Isolation, identification, and Drug sensitivity of *Clostridium perfringens* from Chickens with Necrotic Enteritis

By

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PREFACE

This work has been carried out at the Department of Avian Pathology and Diagnosis in the Central Veterinary Research Laboratories, Soba under the supervision of Prof. S. M. EL-Sanousi and Co-supervision of Dr. Zakia Abbas Mohammed.

All the materials entered here are original and have not been submitted to any other institutions.
DEDICATION

To the soul of my brother Hisham…..

To my daughter Rash with love……..
ACKNOWLEDGEMENT

First praise be to almighty Allah who gave me health and ability to complete this work.

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Abstract

Out of 400 samples (340 intestines and 60 cloacal swabs) from cases with signs and lesions of necrotic enteritis, 40 (10%) isolates of *C. perfringens* were obtained (30 isolates from intestines and 10 from cloacal swabs).

Identification of the isolates was performed using classical bacteriological methods which included morphology, Gram stain reactions and biochemical tests.

Polymerase chain reaction (PCR) technique was used for typing of identified *C. perfringens* isolates in order to determine the presence of $\alpha$, $B$, $B_2$, $\varepsilon$, $\iota$, and enterotoxin genes. All isolates carried $\alpha$ toxin gene and this confirmed the identity of *C. perfringens*. Also enterotoxin gene was detected in all isolates.

All *C. perfringens* isolates were found to be type A and enterotoxin gene positive.

Sensitivity tests were conducted on 30 of the isolates using 9 antimicrobial agents ampicillin, furazolidone, penicillin, cloxacillin, bacitracin, erythromycin, lincomycin, gentamycin and kanamycin.
All isolates had high susceptibility to ampicillin and furazolidone (100%) followed by penicillin (90%), cloxacillin (80%), bacitracin (73%), erythromycin (70%) and lincomycin (50%).

All isolates were resistant to gentamycin and kanamycin.

All isolates had high susceptibility to ampicillin and furazolidone (100%) followed by penicillin (90%), cloxacillin (80%), bacitracin (73%), erythromycin (70%) and lincomycin (50%).

All isolates were resistant to gentamycin and kanamycin.
The culture of C. perfringens showed sensitivity to penicillin (90%)
ampicillin (100%), furazolidone, and amoxicillin (80%). Gentamycin and kanamycin
were also effective, with 73% and 70% effectiveness, respectively.

Kanamycin and Gentamycin
Introduction

*Clostridium perfringens* is a Gram-positive anaerobic bacterium that is able to form spores. It is widespread in the environment and is commonly found in the intestines of animals and humans, where it is pathogenic in certain circumstances. In humans, it can cause gangrene and gastrointestinal diseases, whereas in other animals, gastrointestinal and enterotoxemic diseases occur more frequently. *Clostridium perfringens* does not invade healthy cells but produces various toxins and enzymes that are responsible for the associated lesions and symptoms. The toxins produced depend on the *Clostridium.perfringens* strain involved, and each type of toxin induces a specific syndrome (*Petit et al., 1999*). Classification of *Clostridium.perfringens* into types A, B, C, D or E, is based on major toxin production depending on the toxin profile (McClane *et al.*, 2000).

*Clostridium perfringens* plays a significant role in food-borne human disease (Mead *et al.*, 1999). *Clostridium.perfringens* enteritis negatively impacts the integrated system of poultry at high levels (Tschirdewahn *et al.*, 1991). However, its overgrowth in fowl can be considered an imbalance of the gut ecosystem at the microbial level resulting in necrotic enteritis (Wages and Opergant, 2003).

Poultry necrotic enteritis is associated with predisposing factors (Elwinger *et al.*, 1992), and is caused by *C.perfringens* types A or C. The organisms replicates readily in the intestinal tract where it produces α toxin which are believed to be responsible for the mucosal necrosis (Ficken *etal.*, 1997) and it is a key virulence determinant (Sheedy *et al.*, 2003).
Molecular typing of the major toxins by a multiplex polymerase chain reaction (PCR) is one useful method (Yoo et al., 1997) for grouping of *C. perfringens* isolates into toxin types without inoculation in animals (Engstrom et al., 2003).

The main objective of the present work was to determine the incidence of *C. perfringens* in the intestines of chickens and to perform toxinotyping of *C. perfringens* by PCR assay for the presence of α-B-B2-ε-ι and enterotoxin genes. Also to determine the in vitro susceptibility of *C. perfringens* to different antimicrobials.
Chapter One

Literature review

1.1. Genus *Clostridium*

The genus *Clostridium* includes Gram-positive, spore forming, catalase-negative bacilli. More than 100 species of clostridium have been described, and many have been recovered from environmental specimens of wild and domestic avian species. Pathogenicity of clostridia is mediated primarily through potent exotoxin, clostridial diseases may occur as soft tissue infections, intoxications and toxico-infections (Miller, 1998).

Although some species of *Clostridium* do grow in the presence of air, they can be distinguished from *bacillus* species by their lack of catalase (Smith and Williams, 1984).

Members of the genus are found in soil, sewage, marine sediments, and the intestinal tracts of human and other animals and in decaying animal and plants products. (Cato *et al.*, 1986). Because of the ubiquity of these organisms, they find their way into wounds, foods, and feeds and are often responsible for serious illness, usually mediated by their toxins (Cato *et al.*, 1986).

1.2. *Clostridium perfringens*:

*Clostridium perfringens* is the most widely occurring pathogenic bacterium (Smith and Williams, 1984). It is readily isolated from the intestine of birds and mammals (Hofshagen and Stenwig, 1992). Spores of *C. perfringens* usually are rare or not observed in cultures grown in ordinary media, so it must be recognized by its other characteristics (Holdeman *et al.*, 1977). A characteristic feature of *C. perfringens* is the large number of
toxins this organism is capable of producing: up to 17 different exotoxins, of which some are more commonly produced than others. In addition, some *C. perfringens* also produces an enterotoxin, CPE (Songer, 1996)

1.2.1. Basic characteristics:

*Clostridium perfringens* is a Gram-positive, rod-shaped anaerobe that forms oval subterminal spores. It differs from most other clostridia in that they are relatively large rods (0.6 – 2.4 X 1.3 – 9.0 µm) encapsulated and non motile (Cato, George and Finegold; 1986). The colonies are smooth, round, glistening, surrounded by an inner zone of complete haemolysis caused by the theta-toxin and an outer zone of incomplete haemolysis caused by the α toxin (Quinn *et al*., 1994).

*Clostridium perfringens* is classified as an anaerobe, although the bacteria will survive and occasionally grow in the presence of oxygen (Quinn *et al*., 1994). Growth occurs within the temperature range of 12-50°C, though very slowly below 20°C (Adams and Moss, 1995).

Under optimal conditions, 43-47°C, *C. perfringens* grows extremely rapidly, with a generation time of 8-10 min, and growth is accompanied by abundant gas production (Bryant and Stevens, 1997). The bacterium requires 13 essential amino acids for growth (Cato, George and Finegold, 1986).

1.2.2. Genetic properties:

Among the toxigenic clostridial species, *C. perfringens* is the paradigm species for genetic studies, because of its tolerance to oxygen, high growth rate, and ability to genetic manipulation (Rood, 1998). In 2002 the complete genome of *C. perfringens* strain 13 was published (Shimizu *et al*., 2002).
When *C. perfringens* genome is compared with the genome of the non-pathogenic bacterium, *C. acetobutylicum* (Nalling et al., 2001), the most obvious difference is that virulence-related genes are present in the former, but not in the latter. In addition to the known virulence genes, Shimizu found many virulence-associated genes in *C. perfringens* genome (Shimizu et al., 2002). Five putative haemolysin genes were identified, based on their similarity to haemolysins previously described in other bacterial species. Two types of Putative fibronectin-binding protein genes sharing similarities with genes from *listeria monocytogenes* (Gilot, Jossin and Content, 2000) and *Bacillus subtilis* (Kunst et al., 1977) were also detected, indicating involvement in pathogenesis as colonization factors (Shimizu et al., 2002).

The genes encoding α-toxin and theta toxin are located on the chromosome, many other toxin encoding genes are located on large plasmids (Table 1). The enterotoxin gene (cpe) can be either chromosomal or plasmid encoded. In food poisoning isolates, the cpe gene is located on the chromosome (Brynestad and Granum, 1999).

However, non-food-poisoning human gastro-intestinal disease isolates and veterinary isolates carry the cpe gene on large plasmids (Comillot et al., 1995).

Expression of alpha-toxin and theta-toxin is regulated by a two-component signal transduction system (virR/virS) consisting of two components, a sensor histidine kinase, virS, and a response element, VirR. Regulation occurs at the transcriptional level and mutations can alter the productions of both alpha-toxin and theta-toxin (Rood, 1998).
1.2.3. *Clostridium perfringens* toxins:

1.2.3.1. Major toxins:

Members of the species *C. perfringens* can be subtyped into five toxin types (A, B, C, D and E) based on the production of four major toxins: alpha, beta, epsilon and iota (Songer, 1996) *C. perfringens* is unique not only in terms of the number of toxins produced, but also in terms of their toxicity and lethal activity.

Table (I)

**The most important *C. perfringens* toxins**

(Modified from Petit, et al 1999)

<table>
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<tr>
<th>Toxin</th>
<th>Gene</th>
<th>Gene location</th>
<th>Biological activity</th>
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<tr>
<td>Alpha toxin</td>
<td>Plc</td>
<td>Chromosome</td>
<td>Cytolytic, haemolytic, demonecrotic, lethal</td>
</tr>
<tr>
<td>Beta toxin</td>
<td>cpb1</td>
<td>Plasmid</td>
<td>Cytolytic, dermonecrotic, lethal</td>
</tr>
<tr>
<td>Epsilon toxin</td>
<td>Etx</td>
<td>Plasmid</td>
<td>Oedema in various organs, liver, kidney and C.N.S.</td>
</tr>
<tr>
<td>Iota toxin <em>I</em></td>
<td>iap/</td>
<td>Plasmid</td>
<td>Disruption of cytoskeleton action and cell barrier integrity</td>
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<tr>
<td>And <em>I</em></td>
<td>lbp</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beta2 toxin</td>
<td>cpb2</td>
<td>Plasmid</td>
<td>Cytolytic, lethal</td>
</tr>
<tr>
<td>Entero toxin</td>
<td>Cpe</td>
<td>Chromosome</td>
<td>Cytotoxin, lethal, causes diarrhea by leakage of water and ions</td>
</tr>
<tr>
<td></td>
<td></td>
<td>plasmid</td>
<td></td>
</tr>
<tr>
<td>Theta toxin</td>
<td>pfoA</td>
<td>Chromosome</td>
<td>Lyses red blood cells and modulates the host inflammatory response</td>
</tr>
</tbody>
</table>
1.2.3.1.1. Alpha toxin:

In 1941 Mac Forlane and Knight showed that the alpha-toxin of *C. perfringens* possessed phospholipase *C. enzymatic* properties. Alpha-toxin, the main lethal toxin of *C. perfringens* is a multifunctional phospholipase, produced in varying amounts by almost all *C. perfringens* isolates. It causes hydrolysis of membrane phospholipids in different cells, resulting in lysis or other forms of cytotoxicity (Songer, 1997; Tiball, 1993).

Alpha-toxin is believed to be the major factor responsible for the tissue pathology in myonecrosis (gas gangrene) caused by this organism. Immunization of guinea pigs with alpha toxin protects them against gas gangrene when challenged with *C. perfringens* and toxin (Kameyama *et al.*, 1972).

