3. MATERIAL AND METHODS

3.1. Material

3.1.1. Samples:

Nineteen rabbits farms located in eight Egyptian governorates representing Port-Said, Giza, Cairo, Beni Suef, Fayoum, El-Qaliubiya, El-Sharkea and El-Menoufia were investigated to detect the presence of ***Clostridial*** infections in their weaned rabbits and environment. Three hundred and twenty nine weaned rabbits representing 95 apparently healthy, 204 diseased with signs of diarrhea, bloat, inappetance and ruffled fur and 30 freshly dead ones were examined. The total number of the examined samples from rabbits was 676 including 582 rectal swabs, 60 intestines and 34 livers. Moreover, 38 samples were collected from the farm's environment as 18 sample from water and 20 from feed. The total number of all animals and environmental samples were 714.

3.1.2. Media used for isolation and identification of *Clostridium* spp.:-

3.1.2.1. Liquid media:-

a- Robertson's cooked meat medium (Oxoid):

This medium was used to support the growth of pathogenic ***Clostridium*** spp. and to detect their ability to digest meat, as it provides a range of complex nutrients and suitable reducing conditions.

b- Brain heart infusion broth (Oxoid):

This medium was used to support growth of ***Clostridium*** spp.

c- Toxin production medium:

It was prepared according to (**Roberts et al. 1970)**. This medium was used for the production of ***C. perfringens*** exotoxins.

d- Muller Hinton broth (Oxoid):

It was used for testing the *in-vitro* sensitivity of ***Clostridium*** spp. against different antibiotics.

3.1.2.2. Solid media:

a- Sheep blood agar medium:

It was prepared according to the method described by **(Carter and Cole.1990)**. This medium was utilized for cultivation of C***lostridium*** organisms and detection of their hemolysis.

b- Neomycin sulphate sheep blood agar medium:

It was prepared according to **(Carter and Cole. 1990)**.

It was prepared for detection and purification of the ***Clostridial*** isolates.

c- Egg yolk agar medium:

It was prepared according to the methods stated by **(Cruickshank et al. 1975).** This medium was used for detection of lecithinase activity of the toxigenic ***Clostridial*** isolates.

d- Muller Hinton agar medium (Oxoid):

It was used for testing the *in-vitro* sensitivity of ***Clostridium*** spp. against different antibiotics.

3.1.2.3. Media used for biochemical tests:

a- Sugar fermentation medium:

It consisted of 1% peptone water containing 1% of testing sugar (mannitol, sucrose, lactose, maltose and glucose) and it was prepared to detect the ability of ***Clostridial*** organisms to ferment sugars.

b- Gelatin liquefaction medium (Oxoid):

It was used for detection of gelatin liquefaction activity of the tested ***Clostridial*** isolates.

c- Indole production medium (Oxoid):

It is used for testing the ability of ***Clostridial*** microorganisms to catabolize tryptophane amino acids to indole.

d- Christensen's urea agar slant medium (Oxoid):

It was used to detect urease activity of suspected ***Clostridial*** isolates.

3.1.3. Indicators and reagents:

a- Andrade's indictor:

It was prepared by adding 1% sodium hydroxide (NaOH) to 0.5% solution of acids fuchsin until the color just becomes yellow. It was used at final concentration of 1% in the medium to determine the fermentation reaction of the tested ***Clostridial*** isolates.

b- Kovac's reagent :

It was used to detect the production of indole by ***Clostridial*** organisms.

c- Hydrogen peroxide:

Freshly prepared solution of hydrogen peroxide (H_2O_2) 3% was used for testing the catalase activity of ***Clostridial*** isolates. It should be

stored in dark bottle, avoid exposure to light and kept in refrigerator all times when it was not used.

3.1.4. Stains:

a- Gram's stain was used routinely for microscopical identification of ***Clostridial*** organisms.

b- Haemotoxylin and eosin were used for histopathological examination of organs collected from ***Clostridial*** micro-organisms naturally and experimentally infected rabbits.

3.1.5. Antibiotics:

a- Neomycin sulphate:

It was used as 700.000 units / gram and added to the sheep blood agar plates for inhibition of all organisms other than ***C. perfringens*** during its isolation.

b- Antibacterial sensitivity discs (Oxoid):

Discs containing antimicrobials with different concentrations that used for testing the sensitivity of ***Clostridial*** strains *in-vitro*.

