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PRESENT STATUS OF CLOSTRIDIUM PERFRINGENS INFECTION IN BROILER CHICKENS

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SUMMARY

This study was done on 728 intestinal and liver samples collected from 84 commercial broiler chicken farms aged 1-11 weeks-old located at Behera, Kafr El-Sheikh and El-Gharbia governorates. Anaerobic bacteriological examination revealed isolation of 430 Clostridial strains that identified as; 236 (54.9%) *C.perfringens* and 194 (45.1%) other clostridial species. Out of the 236 isolated *C.perfringens*; 61 isolates were toxigenic that subtyped into types A (83.6%), B (1.6%), C (1.6%), and D (13.1 %). *C.perfringens* also recovered from waterers, feeders and litter surrounding the birds with a percentage of 27.3%, 50% and 22.7%; respectively. The mortality rate reached 100% in 5 day-old broiler chicks when intramuscularly inoculated with broth culture of *C.perfringens* type A,D or b and C toxins of *C.perfringens* in an arrangement identical to type A,B,C and D. These results suggested that the multiplex-PCR as a rapid , accurate and humanitarian assay could replace the seroneutralization methods with mice and guinea pigs in detection and typing of toxigenic *C.perfringens* strains.

INTRODUCTION

Necrotic enteritis (NE) in chickens had been well documented since it was first diagnosed in 1961 by Parish. The disease is caused by entero- pathogenic *Clostridium perfringens* (CP) which is a normal inhabitant in the intestinal tract of apparently healthy birds (Barnes et al., 1972) with the aid of one or several predisposing factors which may be required to elicit the clinical signs and lesions of NE (Kaldhusdal et al., 1999). The most prevalent and important factor of them was being the damage of the intestinal mucosa caused by

coccidial pathogen (Petit et al., 1999), even with coccidiasis resulting from vaccination as reported by Hofacre et al., (1998); (2003) and Jackson et al., (2003). Highly energetic diets play a role in aggravation of the condition (Branton et al., 1987 and Kocher, 2003). Immunosuppressive diseases as infectious bursal disease, chicken anemia virus infection might act as predisposing factors (Kim et al., 1996a and Wages and Opengart, 2003). Mechanical irritation of the gut and sudden changes of gut micro-flora (Ficken and Wages, 1997 and Apajalahti and Bedford, 2000) could enhance the growth of these pathogens.

C.perfringens could be transmitted through contaminated litter, feed, water, egg shell fragments, chicken fluff, paper pads in the hatcheries; contaminated intestinal droppings of wild birds (Craven et al., 2001a,b) and also by house flies (Musca domestica) (Dhillon et al., 2004).

The infection of *C.perfringens* in poultry might present as acute (clinical) or sub-acute (subclinical) disease commonly present at 2-5 weeks of age in well fleshed birds (Narin and Bamford, 1967). The acute form lead to increase the mortality in broiler flocks. This can account for 1% losses per day for several consecutive days during the last weeks of the rearing period (Kaldhusdal and Lovland, 2000). In the sub-acute form, the damage of intestinal mucosa caused by *C. perfringens* lead to decrease the digestion and absorption, reduce weight gain and subsequently, reflects on the feed conversion ratio (Elwinger et al., 1992 and Kaldhusdal et al., 2001). Cholangiohepatitis could occur in the sub-acute form of *C. perfringens* infections with increase in number of condemnations at processing due to liver lesions (Lovland and Kaldhusdal, 1999).

C.perfringens is considered one of the most frequently human food born pathogens (Buzby and Roberts, 1997).

The present work is design to: a) Estimate the incidence of some different Clostridia microorganisms isolated from both apparently healthy and diseased broiler chickens obtained from Behera, El-Gharbia and Kafr El-Sheikh governorates. b) Study the epidemiology of *C. perfringens* infection (Necrotic Enteritis) and trial to isolate *C.perfringens* (toxigenic type) from poultry environment including litter, feeder and waterer in some broiler studied flocks at the previous localities. c) Serotyping of toxigenic *C.perfringens* isolated from chickens then environmental samples and performing strains' pathogenicity of the most toxigenic *C.perfringens* isolates. d) Finally, evaluation the polymerase chain reaction (PCR) technique in the identification and differentiation of the isolated strains of *C.perfringens* obtained from some outbreaks at different localities.

MATERIAL AND METHODS:

1. Samples:

A total of 367 chickens aging 1-11 weeks of age

were obtained from 84 high breed commercial and balady broiler flocks at Behera, El-Gharbia and Kafr El-Sheikh governorates from summer 2002 till summer 2005 were collected. These collected birds were from apparently healthy (126) or diseased (241) birds showing darkly grayish to bloody stained diarrhea, ruffled feathers and inability to walk. Representative collected part from small intestine (upper, middle and lower) and liver of each scarified bird were collected separately and labeled for complete identification. The samples are shown in tables (1 and 2).

Table (1): History of the examined samples.

Serial No. #	Age	Examined birds/total No.	No. of Exam. samples	Signs	Gross lesion scores	Farm hygiene	History of anticlost. Drugs	Morb. Rate ‰ a	Mort. Rate ‰ b
1*	37	9/6000	18	0	+	M	Yes	2	0.8
2*	37	9/6000	18	√	+++	B	Yes	2	0.6
3*	21	9/3000	18	√	+++	M	Yes	2	0.5
4*	57	8/7000	16	√	+++	M	No	5	1
5*	44	6/3000	12	√	+++	B	No	2	0.5
6	43	3/5000	6	√	++	M	No	2	0.3
7	27	3/5500	6	√	+	M	No	2	0.5
8	24	6/4500	12	0	+	G	No	2	0.7
9	22	3/5500	6	0	+	G	No	2	0.5
10	30	4/5000	8	√	+++	B	No	5	1.0
11	36	3/5500	6	0	++	M	No	2	0.5
12	38	10/3500	20	√	+++	B	Yes	2	0.5

13	25	5/4500	10	0	+	M	No	2	0.6
14	25	8/5000	16	0	++	G	No	2	0.4
15	32	3/5000	6	√	++	G	No	5	1.2
16	32	4/4000	8	√	+++	B	Yes	2	0.5
17	39	6/4000	12	√	+++	B	No	5	1.5
18	37	4/4000	8	√	+++	B	No	2	0.4
19	32	2/4000	4	√	+++	B	No	5	2
20	32	7/5000	14	√	++	M	No	2	0.5
21	39	3/5000	6	0	++	M	No	2	0.6
22	25	8/4000	16	√	+++	B	No	5	1.3
23	40	2/4000	4	√	+++	B	No	2	0.5
24	35	2/8000	4	√	+	M	No	2	0.4
25	20	4/4000	8	0	+	G	No	2	0.5
26	25	5/5000	10	√	+++	G	No	2	0.5
27	30	6/5000	12	√	+++	B	No	2	0.7
28	34	2/3000	4	√	+++	B	No	2	0.5
29	87	2/1000	4	√	+++	M	No	5	1.6
30	25	5/5000	10	0	++	M	No	2	0.7
31*	13	6/3000	12	0	+	G	Yes	2	0.5
32*	21	7/3000	14	0	+	G	No	2	0.5
33*	35	9/6500	18	√	+++	M	Yes	5	2
34*	53	3/3500	6	√	+++	M	No	2	0.8
35*	11	2/4000	4	0	++	G	No	2	0.7
36*	14	3/4000	6	0	++	G	Yes	2	0.4
37*	54	4/15000	8	√	+++	M	No	2	0.9
38*	65	3/5200	6	√	+++	M	Yes	2	0.5
39*	40	4/9000	8	√	+++	B	No	2	0.6
40*	74	3/13800	6	√	+++	M	Yes	2	0.5
41*	75	5/5900	10	0	++	M	Yes	5	1.2
42*	65	4/5200	8	√	+++	B	No	2	0.5
43*	65	3/5200	6	0	++	M	No	2	0.4
44*	75	3/4700	6	√	+++	M	Yes	2	1.5
45*	40	2/10000	4	√	+++	B	Yes	2	0.5
46*	40	4/9000	8	0	++	M	Yes	2	0.5
47*	75	3/4700	6	√	+++	M	Yes	2	0.4
48*	54	4/5000	8	0	++	M	Yes	2	0.5
49*	57	4/9000	8	√	+++	B	Yes	2	0.8
50*	74	2/7000	4	√	+++	B	Yes	5	1.2
51	15	6/4500	12	0	+	G	No	2	0.5
52	43	6/8000	12	√	++	M	No	2	0.5
53	40	4/4000	8	√	+++	M	No	5	0.6
54	29	5/6000	10	√	++	M	No	2	0.5
55	42	3/5000	6	√	++	M	Yes	2	0.4

