Genotypic Characterization of *Clostridium Perfringens* and Associated Histopathological Changes in Broiler

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Abstract

In the present study, 6 *C.perfringens* strains (4 were type A and 2 were type D) were recovered from intestinal and liver samples of 6 diseased broiler. PCR used for the detection of alpha and epsilon toxin . Multiplex PCR was done to *C.perfringen*s isolates which had the gene gave a characteristic` band at 402 bp and 541 bp where all of the 6 isolates were positive for alpha toxin genes.The extracted toxins were purified by ammonium sulfate precipitation, then profiling of the exotoxin proteins by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).SDS-PAGE has become an important tool for protein profiling which reflect the genetic identity to *C.perfringense* type A and D. Our study revealed the histopathological changes of intestinal samples of the diseased birds with necrotic enteritis showed Intestine hyaline degeneration of the muscle, fragmentation and edema in between the muscle infiltrated with inflammatory cells. Liver showing fibrous connective tissue in the portal area particularly around intrahepatic bile duct and hyperplastic proliferation of biliary epithelium toward the lumen associated with central vein.

INTRODUCTION

Enteritic disease in poultry are caused by a wide variety of infectious agents where clostridium are the most important diseases. Necrotic enteritis(NE) and the subclinical form of Clostridium perfringens infection in poultry are caused by *Clostridium perfringens* type A, producing alpha toxin ***(Van immerseel et al, 2004)*** . *Clostridium perfringens* is Gram +ve ,sporulated anaerobic bacterium which is wide spreed in environment (soil, water) and intestine of human and animal ***(Petit et al, 1999)*** .Alpha toxin is 370 amino acid protein which shows not only phospholipase C activity but also hemolytic and platelet activities.Alpha-toxin is active towards several phospholipids such as phosphatidyl choline and sphingomyelin. The toxicity of phospholipase can be broadly correlated with their hemolytic activites ***(Tibtall et al, 1999).*** *C.perfringens* types B and D produce Epsilon toxin as a protoxin that activated by a protease ***(Cole, 1995)***. Exotoxin released by *Clostridium*

perfringens are responsible for cell necrosis ***(Hofshang and Stenwig, 1992).***

Multiplex PCR has been applied for detection of the genes encoding major toxin of *Clostridium perfringens* .This method is sensitive, specific, more accurate and faster than seroneutralization with mice or guinia pigs.SDS-PAGE is widely used technique for analysis of complex mixture of poly peptide .NE in broilers caused intestinal lesion which seemed to be friable and often distended with gas.The Liver was enlarged ,congested and necrotizing ***(Jansen & Swifit, 1992)***. This study aimed to Isolate *C. perfringens* on specific media with identification of *C. perfringens* toxins gene (alpha, epsilon toxin gene) isolated from the collected samples by multiplex PCR, this is addition to to electrophoretic profile of *C. perfringens* toxins by SDS-PAGE and histopathological examinations of naturally infected broiler.

Material And Method:

Six C.perfringens strains (4 were type A and 2 were type D were used in this study.

Isolation and identification the microorganism:

Sample were inoculated into cooked meat medium (CMM) then cultured onto 10% sheep blood agar medium with neomycin sulphate incubated anaerobically at 37C for 48hr, ***(Smith and Holdeman, 1968).*** Suspected colonies were subjected to Gram staining and were biochemically identified ***(Koneman et al., 1992).*** Determination of lecithinase activity (Nagler's test by half antitoxin plate as mentioned by ***(Smith and Holdeman, 1968).*** Isolates of *C. perfringens* Typed by dermonecrotic test in albino guinea pigs according to ***Quinn et al (2002).***

Concentration of alpha and epsilon toxin *(El-Idrissi and Ward, 1992):*

The pure isolates of *C. perfringens* types A and D were cultured into thioglycolate medium incubated anaerobically at 37C .After 24hrs of culture growth ,10ml of culture were transferred to 500ml of toxin production medium as previously described by ***(Roberts et al, 1970)*** and incubated at 37c for 8 and 48 hrs in anaerobic condition for alpha and Epslion toxin respectively . The culture medium was centrifuged at 10000 xg , at 4C ,for 30 min.The supernatant crude toxin was separated (epsilon protoxin was treated with 0.1%trypsin and incubated at 37C for 45 min), then the culture supernatant were collected at which ammonium sulphat 35-40%(wt/Vot.)was added and left overnight at 4c.The precipitate was sedimented by centrifugation ,dissolved in 0.01M PBS PH 7.4 then dialysed against the same buffer for 1 week to getrid of ammonium sulphat residue then concentration of the toxin was taken place using polyethylene glycol.

