The Prevalence of *Clostridium Perfringens* in Healthy and Diseased Field Chickens with Necrotic Enteritis

Eman R. Hassan[b] Magdy F. El Kady[a] Ismail Abd EL-Hafeez Radwan[a]. Nagwa, S. Rabie[b] and Mohamed M. Ahmed[b]

[a] Poultry Disease Department, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef, Egypt
[b]Poultry Disease Department, Veterinary Research Division, National Research Center, Cairo, Egypt
prof_emy@yahoo.com

Abstract: This study was conducted to a detection of *Clostridium perfringens* (*C. perfringens*) infection in chickens which cause necrotic enteritis (NE) disease from field cases collected from kaluobia, El-menia, Fayoum and Giza governorates. A total of 360 chickens are examined (150 local breed chickens out of these 50 chickens were apparently healthy and 100 diseased chickens showing clinical signs suspected to be NE) and 210 Foreign breed chickens (hubbard and cubb breed chickens) apparently healthy and diseased were collected for 4years from 2006-2009 at different seasons *C. perfringens* was identified by biochemical test and Polymerase Chain Reaction PCR). The result indicated that the incidence was higher in winter and autumn then spring and summer. The rate of isolation of *C. perfringens* is higher in imported breed than local breed along 4 years Out of 15 *C. perfringens* isolates, from apparently healthy in the intestine 16.7% isolates were toxigenic (type A) and 83.3%isolates were non toxigenic .In liver there is no toxigenic isolates but there is 100% non toxigenic isolates. From diseased chickens with NE that out of 60 *C. perfringens* isolates from the intestine 79.3%isolates were toxigenic type A and 20.6% isolates were non toxigenic.In the liver 100% isolates were toxigenic and 0% isolates nontoxigenic
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1. Introduction

Necrotic Enteritis (NE) is an enterotoxaemic disease in chickens, caused by toxin of *C. perfringens* types A and C. NE is a worldwide poultry disease caused by the alpha toxin-producing bacterium *C. perfringens* ***(Takeda et al., 1995)*** including Egypt ***(Awaad et al., 1976; El-Sisi and Hussein, 1976; Merati, 2010)***.

C. perfringens is a Gram-positive toxin producing anaerobic bacterium ***(Saif et al., 2003***), which occurs normally in the intestine, a high numbers of such bacteria coincide with a damaged intestinal mucosa ***(Al-Sheikhly and Truscott, 1977a)***, the disease results from the high frequency of adhesion by *C. perfringens* to the damaged mucosa ***(Kageyama et al., 1987; Baba et al., 1992 a)***, facilitating bacterial proliferation and toxin production. *C. perfringens* toxigenic strains were isolated from both diseased and healthy chickens ***(Timbermont et al., 2009)*** NE is a globally important welfare and economic problem ***(Copper et al, 2009)*** in chickens causing economic losses due to mortalities, low growth rate and feed conversion ***(Lovland and Kaldhusdal, 2001)*** as well as costs associated with disease prevention. In the other hand; it is difficult to determine the prevalence of the mild infection in chickens that cause higher condemnation rates in broilers due hepatitis ***(Lovland and Kaldhusdal, 1999).***

1.***Material and Methods***

. Isolation and identification

A total of 360 chickens (apparently healthy and diseased) were collected in different years seasons. These flocks were reared in EL- kaluobia, El-menia, Fayoum and Giza Governorates.

150 examined local breed chickens (50 chickens were apparently healthy and 100 diseased chickens showing clinical signs suspected to be NE.)

and 210 examined hubbard and cubb breed chickens (70 chickens were apparently healthy and 140 diseased chickens showing clinical signs suspected to be NE)

Parts of small intestine and liver were collected from sacrified and freshly dead chickens flocks showed postmortem lesions of NE, each collected sample was inoculated into tubes of cooked meat medium and incubated anaerobically for 24 hrs at 37°C.

