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Studies on clostridial infections in broiler chicken and ducks in Damietta governorate

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Abstract

Clostridial infections are important concern to poultry industry because of severe economic losses and increased mortalities. This study was carried to investigate the current situation of C. *Perfringens*infection in broilers and ducks in Damietta governorate, Egypt.A total of 141 intestinal and liver samples were collected from diseased and deadbroiler chickenswith diarrheic chickens (n=129)and ducks (n=12) from private farms to be examined for *Clostridium perfringens*.The rate of isolation was 48.22% while isolation rate in broiler chickens and ducks was 47.22% and 58.33% respectively .In-vitro sensitivity test was made against different types of antibiotics showing that the examined isolates were highly sensitive to amoxicillin ,ampicillin n and doxycycline.Detection of toxins by animal inoculation by using Guinea pigs and Identification of the isolates using PCR were done .Experimental infection was done in broiler chickens .Signs and post mortumlesions were recorded also reisolation was done as well as histopathological findings were recorded.

Key words :C.perfringens– Necrotic enteritis – Experimental infection –Histopathological findings – Cooked meat media – PCR .

Introduction

Clostridia are commonly found in the environment, occurring in soil, sewage, and water , as well as in the intestine of both man and animal. Members of the genus clostridium are recognized as enteric pathogen for man ,domestic animals and wild life (songer , 1996). Clostridium perfringens (Cl.perfringens) is a part of normal gut flora ,which commonly involved in diseases in most domestic animals and some wildlife including horses , poultry, rabbits , sheep ,goats ,cattle ,mink , ostrich ,dogs and cats (Nillo, 1993). Necrotic enteritis is an important sporadic disease of broiler chicken , that first reported by (Parish, 1961) to be caused mainly by C.perfringens(Prukner*et al* .,1995).

Mucosal damage inducing factors such as coccidiosis (parasitism) ,high fiber litters ,dietary changes and poor hygienic and housing conditions are predisposing factors. These may produce a favorable growth environment for C.perfringens ,resulting in its over growth and production of potant toxins that leads to necrotic enteritis (Vissiennen*et al* .,1994). Necrotic enteritis (NE) in poultry is caused by C. perfringens type A or C (Ficken, 1991). Necrotic enteritis occurs when there is an increase in the intestinal population of C.perfringens following ingestion of a large number of bacteria (Riddell and Kong, 1992) or an upset in the normal intestinal microflora (Fukata*et al.*, 1987).

Materials and methods

Samples :A total 141 samples (intestine n =90 and liver n =51) were collected fromdiseased broiler chickens with diarrheic chickens (n=129) and ducks (n=12) from private farms to be examined for Clostridium perfringens.

Isolation and Identification :Was done according to Willis (1997) the collectedsamples were inoculated into tubes of freshly prepared boiled and cooled cooked meat medium (Oxoid) and incubated anaerobically for 24 hours at 37°C. A loopful of inoculated fluid medium was streaked onto neomycin sulphate sheep blood agar plates according to Carter and Cole(1999) .The streaked plates were incubated anaerobically for 24 hours at 37 C using a gaspak anaerobic jar .

Biochemical identification:according to the schem of Koneman et al.,(1992) and Macfaddin, (2000) .The C.perfringens isolates were subjected to biochemical identification patterns , including haemolytic activity on sheep blood agar ,lecithinase activity on egg yolk agar , catalase test ,indole test ,urease test ,sugar fermentation (lactose,glucose,sucrose and maltose) and liquefaction test .

Typing of C.perfringens toxins by dermonecrotic test in albino Guinea pigs:preparation of the toxins was done

according to Bullen, (1952) Application and interpretation of the test in albino Guinea pigs were adopted after Oakley and Warrak,(1953) and Quinn et al., (2002)

In vitro antibiotic sensitivity test: The disc diffusion technique was applied according to National Committee for Clinical Laboratory Standards (NCCLS) , (1998).

