Zag. Vet. J. (ISSN. 1110-1458) Vol. 41 No. 1 (2013) pp.169-175

Molecular Studies on *Clostridium Perfringens* Resistant to Tetracycline Group

Attia A M*, Basma S Mahmoud and Amira S M Elrafie*****
*Bacteriology, Mycology and Immunology Department, Faculty of Vet. Medicine, Zagazig Uni.
** Anaerobic Unit, Bacteriology Research Dept., Animal Health Research Institute, Dokki, Giza
*** Bacteriology Department, Animal Health Research Institute, Zagazig branch, Sharkia

ABSTRACT

Clostridia are spore-forming bacteria causing health hazards for human beings and animals ranging from wound infection to necrotic enteritis. In the present work, 60 dropping samples were collected from diarrhoeic broilers of 7 different farms in Sharkia and Cairo Governorates for isolation and identification of *C.perfringens* by conventional methods. The results showed that incidence of *C. perfringens* was 41% (25/60), 23 out of them were proved to be *C. perfringens* type A, while only two isolates were type C. Antibiotic susceptibility test revealed that 88% of *C. perfringens* isolates were sensitive to amoxicillin, 84% were sensitive to cefotaxime, cefuroxime, cefotraxion, piperacillin and bacitracin, 80% were sensitive to erythromycin, 76% were sensitive to fuscidic acid, 64% were sensitive to tetracycline, doxycycline and clindamycin, while 24% of the isolates were sensitive to lincomycin. Furthermore, MICs for tetracycline resistant isolates were ranged from 8-32 µg/ml. PCR was applied to detect α-toxin gene of *C. perfringens* and tetracycline resistant genes (*tetB*, *tetM* and *tetK*). All isolates gave positive amplification for alpha toxin gene, while *tetB* gene was detected in two out of 5 isolates , but *tetK* and *tetM* genes weren't detected in any isolates.

INTRODUCTION

Clostridium perfringens is anaerobic microorganism responsible for a wide range of diseases in animals and humans *(1)*. Necrotic enteritis (NE) in poultry is caused by *Clostridium perfringens* types A and C *(2)*. *C.perfringens* type A is common in the environment and can be isolated from intestinal contents of birds and mammals *(3)*. Mucosal damage including factors such as coccidiosis, high fiber, litters, dietary changes, poor hygienic and housing conditions may produce a favorable growth environment for *C. perfringens* resulting in its overgrowth and production of potent toxins that lead to necrotic enteritis *(4)*. The virulence of the organism is associated with the production of several toxins; among them, four are known as the major lethal toxins (α, β, ε and ι), the most common one is α toxin *(5)*. The addition of antimicrobial growth promoters in low doses in chicken feed resulting in increase weight gain and feed efficacy. However, its continuous use is thought to contribute to the development of antibiotic resistant bacteria and antibiotic residues *(6)*.

The aim of this work was planned for molecular detection of tetracycline resistant genes in *Clostridium perfringens* isolates recovered from diarrhoeic broilers.

MATERIAL AND METHODS

Sampling

Sixty dropping samples were collected from 7 farms of diarrhoeic broilers in Sharkia (40 samples from 5 farms) and Cairo (20 samples from 2 farms) Governorates at the period from 2007 to 2008.

Isolation of *C. perfringens*

Samples were inoculated into sterile freshly prepared cooked meat broth and incubated anaerobically at 37°C for 24-48 hours. A loopful from each incubated tube was streaked onto the surface of 10% sheep blood agar with neomycin sulphate (200 µg/ml) and incubated anaerobically at 37°C for 24-48 hours *(7)*.

Identification of *C. perfringens* isolates

It is based mainly on colonial appearance *(8)*, microscopical examination *(9)*, biochemical tests (fermentation of sugars, gelatin liquefaction, catalase, litmus milk and indole tests) *(10)*, Nagler's test by half antitoxin plate *(7)* and finally typing of *C. perfringens* toxin by dermonecrotic test in albino guinea pigs *(11, 12)* and interpretation of the results according to colour degree of the dermonecrotic reaction and neutralization *(13)*.

Antimicrobial susceptibility testing

Disc diffusion method

It was applied using previously published protocol *(14)* and the diameter of inhibition zone for each antimicrobial agent was measured and interpreted according to CLSI standards *(15)*. The following antimicrobial discs were used (Oxoid): amoxicillin, cefotaxime, cefotraxion, cefuroxime, piperacillin, bacitracin, fuscidic acid, lincomycin, erythromycin, tetracycline, doxycycline and clindamycin.

