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RESEARCH ARTICLE

Characterization of Toxigenic Strains of Clostridium Perfringens Type A From Broiler Chicken With Necrotic Enteritis.

Fayez, M. M[1], Abdelaziz, A. M[2]. , Al Musallam A.[3]

1. Serum and Vaccine Research Institute, Cairo, Egypt.
2. Department of Avian and Rabbit Medicine, Faculty of Veterinary Medicine,Zagazig University
3. Veterinary Diagnostic Laboratory – Al Ahsa, KSA.

Manuscript Info *Abstract*

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Clostridium perfringens is a ubiquitous and versatile pathogenic bacterium and is implicated in the etiology of the poultry diseases necrotic enteritis (NE) .In this work, four outbreaks of necrotic enteritis were investigated in broiler farms in the AL-Hasa governorate, KSA. The clinically affected birds showed moderate to severe depression, diarrhoea and death. At necropsy, the birds generally showed good bodily conditions, but severely dehydrated. The striking gross morbid lesions were located at small intestine , where it was found markedly dilated with patches of multifocal hemorrhage in the inner wall. Intestinal impression smears showed a plenty of Gram positive bacilli. Eleven Clostridial isolates were characterized phenotypically and biochemically. All isolates exhibited characteristics of Clostridium perfringens. Culture supernatants from all isolates were toxigenic for mice. ELISA and mouse neutralization test revealed the detection of alpha toxin from all isolates. All the Eleven isolates proved the alpha toxin producing strains of C. perfringens when they were subjected to alpha toxin specific PCR. The antimicrobial susceptibility test showed that All isolates were susceptible to Amoxicillin, Amoxicillin- Clavulanic acid, Cefoxtin, Cephalexin , and Metronidazol. On the other hand, all isolates were resistant to Sulfaquinoxalin (100%), 10 isolates (90.4%) to Erythromycin, cephalexin and 4 (36.3%)isolates were resistant to Oxytetracyclin. In conclusion, alpha toxin specific PCR is highly useful for detection of toxigenic strains of Clostridium perfringens in poultry, A continuous monitoring of the virulence factors and the antimicrobial susceptibility profile of C. perfringens from animal origin, particularly, poultry are necessary for a better prevention and treatment of the necrotic enteritis in avian and new control and prevention strategies are needed.

Introduction

Clostridium perfringens (CP) is a Gram positive, anaerobic, rod-shape, spore forming bacterium that is capable of causing a broad spectrum of diseases in both human and animals. It is an environmentally dominat that can found in soil , dust , waste water , feces , feed , poultry litter and also intestinal tract of human and animals (Hatheway, 1990 ; Rood and Cole, 1991 ; Ficken , and Wages, 1997).

CP is a common inhabitant of the intestine of healthy broiler chickens belonging to the resident of microbiota , and it can cause necrotic enteritis (NE) in many species of poultry especially in broiler and turkey flocks (Engstrom et al., 2003; Sengupta, et al., 2011).

Infection with CP is not the sole factor for development of NE disease ; predisposing factors such as intestinal damage caused by coccidial pathogen , dietary protein and carbohydrate proportion and properties has also been shown to

strongly influence the incidence of NE in broilers (Branton et al., 1997 ; Collier et al., 2008 ; Branton , et al., 1987 ; Wu et al., 2010) .

CP is grouped into five toxinotypes, A, B, C, D and E based on production of four major toxins (alpha , beta , epsilon and iota toxins) (Rood, and Cole, 1991 ; Yoo, et al., 1997).

NE of poultry was first described by Parish in 1961 and has since been found in almost all poultry producing countries (Bains, 1968 ; Ficken, and Wages, 1997). It is a wide spread disease in broilers imposing a significant economic burden on poultry industry worldwide. The total global economic loss as a consequence of NE outbreaks in broiler farms is estimated to be over 2 billion dollar annually.(Van der Sluis, 2000 ; Lovland , and Kaldhusdal , 2001).

NE of poultry characterized by sever necrosis of the small intestinal mucosa in the proximal jejunum region and it is associated with high mortality rate (Long, 1973). On the other hand, subclinical disease lead to decrease performance due to extensive mucosal damage, (Lovland , and Kaldhusdal , 2001).

The alpha toxin has been implicated in several diseases including NE in chickens. The toxin destruction of mucosal tissue manifests as macroscopic lesions that are usually seen in jejunum and ileum but can also appear in duodenum (Rood, 1998) .

The polymerase chain reaction assay (PCR) was used for detection of alpha toxigenic strains of C. perfringens .(Engström et al., 2003; Baums, et al., 2004).