Protein determination :

Protein contents of *C. perfringens* toxin (alpha and epsilon) were measured by the method of **Lowry et al (1951).**

Sodium dedocyle sulphate polyacrylamide gel electrophoresis(SDS-PAGE):

Alpha and epsilon toxins of *C.perfringens* type A and D were assayed for their purities by vertical electrophoresis in 10% polyacrylamide gel as described by ***Oconnor (2006):*** protein bands were determind by staining with coomassi brilliant blue.The devoloped protein bands in the gels were scanned and analyzed using image denoitometer (Model-G.S-

700,Bio-Rad laboratories , Richmond, CA, USA: and Gel-pro-analyzer soft ware ver ,3.0media cybemetics, Bethesda,MD,USA.

PCR for genotyping of *C.perfringens*:-

DNA extraction and purfication from samples was performed using the QIa amp DNa minikit (**Cat. No. 51304** Qiagen,Germany, GmbH)with modifications from the manufacture ، recommendations .Briefly,200micro litter of the sample suspension was incubated with 10 ml of proteinase K and 200µl of lysis buffer at 56c for 10min . After incubation , 200µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer recommendations Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

Oligonucleotide primer. primers used were supplied from **Midland Certified Reagent oilgos (USA)** are listed in table(1)

PCR amplification primers were utilized in a 50- ML reaction containing 12.5 µl of Emeral-dAmp Max pcr master Mix (Takara, Japan)1 ML of each primer of 20 pmol concentration , 6.5 µl of water and 6.5 µl of DNA template the reaction was performed in a T3 Biometra thermal cycler as following: initial denaturation at 94°C for 3 minutes then 30 cycles consisting of (denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 1 minute) thus followed by final extension at 72°C for 5 minutes.

Analysis of the PCR products:

At room temperature, PCR products were separated by electrophoresis on 1-1.5% agarose gel (Applichem, Germany, GMbH) in 1x TBE buffer using gradients of 5 V/Cm for gel analysis , each gel slot was loaded by15 µl of the products . A100bp DNA ladder (Qiagen,Germany,GmbH) was used to determine the fragment sizes . The gel was photographed by agel documentation system (Alpha innotech,Biometra) and the data was analyzed through computer software.

Histopathological examination:

Specimen of liver and intestine from diseased broilers with necrotic enteritis were fixed in 10% neutral buffered formalin for at least 24 hours and then routinely processed.The tissues were paraffin embedded and sectioned at 4-6µ thickness, then the sections were stained with haemetoxylin and eosin stain according to ***Bancroft et al (1994)***

Table (1): Oligonucleotide primers sequences

Toxin	Primer	Sequence	Amplified product
Alpha toxin	*F*	GTTGATAGCGCAGGACATGTTAAG	402 bp
	R	CATGTAGTCATCTGTTCCAGCATC	
Beta toxin	*F*	ACTATACAGACAGATCATTCAACC	236 bp
	R	TTAGGAGCAGTTAGAACTACAGAC	
Epsilon toxin	*F*	ACTGCAACTACTACTCATACTGTG	541 bp
	R	CTGGTGCCTTAATAGAAAGACTCC	

Result

Isolation *C. perfringens* on specific media with identification of *C. perfringens* toxins gene (alpha, epsilon toxin gene) isolated from the collected samples by

typing of *C.perfringens* by dermonecrotic reaction in albino guinea pigs revealed that ,4 isolates were type A and 2 were type D.

Results of multiplex PCR:

PCR revealed that, the specificity of the oligonucleotide primers was confirmed by the positive amplification of 402 bp fragments from the extracted DNA of 6 strains of *C. perfringens* type A and 541bp fragments from the extracted DNA of 2 strains of *C.perfringens* type D. Six *C.perfringens* isolates were negative amplification primer of 236 bp fragment from extracted DNA of *C.perfringens* type B .(photo1)

Results of SDS-PAGE of *C. perfringens* alpha end epsilon toxins:

Analysis of alpha toxin reveled that alpha toxin contained about 6 protein bands by SDS-PAGE stained with silver nitrate with molecular weight ranged from 11.05-34 KDa. On the other hand, the protein profile of epsilon toxin has 5 bands ranged from 11.9-34.1 KDa.(photo 2)

Histopathological findings:

