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ASSOCIATED PROFILES OF VIRULENCE GENE MARKERS IN *CLOSTRIDIUM PERFRINGENS* STRAINS ISOLATED FROM HEALTHY AND DISEASED BROILER CHICKENS WITH NECROTIC ENTERITIS

(With 3 Tables and 4 Figures)

By

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البروفيل المصاحب لدلالات جينات الضرواة لعترات الكلوستيريديم بيرفيرنجينز المعزولة من دجاج التسمين السليم والمصاب بمرض الالتهاب المعوى النخرى

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تعتبر الكلوستيريديم بيرفيرنجينز المسببة لمرض الالتهاب المعوى النخرى من المخاطر الكبيرة لصناعة دجاج التسمين لما تسببه من احداث وفيات عالية ونقص فى معدل النمو بالاضافة الى الاصابة بالتسمم المعوى فى الانسان. وجد أن أفراز السموم من ميكروبات الكلوستيريديم بيرفيرنجينز من العوامل الاساسية لأحداث المرض. أجرى هذا البحث لمعرفة نسبة تواجد الكلوستيريديم بيرفيرنجينز فى دجاج التسمين السليم والمصاب بالالتهاب المعوى النخرى، وكذلك مدى تواجد الانواع المختلفة لهذا لميكروب عن طريق الكشف عن دلالات الجينات السمية (alpha (*cpa*), beta (*cpb*), epsilon (*etx*), iota (*iAp*)) وقد شملت الدراسة أيضا مدى انتشار ثلاث من أهم جينات السموم لميكروب الكلوستيريدم وهم (*cpe*, *cpb2* and *netB*). تم فحص عدد ٥٦ عينة مجمعة (n=4) من امعاء وكبد الطيور المصابة و ٢٧ عينة مجمعة من المسحات الشرجية لدجاج التسمين السليم.أوضحت نتائج العزل وجود عدد ٤٧ عترة من الكلوستيريديم بيرفيرنجينز من دجاج التسمين المصاب بنسبة٨٣،٩% و١٤ عترة بنسبة٥١،٨٥% من الدجاج السليم.وللتفريق بين أنواع الكلوستيريديم المعزولة تم استخدام تفاعل البلمرة المتسلسل المتعدد حيث ثبت ناجحه فى التفريق بين أنواع الكلوتيريديم المختلفة واختصاره للوقت وتوفيره للنفاقات اذا اتخدمت الطرق التقليدية(Mouse Toxin Neuratlization Test). وجد أن كل العترات تحمل جين *cpa* مما دل على أن جميع العترات من النوعA. وفى خطوة تعتبر الاولى من نوعها فى مصر تم تحديد البروفيل الخاص بجينات الضرواة للعترات المعزولة من

ال*C. perfringens*. وجد أن البروفيل الجينى بالنسبة لجين التسمم المعوى *cpe* هو عدم امتلاك اى من العترات المصاحبة للدجاج السليم أو المصاب لهذا الجين. على العكس وجد أن جين السموم القبيةال*netB* عالي التواجد فى عترات الكلوستيريديم بيرفيرنجينز المعزولة من الطيور المصابة عنها فى الطيور السليمة مما يعطى دلالة على ايجابية مشاركة هذا الجين فى أحداث المرض. أعطيت هذة الدراسة نظرة جديدة على التكوين الجينى لعترات الكلوستيريدم المعزولة من دجاج التسمين بأسيوط الذى بدوره سيساعد على فهم كيفية التغلب على هذا المرض والسيطرة عليه عن طريق عمل اللقاحات المناسبة لمنع انتشاره بين الطيور والانسان.