A loopful of inoculated fluid media was streaked onto Neomycin sulphate sheep blood agar plates and incubated anaerobically at 37° C for 24hs.

Suspected colonies of were subcultured onto two plates of 10% sheep blood agar and egg yolk agar plates. One plate was incubated aerobically and the other plates were incubated anaerobically. The colonies grew in anaerobic condition and lecithinase producer were picked up and tested by catalase test.

Colonies that were Catalase negative and lecithinase producer colonies were purified. The obtained isolates were tested for biochemical reaction: suger fermentation test, gelatin test , indol test and urease test **(Murray, et al.,(2003)** and Multiplex PCR for genotyping of *C. perfringens* isolated from diseased and apparently healthy chickens blood agar and egg yolk agar plates. One plate was incubated aerobically and the other plates were incubated anaerobically. The

colonies grew in anaerobic condition and lecithinase producer were picked up and tested by catalase test.

2. Material and Method

Running of Polymerase chain reaction PCR according to *(Yoo et al., 1997):*

The amplified reactions were performed in 50 ul volumes in microamplification PCR tubes. The reaction mixture consisted of 1µl (200 ng) of extracted DNA template from bacterial cultures, 25 µl of master mix, 1µl (50 pmol) from each primer pairs and the volume of the reaction mixture was completed to 50 µl using DDW. 40 µl paraffin oil was added and the thermal cycler was adjusted as follow:

Initial denaturation: 94^{0}C/5 minutes

First cycle:

Denaturation---------- 94^{0}C/1 minute.
Annealing---------- 55ºC / 1 minute.
Extension---------- 72^{0}C/1 minute.

The first cycle repeated 30 times.

Final extension: 72^{0}C/10 minutes

The PCR products were stored in the thermal cycler at 4^{0}C until they were collected.

Oligonucleotide primers: Used for amplification of DNA recovered from bacterial isolates *(Yoo et al., 1997).*

Specific oligonucleotide primers for the four toxin genes α-, β-,ε- and iota of *C. perfringens* were selected.

Table1. Nucleotide sequences of primers used in this study:

Primer (direction)	Nucleotide sequence	location	Size(Pb) of Amplified products
CPA (alpha toxin) forward Reverse	5́-GTTGATAGCGCAGGACATGTTAAG-3́ 5́-CATGTAGTCATCTGTTCCAGCATC-3́	511-535 913-889	402
CPB (beta toxin) forward Reverse	5́-ACTATACAGACAGATCATTCAACC-3́ 5́-TTAGGAGCAGTTAGAACTACAGAC-3́	589-613 824-801	236
CPE (epsilon toxin) forward Reverse	5́-ACTGCAACTACTACTCATACTGTG-3́ 5́-CTGGTGCCTTAATAGAAAGACTCC-3́	436-459 976-953	541
CPI (iota toxin) forward Reverse	5́-GCGATGAAAAGCCTACACCACTAC-3́ 5́-GGTATATCCTCCACGCATATAGTC-3́	563-583 879-856	317

Results are shown in tables (2-5), Plates (1-4) and figs (1and 2)

3. Results and Discussion:

There were no marked clinical signs or mortality detected during the isolation, this finding agreed with ***Cowen et al., (1987)*** who reported only a small incidence of NE in some chickens challenged with C *perfringens* but failed to induce signs of NE in others.

