of cause effect relationship (Garmory et al., 2000).

Furthermore, the results of ligated loops assay in claves inoculated with pure and mixed culture of four C. perfringens (α-B2, α-enterotoxin and α-alone), demonstrated macro-and microscopically lesions of haemorrhagic enteritis. These lesions were more pronounced in loops inoculated with α and B2 toxins suggesting the in vivo synergistic role of α and B2 toxins in the production of necrotic and haemorrhagic lesions in small intestine (Manteca et al., 2002).

Though, type D. C. perfringens, which was rare occurrence in current study, pose the greatest danger to livestock industry (Niilo, 1993). This pathogen being widely regarded as causal agent of acute fatal disease of ungulate particularly cattle, goats and sheep. In the latter the epsilon toxin, which is the major toxin of type D, is responsible for the systemic effect and occasional enteritis (Blood et al, 1979; Niilo, 1980 and Songer, 1996). However, other workers (Bullen and Battery, 1957 and Bullen, 1970) have shown that epsilon-toxin increase the permeablity of gut wall, enhanced its own absorption and caused mucoid diarrhoea. Moreover, the cardinal clinical symptom, of acute and chronic type D. C. perfringens in goats being watery diarrhoea intermingled with blood and shreds of intestinal mucosal epithelium (Oxes, 1959; Blackwell and Butler, 1992; Smith and Sherman, 1994; Uzal et al., 1994 and Uzal and Kelly, 1996). Experimental infusion of ligated intestinal loops of sheep and goats with type D culture supernatant resulted in excess mucous in the intestinal lumen (Uzal, Ghoddusi, Kelly
and Rozamec, 1999). Diarrhoea was, also, described in type D toxaemia in suckling camels (ElSanousi and Gameel, 1993); adult camels (Chauhan, et al, 1985 and Wenery et al., 1992) and dogs (Odendaal, Aloeck and Cramer, 1998).

Shahidi and Ferguson (1971) described a suitable media for cultivation and enumeration of *C. perfringe*. This media was used throughout the experimental work in this study. However, this media have some limitation. Among these was growth of some facultative anaerobe such as streptocci spp. Also hydrogen sulphid producing bacilli were found to grow in this medium resulting in contamination of *C. perfringens* cultures. Accordingly, blood agar medium was used to demonstrate dural haemolysis which was phonomen of *C. perfringens* (Cruickshank et al., 1975 and Buxton and Fraser, 1977).

The black colonies obtained in SFP agar were further confirmed *C. perfringes* with LM agar proposed by Shahidi and Ferguson (1971) in conjunction with SFP agar. The tested organisms were expected to demonstrate lactose fermentation and negative motility. This media was satisfactory for lactose fermentation but did not demonstrate the motility. This because of the stormy growth of organism and extensive production of gas, which disrupted the media, resulted in dissemination of the colonies throughout the medium. Hence, the recovered pathogen in SFP agar were tested for lactose fermentation and motility in separate test. Using LM agar and hanging drop method for lactose fermentation and mortality respectively. Non-mortality was a character of all types of *C. perfringens* isolated in this investigation. This confirmed the finding of Cruickshank et al. (1975) and Buxton and Fraser, (1977).

An other disadvantage of SFP agar is that it is tedious to prepared and the ingredient are not easy to find. This media was of limited value (Harford, 1974) because the pathogen can be isolated only when it is the predominant organism within sample.

Colonical morphology of the current isolates were similar to that described previously (Willson and Millers, 1975 and Crickshank et al, 1975).