Intestine showed mucinous degeneration and necrosis of the epithealial cells lining the intestinal villi lamina propria and submucosa were infliterated with inflammatory cells (Photo 3). The intestinal glands revealed mucinous degeneration and necrosis in addation to hyperplasia of goblet cells of the intestinal gland. Edema and hyaline degeneration of the tunica muscularis were evidence (Photo 4).The Liver showed vacuolar degeneration and multifocal area of coagulative necrosis of hepatocytes (Photo 5).The portal area revealed fibrosis connective tissue proliferation with focal inflammatory cells aggregation(Photo 6)

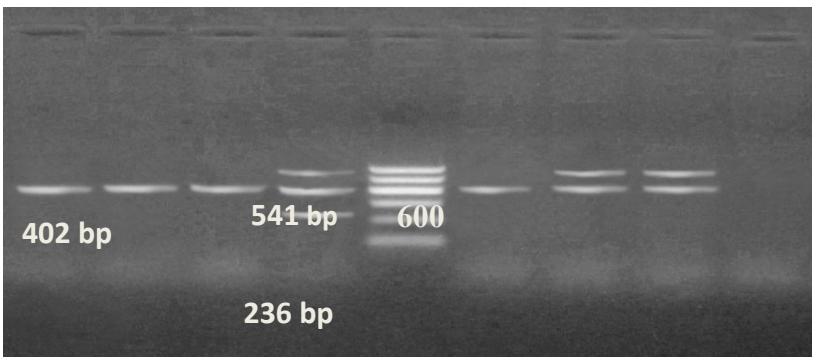


Photo (1): *Agarose gel electrophoresis of C. perfringens alpha and epsilon toxin genes amplicons*

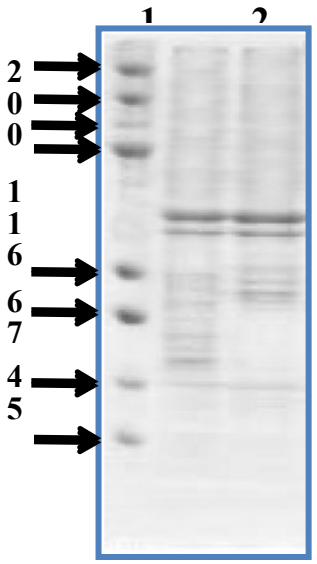
Lane 5: 100 bp ladder (Pharmacia).

Lane1,2,3,6: positive for *C. perfringens* alpha toxin gene field isolates.

Lane 7,8 : positive for *C. perfringens* alpha and epsilon toxin genes

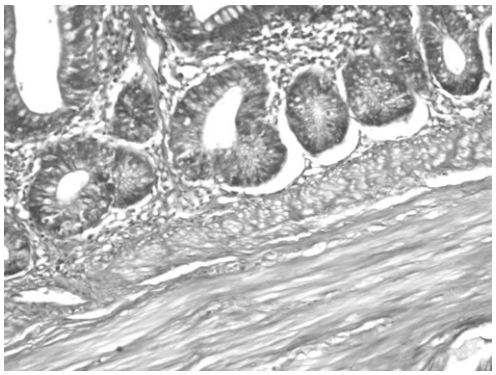
Lane 4: Positive control for alpha toxin at 402bp,beta toxin at 236 bp and epsilon toxin at 536 bp .

Lane 9: Negative control for alpha,beta,epsilon toxin gene.



Lane 1: Standard protein marker
Lane 2: *C.perfringens* type A.
Lane 3: *C.perfringens* type D.

Photo (2): *The electrophoretic patter of alpha and epsilon toxin of C.perfringens type A and D field isolates.*



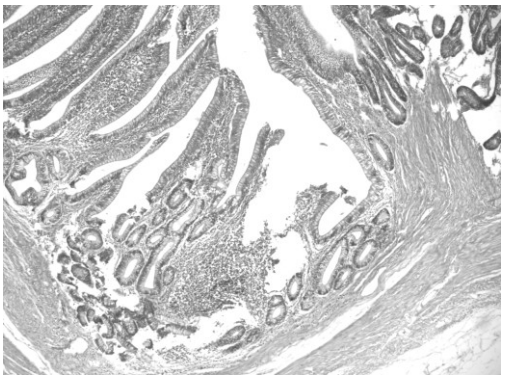
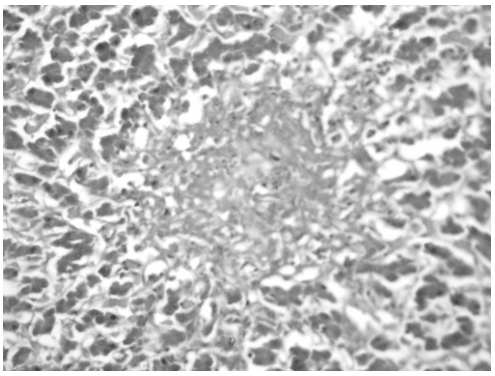