Suspected lesions were seen in middle intestine that had friable wall and distended with gases. Intestinal mucosa was covered by a loose or adherent yellow to green necrotic membrane with or without haemorrhages plate(1). ***Ficken and Wages, 1997,*** recorded that , the disease can be divided into 2 categories, clinical and subclinical NE. Clinical signs of NE include depression, decreased appetite, diarrhea, and severe necrosis of the intestinal tract. This intestinal damage will result in release of plasma proteins into the lumen of the intestinal tract. Since the minimal requirements for growth of C. *perfringens* include more than 11 amino acides besides many factors and vitamins ***(Boyd et al., 1948; Petit et al., 1999)***, leaking of plasma to the intestinal lumen can provide a necessary growth substrate for extensive proliferation of these bacteria. It is also proven that diet strongly influences the incidence of NE, diets with high levels of indigestible, water-soluble- non-starch polysaccharides, known to increase the viscosity of the intestinal contents, predispose to NE. This viscosity of the diets rich in rye, wheat and barely relative to diets rich in corn ***(Branton et al., 1987; Kaldhusdal and Hofshagen, 1992*; *Riddell and kong, 1992; Kocher, 2003*)** also, diet rich in fish meal predispose to necrotic enteritis ***(Kocher, 2003).*** Poor hygienic and housing conditions such as rising on litter floor are greatly associated with NE disease ***(Frame and Bickford., 1986).*** Feed stuffs rich in zink contributes to the protection of alpha toxin from proteolysis by trypsin ***(Sato et al., 1978; Baba et al., 1997)*** now explained by the finding that alpha toxin is a zinc-metalloenzyme **(*Naylor et al., 1998).***

In the present work, (table 2) and(fig. 1) the seasonal distribution of *C.perfringens* isolation from samples collected from field cases of chickens was 23 in autumn, 31 in winter, 14 in spring and 10 in summer ;respectively. The result indicated that the incidence was higher in winter and autumn then spring and summer .these result agree with the finding of ***Berinier et al., (1974)*** who recorded sudden onset of NE in Québec with high mortality 75% occurred in chicks between 2 and 4 weeks of age and with greatest incidence observed between May and November.

Also, ***Cygan and Nawak, (1974)*** reported NE with high mortality rate, mostly chicks aged *4*-7 weeks, with high incidence between April and October.

The isolation of *C. perfringens* with relation to season in broilers was 17 in autumn, 12 in winter, 9 in spring and 7 in summer; respectively. While in layer was 4 in autumns , 19 in winter, 4 in spring and 3 in summer; respectively. So there is significant difference in the incidence of *C.perfringens* according to season as previously mentioned if compared with the difference in the breed.

The rate of isolation of *C. perfringens* is higher in imported breed than local breed along 4 years (table3). Bacteriological examination of 120 samples of apparently healthy chickens (56 samples from local breed and 64 from broiler chickens); resulted in isolation 6.25% (10% from the intestine and 2.5%; from the liver). Examination of 240 samples from diseased chickens (102 samples from local breed and 138 samples from broiler chickens) with NE was resulted in isolation of 12.5% (24.17% from the intestine and %0.83 from the liver) (table 4)this result agreed with ***Sasaki et al., (2000)***who found that45 broiler carcasses with liver lesions. All affected chickens showed the liver enlargement with discoloration. It was suggested that *C. perfringens* may be important in the pathogenesis of cholangio-hepatitis in broiler chickens. Results of this study reveal that the *C. perfringens* type A was isolated from broiler chickens with higher prevalence than local breed this due to presence of antibiotic (flavomycin) in ration used in these farms.

Out of 15 *C. perfringens* isolates, from apparently healthy in the intestine 16.7% isolates were toxigenic (type A) and 83.3%isolates were non toxigenic .In liver there is no toxigenic isolates but there is 100% non toxigenic isolates.

From diseased chickens with NE that out of 60 *C. perfringens* isolates from the intestine 79.3%isolates were toxigenic type A and 20.6% isolates were non toxigenic.In the liver 100% isolates were toxigenic and 0% isolates nontoxigenic (table 5) this result agreed with ***Sedeek-Dalia, (2010)*** who isolated 25 *C. perfringens* isolates from 120 intestinal and liver samples of apparently healthy chickens and found that only 10 isolates were toxigenic type A. while 36 *C. perfringens* isolates from 80 intestinal and liver samples of diseased chickens were typed to 25 isolates toxigenic type A.

