Rapid methods of diagnosis of *Clostridium* in broilers in Sharkia governorate.

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A B S T R A C T

A total 100 samples of liver and intestine from broilers (apparently diseased) from 13 poultry farms were analyzed to detect the incidence of *C. perfringes*. The samples were examined bacteriologically by cultivating on CMC and blood agar, typical colonies were identified and biochemically confirmed. The total number of positive samples was 46 (46%). *C. perfringens* showed very high sensitivity to enrofloxacin, high sensitivity to amoxicillin and cefoperazone, but resistant for mupirocin and azteronam. By making artificial infection for mice, the mice died after 24 hours and by examination histopathologicaly, it showed liver degeneration and desquamation of epithelial cells of intestine. Multiplex polymerase chain reaction method (PCR) was amplified for toxin genotyping by using primer for *clostridium perfringes* alpha toxin which amplified at 402 bp.

Keywords: Clostridium perfringes, Broiler, Sharkia, Liver, Intestine.

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1. INTRODUCTION

Clostridium perfringens is a widespread spore-forming, Gram-positive, anaerobic, non-motile rod. It is recognized as an enteric bacterial pathogen in humans, poultry, other farm animals and wildlife worldwide (Songer 1996 and Craven *et al.* 2003). It is often found in the intestinal tract of healthy birds but it can cause necrotic enteritis (NE) in many species of poultry and especially in broiler and turkey flocks (Engström et al., 2003). At low population levels (< 104Cfu) the organism is non-pathogenic. The pathogenicity of the organism is associated with several toxins. *C. perfringens* is usually classified into five types (A - E) on the basis of its ability to produce the major lethal toxins α, β, ε and ι (Yoo et al., 1997) and according to specific animal hosts. Only *C. perfringens* types A and C are pathogenic for poultry. However, the presence of *C. perfringens* does not lead directly to the disease. Although 75 - 95% of birds are colonized by *C. perfringens*, only a small proportion of these ever shows symptoms of the disease (McDevitt et al., 2006). The disease in poultry is called necrotic enteritis and is presented in an acute or subclinical form. The acute form leads to increased mortality in the last weeks of the rearing period. Typical clinical signs include depression, dehydration, somnolence, ruffled feathers, diarrhoea and decreased feed consumption. The subclinical form is characterized by damage to the intestinal mucosa that decreases digestion, absorption and reduces weight gains. Subclinical *C. perfringens* infection is also associated with hepatitis and cholangio-hepatitis. The gut epithelial damage associated with NE coincides with infection by the coccidian genus *Eimeria*, and so NE and coccidiosis are often linked

as one set of similar symptoms (Williams 2005).

The major lethal effects of toxins associated with α-toxin are necrotic enteritis and enterotoxemia in animals (Siragusa et al. 2006). α-toxin (phospholipase C) is commonly produced by all 5 types. It can hydrolyze lecithin into phosphorylcholine and diglyceride, which leads to tissue damage. β-toxin is produced by *C. perfringens*type B and type C strains and it causes a necrotic enteritis characterized by haemorrhagic mucosal ulcerationor superficial mucosal necrosis of the small intestine in animals (types B and C) (Smedley III. *et al*. 2004). Toxins ε and ι play no role in pathogenesis in poultry diseases. All types of *C. perfringens* can produce enterotoxin that is responsible for provoking disease in both man and animals (Van Immerseel et al., 2004). Enterotoxin relates to food poisoning and is produced during sporulation in the infected host intestine, where it binds to the intestinal epithelium, forms pores and causes diarrhoea (Nakamura et al., 2004) in Sharkia governorate.

2. MATERIAL AND METHODS

2.1. Profiles of farms

In Sharkia governorate the length of rearing periode between 21 - 37 days, deep litter of straw, commercial pelleted feed mix and all-in-all-out technology are typical for these farms. The feed mix contained coccidiostats. In all farms were used only two broiler lines: Cobb, Ross, Habbered and Saso.