Genotyping of C.perfringens using polymerase chain reaction (PCR) : Oligonucleotide primers used for detection of alpha and beta toxins by conventional PCRwas carried out accordingto Yooet al. (1997).

Table (1): Oligonucleotide primers sequences
Source:Midland Certified Reagent Company_ oilgos (USA).

Toxin	Primer	Sequence	Amplified product
Alpha toxin	F	GTTGATAGCGCAGGACATGTTAAG	402bp
	R	CATGTAGTCATCTGTTCCAGCATC	
Beta toxin	F	ACTATACAGACAGATCATTCAACC	236 bp
	R	TTAGGAGCAGTTAGAACTACAGAC	

Extraction ofDNA was done According to QIAamp DNA mini kit instructions.

Preparation of PCR master mix :was done according to according to Emerald Amp GT PCR mastermix (Takara) Code No. RR310A kit

DNA samples were amplified in a total volume of 25µl of the following reaction mixture: 12.5µl of PCR master mix, 1µl of both of forward and reverse primer for each toxin gene (alpha and beta), 4.5µl of PCR grade water, and 6µl of the template DNA. Thermal cycling designed as initial denaturation for 5 min at 94°C, the samples were subjected to 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min. After the last cycle, a final extension was performed for 10 min. at 72°C. The detection of amplified products were analyzed by electrophoresis on agarose gel which supplemented with ethidium bromide in order to visualize the DNA on an UV transilliminator .(Sambrooket al ., 1989).

Experimental induction of NE in broiler chickens:

A total number of 40 Cobb broiler chicks obtained at 1 day of age were reared for experimental study .They were housed in the same room and chickens had ad libitum access to drinking water and feed .At 20th day of age they were divided into two groups ,group 1 containinig11 chicks were kept without inoculation as negative control group and group 2 containing 29 chicks were inoculated each orally by (1ml × 10^8cfu)of 24 hrs. cooked meat broth culture of the identified toxigenic Cl.perfringens isolates .The plate count technique was carried out according to Cruickshank et al., (1975) for the determination of the viable count of cell per ml suspension. Each bird in infected group was inoculated orally 1ml (1× 10^8cfu) with clostridial isolate according to Abd El-GwadandAbd El-Kader (2001). Both groups were kept for 10 days with daily observation for clinical signs .At the end of the observation period, all experimental and control birds were slaughtered and subjected to postmortem and bacteriological examination for reisolation of the inoculated organisms. The presence of C.perfringenswas confirmed by colony morphology, Gram-staining,negative reaction in catalase, indole, and urease test , as well as histopathological examination .Samples were taken from intestine and liver at 3,5,and 7 day post infection . Feed consumption and average body weight were recorded in control and infected group as shown in

Results and discussion

Clinical signs of diseased birds and post-mortem examination of dead ones: The diseased birds exhibited general signs in the form ofdepression,

reluctance to move, pronounced apathy, ruffledfeather and watery diarrhoea Moreover, the pathological lesions of dead birds revealed friable small intestine (duodenum and jejunum) distendedwith gas. The intestinal mucosas were covered by yellowish or greenpseudo membrane.

A total of 141 samples collected from freshly dead broilers, and ducks were investigated for the presence ofC.perfringens ,out of which 68 isolates were detected (48.22%) Abd El- Hamid et al ., (2015) .The isolation from broilers was (47.28%) and ducks (58.33%)that results were relatively similar to results obtained by Abd El-Hamid et al .,(2015) who recorded isolation from broilers (77.4%) and while El-Jakeeet al ., (2013) recorded isolation from broilers (75%) and ducks (66.6%).

Concerning the incidence of C.perfringens among dead birds from intestine and liver samples the rate of isolation was 51.11% and 43.13% respectively as shown in table (2)and table(3). This table indicated that there were significant rise in isolation rate of C.perfringens from intestinal samples compared with liver samples and this could be attributed to the fact that C.perfringensis a normal inhabitant in intestine and is considered as mean predisposing factor to necrotic enteritis (Silva, et al., 2009).This results partially agreed with Abd El-Hamid et al ., (2015)who recorded that isolation from intestine and liver was (79%) and (41.3%) respectively . The present results were higher than results obtained by El-Refaey (1999); Craven et al., (2001) who recorded that the incidence of C.perfringens isolation from intestine was 33.3% and 20%, respectively.