56	65	5/8000	10	√	+++	M	No	2	0.7
57	36	4/3000	8	√	+++	M	No	2	0.5
58	30	4/5000	8	0	++	M	No	2	0.5
59	43	3/5000	6	√	+++	M	No	2	0.6
60	27	4/5500	8	0	+	G	No	2	0.3
61*	44	5/4000	10	√	+++	M	No	5	1.3
62*	44	4/4000	8	√	+++	M	No	5	1
63*	45	5/4400	10	√	+++	M	No	2	0.9
64*	43	4/2400	8	√	++	M	No	2	0.5
65*	25	4/1000	8	√	++	G	No	2	0.5
66*	14	3/4500	6	0	+	G	Yes	2	0.8
67	31	2/4500	4	√	++ /	M	No	2	0.7
68	25	2/6000	4	0	+	G	No	2	0.4
69	34	6/19000	12	√	++	G	Yes	2 /	0.4
70	37	2/5000	4	0	+	G	Yes	5	0.3
71	32	7/5000	14	√	++	M	No	2	1.56
72	28	3/6000	6	0	+	G	No	2	0.2
73	34	3/4500	6	√	++	M	No	2	0.4
74	23	2/5000	4	0	+	G	No	2	0.2
75	27	2/5000	4	0	++	G	No	2	0.3
76	34	2/8000	4	0	++	G	No	2	0.5
77	35	2/4500	4	√	++	M	No	5	1.8
78	28	6/6000	6	√	++	M	No	2	0.7
79*	23	7/10000	14	√	++	G	Yes	20 c	16 c
80*	29	5/5500	10	√	+	G	Yes	2	0.2
81*	41	4/6500	8	0	++	M	Yes	2	0.3
82*	19	4/5000	8	0	+	G	Yes	2	0.7
83*	29	7/6500	14	√	++	G	No	2	0.3
84*	9	3/9000	6	0	+	G	Yes	2	0.8

#= Total numbers (No.) of: Examined farms = 84.- Examined birds =367 broiler chickens - Examined samples = 728.

- Intestinal lesion scores:[+= 69; ++ = 215; +++ = 83] Morb.= Morbidity. Mort.= Mortality.

a= percentage was calculated according the average at the last three days of sampling.

-b= percentage was calculated according the observation at day of sampling.

-b= percentage was higher due to other diseases.

*= environmental samples (water, feed, litte) were collected from these farms.

/ = means that those collected birds showed signs of ruffled feather, difficulty to walk and diarrhea.

0= apparently healthy birds.

- Inteestinal lesion scoring (El-Seedy, 1990):

+= slight enteritis

++= moderate necrosis, thickened wall, velvet texture mucosa.

+++= severe necrosis with ulcerations, hemorrhages, bloody stained ingesta.

-Farm Hygiene: (according to quality of litter, level of ammonia and degree of ventilation)

-G = good degree. - M = medium degree. - B = bad degree.

Table (2): Types and numbers of samples collected from broiler chickens at different governorates.

Locality	No. of Exam. farms	No. of Exam. birds	No. of Exam. samples	Types of examined samples			
				Apparently healthy chickens (126)		Diseased chickens (241)	
				Intestine	Liver	Intestine	Liver
Behera	33	173	346	64	64	109	109
Kafr El-Sheikh	27	100	200	35	35	65	65
El-Gharbia	24	94	182	27	27	67	61
Total	84	367	728	126	126	241	235

For the epidemiological study a total of 111 samples consisting of 37 feed from feeder, 37 litter and 37 drinking water from waters were collected from each of 37 broiler chickens farms during the production period.

Media: Cooked meat medium, toxin production medium, Muller Hinton Broth, sheep blood agar medium (for detection of the toxigenic isolates), Muller Hinton agar, and gelatin agar medium, indol production and urea agar slant medium were used.

Indicators: Andrade's indicator, Kovac's reagent, Gram's stain. and hydrogen peroxide.

Diagnostic antisera: Antisera of C.perfringens types A, B, C, D and E were obtained from Burroughs Welcome Research Laboratories, Beckenham, England.

Experimental animals:

a- **Albino guinea pigs:** Local breeds of albino guinea pigs weighing 350-450 gm were used for dermonecrotic reaction as well as typing of C.perfringens isolates. They were kept under observation for 2 weeks before use (Willis, 1977).

b- **Swiss mice**: Mice with an average weight 15-25 grams were used for the determination of toxigencity and typing of C.perfringens isolates. (Smith and Holdeman, 1968).

c- **Chickens:** One day-old Hubbard chicks obtained from a commercial chicken farm were used for detection of pathogenecity of toxigenic *C.perfringens* isolates.

Diet: A commercial ration that not contain any anticoccidial or anticlostridial drugs prepared by the poultry organization in Egypt was used to feed the purchased chicks during the period of the experiment.

Materials for DNA extraction: Toxigenic isolates of *C.perfringens,* Tris EDTA (TE) buffer

(10 mM Tris-HCl, 1mM EDTA [pH 8.0]), 10 % sodium dodecyl sulfate (SDS) (Sigma), proetinase k (20 mg/ml in distilled water) (Sigma), 5 M NaCl, CTAB-NaCl solution (10% cetyl trimethyl ammonium bromide in 0.7 M NaCl buffer) [CTAB/NaCl] (sigma), Phenol-chloroform-isoamyl alcohol (Ratio: 25 : 24 : 1) solution (Merek) for DNA purification by removing the endogenous proteins, Absolute alcohol (ADWIC) used for washing the DNA pellet and RNAse (1ug/ml).

Buffers and reagents:10x PCR buffer (Bioneer), Magnesium chloride (MgCl2), 3- Deoxyribonucleotides (dNTPs) (Bioneer), Taq DNA polymerase (Bioneer), Paraffin oil and Agarose gel electrophoresis buffers and reagents(Amrasco).

Primers: Specific primer corresponding to each toxin were designed by using the sequence data obtained from GenBank (Bioneer,Comp.), and were synthesized with a DNA synthesizer. They were used for amplification of DNA recovered from C.perfringens isolates. Oligonucleotide primers sequences used are showed in table (3).

Table (3): Oligonucleotide sequences of primers used in the study according to Yoo, (1997):

Primer direction	Sequence	Location	Size (bp) of amplifiedproducts
CPA: alpha-toxin			
Forward	5-GTTGATAGCGCAGGACATGTTAAG-3	511-535	402
Reverse	5-CATGTAGTCATCTGTTCCAGCATC-3	913-889	
CPB: beta-toxin			
ForwarD	5-ACTATACAGACAGATCATTCAACC-3	589-613	236
Reverse	5-TTAGGAGCAGTTAGAACTACAGAC-3	824-801	
CPE:epsilon-toxin			
Forward	5-ACTGCAACTACTACTCATACTGTG-3	436-459	541
Reverse	5-CTGGTGCCTTAATAGAAAGACTCC-3	976-953	

Culture procedure, according to Willis, (1975): Each collected sample (2 gm of intestine or liver as well as 0.5 ml from each sample suspension of feed, litter, water) were inoculated individually into 2 tubes of freshly prepared cooked meat broth medium and incubated anaerobically at 37°C for 24 hrs. A loop-full from the first tube (T1) were streaked into neomycin sulphate sheep blood agar plates (for the isolation of *C.perfringens*) and incubated anaerobically at 37°C for 24 hrs. Suspected colonies of C.perfringens that were characterized by double zone of haemolysis were subcultured onto 2 plates of each 10% sheep blood agar and egg yolk agar plates. One plate from each inoculated solid media was incubated aerobically (as a control plate) and the other plate was incubated anaerobically. The colonies that grown only in an-

aerobic condition and lecithinase producer were picked up and tested by catalase test. Colonies that were catalase negative, lecithinase producer and showed double zone of haemolysis were purified and kept in cooked meat broth for further identification.

The second inoculated tube of cooked meat broth with sample (T2) was heated in water bath at 80 0C for 10 minutes (for the isolation of other spore forming Clostridia species). A loop-full was streaked onto sheep blood agar without neomycin and incubated anaerobically. The suspected colonies of catalase negative character were picked up, purified and maintained in cooked meat broth for further identification.

Identifiaction of the isolated bacteria:

a- Microscopical examination: After Gram stained, films from the purified isolates were examined microscopically for detecting their stain reaction, morphological characters and spore formation (Cruickshank et al., 1975).

b- Biochemical identification: Suspected purified isolates that obtained were identified according to Cruickshank et al., (1975) and Koneman et al., (1988) using the following tests: Fermentation of sugars, Lecithinase activity test, Gelatin liquefaction test, Indole test, Motility test and Urease test.

Determination of toxigenic isolates of C.perfringens:

a- Nagler's reaction: (Willis and Hobbs, 1959). The test was done by spreading the antitoxin serum of *C.perfringens* type A, B, C, D and E on half of the egg yolk agar plate which allowed for 30 minuets to dry in the incubator , then the culture was applied and incubated anaerobically at 37°C for 24 hrs. Positive results was expressed by the zone of opacity surrounding the toxigenic *C.perfringens* colonies in the half of the plate without antitoxin while nothing changed on the other half containing antitoxin as demonstrated.

b- Pathogenicity to guinea pigs (Willis, 1977): Two albino guinea pigs were experimentally inoculated intramuscularly with 0.5 ml (containing 10^7 CFU / ml) of overnight old cooked meat broth of C.perfringens mixed with 0.5 ml of 5% calcium chloride solution. Guinea pigs were kept under observation for 3 days. Post-mortem examinations of dead animals were done and cultures from heart blood, liver and exudates were inoculated onto blood agar plates and cooked meat broth to re-isolate the inoculated organism.