Multiplex PCR was done with the primers mixture of alpha, beta, epsilon and iota toxins genes to determine the type of toxin of the 50 isolates of *C. perfringens* recovered from the intestine and the liver samples (48 from diseased chickens with necrotic enteritis and 2 from apparently healthy chickens). All examined 50 isolates were positive for α-toxin gene with amplified PCR product of 396 bp in comparison with the standard molecular size marker, 100 bp (plate 4) ***Dahillon et al., (2004)***recorded that NE was diagnosed as the cause of increased mortality ,the bacterium isolated on anaerobic culture was identified as *C. perfringens* by PCR.

In conclusion we found that ;

1. The incidence of NE was higher in autumn and winter than summer and springthe rate of isolation of *C. perfringens* is higher in imported breed than local breed along 4 years
2. The typed isolates were represented only by type A with higher prevalence from disease than healthy birds and from intestine more than liver.

Plate (1): Parts of chicken intestine of field cases with lesions suspected to be NE.

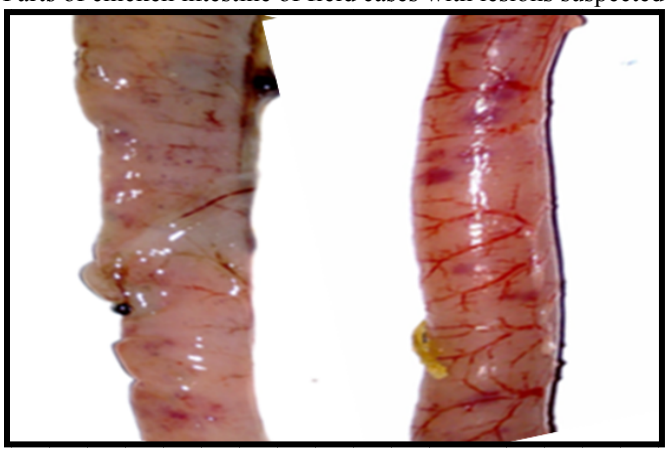

Table (2): No. of chicken flocks, age, mortality rate and no. of samples/flock in Year and Season

Year	Season	Diseased chickens								Apparently healthy chickens						
		Flock No.	Breed	Age/Day	Dis.mort. %/ Day	Sample No. /farm	+ Ve	%		Flock No.	Breed	Age/day	Dis.mort. %/Day	Sample No./ farm	+ Ve	%
2006	Winter	1	L	18	1.5	15	6	40		1	B	20	1.5	5	0	0
		2	L	30	3	10	8	80		2	B	30	0	5		
		Total				25	14	56						10		
	Spring	1	B	40	3	10	1	10		1	L	40	1	2		
		2	L	46	1	10	1	10		2	L	46	1	3		
		Total				20	2	10						5		
	Autumn	1	B	35	2	5	3	60		1	B	35	0	3		
		2	B	45	2.5	5	2	40		2	L	45	0	2		
		Total				10	5	50						5		
	Summer	1	B	20	4	5	1	20		1	B	28	2	3		
		2	B	35	1.5	5	1	20		2	B	30	2	2		
		Total				10	2	20						5		
Total						65	23	35.4						25		
2007	Winter	1	B	45	0.3	5	2	40		1	L	45	2	7	1	14.28
		2	L	46	1	6	2	33.3								
		3	B	49	1.5	5	1	20								
		4	B	45	2	4	1	25								
		Total				20	6	30						7	1	14.28
	Spring	1	B	25	0.1	5	1	20		1	B	35	1	9	2	33.33
		2	B	35	0.5	10	1	10								
		Total				15	2	13.3						9	2	33.33
	Autumn	1	B	20	1.5	2	2	100		1	B	25	2	2	1	50
		2	L	30	2.5	10	1	10		2	B	30	3.5	2	1	50
		Total				12	3	25						4	2	50
	Summer	1	B	30	1	7	1	14.3		1	L	35	2	5	2	40
		2	L	38	1.5	3	0	0								
		Total				10	1	10						5	2	40
Total						57	12	21						25	8	32