SUMMARY

Clostridium perfringens induced necrotic enteritis (NE) and subclinical disease have become important threats to poultry health and is one of the main causes of losses in broiler flocks due to high mortalities and reduction in growth rate as well as enterotoxemias in domestic animals and humans . The mechanism of virulence of *C. perfringens*, a bacterium causing necrotic enteritis in birds, results largely from its ability to produce toxins. A study was set up to look the rate of carriage of *C. perfringens* among broilers with different heath status, incidence rate of the different toxin genotypes of *C. perfringens* in healthy and diseased birds and, lastly, the relative abundance of *cpe*, *cpb2* and *netB* virulence genes. Broiler chickens from healthy flocks were sampled by cloacal swabs, while intestinal and liver samples of birds suffering from necrotic enteritis were analysed. A total of 47 (83.9%) isolates was obtained from 56 examined pooled samples (n=4) of broiler chickens with clinical problems and 14 (51.85%) isolates were obtained from 27 pooled samples (n=4) from broiler chickens without clinical problems. Seroneutralization with mice or guinea pigs has been used to type each toxin, but this conventional method has some disadvantages. Therefore, we used a multiplex PCR (m-PCR) assay with primers amplifying fragments of alpha (*cpa*), beta- (*cpb*), epsilon (*etx*), iota (*iap*), for genotyping of isolated *C. perfringens* strains. All 61 broiler chicken *C. perfringens* isolates were shown to carry the *cpa* gene but not *cpb, etx and iA* genes, signifying that all isolates represented type A. For the first time the associated profiles of the following virulence genes [*cpe, cpb2* (beta-2 toxin) and the newly described pore forming toxin gene (*netB)*] were determined among Egyptian isolated *C. perfringens* strains. None of the isolates contained the enterotoxin gene that may indicate the enterotoxin of *C. perfringens* does not play important role in the occurrence of Necrotic enteritis in broiler chickens. *netB* was almost always found in outbreak isolates, suggesting a positive correlation of

netB toxin gene with the diseased status that may explain its role in the pathogenesis of the disease. Whereas *cpb2* was found in healthy and diseased bird isolates that suggest there is a weak or no relationship between beta2 toxin and necrotic enteritis in birds. So this study throw novel insights into the presence and distribution of *C. perfringens* types as well as virulence-associated genes in field strains, which will help us to understand the pathogenesis of disease in poultry and more comprehensively characterize *C. perfringens* in future studies to put a suitable strategy for prevention and control.

Key words: *Clostridium perferingens, broiler chickens, virulence and m-PCR.*

INTRODUCTION

Enteric diseases are an important concern to the poultry industry because of lost productivity, increased mortality, reduced welfare of birds and the associated contamination of poultry products for human consumption (Dahyia *et al.,* 2006).

One of the most important enteric diseases that affected broilers is necrotic enteritis (NE). NE was first described by Parish in 1961 (Parish, 1961), and since then it has been reported to occur in almost all poultry-producing countries. NE in chickens gets in two forms, as an acute or chronic enterotoxemia (Van Immerseel *et al.,* 2004). The acute disease results in significant levels of mortality whereas the chronic disease leads to loss of productivity and welfare concerns. It has been estimated that the disease costs the international poultry industry in excess of $US 2 billion per year based on average damage of $0.05 per bird (McReynolds *et al.,* 2004).

NE is an enteric disease that is caused predominantly by *Clostridium perfringens* type A and to a lesser extent by type C strains (Songer, 1996). Mucosal damage inducing factors such as coccidiosis (parasitism), high fiber litters, dietary changes, poor hygienic and housing conditions are considered predisposing factors for NE. The NE is thought to occur when these factors create a favorable environment for *C. perfringens* to multiply rapidly into much higher numbers in the small intestine, leading to the production of potent lethal toxins that damage the intestine. Moreover, excessive uses of growth promoting antibiotics enhance *C. perfringens* to induce NE and sub clinical infections, important threats to poultry health (Songer, 1996).

C. perfringens in poultry constitutes a risk for transmission to humans through the food chain. *C. perfringens* is one of the most frequently isolated bacterial pathogens in foodborne disease outbreaks in

humans, after some other pathogens such as *Campylobacter* and *Salmonella* (Buzby and Roberts, 1997).

The pathogenicity of the organism is associated with several toxins which are used for toxin typing of the bacteria. The alpha (α), beta (β), iota (ι) and epsilon (ε) are the major lethal toxins produced by the organism that are closely related to its virulence (Petit *et al.*, 1999). 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 ulceration or superficial mucosal necrosis of the small intestine in animals (types B and C) (Smedley III *et al.*, 2004).

Clostridium perfringens is classified into five types (A–E) on the basis of their ability to produce major lethal toxins. Within these five types, all *C. perfringens* produce α toxin. In addition, type B strains produce β and ε toxins, type C produces β toxin, type D produces ε toxin and type E produces ι toxin. In addition to the major lethal toxins, a minority of *C. perfringens* strains produce a *C. perfringens* enterotoxin (CPE), which is responsible for the symptoms of common *C. perfringens* type A food poisoning. Different meats, including poultry meat, have been frequently reported as the most common food vehicles (Eisgruber and Hauner 2001; McClane 2001).