Typing of the toxigenic *C.perfringens* isolates:-

1. Neutralization test in mice:- (Smith and Holdeman, 1968). 0.1 ml of specific antiserum of each type of *C.perfringens* A, B, C or E was added to 0.3 ml of the supernatant fluid of centrifuged (3000 rpm for 15 minutes) cooked meat broth sub-cultured anaerobically with the toxi-

genic *C.perfringens* isolates. The supernatant fluid of type D only, was treated with 0.1% trypsin at 37°C for 45 minutes to activate the toxin before adding its specific antitoxin. On the other hand, the centrifuged supernatant fluid of cooked meat broth was mixed with cooked meat cultures of *C.perfringens* without adding the specific antiserum that used as a control. The prepared mixtures were left at 37°C for 30 minutes before their injection. Two mice were used for every mixture. Each mice received 0.4 ml of mixture intravenously. The inoculated animals were kept under observation for 3 days.

2. Dermonecrotic test in guinea pigs:

a- Preparation of toxins and their treatments according to Bullen, (1952) and Roberts et al., (1970)

Stirred parts of the hair on the back and sides of albino guinea pigs were carefully marked longitudinally into both sides. On the right side, 0.2 ml of the 5 hours or trypsinzed 48 hrs supernatant of each culture was injected intradermally. Meanwhile, 0.2 ml of supernatant antiserum mixtures was injected by the same manner and arrangement on the left side. The injected guinea pigs were kept under observation for 48-72 hrs to determine any dermal reaction.

The results were interpreted by the degree of the dermonecrotic reaction and its neutralization according to Stern and Batty (1975).

Experimental infections of baby chicks with types A and D of *C.perfringens* recovered from broiler chickens according to Meghawery(1993): Forty, 5 days-old commercial broiler chicks were divided into four groups, 10 chicks each were used as follows:-

Group I: chicks were inoculated intramuscularly by 0.5 ml of 24 hours cooked meat broth contains C.perfringens type A isolate (containing 10^7 CFU / ml).

Group II: chicks were inoculated intramuscularly by 0.5 ml of 24 hours cooked meat broth contain C.perfringens type D isolate (containing 10^7 CFU / ml).

Group III: chicks were inoculated intramuscularly by 0.5 ml of 24 hours cooked meat broth contain C.perfringens type A and D isolates (containing 10^7 CFU / ml).

Group IV: chicks were kept without treatment as control.

Chicks of all groups were kept under observation. Signs, lesions and moralities were recorded, as well as subjected for re-isolation of the inoculated organism.

Multiplex PCR for typing of toxigenic *C.perfringens* isolates according to Yoo et al. (1997):

a- Isolation of total DNA:- A suspension of each isolate having 10^7 CFU / ml was prepared in TE buffer by adjusting its turbidity with McFarland tubes. A total of 10 ul of this suspension was put onto a then walled microtubes

and was placed into boiling water and resuspended in 567 ul of TE buffer (10mM tris-HCl, 1mM EDTA [pH 8.0]). The solution was incubated at 37°C for one hour after the addition of 30ul of 10% sodium dodecyl sulfate and 3ul of proetinase-k (20mg/ml in distilled water) after incubation of the reaction mixture with 100ul of 5M NaCl and 80ul of CTAB-NaCl solution (10% cetyl trimethyl ammonium bromide in 0.7 M NaCl) at 65°C for 10 minutes. The solution was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) solution and DNA was precipitated with absolute ethanol, and resuspended in 100ul of TE buffer after it was vacuum dried. RNA was removed by incubation of the solution with RNAse (1ug/ml) at 37 °C for one hour. DNA solution was kept at -20 °C until use. The concentration of the pure of extracted DNA was measured by estimating the optical density at wave length at 260 nm using the spectrophotometer.

The concentration was calculated as follows:1 OD 260 = 50 ug/ml

b- PCR running:-

A one ul template DNA was added to a 50ul reaction mixture with the following reagents: 50 mM KCl, 10 mM Tris HCl (pH 8.3), 2.5 mM MgCl2, 0.1 mM of each deoxynucleotide, 1U of Ampli-taq Gold DNA polymerase (Applied biosystems, Foster city, USA), 25 nM of each *C.perfringens* A (α-toxin) primer, 25 nM of each *C.perfringens* B (B-toxin) primer, 250 nM of each *C.perfringens* E (8-toxin) primer. 40µl paraffin oil was added and the hermal cycle was adjusted. The thermo-cycling (incubations for 1 minutes at 95°C, 55°C and 72°C respectively was repeated 35 times) was preceded by incubation for 5 minutes at 94°C for initial denaturation and followed by final extension at 72°C for 10 minutes. Ten microliter of amplicons was separated by electrophoresis in a 1.5% agarose gels according to standard procedures.

c- PCR protocol:- Step 1: Initial denaturation at 94°C for 5 minutes, Step 2: Denaturation at 95 °C for 1 minute, Step 3: Annealing at 55°C was adjusted for 1 minute, Step 4: Extension at 72°C for 1 minute and Step 5: Final extension at 72°C for 10 minutes.

The PCR products were stored in the thermal cycler at 4°C until they were collected.

d- Screening of PCR products: The gel tray was trapped and the warm agarose was poured. The comb was inserted in the agarose which was left to polymerize. After hardening, the tray was untrapped, the comb was removed and the gel was applied to the electrophoresis cell. The cell was filled with TAE buffer. Ten micro-liter of each amplicons were applied to the gel along with 5ul molecular weight marker after mixing each with 1ul loading buffer on a piece of paraffin. The electrophoresis cell was covered and the power supply was turned on and adjusted at 10 volt/cm. The gel was taken out from the cell and examined under

short wave UV trans-illuminator.

c- **Photographing of the gel:** The gel was photographed in order to obtain a permanent record using a Polaroid Camera (Kodak, WA).

RESULTS

Out of 728 examined samples collected from 84 broiler chicken farms; 430 Gram positive isolates, recovered under anaerobic condition, were recorded to be Clostridia microorganisms with a percentage of (59.1%). The biochemical reactions, with the 430 isolates were differentiated into 6 clostridia species, whereas, *C.perfringens* was 236/430 (54.9%). The highest recovery rate was in El-Gharbia (66.3%) followed by Behera (55.1%), while the lowest rate was at Kafr El-Sheikh 41.6% (Table : 4).

Table (4): Results of Biochemical reactions:-

No. of reacted Isolates	Meat dig.	Motility	Indole	Urease	Gelatin Liquef.	Lecithinase	Acid production from				
							Glucose	Sucrose	Lactose	Mannitol	Salicin
236	+	-	-	-	+	+	+	+	+	-	+

Fig. (1): Observed clinical signs and gross lesions on the examined naturally infected chickens:

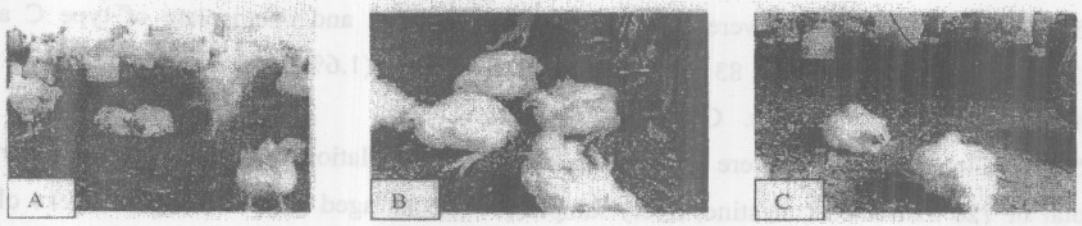

Fig. (1a) : Showed signs of NE (A, B, C); inappetence, unable to walk, marked depression, ruffled feather in well fleshed and nourished 39 day old Hubbard-broiler chickens (acute form).

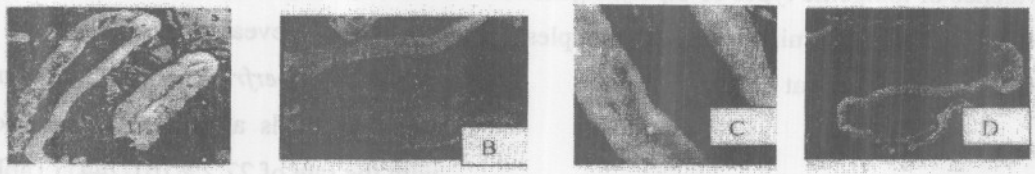

Fig. (1b): Showed intestinal gross lesions of NE that restricted at the small intestine(from A to D); ballooned intestine with thickened wall, the mucosa showed different degrees of necrosis with loosely attached diphtheritic membrane(in 39 day old commercial broiler chickens, A and B). The intestinal lumen in more severe cases was filled with bloody stained ingesta or blood (darkly stained) with severe degrees of necrosis, hemorrhages and ulceration (in a 8 week old balady broiler chicken, C and D).

A total of 102 (40.5%) Clostridia isolates were recovered from 252 examined samples collected from apparently healthy birds, 55 (21.8%) isolates of them were *C.perfringens*. From them, sixty four (50.8%) Clostridia isolates were recovered from 126 examined intestinal samples, 34 (27%) isolates of them were *C.perfringens*. While, 38 (30.2%) Clostridia isolates were recovered from 126 examined liver samples, 21 (16.7%) isolates of them were *C.perfringens*..