Table (2): Incidence of C.perfringens in examined intestinal samples from broiler chickens and ducks in Damietta govenorate.

* Incidence was calculated according to total samples number.

No. of examined farms		No. of examined intestinal samples	No. of +veC.perfringens isolates	* Incidence of C.perfringens in examined farms (%)
broilers	٨٤	84 pool samples	43	51.19
ducks	٦	6 pool samples	٣	50
Total samples		٩٠	٤٦	51.11

Table (3): Incidence of C.perfringens in examined liver samples from broiler chickens and ducks .

No. of examined farms		No. of examined liver samples	No. of +veC.perfringens isolates	*Incidence of C.perfringens in examined farms (%)
broilers	٨٤	45 pool samples	18	40%
ducks	٦	6 pool samples	4	66.6%
Total samples		٥١	22	٤٣.١٣

Characterization of bacterial isolates: All bacterial isolates exhibited the characteristic features of C. perfringens. The colonial characters on blood agar
showed dew drops smooth greyish convex colonies with a double zone of haemolysis. Microscopic characters revealed gram positive non motile rods.

Biochemical identification of the isolates showed catalase,indole ,urease negative while lecithinase,gelatinase were positive and a haemolytic activity onsheep blood agar showing double zone of haemolysis.Gas and acid from lactose,glucose,sucrose and maltosewere seen,.Negler's reaction was positive.

Dermonecrotic reaction in albino Guinea pigs was used for differentiation of different C.perfringenstypes,the result was type A showed An irregular area of yellowish to greenish necrosis and the lesion tend to spread downward (alpha toxin).

In vitro sensitivity ofC.perfringens isolates to different antimicrobial agent: Senstivity of C.perfringensisolates to antimicrobial agents was studied. C.perfringens isolates were sensitive to amoxycillin ,ampicillin, penicillin and doxycycline while, resistant to gentamicin,streptomicin and colistin but they intermediate sensitivity to Sulphaquinoxalin/Trimethprim and Ceftriaxone .These results agreed with Abd-El Gwad and Abd-El Kader (2001) who said that C. Perfringensisolates were highly sensitive to ampicillin, ciprofloxacin, amoxicillin, colistine, lincomycin and moderately sensitive to enerofloxacin, chloramephenicol, erythromycin, oxytetracycline, nalidixicacid, while neomycin, gentamycin, streptomycin had no effect at all. Such data was partialy agree also with Silva et al., (2009) who revealed that C.perfringensisolates were sensitive to penicillin, avilamycin, monensin and narasin but resistant to tetracycline and bacitracin. These findings relatively came with that recorded by Heba (2012) who found amoxicillin and ampicillin were the most effective therapy and prevention of C. perfringens infection while enrofloxacin and oxytetracycline were intermediate sensitivity.

Result of PCR

In this study we used PCR technique as a recent, rapid, reliable and accurate diagnostic tool for

characterization proved that 12 examined field isolates were C.perfringens type A(Alpha toxin) at 402bp as inFig no.(3)and all isolates were negative for beta toxins. This agreed with Effat et al. (2007) who reported that the same single amplicon, with the same size representing the alpha toxin encoding gene was amplified from all C. Perfringensfield isolates causing outbreaks of severe NE during the winter of 2006 in Egypt.This result is hand to hand toAbd El-Hamid et al ., (2015).

Experimental infection was done in broiler chickens by using forty –one day old baby chicks obtained from commercial hatchery . The infectious dose was used according to Abd El-Gwad and Abd El-Kader (2001) with 1ml (1×10^8cfu) of the identified toxigenic C.perfringens isolate orally. The chicks were experimentally infection at 20days old.