The classification of *C. perfringens* isolates into toxigenic types has been traditionally performed by toxin neutralization test in mice. This procedure consumes a lot of antisera and experimental animals. Additionally it is time consuming. Nowadays, with the advent of highly efficient detection chemistries, sensitive instrumentation and optimized assays the number molecular techniques such as polymerase chain reaction (PCR) have been used to type *C. perfringens* (Gkiourtzidis *et al.*, 2001; Baums *et al.*, 2004; Gholamiandekhordi *et al.*, 2006) according to the presence of toxin genes.

The virulence factors which lead to the development of NE have yet to be fully understood. Based on experimental studies, early investigators suggested that alpha toxin is the major toxin involved in causing NE (Fukata *et al.*, 1988), and there are recent reports that vaccination against alpha toxin is at least partially protective against the disease (Lovland *et al.*, 2004; Copper *et al.*, 2009). However, Keyburn *et al.* (2006) have reported that alpha toxin knockout mutants of *C. perfringens* were able to produce necrotic enteritis in an experimental model.

Keyburn *et al.* (2008) reported the discovery of pore forming toxin of *C. perfringens* which they named NetB. *netB* gene was only identified in strains recovered from chickens with NE and not found in isolates from chickens which did not have NE. Keyburn *et al.* (2008) also mentioned that *netB* knockout mutants failed to produce NE in chickens, while such mutants complemented with the wild type *netB* gene did cause NE. Clearly, the identification of the toxin responsible for NE would represent a major advance in knowledge, so there remains a need to examine isolates from other countries like Egypt for the presence of *netB*.

Prophylaxis and control of necrotic enteritis is essential in broiler flocks, and the primary approach to this has been vaccination. For this to be successful, the vaccine must be based upon strains which are clinically relevant. Thus, identification of *C. perfringens* toxinotypes using a robust genetic test for the presence of toxin-encoding genes is critical for both epidemiological studies and for development and effective use of preventative measures, including vaccination.

However, there are no published data on molecular typing of *C. perfringens* in broilers in Assiut, Egypt. So, we report here for the first time in Egypt esp. in Assiut and Upper Egypt, testing of a multiplex polymerase chain reaction (PCR) assay for the simultaneous identification of the *C. perfringens* toxin genotyping as well as molecular analysis of the virulence genes among this pathogen and the its association with the disease to undertake the development of control measures, especially for the formulation of cost effecting vaccine.

MATERIALS and METHODS

History of broiler chickens and sample collection:

Cases of enteric disorders compatible with necrotic enteritis in 21 to 32 days old broiler chickens reared in different poultry farms in Assiut province, Egypt occurred in the months of September 2009 to Novemeber 2009 were investigated. These farms previously exposed to enteric infection, mainly with coccidiosis.

A daily mortality rate of 1.5 to 2% was recorded. The mortality peak was terminated by a 3-5 day antibiotic treatment with amoxicillin. In every case, postmortem was performed as soon as possible after death. All the internal organs were thoroughly examined and any macroscopic and gross lesions observed were recorded.

Intestinal contents esp. duodenum (n=168) and gall bladder of affected liver samples (n=56) from diseased birds as well as 108 samples of cloacal swabs from healthy birds were collected for microbiological investigation.

Culture of *C. perfringens*

Pool samples (n=4) were inoculated in sterile cooked meat medium broth (Difco), then overlaid with mineral oil and incubated at 37°C for 24hr.

The inoculums from each cooked meat broth media were seeded onto 5% citrated sheep blood agar containing 12 mg kanamycin sulphate and 30,000 U polymyxin B sulphate/Liter then incubated anaerobically with an anaerobic gas-pack system (BBL Microbiology Systems Cockeysville, Md.; Div. Becton Dickinson and Co.) for 48hr at 37°C. Pure colonies were obtained by plating subcultures on *Brucella* agar and incubating them anaerobically for 24 hours.

The identity of isolates was confirmed by bacterial colonies characteristics (size, shape, color and patterns of haemolysis on blood agar produced a double zone of hemolysis), motility test, Gram's and malachite spore staining, litmus milk test, gelatinase, lecithinase and fermentation of glucose and lactose (Holt *et al.*, 1994).