2.2. Sample collection

Samples (liver and intestine) were collected from 13 poultry farms in the period from May 2012 to September 2013 (Table 1). The chicken suffered from bloody diarrhea, drooping wings and head, decreased appetite, severe depression, ruffled feathers, and reluctance to move. These symptoms

Table (1) Sample collection

Total samples	Location of the farm	Density of the farm	Type of broiler	Age of birds
7	Menia el kamh	6000	Cobb	33
4	Menia el kamh	4000	Cobb	33
2	Menia el kamh	3000	Cobb	33
8	Belbeis	4000	SASO	24
12	Menia el kamh	8000	Hubbered	22
9	Belbeis	12000	Hubbered	21
7	Belbeis	8000	Ross	25
5	Belbeis	15000	Cobb	37
16	Zagazig	30000	Cobb	24
8	Ebrahemia	5000	Cobb	30
6	Gelfina	5000	Cobb	28
7	Belbeis	8000	Cobb	25
9	Al salhia	15000	Cobb	22

are thus rather a specific. Retarded growth rate and increased feed conversion ratio (case history). The post-mortem lesions revealed: The gut of affected broiler is described to be thin-walled, friable and distended with gas, with foul-smelling brown liquid contents. Most prominent features are necrotic lesions, ranging from a few spots of a few mm in size to necrotic patches of a few cm in size. The affected mucosa may be covered with a loosely to tightly adherent pseudomembrane of dead enterocytes trapped in fibrin (figure 1, 2).

The samples were kept in sterile separate polyethylene bags, labeled and carried on ice tank to be transferred with a minimum delay to the laboratory for bacteriological examination.

2.3. Microbiological methods

The sample was inoculated onto a tube of sterile freshly prepared cooked meat medium **(**Oxoid, Basingstoke, UK), then

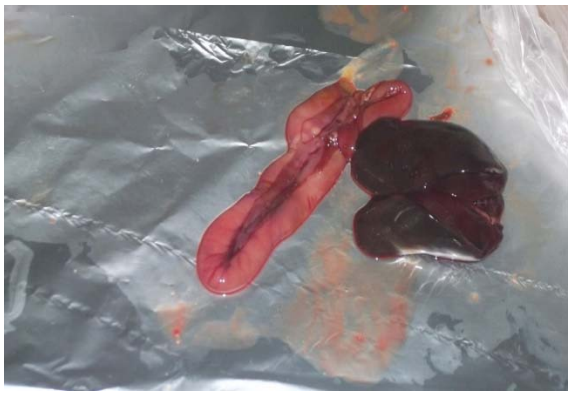

Figure1. Thin-walled, friable intestine and distended with gas, with foul-smelling brown liquid contents, enlarged friable liver

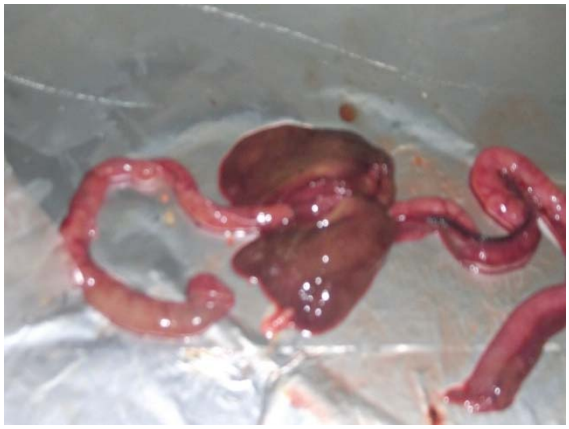

Figure2. Necrotic lesions, ranging from a few spots of a few mm in size to necrotic patches of a few cm in size

the tube was incubated anaerobically in anaerobic jar using anaerobic gas generating kits at 37°C for 24-48 hours, For isolation of *C.perfringens*, a loopful from the previously incubated tube was streaked onto the surface of 10% sheep blood agar with Neomycin sulphate (200 µg /ml). The plate was incubated anaerobically at 37°C for 24-48 hours. The suspected colonies of *C.perfringens* were picked up and examined for their morphological and cultural characters. Subcultures were restored for purification and further identification. Isolated colonies with a typical double zone of haemolysis were then biochemically tested catalase, gelatin liquefaction, indole, glucose, lactose and sucrose fermenters and Nagler's reaction.