Photo (3): *Intestine of broiler infected by C.perfringens showing hyperplasia of goblet cells of the infiltrations in the lamina propria and submucosa, edema and epithelial mucinous degeneration of the muscle layer. H&E stain x 400*

Photo (4): *Intestine of broiler infected by C.perfringens showing hyperplasia of goblet cells of intestinal gland . H&E stain x 100*



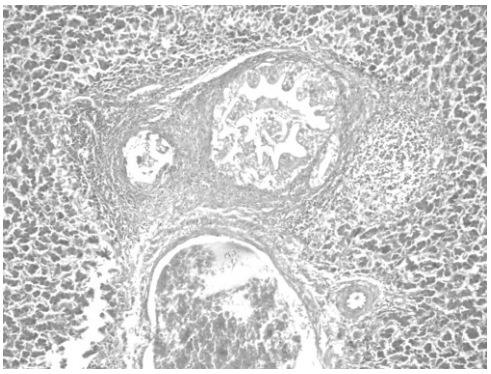


Photo (5): *Liver of broiler infected by C.perfringens showing focal area of coagulative necrosis of hepatocytes represented by deeply eosinophilic homogenous structurless areaH&E stainx400 .*
Photo (6) *Liver of broiler infected by C.perfringens showing fibrous connective tissue proliferation in the portal area with focal inflammatory cells infiltrations and hyperplastic proliferation of biliary epitheliumH&E stain x 200*

Disscusion

Necrotic enteritis and subclinical form of *C. perfringens* infection in poultry are caused by *C. perfringens* type A,That produce the alpha toxin, and type C ,that produce both alpha and beta toxin ***(Van Immerseel et al, 2004);*** resulting in decreased production efficiency parameters and mortality as high as 50% within a flock ***(Cooper et al, 2009).*** Polymerase chain reaction has been applied in several areas since the late 1980. This method has been highlighted as a rapid and accurate method for the detection of low copy numbers of genes.Six *C.perfringens* isolates were subjected for multiplex PCR analysis . The result in photo (1) show development of PCR band at 402 bp and at 541 bp for alpha and epsilon gene.Four isolates (lane 1,2,3,6,) were classified as type A and 2 isolates(lane 7,8) were type D, which all were pcr negative for B.

The present results agree with reports of other investigators for accurate typing of *C. perfringens* with PCR ***(Yamagishi et al, 1997; Yoo et al, 1997; Eman, 2009 and Sally, 2010)*** who used PCR as a new method for typing and diagnosis of *C. perfringens* type A and D in broiler and ostriches where symptoms and gross lesions are not very characteristic The studies of ***Das et al (1997), Heikinheimo and Korkeala (2005) and Gholamiandekhordi et al (2006)*** depend on multiplex PCR for typing of *C.perfringens* toxin genes and all these results agreed with the *C. perfringens* type A which was the most predominant type isolated from necrotic enteritis in the broiler in the present study. In this study to describe the *C.perfringens* type A and D toxins by SDS-PAGE (Photo 2 and Table 1),SDS was a successful aid to identify protective antigens and classify various bacterial species. The genus clostridium is well known for numerous and diverse toxins produced by its members. For confirmation on the presence of alpha and epsilon toxins in culture supernatants of *C.perfringens* type A, SDS-PAGE was performed for culture filtrate of both types. Analysis of alpha toxin reveled that alpha toxin contained

about 6 protein bands by SDS-PAGE stained with silver nitrate with molecular weight ranged from 11.05-34 KDa. On the other hand, the protein profile of epsilon toxin has 5 bands ranged from 11.9-34.1 KDa.

The results are in accordance with that recorded by ***Mona and Eman (2009)*** who analyzed alpha toxin contained about 10 protein bands ranged from 19.5 to 107.95 KDa and epsilon toxin that has 11 bands ranged from 23.36 to 107.95 KDa.