The most effective method to prevent or to control the outbreak of necrotic enteritis is the use of antimicrobials mixed to feed and water, although, bacterial resistance to bacitracin, tetracycline, clindamycin, lincomycin and erythromycin has been reported in several countries, such as, Denmark, Switzerland, Norway, Belgium, Jordan and Brazil . (Silva, et al., 2009).

The present study reports for the first time the occurrence of NE due to C P in commercial broilers in AL-Hasagovernorate KSA with subsequent toxinotyping and antimicrobial susceptibility of the isolated CP strains.

Material and Methods

1- Samples :

Samples were obtained from four commercially broiler farms (HA01, HA02, HA03, and HA04) located in Al-Hasa governorate , KSA. Data shown in Table (1).

A total of 25 apparently healthy birds and 116 birds with symptoms of NE were subjected to necropsy following the standard procedures described by (Charlton, 2000). Intestine showed macroscopic lesions were scientifically processed for microbiological investigation. A Gram stained impression smears were prepared from the collected samples after the methods of(McLeod, 1981).

2- Bacterial isolation and identification :

Intestinal contents after scarping were inoculated into tubes containing cooked meat medium (Difco) and incubated in anaerobic jar for 48 hours at 37 C^o. Aliquots of 0.1 ml were streaked 5% sheep blood agar supplemented with neomycin sulfate (200ug/ ml) ; plates were incubated anaerobically for 48 hours. suspected colonies were identified after the methods of (Russo, and Gorbach,1987).

3- Mouse bioassays:

Following culture of the isolated C. perfringens strains in cooked meat broth medium, the cells were harvested by centrifugation at 3000 rpm for 15 min and the cell-free culture supernatants were recovered. White mice (25 - 40 g) were injected intraperitonealy with 0.3 ml of the culture supernatant and then observed over a period of three days for either death or disease symptoms (Sterne and Batty, 1975) . Control group of mice were injected with broth culture without bacteria.

4- Toxinotyping of CP isolates :

The culture supernatants from all CP isolates were subjected to:

a- Mouse neutralization test :

The test was performed according to (Sterne and Batty, 1975)using alpha , beta, and epsilon antitoxins.

ELISA test :

Bio-X Enterotoxaemia ELISA kit was used for toxin typing of CP isolates. The test and interpretation were according to the kit manufacture

5- Identification of toxigenic strains by Polymerase Chain Reaction (PCR):

Cell lysates were prepared after the methods of,(Baums, et al., 2004). The DNA samples were analyzed spectrophotometrically at 260 and 280 nm to check the presence of DNA and its purity. Samples were also subjected to electrophoresis on a 1.5% (w/v) agarose gel in 1 × TBE buffer to ensure the presence of intact DNA. (Maniatis, et al., 1982).

Primers used in this study were designed according to,(Baums, et al., 2004). Forward primer: (AGT CTA CGC TTG GGA TGG AA) and Reverse primer: (TTT CCT GGG TTG TCC ATTTC), which flanked 900 base pair DNAsequence.

To perform the PCR, 2 ul template DNA, was added to a 50 ul reaction mixture with the following reagents 1.25 U Taq DNA polymerase, 50 mMPottassium chloride, 30 mMTris-Hcl, 1.5 mM $Mg2^{++,}$ 200 µM of each dNTPand 50 picomoles of each primer. The thermocycling (incubations for 1 min at 95oC, 55oC and 72oC respectively was 35 times) was preceded by incubation for 2min 30 seconds at 95oC. Six microlitre of the amplicons was separated on 1.5% agarose gel according to standard procedure.

6- Antimicrobial susceptibility testing :

Antimicrobial susceptibility testing of CP isolates was performed using disc diffusion test as described by the National Committee for Clinical Laboratory Standards **(NCCLS) .** The antibiotics used were Amoxicillin , Amoxicillin- Clavulanic acid , Cefoxtin , Cephalexin , Clindamycin , Erythromycin , Oxytetracyclin , Enrofloxacin, Metronidazol, Floramphenicol, and Sulfaquinoxalin. All isolates were grow overnight in cooked meat broth medium , then cultures were suspended in 0.85% Nacl to an optical density equivalentto that of MacFarland 0.5 standards; each isolate was then inoculated into 5% sheep blood agar , 15 minutes later , antibiotic discs were applied . the plate were incubated at 37 C^o for 24 hours and the interpretation was performed according to the manufacture of the antibiotic disc.