2.4. Material used for antibiotic sensitivity test

The used antibiotic discs (Oxoid), their potency and manufacture, used for the *in-vitro* studying the antibiogram pattern of *c.perfringes* (Table 2).

Table (2) antibiogram pattern of *c.perfringes*

Antibiotic discs*	Disc concentration
Cephraden	30mg
Amikacin	30mg
Azteronam	30mg
Ampiclox	30mg
Amoxicillin	10mg
Enrofoxacin	5mg
Cefoperazone	70mg
Vancomycin	30mg
Penicillin	10mg
Mupirocin	5mg

*manufactured by Oxoid

2.5. Typing of C.perfringens toxins by dermonecrotic test in albino guinea pigs

Preparation of toxins and their treatment (Bullen, 1952): 1) Addition of 1 ml of 60% glucose to 50 ml of toxin production medium to ensure anaerobiosis, the medium was inoculated with 5 ml of 24 hours cooked meat cultures of toxigenic strains of *C.perfringens* and incubated in water bath at 37° C for 6 hours. During the period of incubation, pH was adjusted each hour to 7.2. 2) After six hours of incubation, half of the culture was symphonized and centrifuged at 3000 rpm for 20 minutes. The clear supernatant fluid was divided into 4 portions. A) The first portion (0.3 ml) was neutralized with 0.1 ml of type "A" diagnostic antiserum. B) The second portion (0.3 ml) was neutralized with 0.1 ml of type "B" diagnostic antiserum. C) The third portion (0.3 ml) was neutralized with 0.1 ml of type "C" diagnostic antiserum. D) The fourth portion (0.3 ml) was added to 0.1 ml of saline as control. 3) The other half of the same culture was incubated at 37° C for 48 hours anaerobically; pH was adjusted to 7.5 twice daily and centrifuged at 3000 rpm for 20 minutes. The supernatant was trypsinized to a final concentration of 0.1% then incubated at 37°C for an hour and neutralized by type D and E diagnostic antisera in the same ratio (3:1) toxin antitoxin respectively.

2.6. Application of dermonecrotic test Quinn et al., (2002)

A) The hair of the back and two sides of albino guinea pigs were shaved carefully and marked longitudinally onto both sides.

B) On the right side, 0.2 ml of 6 hours or trypsinized 48 hours supernatant of each culture was injected intradermally and the neutralized one was injected in the left side by the same manner and arrangement.

C) The injected guinea pigs were kept under observation for 24 hours for any dermal reaction.

2.7. Artificial infection of laboratory animals

Following culture of the isolated C.perfringens strains in cooked meat broth medium, the cells were harvested by centrifugation at 3000 rpm for 15 min and the cell-free culture supernatants were recovered. White mice (25 - 40 g) were injected intraperitonealy with 0.3 ml of the culture supernatant and then observed over a period of three days for either death or disease symptoms Sterne and Batty, (1975). Control group of mice were injected with broth culture without bacteria. The mice which infected with clostridium dead after 24 hours and the liver and intestine examined histopathologically.

2.8. PCR detection

Extraction of DNA was performed according to QIAamp DNA mini kit instructions. 20µl Qiagen protease was pipetted into the bottom of a 1.5ml microcentrifuge tube. 200µl of the tracheal swab pool or the lung wash were added. 200µl buffer AL was added to the sample, mixed by pulse vortexing for 15 seconds. The mixture was incubated at 56˚C for 10 min.