All isolates demonstrated clear double zone of haemolysis on sheep blood agar media. This dural haemolysis was attributed to the production, by the pathogen, of alpha and theta toxins which were haemolytic, histotoxinice and lethal (Hatheway, 1991}
The alpha toxin is a phospholipase c, sphingomyelinase that hydrolyze phospholipids of cell membrane and promotes membrane disorganization leading to haemolysis of erythrocytes, whereas the theta toxin causes complete haemolysis of erythrocytes by forming oligomers, which subsequently form pores through the cell membrane (Petit et al., 1999). These two toxins were found not to produce the same pattern of haemolysis of erythrocytes of other animals. This is because their action mainly dependant upon the affinity of the toxin, which is protein, to bind with the erythrocytes walls (Kadis et al., 1971).

Lecithinase was found to be produced by all C. perfringens isolates. 89.3% of the isolates were completely inhibited by C. perfringens type A antisera while 10.7% of the isolates presented incomplete inhibition of C. perfringens type A antisera. This possibly because some stain produced too high concentration of the toxin to be inhibited by the antisera or the organism require higher concentration of antitoxin.

The importance of C. perfringens is due to production of multiple toxin which are harmful for man, animals and avian. The detection of these toxins in contaminated food, intestinal content and faeces of victum is essential for diagnosis of C. perfringens infection. The mice lethality test, which is the conventional method, have been used in this work to detect the toxin from culture supernatant. The tested strain in this study were found fatal causing death for all inoculated mice except 6 which showed unthriftiness followed by complete recovery 24 hours post inoculation.

The inoculated mice showed dullness, variety of neurological manifestation such as restlessness, staggering gait, paralysis of hind quarters and sometimes tremors. At the terminal stages, just prior to death the mice stretched its body having tendency for flaccid paralysis.

Though this method is sensitive but have some limitation. Using living animals, which has inaccuracy of biological assay such as variation in individual sensitivity; non specific toxicity from others substance, which may be present in the intestinal content (Henderson, 1984); difavour on humanitarian ground and animals well fare.
The protocol used here provided suitable or reasonable model for successful production of enterotoxaemia in camel calves and inducing pathological changes comparable to those of natural cases.

Severe vasculitis with disrupted endothelial lining, accumulation of pericardial, pleural and peritoneal fluid, oedema of lung and brain, haemorrhages and congestion involving all organs were the predominant lesions encountered in camel calves inoculated with type D culture.

The cerebral vasogenic oedema and the presence of oedematous fluid in body cavities described here were the characteristic and most common manifestation of *C. perfringens* type D (Barker *et al*., 1983; Pathak and Parihar, 1996 and Uzal *et al*., 1997). This changes were possibly due to vascular endothelial damage. Previous investigations showed that the parental administration of epsilon toxin resulted in increase vascular permeability (Griner and Carlson, 1961), severe generalized vascular endothelial damage (Gardner, 1973) with subsequent effusion of fluid and plasma protein. Using horseradish peroxidase (HRP) (Gardner, 1974 and Morgan *et al*., 1975) radioactive iodine (Grinder and Carlson, 1961 and Worthington and Mulder, 1975) as a tracer for vascular integrity, showed that the endothelial damage allowed fluid and protein to escape from the vascular lumen into the extracellular spaces of brain. Furthermore, ultrastructural examination of brain tissue of inoculated animals with epsilon toxin revealed that the tight junctions in the vascular endothelium degenerate (Buxton and Morgan, 1976), causing perivascular astrocyte processes to swell and rupture (Finnie, 1984a) leading to an increase in capillary permeability (Finne, 1984b) and loss of plasma protein (Finnie and Hajducl, 1992).

The present study, to my knowledge, recorded the first report of neurological lesion in camel. The vasogenic oedema and the malacic lesion was comparable to those described in sheep, cattle and goats (Hartley, 1956; Griner, 1961; Buxton *et al*.,1978 and 1981; Uzal *et al*., 1997 and Uzal and Kelly, 1998). It has been suggested that vasogenic oedema progressed to malacic lesions which were focal and
bilaterally symmetrical in distribution (Hartley, 1956; Finnie, 1984a&amp;b; Griner, 1961 and Uzal et al, 1994).