Ahmed (2004) reported that alpha toxin has a complex protein profile with 10 bands of molecular weight ranged from 17-164 KDa while analysis of epsilon toxin showed 11 bands ranged from 14-200 KDa.

Histopathological examination showed the inflammatory cells infiltration in the mucosa and sub mucosa of intestine , hyperplasia of goblet cells of the intestinal glands, edema and hyaline degeneration of the muscle layer as shown in (photo 3,4).

Hepatic changes including randomly scattered multifocal area of coagulative necrosis of hepatocytes and fibrous connective tissue proliferation in the portal area with focal inflammatory cells aggregation as shown in (Photo5,6). The histopathological lesions described in this study corresponded with the findings of other investigators ***(Shamimuzzaman, 1999; Samad, 2005 and Keyburn et al, 2008).***

This study was concluded that *C. perfringens* is considered to be one of the most common pathogenic bacteria in nature which production of several exotoxins as alpha, beta, epsilon and iota toxin.Polymerase chain reaction (PCR) has been proved to be a reliable, sensitive and specific protocol for detection of a very low numbers of toxin genes.Six *C. perfringens* isolates had alpha toxin gene which gave characteristic bands at 402 bp and episilon toxin gene which gave characteristic bands at 541 bp in 2 isolates and identified as *C. perfringens* type A and D. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of *C. perfringens* type A and D recovered from diseased broilers with necrotic enterits was designated to determine the protein analysis of alpha and epsilon toxin. Pathological examination of slides affected intestine and liver from diseased broiler showed Intestine mucinous degeneration and necrosis of the epithealial cells lining the intestinal villi lamina propria and submucosa were infliterated with inflammatory cells. The intestinal glands revealed mucinous degeneration and necrosis in addation to hyperplasia of goblet cells of the intestinal gland. Edema and hyaline degeneration of the tunica muscularis were evidence .The Liver showed vacuolar degeneration and multifocal area of coagulative necrosis of hepatocytes.The portal area revealed fibrosis connective tissue proliferation with focal inflammatory cells aggregation.

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الملخص العربى

التوصيف الجينى للكولستريديم بيرفيرنجينز والتغيرات الهستوباثولوجية للنزلات المعوية التنكرزية فى بدارى التسمين

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**قسم البكتريولوجيا والمناعه والفطريات-كلية الطب البيطرى -جامعة قناه السويس **قسم البكتريولوجيا والمناعه والفطريات-كلية الطب البيطرى-جامعة الزقازيق ***وحدة اللاهوائيات - قسم بحوث البكتريولوجيا معهد بحوث صحة الحيوان-الدقى-الجيزه ****قسم الباثولوجيا- قسم بحوث البكتريولوجيا معهد بحوث صحة الحيوان-الدقى-الجيزة *****قسم الباثولوجيا- قسم بحوث البكتريولوجيا معهد بحوث صحة الحيوان-فرع الاسماعيلية*

تم اجراء هده الدراسه على عدد٦ عترات من الكوليستريديم بيرفيرنجينز (٤لنوعAو٢ لنوعD) والمعزولات تم تجميعها لامن عينات امعاء وكبد من ٦ بدارى تسمين مريضة . وتم استخدام تفاعل البلمره المتسلسل لتحديد سم الالفا والابيسيلون. تم عمل تفاعل البلمره المتسلسل المتعدد لعددى٦ معزولات من الكوليستريديم بيرفيرينجينز (٤ من النوع A و٢ اخرين من النوع D)ووجد مناطق مميزه عند الوزن الجزيى ٤٠٢ و٥٤١ قاعده مزدوجه لكل من ال٦ معزولات اعطت ايجابيه لوجود جينات لسم الالفا . وتم تنقيه السم المستخلص عن طريق ترسيب املاح سلفات الامنيوم وتحليل البروتين باستخدام الفصل الكهربى. واوضحت الدراسه ان التحليل الكهربى من الادوات المهمه للتعريف الروتينى اللدى يعكس المعرفه الجينيه لكل من نوع Aو Dللكولسيتريديم بيرفرينجينز. وتم عمل الفحص الهستوباثولوجى لعينات الامعاء اظهرت تنكس هيالينى وتشدف وانتفاخ بين العضلات الممتله بخلايا ملتهبه.اظهر الكبد تكاثر نسيج ليفى ضام جزئيا فى الوريد البابى حول القناه الصفراوية داخل الكبد وتكاثر مفرط للتنسج لظهاره صفراوية متجه للمعه مصاحبه بتوسع جيبى كبدى.