Minimum inhibitory concentration (MIC) *(16)*

MIC of tetracycline against *C. perfringens* isolates was determined by broth macrodilution method (concentration of stock solution should be 1000mg/L or Greater) then a double fold serial dilution was made starting from a concentration of 1024 µg/ml. The results were interpreted as susceptible, intermediate, or resistant according to CLSI standards *(15)*.

PCR for detection of *C. perfringens* alpha toxin gene and tetracycline resistant genes

Template DNA preparation *(17)*

Pure colonies of *C. perfringens* were grown overnight in 5 ml brain heart infusion supplemented with 1% sodium thioglycolate at 37°C under anaerobic condition. One ml of culture was centrifuged at 5000 xg for 15 minutes in a heat block for cell lysis then cooled on refrigerator for 15 minutes. Finally, the suspension was centrifuged at 13000 xg for 2 minutes. Ten microliter of supernatant fluid was used as template DNA.

Oligonucleotide primers (Sigma)

Primer for *C. perfringens α-toxin gene (18)*

F 5`GTTGATAGCGCAGGACATGTTAAG3`
R 5` CATGTAGTCATCTGTTCCAGCATC3`

Primer for tetracycline resistant genes *(19, 20)*

Tet M F5`-ACAGAAAGCTTATTATATAAC-3`
R 5`-TGGCGTGCTAGAGTCAC3`

Tet B F 5`AAA ACT TAT TAT ATA ATG GTG3`
R 5` TGG AGT ATC AAT AAA TTCAC3`

Tet K F 5`-TTATGCTGCTTGTAGCTAGAAA-3`
R5`AAAGGGTTAGAAACTCTTGAAA-3`

C. PCR amplification and cycling protocol

1.Uniplex PCR for amplification of alpha toxin gene of *C. perfringens (21)*

DNA samples were amplified in a total of 25 µl of the following reaction mixture 5 µl 10x buffer, 1 µl $MgCl_2$, 1µl dNTPs, 0.5 µl taq polymerase enzyme, 0.25 µl of primer, 12 µl DNAse-RNase-free deionized water and 5 µl of template DNA. PCR cycling program was performed in ptc-100 peltier thermal cycler as following: initial denaturation at 94°C for 5 minutes then 35 cycles consisting of denaturation at 94°C for one minute, annealing at 55°C for one minute and extension at 72°C for one minute and final extension at 72°C for 5 minutes.

2.Multiplex PCR for amplification of tetracycline resistant genes *(22)*

DNA samples were amplified in a reaction volume of 50 µm containing 1.5 µm $MgCl_2$, 2.5 µ *Taq* DNA polymerase, 200 µm of each dNTPs, pmol of each primer, 25 µl DNA samples. PCR running condition consisted of an initial cycle of 5 minutes of denaturation at 94°C/ min, annealing at 55°C for one minute and extension at 72°C for one minute thus

followed by final extension at 72ºC for 5 minutes.

Detection of PCR products *(23)*

Aliquot of each amplicon was loaded on 2% agarose gel containing 0.5 µg/ml of ethidium bromide. A 100 bp DNA ladder (Fermentas, Catalogue No. SM0328, Lot: 00049161) was used as a molecular weight standard The samples were electrophoresed at 90 V for 90 minutes on a mini horizontal electrophoresis unit (model EC370M ,BIO-RAD, USA); the gel was visualized under UV transilluminator (Spectroylyne Model TR-312 A, USA) and photographed.

RESULTS

Incidence of *C. perfringens* in diarrhoeic broilers

Incidence of *C. perfringens* in diarrhoeic broilers was 37.5% (15/40) in Sharkia and 50% (10/20) in Cairo Governorate.

Typing of *C. perfringens* isolates

C. perfringens type A (alpha toxin) appeared as an irregular area of yellowish necrosis tended to spread downward. While type C (beta toxin) produced a reaction which is intensively purplish yellowish haemorrhagic necrosis. Twenty three of examined isolates were considered as *C. perfringens* type A (had α-toxin) except two isolates only were *C. perfringens* type C (had α and β-toxin)

Antimicrobial susceptibility testing

Results of disc diffusion test of *C. perfringens* isolates showed that 22out of 25 samples (88%) of the isolates were sensitive to amoxicillin, 21 out of 25 (84%) were sensitive to cefotaxime, cefuroxime, cefotraxion, piperacillin and bacitracin, 20 out of 25 (80%) were sensitive to erythromycin, 19 out of 25 (76%) were sensitive to fuscidic acid, 16 out of 25 (64%) were sensitive to tetracycline, doxycycline and clindamycin, while 6 out of 25 (24%) of the isolates were sensitive to lincomycin.