Detection of *Clostridium perferingens* types by Multiplex PCR of toxin genes:

DNA extraction

A liquots (300 uL) of stored culture suspensions were transferred to microfuge tubes, boiled for 10 min in a low power microwave, centrifuged at 13,000 xg for 5 min (Ferrarezi *et al.*, 2008), and DNA precipitated with 1mL absolute ethanol for 3 h at - 60C. After centrifugation, pellets were dissolved in 50 uL ultrapure water (Ultra Pure DistilledWater, Invitrogen) and incubated at 56C for 20 min. An aliquot (5 uL) of this extracted DNA was used as template in PCR genotyping.

Multiplex PCR assay

Two multiplex PCR were used: one for detection of *cpa*, *cpb1*, *cpetx*, and *cpi* genes and one for detection of *cpb2*, *cpe* and *netB* genes. PCR Primers, and references to the sequences from which they were derived, are presented in Table 1.

The multiplex PCR assay was performed by a modification of a previously described method (Songer and Bueschel, 1999). Briefly, each 50 uL reaction contained 25 µl 2× QIAGEN Multiplex PCR Kit (Qiagen)

containing HotStart *Taq* polymerase, multiplex PCR buffer, dNTP mix and 3 mM $MgCl_2$, a final concentration of 0.4 µM for each primer (1µl of each primer), 8 µl sterile ..Nase-free water and 5 µl of test or control DNA.

Amplification was carried out in a Techne Cyclogene Thermocycler, using a program composed of 5 min at 94°C, 35 cycles consisting of 1 min at 94°C as initial denaturation, 1 min at 55°C, and 1 min at 70°C), and a final incubation for 10 min at 72°C as final elongation cycle. Negative (ultrapure water) controls were included. PCR products were examined by electrophoresis in a 1.2% agarose gel with 5 µL/100 mL of ethidium bromide and visualized by UV transillumination, then documented with a gel documentation system (BioRad, Munich, Germany). Molecular weight marker (1 kb Plus DNA Ladder) was added to agarose gels before electrophoresis.

Table 1: Target toxin gene, oligonucleotide primer sequences and length of amplification products of the *C. perfringens* multiplex PCR.

Primers of toxin gene	Amplicon Size (bp)	Nucleotide sequence(5-3)	Referance	Types of *C. perferingens*
cpa (α toxin)	900	AGTCTACGCTTGGGATGGAA TTTCCTGGGTTGTCCATTTC	Songer and Bueschel, 1999	[a]A, B, C, D and E
cpb (β toxin)	611	TCCTTTCTTGAGGGAGGATAAA TGAACCTCCTATTTTGTATCCCA	Songer and Bueschel, 1999	B and C
etx (ε toxin)	396	TGGGAACTTCGATACAAGCA TTAACTCATCTCCCATAACTGCAC	Songer and Bueschel, 1999	B and D
iap (ι toxin)	293	AAACGCATTAAAGCTCACACC CTGCATAACCTGGAATGGCT	Songer and Bueschel, 1999	E
cpe (Enterotoxin)	506	GGGGAACCCTCAGTAGTTTCA ACCAGCTGGATTTGAGTTTAATG	Songer and Bueschel, 1999	Untyped
cpb2 (B2 toxin)	200	CAAGCAATTGGGGGAGTTTA GCAGAATCAGGATTTTGACCA	Songer and Bueschel, 1999	Untyped
net B (Toxin B)	670	TAATTAGTACAAGCCTTTTTTC TATTACAGATTCTTTAGCATTT	Designed in this Study from the *C. perfringens* toxin type A sequence of *netB* (EU143239.1) supplied by Keyburn *et al.*, 2008	Untyped

[a]Definition of *Clostridium* genotype is based upon amplification of a toxin gene fragment. Type A has *cpa* alone, type B has *cpa*, *cpb*, and *etx*, type C has *cpa* and *cpb*, type D has *cpa* and *etx*, and type E has *cpa* and *i*A.

RESULTS

Clinical signs and postmortem lesions

During appearance of necrotic enteritis, broiler chickens showed severe depression, decreased appetite, ruffled feathers and diarrhea tinged with blood prior to death. Post mortem examination showed that birds were dehydrated, mid-small intestine (Fig. 1), where the enteric mucosa was abnormally thickened like yellow brownish diphtheritic membrane (Fig. 1), and varying degrees of haemorrhages. Focal necrosis and hemorrhages on the liver surface was also noticed in some chickens. Rest other organs appeared apparently healthy.