The 1.5ml microcentrifuge tube was centrifugated to remove drops from the inside of the lid.
200µl ethanol (96%) were added to the sample, and mixed again by pulse vortexing for 15 seconds. After mixing, the 1.5 ml microcentrifuge tube was briefly centrifugated to remove drops from the inside of the lid.
The mixture from step 6 was carefully applied to the QIAamp mini spin column (in a 2ml collecting tube) without wetting the rim. The cap was closed, and centrifugated at 8000 rpm for 1 min. The QIAamp mini spin column was placed in a clean 2 ml collection tube, and the tube containing the filtrate was discarded.
The QIAamp mini spin column was carefully opened and 500 ml buffer AW1 was added without wetting the rim. The cap was closed, and centrifugated at 8000 rpm for 1min. The QIAamp mini spin column was placed in a clean 2 ml collection tube, and the tube containing the filtrate was discarded. The QIAamp mini spin column was carefully opened and 500 ml buffer AW2 was added without wetting the rim. The cap was closed, and centrifugated at full speed for 3min.
The QIAamp mini spin column was placed in a new 2ml collection tube and the old collection tube was discarded with the filtrate. Centrifugation at full speed for 1 min was done. The QIAamp mini spin column was placed in a clean 1.5 ml microcentrifuge tube, and the collection tube containing the filtrate was discarded. The QIAamp mini spin column was carefully opened and 100 µl buffer AE were added. The QIAamp mini spin column was incubated at room temperature (15-25˚C) for 1min, and then centrifugated at 8000 rpm for 1min.

2.9. Preparation of PCR Master Mix

It was prepared according to Emerald Amp GT PCR master mix (Takara) Code No. RR310A kit as shown in table (3)

2.10. Cycling conditions of the primers during cPCR

Temperature and time conditions of the two primers during PCR are primary denaturation at 94°C for 5min, secondary denaturation at 94°C for 1min, annealing at 55°C for 5min, extension at 72°C for 1min

Table 3: Preparation of PCR Master Mix

Volume/reaction	Component
12.5µl	Emerald Amp GT PCR mastermix (2x premix)
4.5µl	PCR grade water
1µl	Forward primer (20 pmol)
1µl	Reverse primer (20 pmol)
6µl	Template DNA
25 µl	Total

for each cycle till the 35th cycle with final extension at 72°C for 10min according to specific authors and Emerald Amp GT PCR master mix (Takara) kit.

2.11. DNA Molecular weight marker

The ladder was mixed gently by pipetting up and down. A 6µl of the required ladder were directly loaded.

2.12. Agarose gel electrophoreses (Sambrooket al., 1989) with modification

Electrophoresis grade agarose (2g) was prepared in 100 ml TBE buffer in a sterile flask, it was heated in microwave to dissolve all granules with agitation, and allowed to cool at 70˚C, then 0.5µg/ml ethidium bromide was added and mixed thoroughly. The warm agarose was poured directly in gel casting apparatus with desired comb in apposition and left at room temperature for polymerization. The comb was then removed, and the electrophoresis tank was filled with TBE buffer. Ten to fifteen µl of each PCR product samples, negative control and positive control were loaded to the gel. The power supply was 1-5 volts/cm of the tank length. The run was stopped after about 30 min and the gel was transferred to UV cabinet. The gel was photographed by a gel documentation system and the data was analyzed through computer software.

DNA was isolated using Midland Certified Reagent Company-Oilgos (USA) according to Yoo et al., 1997.

3. RESULTS

3.1. Isolation and identification of C. perfringens

On Neomycin sulphate sheep blood agar, *C. perfringens* colonies were 2-3 mm in diameter, rounded, raised and showed double zones of hemolysis (figure 3, 4). *C. perfringens* isolates was 46% as in Table 4 appear as Gram-positive short plump bacilli, which rarely had central oval non-bluging endospores. Biochemical characterization of suspected isolates revealed that all *C. perfringens* isolates were catalase negative, gelatine liquefier, indole negative, glucose, lactose and sucrose fermenters and they gave stormy fermentation with litmus milk medium.