3.1.6. Solutions:

a- Physiological saline solution (0.85% sodium chloride):

It was sterilized by autoclaving at 121°C for 15 min. and used in typing of ***C. perfringens*** as it was intravenously inoculated in negative control mice in serum neutralization test.

b- Trypsin:

It was used in a concentration of 0.1% for activation of epsilon and iota toxins of ***C. perfringens***.

c- Glucose solution (60%) in distilled water:

It was used for the preparation of toxin production medium for detection of ***Clostridial*** toxins.

d- Sodium hydroxide solution (5N):

It was used to adjust the pH of toxin production medium for detection of ***Clostridial*** toxins.

3.1.7. Apparatus for anaerobiosis (Oxoid and PPL):

Gas packs anaerobic jar and Gas pack anaerobic kits were used for production of anaerobic condition which is important for ***Clostridium*** spp. growth.

3.1.8. Diagnostic antitoxins:

Diagnostic ***C. perfringens*** antisera types (A, B, C, D and E) as well as antitoxins (alpha, beta, epsilon and iota) were obtained from Veterinary Serum and Vaccine Research Institute (Abbassia, Egypt). They were used in serological identification of ***C. perfringens***.

3.1.9. Animals used for *C. perfringens* typing and experimental infection:

3.1.9.1. Laboratory animals

3.1.9.1.1. Albino Guinea pigs:

Local breeds of 65 albino Guinea pigs about 6-8 weeks old and weighing 350-450 gm were used for dermonecrotic reaction as well as typing of ***C. perfringens*** isolates.

3.1.9.1.2. Swiss mice:

A total 200 Swiss mice with an average weight 15-25 gm were used for the determination of toxigencity and typing of ***C. perfringens*** isolates.

3.1.9.2. Rabbits:

Four to six weeks old, sixty five Newzeland newly weaned rabbits of mixed sex that were obtained from a commercial rabbit's farm were used for experimental infection using the most prevalent isolated ***Clostridial*** strains. They were kept under complete daily observation

for one week for adaptation and detection of any abnormal signs or mortalities before they were inoculated with ***Clostridial*** organisms.

3.1.10. Ration:

3.1.10.1. A commercial balanced pelleted rabbit's ration without feed additives was used to feed the purchased rabbits during the period of the experiment.

3.1.10.2. A commercial balanced pelleted laboratory animal's ration was used to feed the purchased Guinea pigs and mice during the period of the experiment.

3.1.11. Vaccines:

3.1.11.1: Rabbit's viral haemorrhagic septicaemia vaccine:

It was obtained from Vaccine and Antiserum Research Institute (Abbassia, Egypt), with batch No. 32. It was used for 2 months old rabbits at a dose of 0.5 ml/rabbit and it was given subcutaneously at the back of the neck.

3.1.11.2: Formalized polyvalent rabbit's pasteurellosis vaccine:

It was obtained from Vaccine and Antiserum Research Institute, (Abbassia, Egypt), with batch No. 28. It was used in a priming dose of 1 ml/animal for 2 months aged rabbits and boostered with a dose of 2 ml/animal for 4 months aged rabbits. Both doses were given subcutaneously at the back of the neck.

3.1.12. Materials and equipment used for histopathological examination.

10% formol saline, Serial dilutions of alcohol (methyl, ethyl and absolute ethyl), Xylene, Paraffin, Hematoxylin and eosin stains, Slidge microtome, Electric light microscope and Glass slides.

3.1.13. Materials used for polymerase chain reaction (PCR)

3.1.13.1. Buffers and reagents used for conventional PCR:

1) Tris acetic EDTA (TAE) 5x:

It was used for DNA extraction and as a buffer for PCR products visualization in agarose.

2) 10 X buffer (Gibco/BRL, Grand Island, N. Y.).

3) $MgCl_2$ (2.5 mM) (Applied biosystems PCR mix, USA, Catalogue No. C07954).

4) dNTPs (10x) (10 mM) (Gibco).

5) *Taq* thermostable DNA polymerase (Biometra) (2 Units).

6) Template DNA (*C. perfringens* alpha toxin genomes).

7) Oligonucleotide primers (100 pmol).

Primer design used for detection of alpha toxin gene of ***C. perfringens*** (**Augustynowicz *et al.*, 2000**) is shown in **Table (1).**

Table (1): Primer of alpha toxin gene of *C. perfringens* used in conventional PCR

Primer name and direction	Nucleotide sequence
***cpa*: Forward**	GCTAATGTTACTGCCGTTGA
Reverse	CCTCTGATACATCGTGTAAG`

Cpa*: *C. perfringens alpha toxin

8) Paraffin oil:

Light mineral oil was used to cover the reaction to prevent evaporation.