On the other hand, results of isolation of diseased broiler chickens was 68.9% (A total of 328) Clostridia isolates were recovered from 476 examined samples, 181 (38%) isolates of them were *C.perfringens*. One hundred and ninety-seven (81.7%) Clostridia isolates were recovered from 241 examined intestinal samples, 98 (40.7%) isolates of them were *C.perfringens*. While, 131 (55.7%) Clostridia isolates were recovered from 235 examined liver samples, 83 (35.3%) isolates of them were *C.perfringens*. Collectively, 430 (59.1%) Clostridia isolates were recovered from a total of 728 examined (intestine, liver) samples, 236 (32.4%) isolates of them were *C.perfringens*. Prevalence of toxigenic types of *C.perfringens* recovered from the examined chicken samples at different localities and at different ages:

Out of 236 tested *C.perfringens* isolates; 61 (25.9%) isolates were toxigenic and 175 (74.1%) isolates were non-toxigenic by using Nagler's reaction and the pathogenicity test on albino mice. Both tests gave positive results with the 61 isolates only. They were differentiated according to dermonecrotic test on albino guinea pigs into 51 (83.6%), 8 (13.1%), one (1.6%) and another one (1.6%) isolates of A, D, B and C types of toxigenic *C.perfringens;* respectively. The highest rate of *C.perfringens* type A was recorded at Behera followed by El-Gharbia then Kafr El-Sheikh governorate with the records of 29, 15, and 7 isolates; respectively. Whereas, The highest rate of *C.perfringens* type D was recovered at Kafr El-Sheikh (5 isolates) followed by El-Gharbia (3 isolates), while no recovery for type D was recorded at Behera governorate. There was one isolate only was recorded for type B (1.6%) at El-Gharbia and one isolate of type C at Kafr El-Sheikh (1.6%) governorate.

The isolation rate of *C.perfringens* from broiler flocks aged more than two weeks old was 224 (36.8%) isolates, while it was 12 (10%) isolates from younger ages(less than two weeks old). The results revealed that all of the recorded 61 toxigenic *C.perfringens* isolates were recovered from the birds aged more than two weeks old with the rate of 27.2% (61/244) (Table 5).

Table (5): Prevalence of toxigenic *C. perfringens* at different ages of examined chickens:

Groups of Birds	No. of exam. Farms	No. of exam. Birds	Examined Samples			C.perfringens isolates			
						Toxigenic		Non-toxigenic	
			No.	+ve	% #	No.	% *	No.	% *
Less than two weeks	13	60	120	12	10	0	0.00	12	100
More than two weeks	71	307	608	224	36.8	61	27.2	163	72.8
Total	84	367	728	236	32.4	61	25.9	175	74.1

: Percentage was calculated according to the number (No.) of the examined samples.

*: Percentage was calculated according to the number (No.) of the positive (+ve) samples.

Epidemiology of *C.perfringens* at different localities:

Our results revealed that, 22 (19.8%) *C.perfringens* isolates were recovered from a total of 111 examined environmental samples collected from 37 examined broiler chicken farms. Out of the 22 isolates of *C.perfringens* recovered from the environmental samples; 11 (50%), 6 (27.3%) and 5 (22.7%) isolates were recovered from feeder, waterer and litter samples; respectively (Table 6).

Table (6): Prevalence of *C. perfringens* at the environment surrounding the birds at the examined 37 farms:

Total No. of the examined samples			isolation from the examined environmental samples								
			Waterer			Feeder			Litter		
No.	+ve	%	No.	+ve	%	No.	+ve	%	No.	+ve	%
111	22	19.8	37	6	27.3	37	11	50	37	5	22.7

%= percentage was calculated according to total number (No.) of positive (+ve) examined samples.

Prevalence of toxigenic types of *C.perfringens* recovered from examined environmental samples: Five isolates out of the 22 tested *C.perfringens* isolates that recovered from the environmental samples were detected to be of toxigenic and all of them were type D by using dermonecrotic test on guinea pigs with the rate of (22.7%). They were recorded from litter [2 (40%)], feeders [2 (18.2%)] and water [1 (16.7%)].

Experimental infections of baby chicks by single or mixed *C.perfringens* type A and / or D obtained from natural infections: as shown in table (7), 100% mortality rate was recorded to be the highest rate of moralities after 24-48 hours of intra-muscular inoculation with mixed inoculums of both A and D types (3rd group) of *C.perfringens* followed by 90% moralities for type A (1stgroup), then 80% for type D (2nd group). While, the control (4th) group did not record any deaths. The live chicks at the first three groups showed signs of diarrhea, gradual paralysis and tremors. The postmortem lesions were ballooned intestine, with thickened congested internal organs and distended gall bladders. The organism of *C. perfringens* has been recovered from chicks by reisolation from the 1st three groups. The chicks of the control group were apparently healthy without postmortem lesions or mortalities.

Table (7): Results of experimental reproduction of NE in 5-days old chicks after Intramuscular inoculation with single or mixed toxigenic types of *C.perfringens* recovered from natural infections:

Type of inoculums	Dose (ml) of inoculums (10^7 CFU / ml)	No. infected chicks	Mortality Rate	
			No.	% a
C.perfringens type A	0.5	10	9	90
C.perfringens type D	0.5	10	8	80
C.perfringens. types A&D	0.5	10	10	100
Control	Not infected	10	0	0.00

a = percentage was calculated according to the number (No.)of inoculated chicks.

Electrophoretic analysis of the isolated C.perfringens toxin gene amplified by a multiplex PCR:- As shown in figure (2) and figure (3), the screened agarose gel electrophoresis showed amplification of alpha toxin at 402 base pair fragments and beta toxin at 236 base pair fragments and epsilon toxin at 541 base pair fragments only in an arrangement typical to that of type A as shown in lanes 2, 3, 4, 5, 6, 7 and 8 at figure (8) and lanes 3 and 4 at figure (9), lane 8 of type B, lane 5 of type C and lanes 6 and 7 of type D at figure (3). While lane 9 at figure (2) and lane 2 at figure (3) were represented a negative control (no template of DNA). Lane 1 at both figures (2,3) were a DNA size marker (1kb ladder).

Comparison between traditional (dermonecrotic test) and recent (Multiplex-PCR) techniques in biotyping of all toxigenic *C.perfringens* isolates (66) obtained from different sources of samples:-

Both dermonecrotic and recent multiplex-PCR techniques could detect and differentiate all the 66 *C.perfringens* isolates obtained from different sources of samples (chickens and environment) into 51, 1, 1, and 13 of A, B, C, and D types; respectively.

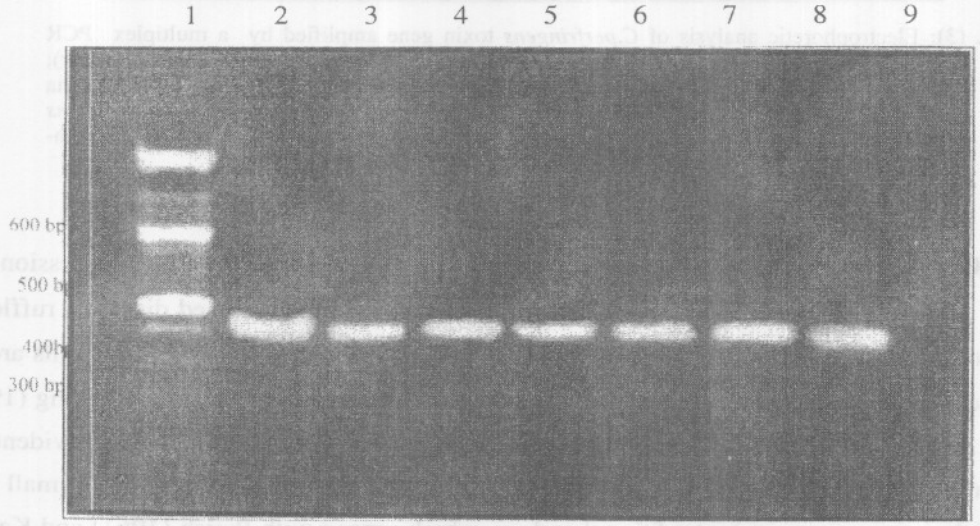

Fig. (2): Electrophoretic analysis of *C.perfringens* toxin gene amplified by a multiplex PCR (*C.perfringens* type A). Lane 1, DNA size marker (1-kb ladder); Lanes (2-8). *C.perfringens* type A (alpha toxin). Lane 9, negative control (no template DNA).