Table (2): Continue

Year	Season	Diseased chickens							Apparently healthy chickens						
		No. of flocks	Bread	Age/Day	Dis.mort. rate /Day	Sample No./ flock	+ Ve	%	No. of flocks	Bread	Age/Day	Dis.mort. Rate /Day	Sample No./ flock	+ Ve	%
2008	Winter	1	B	27	3	10	2	20	1	B	27	3	8	1	12.5
		2	L	35	3.5	6	1	16.7	2	B	38	3.5	8	1	12.5
		Total				16	3	18,8					16	2	12.5
	Spring	1	L	45	2	4	0	0	1	L	45	2.5	8	1	12.5
		2	B	50	2.5	6	2	33.3	2	B	47	2.5	7	1	14.3
		Total				10	2	20					15	2	13.3
	Autumn	1	B	35	2	7	2	28.6	1	L	38	3	8	1	12.5
		2	L	40	2.5	10	1	10							
		Total				17	3	17.6					8	1	12.5
	Summer	1	B	25	2	5	1	20	1	B	25	2.5	5	1	20
		2	B	35	3	5	1	20							
		Total				10	2	20					5	1	20
	Total					53	10	18.9					44	6	13.6
2009	Winter	1	B	30	2	12	4	33.3	1	L	42	3.5	6	1	16.7
		Total				12	4	33.3					6	1	16.7
	Spring	1	L	25	1	8	1	12.5	1	L	30	1	8	1	12.5
		2	B	30	1.5	7	1	14.3							
		Total				15	2	13.3					8	1	12.5
	Autumn	1	B	29	0.2	18	6	33.3	1	L	35	1.5	7	0	0
		2	L	39	1	10	1	10							
		Total				28	7	28.6					7	0	0
	Summer	1	L	35	0.5	10	1	10	1	B	40	2	5	1	20
		Total				10	1	10					5	1	20
Total						65	14	21.5					26	3	11.5

L: local bread
B: broiler chicken

Figure (1): Percentage of *C. perfringens* from examined field cases from 2006-2009 in relation to season.

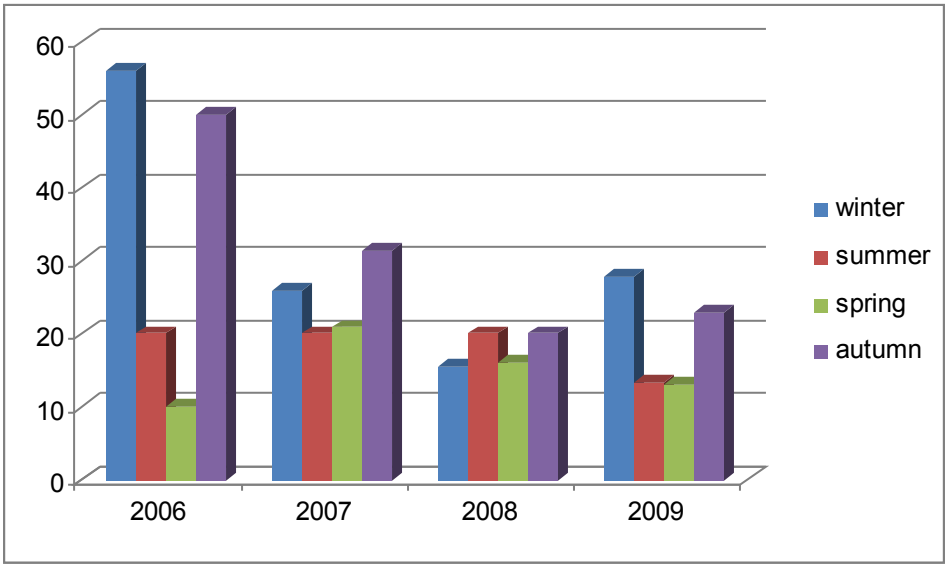

Table (3): Incidence of *C. perfringens* isolated from local and imported chicken breeds.

year	Local breeds.		Imported breeds	
	+Ve	%	+Ve	%
2006	15/23	30.4	8/23	69.6
2007	5/18	33.3	13/18	77.8
2008	4/16	25	12/16	75
2009	5/17	27.8	12/17	94.1

Table (4): Prevalence rate of *C. perfringens* isolated from apparently healthy and diseased chickens.