3.1.13.2. Buffers and reagents used for multiplex PCR:

1) Tris acetic EDTA (TAE):

It was used for DNA extraction and as a buffer for PCR products visualization in agarose.

2) 10 X buffer (Gibco/BRL, Grand Island, N. Y.).

3) MgCl2 (2.5 mM) (Applied biosystems PCR mix, USA, Catalogue NO. C07954).

4) dNTPs (10x) (10mM) (Gibco).

5) Taq thermostable DNA polymerase (Biometra) (2U).

6) Template DNA (C. perfringens alpha and epsilon toxin genomes).

7) Oligonucleotide primers (100 pmol).

Primers design used for detection of the four toxin genes (alpha, beta, epsilon and iota) of ***C. perfringens*** (**Augustynowicz *et al.*, 2000**) is shown in **Table (2)**.

8) Paraffin oil:

Light mineral oil was used to cover the reaction to prevent evaporation**.**

Table (2): Primers for the four toxins genes of *C. perfringens* used in multiplex PCR

Primer name and direction		Nucleotide sequence
***cpa*:**	**Forward**	GCTAATGTTACTGCCGTTGA
	Reverse	CCTCTGATACATCGTGTAAG`
***cpb*:**	**Forward**	GCGAATATGCTGAATCATCTA
	Reverse	GCAGGAACATTAGTATATCTTC`
***etx*:**	**Forward**	GCGGTGATATCCATCTATTC
	Reverse	CCACTTACTTGTCCTACTAAC
***iA*:**	**Forward**	ACTACTCTCAGACAAGACAG
	Reverse	CTTTCCTTCTATTACTATACG`

Cpa*: *C. perfringens alpha toxin**, *Cpb*: *C. perfringens*** beta toxin**,**
etx*: *C. perfringens epsilon toxin**, *iA*: *C. perfringens*** iota toxin

3.1.13.3. Agarose gel electrophoresis buffers and reagents (Sambrook *et al.*, 1989):

1) Agarose gel (1.5%)

Agarose powder	0.6 g
TBE	40 ml

2) Tris acetic EDTA.

3) Tris boric EDTA.

4) Ethedium bromide solution [Stock solution]:

It was added to melted agarose to reach a final concentration of 0.1 - 0.5 μg/ml. It was used as a fluorescent dye to stain the DNA during examination by ultra violet (UV) transilluminator.

5) Gel loading buffer (Sample buffer) (6X stock):

Bromophenol blue	0.25 %
Xylene cyanol	0.25 %
Glycerol	30.0 %

Components were dissolved in sterile distilled water and stored covered with aluminum foil at room temperature.

6) DNA ladder (100 bp, Pharmacia, Catalogue number 27-4001-01, USA).

3.1.13.3. Apparatus and equipment used for PCR:

1) Thermocycler

2) Power supply (BIO RAD, Power PAC 1000).

3) Ultra violet transillumination (Spectroylyne Model TR-312 A).

4) Safety hood (CN-Vilber Burmat, France).

5) Heat block (Technie).

6) Mini slab horizontal electrophoresis tank (Submarine mini cell gel system E-C model EC370 - BIO-RAD, USA).

7) Microwave (Sharp, Japan).

8) Digital camera (Canon, Model A530, Japan).

9) Laminar air flow (AUAIR).

10) Automatic micropipettes (Jencon, England) (10, 20, 200, 1000 µl).

11) Micropipette tips (White, yellow and blue).

12) Glass wares.

13) Eppendorf tubes (200, 500, 1000µl).

14) Ultra-violet spectrophotometer

3.1.14. Others chemicals and instruments:

- **Sterile cotton swabs:** They were used for collection of rectal swabs.
- **Glycerin:** It was used for long time preservation of the ***Clostridial*** strains (more than one year).
- **Glass wares:**
- Screw capped test tubes.
- Cylinders (50, 100, 500) ml. and flasks (100, 250, 500 and 1000) ml.
- Glass slides: They were used for preparation of bacterial films and detection of catalase test positive reaction.
- Test tubes: They were used in biochemical reactions.
- Pipettes of different sizes.
- Petri dishes of different sizes.