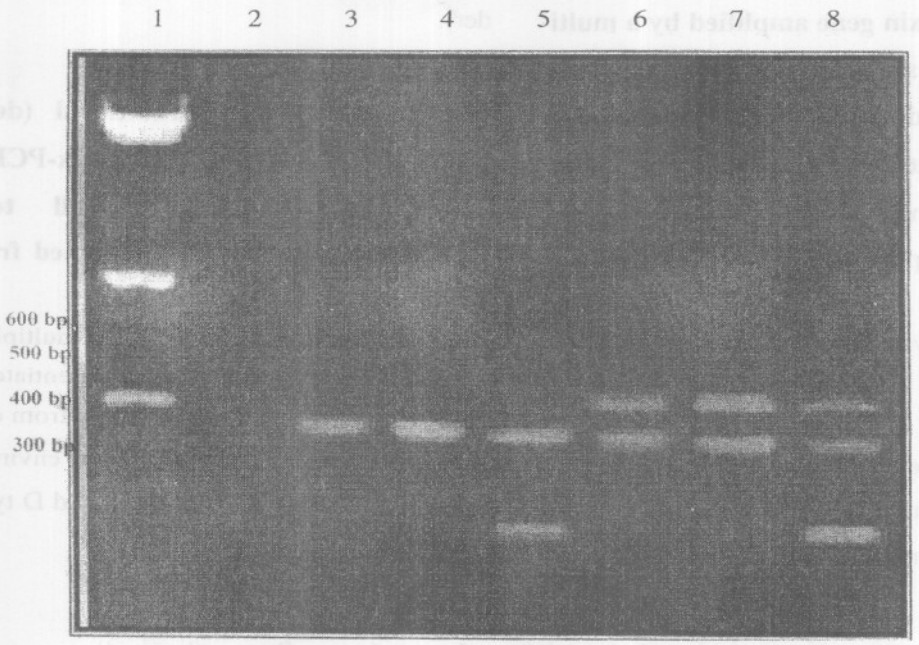

Fig. (3): Electrophoretic analysis of *C.perfringens* toxin gene amplified by a multiplex PCR (*C.perfringens* types A, B, C and D toxins). Lane 1, DNA size marker (1-kb ladder); Lane 2, negative control (no template DNA); Lanes (3-4), *C.perfringens* type A (alpha toxin); Lane 5, *C.perfringens* type C (alpha and beta toxin); Lane (6,7), *C.perfringens* type D (alpha and epsilon toxins) recovered from chicken and environmental samples; Lane 8, *C.perfringens* type B (alpha, beta, and epsilon toxins)

DISCUSSION

The present study was planned to through light on the incidence, and epidemiology of *C. perfringens* at 3 localities (Behera, Kafr-El-Sheikh, and El-Gharbia governorates), isolation and identification of the microorganism by traditional and recent methods of diagnosis by using polymerase chain reaction (PCR), finally, experimental induction of the microorganism.

The recorded signs on the diseased chickens in the examined farms were depression, darkly grayish to bloody stained diarrhea, ruffled feathers and inability to walk. These signs are similar to that recorded by Riddell and Kong (1992) and Kaldhusdal et al., (2001). Most evident macroscopically lesions occurred in the small intestine which agreed with Parish (1961) and Kaldhusdal and Hofshagen (1992). Intestinal gross lesion scores varied from slight enteritis, to moderate degree of necrosis, thickened wall, velvet texture mucosa, while in severe degree showed severe necrosis with ulcerations, hemorrhage and

bloody stained ingesta. These lesions were declared as lesions recorded by Riddell (1987).

It is evident that the recovery rate of Clostridia microorganisms was 59.1% obtained from both apparently healthy and diseased broiler chickens. These results agreed with those recorded by Hussein (1972) 48.4%, El-Ged and Hegazy (1985) 62.5%, Meghawery (1993) 58%, Hikal (1999) 62.4%, Ibrahim et al. (2001) 49.1% and Abd- El-Wahab (2002) 59.8%. A much higher incidence was recorded by El-Refaey (1999) 79%, Mustafa (2000) 66% and Ahmad and Abd El-Latif (2004) 82%. On the other hand, a much lower incidence was recorded by Kohler et al., (1977) 12.4%, Prukner and Milakovic (1991) 0.21%, Azzam and EL-Bardiesy (1998) 15% and Kuliler and Pilsl (2001) 4.7%.

On the basis of biochemical tests C.perfringens was the most predominant Clostridium isolate from a total of 430 isolates (54.9%). Nearly finding had been recorded by previous authors; 50% by Ibrahim (1979), 48.9% by Prukner and Milakovic (1991), 51.9% by El-Refaey (1999), 50.6% by Kuliler and Pilsil (2001), 54.4% by Abd El-Wahab (2002) and 56% by Ahmad and Abd El-Latif (2004). While, a much lower incidence were recorded by Mustafa (2000) 40% and Abd El-Gwad and Abd El-Kader (2001) 44.4%. A much higher incidence was recorded by Awad et al., (1976) 97.4%, Kohler et al., (1977) 69.2%, El-Ged and Hegazy (1985) 78%, Abd El-Gaber et al., (1994) 78.9%, Azzam and El-Bardiesy (1998) 92.8% , Hikal (1999) 74.5% and Monazi (2000) 71.9%.

Among the governorates, variations in the incidence of Clostridia species were seen. The highest recovery rate was recorded at farms located in Behera governorates (70.2%) followed by EL-Gharbia (53.8%), then Kafr El-Sheikh (44.5%). These results were in accordance with that recorded by Meghawery (1993), who recorded this variations with a percentage of 65.7% at Nobaria, 60% at Kalyobia and 15.4% at Giza farms.

Our results indicated that the incidence of Clostridia species from apparently healthy and diseased chickens were 40.5% and 68.9%; respectively. The same results were detected by El-Ged and Hegazy (1985) 50% and 63% as well as Abd El-Wahab (2002) 30.5% and 89% from apparently healthy and diseased chickens; respectively.

The isolation rate of *C.perfringens* from the examined intestinal samples collected from apparently healthy chickens was in a lower rate (27%), than from diseased chickens (40.7%). This observation was recorded by Shane et al., (1984), El-Ged and Hegazy (1985) Tschirdewahn et al., (1992), and Craven et al., (2001a and b), Abd El-Wahab (2002) and Craven et al.,

(2003).

Although C.perfringens present commensally in the intestine, it could be responsible for enteritis in chickens. The variations in the rate of the intestinal lesions due to of *C.perfringens* in apparently healthy and diseased chickens could be attributed to many predisposing factors (Wages and Opengart, 2003).

Hepatic lesions in examined liver samples represented by congestion with focal necrosis and distention of gall bladder might explain the high recovery rate of C.perfringens (35.3%) from the diseased chickens, while it was 16.7% from apparently healthy birds. These lesions might be due to the bacteraemia and toxaemia as postulated by Lovland and Kaldhusdal (1999 and 2001). Abd El-Wahab (2002) isolated C.perfringens from the examined liver samples of the apparently healthy and diseased chickens at a rate of 8% and 41%; respectively.

With the importance of toxigenic types of *C.perfringens* in chicken necrotic enteritis (NE), 61 toxigenic isolates were identified from a total of 236 tested C.perfringens isolates with a percentage of 25.9%. These results were close to that recorded by Latinovic (1983) 29% and Tschirdewhan et al., (1992) 10%.

C.perfringens toxins are alpha, beta, epsilon and iota according to types of *C.perfringens* isolates (A to E). The alpha toxin is a phospholipase C and a lecithinase that has been implicated in several diseases including necrotic enteritis in chickens due to its lethal and hemolytic effects (Julian, 1998).

On typing of the recovered 61 toxigenic *C.perfringens* by using neutralization test in mice and dermonecrotic reaction in guinea pigs, 51 (83.6%) isolates proved to be type A, one isolate (1.6%) belonged to type B, one isolate (1.6%) was type C and the remaining eight isolates (13.1%) belonged to type D.

In this concern, this study affirmed that C.perfringens type A was the most prevalent type in chickens showed signs and lesions of necrotic enteritis. This agreed with Songer and Meer (1996), Heikinheimo and Korkeala (2005) and Siragusa et al., (2006). Type A was widely distributed in the examined broiler chickens up to the age of marketing which may give a wide chance for rapid transmission of the infection from birds to human beings, causing food poisoning (Buzby and Robert, 1997 and Johansson et al., 2006).

In comparison with other literature, some researchers demonstrated high incidence of toxigenic type A of C.perfringens (81.3%) in chickens suffered from NE (Awad et al., 1976). Also,

Sinha et al., (1975), Hikal (1999), Mustafa (2000), Younes (2005) and Neumann et al., (2007) cited on a higher incidence; 89.7%, 76%, 100%, 90% and 100%; respectively. On the other hand, other researchers revealed a lower incidence of toxigenic type A than our results, 50%, 66.7%, 62.1%, 50.5%, 46.9%, 51.5%, 60.3%, 58.3% and 60% as investigated by El-Refaey (1999), Ibrahim et al., (2001) and Abd El-Wahab (2002) and El-Refaey (2006); respectively. In addition, Azzam and El-Bardiesy (1998) and Abd El-Salam (2000) recorded a much lower ratio (15.3%, 22.1%, 23.3% and 36.1%; respectively). C.perfringens types B and C recorded at higher rates by El-Ged and Hegazy (1985) 16.9%, 14.4% and El-Refaey (1999) 7.6% and 36.4%; respectively compared with our results (1.6% for each type). A higher rates were also, recorded by Awad et al., (1976) 18.2%, Farid et al., (1978) 82.9%, Latinovic (1983) 16.7%, El-Seedy (1990) 24.1%, Meghawery (1993) 17.2%, Azzam and El-Bardiesy (1998) 55.6%, Abd El-Salam (2000) 30%, Monazi (2000) 26.7%, Abd El-Wahab (2002) 20% and El-Refaey (2006) 40% for type C. A much lesser extent of recovery rate of type B was recorded 3.5% by Sinha et al., (1975).