Alpha-toxin has been shown to affect myocardial function, causing hypotension and brady-cardia, resulting in shock, a common and after fatal feature of gas gangrene (Stevens *et al.*, 1988).

Recently, three laboratories almost simultaneously reported cloning the alpha-toxin gene from *C. perfringens*. Titball *et al.*, in England (Titball *et al.*, 1989) incorporated fragments of the *C. perfringens* genome into an *E. coli* plasmid and detected organisms carrying the recombinant plasmid containing the gene by their lecithinase reaction on egg yolk agar plates. The gene coded for a protein that had a molecular mass of 44.5 KDa and appeared to be identical to the alpha-toxin produced by the donor strain. Tso and Seibel (1989) in the United States cloned the gene by similar methods.

Alpha-toxin production of plasmid-bearing organisms was detected by the hemolytic reaction on blood agar plates. They found a 2-kb fragment inserted into the plasmid that contained 1, 197-nucleotide sequence from *C.
*perfringens* that coded for the 399-amino-acid peptide with a molecular mass of 43 KDa. The peptide had biological activities similar, it not identical, to those of the α-toxin of the donor strain. These researchers also cloned the gene for *C. bijermentans* phospholipase c and found it to be 64% homologous in coding for sequence with the *C. perfringens* α-toxin gene. The gene product had 1/50 the activity of the native *C. perfringens* product and of the cloned *C. perfringens* gene product as measured by hemolysis or by phosphatidylcholine hydrolysis (Hatheway, 1990). Okabe et al. in Japan also successfully cloned the *C. perfringens* α-toxin gene and have published nucleotide and amino acid sequences identical to those of the other two studies (Okabe et al., 1989).

1.2.3.1.2. Beta toxin:

Beta-toxin is a major lethal toxin produced by both type B and C strains of *C. perfringens*. It is responsible for the lesions of necrotic enteritis of pig-bel (Murrell et al., 1966). Lawrence and Cooke (1980) showed that beta-toxin of type C organisms will produce necrotic lesions in the small intestines of guinea pigs only when protease inhibitor is introduced into the gut. The lesions in the guinea pigs were similar to those observed in human pig-bel patients. The experimental results supported the following hypothesis: that pig-bel occurs because the victims have low protease activity in their intestines due to a normally low protein diet and that the low level of protease activity is further inhibited by protease inhibitors present in sweet potatoes that are consumed in large quantities at the New Guinea feasts, along with the pork that serves as the source of the pathogen (Murrell et al., 1966). Immunization of guinea pigs with beta-toxin protects them from the pig-bel like disease after challenge with either toxin or organism and the protease inhibitors (Hatheway, 1990). Beta-toxin can be recovered from
culture fluid and purified by affinity chromatography on hydrophilic vinyl resin (Sakurai and Fujii, 1987).

The purified toxin has a molecular mass of 40 KDa and an isoelectric PH of 5.6. These properties are similar to those of alpha toxin, and for this reason it is difficult to separate the two toxins. Administered I.V. into rats, beta-toxin causes a rise in blood pressure and a fall in heart rate. The toxin apparently induces the release of catecholamines that are responsible for the increased blood pressure (Sakurai et al., 1984 and Sakurai et al., 1981).

When injected i.d. into guinea pigs, a minimum doses of 2ng causes purple areas of dermonecrosis. The 50% lethal dose for adult mice are 310 and 4,500 ng/kg i.v. and intraperitoneally respectively (Sakurai and Fujii, 1987).

1.2.3.1.3. Beta$_2$ toxin:

A novel toxin (B$_2$) has recently been identified from a strain isolated from a piglet that died of necrotic enteritis (Petit et al., 1999), enterocolitis in horses (Garmony et al., 2000), enterotoxemia in calves (Manteca et al., 2002) and clostridial dysentery in lambs (Miserez et al., 1998).

Purified CPB$_2$ was reported to be cytotoxic for chinese hamster ovary cells and to induce hemorrhagic necrosis of the intestinal mucosa in a guinea pig ligated intestinal loop (Jolivet et al., 1986). In 1997, the gene encoding this 28-KDa protein was cloned from C. perfringens type C strain (CWC245, and its nucleotide sequence was determined (Gilbert et al., 1997). Since the deduced amino acid sequence showed no significant homology with beta toxin, the respective protein corresponded to a new C. perfringens toxin, referred to as CPB$_2$. 
1.2.3.1.4. Epsilon – toxin:

Both type B and D strains of *C. perfringens* produce epsilon-toxin. It is produced as a proto toxin that is activated by proteolytic enzymes produced by the same organism (the Keappa – and lambda-toxin) (Willis 1969), or it can be activated by adding trypsin to the culture. The prototoxin is a protein consisting of 311 amino acids, with a molecular mass of 34.25 KDa based on sedimentation coefficient (Habeeb, 1975). Activation of the prototoxin with trypsin involves breaking of the peptide bond between the 14th and 15th amino acids from the amino terminus (Lys-14-Ala-15) and releasing an activation peptide of 14 residues (Bhown and Habeeb, 1977). Epsilon toxin primarily affects the intestine by increasing permeability of the gut wall, thus enhancing its own uptake, and acts systematically as a lethal toxin (Buxton, 1978). After entering the circulation, it causes swollen hyperemic kidneys, edema in the lungs, and excess pericardial fluid (McDonel, 1980). The effect of increasing vascular permeability can be demonstrated by i.d injection of the toxin, after which the injected site will be permeable to circulating Evans blue (Buxton, 1978). An enzyme-linked immunosorbent assay for epsilon-toxin has been described and proposed as an alternative to mouse lethality and specific antibody protection tests (Naylor et al., 1987).

1.2.3.1.5. Iota toxin:

Iota toxin was first described more than four decades ago, it is produced by type E only (Bosworth, 1943). Iota toxin is a binary toxin which is composed of two independent poly peptide chains called iota\(_a\) (I\(_a\)) and iota\(_b\) (I\(_b\)), which are not associated by either covalent or noncovalent bonds (Wilkins *et al.*, 1985). The individual chains possess relatively little toxicity, but the combination of chains is very potent (Simpson *et al.*, 1987).
In addition to mouse lethality after i.v. injection, iota-toxin has dermonecrotic activity (Bosworth, 1943), at subnecrotizing doses, iota-toxin injected intracutaneously increases vascular permeability (Craig and Miles, 1961).

Iota toxin appears to share certain properties with the botulinum binary toxin, inducing the ability to promote the movement of fluids across membrane (Craig and Miles, 1961; Jensen and Duncan, 1980; Ohishi, 1983; and Simpson, 1982). There are also the obvious similarities that both toxins are clostridial in origin and both can be released into the gut of a host that has succumbed to an opportunistic infection. These similarities suggest that the two toxins should be compared in terms of cellular and molecular action (Simpson et al., 1987).

1.2.3.1.6. Enterotoxin:

*Clostridium.perfringens* enterotoxin was isolated and purified (Hauschild and Hilsheimer, 1971) and found to be a protein with a molecular mass of 35 KDa and an isoelectric pH of 4.3. The amino acid sequence has been determined (Granum, 1986; Richardson and Granum, 1985). The toxin consists of one peptide of 309 amino acids with a molecular weight of 34.262. The peptide has one free sulfhydryl group. The activity of the enterotoxin is enhanced three fold by treatment with trypsin (Granum et al., 1981). Trypsin cleaves at two sites, each involving a lysine residue, cleaving 15 or an additional 10 amino acids from the amino-terminal end of the toxin (Richardson and Granum, 1983).

The mechanism of action of the enterotoxin seems to involve direct binding of the toxin receptors on the surface of intestinal epithelial cells (Hatheway, 1990) Wnek and McClane, 1983 isolated a 50-KDa protein from rabbit intestinal brush border membranes that specifically inhibited
cytotoxicity for vero cells. This molecule may be the specific receptor for the enterotoxin. Binding is followed by insertion of the entire molecule into cell membrane, but no internalization into the cell (McClane et al., 1988). A sudden change of ion fluxes occurs, affecting cellular metabolism and macromolecular synthesis. As intracellular calcium ion levels increase, morphological damage occurs, resulting in greatly altered membrane permeability and loss of cellular fluid and ions and moderate-sized molecules up to 3.5 KDa (McClane et al., 1988). Under some conditions a loss of protein molecules may occur, but this may reflect cell death (McClane et al., 1988). Based on rabbit studies, the enterotoxin is most active in the ileum moderately active in the jejunum, and essentially inactive in the duodenum (McDonel, 1980). Enteroxin has been studied almost exclusively in type A strains. It has been established that type C and D strains also can produce enterotoxin (McDonel, et al 1980).

1.2.3.2. Minor Toxins:

At least some strains produce toxins of all types eg: theta, Kappa, mu, nu and neuraminidase. Delta-toxin is a hemolysin produced by type B and C strains, but not by type A, D and E strains. Lambda has not been detected in type A and C strains. It is a proteinase that digests gelatin, hemoglobin, and casein to some extent, but not collagen (Smith and Williams, 1984). Strains of any toxin type may be encountered which fail to produce one or more of the indicated minor toxins. The minor toxins have been of primary interest in the early attempts at classifying members of the species. Kappa-toxin (Collagenase) mu-toxin (hyaluronidase), and lambda-toxin (protease) appear to play roles in pathogenesis because of their ability to break down host tissues Gamma-and eta-toxin have been proposed to account for discrepancies in neutralizing results obtained with specific antisera, but
specific active substances related to those supposed entities have not yet been isolated (McDonel, 1980).

1.2.3.2.1. Delta Toxin:

Delta-toxin has been recovered from \textit{C. perfringens} type C and purified (Alouf and Jolivet, 1981; Tixier and Alouf, 1976). It has a molecular mass of 42 KDa and an isoelectric PH of 9%. It has high hemolytic activity for erythrocytes from sheep, goats and pigs but is relatively inactive against those from humans, horses, rabbits, mice and other mammals. The activity is inhibited by gangliosides (especially GM2), but not by other lipid compounds such as sphingomyelin, lecithin, or cholesterol (Hatheway, 1990).

1.2.3.2.2. Theta toxin:

Theta-toxin, also known as perfringolysin O, is responsible for the clear zone of hemolysis produced by at least some strains of all toxin types (McDonel, 1980; Smith and Williams, 1984). It is an oxygen-labile, thiolactivated cytolysin, similar but not identical to some hemolysins produced by other species (\textit{Streptococcus pyogenes}, \textit{Streptococcus pneumonia}, \textit{C. tetani}, and \textit{C. novyi}) (Smith and William, 1984; Bernheimer, 1976). Theta toxin has been purified by anion-exchange and gel permeation chromatography (Yamakawa and Sato, 1977) and has a molecular size of 51 KDa by polyacrylamide gel electrophoretic analysis. This estimate agrees with amino acid analysis. Theta-toxin, activated by cysteine, is lethal for mice (i.v. injection), and the lethality is reportedly due to the cardio depressant effect of toxin-induced release of endogenous mediators such as platelet-activating factor (Stevens \textit{et al}., 1988).