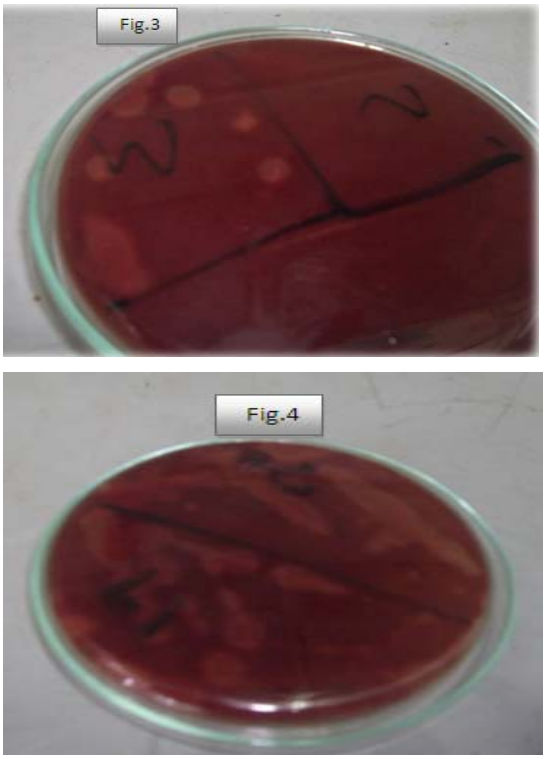

Figure 3, 4: *C. perfringens* colonies were 2-3 mm in diameter, rounded, raised and showed double zones of hemolysis on neomycin sulphate sheep blood agar.

Table 4: Incidence of *C.perfringens* in broiler chicken in Sharkia governorate

Total samples	Positive samples	Negative samples	Incidence of positive samples
100	46	54	46%

3.2. Typing of C. perfringens isolates by dermonecrotic test

Typing of *C. perfringens* isolates revealed that type A was the most predominant one, the action of *C. perfringens* type A (alpha toxin) appeared as an irregular area of yellowish necrosis tended to spread downward as clear in figure 5.

3.3. Antibiotic sensitivity test against C.perfringens

It showed very high sensitivity to Enrofloxacin, high sensitivity to Amoxicillin and Cefoperazone, but resistant for Mupirocin and Azteronam (table 5).

Figure 5. *C.perfringens* type A (alpha toxin) appeared as an irrigular area of yellowish necrosis tended to spread downward as referred by black arrow.

Table (2) Antibiogram of selected isolate against chemotherapeutic agents

Antibiotic discs	Degree of sensitivity
Cephraden	++ve
Amikacin	++ve
Azteronam	-ve
Ampiclox	++ve
Amoxicillin	+++ve
Enrofoxacin	++++ve
Cefoperazone	+++ve
Vancomycin	++ve
Penicillin	+ve
Mupirocin	-ve

Negative, -ve, resistant, +ve slightly sensitive;++ve Moderately sensitive; +++ve highly sensitive; ++++ve very highly sensitive

3.4. Atrficial infection of lab animal

Sever degeneration of intestinal mucosa and intestinal lumen filled with necrotic fibrinous exudate, multinucleated inflammatory cells and red blood cells.

1. Sever necrosis of intestinal mucosa and desquamation of epithelial lining of intestinal gland.
2. Ileum showed severs and diffuse coagulative necrosis of intestinal mucosa especially in intestinal villi.
3. The muscularis mucosa showed degenerative changes associated with edema between muscle fiber.

Liver showed dilated portal, central vein and hepatic sinusoids with infiltration of multinucleated inflammatory cells inside hepatic sinusoids and central vein as well as activation of Kupffer cells. Hepatocyte around central vein showed vacuolar degenerative changes and in some cases showed coagulative necrosis.

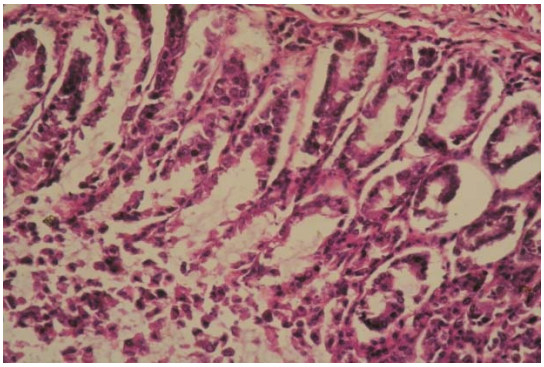

Figure 6. Sever degeneration of intestinal mucosa of artificial infected mice. H&E stain

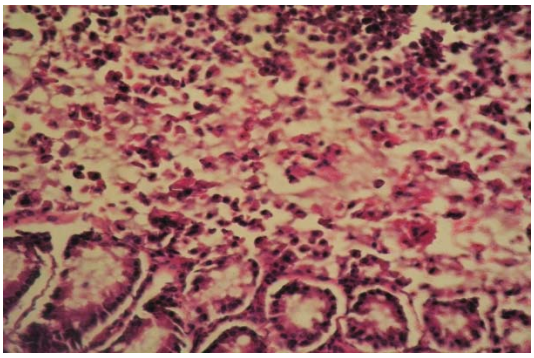

Figure 7. desquamation of epithelium lining of intestinal gland and mixed with inflammatory cells of artificial infected mice H&E stain