Culture of *Clostridium perfringens*

A total of 56 pooled samples (n=4) from diseased birds (42 intestinal pooled samples and 14 gall bladder of affected liver samples) were examined for the presence of *C. perfringens*. 47 (83.9%) isolates of *C. perfringens* out of 56 examined pooled samples were isolated and identified by colonies characteristics on blood agar as shown in fig.2 as well as biochemical tests as described in Bergey's manual (Holt *et al.*, 1994) like positive fermentation of glucose, lactose, sucrose, and maltose; hydrolysis of gelatin; production of lecithinase.

Bacteriological investigation was carried out also on 27 pooled samples of cloacal swabs from healthy birds for the presence of *C. perfringens*, 14 (51.85%) *C. perfringens* strains were isolated and identified (Table 2).

Table 2: Number of positive samples for *C. perfringens* isolates examined from broiler chickens.

Condition of birds	Small intestine	Liver (gall bladder)	Cloacal swabs	Total
Diseased birds	42[a] (41[b])	14[a] (6[b])	-[c]	56[a](47[b])
Healthy Birds	-[c]	-[c]	27[a](14[b])	27[a](14[b])
Total				83[a](61[b])

[a] Number of examined samples
[b] Number of positive samples
[c] Not subjected for examination

Toxin typing by PCR

All 61 isolates which had been tentatively identified as *C. perfringens* from diseased and healthy broiler chickens, were examined for the presence of cpa, cpb, iA, etx, cpb2 and cpe which encode respectively for alpha toxin, beta toxin, iota toxin, epsilon toxin, beta2 toxin and enterotoxin of *C. perfringens*, by using a multiplex PCR method.

In all *C. perfringens* isolated from diseased and healthy birds harboring the *cpa* gene of 900 bp fragment (Fig. 3), so all the isolates were confirmed as *C. perfringens* type A. However, the *cpb, etx, iap and cpe* were not detected in any of the examined isolates (Table 2).

A 27/61 (44.3%) were positive for the presence of *cpb2* (b2 toxin) gene of 200 bp fragment (Fig.4). Its presence is particularly high among isolates from affected liver (50%) (Table 3).

Of the 61 *C. perfringens* isolates which had been recovered from diseased and healthy chickens, 37 (60.6%) were *netB* positive (Fig. 4 and Table 3) 35 out of 47 (74.4%) were positive for *netB* gene. Conversely, 2 out of 14 (14.3%) isolates from healthy chickens, were *netB* positive (Table 3).

Table 3: Analysis of virulence factors associated with *C. perfringens* isolates from healthy and diseased broiler chickens

Birds Examined			*C. perfringens* toxin-encoding genes							
Birds Condition	Sample Nature	No. of Positive	*cpa* (α toxin)	*cpb* (β toxin)	*etx* (ε toxin)	*iap* (ι toxin)	*cpe* (enterotoxin)	*cpb2* (B2 toxin)	*net B* (Toxin B)	Isolated Type
Diseasesd	Intestine	41	+	0	0	0	0	19 (46.3%)	31 (75.6%)	A
	Liver (gall Bladder)	6	+	0	0	0	0	3 (50%)	4 (66.6%)	
Healthy	Cloacal swabs	14	+	0	0	0	0	5 (35.7%)	2 (14.3%)	A
Total		61	61	0	0	0	0	27 (44.3%)	37 (60.6%)	

DISCUSSION

Clostridium perfringens is an important pathogen of many animals and can cause a disease known as necrotic enteritis (NE) in avian species. The disease results from extracellular toxin production by the bacterium in the intestinal tract and leads to tissue necrosis and malabsorption and is often fatal (Songer, 1996).

So, in this study we tried to get a broad idea on the situation of *C. perfringens* incidence and types on Assiut broiler chicken farms. Our observations revealed that necrotic enteritis caused 3% mortality within two days in the broiler chickens of age between three to four weeks old. Broussard *et al.* (1986) reported that clinical NE cause higher mortality in 2 to 4 weeks old chicken which sometimes exceeds 1% daily. Severe depression, decreased appetite, diarrhea, reluctance to move and ruffled feathers also reported earlier (Songer, 1996; Das *et al.*, 1997).

In post mortem examination, chickens were found dehydrated and produced fetid odor and had severe lesions and necrosis on the wall of the small intestine and in some cases focal necrosis and hemorrhages on the upper surface of liver (Fig. 1) was also observed. In similar observation, the gross pathological changes were characterized by severe lesions and diffused mucosal necrosis in the small intestine and even in caecum, liver and kidney (Long, 1974; Broussard *et al.*, 1986; Lovland and Kaldhusdal, 2001).