Tweten (1988) recently has cloned and expressed the gene for theta-toxin in \textit{E. coli}. The gene product was identical to the theta-toxin produced
by the donor strain in amino-terminal sequence and in sodium dodecyl sulfate-gel electrophoretic analysis. Both had an estimated molecular mass of 54 KDa and were of comparable hemolytic activity. The 1.8-kb chromosomal fragment generated a peptide of 499 amino acids that included a 27-residue signal peptide. The secreted form of the theta-toxin has a calculated molecular weight of 52,469. It has 42% homology with streptolysin O and 42% with pneumolysin. All three hemolysins share an identical region of 12 amino acid residues that include the single cysteine residue of the molecule, which is involved in the thiol activation.

1.2.3.2.3. Neuraminidase:

Neuraminidase, or siolidase, serves many microorganisms as a pathogenicity factor in a variety of ways (Mueller, 1976).

Its action on erythrocytes may render them panagglutinable, resulting in increase of blood viscosity and promoting capillary thrombosis. Its modification of ganglio sites on host cell surfaces may allow more direct contact of pathogens with the host, or it can provide suitable receptors for other toxins produced by the same or other microorganisms. (Hathway, 1990).

1.2.4. Antimicrobial susceptibility:

In vitro studies of the susceptibility of *C. perfringens* to chemotherapeutic agents are numerous. Penicillin is known to be particularly active against *C. perfringens*. Resistance to penicillin is very rare and *B. lactamase* has not been demonstrated in *C. perfringens*. Tetracycline resistance is the most common antibiotic resistance trait found in *C. perfringens* (Lyras and Rood, 1996). It has been reported that *C. perfringens* can carry the tetracycline-resistance genes tet A (P), tet B (P), tet
A 408(P), tet M and tet Q (Kather, Marks and Foley, 2006; Lyras and Rood, 1996; Martel et al., 2004; Sasaki et al., 2001).

Resistance to chloramphenicol in *C. perfringens* is not common, though several resistant isolates have been identified (Bannam and Rood, 1991). No resistance to the ionophorous anticoccidial (IAC) narasin, an important substance used to control clostridiosis in the Nordic countries, has been found (Martel et al., 2004; Watkins et al., 1997).

**1.2.5. Toxinotyping of *C. perfringens***:

Detection of *C. perfringens* toxin types and subtypes is critical for a better understanding of the epidemiology of *C. perfringens* infections and may be helpful in the development of effective preventive measures. (Baums et al., 2004).

The typing of *C. perfringens* strains was originally established based on neutralization of the pathological effect of each major toxin, both trypsin treated and untreated, with appropriate antisera in laboratory animal models (Sterene and Batty, 1975). This toxino-typing requires a continuous supply of laboratory animals and the use of monovalent diagnostic sera which are increasingly difficult to find and are extremely expensive. Moreover, the result of the toxin-typing can not be obtained until 24 or even 48h of observation have elapsed. (Kadra et al., 1999). In diagnostic laboratories this differentiation has been replaced by rapid and easy to use enzyme linked immunosorbent assays (ELISA). (Nagahama et al., 1991). Although the ELISA allows reliable typing of *C. perfringens* isolates, the options for subtyping are limited. For example, so far no ELISA is available to detect the B2-toxin. In addition high levels of enterotoxin have been shown to be present during sporulation only (Czeczulin et al., 1993). As a consequence sporulation of *C. perfringens* isolates has to be induced via specific
cultivation methods to detect enterotoxin producing strains (Duncan and Strong, 1986).

These problems have been solved by genotype *C. perfringens* isolates. Various PCR protocols including multiplex PCR assays had been established to genotype *C. perfringens* isolates with respect to the genes cpa, cpb, etx, iap, cpe and cpb₂, encoding the α-, B-, ε, I, entero – and B₂-toxin, respectively (Daube *et al.*, 1994; Songer and Meer, 1996 and Songer, 1997; Yoo *et al.*, 1997; Kanakaraj *et al.*, 1998).
**Table (2)**

**Diseases produced by toxigenic types of *C. perfringens***:

(Data taken from Maclennan, 1962; Møller *et al.*, 1963 and Nillo, 1993).

<table>
<thead>
<tr>
<th>Toxin type</th>
<th>Major toxin(s)</th>
<th>Diseases</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>$\alpha$</td>
<td>Myonecrosis, food poisoning, necrotic enteritis in fowl, enterotoxemia in cattle and lambs, necrotizing enterocolitis in piglets; possibly equine colitis, canine hemorrhagic gastroenteritis</td>
</tr>
<tr>
<td>B</td>
<td>$\alpha$, $B$, $\varepsilon$</td>
<td>Dysentery in newborn lambs, chronic enteritis in older lambs (pine), hemorrhagic enteritis in neonatal calves and foals, hemorrhagic enterotoxemia in adult sheep</td>
</tr>
<tr>
<td>C</td>
<td>$\alpha$, $B$</td>
<td>Enteritis necroticans(pigbel) in humans; necrotic enteritis in fowl; hemorrhagic or necrotic enterotoxemia in neonatal pigs, lambs, calves, goats, foals; acute enterotoxemia (struck) in adult sheep</td>
</tr>
<tr>
<td>D</td>
<td>$\alpha$, $\varepsilon$</td>
<td>Enterotoxemia in lambs (Pulpy kidney) and calves, enterocolitis in neonatal and adult goats, possibly enterotoxemia in adult cattle</td>
</tr>
<tr>
<td>E</td>
<td>$\alpha$, $i$</td>
<td>Enterotoxemia likely in calves and lambs, enteritis in rabbits; host range and disease type unclear</td>
</tr>
</tbody>
</table>
1.2.6. Types of *C. perfringens*:

Classification of *C. perfringens* into one of the five toxin types A-E is based on the production of the major toxins $\alpha$, $\beta$, $\varepsilon$, and $\iota$ (Petit *et al.*, 1999), each type carries a different combination of the toxin gene (Khaldi *et al.*, 2004).

In 1931, Wilsdon established four toxin types, A, B, C, and D, of *C. perfringens* by preparing toxin culture filtrates and their corresponding antitoxin sera and observing the differential mouse protection by the sera. Wilsdon found that type A antiserum neutralized only type A cultures, type B neutralized all four types, type C neutralized all but type D, and type D neutralized filtrates contained multiple antigens. These antigens were later designated as alpha, present in all four types, beta present in types B and C, and epsilon, present in types B and D. (Sterne and Warrack, 1964). A fifth type (type E) was established by Bosworth in 1943 when he isolated a strain from a calf that produced a lethal toxin that could not be neutralized by type A, B, C, or D antitoxin or any combination. Type E antitoxin neutralized type A toxin filtrates as well as those of the homologous strain but not those of type B, C, or D. Bosworth proposed that the unique lethal factor in type E cultures be designated as iota. Type F had been established for a strain isolated from an outbreak of human necrotic enteritis in Germany (Zeissler and Rassfeld, 1949).

The organisms produced alpha-and beta-toxins and would have been classified as type C on that basis, but was unusually heat resistant and lacked

<table>
<thead>
<tr>
<th>A-E</th>
<th>Enterotoxin</th>
<th>Canine and porcine enteritis</th>
</tr>
</thead>
</table>
three minor antigens (delta, theta, and kapa) found with the classic type C strains (Sterne and Warrack, 1964).

This distinction was subsequently deemed unjustified because discrepancies in ancillary characteristics among other strains were noted, and establishing new types to denote such differences would become unmanageable (Sterne and Warrack, 1964).

1.2.6.1. Type A:

Strains of type A are ubiquitous, are the most common toxinotype in the environment (Petit et al., 1999), being found in most soils that are specifically cultured for *C. perfringens* (Smith and Williams, 1984). Well known as cause of wound contamination, anaerobic cellulites, and gas gangrene (Hatheway, 1990), strains of type A also cause enteric disease. Enterotoxemia in lambs, known as yellow lamb disease, occurs primarily in spring in California and Oregon, when the population of nursing lambs is high (McGowan et al., 1958 and Fleming, 1985).

Affected animals exhibit depression, anemia, icterus, and hemoglobinuria, dying after a clinical course of 6 to 12 h (Russell, 1970). Type A strains have been isolated frequently in the western United States from calves with tympany, abomastitis, and abomasal ulceration. Gross lesions include hemorrhage and ulceration of the abomasal mucosa. Severe diarrhea may occur in calves, but enteric lesions can be obscured by rapid autolysis. Gram-positive bacilli are often found on the mucosa and in the submucosa (Daube et al., 1994 and Roeder et al., 1988).

Necrotic enteritis, a disease of domestic chickens worldwides is usually caused by *C. perfringens* type A (Al-Sheikhly and Truscott, 1977) or type C (Mulaney et al., 1984).
Intestinal clostridiosis in adult horses occurs as profuse watery diarrhea with high mortality, and large numbers of type A organisms are demonstrable in the gut (Princewill and Oakley, 1976; Wierup, 1977).

Type A has been linked to enteric disease in suckling and feeder pigs with mild necrotizing enterocolitis and villous atrophy (Popoff and Jestin, 1985; Johannsen et al., 1993). Other reports of enteric disease associated with *C. perfringens* type A include enterotoxemia in minks (macarie et al., 1980), muskrats (Vustina, 1988), and racing camels (Seifent et al., 1992), gastroenteritis in black-footed ferrets (Schulman, 1993) and acute toxemia in water buffaloes (Worrall et al., 1987).

### 1.2.6.2. Type B:

*C. perfringens* type B is the etiologic agent of dysentery in newborn lambs. Disease occurs primarily in the border country between England and Scotland, as well as in Wales, South Africa, and the Middle East (Timoney et al., 1988).

A similar disease was reported in Montana (Tunnicliff, 1933) although isolates of type B are relatively rare in North America. Lamb dysentery usually develops during the first few days of life, although older lambs may be involved as an outbreak progress (Dalling, 1926). After the infection is acquired from the dam or the environment, organisms in the gut increase in numbers, especially with heavy lactation by the dam. The result is enterotoxemia accompanied by enteritis and extensive hemorrhage and ulceration of the small intestine (Frank, 1956 and Roberts, 1958). The primary sign is sudden death, without forewarning in peracute cases. In acute incidents, cessation of feeding and severe abdominal pain are accompanied by bloody diarrhea, with recumbency, coma, and death less than 24 h after on set. Incidence is often as high as 30%, with case fatality
rates approaching 100%. Chronic disease (Called pine) in older lambs manifests as chronic abdominal pain without diarrhea. Type B may also be associated with hemorrhagic enteritis in goats, calves and foals (Frank, 1956 and Roberts, 1958). There has been little experimental study of the pathogenesis of infections by type B (Jansen, 1961).

1.2.6.3. Type C:

Infections by *C. perfringens* type C have been reported in pigs, cattle, sheep, horses, chickens, humans, and dogs in the United States, Denmark, Great Britain, Japan, and elsewhere (Mackinnon, 1989). Newborn animals are typically most susceptible, perhaps because of ready colonization of the gut by *C. perfringens* in the absence of well-established normal intestinal flora. Alteration of the flora by sudden dietary changes may also be an inciting factor in type C infections (Timoney et al., 1988).

Piglets are perhaps more commonly affected by type C than are other domestic animals (Fitzgerald et al., 1988; Johnson et al., 1992). Type C is the most commonly reported clostridial enteric pathogen of foals in North America (Dickie et al., 1978; Drolet et al., 1990; Howard-Martin et al., 1986). Clinical signs include depression, severe hemorrhagic diarrhea, dehydration, and occasionally colic (Howard-Martin et al., 1986).

At least one type C isolate has been obtained from peracute to acute, lethal, hemorrhagic enteritis in dogs (Argenti et al., 1987; Lage et al., 1992). Untyped isolates, associated with a similar but milder necrotic process, were also obtained from a high proportion of dogs with a primary parvovirus infection (Turk et al., 1992).