3.5. Polymerase chain reaction for C. perfringens-alpha toxin

PCR made by using alpha toxin from *C. perfringens* which was amplified with the primer; DNA was electrophoresed on 1.8%

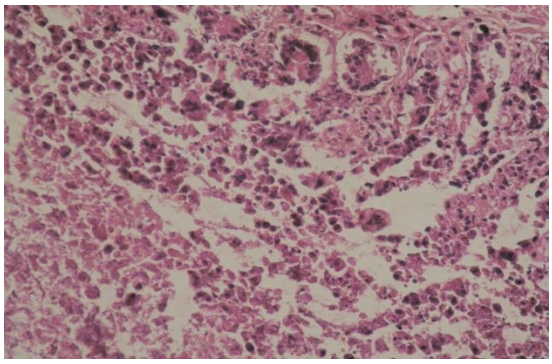

Figure 8. Ileum showed coagulative necrosis of intestinal mucosa especially in intestinal epithelial cell lining villi of artificial infected mice H&E stain.

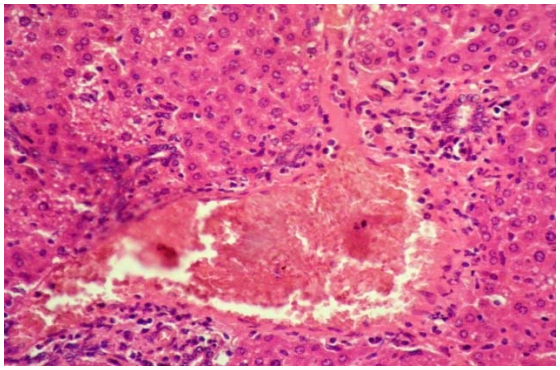

Figure 9. Liver showed dilated portal vein and hepatic sinusoids with mild infilteration of inflammatory cells in portal area H&E stain

agarose gel. The 402 bp amplified product matches with *C.perfringens* as clear in figure (10).

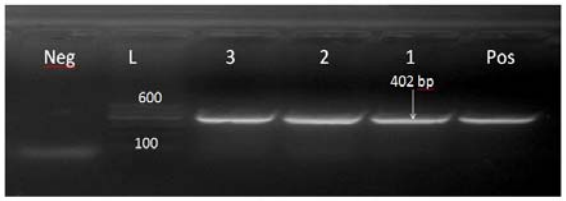

Figure (10) PCR analysis of *C. perfringens* alpha toxin.L 100bp DNA lader, Neg: negative control, pos: positive for *C. perfringens* at 402 bp., lane 1-3, Marker 600 bp lane 5 (negative, positive and 3 samples) positive at 402 bp.