The clinical signs were recorded as depression , ruffled feathers and pasty brown diarrhea that agree with Al-Sheikhly and Al-Saieg, (1980) and Gadzinski and Julian, (1992) .

The post mortum lesions were recorded as liver showed congestion , friability and subcapsular hemorrhage as Fig (4)while intestine showed enteritisand duodenum, jejunum and ileum, are thin walled, friable, dilated and filled with gas as Fig(5) and (6) this lesion is hand to hand with those recorded by Broussard et al., (1986) , Mostafa, (1992), andAbd El-Gwadand Abd El-Kader (2001)

Reisolation was done from the intestine and liver with rates (68.9%),(48.27%) respectively as Table ( ) neighbor joining study with Abd El-Hamid et al ., (2015).

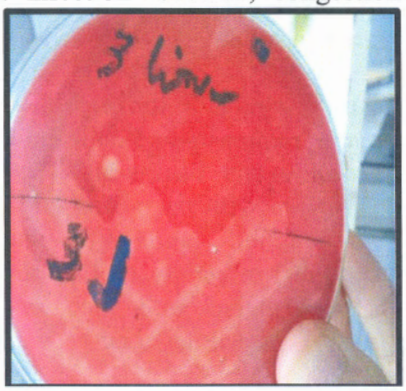
Organs for histopathlogical examinations were collected from intestine and liver .The examination showing of liver 3 & 5 and 7 days post infection revealed, congestion of central vein and hepatic sinusoids, many cases showed periportal inflammatory cells infiltration ranged in severity from mild to heavy infiltration either with mononuclear cells, heterophils or bothwith different severity,similar findings were recorded byPrescott (1977) and Heba (2012).

Histopathological examination of intestines 3 days post infection showed heavy mononuclear and heterophilic cells infiltration of lamina propriaIntestinal lesions became more obvious and severe in 5 day posy infection, some cases showed severe intestinal congestion and hemorrhagewith mononuclear cells infiltration and necrosis in the intestinal glands. Heterophilic cells infiltration of lamina propria was also detected in many cases.Many other cases showed severe necrotic enteritis manifested by complete destruction and necrosis of lamina epithelialis.Thereforhistopathological examination of intestine 7 days post infection showed necrotic enteritis in many cases with complete destruction of intestinal glands similar histopathological alterations were also observed previously in broiler chickens by Hofshagen and Stenwig, (1992) and in rabbits by Heba(2012)Fig(7) and Fig(8).

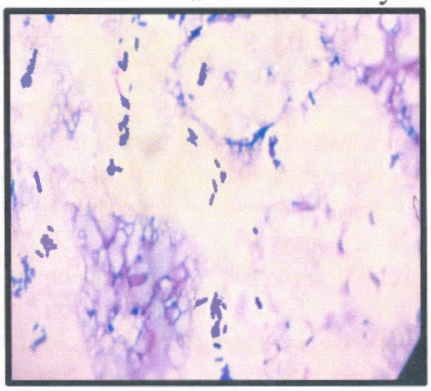
Concerning body weight the infected group has lower body weight than control group and this observed result was agree with Al-Sheikhly and Al-Saieg, 1980 and Gadzinski and Julian, 1992).