1.2.6.4. Type D:

Type D strains cause enterotoxemia, also known as sudden death or overeating, in sheep of all ages except newborns (Timoney et al., 1988).
It is probably most prevalent in lambs 3 to 10 weeks old, often suckling heavily lactating ewes grazed on luxuriant pastures. Enterotoxemia is also a predominant cause of death in weaned animals up to 10 months of age, usually those fed rich rations of grain in feedlots. Disease is often associated with upsets in the gut flora, such as result from sudden changes to a rich diet or from continuous feeding of high levels of feed concentrates (Popoff, 1984). Appropriate microenvironments lead to rapid multiplication of type D and production of toxin, almost certainly favored by the presence of excess dietary starch in the small intestine (Bullen, 1970). A high concentration of toxin facilitates its absorption (Nillo, 1993).

Pulpy kidney, another common name for type D enterotoxemia, is derived from one of the hallmark lesions in affected sheep, a result of postmortem autolysis, which occurs rapidly in hyperemic, toxin damaged tissue (Songer, 1996). Type D enterotoxemia is also important in calves (Griner et al., 1956; Munday et al., 1973), in goats (Oxer, 1956), and rarely in adult cattle (Mumford, 1961), deer, domesticated camels, and horses (Carrol et al., 1987).

Enterotoxemia in suckling calves is similar to the disease in sheep (Griner et al., 1956; Lulov and Angelov, 1986). In neonatal and adult goats, however, catarrhal, fibrinous, or hemorrhagic enterocolitis is a consistent lesion (Blackwell and Butler, 1992; Blackwell et al., 1991), and the classic pulpy kidney is absent. Small amounts of toxin detected in the gut of normal animals are considered innocuous, but persistence of high concentrations of toxin leads to an increase in permeability and to absorption of toxin into the circulation (Bullen, 1970). A primary target of toxin is the central nervous system, where it produces foci of liquefy active necrosis, perivascular edema, and hemorrhage, especially in the meanings (Buxton et al., 1978).
The extent of clinical signs of central nervous system derangement, including in coordination and convulsions, is directly related to the severity of lesions (Griner, 1961). Hemorrhage is not a hallmark of typical type D induced disease in sheep or cattle, although hemorrhagic areas in the small intestine and petechial hemorrhages of the endocardium can be present, as can subendocardial hemorrhage around the mitral valve (Jubb et al., 1993). Disease in goats is present frequently as hemorrhagic enterocolitis, which can be chronic, it is often associated with lactation and high food intake. The basis for this species to species difference in clinical picture is unknown (Blackwell et al., 1991). Peritoneal and pericardial effusions are typical in sheep (Jubb et al., 1993). Hyperglycemia and glycosuria are pathognomonic for type D enterotoxemia (Gardner, 1973; Nillo, 1993). These events apparently occur very rapidly, probably in the first hour after toxin enters the circulation (Songer, 1996).

1.2.6.5. Type E:

Type E is an apparently uncommon cause of enterotoxemia of lambs, calves, and rabbits (Baskerville et al., 1980). I enterotoxemia in a calf and lambs was reported 50 years ago in Britain, but it is uncertain if published accounts since that time of hemorrhagic, necrotic enteritis of calves in Australia (Hart and Hooper, 1967) and of detection of the organism and I toxin in ovine or bovine intestines postmortem in the absence of signs of clostridial enterotoxemia (Sterne and Thomson, 1963) represent genuine cases. Recent experimental work demonstrated the ADP-ribosylating nature of I toxin, and characterization of the genes will provide a further basis for study of this toxin’s role in the pathogenesis of intestinal infections. The functional similarity of \textit{C. perfringens} I toxin to the toxin of \textit{C. spiroforme} suggests that infection by the latter may be mistaken for infection by the
former and vice versa if detection of toxin in the gut is the sole diagnostic measure. Further experimental work is needed to clarify the role of each of these organisms as etiologic agents of disease in domestic animals (Songer, 1996).

1.2.7. Economical significance:

Loveland and Kaldhusdal (2001) studied the association between Clostridium perfringens infection and production performance in broiler flocks during a 2.5 years time period when clinical and subclinical necrotic enteritis was frequently seen. They found that the farmer’s profit on average was reduced by 33% when comparing flocks with high and low levels of the disease. Impaired feed conversion, reduced live weight at slaughter and increased condemnation percentage were major causes of production losses associated with Clostridium perfringens infection. Subclinical clostridial enteritis has also been associated with impaired feed conversion and retarded growth in a pen trial (Kaldhusdal and Hofshagen, 1992).

1.2.8. C. perfringens food borne illness:

The earliest report of food-borne illness outbreaks due to C. perfringens was provided by McClung in 1945. In four episodes, patients experienced nausea, intestinal cramps, and a pronounced diarrhea 8 to 12 h after consuming chicken dishes prepared on one day and served the following day samples of the foods showed heavy contamination with Gram-positive rods, which were identified as C. perfringens (Hatheway, 1990).

Hobbs et al., 1953 observed similar outbreaks in England; the causative organisms were of toxin type A. They noted that the spores of implicated strains were considerably more heat resistant and the organisms
were less hemolytic than the classic strains. Outbreak stains in the United States were usually more like the classic strains, with spore death within 10 min at 100°C, rather than spore resistance to 100°C for more than 1 h as observed with the British Strains. Sutton and Hobbs, 1965 later found that both heat-susceptible and heat-resistant strains were implicated in England. Symptoms of the illness occurred in human volunteers after ingestion of a large number of viable vegetative bacteria (more than $5 \times 10^8$), but not after ingestion of spores or cell-free culture fluid (Diche and Eleck, 1957; Hauschild and Thatcher, 1967).

An enterotoxin released from the cells during sporulation was responsible for the diarrheal illness (Duncan and Strong, 1969, Duncan et al., 1972). It has been generally assumed that enterotoxin is a byproduct of sporulation, perhaps a spore coat protein, but synthesis of substantial amounts of enterotoxin by nonsporulating cultures has been demonstrated convincingly (Goldner et al., 1986).

*C. perfringens* food borne illness undoubtedly occurs frequently, but because of the mildness of the disease it is often not reported. Between 1970 and 1980, 567 outbreaks of food borne illness due to *C. perfringens* were confirmed in England (Stringer, 1985). In the United States, about 25 outbreaks are reported each year (Hatheway et al., 1980).

**1.2.7. Clostridium Perfringens diseases in Poultry:**

Clostridial infections associated with four disease conditions in poultry or game birds, clostridium colinum is the cause of ulcerative enteritis, *C. perfringens* and *C. septicum* have been isolated from cases of necrotic enteritis or gangrenous dermatitis, and *C. botulinum* is the etiological agents of botulism (Barnes, 2003).
C. perfringens also is being increasingly recognized as a cause of cholangio hepatitis in chickens (Kaldhusdal et al., 2001), and it has been associated with cellulites involving the tail of turkeys (Carr et al., 1996) and gizzard erosions in neonatal chicks (Jordan, 1996). Possible vertical transmission of C. perfringens has been shown (Shane et al., 1984).

1.3. Necrotic enteritis:

The etiologic agent of NE is Clostridium perfringens types A and C (David, 1998). It was first recorded by Bennetts (1930) in Australia, and fully characterized by Parish (1961a, b, c) in the United Kingdom. Interestingly, NE did not appear in a 1964 guide to common poultry diseases in the UK (British Oil and Cake Mills, 1964), nor in 1979 global disease survey (Biggs, 1982). Even as recently as 1997, it did not meet special attention in an international poultry disease directory (Van der Sluis, 1997). Soon afterwards however, NE emerged as a worldwide problem (Van der Sluis, 2000a b, Van Immerseel et al., 2004). Furthermore, the occurrence of the causative agent of NE, Clostridium perfringens, in poultry meat is an important threat to public health (Van Immerseel et al., 2004).

1.3.1. Host:

Necrotic enteritis has been reported in many bird species. Among chickens the disease is by far most common in broilers, but outbreaks in layer pullets and adult layer strain chickens have also been reported. (Kaldhusdal, 2002).

1.3.2. Predisposing factors:
*Clostridium perfringens* is a common inhabitant of the chicken gut and its presence does not lead to disease without the presence of predisposing factors (Gholamiandekhordi *et al.*, 2006).

Coccidial infections are assumed to be a predisposing factor in many cases. These organisms invade and damage intact mucosal gut tissue, which is likely to favour the establishment of a non-invasive bacterium like *Clostridium perfringens* (Kaldhusdal, 2002).

Also concurrent coccidio infection has been shown experimentally to predispose to the disease (Shanel *et al.*, 1985).

At the molecular level, one or several factors triggering the alpha toxin production of *Clostridium perfringens* may be essential. One the bacteria produce significant amount of toxins, these toxins eventually induce gut tissue necrosis, favouring proliferation of *Clostridium perfringens* and further tissue damage within the mucosa. (Kaldhusdal, 2002).

Diet is believed to be an important determinant of the disease. Manipulation of the diet can affect the numbers of *C. perfringens* in the intestinal tract (Buranton *et al.*, 1997). High levels of wheat and barley have been associated with NE in experimental and epidemiological studies (Branton *et al.*, 1987; Ridded *et al.*, 1992; Kaldhusdal *et al.*, 1992; Kaldhusdal *et al.*, 1996). Also diets rich in fish meat predispose to necrotic enteritis (Kocher, 2003) Feed and litter contaminated with clostridial spores have been incriminated as source of infection. (Wicker *et al.*, 1977 and Ficken *et al.*, 1977) and litter high in fiber content has been associated with necrotic enteritis (Truscott *et al.*, 1977).

The viscosity of the intestinal contents will affect clostridial proliferation in the proximal intestinal tract, and the inclusion of enzymes to
off set the viscosity of wheat and barley is an important control factor (Smit, 1996).

1.3.3. Clinical signs and lesions:

The prominent feature of necrotic enteritis is acute death, with mortality rates that can reach 50% (Riddell and Kong, 1992).

Clinical signs in naturally occurring outbreaks include marked to severe depression, decreased appetite, reluctance to move, diarrhea and ruffled feathers. (Bains, 1978; Helmboldt and Bryant 1971). Most evident macroscopical lesions can be seen in the small intestine, but lesions can also be detected in other organs, such as the caeca, liver and kidney. The duodenum, jejunum and ileum and some times also the caeca, are thin walled, friable, dilated and filled with gas (Broussard et al., 1986). Mucosal surfaces are covered with a grey brown to yellow-green diphtheric membrane or pseudomembrane (Gadzinski and Julian, 1992).

Microscopic changes in natural outbreaks are characterized primarily by severe necrosis of the intestinal mucosa with an abundance of clinical signs fibrin admixed with cellular debris adherent to the necrotic mucosa (Nairn and Bamford, 1967., Helmboldt and Bryant, 1971., Long et al., 1974,). Initial lesions develop at the apices of villi and are characterized by sloughing of epithelium and colonization of exposed lamina proria with bacilli, accompanied by coagulation necrosis. Areas of necrosis are surrounded by heterophils. Progression of lesions usually occurs from villi apices to crypts. Necrosis may extend into the submucosa and muscular layers of the intestine. Numerous large bacilli often are observed attached to cellular debris. In birds that survive, regenerative changes consist of crypt epithelial cell proliferation with a corresponding increase in mitotic figures. Epithelid cells are primarily cuboidal, with a relative decrease in goblet and
columnar epithelial cells. Villi are relatively short and flat. In many outbreaks various sexual and asexual stages of coccidia are also found in the intestine (Hemboldt and Bryant, 1971, Long et al., 1974, Nairn and Bamford, 1967).