3.1.15. Equipment:

- Laminar air flow (Ps.3A)
- Electric shaker (Ps.3A).
- Automatic micropipettes.
- Water bath (TGL).
- Incubator (TITANOX).
- Autoclave (Raypa).
- Antibiotic discs dispenser.
- Deep freezer (Toshiba).
- Electronic balance (Ps.3A).
- Refrigerator (Toshiba).
- Hot air oven (Ps.3A).

3.2. Methods

3.2.1. Selection of weaned rabbits for sampling:-

Clinical examination of apparently healthy and diseased weaned rabbits was done according to **Merck Veterinary Manual (2006)** as:

- Visual examination:

  Rabbits were visually inspected for general body configuration and presence of any external abnormalities or lesions.

- Physical examination:

  Palpation of the rabbit's abdomens was carried out carefully for detection of any external abnormalities as presence of abnormal content or excessive gases.

3.2.2. Sampling (Cruickshank *et al.*, 1975):

Nearly equal two parts from the liver and intestine as well as two rectal swabs were collected from apparent healthy, diseased or freshly dead weaned rabbits in separate sterile bags with serial numbers corresponding to each flock. Two feed and two water samples were collected in identified and labeled sterile plastic cups with a serial number corresponding to each flock. All samples were rapidly transferred to the laboratory on ice for bacteriological examination.

3.2.3. Bacteriological examination.

a- Isolation of *Clostridium* spp. (Smith and Holdman, 1968):

Each sample was transferred aseptically into two separate sterile test tubes containing cooked meat media. The media were previously heated in boiling water bath for 10 min. to drive off any dissolved oxygen and then rapidly cooled in a cold bath just prior to their inoculation with the samples. Immediate inoculation of samples was done to ensure that cultures were placed under anaerobic conditions.

One of the inoculated tubes was heated at 80º C for 10 min. in a water bath with a depth of water more than the level of the tube content to eliminate non spore forming aerobes and allow heat resistant spore former ***Clostridium*** spp. to grow, while the other tube was left unheated.

Both heated and unheated inoculated tubes were incubated at 37º C for 48 hrs. under anaerobic conditions (Gas Pack Jar).

A loopful from unheated tubes was then streaked on neomycin sulphate blood agar plate, while the other loopful was taken from heated culture and streaked onto 10% sheep blood agar plates.

All the inoculated plates were incubated anaerobically at 37ºC for 24-48 hrs. Sub-culturing of the identified culture was restored in cooked meat media and then kept in the refrigerator for purification and further identification.

b- Identification of *Clostridial* isolates:

1- Colonial morphology:

Suspected different ***Clostridial*** colonies were examined morphologically **(Vaikosen and Muller, 2001)**.

2- Microscopical examination:

Smears from suspected ***Clostridial*** colonies were stained with Gram's stain and examined microscopically for detection of morphological character of ***Clostridial*** microorganisms **(Cruickshank *et al.*, 1975)**.

3- Biochemical reactions:

Suspected purified ***Clostridial*** isolates were identified according to the schemes of **Koneman *et al.*, (1992**) and **Macfaddin, (2000)** using the following tests:

- **Catalase test:**

A loopful of 24 hrs. Culture was taken over a clear glass slide then a drop of 3% H_2O_2 was added to the portion of the colony. Positive result of the test was indicated by gas evolution and foaming of the reagent.

- **Sugar fermentation test:**

Peptone water 1% plus 1% Andrad's indicator was added to 1% of each of glucose, sucrose, lactose, mannitol and maltose. The prepared medium was sterilized by tyndallization. Each tube was inoculated with 1 ml of fluid culture of cooked meat media followed by incubation at 37°C for 24-48 hrs. under anaerobic condition. The positive result was any change of the tube color.

- **Gelatin liquefaction test:**

Stabbing of the pure culture of the organism was done into gelatin liquefaction medium, kept in the incubator at 37°C for 1-7 days under anaerobic condition and examined daily by putting in refrigerator for 15 min. Positive result was indicated by liquefaction of the gelatin medium.

- **Indole test:**

An inoculum was taken from the suspected culture and inoculated into treptophan or peptone broth. The culture medium was incubated at 37°C for 48 hrs. under anaerobic condition. Five drops of Kovac's reagent was added to each of the incubated tube and then the tube was shaken gently. Positive result was indicated within seconds as red ring at the interface of the medium in the lower portion, while negative result was indicated by cloudy ring that take the color of Kovac's reagent.

- **Lecithinase test:**

Egg yolk agar plates were inoculated with the isolated organisms and incubated anaerobically at 37º C for 24 hrs. The plates were examined

for opalescence (shinning) and formation of pearly of streaks. The growing organisms were divided into 2 groups; lecithinase positive and lecithinase negative ones.