The recorded result for type D, was 13.1% that in accordance with Latinovic (1983) 16.7%, El-Ged and Hegazy (1985) 15.6%, El-Seedy (1990) 10.3%, Abd El-Salam (2000) 18.6%, Monazi (2000) 13% Abd El-Wahab (2002) 13.3% and Younes (2005) 10%. A much higher incidence was recorded by Meghawery (1993) and Abd EL-Gaber et al., (1994) 35.4% and Azzam and El-Bardiesy (1998) 21.1%. A lower incidence was recorded by Awad et al., (1976) 0.44%, Sinha et al., (1975) 7% and El-Refaey (1999) 1.5%.

This study proved that broiler chickens were frequently affected by C.perfringens-associated NE at ages over than two weeks old as it recovered at a rate of 36.8% from birds aged more than two weeks old. As well as all of the 61 toxigenic isolates recorded above 2 weeks of age with a percentage of 27.2%. These findings agreed with Narin and Bamford (1967), Bains (1968), Bernier and Filion (1971), Long (1973) and Wilkie et al., (2006). Non-toxigenic C.perfringens (12 isolates) isolated from chickens below two weeks old which was in agreement with by Craven et al., (2001a,b and 2003) and Abd El-Wahab (2002).

In regard of epidemiological studies, it is clearly evident that *C.perfringens* was recovered from waterers, feeders and litters as environment surrounding the broiler chickens with an incidence of 27.3%, 50% and 22.7%; respectively. These results illustrated the relationship of isolation of C.perfringens between environment and birds samples among the examined broiler chicken farms that elucidated a percentage 24.3% for group a (both samples were positive) 4.5% for

group b (positive environmental samples only) and 40.5% for group c (positive birds samples only) with a total percentage in the three positive groups (a, b and c) of 70.3%. While the remainder 29.7% were found to be negative for both environmental and bird samples. These results were announced that excretion of *C.perfringens* organism in droppings of the infected birds was the most prevalent source of poultry environmental (feed, litter, water) contamination with a consequent facilitation of the spreading of the infections among chicken farms that agreed with Cowen et al., (1987) and Click to search for citations by this author." Olkowski et al., (2006).

These findings agreed with that recorded by Craven et al., (2001a) who recovered *C.perfringens* from litter, feeders and waterers samples with a percentage of 23%, 17% and 12%; respectively. A higher percentage were recorded by Dosoky (1990) with a rate of 90% from litter, 80% from ration and 75% from water samples collected from outbreaks of NE disease in broilers.

A higher recovery rate of *C.perfringens* from feeders (50%) indicated that poultry feed was one of the major sources of infective spores. These results are similar to Dosoky (1990), Kohler (2000), Abd El-Rahman et al., (2001) and Heijnen (2004).

On determining and typing of the toxigenic *C.perfringens* from the recovered 22 environmental isolates, 5 isolates of type D only were detected to be toxigenic with a percentage of 22.7%. One isolate of them was detected from waterers (16.7%), two isolates from feeders (18.1%) and two isolates from litter (40%). This throw light on the importance of managemental and hygienic measures inside chicken farms before and after marketing of the flock. From our results the most prevalent toxigenic *C.perfringens* types were A and D. So, our experimental study focused on these two types of toxigenic *C.perfringens.*

On studying the experimental infection, the highest mortality rate occurred within 24-48 hours post-inoculation in group 3 inoculated with 0.5 ml of mixed broth culture of types A and D with a percentage of 100% followed by group 1 of type A (90% mortalities) then group 2 of type D (80%). These results agreed with that declared by Meghawery (1993) and Mustafa (2000) who could produce NE in intramuscularly inoculated chicks with type A and D either singly or mixed. These higher rates of mortalities were attributed to the route of inoculation which gave the organism more suitable anaerobic condition for growth and secretion of high quantity of C.perfringens toxins although, it is a localized intestinal organism as reported also by Katitch et al., (1966) and Hofshagen and Stenwig (1992). Moreover, this toxigenic types cause

necrosis of the intestinal mucosa that exhibited the clinical illness and deaths. These findings agreed with Fukata et al., (1988) and Meghawery (1993).

Many authors used PCR technique as a rapid and accurate diagnostic tool in subtyping of C.perfringens isolates (Gholamiandekhordi et al., (2005), Mark and Siragusa, 2005, El-Refaey, 2006, Siragusa et al., 2006 and Neumann et al., 2007). The pathogencity of chicken NE; that is closely related to the production of major lethal toxins of C.perfringens (alpha, beta, epsilon and iota). A multiplex polymerase chain reaction (PCR) was applied as a confirmatory test for the 66 toxigenic isolates of C.perfringens (61 isolates from birds and 5 isolates from environment) by using specific primers that designed for each toxin gene.

The screened agarose gel electrophoresis showed amplification of the alpha toxin gene at 402 base pair fragments and beta toxin gene at 236 base pair fragments and epsilon toxin gene at 541 base pair fragments; only in an arrangement typical to that of type A as shown in lanes 2, 3, 4 , 5, 6, 7 and 8 in figure (8) and lanes, 3 and 4 at figure (9); type B as in lane 8 at figure (9); type C as in lane 5 at figure (9) and type D as in lanes 6 and 7 at figure (9). On typing of the 66 isolates of C.perfringens by multiplex PCR; 51 isolates belonged to type A, one isolate of type B, one isolate of type C and 13 isolates of type D, which extremely agreed with that detected by traditional method of typing (dermonecrotic test in guinea pigs) in our results, as shown in table (16). So that, C.perfringens isolates could be typed by multiplex polymerase chain reaction (PCR) which is a simple, rapid, accurate and humanitarian method than the traditional method of typing. These results were in agreement with that recorded by Miwa et al., (1996 and 1997), Yoo et al., (1997), Engstrom et al., (2003), Baums et al., (2004) and Heikinheimo and." Korkeala (2005) who typed the five types of C.perfringens (A, B, C, D, and E) by multiplex PCR, as well as with that recorded by Younes (2005) who referred that the two types of toxigenic *C.perfringens* (A and D) were the major toxgenic types recovered from chickens affected with NE.

The isolates that recovered from examined birds and environmental samples, showed identical lanes 6 and 7 of type D. These results were indicated that a multiplex PCR method could be used in typing the prevalent types of *C.perfringens* during the epidemiological studies of chickens NE that agreed with Nauerby et al., (2003), Johansson et al., (2006) and Siragusa et al., (2006). Reviewing available literature; it seems that this is the first study in Egypt which deals with the using of multiplex PCR in detection of toxigenic C.perfringens in the environmental samples. Whereas, Lu et al., (2003), Sira-

gusa et al., (2006) and Johansson et al., (2006) corroborated that PCR technique play a pivotal role in detection of C.perfringens in broiler chicken environmental samples.

REFERENCES

Abd El-Gaber, G., Abd El-Rahman, M., Akeila M., Torky, H. and Meghawery, M. (1994): Prevalence of anaerobic organisms in necrotic enteritis of chickens. Egypt. Vet. Med. Ass. J., 54 (4): 289-301.

Abd El-Gwad, A.M. and Abd El-Kader, H.A. (2001): The occurrence of Clostridium perfringens in the intestine of broiler chickens in Assiut Governorate. Assiut Univ. Bullet. Environ. Res., Vol.(4), No.(2).[Abstract]

Abd El-Rahman, A.A., Shoeib, H.K., Ahmad, M.M. and Abd El-Fattah, A.M. (2001): Health and Economic Hazards of Microbiological contamination of broiler poultry rations and ingredients with special interest to some toxigenic fungi. Assiut Univ. Bullet. Environ. Res., Vol.(4), No.(1).[Abstract]

Abd El-Salam, W.M. (2000): Microbiological studies on anaerobic microorganisms causing diseases in poultry. M.V.Sc. Thesis, Cairo. Uni.

Abd El-Wahab, Hanan, S. (2002): Studies on clostridium microorganisms in newly hatched poultry. M.V.Sc. Thesis, Cairo Uni.

Ahmad, A. and Abd El-Latif, M. (2004): Some studies on clostridia in the balady chickens in Dakahlia province. Ass. Vet. Med. J., 50 (103): 106-119.

Apajalahti, J. and Bedford, M. (2000): Impact of dietary and environmental factors on microbial communities of the avian GI tract. Online 4/8/2002. http:// www.poultry-health.com/fora/inthelth/apabed01.htm.

Awad, F.I.; Bassiouni, A.A., Gadalla, M.S., El-Sisi, M.A. and Hussein, A.Z. (1976): Studies on poultry anaerobes in Egypt. 1. An attempt to isolate anaerobic bacteria from intestinal tract of normal and dead chickens, 2. the effect of alpha and beta toxins of clostridium perfringens type ëAí and ëC" introduced by different routes, 3. the effect of ration on chickens infected with clostridium perfringens typo ëC". Egypt. J. Vet. Sci., 13 (1): 1-22.

Azzam, A.H. and El-Bardiesy, M.M. (1998): Further studies on ulcerative and necrotic enteritis in chickens. BeniSuef Vet. Med. Res., 8 (2): 271-284.

Bains, B.S. (1968): Necrotic enteritis of chickens. Aust. Vet. J.,44: 40.

Barnes, E.M., Mead, G.C., Barnum, D.A. and Harry, E.G. (1972): The intestinal flora of chicken in the period 2 to 6 weeks of age, with particular reference to the anaerobic bacteria. British Poult. Sci., 13: 311-326.