Moreover, MIC for tetracycline resistant *C. perfringens* isolates was ranged from 8 to 32 µg/ml while MIC for tetracycline sensitive *C. perfringens* isolates ranged from 0.25 to 4 µg/ml.

Results of polymerase chain reaction

Uniplex PCR for alpha toxin gene of *C. perfringens*

All the examined isolates (5 isolates) were proved to have ά toxin gene which gave a characteristic band at 402 bp fragment (Fig.1).

Multiplex PCR for tetracycline resistant genes

Two isolates only have tet B gene which gave a characteristic band at 169 bp while tetK and tetM genes weren't detected on any of the isolate (Fig.2).

Fig.1. α toxin gene of C.perfringens Gave a characteristic band at 402 bp

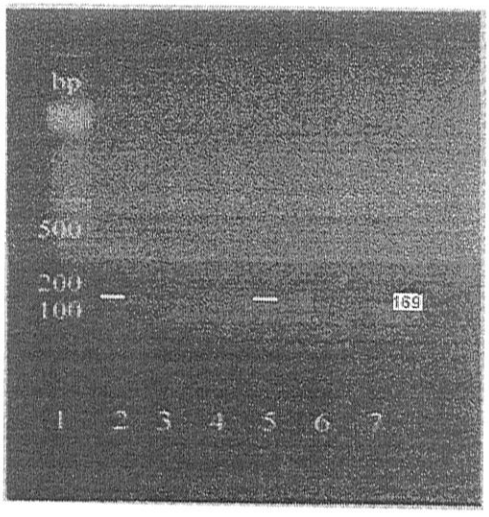

Fig. 2. *tetB* gene of examined *C.perfringens* isolates gave a Characteristic band at 169 bp

DISCUSSION

Clostridium perfringens is generally found in gastrointestinal tract of man and animals and is usually presented as mixed infection in which the primary pathogen has paved the way for the anaerobe by damaging the tissue and causing anaerobiosis *(24)*.

Incidence of *C. perfringens* recovered from diarrhoeic broilers was 41.6%. A nearly similar result was recorded in previous study *(25)*.

Results of antimicrobial susceptibility patterns of *C. perfringens* isolates showed that amoxicillin, cefotaxime, cefuroxime, ceftraxione, piperacillin and bacitracin were more effective against the microorganism under the study, while lincomycin was less effective. Nearly similar results were reported in another study *(26)* where higher susceptibility of *C. perfringens* to amoxicillin, cefotaxime, cefotraxion and bacitracin was detected.

C. perfringens isolates were resistant to tetracycline with a percentage 33.4%. Similar result was previously reported **(27)**, while higher result was recorded in another study *(28)*. On the contrary, tetracycline, penicillin, cephalosporin and rifampicin were the most active antibiotics against clostridia *(29)*.

The results of MIC test value shown that MIC of tetracycline for *C. perfringens* isolates ranged from 8 to 32 µg/ml. Those obtained results go in hand with those obtained previously *(22, 30)*.

Polymerase chain reaction (PCR) allows the specific and exponential synthesis of a predetermined DNA region via the use of two small and specificity designed fragments of DNA primers, which form the two termini of the nucleic acid molecule to be amplified.

In this study all the tested strains had α toxin gene and gave a characteristic band at 402 bp which is similar with that previously recorded *(18)*.

In this study ,*tet B* gene was detected only in 2 out of 5 examined *C.perfringens* isolates by multiplex PCR as detected in previously published paper *(31)*, while *tetK* and *tetM* genes weren't detected in any isolates *(27)*.