Disseminated haemorrhages in brain, heart, lung, kidney, intestine, adrenal gland and pancreas and pink proteinaceous material in lung alveoli, kidney glomeruli and adrenal glands described here were possibly subsequent to vasculitis with disrupted endothelial lining. The damage of the endothelial lining cells resulted in leakage of fluid, protein and erythrocyte from the vascular lumina.

Inoculated camel calves with classical type A strain 95, which was positive for α toxin gene (cpa) only, developed clinical symptom similar to those described for sheep (Rose and Edger, 1936). However, they observed yellowish mucous membrane. Jaundice, which was not encounter in this study.

The most striking necropsy findings were accumulation of oedematous fluid in body cavities, haemorrhage and congestion in all organs. However, more pronounced pathological changes were demonstrated by animal No 7 which infused with low dose (table 6) and survived for 30 HPI. These gross lesions were confirmed histologically. In addition, there were haemorrhages and vascogenic oedemes in this brain. This results are in general consistent with those of Chauhan et al. (1985), who however, did not described the prevascular proteinaceous oedema in brain section. He observed, only, haemorrhages and neuron degeneration and subpleural haemorrhages were previously detected in camel (Dashdaua and Ipatenko, 1967 and Wernery et al., 1992).

The presence of fluid in body cavities together with endocardial and or epicardial haemorrhages were the characteristic sheep, cattle and goat enterotoxaema (Rose and Edger 1936, Macrae et al., 1943 and Uzal et al., 1994) This pathological change, were possibly due to vasculitis with disrupted endothelial linging observed in current study and to increase in vascular permeability induced by alpha toxin (Elder and Miles, 1957 and Sugahara et al., 1977). It has been shown that intravenous inoculation with alpha toxin resulted in damage to capillary endothelial cell throughout the body, with on increase in permeability (Smith, 1979). The change in vascular permeability resulted in effusion of fluid, protein and erythorcytes from vascular lumen to the exteria. Though, the changes in
the vascular permeability due to type D. *C. perfringens* have be confirmed using HRP (Gardner, 1974 and Moprgan *et al.*, 1975) and radioactive iodine (Grinder and Carlson, 1961 and Worthington and Mulder, 1975) as traces for vascular integrity, similar studied for alpha toxin were lacking in literature. Previous work (Strandberg, Mollby and Walstrom, 1974) confirmed that phospholipase C (alpha–toxin) release histamine from mast cell which may contribute in the increase of vascular permeability.

Inoculated animals with type A strain 80, which was positive for alpha toxin (cpa) and Beta 2 toxin (cpb2) gene, culture showed pathogloical change comparable to those initiated by type A strain 95. These changes included severe vasculatis with damage endothelial cells, haemorrhages and congestion throughout the body, oedematous fluid body cavities and vaso genic oedema. However, strain 80 showed more advance Malacic lesion in the brain. More marked haemorrhages in endo-myo and pericardium, alveoli, subpleual and dilated sinusoid were observed in section of animal effusion by strain 80. Furthermore, more extensive necrotic foci were caused by the same strain in myocardial and lives.

The collection of fluid in body cavities and perivascular area haemorrhage and congestion were probably causes by the damage endothelium and increase vascular permeably initiated by alpha toxin (Elder and Miles, 1957, Sugahara *et al.*, 1977 and Smith, 1979). The necrosis and dissolution of tissue of some organ such brain, heart, liver were possibly due to the action of alpha toxin. Smith (1979) concluded that the ability of alpha toxin to hydrolyze lecithin and sphingomyelin of cell membranes render alpha toxin lethal to a variety of tissue. The contribution of B₂ toxin in necrotic process could not be excluded since this toxin was found lethal to mice and necrotizing (Gibert, *et al.*, 1997). B₂ toxin was found to induce hemorrhage necrosis of intestinal mucosa in experimental intestinal loops (Lawrence and Cooke, 1980) and cytotoxic to cell culture (Gibert *et al.*, 1997). In other experiment of legated intestinal loop. Manteca *et al.*, (2002) observed, macroscopically, haemorrhages in intestinal wall, necrosis and haemorrhages of intestinal content, and microscopic lesions of necrosis with polymorhonuclear and mononucler cell infiltration of intestinal with in loops inoculated with alpha and B₂
toxigenic *C. perfringens* isolates. He suggested in vivo synergistic role of the $\alpha$ and $B_2$ toxins in production of necrotic and haemorrhagic lesions of the small intestine in cases of bovine enterotoxaemia. Hence, the extensive and haemorrhage and necrosis produced by strain 80, in the present study were possibly due to the synergistic reaction of $\alpha$ and $B_2$ toxin.
CHAPTER VI