Conclusion

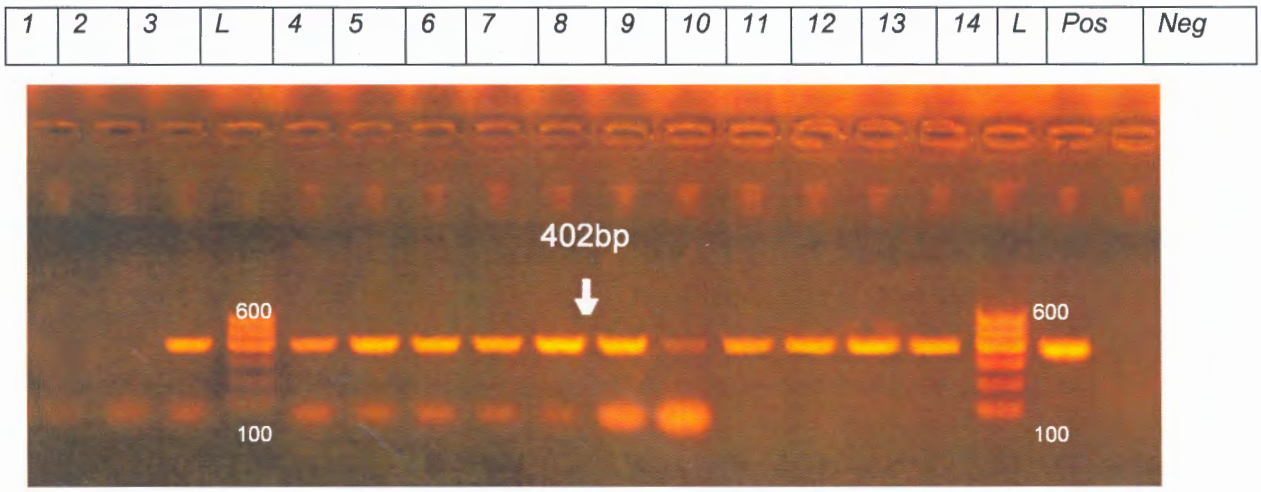
C.perfringens has a highly pathological effect on broiler and ducks .C. Perfringenstype A was the most predominant etiology of necrotic enteritis in the examined chicken flocks in Dameitta governorate.Good concern should be taken to prevent such infection in poultry farms through biosecurity and predisposing factors such as coccidiaovercrowding, badlitter, increase ammonia and mycotoxins.



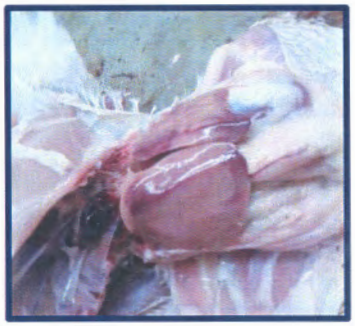
Fig(1): The isolates grow on 10% sheep blood agar medium showing the double zone of haemolysis



Fig(2): Gram +ve bacilli of isolates showing small straight bacilli with rounded end & parallel sides and spores were usually absent.



Fig(3): Agarose gel electrophoresis for detection of C.perfringens alpha-toxin gene .



Fig(4): Liver showing congestionand friability

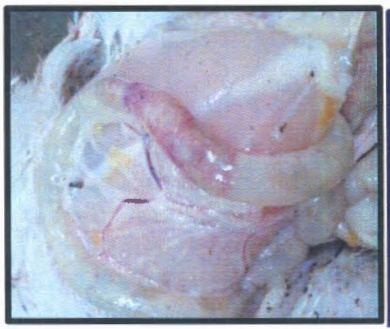


Fig (5): Ballooning of small intestine

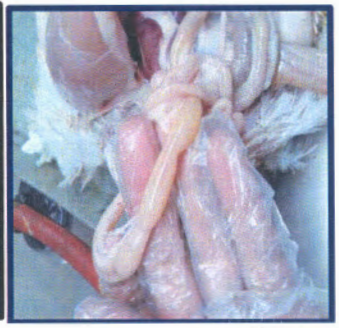


Fig (6) Intestine showing inflammation

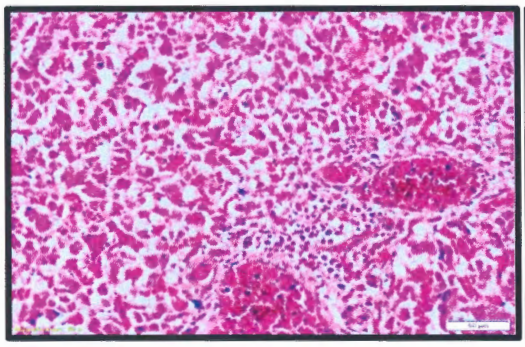


Figure (7): Liver of chicken 7 days post infection, showing severe congestion and necrosis of hepatocytes (H&E)