- **Urease test:**

Christensen's urea agar slant media were inoculated with isolated organisms and incubated anaerobically at 37º C for 24 hrs. to detect urease activity of the tested isolates. Positive sample were indicated by changing of agar color into pink color.

- **Motility test:**

Stabbing of the pure culture of the organism was done into semi solid nutrient agar media and incubated anaerobically at 37º C for 24 hrs. Positive samples showed growth spread along and around the stabbing line.

- **Meat digestion:**

Pure culture was inoculated into cooked meat media and incubated anaerobically at 37º C for 24 hrs. Positive samples digested meat particles completely.

3.2.4. Histopathological examination:

Autopsy samples were taken from livers as well as small and large intestine of naturally infected weaned rabbits. Liver, small intestine, large intestine and kidneys were also collected from experimentally infected rabbits with ***Clostridial*** microorganisms at different groups. All these samples examined according to **(Banchroft *et al.*, 1996)**.

3.2.5. Determination of toxigenic strains of *Clostridium* spp. by intravenous inoculation in Swiss mice (Mariano *et al.*, 2007):

From freshly dead rabbits, intestine were collected and added to equal volume of saline then centrifugated at 3000 rpm/ 5 min. supernatant fluid were collected

and 0.1 ml injected directly into tail of Swiss mice using insulin syringe, in case of toxigenic stains, mice died shortly after inoculation.

3.2.5.1. Determination of pathogenicity of isolated *C. perfringens* by intravenous inoculation in Swiss mice (Mariano *et al.*, 2007):

- Addition of 1 ml of 60% glucose to 50 ml of toxin production medium was made to ensure anaerobiosis, and then the medium was inoculated with 5 ml of 24 hrs cooked meat cultures of toxigenic strains of ***C. perfringens*** and incubated at 37° C for 6 hrs. During the period of incubation, the pH was adjusted each hour to 7.2.

- After six hrs of incubation, half of the culture was centrifuged at 3000 rpm for 20 min.

- Half ml from the clear supernatant fluid was injected intravenously in the tail vain of each of Swiss mouse.

- The other half of the same culture was incubated at 37° C for 48 hrs anaerobically; the pH was adjusted to 7.5 twice daily and centrifuged at 3000 rpm for 20 min. The supernatant was trypsinized to a final concentration of 0.1% and then incubated at 37° C for an hour.

- From the clear trypsinized supernatant fluid, 0.1 ml was injected intravenously in Swiss mice.

Interpretation of the results:-

Mice were kept under observation for 48 hr.

If the mice died during 48 hr observation period, it considered a highly toxinogenic ***C. perfringens***:

If the mice still a life after 48 hr. observation period, It considered non- toxinogenic ***C. perfringens***.

3.2.5.2. Nagler's test by half antitoxin plate:

This test was carried out according to the method of **Smith and Holdman, (1968)**. It was done by spreading ***C. perfringens*** types A, B, C, D and E antitoxin separately on half of egg yolk agar plate and allowed to dry in incubator for half an hr. The suspected colonies were streaked across the plate starting from the half of plate without antitoxin and ending to side containing antitoxin. The plate was incubated anaerobically at 37º C for 24 hrs.

The release of alpha toxin that produced by all types of ***C. perfringens*** on lecithin was inhibited by the alpha antitoxin. In positive cases; opalescence should be clear on the side of the plate without antitoxin.

3.2.5.3. Typing of *C. perfringens* toxins by dermonecrotic test in albino Guinea pigs (Bullen, 1952):

I- Preparation of the toxins:

1) Addition of 1 ml of 60% glucose to 50 ml of toxin production medium was made to ensure anaerobiosis, and then the medium was inoculated with 5 ml of 24 hrs cooked meat cultures of toxigenic strains of ***C. perfringens*** and incubated at 37° C for 6 hrs. During the period of incubation, the pH was adjusted each hour to 7.2.

2) After six hrs of incubation, half of the culture was syphonized and centrifuged at 3000 rpm for 20 min. The clear supernatant fluid was divided into 4 portions.

3) From the 1st, 2nd, 3rd and 4th portion, 0.3 ml was neutralized with 0.1 ml of each type A, B, C antitoxins and saline, respectively.