In this study, *C. perfringens* was typically isolated from the duodenal samples of NE-affected birds, whereas isolates from healthy birds could be recovered frequently from cloacal samples only. This has been previously observed in other studies, in which concentrations of *C. perfringens* are higher in the intestinal tract of NE-affected birds, where the bacteria have proliferated upward into the duodenum and are often associated with coccidial coinfection (Shane *et al.*, 1986).

The patterns of production of the toxins are different, depending on the *C. perfringens* type. Therefore, the patterns have been used to type the bacterium into types A, B, C, D, and E. Some strains of *C. perfringens* may not be able to produce toxin in measurable amounts under laboratory conditions and this causes an obstacle for typing by classical methods. In recent years, PCR techniques have been used to determine *C. perfringens* toxin types (Songer and Meer, 1996; Baums *et al.*, 2004).

Herein, we describe a simple and rapid multiplex PCR for detection of *C. perfringens* toxin. In the study, all the isolated *C. perfringens* strains were successfully typed by the multiplex PCR.

C. perfringens type A, have been isolated from necrotic enteritis cases as well as healthy birds (Fig. 4). In agreement with our study, several studies reported that type A is the predominant type in poultry (Songer 1996; Van Immerseel, *et al.*, 2004). Our Results confirm the most recent results reported from different countries and the data suggest that the role of *C. perfringens* type C should be revaluated in the pathogenesis of NE.

The pathogenicity of *C. perfringens* is closely related to the production of major lethal toxins (alpha, beta, epsilon, and iota toxins) and other toxins, including enterotoxin (Hatheway, 1990). Recently, others (Keyburn *et al.*, 2008) described NetB, a virulence factor likely involved in the pathogenesis of NE. In a strong support of this, they reported that netB knockout mutants failed to produce NE in chickens, while such mutants complemented with the wild type *netB* gene, caused NE.

In a trial to extend the findings of Keyburn *et al.* (2008) by investigating the occurrence of the *netB* gene among Egyptian *C. perfringens* isolates and to examine its association with NE. In opposite to the findings of Keyburn *et al.* (2008), our results revealed that *netB* was found in 35 out of 47 isolates (74.4%) from diseased chickens, however 2 out of 14 (14.28%) from healthy chickens (Table).

Although *netB* was detected in the majority of isolated strains from diseased broilers, it was found healthy birds as well but in a low percentage. (Table 3). Overall, this study provided a good opportunity to observe the prevalence of the *netB* gene in a field situation where NE- and non-NE-associated isolates were used. The data suggest that this toxin is an important factor in *C. perfringens* infection, facilitating the development of NE. However, our results also show that it is neither a necessary nor a sufficient cause for the disease to occur. As the authors of the *netB* discovery stated (Keyburn *et al.*, 2008), there may be many other unidentified factors which are important to allow *C. perfringens* type A to compete effectively in the gut and produce tissue damage consistent with NE.

None of the broiler chicken isolates tested in the study were Enterotoxin(*cpe*)-positive. This result indicates that the enterotoxin is not involved in disease in broiler chickens investigated in the present study. This is in accordance with previous studies, as the *cpe* gene has been estimated to be present in <5% of global *C. perfringens* isolates (Engstrom *et al.*, 2003).

Beta2 toxin (CPB2) may have a role in the pathogenesis of NE, several studies on the CPB2 toxin strongly suggest an association between this toxin and clostridial gastrointestinal diseases in some domestic animals, including typhlocolitis in horses and necrotic enteritis in piglets (Herholz *et al.*, 1999 and Waters *et al.*, 2003).

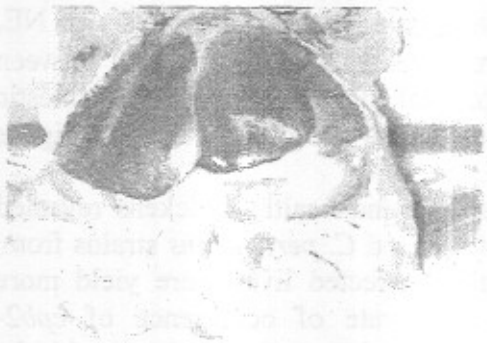
Our study surveillance of diseased and healthy chickens revealed the *cpb*2 gene was found in 44.3% of isolated *C. perfringens* strains from, but the prevalence varied substantially. Affected liver were yield more *cpb*2-positive isolates (50%). This high rate of occurrence of *cpb*2-positivity among strains isolated from affected livers is consistent with the contention of other workers, that beta2 plays a role in pathogenesis of *C. perfringens*-associated hepatitis (Engstrom *et al.*, 2003).