1.3.4. Diagnosis:

Diagnosis of NE can be made based on typical gross and microscopic lesions and isolation of the causative agent. In field cases of N.E., *C. perfringens* can be isolated readily from intestinal contents, the scraping of intestinal wall, or hemorrhagic lymphoid nodules by anaerobic incubation overnight at 37°C on blood agar plates (Fickon and Berkhoff, 1989). Identification of *C. perfringens* can be done. Some commercially available media may not be formulated adequately to selective cultivation and enumeration of *C. perfringens* unless used in combination with other tests for specific identification (Dafwang et al., 1987).

Diseases that must be differentiated from NE are ulcerative enteritis (UE) and *Eimeria brunette* infection. Ulcerative enteritis is caused by *C. colinum*; characteristic gross lesions are multiple areas of necrosis and ulceration in the distal small intestine and ceca and areas of necrosis in the liver. Lesions of NE usually are confined to jejunum and ileum with little or no involvement of ceca or liver. These distinguishing characteristics should allow differentiation of NE and UE. Isolation and identification the causative agent will confirm the diagnosis (Wages and Opengart, 2003).

1.3.5. Treatment:

In field cases the disease normally responds well in 24 to 48 hours to oral treatment with one of a variety of antibiotics (long, 1973). Several
workers have reported the results of in vitro sensitivity tests on *C. perfringens* isolates with rare exception (Narin and Bamford, 1967).

Smith 1972 dosed day old chicks orally with *C. perfringens* isolates from cases of necrotic enteritis and conducted in vivo sensitivity tests. He found that a number of antimicrobial agents when added to the ration decreased the clostridial count in the feces of birds after they consumed the medicated feed from one day.

In some areas at the present time, an antibiotic is added to the feed of broiler chickens for the first month of life to prevent necrotic enteritis (Long, 1973).

**1.3.6. Control:**

The use of most antimicrobial growth promoters leads to a dramatic decrease in the incidence of necrotic enteritis in poultry, relative to non-medicated animals (Van Immersal *et al.*, 2004). Necrotic enteritis in different mammalian species including humans can be prevented by vaccination (Lawrence *et al.*, 1990), also vaccination against coccidiosis may indirectly prevent losses from clostridium (Williams, 2002).
Chapter Two

Materials and Methods

2.1. Sterilization:

2.1.1. Hot-air oven (160°C for one hour)

Used for sterilization of glassware like Petri dishes, pipettes, tubes, flasks and glass-rods. These were sterilized in the hot-air oven at 160°C for one hour.

2.1.2. Autoclaving: (121°C – 1516 square inch)

Media, solutions, screw capped bottles, rubber-stoppered flasks were sterilized by autoclaving at 121°C for 15 minutes and 110°C for 5 minutes for sugar media.

2.1.3. Irradiation and disinfectants:

Used for aseptic work, addition of supplements and pouring plates. Phenolic disinfectants and absolute alcohol were used for disinfecting floors and stage of a U.V room followed by U.V irradiation for 20 minutes.

2.2 Collection of blood:
Blood for enrichment of media was collected by veni-puncture of the Jugular Vein of a healthy donor sheep. It was defibrinated by shaking the sterile flask containing glass beads.

The flask was transferred to the laboratory where the defibrinated blood was aseptically distributed in 20ml aliquots into sterile McCartney's bottles.

2.3. Reagents and media additives:

2.3.1. Distilled water (D.W)

Tap water was distilled using distiller.

2.3.2. Normal saline:

Normal saline was prepared according to CruichShank, (1975).

Ingredients:

Sodium chloride (NaCl) 8.5g
D.W. 1000ml

The sodium chloride was dissolved in distilled water, the solution was distributed in bottles of different sizes and autoclaved at 121°C for 15 minutes and stored at 4°C.

2.3.3. Staining reagents:

2.3.3.1. Crystal violet

Crystal violet 1.0g
D.W. 100.0ml

2.3.3.2. Lugol’s iodine

Iodine 1.0g
Potassium iodide 2.0g
2.3.3.3. Carbol fuchsin:

A. Basic fucshin
   Absolute ethanol
   D.W.

B. Phenol
   D.W.

Mixed A and B

2.4. Reagent in PCR:

2.4.1. Saturated bromophenol blue:

Bromophenol blue 10%
Bromophenol blue 5 g
D.W. 50ml

2.4.2. Ethidium bromide solution:

10mg/ml used to visualize DNA
Ethidium bromide 0.2g
D.W. 20.0ml

Mixed well and stored at 4°C in the dark (Brown or foil covered bottles).
It was handled with gloves and was avoided inhalation.

2.4.3. Gel loading buffer used to load DNA samples in gels:

Glycerol 5.00ml
TBE (5x) 250.00ml
Saturated bromophenol blue 1.00 ml
Xylene cyanol (10% suspension) 1.00ml
D.W. 2.75ml

Divided to 1ml aliquots and stored at -20°C.
2.4.4. TBE buffer (Tris base Boric Edta):

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boric acid</td>
<td>14g</td>
</tr>
<tr>
<td>Edta</td>
<td>0.93g</td>
</tr>
<tr>
<td>Tris base</td>
<td>27.5g</td>
</tr>
<tr>
<td>D.W.</td>
<td>250ml</td>
</tr>
</tbody>
</table>

The buffer was adjusted to pH 8.3

2.4.5. Preparation of running buffer:

<table>
<thead>
<tr>
<th>Component (5x)</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBE</td>
<td>1ml</td>
</tr>
<tr>
<td>D.W.</td>
<td>300ml</td>
</tr>
</tbody>
</table>

2.4.6. TE buffer (tris/EDTA)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris 10mM pH 7.4</td>
<td>0.5ml of 2M stock</td>
</tr>
<tr>
<td>0.1mM EDTA, pH 8</td>
<td>20µl of 0.5M stock H2O</td>
</tr>
</tbody>
</table>

To make 99.3ml

**Total 100.0ml**

It was stored at room temperature.

2.4.7. Agarose gel:

1.5% agarose containing 5mg/ml ethidium bromide

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose</td>
<td>0.75g</td>
</tr>
<tr>
<td>TBE</td>
<td>50.00ml</td>
</tr>
</tbody>
</table>

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

2.5. Preparation of media:
2.5.1. Blood agar medium:

Forty grams of (Oxoid CM55) blood agar base were suspended in a litre of distilled water the pH was adjusted to 7.4. It was then sterilized by autoclaving at 121°C for 15 minutes. After cooling to about 46°C, defibrinated sheep blood was added aseptically in 7-10 concentration before pouring plates.

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

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

One litre of SFP agar contained:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptose (Difco)</td>
<td>15g Yeast</td>
</tr>
<tr>
<td>extract (Difco)</td>
<td>5g</td>
</tr>
<tr>
<td>Ferric ammonium citrate</td>
<td>1g</td>
</tr>
<tr>
<td>Sodium metabisulfite (Na₂S₂O₂)</td>
<td>1g</td>
</tr>
<tr>
<td>Polymyxin B sulfate (Antimicrobial vial P, Difco)</td>
<td>30,000 units</td>
</tr>
<tr>
<td>Kanamycin sulfate</td>
<td>0.012g</td>
</tr>
<tr>
<td>Agar</td>
<td>20g</td>
</tr>
<tr>
<td>Water</td>
<td>900ml</td>
</tr>
</tbody>
</table>

Egg yolk emulsion was dissolved in water. After thorough mixing, the pH was adjusted to 7.6. The basal medium was autoclaved in 900ml portions at 121°C for 10 min. After cooling to about 50°C, 100ml of the 50% egg yolk emulsion was added to each 900ml of the basal medium thoroughly mixed, and added aseptically in 10-20ml amounts to Petri dishes. The medium was allowed to solidify, incubated overnight at 35°C, and examined the following day for dryness and sterility.

2.5.3. 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 eggs shell was opened and the egg white was aseptically discarded. The eggs Yolk were then collected aseptically into sterile beaker. An equal volume of sterile normal saline was added to the egg yolk, mixed with shaking or stirring with sterile glass-rod. The egg yolk emulsion was then distributed in sterile 100ml bottles and kept at 20°C for further use.

2.5.4. Egg yolk medium:

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

2.5.5. Lactose motility agar:

Lactose motility agar was prepared with tryptose, 15g; yeast extract, 10g, lactose, 10g; sodium phosphate (dibasic), 5g; phenol red, 0.05g; agar, 3g; and distilled water, 1,000ml. The ingredients were dissolved in the water, the pH was adjusted to 7.5, and the lactose and phenol red were added. Ten milliliters of the medium was dispensed into tubes. The cotton plugged tubes containing the medium were autoclaved at 118°C for 15 min.

Before use, the medium was boiled for 10 min and coded immediately.
2.5.6. Reinforced Clostridial medium (RCM)

Thirty-eight grams of RCM (Oxoid, CM149) was suspended in 1 litre of distilled water (DW), and heated in a water bath then sterilized by autoclaving at 121°C for 15 minutes.

2.5.7. Cooked meat media:

Ten grams of granules of cooked meat medium (Oxoid, CM82) was transferred into McCartney bottles and 10ml of DW was added. The medium was then autoclaved at 121°C for 15 minutes for sterilization.

2.5.8. Hugh and Leifson’s (O.F) medium:

Peptone, 2g; NaCl, 5g; K2HPO4; 0.3g; agar, 3g; and distilled water 1000ml, bromothymol blue 0.25% aqueous solution 15ml, steamed to dissolve, the pH was adjusted to 7.1. Indicator was added to the medium which was then sterilized by autoclaving at 115°C for 20 minutes. Ten ml of sterile glucose solution was aseptically added to 90ml of the medium to give a final concentration of 1%. It was then distributed into test tubes each contained 10ml.

2.5.9. Peptone water

Fifteen grams of dehydrated (Oxoid CM9) peptone water were rehydrated in a litre of distilled water, mixed well. The pH was adjusted to 7.2 and then autoclaved at 121°C for 15 minutes.

2.6. Collection of samples:

Three hundred and forty intestines were collected, one 140 from field cases which were brought to the department of Avian Pathology and Diagnosis (C.V.R.L.) for investigation, and the remaining 200 from abattoirs
in (Khartoum north) Bahry. All intestinal samples had lesions of necrotic enteritis.

Sixty cloacal swabs were taken from chickens with avian influenza and had diarrhea.

2.7. Isolation Procedure:

Under aseptic conditions, the intestines were opened with sterile blades, parts of the intestinal mucosa were scraped with a wire loop and cultured into reinforced clostridia medium, also cloacal swabs were cultivated in the same medium.

Inoculated RCM were heated at 80°C for 10 minutes to destroy vegetative organisms followed by anaerobic incubation using anaerobic jar’s with gas bag generating kits at 37°C for 24-48 hours. After that subcultures were made on blood agar plates for 24-48 hours, then cultures were examined with the naked eye for growth, colonial morphology and haemolysis. Smears were made from colonies surrounded by double zones of haemolysis, and stained with Gram stain. After purification of isolates biochemical tests were done to identify the bacteria.

2.8. Preservation of the cultures:

Pure, full identified colonies were inoculated on cooked-meat medium and then incubated at 37°C for 24-48 hours and then stored at 4°C in refrigerator.