4) The other half of the same culture was incubated at 37° C for 48 hrs anaerobically; the pH was adjusted to 7.5 twice daily and centrifuged at

3000 rpm for 20 min. The supernatant was trypsinized to a final concentration of 0.1% then incubated at 37° C for an hour and then neutralized by types D and E diagnostic antitoxins in the ratio of 3 toxin: 1 antitoxin.

II- Application of dermonecrotic test (Oakley and Warrack, 1953 and Quinn *et al.*, 2002):

1) The hair of the back and two sides of albino Guinea pigs was shaved carefully and marked longitudinally onto both sides.

2) On the right side, 0.1 ml of 6 hrs or trypsinized 48 hrs incubated supernatant fluid from each ***C. perfringens*** culture was inoculated intradermally and 0.2 ml from the neutralized one was injected at the left side by the same manner and arrangement.

3) The inoculated Guinea pigs were kept under observation for 24 hrs for any dermal reaction.

III- Interpretation of the results:

The results were interpreted by the color degree of the dermonecrotic reaction and its neutralization according to **Stern and Batty (1975)** as in **Table (3)**.

Table (3): Interpretation of the results of dermonecrotic test in albino Guinea pigs:

Type of *C. perfringens*	Expected dermonecrotic reactions produced by toxin
A	An irregular area of yellowish to greenish necrosis and the lesion tends to spread downward (alpha toxin).
B	Purplish yellow hemorrhagic necrosis (beta toxin).
C	The reaction will be intensively more purplish than that produced by type "B" filtrates.
D	A circular white necrosis which will be fully developed in 24 hrs (epsilon toxin). Few small areas of purplish hemorrhagic necrosis.
E	The reaction will be similar to that elicited by type "D" but with irregular outline and more marked purplish hemorrhagic appearance (iota toxin).
Non -toxigenic	No reaction at the site of injection.

3.2.5.4. Toxin antitoxin serum neutralization test (SNT) in Swiss mice (Smith and Holdman, 1968):

1. Specific ***Clostridial*** antiserum (0.1 ml) was added to 0.3 ml of centrifugated supernatant of toxin produced in toxin production media for types A, B and C.
2. Toxin production medium of ***C. perfringens*** types D and E was treated with 0.1% trypsin.
3. The toxin antitoxin mixture was kept at 37º C for 30 min.
4. About 0.2 ml of previously prepared mixture was inoculated intraveneously into a mouse tail's vein, while other 0.5 ml was

inoculated intraperitoneally in the other mouse and then both mice were kept under daily observation for 1-3 days.

5. If injected the mice were still life after 3 days, it was considered as positive results.

6. 0.1 ml from the supernatant fluid of toxin production medium without adding antiserum was inoculated in mouse as positive control, while 0.1 ml from sterile saline was inoculated into another mouse as negative control one.

3.2.6. Genotyping of *C. perfringens* using PCR:

3.2.6.1. DNA extraction and purification (Sheedy *et al.*, 2004):

A) Extraction of *C. perfringens* DNA:

Identified pure colonies of ***C. perfringens*** were grown over night in 5 ml brain heart infusion broth supplemented with 1% sodium thioglycolate at 37° C under anaerobic condition. One ml of the culture was centrifuged at 5000 xg for 15 min., the cell pellet was washed twice with 500µl TE and then resuspended in 200µl TE. The mixture was vortexed, heated directly at 100°C for 10 min. in a heat block for cell lysis and then cooled on refrigerator for 5 min. Finally the suspension was centrifuged at 13.000 xg for 2 min. and the supernatant fluid was used as a template DNA.

B) Purity assessment:

The concentration of DNA in µg /ml was measured at 260 and 280 nanometer (nm) by ultra-violet spectrophotometer and then the ratio of 260/280 was calculated. Pure DNA should have ratio of >1:8 that contamination with protein resulted in a significantly lower value. The DNA solution was kept at -20° C until used.

3.2.6.2. PCR amplification and cycling protocol:

A) For conventional PCR (Buogo *et al.*, 1995; Yamagishi *et al.*, 1997 and Tong and Labbe, 2003):

DNA samples were amplified in a total of 25 µl of the following reaction mixture: 5µl 10X buffer, 1 µl MgCl2, 1µl dNTPs, 0.5µl *Taq* polymerase, 0.25µl of the primer and 5µl template DNA that completed to 12 µl by DNase-RNase-free deionized water. Cycling program of PCR was performed in the thermal cycler as in **Table (4)**.

Table (4): PCR cycling protocol for alpha gene of *C. perfringens* using a conventional PCR.