The percentage of *cpb2*- positive *C. perfringens* strains in chickens is higher than that reported by previously mentioned by it was reported from Swedish broiler chicken farms (Chalmers *et al.*, 2008) that only 2 of 53 isolates (4%) were beta2-toxin gene positive this difference may be owing to geographic location. Furthermore, this is the first report of detection of this gene in *C. perfringens* isolated from other avian species.

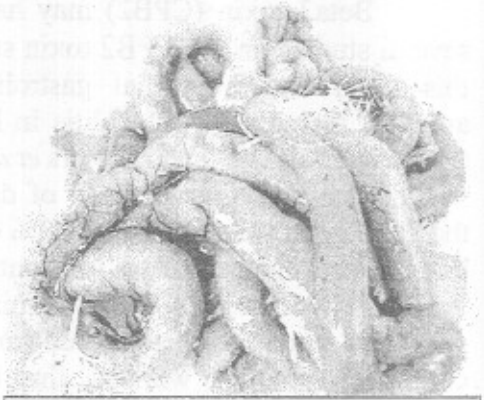
In conclusion, the multiplex PCR protocol described by Songer and Bueschel, 1999 was successfully applied in this study and could be used as a new method for typing avian *C. perfringens* isolates that led to avoidance of long time and financial costs if we use the conventional method for typing by toxin neutralization test in mice. The resulting data revealed that the *C. perfringens* isolates included in the study were of toxin type A which throw the light that broiler chickens can act as important source for human infection with *C. perferingens* that lead to food poisoning. Though a good management practices, awareness regarding the disease is in first priority.

We demonstrated for the first time that *C. perfringens* genotypes corresponded to *cpa, cpb iap, etx*, *cpe* and *cpb2* toxin producing strains from healthy and diseased broiler chickens in Assiut-Egypt. The genotyping results demonstrated that there were differences in the toxin genotype between diseased and healthy broiler chickens that a relatively high percentage of isolates from diseased birds carry the *netB* and *beta2* toxin genes than from healthy ones.

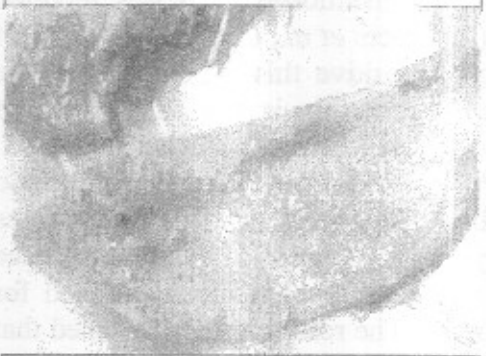
These findings can be used for epidemiologic studies, prophylaxis programs, and to formulate strategies for correct use of vaccines and suggest a more work needed to elucidate *C. perfringens* pathogenic mechanisms in necrotic enteritis etiology alone and with other predisposing factors like (nutrition, concomitant diseases).



A. Opened Carcass showing yellow mottling and fatty degenrtaion of liver in broiler chicken infected with *C. perfringens*



B. Close up view of of small intestine showing ballooning and serosal hemorrhages in birds affected with necrotic enteritis



C. Close up view showing thickened and fissured intestinal mucosal membrane like Turkish towel surface

D. Extensive necrosis in the mucosal membrane of small intestine in the affected birds.

Fig. 1 Different postmortem lesion from liver and intestine of the affected birds

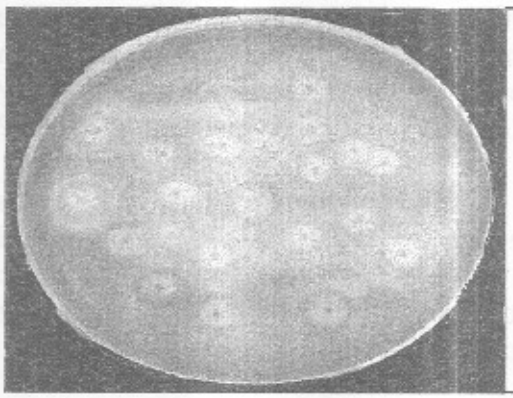


Fig. 2 Blood agar cultivated with *C. perferingens* showing dual hemolysis. The colonies are smooth, round, glistening, surrounded by an inner zone of complete haemolysis and an outer zone of incomplete haemolysis caused by the alpha-toxin