GENERAL DISCUSSION AND RECOMMENDATIONS

The results of this study elucidate that \textit{C. perfringens} played significant role in camel-calve diarrhoea with overall prevalence 40.7%. The regional distribution was 50%, 63.8%, 45.4%, 55.6% and 21.3 in Kassala, ElGedarif, Butana, Nahr Elniel and Kordofan respectively. Type A, which was the most prevalent type containing \(\alpha\)-toxin, represented 97.9\% (N=95), followed by type D, which characterized by \(\alpha\)- and E-toxin, was isolated in 2.06\% (N=2). However, \textit{C. perfringens} type B and C, which produced B-toxin and harbouring \text{cpb} gene, were not isolated in this survey. The occurrence of these types may not be excluded and loss of \text{cpb} bearing plasmid on vitro passages and/or long storage was expected, since both types were unstable and heat labile. Similarly \textit{C.}
*perfringens* type E which produced I toxin and harbouring Ai gene was not isolated corresponding to finding of Songer (1997) who showed that *C. perfringens* type E seems very rare to find.

The enterotoxigentic type A *C. perfringens* harbouring α and cpe toxin gene, which implicated as major virulence factor in the pathogenesis of gastrointestinal illness of both human and animal (Sarker *et al.*, 1999), represented 49.4% (N=47) of total type A isolates (N=95). 93.6% isolates of enterotoxigenic type A (N=44) were from the eastern states, which consisted of Kassala, ElGedarif and Butana. The high prevalence of the enterotoxigenic type A of in Eastern region may reflect the importance of these isolates as virulent factor in pathogenesis of camel calves gastrointestinal illness (diarrhoea).

The novel toxin B₂ toxigenic type A was represented in 7.4% (N=7). The prevalence of this B₂ toxigenic type A were 40%, 12% and 9.5% in Nahr Elniel, Butana and Kordofan respectively. Though, few *C. perfringens* isolates were obtained from Nahr Elniel (N=5), there was high prevalence of B2 toxigenic type A represented 40% (N=2). This strain, whose role in pig enteritis has been documented (Garmory *et al.*, 2000), may play significant role in camel diarrhoea.

Intraduodenal inoculation, used in this study, presented a reasonable model for successful production of enterotoxaemia in animals and induced pathological changes comparable to that of natural infection.

The pathological changes recorded here indicated that haemorrhages and congestion were hallmark of type a and D *C. perfringens* enterotoxaemia in camel. However, these lesions were not considered as hallmark of typical type A and D enterotoxaemia in sheep, goats and cattle, despite that these animals showed areas of haemorrhage in small intestine and petechial haemorrhages of endocardium, subendocardium and around the mital value (Barker *et al.*, 1985 and Songer, 1996). The generalized haemorrhages, congestion and vasculitis with disrupted endothelial lining may suggest that the cardiovascular system of camel is more sensitive to *C. perfringens* toxin than those of sheep, goat and cattle.
The vasogenic or prevascular oedema previously considered (Buxton et al., 1978; Barker et al., 1985 and Uzal et al., 1997) as pathognomonic for type D epsilon toxin (Griner, 1961 and Grander, 1974) was produced here by both type A and D C. perfringens. This may elucidate the role of alpha toxin in the pathogens of brain oedema, which was previously considered pathognomonic of type D epsilon toxin (Grander, 1974), this possibly because alpha toxin increase the vasular permeability (Elder and Miles, 1957 and Sugahara et al., 1977) release histamine from mast cells (Stradberg et al., 1974) and damage the endothelial cell throughout the body (Smith, 1979). This results showed that both type A and D C. perfringens were capable to induced lung oedema, which was considered (Kellaway, 1940; Niilo et al., 1963; Moebuu et al., 1967 and Uzal and Kelly, 1998) as characteristic for type D, in variance with Gordon et al., (1940) who did not detect oedematous fluid in lung of sheep infected with type C or D.