Baums, C.G., Schotte, U., Amtsberg, G. and Goethe, R. (2004): Diagnostic multiplex PCR for toxin genotyping of clostridium perfringens isolates. Vet. Microbiol., 100: 11-16.

Bernier, G., and Filion, R. (1971): Necrotic enteritis in broiler chickens. Journal of the Am. Vet. Med.Assoc., 158(11): 1869-1897.

Branton, S.L., Reece, F.N. and Hagler, W.M. (1987): Influence of a wheat diet on mortality of broiler chickens associated with necrotic enteritis. Poult. Sci., 66: 1326-1330.

Bullen, J.J. (1952): Enterotoxaemia of sheep-

C.perfringens type D in alimentary tract of normal animals. J. Path. Bact., 64:201-206.

Buzby, J.C. and Roberts, T. (1997): Economic costs and trade impacts of microbial food-borne illness. World Health Status Quarterly, 50: 57-66.

Cowen, B.S., Schwartz, L.D., Wilson, R.A. and Ambrus, S.I. (1987): Experimentally induced necrotic enteritis in chicken. Avian Dis., 31(4): 904- 906.

Craven, S.E., Stern, N.J., Bailey, J.S. and Cox, N.A. (2001a): Incidence of Clostridium perfringens in broiler chickens and their environment during production and processing. Avian. Dis., 45: 887-896.

Craven, S.E., Cox, N.A., Stern, N.J. and Mauldin, J.M. (2001b): Prevalence of Clostridium perfringens in commercial broiler hatcheries. Avian Dis., 45: 1050-1053.

Craven, S.E., Cox, N.A., Bailey, J.S. and Cosby, D.E. (2003): Incidence and tracking of Clostridium perfringrns through an integrated broiler chickens operation. Avian Dis., 47: 707-711.

Cruickshank, R., Duguid, J.P., Marmio, B.R. and Swain, R.H. (1975): Medical Microbiology. Vol. 2, 12th Ed., Livingstone, London, and New York.

Dhillon, A.S., Roy, P., Lauerman, L., Schaberg, D., Weber, S., Bandli, D. and Wier, F. (2004): High mortality in egg layers as a result of necrotic enteritis. Avian Dis., 48: 675-680.

Dosoky, R.M. (1990): The role of the environment in the occurrence of clostridia infection among fowls. Assiut Vet. Med. J., 24 (47): 165-171.

El-Ged, A. and Hegazy, A.M. (1985): Studies on the incidence of different types of clostridia in chickens. In., Egypt. Anntls of, Agriculture, Science, Moshtohor 23 (2): 939-945.

El-Refaey, T.M. (1999): Bacteriological studies on Clostridium microorganisms in poultry. M.V.Sc. Thesis, Cairo Uni.

El-Refaey, T.M. (2006): Further Bacteriological and immunological studies on Clostridial microorganisms in poultry. Ph.D. Vet. Thesis, Cairo Uni.

El-Seedy, F.R. (1990): Studies on necrotic enteritis in chickens. Vet. Med. J. Giza, 38 (3): 407-407.

Elwinger, K., Engstrom, B.E., Berndtson, E., Fossum, O. and Teglof, B. (1992): The effects of narasin on clostridium perfringens in caeca and the occurrence of necrotic enteritis in broiler chickens. In, Proceeding XIX World Poultry Cong., Vol. (13), Amesterdam The Netherlands, PP.: 580-584.

Engstrom, B.E., Fermer, C., Lindberg, A., Saarinen, E., Baverud, V. and Gunnarsson, A. (2003): Molecular typing isolates of clostridium perfringens from healthy and diseases poultry. Vet. Microbiol., 24: 225-235.

Farid, A., Oof, F. and Abd El-Ghani, M. (1978): A note on necrotic enteritis in chickens in Egypt. Vet. Med. J., Giza, 26: 281-287.

Ficken, M.D. and Wages, D.P. (1997): Necrotic enteritis. In, B.W. Calnek (Ed.), Diseases of Poultry 10th edn (pp.261-264). Ames, IA: Mosby-Wolfe.

Fukata, T., Hadate, Y., Baba, E., Uemura, T. and Arakawa, A. (1988): Influence of Clostridium perfringens and its toxin in germ-free chickens. Research in Vet. Sci., 44: 68-70.

Gholamiandekhordi, A.R., Ducatelle R., Heyndrickx M., Haesebrouck F., Van Immerseel, F. (2005): Molecular and phenotypical characterization of Clostridium per-

fringens isolates from poultry flocks with different disease status. Vet. Microbiol. [Abstract

Heijnen, G. (2004): Conditioning and pelleting. Heikinheimo, A . and Korkeala, H . (2005): Multiplex PCR assay for toxinotyping Clostridium perfringens isolates obtained from Finnish broiler chickens. Lett. Appl. Microbiol. , 40(6):407-411.

Hikal, M. A. (1999): Studies on necrotic in chickens. M.V.Sc., Thesis, Zagazig Uni.

Hofacre, C.L., Froyman, R., Gautrias, B., George, B., Goodwin, M.A. and Brwon, J. (1998): Use of Aviguard and other intestinal byproducts in experimental Clostridium perfringens-associated necrotizing enteritis in broiler chickens. Avian Dis., 42 (3): 579-584.

Hofacre, C.L., Beacorn, T., Collett, S. and Mathis, G. (2003): Using of competitive exclusion, mannan-oilgosacchardie and other intestinal products to control necrotic enteritis. J. Appl. Poult. Res., 12:60-69.

Hofshagen, M. and Stenwig, H. (1992): Toxin production by Clostridium perfringens isolated from broiler chickens and Capercaullies (Tetrao urogallus) with and without necrotizing enteritis. Avian Dis., 36: 837-843.

Hussein, A.E.Z. (1972): Studies on poultry anaerobes. M.V.Sc. Thesis, Cairo Uni.

Ibrahim, A.A. (1979): Anaerobic microflora of the small intestine of chickens. M.V.Sc. Thesis, Assiut Uni.

Ibrahim, R.S., Monazi, Ibthal, L.M. and Soliman, A.M. (2001): Epidemiological studies on intestinal Clostridial infection in broiler chickens in Assiut and El-Menia governorates. Assiut Vet. Med. J., 44 (88):291-205.

Jackson, M.E., Anderson, D.M., Mathis, G.F. and Fodge, D.W. (2003): Beneficial effect of fl-mannanase feed enzyme on performance of chickens challenged with Eimeria species and Clostridium perfringens. Avian Dis., 47: 759-763.

Johansson A., Anna Aspan A., Bagge E, BÂverud V., Engstrom B.E., and Johansson K.E. (2006): Genetic diversity of Clostridium perfringens type A isolates from animals, food poisoning outbreaks and sludge. BMC Microbiology, 6:47, doi:10.1186/1471-2180-6-47.

Julian, I. Rood (1998): Virulence genes of Clostridium perfringens. In, Annu. Rev. Microbiol., 52: 333-360.

Kaldhusdal, M. and Hofshagen, M. (1992): Barley inclusion and avoparcin supplementation in broiler diets, 2. clinical, pathological and bacteriological findings in a mild form of necrotic enteritis. Poult. Sci., 71: 1145-1153.

Kaldhusdal M. and Lovland, A. (2000): The economical impact of Clostridium perfringens in greater than anticipated. World poultry, 16: 50-51.

Kaldhusdal, M., Hofshogen, M., Lovland, A., Langstrand, H. and Redhead, K. (1999): Necrotic enteritis challenge models with broiler chickens raised on litter. Evaluation of preconditions, Clostridium perfringens strains and outcome variables. FEMS microbial. Med. Immunol., 24: 337-343.

Kaldhusdal M., Schneitz, C., Hofshagen, M. and Skjeve, E. (2001): Reduced incidence of Clostridium performance-associated lesions and improved performance in broiler chickens treated with normal intestinal bacteria from adult fowl. Avain Dis., 45, 149-156.

Katitch, R., Chibalitch, S., Kositch, L.J., Voukitchevitch, Z., Djoukitch, B. and Tomanovitch, B. (1966): Role of

E.tenella and E.necatrix in the epidemiology of enteritis caused by C.perfringens type C in chicks. Bull. Acad. Vet., 39: 101-107.

Kim, H.J., Kang, M.I. and Chung, U.I. (1996a): Effect of infectious bursal disease virus infection on the pathogenesis of necrotic enteritis in chickens. Korean J. of Vet. Res., 36(4): 997-1005.

Kim, H.J., kang, M.I. and Chung, U.I. (1996b): Survey of enteric diseases of chickens. Korean J. of Vet. Res., 36 (4): 1007-1012.

Kocher, A. (2003): Nutritional manipulation of necrotic outbreak in broiler. Recent Adv. in Animal Nutrition in Australia, 14: 111-116.

Kohler, B. (2000): Clostridium perfringens intoxication affects bird performance. World Poultry, 16 (5), 57 ñ 59.

Kohler, B., Vogel, K. and Starost, P. (1977): Necrotic and Ulcerative enteritis in fattening and laying fowls under condition of intensive poultry production. Monatshelte f‹r veterinármedizin, 29 Heft (18): 704-711.

Koneman, E.W., Allen, B.D., Dowell, V.R. and Sommers, H.M. (1988): Color Atlas and Text Book of Diagnostic Microbiology. Ed., J.B. Lip. Co., New York and London.