Farm	Breed	Breeding system	Pervious history	Population of birds	Morbidity	Mortality	Samples
HA01	broiler	litter	coccidia	300	70 (23.3%)	50 (16.6%)	30
HA02	broiler	Litter	Coccidia ND	800	300 (37.5%)	300 (37.5%)	25
HA03	broiler	Litter	-	1000	350 (35%)	200 (20%)	33
HA04	broiler	litter	-	30,000	200 (0.66%)	100 (0.33%)	28

Table (1) Data of the broiler farms and sampling.

Result

1- Symptoms and necropsy findings :

Broiler chicken showed severe depression, decreased appetite, diarrhea, reluctance to move and sudden death.

At necropsy, the birds generally showed good bodily conditions, but severely dehydrated. The striking gross morbid lesions were located at small intestine , where it was found markedly dilated with patches of multifocal hemorrhage in the inner wall .

Large amount of necrotic entero-epithelial debris in the lumen including flecks of blood. Deposition of gas, bile contents in jejunum and ilium were also noted . The intestine was friable and easily tore off when handled. The other organs appeared apparently normal.

2- Bacteriological Findings :

Gram stained impression smears from intestinal samples showed large number of Gram positive bacilli.

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, lecithinase positive and a haemolytic activity on sheep blood agar showing double zone of haemolysis. Gas and acid from glucose, fructose, lactose sucrose and mannitol were seen, urease negative and gelatinase positive.

Out of 116 examined intestinal samples, 11 CP strains were isolated (9.4 %). Bacteriological examination of 25 apparently healthy chickens not revealed isolation of any CP isolates.

3- Mouse Bioassay :

Results of pathogenicity test in white mice were observed during 3 days which ends with death. All mice injected with the bacterial culture filtrate died and those injected with control broth without bacteria were alive.

4- Toxinotyping of CP isolates :

As shown in Table (2), typing procedures performed by both ELISA and mouse neutralization revealed the detection of alpha toxin in the culture supernatants of all isolates; on the other hand neither beta nor epsilon toxins were detected.

PCR products for the alpha toxin gene (900 bp) of C. perfringens are shown in the Figure (1).

5- Antimicrobial susceptibility test :

Susceptibility and resistance to the different antibiotics are shown in Table (3). All isolates were susceptible to Amoxicillin, Amoxicillin- Clavulanic acid, Cefoxtin, Cephalexin, and Metronidazol. On the other hand, all isolates were resistant to Sulfaquinoxalin (100%), 10 isolates (90.4%) to Erythromycin, cephalexin and 4 (36.3%)isolates were resistant to Oxytetracyclin.

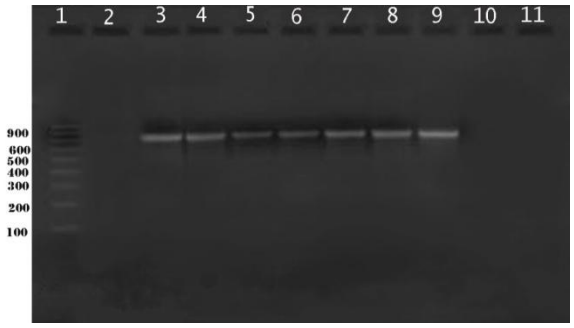

Figure (1): An agarose gel stained with ethidium bromide with PCR amplification products of C. perfringens isolates. Lane 1: molecular size markers 100bp; Lanes 2 negative control , lane 3- 9: C. perfringens isolates.

Farm	No of samples	No of isolates	%	Mouse neutralization test			ELISA		
				alpha	beta	Epsilon	alpha	beta	Epsilon
HA01	30	6	20	+	-	-	+	-	-
HA02	25	2	8	+	-	-	+	-	-
HA03	33	2	6	+			+		-
HA04	28	1	5	+	-	-	+	-	-

Table (2) Results of ELISA and mouse neutralization test for toxinotyping of CP isolates.

Antibiotic	Sensitive	Intermediate	Resistant
Amoxicillin	11 (100%)	0	0
Amoxicillin- Clavulanic acid	11 (100%)	0	0
Cefoxtin	11 (100%)	0	0
Cephalexin	0	1 (9.09%)	10 (90.9%)
Clindamycin	7 (63.6%)	3 (27.2 %)	1 (9.09 %)
Erythromycin	0	1 (9.09%)	10 (90.9%)
Oxytetracyclin	2(18.18 %)	5 (45.4%)	4 (36.3%)
Enrofloxacin	11(100%)	0	0
Metronidazol	11(100%)	0	0
Floramphenicol	10(90.9%)	1(9.09%)	0
Sulfaquinoxalin	0	0	11(100%)

Table (3) Results of antimicrobial susceptibility test.