The accumulation of pink proteinaceous maternal in the dilated Bowmans’ space of kidney were in agreement with the finding of ElSanousi and Gameel (1993). This maternal was possibly oedematous fluid resulted from vascular endothelial damage.

The adrenal cortical necrosis and oedema fluid in adrenal gland observed here were similar to those described for type C. perfringens in pig (Bergeland, 1967) at variant, the oedema fluid was not seen in pigs enterotoxamia. It is was found that C. perfringene exotoxin, except the I-toxin interact with cell membrane leading to membrane disruption or pore formation (Petit et al., 1999). Accordingly, one may suggest that all types of C. perfringens produced similar pathological changes with different in intensity of reaction.

C. perfringens produced major toxin (α, B, E and z) and several minor toxin such theta toxin θ, which is haemolytic and form pores through the cell membrane. Together with alpha toxin, the theta toxi contribute to the dysregulation of endothelial cells (Petit, 1999). The contribution and/or the synergistic reaction of these toxins with alpha and/or epsilon toxin may not be unexpected.

Camels used to drink from water pools, where other animals are watered and stand. Traditionally, the camel owner used to construct water trough from mud at the
edge of the water pool. The camel first enter the pool, they rest nearby during which time the mud tray is prepared and the salt added. Then they allowed to drink in small number. Although, no trials has been made to isolate *C. perfringens* from these pools, they hold source of infection because they contained stagnant and contaminated water. In addition, when the diarrhoeic calves were allowed to enter the water pool, they may contaminate the waker pools by their soiled hoof and by shedding the organism with faecis resulting in further contamination. Furthermore, it has been recorded that *C. perfringens* is the most wide spread in the environment, paddock soil (Niilo, 1980, Itodo, A destyun, Adekeye and Umoh, 1986). It has been agreed that the main portal of entry to clostridial infection is the mouth (Wijewanta, 1964; Cruickshank, 1975 and Buxton and Fraser, 1977). The study of Wijewanta (1964) showed that chicken fed on manure soil revealed high incidence of clostridial infection than those fed on clean soil consequently they shed the pathogen in faecis leading to further contamination.

The nomad camels owner used to complain that the calves sucked too much milk, particularly on hot midday, leading to ruminal embaction, tympany and eventually diarrhoea. The excess milk may be predisposing factor for enterotoxaemia. It has been agreed that over feeding is one of the predisposing factor for *C. perfringens* infection (Bullen, 1970 and Niilo, 1980). Previous epidemiological study, in Sudan, showed that excess milk is one of causes, of camel calves diarrhoea. It is a real problem they clamied, it is too difficult to separate the calves from their dams. These calves ought to be separated from the dams and allows to suck during the milk led down.

Though, this is the first report of genotyping of *C. perfringens* associated with diarrhoeic camel calves in the Sudan, adesign of prevention measures against *C. perfringens* enterotoxaemia will only be advisable after thorough study on the aetiology. Survey of this disease in other animals at different location should be conducted. The demonstration of *C. perfringens* toxin in intestinal content and faecal specimens of affected animals is very essential for the diagnosis of this disease. However, the economic impact of this disease has been documented nation wide.
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