Kuliler, B. and Pilsl, H. (2001): Investigation about occurrence, etiology, experimental reproduction and control of toxi-infections caused by clostridium perfringens in chickens. Abstract in proceedings XII Int. cong. World Poult. Ass., pp: 219.

Latinovic, V.C. (1983): Study of characteristics of Clostridium perfringens strains isolated from broiler with enteritis. Veterinaria (Yugoslavia), 32 (2): 267-275.

Long, J.R. (1973): Necrotic enteritis in broiler chickens. I. A review of the literature and the prevalence of the disease in Ontario. Can. J. Comp. Med., 37: 302-308.

Lovland, A. and Kaldhusdal, M. (1999): Liver lesions seen at slaughter as an indicator of necrotic enteritis in broiler flocks. FEMS Immunol. Med. Microbial., 24: 345-351.

Lovland, A. and Kaldhusdal, M. (2001): Severely impaired production performance in broiler flocks with high incidence of Clostridium perfringens associated with hepatitis. Avian Pathol., 30: 73-81.

Lu, J., Sanchez, S., Hobacre, C. Maurer, J.J., Haron, B.G. and Lee M.D. (2003): Evaluation of broiler litter with reference to the microbial composition as assessed by using 16S rRNA and functional gene markers. Appl. and Environ. Microb., pp: 901- 908.

Mark, G.W. and Siragusa, G.R. (2005): Quantitative Detection of Clostridium perfringens in the Broiler Fowl Gastrointestinal Tract by Real-Time PCR. Appl. Environ. Microbiol., 71(7): 3911-3916.

Meghawery, M.A. (1993): Characterization of anaerobic microorganisms isolated from chickens suffering from necrotic dermatitis and necrotic enteritis. M.V.Sc. Thesis, Alexandria Uni.

Miwa, N., Nishina, T., Kubo, S. and fujikura, K. (1996): Nested polymerase chain reaction for detection of low levels of enteroxigenic Clostridium perfringens in animals faeces and meat. J.of Vet. Med. Sci., 58: 197-203

Miwa, N., Nishima, T., kubo, S. and Atsumi, M.C. (1997): Most probable number method combined with nested polymerase chain reaction for detection and enumeration of enterotoxigenic Clostridium perfringens in intestinal contents of cattle, pig and chickens. J. of Vet.

Med. Sci., 59 (2): 89-92.

Monazi, Ebtehal, M. (2000): Studies on intestinal Clostridial infections in broiler chickens. M.V.Sc. Thesis, Assiut Uni.

Mustafa, M.G. (2000): Some studies on Clostridial infection in birds. M.V.Sc. Thesis, Zagazig Uni.

Narin, M.E. and Bamford, V.M. (1967): Necrotic enteritis of broiler chickens in Western Australia. Aust. Vet., 43: 49-54.

Nauerby, B, Pedersen K, Madsen M. (2003): Analysis by pulsed-field gel electrophoresis of the genetic diversity among Clostridium perfringens isolates from chickens. Vet. Microbiol., 94:257-266.

Neumann, T., Dunham, S., Skalecki, J. and Rehberger, T. (2007): RAPD comparison of Clostridium perfringens isolated from cases of Necrotic Enteritis and Gangrenous Dermatitis. Abstract, In; International Poultry Scientific Forum, pp:52; Georgia World Congress Center, Atlanta, Georgia.

Olkowski, A.A.,Wojnarowicz, C .,Chirino-Trejo, M . and Drew, M.D . (2006): Responses of broiler chickens orally challenged with Clostridium perfringens isolated from field cases of necrotic enteritis. Res. Vet. Sci ., 81(1):99-108.

Parish, W.E (1961): Necrotic enteritis in the fowl (Gallus gallus domesticus). 1. Histopathology of the disease and isolation of the strain of Clostridium welchii. 11.The experimental disease. 111.Examination of the causal Clostridium welchii. J. Comp. Pathol., 71: 377-414.

Petit, L., Gilbert, M. and Popoff, M.R. (1999): Clostridium perfringens: toxinotype and genotype. Trends in Microbiology, 7: 104-110.

Prukner, R.E. and Milakovic, N.I. (1991): Prevalence and epidemiological significance of Clostridium bacteria in poultry. Veterinarska Stanico, 22(5): 259-264.

Riddell, C. (1987): Avian Histopathology, Pennsylvania, PA: American Association of Avian pathologists.

Riddell, C. and Kong, X.M. (1992): The influence of diet on necrotic enteritis in broiler chickens. Avian Dis., 36: 499-503.

Roberts, R.S., Guven, S. and Worrall, E.E. (1970): Studies on clostridium oedematiens. J. Comp. Path., 80: 9-18.

Shane, S.M., Koetting, D.G. and Harringotn, K.S. (1984): The occurrence of C.perfringens in the intestine of chicks. Avian Dis., 28 (4): 1120-1124.

Sinha, S.M., Willinger, H. and Trcka, J. (1975): Studies on the occurrence of Clostridium perfringens in fowls. Wiener Tieráztliche Monatacgrift, 62 (5): 163-169.

Siragusa, G.R., DanyLuk, M.D., Hiett, K.L., Wise, M.G. and Craven, S.E. (2006): Molecular subtyping of Poultry-Associated Type A Clostridium perfringens isolates by Repetitive-Element PCR. Journal of Clinical Microbiology, Vol. 44 (3): 1065-1073.

Smith, L.D. (1975): "The pathogenic anaerobic bacteria". 2nd Ed., Charles C- Thomas publisher, U.S.A

Smith, L.D. and Holdeman, L.V. (1968): The pathogenic anaerobic bacteria. 1st Ed., Charles C- Thomas Publishers, U.S.A., pp.201-205.

Songer, J.G. and Meer, R.R. (1996): Genotyping of Clostridium perfringens by polymerase chain reaction is a useful adjunct to diagnosis of Clostridial enteric disease in animals. Anaerobe, 2: 197-203.

Stern, M. and Batty, I. (1975): Pathogenic Clostridia. Bu-

terworths, London, Boston.

Tschirdewahn, B., Notermans, S., Wernars, K. and Untermann, F. (1992): The presence of enterotoxigenic Clostridium perfringens strains in feces of various animals. Int. J. Food Microbiol., 14 (2): 175-178.

Wages, D. and Opengart, K. (2003): Necrotic Enteritis. 11th Ed., In, Diseases of poultry. Iowa state press, pp: 775-795.

Wilkie, D.C., Van Kessel, A.G., Dumonceaux, T.J. and Drew, M.D. (2006): The effect of hen-egg antibodies on Clostridium perfringens colonization in the gastrointestinal tract of broiler chickens. 74 (4): 279-292.

Willis, A.T. (1977): Anaerobic Bacteriology in clinical Medicine. 2nd Ed., London, Butterworths.

Willis, A.T. and Hobbs, G. (1959): Some new media for the isolation and identification of Clostridia. J. Path. Bact., ZZ: 511.

Yoo, H. S., Lee, S.U., Park, K.Y. and Park, Y.H. (1997): Molecular typing and epidemiological survey of prevalence of Clostridium perfringens types by multiplex PCR. J. Clin. Microbiol., 35(1):228-232.

Younes, A.M.A. (2005): Use of polymerase chain reaction (PCR) for diagnosis of Clostridium perfringens in chickens. M.V.Sc. Thesis, Cairo Uni.

الوضع الحالى لعدوى كلستريديم بيرفرنجينز في كتاكيت التسمين

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أجريت هذه الدراسة علي 728 عينة من الكبد و الأمعاء ، جمعت من 84 مزرعة دجاج تسمين تجاري و سلالات محلية مهجنة من أعمار 1- 11 أسبوع في محافظات البحيرة و كفر الشيخ و الغربية . أظهرت نتائج الفحص البكتريولوجي اللا هوائي عزل 430 معزولة من الكلستريديم ، والتي تم تصنيفها إلي كلستريديم بيرفرنجينز (54.9%) و أنواع كلستريديم أخرى (45.1%). وجد أن عدد 61 من إل 236 معزولة للكلستريديم بيرفرنجينز كانت من النوع الممرض ، و التي تم تصنيفها إلي الأنواع أ (83.6%) و ب (1.6%) وج (1.6%) و د (13.1%) . تم عزل الكلستريديم بيرفرنجينز كذلك من مياه الشرب والعلف و الفرشة المحيطة بالطيور بنسبة 27.3% و 50% و 22.7% علي التوالي (5 معزولات منهم وجدت ممرضة من النوع د) . وصل معدل النفوق إلي 100% نتيجة الحقن العضلي بمستنبت الشوربة الذي يحوي خليط من النوعين أ و د لكتاكيت دجاج تسمين عمر 5 يوم . نجح اختبار تفاعل البلمرة المتسلسل في الكشف عن كل من الجينات الخاصة بكلّ من ألفا و بيتا و إبسيلون في تفاعل واحد و كذلك تصنيف عترات الكلستريديم بيرفرنجينز بصورة مطابقة للأنواع أ و ب و ج و د . أوضحت هذه النتائج أن اختبار تفاعل البلمرة المتسلسل السريع و الدقيق و ذا البعد الإنساني يمكنه أن يحل محل اختبار التعادل المصلي في الفئران أو الحقن في خنازيرغينيا في التعرف و التصنيف للأنواع الممرضة من الكلستريديم بيرفرنجينز .