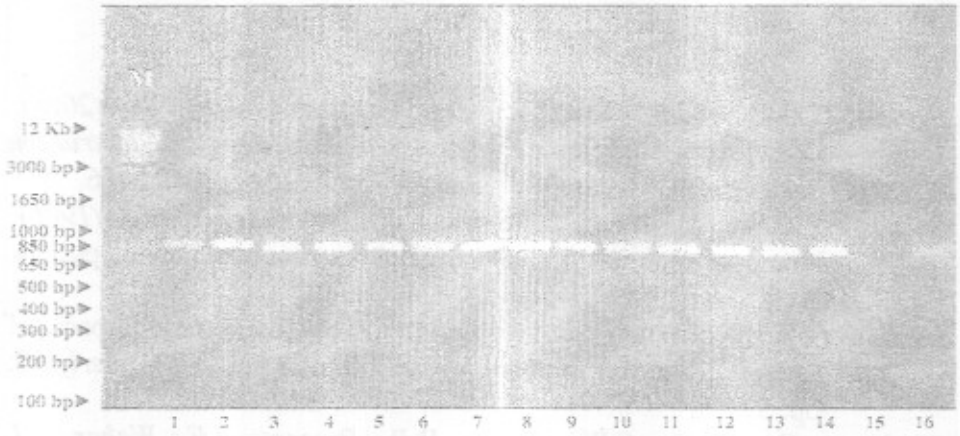


Fig. 3: Amplification of *C. perfringens* toxin genes by the multiplex PCR. Chromosomal DNA was isolated from the bacteria listed in Materials and Methods, and multiplex PCR was performed with template DNA under the conditions described in Materials and Methods. Amplified PCR products were analyzed by electrophoresis on 1.2% agarose gel. Lane M, DNA size marker (1-kb plus ladder); lane 1-16, *C. perfringens* type A (alpha toxin gene (*cpa*) amplification).

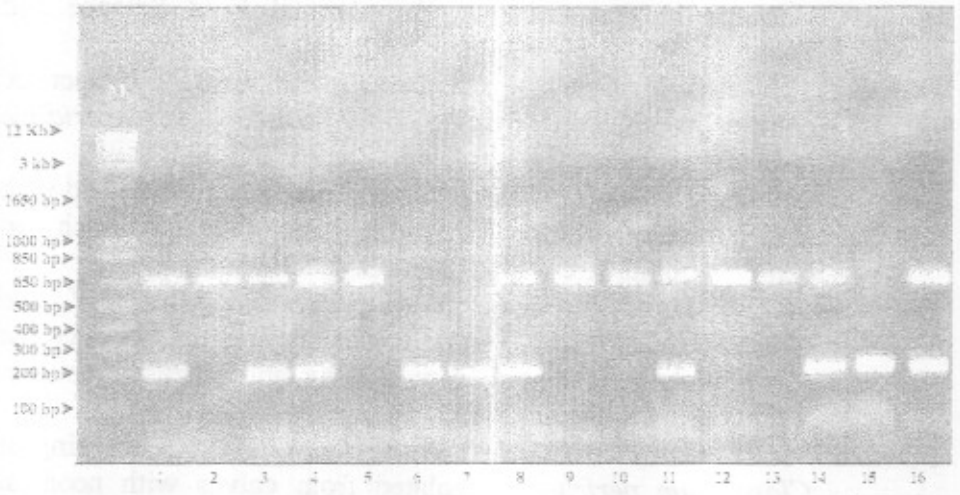


Fig. 4: Multiplex PCR subtyping amplification products for a representative number of *C. perfringens* type A strains. Lane M, 1 Kb plus DNA ladder; lanes 1, 3-4, 8, 11, 14 and 16 *C. perfringens* (*netB*$^{+}$, *cpb2*$^{+}$ type A); lanes 2, 5, 9-10 and 12-13 *C. perferingens* (*netB*$^{-}$, *cpb2*$^{+}$ type A); Lanes 6-7 (*netB*$^{+}$ and *cpb2*$^{-}$ type A).

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