Amplified DNA	Initial denaturation	Actual cycles	Final extension	Amplified product size (bp)
C. perfringens **(alpha) toxin gene**	94° C for 5 min.	35 cycles of : *Denaturation*: 94° C for 1 min. *Annealing*: 53° C for 1 min. *Extension:* 72° C for 1 min.	72° C for 5 min.	*cpa*: 324

B) For multiplex PCR (Augustynowicz *et al.*, 2000):

DNA samples were amplified in a total of 50 µl of the following reaction mixture: 5µl 10X buffer, 1.5µl MgCl2, 4µl dNTPs, 1µl *Taq* polymerase, 0.5µl of each primers and 5µl template DNA that completed to 50 µl by DNase-RNase-free deionized water. Cycling program of PCR was performed in the thermal cycler as in **Table (5)**.

Table (5): PCR cycling protocol for the four toxins genes of *C. perfringens* using a multiplex PCR.

Amplified DNA	Initial denaturation	Actual cycles	Final extension	Amplified product size (bp)
***C. perfringens* toxin genes (alpha, beta, epsilon and iota)**	94° C for 3 min.	30 cycles of : *Denaturation:* 94° C for 1 min. *Annealing:* 55° C for 1 min. *Extension:* 72° C for 1 min.	72° C for 5 min.	*cpa*: 324 *cpb*: 196 *etx*: 655 *iA*: 446

3.2.6.3. Detection of PCR products (Augustynowicz *et al.*, 2000):

Aliquots of amplified PCR products were mixed with gel loading buffer and electrophoresed in 1.5% agarose gel as in the following steps:

1) The prepared agarose was melted using microwave, left to cool till 55ºC, then ethidium bromide was added by 0.5 µg/ml and poured into the assembled horizontal gel tray after proper installation of the desired comb.

2) The gel was allowed to be solidified at room temperature.

3) Buffers of TBE or TAE that used for preparation of the gel were added into the tank to a level 1-2 mm above the gel layer.

4) The samples and a 100 bp DNA ladder (marker) were loaded in the wells, the tank was closed and the power supply was attached.

5) A current of 80 Volt for 1 hr. was passed on the mini horizontal electrophoresis unit and bromophenol blue was allowed to run 2/3 of the gel length before terminating the run.

6) Specific amplicons were observed under ultraviolet transillumination, compared with the marker and photographed by a digital camera.

3.2.7. Antibiotic sensitivity test:

The disc diffusion technique was applied according **National Committee For Clinical Laboratory Standards (NCCLS), (1998)** as follow:

(A) Preparation of standardized inoculum:

Pure colonies of ***C. perfringens*** were picked up from an overnight blood agar plate culture using sterile loop, inoculated into tubes containing 5 ml of Muller-Hinton broth and then incubated at 37º C for 2-8 hrs. anaerobically. The broth turbidity was matched with standard McFarland tube No. 0.5

(B) Inoculation of the plates:

By using sterile cotton swab, the dried surface of Muller-Hinton agar plate was streaked with ***C. perfringens*** culture and then the plate was allowed to dry for 15 min. in a flat undisturbed level before addition of the antibiotic discs.

(C) Placement of the antibiotic discs:

- Using the antibiotic disc dispenser, the antibiotic discs were distributed onto the dried agar surface. (Ensure complete contact of discs with the agar using sterile forceps).
- The discs were distributed evenly in a manner of minimum spacing of 24 mm between discs and 15 mm between the disc and the edge of the plate.
- The plates were incubated with discs anaerobically at 37 ºC for 18-24 hrs. till reading the result.

(D) Reading and interpretation of the result:

- The degree of the organism sensitivity is present in **Table (6)**. The organism sensitivity was determined by measuring the easily visible

clear zone of growth inhibition that produced by diffusion of the antibacterial agents.

Table (6): Interpretation of the inhibition zone standards of the used antibiotic discs (Oxoid).