REFERENCES

1. ***Niilo L (1993):*** Pathogenesis of bacterial infections in animals. 2nd Ed., In: Gyless C.L. and Thoen C.O. (Ed. Iowa State University, press Ames, 114-123.
2. ***Fiken M D (1991):*** Necrotic enteritis in diseases of poultry. Calnek B.W., John Barners H., Beaed C.W., Reid W.M. and Yoder H.W. 9th Ed., pp. 264-275. Iowa State, University press, Ams, Iowa, USA.
3. ***Timoney J F, Gillespie J H, Scott F W and Bralough J K (1988):*** The Genetics of Virulence and Antibiotic Resistance. Hagan and Bruners Microbiology and Infectious Diseases of Domestic Animals. 8th Ed., Cornell University press, Ithaca and London. pp. 223-229.
4. ***Vissiennon T, Johannsen U and Kohler B (1994):*** Pathology and pathogenesis of *C. perfringens* type A enterotoxaemia in fowls experimental reproduction clinical picture and mortality rate. Monatsheft fur Veterinarmedizin, 49 (1): 23-28.
5. ***Petit L, Gibert M and Popoff MR (1999):*** *Clostridium perfringens*: toxinotype and genotype. Trends Microbiol., 7 (3): 104-110.
6. ***Tauber M, Meile L and Schwarz F (1999):*** Acquired antibiotic resistance in lactic acid bacteria from food. Antonie Ieeu Wenhoek., 76: 115-137.
7. ***Smith L D S and Holdman (1968):*** The pathogenic anaerobic bacteria. 1st Ed., Charles Thomas publisher, USA, 201-255.
8. ***Vaikosen E S and Miller W (2001):*** Evaluating biochemical tests for isolation/identification of *C. perfringens* in faecal samples of small ruminant in Nigeria. Bulletin of Animal Health and Production in Africa, 49 (4): 244-248.
9. ***Wilson E S and Muller W (2001):*** Principle of bacteriology, virology and immunology. 6th Ed. Vol. I and Vol. II, Baltimore, Williams and Wilkin.
10. ***Koneman E W, Allen S D, Dowell V R and Summers H W. (1992):*** Color Atlas and Textbook of Diagnostic Microbiology 4th Ed. J.B. Lippencott, New York, London.
11. ***Bullen J J (1952):*** *C. perfringens* in the alimentary tract in normal sheep. J. Path. Bact., 64: 201-210.
12. ***Quinn P J, Markey B K, Carter M F, Donnelly W J, Leonard F C and Maguire D (2002):*** Veterinary Microbiology and Microbial Diseases. 2nd Ed. Blackwell Science, 84-96.
13. ***Stern M and Batty I (1975):*** Pathogenic clostridia. Butter Worth, London, Boston.
14. ***Ortez H J (2005):*** Test methods: disk diffusion testing, In, Coyle B M (Ed.): Manual of antimicrobial susceptibility testing, American Society for Microbiology, P. 39-52.
15. ***Clinical and Laboratory Standards Institute (CLSI) (2011):*** Performance standards for antimicrobial susceptibility testing; twenty-first informational supplement, CLSI document M100-S21, Wayne, Pennsylvania, USA, 30 (15): 42-46.
16. ***Rankin D I (2005):*** Test methods: MIC testing, In, Coyle B. M. (Ed.): Manual of Antimicrobial Susceptibility Testing, American Society for Microbiology, 53-62.
17. ***Sheedy S A, Ingham A B, Rood J I and Moore R J (2004):*** Highly conserved alpha-toxin sequences of avian isolates of

Clostridium perfringens. J. Clin. Microbiol., 42 (3): 1345-7.

18. ***Augustynowicz E, Gzyl A and Slusarczyk J (2000):*** Molecular epidemiology survey of toxinogenic Clostridium perfringens strain types by multiplex PCR. Scand. J. Infect. Dis., 32 (6): 637-41.

19. ***Aminove R I, Garrigues-Jeanhean N and Mackie R I (2001):*** Molecular ecology of tetracycline resistance: development and validation of priers for detection of tetracycline resistance genes encoding ribosomal protection proteins. Appl. Environ. Microbiol., 67: 22-32.

20. ***Trzcinski K, Cooper B S, Hryniewicz W and Dowson C G (2001):*** Expression of resistance to tetracyclines in strains of methicillin-resistant *Staphylococcus aureus*. J. Antimicrob. Chemother., 45: 763-770.

21. ***Yamagishi T, Sugitani K, Tanishima K and Nakamura S. (1997):***Polymerase chain reaction test for differentiation of five toxin types of Clostridium perfringens. Microbiol. Immunol., 41 (4): 295-299.

22. ***Martel A , Deviriese L A , Cauwerts K , DeGussem K , Decostere A and Haesebrouk (2009):*** susceptibility of *C.perfringens* strains from broiler chicken to antibiotics and anticocidiosis . Avian Pathol., 33(1):3-7.

23. ***Sambrook J, Fritsch E F and Maniates T (1989):*** Purification of closed circular DNA by equilibrium centrifugation in Cs-Cl ethidium bromide gradients. Molecular Cloning: a laboratory manual, 2nd edition, cold spring Harbor Laboratory Press, New York.