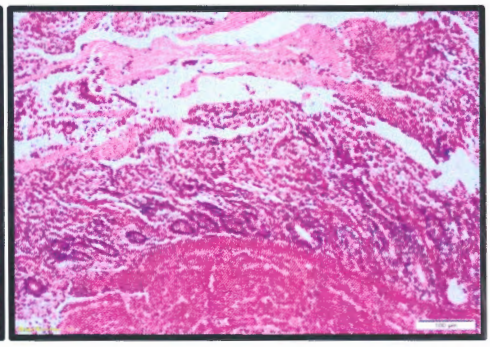


Figure (8): Intestine of chicken 7 days post infection, showing severe necrotic enteritis (H&E)

Acknowledgment

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المستخلص العربي

في هذه الرسالة تم فحص عدد ١٤١ عينة من أمعاء وكبد دجاج تسمين وبط نافقة حديثا حيث تم جمعها من مزارع خاصة فى محافظة دمياط .حيث تم عزل ٦١ عينة للكلوستردیوم بيرفرينجينز من ٨٤ مزرعة دجاج التسمين بإجمالي ١٢٩ عينة أمعاء وكبد (٨٤)،(٤٥)على الترتيب حيث كانت نسبة العزل الإجمالية(٤٧.٢٨%) بينما من الأمعاء (٤٣)بنسبة(٥١.١٩%) والكبد(١٨)بنسبة(٤٠%) بينما تم عزل ٧ عينات لنفس الميكروب من ٦ مزارع بط بإجمالى ١٢ عينة أمعاء وكبد (٦)،(٦) على الترتيب حيث كانت نسبة العزل الإجمالية (٥٨.٣٣%) بينما من الأمعاء (٣) بنسبة(٥٠%) والكبد (٤) بنسبة (٦٦.٦٦%) وهذ العينات كانت تعاني من أفات مرضية و تشريحية لعدوى الكلوستردیوم بيرفرينجينز. وقد تم تطبيق تفاعل الناجلرز على عترات الكلوستردیوم بيرفرينجينز المعزولة لمعرفة تأثير الليسيسين لسم الألفا لمح البيض بإستخدام أجار مح البيض الغني وأوضحت النتائج تكون طبقة تماثل اللؤلؤمحيطة بالمستعمرات وقد تم أضا فى هذه الدراسة تصنيف عترات الكلوستردیوم بيرفرينجينزالمعزولة بإستخدام تفاعلات تنكرز الجلد (بالحقن داخل الجلد فى خنازير غينيا الألبينو) وأوضحت النتائج عند مكان الحق ظهرت منطقة تتراوح من اللون الإصفر إلى اللون الأخضر تماثل لسم الألفا.وبإجراء إختبار الحساسية لعدد ٧ عترات معزولة عشوائيا وجد أنها عالية الحساسية لكل من بنسلين ، الأموكسيلسين ، أمبيسلين ،دوكسسسيكلين،أوكسسيكلين بينمل لم يتأثر الجنتاميسين والإستربتومايسين والنيومايسين .وبإجراء تفاعل البلمرة المتسلسل لعدد ١٤ عترات معزولة عشوائيا تم التعرف على ١٢ عترة معزولة لسم الألفا بينما لم تظهر أى من النتائج لسم البيتا .

وبإجراء العدوى الصناعية بهذا الميكروب فى دجاج تسمين عند عمر ٢٠ يوم عن طريق الفم لم يحدث أى وفيات بينما كانت الأفات المرضية والتشريحية تشبه الى حد كبير تلك المسجلة فى العدوى الطبيعية وهذا وقد تم عزل الميكروب مرة أخرة من الطيور بعد ذبحها حيث سجلت نسبة العزل من عينات الأمعاء ٦٨.٩% والكبد ٤٨.٢٧% .