2.9. Staining Technique

2.9.1. Preparation of smears:

Smears were prepared by emulsifying a small part of the colony in a drop of normal saline and the suspensions were spreaded on a clean slide. The smears were allowed to dry in air and then fixed by gentle flaming.

2.9.2. Gram’s stain:
The prepared slides were placed on the rack and flooded with crystal violet-stain for one minute. The slide was left covered with iodine for one minute, rinsed with water, acetone was poured for 15 seconds, and the slides were rinsed with water again. The slides were counter stained with carbol fuchsin for one minute, rinsed with water and Dried by blotting with filter paper.

2.10. Biological and biochemical methods:

2.10.1. Catalase test:

A small amount of culture was picked with the help of a sterile pasteur pipette placed on a clean grease-free slide then a drop of 3% aqueous solution of hydrogen peroxide was placed over the culture and observed for production of gas bubbles.

2.10.2. Oxidase test (Kovacs, 1956)

One percent aqueous solution of tetramethylene-p-phenylene diamine dihydrochloride was used to soak strips of whatman No. 1 filter paper for about 30 seconds, after which the strips were oven dried till white bleaching. For use, a discrete colony was picked and rubbed over the surface of the strip, the development of purple colour was recorded as positive for cytochrome oxidase.

2.10.3. Urease Test (Christensen’s, 1946):

A urea agar slant was streaked with the test culture and incubated at 37°C for 24-48 hours. The development of a pink colour was indicative of production of NH₃. Negative and weak tests were left for a week before discarded.

2.10.4. Sugar fermentation:

This test was carried out on media containing Sucrose, Maltose, Mannitol, Lactose, Fructose, Salicin, Raffinose and Glucose.
concentration of the sugar was 1% in peptone water. Each test culture was inoculated into a set of sugar tubes, incubated at 37°C and examined for acid or gas production for 14 days. When this occurred it was indicated by the colour change to pink, when no acid was produced the medium colour remained unchanged.

2.10.5. Gelatin hydrolysis (or liquefaction)

Inoculated Nutrient gelatin was inoculated with a straight wire loop and incubated at 37°C for up to 14 days and observed every 2-3 days, then placed in a refrigerator for 2h and examined for liquefaction.

2.10.6. Motility tests:

2.10.6.1. Hanging drop test:

A loop full of liquid culture was transferred to a coverslips. The coverslips were put on glass slides with petroleum vasline and examined microscopically.

2.10.6.2. Lactose fermentation and motility test:

Lactose fermentation and motility were both examined in lactose motility agar (LMA) medium as described by Shahidi and Ferguson (1971). 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 under anaerobic incubation at 37°C.

2.10.7. Lecithinase Production:

The isolated organisms were streaked on 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.

2.10.8. Half Plate neutralization test:
Standard *C. perfringens* antiserum type A was spreaded to cover half of the egg-Yolk plate, titled and allowed to dry. Pure colonies of the isolates were streaked in the medium starting from the untreated half of the plate. The plates were then incubated anaerobically at 37°C for 24 hours.

2.12. Multiplex PCR procedure:

2.12.1. DNA Extraction:

Five to ten colonies of *C. perfringens* from blood agar plates suspended in 200ml eppendorf tube using micropipettes. The samples were boiled at 100°C for 20 minutes, snapped cooled on ice for 5 minutes and centrifuged at 13,000 revolutions per minute for 3 minutes. The supernatant was transferred to clean tubes and stored at -20°C.

2.12.2. Oligonucleotides:

PCR primers of Qiagen, 1000 Atlantic Ave, suite 108, Alameda, CA94501, were kindly supplied by Dr. Zakia Abbass head department of Pathology in (C.V.R.L.).

The prepared master mix from Invitrogen Corporation, under U.S. patent Nos. 5, 338, 5, 587, 287 was kindly supplied by Dr. Dia El Din Ahmed Salih (Kassla Regional Research Lab).
### Primers used:

<table>
<thead>
<tr>
<th>Type</th>
<th>Sequence 5 – 3</th>
<th>Amplican size</th>
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</thead>
<tbody>
<tr>
<td><em>C. perfringens</em> A toxin</td>
<td>GCTAATGTTACTGCGTGTGA</td>
<td>324</td>
</tr>
<tr>
<td>forward CPa-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reverse CPa-2</td>
<td>CCTCTGATACATCGTGTAAG</td>
<td></td>
</tr>
<tr>
<td><em>C. perfringens</em>-B toxin</td>
<td>GCGAATATGCTGAATCATCTA</td>
<td>196</td>
</tr>
<tr>
<td>forward CPb-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reverse CPb-2</td>
<td>GCAGGAACATTAGTATATCTTC</td>
<td></td>
</tr>
<tr>
<td><em>C. perfringens</em>-E toxin</td>
<td>GCGGTGATATCCATCTATTC</td>
<td>655</td>
</tr>
<tr>
<td>forward eTX-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reverse Etx-2</td>
<td>CCACCTACTTGTCTACTAAC</td>
<td></td>
</tr>
<tr>
<td><em>C. perfringens</em>-I toxin</td>
<td>ACTACTCTCAGACAAGACAG</td>
<td>446</td>
</tr>
<tr>
<td>forward Iₐ⁻1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reverse Iₐ⁻2</td>
<td>CTTTCCTTCTATTACTATACG</td>
<td></td>
</tr>
<tr>
<td>C. <em>perfringens</em>-e toxin</td>
<td>GGAGATGGTTGGATATTAGG</td>
<td>233</td>
</tr>
<tr>
<td>--------------------------</td>
<td>-----------------------</td>
<td>-----</td>
</tr>
<tr>
<td>forward CPe-1</td>
<td>GGACCAGCAGTTGTAGATA</td>
<td></td>
</tr>
<tr>
<td>Reverse CPb₂-2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>C. <em>perfringens</em>-B₂ toxin</th>
<th>AGATTTTTAAATATGATCCTAAC</th>
<th>567</th>
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<tbody>
<tr>
<td>forward CPb₂-1</td>
<td>CAATACCCCTTCACCAAATACTC</td>
<td></td>
</tr>
<tr>
<td>Reverse CPb₂-2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.12.3. **PCR reaction:**

Two trials were made for PCR the first one optimized in a total of 40µl master mix consisting of 5.0µl of 10 X PCR buffer, 1.2µl of dNTPs, 0.5µl of tag polymerase (5 units/µl), 1.0µl MgCl₂, 26µl DDW and six primers 1.3µl for Ia, 1.2 for PCR, 1.1µl for etx, 0.9µl for cpb, cpe, cpb₂. The master mix for all samples was mixed by vortexing and then pipetted in 5µl PCR tubes 10µl of DNA was added for each PCR reaction. A negative control was included in which 10µl of DDW was added instead of DNA.

In the second trial a prepared master mix was used which consist of 22 mMtris-HCl, 55mm KCL, 1.65mm MgCl₂ 220mmdGTP, 220mmdATP, 220mmdTTP, 220mmdCTP and 22U/ml complexed recombinant tag DNA polymerase with platinum Tag Antibody. This master mix for 50µl reaction uses 45µl of PCR mastermix and 5µl of primer and DNA.

The PCR tubes were then placed in a Biometra DNA thermocycler the reaction was subjected to 35 cycles of amplification consisting of 2 minutes of initial denaturation at 94°C, followed by 1 minute of denaturation at 94°C, 1 minute of annealing at 55°C prior to 1 minute of extension at 72°C.

The presence or absence of the desired band was resolved by running 5ml of PCR product through 1.2% agarose gel stained with 2.5µl ethidium
bromide. Molecular sizes were determined based on a 100 pb ladder molecular mass marker. This was performed at 75V for 45 minutes and the analysis was done by using an automated photo documentation system (Bio. Doc. Analyza, digital).

2.13. Drug sensitivity test:

This test was carried out essentially as described by Bauer, Kirby, Sherris and Truck (1966) and Benson 1980. The procedure followed, briefly known as Kirby-Bauer method, is reliable, accepted method in current use and sanctioned by the U.S. FDA and the subcommittee on Antimicrobial susceptibility testing of the National Committee for Clinical Laboratory Standards, NCCLS (Benson, 1980).

Three to ten colonies were picked from *C. perfringens* grown anaerobically on blood agar (Oxoid), subcultured into RCM (Oxoid) and incubated anaerobically at 37°C for 24 hrs.

For preparation of the bacterial suspension, the inoculated RCM was then diluted with RCM to a degree of turbidity visually equivalent to that of the standard prepared by adding 0.5ml of 1% BCL₂ to 99.5ml of 1% H₂SO₄ (O.36N).

The bacterial suspension was inoculated onto the top of the medium and spread by horizontal rotation on a surface. Excess bacterial suspension was aspirated by a sterile Pasteur pipette. Then the inoculum was allowed 3-5 minutes to dry before the antimicrobial agent disks were placed on and gently pressed. Inoculated plates were incubated at 37°C overnight.

The diameter of the inhibition zone including that of the antimicrobial disk was measured. The inhibition zone produced by antimicrobial drug was compared with its corresponding standard zone of inhibition to determine
whether the specified \textit{C. perfringens} isolate was sensitive or resistant to the drug.

\textbf{Chapter Three}

\textbf{Results}

In this study 40 isolates of \textit{C. perfringens} were isolated from 400 samples, 340 were intestinal specimens and 60 were cloacal swabs as shown in table (3).

One hundred and forty intestinal specimens were taken from field cases came to the department of Avian Pathology and Diagnosis for investigation as shown in table (4), the remaining twenty hundred from abattoirs in Khartoum North (Bahry). Intestinal specimens had lesions of necrotic enteritis fig (1).

Cloacal swabs were from cases of avian influenza as shown in table (5).

\textbf{Table (3)}

\textbf{Source and numbers of specimens.}
Table (4)  
*Clostridium perfringens* isolated from field cases

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Types of chicken</th>
<th>Flock status</th>
<th>Genotype of isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Layer</td>
<td>Tape worms + ND</td>
<td>A(CPe⁺)</td>
</tr>
<tr>
<td>2</td>
<td>Layer</td>
<td>ND</td>
<td>A(CPe⁻)</td>
</tr>
<tr>
<td>3</td>
<td>Layer</td>
<td>ND</td>
<td>A(CPe⁺)</td>
</tr>
<tr>
<td>4</td>
<td>Layer</td>
<td>Marek's</td>
<td>A(CPe⁺)</td>
</tr>
<tr>
<td>5</td>
<td>Layer</td>
<td>Marek's</td>
<td>A(CPe⁻)</td>
</tr>
<tr>
<td>6</td>
<td>Layer</td>
<td>Marek's</td>
<td>A(CPe⁻)</td>
</tr>
<tr>
<td>7</td>
<td>Layer</td>
<td>Coccidia</td>
<td>A(CPe⁺)</td>
</tr>
</tbody>
</table>

### Source of specimens

<table>
<thead>
<tr>
<th>Source of specimens</th>
<th>Organs examined</th>
<th>No.of specimens</th>
<th>No.positive</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Field cases</td>
<td>Intestine</td>
<td>140</td>
<td>16</td>
<td>11.42</td>
</tr>
<tr>
<td>Cases of avian influenza</td>
<td>Cloacal swabs</td>
<td>60</td>
<td>10</td>
<td>16.66</td>
</tr>
<tr>
<td>Abattoir</td>
<td>Intestines</td>
<td>200</td>
<td>14</td>
<td>7</td>
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<tr>
<td>Sample no.</td>
<td>Type of chicken</td>
<td>Clinical Signs</td>
<td>Genotype of isolate</td>
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<tr>
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<td>------------------------</td>
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</tr>
<tr>
<td>1</td>
<td>Layer</td>
<td>Yellowish diarrhea</td>
<td>A(CPe⁺)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Broiler</td>
<td>Diarrhea</td>
<td>A(CPe⁺)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Layer</td>
<td>whitish diarrhea</td>
<td>A(CPe⁺)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Layer</td>
<td>whitish diarrhea</td>
<td>A(CPe⁺)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Layer</td>
<td>whitish diarrhea</td>
<td>A(CPe⁺)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Layer</td>
<td>Diarrhea</td>
<td>A(CPe⁺)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Layer</td>
<td>Diarrhea</td>
<td>A(CPe⁺)</td>
<td></td>
</tr>
</tbody>
</table>

**Table (5)**

*Clostridium. perfringens* isolated from cases of Avian influenza (cloacal swabs)
3.1 Characteristics of isolates:

3.1.1 Colonial morphology:

The organisms produced circular colonies about 2mm. Large, with glistening or finely granular surfaces with entire edges. Some strains developed dull and granular colonies with radial surfaces and dentated edges.