Chickens	Sample	No. of examined samples	+Ve Samples	
			No.	%
Apparently Healthy	Intestine	120	12	10
	Liver	120	3	2.5
Total chickens examined		120	15	6.25
Diseased	Intestine	240	58	24.17
	Liver	240	2	0.83
Total chickens examined		240	60	12.5

Table (5): Typing of *C. perfringens* isolated from apparent healthy and diseased chickens.

Chickens	Types of semples	No. of + ve semples	Types of *C. perfringens*			
			Toxigenic type A		Non-toxigenic	
			N0	%	No.	%
Apparently Healthy	**Intestine**	12	2	16.7	10	83.3
	Liver	3	0	0	3	100
	Total	15	2	13.3	13	86.7
Diseased	**Intestine**	58	46	79.3	12	20.6
	Liver	2	2	100	0	0
	Total	60	48	80	12	20
Over all total		**75**	**50**	**66.7**	**25**	**33.3**

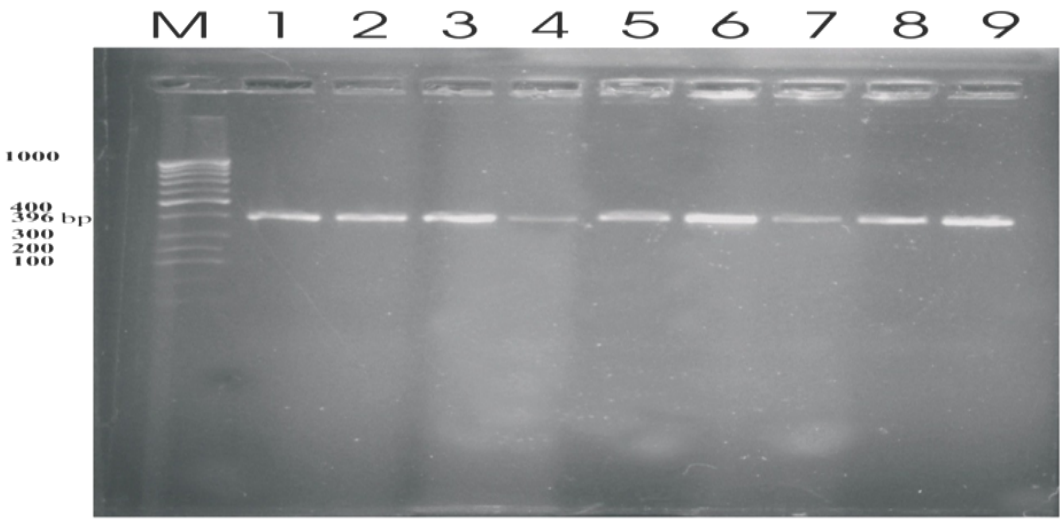

Plate (4): Multiplex PCR for genotyping of *C. perfringens* isolated from diseased and apparently healthy chickens.
M lane: 100 bp ladder
Lan 1 control positive alpha toxin
Lan 2, 3, 4, 5, 6, 7, 8, 9 positive C.*perfringens* field isolates type A

All examined 50 isolates were positive for α-toxin gene with amplified PCR product of 396 bp in comparison with the standard molecular size marker (100 bp).

Corresponding author
Eman R. Hassan
Poultry Disease Department, Veterinary Research Division, National Research Center, Cairo, Egypt
prof_emy@yahoo.com

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