4. DISCUSSION

In the current study, the incidence of *C. perfringens* related necrotic enteritis cases in broiler-farms in Sharkia governorate, Egypt. The diseased birds were suffering from retarded growth rate and decreased feed conversion ratio and increasing the mortality rate in the diseased cases, these symptoms were highly matching with the symptoms reported by Van Immerseel et al. (2004) who reported that the prominent feature of necrotic enteritis is acute death, with mortality rates that can reach 50%. Moreover, they reported that the clinical signs include depression, dehydration, somnolence, ruffled feathers, diarrhea and decreased feed consumption. The post-mortem lesions revealed that the intestine is described to be thin-walled, friable and distended with gas, with foul-smelling brown liquid contents. Most prominent features are necrotic lesions, ranging from a few spots of a few mm in size to necrotic patches of a few cm in size as shown in figure 2&3. On neomycin sulphate sheep blood agar, *C. perfringens* colonies were 2-3 mm in diameter, rounded, raised and showed double zones of hemolysis (figure 4, 5). *C. perfringens* isolates appear as Gram-positive short plump bacilli, which rarely had central oval non-bluging endospores .Biochemical characterization of suspected isolates revealed that all *C. perfringens* isolates were catalase negative, gelatine liquefier, indole negative, glucose, lactose and sucrose fermenters and they gave stormy fermentation with litmus milk medium. Regarding to Nagler's reaction isolates gave opalescence on the side of egg yolk agar medium without antitoxin, while inhibited on the other side of the plate with antitoxin .These general characters of *C. perfringens* were in line with other literatures about general morphological characters of *C. perfringens* (Hatheway, 1990). In addition, Quinn et al., (1999) mentioned that *Clostridium perfringens* on blood agar appear as round, smooth and glistening colonies that are surrounded by a double hemolysis zone, and an outer, partial, non-complete hemolysis zone is produced by alpha toxin. From the information shown in Table 1, it is clear that the major affected breed of broiler chickens were Cobb compared to other breeds like SASO and Hubbard. This observation was also in agreement with Jang et al., (2013), who measured NE susceptibility and host immune response in Cobb, Ross, and Hubbard broilers. Cobb chickens exhibited

increased body weight loss compared with Ross, Hubbard breeds, and greater gut lesion severity compared with Ross chickens. In the current study, *C.perfringens* was confirmed to be positive in 46% of the examined samples as indicated in table 4. This comes in agreement with other reports. For instance, Craven *et al*., (2001) found that the incidence of *C. perfringens* in the intestinal tract and in poultry processed meat is high.

In the current study, *C. perfringens* isolates revealed that type A was the most predominant one, the action of *C. perfringens* type A (alpha toxin) appeared as an irregular area of yellowish necrosis tended to spread downward in dermonecrotic test in albino guinea pigs as clear in figure 5. Similar symptoms in guniea pig detected by Sterne and Batty, (1975) after injection with clostridium perfringes type A

In regard to antibiotic susceptibility of *C. perfringens,* the obtained results showed very high sensitivity to enrofloxacin, high sensitivity to amoxicillin and cefoperazone, moderate sensitivity to cephraden, amikacin, ampiclox, and vancomycin, slightly sensitive to penicillin, but resistant to mupirocin and azteronam as shown in table 4. In line with our results, Teng et al., (2002) showed that *C.perfringens* isolates from Thailand are sensitive to the following antimicrobial agents; ticarcillin, sulbactam, piperacillin, ticarcillin, cefoxitin, cefmetazole, cefoperazone, imipenem, meropenem, moxifloxacin, clindamycin, chloramphenicol, penicillin, ampicillin, piperacillin and metronidazole. Unlikely, Tansuphasiri et al., (2005) recorded that *C.perfringens* isolates from Taiwan showed the highest resistance to tetracycline (56.2%), followed by imipenem (24.9%), metronidazole (9.5%), penicillin G (9%), vancomycin (4.5%), chloramphenicol (3%) and ceftriaxone (1%). A recent report by Marchand-Austin *et al.,* (2014) who tested the sensitivity of *C.perfringens* isolates from Ontario, Canada to common antibiotics used there. They observed that *C.perfringens* strains are sensitive to cefoxitin, meropenem, metronidazole, penicillin and piperacillin-tazobactam, but 3.8% from the isolates were resistant to clindamycin. These variations among antibiotic susceptibility of *C.perfringens* may be attributed to the differences in the geographic locations, environmental factors or emerge of some mutations in the resistant isolates.

The polymerase chain reaction assay (PCR) was used for detection of alpha toxigenic strains of C.perfringensSchoepe, *etal* 2001 Application of PCR analysis of for detection *C.perfringens* type A (alpha toxin) (CPA) gene for biochemically and *clostridium perfringes* typing revealed that all selected strains were positive for (CPA) gene at 402 bpas shown in figure 10. This result goes in line with Aras and Hadimli, (2015). Additionally, similar results have been reported that type A is the dominant type of *C.perfringens* worldwide (Engstrom et al., 2003; Nauerby et al., 2003; Erol et al., 2008). Thus, the results of our study and others highly recommend using of molecular typing for *C.perfringens* as a rapid method for identification of toxigenic strains of *C. perfringens*.

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