3.1.2 Gram’s stain:

All (100%) isolates were Gram-positive rod when freshly examined. Old cultures possessed less affinity to retaining the crystal violet (fig 2).

3.1.3 Catalase test:

All (100%) isolates of *C. perfringens* were catalase negative.

3.1.4 Motility test:

All (100%) isolates were non motile by both hanging drop and L.M agar tests.
3.1.5 Haemolysis test:
All (100%) isolates were haemolytic for sheep blood cells. They developed double zone of haemolysis, colonies were surrounded by inner zone of complete haemolysis and wide zone of incomplete haemolysis (fig 3).

3.1.6 Growth in RCM:
All (100%) isolates propagated in RCM with production of turbidity and gas (fig 4).

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

3.1.8 Growth on blood agar:
All (100%) isolates developed colonies on sheep blood agar with different shapes and sizes.

3.1.9 Growth in cooked meat medium:
All (100%) produced turbidity and gas.

3.1.10 Aerobic growth:
None of the isolates grew on blood agar when incubated aerobically at 37°C for 24 hours.

3.1.11 Anaerobic growth:
All (100%) isolates grew on blood agar under anaerobic conditions.

3.1.12 Lactose fermentation:
All (100%) isolates changed the colour of LM agar from red to yellow and produced gas due to lactose fermentation (fig 5).

3.1.13 Naglar’s reaction:
All (100%) isolates caused opalescence of egg yolk agar and Shahidi and Ferguson perfringens agar (fig 6).

3.1.14 Naglar's half-plate antitoxin:
Inhibition of lecithinase production by \textit{C.\textit{perfringens}} type A antitoxin was noted by \textit{C.\textit{perfringens}}, some isolates showed complete inhibition and others incomplete inhibition.

3.1.15 Gelatin test:
All (100%) isolates liquefied the gelatin.

3.2 Result of PCR:
All 40 isolates of \textit{C.\textit{perfringens}} were analysed using multiplex PCR. Alpha toxin gene cpa and enterotoxin gene were observed in agarose gel as bands at approximately 324bp and 233bp respectively, as shown in Fig (7).

This result showed that all isolate \textit{C.\textit{perfringens}} belonged to toxin type A and were enterotoxin +ve (C\textit{Pe} + typeA). No bands were seen for iota-epsilon-beta-and beta\textsubscript{2} toxin genes.

3.3 Results of sensitivity tests
The concentration of antimicrobial drugs used, and standard zones of inhibition produced by susceptible, intermediate, and resistant \textit{Clostridium perfringens} isolates are shown in table (6). The diameter of growth inhibition zones including those of the drug disks were measured and are shown in Table (7).
Thirty of forty isolates were tested for their susceptibility to different antimicrobial agents. All isolates were sensitive to ampicillin and furazolidone and resistant to gentamycin and kanamycin. Ninety percent of isolates (90%) were susceptible to penicillin while, 80% to cloxacillin, 73% to bacitracin, 70% erythromycin, and 50% lincomycin (Fig. 8, 9, 10, 11 and 12 respectively).

Fig.13 showed the susceptibility of *C. perfringens* to 9 antimicrobial drugs.

Fifteen different patterns of drug sensitivity test were obtained (sensitive, intermediate and resistant) of 30 *C. perfringens* isolates to nine antimicrobial drugs as shown in Table (8).

### Table (6)
**Antimicrobial agents used, Concentrations and the standard zones of growth inhibition of resistant intermediate and susceptible *Clostridium perfringens*.**

<table>
<thead>
<tr>
<th>Name of Antimicrobial</th>
<th>Conc.</th>
<th>Standard zones of growth inhibition in mm</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Resistant</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>10mcg</td>
<td>&lt; 12</td>
</tr>
<tr>
<td>Bacitracin</td>
<td>10units</td>
<td>&lt; 9</td>
</tr>
<tr>
<td>Penicillin</td>
<td>10units</td>
<td>&lt; 12</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>30mcg</td>
<td>&lt; 14</td>
</tr>
<tr>
<td>Furazolidone</td>
<td>100mcg</td>
<td>&lt; 15</td>
</tr>
<tr>
<td>Cloxacillin</td>
<td>5mcg</td>
<td>&lt; 12</td>
</tr>
<tr>
<td>Lincomycin</td>
<td>10mcg</td>
<td>&lt; 17</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>30mcg</td>
<td>&lt; 13</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>Mcg</td>
<td>&lt; 14</td>
</tr>
</tbody>
</table>
Table (7)
The diameter (mm) of growth inhibition zones of nine antimicrobial agents.

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>Amp</th>
<th>Bac</th>
<th>Pen</th>
<th>Ery</th>
<th>Fur</th>
<th>Clo</th>
<th>Lin</th>
<th>Gen</th>
<th>Kan</th>
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<tbody>
<tr>
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<td>22</td>
<td>19</td>
<td>22</td>
<td>18</td>
<td>20</td>
<td>16</td>
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<td>32</td>
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<td>23</td>
<td>15</td>
<td>23</td>
<td>13</td>
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</table>
Table (8)
Sensitivity patterns of *C. perfringens* isolate

<table>
<thead>
<tr>
<th>Sensitivity pattern</th>
<th>No. of isolates</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitive to all 9 antibiotics except gentamycin and Kanamycin</td>
<td>10</td>
<td>33.3%</td>
</tr>
<tr>
<td>Sensitive to ampicillin, penicillin, furazolidone, and cloxacillin and resistant to the lincomycin, gentamycin and a kanamycin.</td>
<td>2</td>
<td>6.7%</td>
</tr>
<tr>
<td>Resistant to gentamycin and kanamycin, intermediately resistant to bacitracin and sensitive to ampicillin, penicillin, erythromycin, furazolidone and cloxacillin and lincomycin.</td>
<td>1</td>
<td>3.3%</td>
</tr>
<tr>
<td>Sensitive to ampicillin, furazolidone, lincomycin, bacitracin, erythromycin, cloxacillin, and intermediately resistant to penicillin and fully resistant to gentamycin and kanamycin.</td>
<td>1</td>
<td>3.3%</td>
</tr>
<tr>
<td>Sensitive to ampicillin, bacitracin and furazolidone, resistant to cloxacillin and sensitive to ampicillin, penicillin, erythromycin, furazolidone, bacitracin and lincomycin.</td>
<td>1</td>
<td>3.3%</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Sensitive to ampicillin, bacitracin and furazolidone, and intermediately resistant to penicillin and erythromycin, resistant to cloxacillin, lincomycin, gentamycin and kanamycin.</td>
<td>1</td>
<td>3.3%</td>
</tr>
<tr>
<td>Sensitive to ampicillin, penicillin, erythromycin, furazolidone, bacitracin and cloxacillin and resistant to lincomycin, gentamycin and kanamycin.</td>
<td>3</td>
<td>10.0%</td>
</tr>
<tr>
<td>Sensitive to ampicillin, penicillin, furazolidone, bacitracin, erythromycin and cloxacillin, and intermediately resistant for lincomycin and resistant to gentamycin and kanamycin.</td>
<td>1</td>
<td>3.3%</td>
</tr>
<tr>
<td>Sensitive to ampicillin, penicillin, furazolidone, cloxacillin and lincomycin and resistant to erythromycin, bacitracin, gentamycin and kanamycin.</td>
<td>1</td>
<td>3.3%</td>
</tr>
<tr>
<td>Sensitive to ampicillin, penicillin, furazolidone and cloxacillin and intermediate for bacitracin, resistant to erythromycin, lincomycin, gentamycin kanamycin.</td>
<td>2</td>
<td>6.7%</td>
</tr>
</tbody>
</table>

| Sensitive to ampicillin, penicillin, furazolidone, bacitracin, cloxacillin and intermediately resistant to erythromycin and resistant to lincomycin, gentamycin and kanamycin | 2 | 6.7% |
| Sensitive to ampicillin, penicillin, furazolidone, erythromycin and cloxacillin and intermediately resistant to bacitracin, resistant to lincomycin, gentamycin and kanamycin | 1 | 3.3% |
| Sensitive to ampicillin, penicillin, furazolidone and erythromycin, resistant to cloxacillin, bacitracin, lincomycin, gentamycin and kanamycin. | 2 | 6.7% |
| Sensitive to ampicillin, furazolidone, erythromycin and bacitracin,intermediately resistant to penicillin resistant to cloxacillin, lincomycin, gentamycin and kanamycin. | 1 | 3.3% |
| Sensitive to ampicillin, penicillin, furazolidone, bacitracin, erythromycin and lincomycin and resistant to cloxacillin, gentamycin and kanamycin | 1 | 3.3% |
Fig. 1. Chicken affected with necrotic enteritis.
The intestines were hemorrhagic with gases.
Fig. 2. Gram stain of a smear from colonies of *C. perfringens*.
**Fig.3.** Growth of *C. perfringens* on blood agar, showing double zones of haemolysis.
**Fig.4.** Growth of *C. perfringens* in RCM medium with turbidity and gas production
Fig. 5. Lactose fermentation by *C. perfringens* and change of colour from red to yellow.
Fig. 6. Growth of *C. perfringens* on egg yolk medium showing Lecithinase production (opacity).
**Fig. 7.** Agarose gel-electrophoresis of the PCR products obtained from amplification of cpa (324 bp) and cpe (233bp) of different *C. perfringens* isolates. Lane 1: 100bp ladder; lane 2: negative control; lanes 3-8: *C. perfringens* isolates.
Fig. 8. Sensitivity of *C. perfringens* to penicillin
Fig. 9. Sensitivity of *C. perfringens* to cloxacillin
Fig. 10. Sensitivity of *C. Perfringens* to erythromycin
Fig. 11. Sensitivity of *C. Perfringens* to bacitracin.
Fig. 12. Sensitivity of *C. perfringens* to lincomycin.
Fig. 13. Sensitivity of *C. perfringens* to different antimicrobials agents.
In this study 40 isolates of *C. perfringens* were recoverd from 400 samples (10%). Intestinal specimens were taken from cases showing lesions of necrotic enteritis. Olumbunmi and Taylor (1985) stated that cultures of lesion material could frequently yield heavy growth of *C. perfringens*.