Active principle	Disc concentration	Interpretative standards of zone diameter in mm		
		R (≤ mm)	I S (mm range)	S (≥ mm)
Amoxicillin / Clavulanic acid (2:1)	30 µg	19	14-17	20
Cloxacillin	5 µg	11	-	12
Erythromycin	15 µg	13	14-17	18
Gentamicin	10 µg	12	13-14	15
Oxytetracycline	30 µg	14	15-18	19
Penicillin G	10 µg	28	-	29
Sulphquinoxaline / Trimethoprim	25 µg	10	11-15	16
Kanamycine	30 µg	≤ 13	14 – 17	≥ 18
Enrofloxacin	5 µg	≤ 15	16 – 20	≥ 21
Doxycycline	30 µg	≤ 12	13 – 15	≥ 16
Ampicillin	10 µg	≤ 11	12 – 14	≥ 15
Colistine	10 µg	≤ 8	9 – 10	≥ 11
Lincomycine	15 µg	≤ 16	17 – 20	≥ 21
Tylosine	5 µg	11	-	12
Tetracycline	30 µg	≤ 14	15 – 18	≥ 19
Chlorotetracycline	30 µg	≤ 14	15 – 18	≥ 19
Nalidixic Acid	30 µg	≤ 13	14 – 18	≥19
Metronidazole	5 µg	11	-	12

R: Resistant IS: Intermediated sensitivity S: Sensitive

3.2.8. Preparation of the *Clostridium* spp. inocula used for experimental infection:

Species of ***C. perfringens*** and ***C. difficile*** inocula were prepared by plate count technique according to **Mackie and McCartny (1960)** as follow:

For oral route ***(C. perfringens*** and ***C. difficile)***;

- Concentration of the inoculum was $3x10^{10}$ colony forming unit (CFU).
- Dose of the inoculum was 2 ml/ each rabbit.

For subcutaneous (S/C) route ***(C. perfringens*** and ***C. difficile)***;

- Concentration of the inoculum was $1x10^{8}$ CFU.
- Dose of the inoculum was1 ml/ each rabbit.

3.2.9. Experimental infection of weaned rabbits with the most prevalent recovered toxigenic *Clostridium* spp. (Mostafa, 1992):

One hundred and twenty five newly weaned Newzeland rabbits of mixed sex that aged 5 weeks were obtained from a commercial farm and housed in thoroughly cleaned and disinfected battaries. Rectal swabs were collected from purchased rabbits at arrival as well as feed and water samples were examined to ensure their freedom of aerobic and anaerobic infections. One hundred and twenty rabbits were randomly divided into 6 equal groups; each containing 20 rabbits. Each group of 1, 2, 3, 4 and 5 was subdivided into 5 equal sub-groups; each containing 10 rabbits while group 6 was kept as control one.

The experimental deign including the groups, sub-groups, types of the inocula and the route and dose of inoculation are summarized in **Table (7)**.

The rabbits received ration without feed additives. Feed and water were given to animals *ad libidum*. The rabbits were kept for one week observation period for adaptation, detection of any abnormal signs or mortality and for bacteriological examination before experimental work.

Table (7): Experimental design of the experiment:

	Group No.	1	2	3	4	5	6
Sub-groups (10 rabbits/group)	Oral route $3x10^{10}$ CFU 2 ml/ each rabbit	*C. perfringens* type A	*C. perfringens* type B	*C. perfringens* type D	*C. perfringens* type E	*C. difficile*	20 rabbits kept as control
	S/C route $3x10^{10}$ CFU 1 ml/ each rabbit	*C. perfringens* type A	*C. perfringens* type B	*C. perfringens* type D	*C. perfringens* type E	*C. difficile*	

Just before inoculation (zero hour) rabbits aged 6 weeks, randomly selected five rabbits were sacrificed and then intestinal and rectal smears were taken to be examined for ensure absence of ***Eimeria*** infection and ***Clostridial*** infection.

Rabbits were kept for six weeks observation period post infection. Mortalities and clinical signs were observed daily till the end of observation period. Feed consumption and average animal body weight in orally infected groups were recorded weekly. Liver and intestinal samples were collected from dead rabbits as well as rectal swabs from living animals for re-isolation of ***C. perfringens*** and ***C. difficile***.

After 2 weeks from the start of experimental infection rabbits vaccinated by Rabbit's viral hemorrhagic septicemia vaccine and after 3 weeks rabbit received Formalized polyvalent rabbit's pasteurellosis vaccine.

Specimens including liver, kidney, small and large intestines were collected form sacrificed rabbits at zero hour as a blank control animals, from dead ones during experiment and form sacrificed rabbits at the end of experiment for histopathological examination.

3.3. Statistical analysis of the results

Data were presented as mean standard deviation (SD) to compare between groups performance (body weight, feed consumption and feed conversion rate (FCR)). Post hoc (least significant difference) LSD test

was used to assess difference between tests. When the significance level set as P value ≤ 0.05, ANOVA test is significant according to **Greenwood and Nikulin (1996)**. Data were analyzed by SPSS © (Statistical Package for Social Sciences) version 16.