Discussion

Enteric diseases are an important concern to the poultry industry because of production losses, increased mortality, reduced welfare of birds and increase the risk of contamination of poultry products for human consumption.

In the present study, the role of CP in necrotic enteritis in commercial broilers was investigated based on clinical, post mortem and microbiological findings.

The clinical sings of affected birds were included dehydration, emaciation ruffled feathers severe depression , decreased appetite , diarrhea , reluctance to move and sudden death ; these signs were more or less similar to those described by(Long, et al 1974 ; Al-Sheikhly, and Truscott, 1977 ; Ficken ,. and Wages, 1997).

Post mortem examination showed that birds were dehydrated and had fetid odor. Gross lesions were restricted to small intestine with severe lesions and necrosis on the wall of small intestine, these results come in accordance with those obtained by (Helmboldt, and Bryant,1971 ; Bernier, et al., 1977 ; Ficken ,. and Wages, 1997).

In the present study, 11 C. perfringens strains were isolated from 116 intestinal samples of broilers; the isolates were reconfirmed on the basis of morphological and cultural characteristics. The obtained colonies were circular, flat, greyish and surrounded by zone of double hemolysis after cultured anaerobically on blood agar at 37°C for 48 hrs. Microscopically all isolates were Gram-positive spore-forming bacilli, biochemical identification of the isolates showed both catalase and lecithinase positive, production of gas and acid from glucose, fructose, lactose, sucrose and mannitol was seen, these results come in accordance with (Quinn et al., 2002 ; El-Jakee et al., 2010)

As shown in Table (2) the incidence was high in farm HA01 (20%) and farm HA02 (8%) , this many contributed to the previous history of coccidiosis where the best known predisposing factor for necrotic enteritis is the mucosal damage caused by coccidial pathogen .(Williams, 2005).

The Gram stained intestinal impression smears showed high number of Gram positive bacilli , these results are documented by (Si, et al., 2007), however, the presence of C. perfringens in the intestinal tract of broiler chickens, even at high numbers, is not sufficient to produce necrotic enteritis(Nauerby, et al., 2003). It is likely that pathogenesis is toxin-mediated, although this has not been definitively demonstrated. The results of mouse bioassay confirmed this hypnosis where all mice injected with culture supernatant were die within 72 hours suggesting the presence of toxin.

Regarding to the results of mouse neutralization and ELISA, it was clear that alpha toxin was detected in the culture supernatant of all isolated strains while none of beta or epsilon toxins were detected. these results come in agree with the results obtained by(Ficken , and Wages, 1997)and as CPA is the only major toxin produced by C. perfringens type A , so the isolated strains were classified as CP type A, the results documented by (Engström et al., 2003).

PCR has been widely used in identifying the toxin genes of C. perfringens because of its high sensitivity. In this study, the primer combination used was reliable and very specific in amplifying 900 bp fragment of the alpha toxin gene- cpa of C. perfringens but not other genes of C. perfringens as proved by , (Baums, et al., 2004)

All the twenty isolates produced the predicted amplification size of 900 bp, with the gene coding for alpha toxin production (Fig .1) these results come in accordance with (Engström et al., 2003 ; Kalender, 2004)

Regarding to the results of antimicrobial susceptibility test, it was clear that Amoxicillin, amoxicillin-clavulanic acid and cefoxitin as well as Enrofloxacin showed an excellent activity against the evaluated CP strains and it is in accordance with studies performed by (Gharaibeh, et al., 2010).

Cephalexin showed low activity against the tested strains and resistance rate of 90.9% was observed; it might be explained by its widespread use in broiler production and due to the low cost. (Allen, et al., 2003).

The results revealed that the resistance of CP isolates were 36.4% to Oxytetracyclin; The resistance to tetracycline is commonly observed in C. perfringens and it is codified by the tetP gene. (Silva, et al 2009).

Sulfonamide is another drug commonly used as feed additives and for the treatment of respiratory diseases in poultry. In this study, all the tested strains were resistant to sulfaquinoxalin in accordance with previous report. (Allen, et al., 2003)

Based on the study, we concluded that 11 toxigenic strain of C. perfringens were isolated from necrotic enteritis cases in poultry farms in Al hasa, KSA. ELISA, Mouse neutralization test and PCR-based assay were used for the detection of C. perfringens. PCR method was rapid, sensitive, and specific. A continuous monitoring of the virulence factors and the antimicrobial susceptibility profile of C. perfringens from animal origin, particularly, poultry are necessary for a better prevention and treatment of the necrotic enteritis in avian and new control and prevention strategies are needed.

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