In severe cases, lesions are longitudinally expanded along the gut; the epithelium is eroded and detached. Gram-positive rods occur in the lesions, (Nairn and Bamford, 1967; Kaldhusdal and Hofshagen, 1992). Gardiner (1967) and Bains (1968) mentioned that *C. perfringens* was considered to be the only bacterium of significance isolated from lesions of necrotic enteritis or seen in smears from intestinal lesions.

Cloacal swabs were taken from chickens suffering from avian influenza and the obvious clinical signs were diarrhoea. This is in agreement with Helmboldt and Bryant (1971) who reported that diarrhoea might be associated with acute necrotic enteritis.

In the present work, *C. perfringens* was isolated from both broilers and layers of different ages. Craven *et al.* (2001) stated that the disease occurred most commonly in broiler chicks but was also observed in older pullets and layers also Ficken and Wages, (1997) mentioned that *C. perfringens* could be isolated in chickens of all ages.

One or several predisposing factors may be required to elicit the clinical signs and lesions of necrotic enteritis. Gut damage caused by coccidial infection some times predisposes birds to clostridial infection and necrotic enteritis (Hermans and Morgan, 2003), also Van immerseel *et al.* (2004)
mentioned that coccidial infection stimulated proliferation of intestinal \textit{C.perfringens}.

In the field, coccidial pathogens are mostly found associated outbreaks of necrotic enteritis (Broussard \textit{et al}., 1986). It is documented in numerous studies that coccidial vaccines and coccidiostatic drugs are able to prevent \textit{C.perfringens} associated necrotic enteritis (Williams \textit{et al}., 2003) and removal of coccidiostat from poultry feed is considered as a predisposing factor for the development of necrotic enteritis (Elwinger \textit{et al}., 1992). The finding of this study confirmed the above mentioned statement. Six isolates were found positive for coccidiosis beside necrotic enteritis.

Also low immunity due to infection with viral diseases predisposes chickens to necrotic enteritis. In this study one isolate of \textit{C.perfringens} was from chickens suffered from Gumboro, 3 isolates were from chickens with Marek's disease and 10 isolates were from cases of avian influenza. This results is in accordance with the finding of Schuring and Van Gils (2001) who stated that exposure to infectious bursal disease, chicken infectious anemia virus and Marek's disease as well as non specific stress, may predispose birds to necrotic enteritis.

The composition of feed may greatly influence the occurrence of necrotic enteritis in chickens. Different reports showed that maize in chicken feed reduces the incidence and severity of necrotic enteritis, as opposed to diets based on wheat, rye and barley (Branton \textit{et al}., 1987; Kaldhusdal and Hofshagen, 1992; Riddell and Kong, 1992). When broiler chickens were feed \textit{C.perfringens} in different feeds for 3 consecutive days, mortality ranged from 0.0 to 12.5% in broilers fed a maize-based diet, while the mortality in animals fed a diet with high amounts of wheat, rye or barley ranged from 26% to 35% (Riddell and Kong, 1992).
High protein content in poultry feed seems to increase the incidence of necrotic enteritis. An increased risk of necrotic enteritis is often associated with animal protein ingredients such as fishmeal or meat and bone meal (Kocher, 2003). Studies of the effects of vegetable protein ingredients, such as soy protein, on the incidence of necrotic enteritis in vivo are lacking (Van Immerseel et al., 2004).

In this study it can be concluded that all isolates of *C. perfringens* found in the intestines carried only the cpa gene and cpe gene, judging from the multiplex PCR, showing that all belonged to toxin type A and this is in agreement with the finding of other authors who established that all *C. perfringens* isolates from chickens to be type A (Hofshagen and Stenwig, 1992., Songer, 1996., Yoo et al., 1997). Engstrom et al. (2003) mentioned that in poultry, almost exclusively *C. perfringens* type A has been isolated, as shown in studies in Scandinavia. Van Immerseel et al. (2004) stated that toxinotypes B, D and E do not play a role in poultry diseases. The presence of the beta2 toxin in *C. perfringens* isolates has been linked with increased incidence of bovine enterotoxaemia (Manteca et al., 2002), intestinal disorders in horses (Herholz et al., 1999), and diarrhea in piglets (Klaasen et al., 1999) and sheep (Garmory et al., 2000). In all these studies, a significantly higher incidence of beta2 toxin positive isolates was detected in animals having intestinal disease. This seems to be in contrast with *C. perfringens* isolates from poultry. In a study by Engstrom et al. (2003) only one *C. perfringens* PFGE types harboured the beta2 toxin.

*Clostridium perfringens* type A bacteria produces the alpha toxin (Titeball, 1993). Kana Karaj (1998) stated that cpa was present in all the strains of *C. perfringens*, making it useful as a target for DNA-based assays to detect the organism. The \( \alpha \)-toxin produced by *C. perfringens* is considered
to be the major virulence factor in the pathogenesis of necrotic enteritis. (AL-Sheikhly and Truscott, 1977), and it is by far the most potent in poultry (Kohler, 2002).

Alpha toxin from *C. perfringens* was the first bacterial protein shown to possess both enzymatic and toxic properties, it is a phospholipase C and exhibits hemolytic, necrotic vascular permeabilization, and platelet aggregating properties (Justin *et al.*, 2002).

Some *C. perfringens* strains form additional toxins that have been proposed to be important for the pathogenesis of intestinal disorders (Baums *et al.*, 2004). Some type A strains produce enterotoxin (CPE) which causes diarrhoea in humans and, most likely in various domestic animals (McClane, 1996., Songer, 1996., Sarker *et al.*, 1999) a result which substantiated by this work. Also McClane *et al.*, 1988 mentioned that CPE production is most often reported in strains of type A, but it can be produced by all of the toxin types of *C. perfringens*.

The CPE appears to be a bifunctional toxin, which first induces cellular damage via its cytotoxic activity, resulting from plasma membrane permeability alterations caused by formation of CPE – containing complex that may corresponds to a pore. CPE then interacts with structural components of the epithelial tight junction, including certain claudins and occludin. These interaction can affect tight junction structure and function, thereby altering paracellular permeability (Singh *et al* .,2000, 2001, McClane, 2000, 2001).

Craven *et al.*, (1999) illustrated that enterotoxigenic strains of *C. perfringens* have been isolated from outbreaks of necrotic enteritis in chickens and enterotoxin could be detected in the jejunum of experimentally
infected chickens, however, the superficial lesions in the mucosa of jejunum caused by enterotoxin might favour growth of *C. perfringens* and production of $\alpha$-toxin (Engstrom *et al.*, 2003).

When a nested PCR reaction was used after enrichment culture of intestinal contents of poultry, Miwa *et al.* (1997) found 40% of all tested intestinal contents samples were positive for the cpe gene, whereas 100% of *C. perfringens* isolates were positive in this study.

The results obtained above disagree with Tschirdewahn *et al.* (1992) who stated that most isolates of *C. perfringens* from animals do not carry the enterotoxin gene and he mentioned that in a study of the presence of enterotoxigenic strains of *C. perfringens* in faeces of various animals, only 10% of *C. perfringens* isolated from poultry carried the enterotoxin gene.

The genotype of cpa (alpha toxin or phospholipase C) with cpe (*Clostridium perfringens* enterotoxin) is generally associated with human food-borne disease strains. Human disease – associated food isolates of *C. perfringens* generally have a chromosomally located cpe gene, while the cpe locus of non – food borne isolates is episomally or plasmid located (Sparks *et al.*, 2001).

*Clostridium perfringens* is one of the most frequently isolated bacterial pathogens in food borne disease outbreaks in humans, after some other pathogens such as *Campylobacter* and *Salmonella* (Buzby and Roberts, 1997).

Outbreaks due to *C. perfringens* can be traced back to different sources, one of which is poultry (Schiemann, 1977., Regan *et al.* 1995., Hook *et al.*, 1996).

Miwa *et al.*, (1997, 1998) stated that chicken, porcine and bovine intestinal samples and meat were analyzed for the presence of CPE positive
strains. The amount of CPE positive samples in both the intestinal contents
and meat of chickens was higher than for cattle and pig samples.

In this study we tried to get a broad idea on the susceptibility of *C. perfringens* to different antimicrobial agents vis: ampicillin, penicillin, furazolidone, cloxacillin, bacitracin, erythromycin, lincomycin, gentamycin and kanamycin.

*Clostridium perfringens* was highly susceptible to ampicillin and furazolidone (100%), followed by penicillin (90%), cloxacillin (80%), erythromycin (73%), bacitracin (70%) and the lower susceptibility was recorded for lincomycin (50%).

All isolates of *C. perfringens* were resistant to Kanamycin and Gentamycin. Rodriguez *et al.* (1980) who tested The sensitivity of *E. coli*, *Staph.aureus* and *C. perfringens* to various drugs under different conditions (including different culture media and incubation atmospheres) found that Gentamicin and kanamycin showed no activity in tests with brain heart infusion agar incubated anaerobically or in 10% CO2.

Experimentally, in vivo, a number of antimicrobial agents placed in the feed reduced the numbers of *C. perfringens* shed in the feces (Stutz *et al.*, 1983). These include virginiamycine, tylosin, penicillin, ampicillin, bacitracin, and furazolidone (Watkins *et al.*, 1981). Outbreaks of necrotic enteritis can be treated effectively by administration of lincomycin (Humdy *et al.*, 1983), bacitracin (Prescott *et al.*, 1971) or Penicillin (Long and Trescott, 1976).

Long (1973) mentioned that enterotoxemia caused by *C.perfringens* responded readily to antibiotics such as penicillin or bacitracin to which virtually all isolates of *C.perfringens* were susceptible in vitro. Also Rood *et al.* (1978) studied the effect of penicillin on *C.perfringens* isolates obtained
from different animals and different sources. He found that penicillin was the most effective antibiotic against *C. perfringens* and recommended that penicillin should be used in the treatment of *C. perfringens* infections.

The study of Sasaki *et al.* (2001) and the present study suggest that *C. perfringens* had a low degree of resistance to ampicillin.

The result obtained in this work disagree with the finding of Heller and Smith (1973) who reported the resistance of *C. perfringens* to furazolidone, whereas in this study *C. perfringens* was highly susceptible (100%) to furazolidone.

Martel *et al.* (2004) stated that all poultry isolates of *C. perfringens* in 1980 were susceptible to erythromycin and this is in agreement with our finding, as in this study 73% susceptibility was obtained to erythromycin. But Smart, Truman and Stringer, (1983) found *C. perfringens* recovered from beef carcasses to be resistant to erythromycin.

In Sudan Abdel Salam (1986) studied the sensitivity of 7 strains of *C. perfringens* from cases of necrotic enteritis, he found high susceptibility to bacitracin.
**Conclusion:**

1. We can conclude that DNA based techniques (PCR) have been developed for *C.perfringens* typing. Genotyping is reliable and practical and allows more accurate and complete determination of *C.perfringens* pathovars than doe’s classical toxinotyping.
2. Bioassays may not be sensitive enough to detect the presence of enterotoxigenic strains in normal animals. The possible use of PCR for detection *C.perfringens* producing enterotoxin may, therefor, offer a considerable advantage over conventional techniques.
3. The availability of a quick method to define exactly the toxin type of *C. perfringens* should shorten and simplify the development of adequate vaccines fitting the epidemiological situation.

**Recommendation:**

This study warrants further studies on the susceptibility of more strains of *C. perfringens*
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Hemorrhagic gastroenteritis in a dog with isolation of Clostridium

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