24. ***Secasiu V, Dumitru M, Stanciu G and Comonici G (1997):*** Anaerobic enterotoxaemia due to Clostridium perfringens in buffalo. Revista Romana de Medicina Veterinara, 7 (1): 39-46.

25. ***El-Rafaey M T (1999):*** Bacteriological studies on Clostridium microorganisms in poultry. M.V.Sc., Thesis (Microbiology) Faculty of Veterinary Medicine, Cairo University.

26. ***Traub W H (1990):*** Comparative in vitro bacterial activity of 24 antimicrobial drugs against *Clostridium perfringens*. Chemotherapy, 36 (2): 127-135.

27. ***Johansson C, Greko B, Engstrom E and Karisson M (2004):*** Antimicrobial susceptibility of Swedish, Norwegian and Danish isolates of *Clostridium perfringens* from poultry and distribution of tetracycline resistance genes. 99 (3-4): 251-257.

28. ***Tansuphasiri V, Matra W and Sangsuk L (2005):*** Antimicrobial resistance of Clostridium perfringens isolated from various sources in Thailand. SE Asian Trop. Med. Pub. Health, 36: 954-961.

29. ***Poliak M S (1975):*** Sensitivity of pathogenic clostridia antibiotics. Antibiotiki., 20 (7): 628-632.

30. ***Rood J I, Maher E A, Somers E B, Campos E and Duncan C L (1978):*** Isolation and characterization of multiply antibiotic-resistant Clostridium perfringens strains from porcine feces. Antibmicrob. Agents Chemother., 13 (5): 817-880.

31. ***Sasaki Y, Yamamoto K, Tamura Y and Takahashi T (2001) :*** Tetracycline – resistant genes of Clostridium perfringens, Clostridium septicum and Clostridium sordelli isolated from cattle affecte with malignant edema Vet. Microbiol ., 83 (1) : 64-69.

الملخص العربى

دراسات جزيئية على الكلوستيرديوم بيرفرنجينز المقاومة لمجموعة التتراسيكلين

عادل عطيه محمد أحمد* ، **بسمة شلبى محمود**** ، **اميرة سمير محمد الرافعى*****
* قسم البكتريولوجيا والفطريات والمناعة – كلية الطب البيطرى – جامعة الزقازيق
** قسم البكتريولوجيا – وحدة اللاهوائيات – معهد بحوث صحة الحيوان – الدقى – جيزة
*** قسم البكتريولوجيا – معهد بحوث صحة الحيوان – الزقازيق-شرقية

تم إجراء هذه الدراسة على ٦٠ عينة براز تم تجميعها من دجاج التسمين مصابة بالاسهال من عام ٢٠٠٧ إلى٢٠٠٨ من محافظتى القاهرة والشرقية وكان تواجد ميكروبات الكلوستريديم بيرفرنجيزفى ٢٥عينة من ٦٠بنسبة (٤١,٦٪)منها٢٣النوع(ا) واثنين النوع (بى).

تم فحص عترات الكلوستريديم بيرفرنجيز المعزولة بإختبارات التصنيف البيوكيميائى وتفاعل الناجلرز وتفاعلات تنكرز الجلد وتم عمل إختبار الحساسية للعترات المعزولة وقد أوضحت النتائج حساسية الكلوستريديم بيرفرنجيز للأموكسيسللين ، السيفوتاكسيم ، السيفروكسيم ، السيفوتركسيون ، البيراسيللين والباستراسين بنسبة ٨٦,٦٪. ثم يليه الارثرومايسين والفيوسيدك اسد بنسبة ٨٠٪ ثم يليه التتراسيكلين والدوكسيسللين والكلنداميسين بنسبة ٦٦,٦٪ وأخيرا يأتى اللينكومايسين بنسبة ٢٦,٦٪. وتم قياس الحد المثبط للتتراسيكلين وكانت تتراوح مابين ٨ إلى ٣٢ ميكروجرام/مللى.

وبإستخدام تفاعل البلمرة المتسلسل أثبت وجود منطقة مميزة لسم الألفا عند الوزن الجزيئى ٤٠٢ قاعدة مزدوجة. كما أثبت تفاعل البلمرة المتسلسل المتعدد وجود منطقة مميزة لجين التتراسيكلين(تيت بى) عند الوزن الجزيئى ١٦٩ قاعدة مزدوجة وقد وجد فى عينتين فقط ولم يوجد منطقة مميزة لجين (تيت ام) وكذلك لجين (تيت ك) .