

Isolation of *Clostridium perfringens* from Chickens and Detection of the Alpha Toxin Gene by Polymerase Chain Reaction (PCR)

Hakan KALENDER

Veterinary Control and Research Institute, 23100, Elazığ - TURKEY
E-mail: hakgg@yahoo.com

Hasan Basri ERTAŞ

Department of Microbiology, Faculty of Veterinary Medicine, Firat University, 23119, Elazığ - TURKEY

Received: 24.03.2004

Abstract: This study was carried out to isolate *Clostridium perfringens* from chickens and to detect the gene encoding the alpha toxin produced by all types of *C. perfringens* by polymerase chain reaction (PCR).

Intestinal contents of 160 slaughtered chickens from 8 different farms in Elazığ province were analyzed. *C. perfringens* was isolated from 8 (5%) of the samples. DNA samples extracted from suspected isolates grown on selective agar were amplified by PCR using a pair of primers derived from the alpha toxin gene. All of 8 suspected isolates were found to be *C. perfringens* by conventional methods and PCR. Isolates were typed by the toxin neutralization test. Of the 8 isolates, 6 were type A and 2 could not be typed.

Since *C. perfringens* type A is associated with food poisoning in humans, the isolation of *C. perfringens* type A from chickens in this study is important for public health.

Key Words: Chicken, *Clostridium perfringens*, isolation, PCR, alpha toxin

Tavuklardan *Clostridium perfringens*'in İzolasyonu ve Alfa Toksin Geninin Polimeraz Zincir Reaksiyonu (PZR) ile Saptanması

Özet: Bu çalışma tavuklardan *Clostridium perfringens*'in izolasyonu ve *C. perfringens*'in tüm tipleri tarafından üretilen alfa toksininin kodlayan genin Polimeraz Zincir Reaksiyonu (PZR) ile saptanması amacıyla yapıldı.

Çalışmada Elazığ ilinde mezbahadan kesilen, 8 işletmeye ait 160 adet tavuğun bağırsak içeriği incelendi ve örneklerin 8'inden (% 5) *C. perfringens* izole edildi. Selektif agarda üreyen şüpheli kolonilerden DNA izolasyonu gerçekleştirildi. İzole edilen DNA'lar alfa toksin geninden türetilen bir çift primer kullanılarak PZR'de çoğaltıldı. Şüpheli 8 izolatin tümünün hem klasik yöntemlerle hem de PZR ile *C. perfringens* olduğu saptandı. Toksin nötralizasyon testi ile yapılan tiplendirmede; 6 suş tip A olarak tiplendirildi ve 2 suş tiplendirilemedi.

C. perfringens tip A insanlarda gıda kaynaklı enfeksiyonlara neden olduğundan, bu çalışmada tavuklardan *C. perfringens* tip A'nın izolasyonu halk sağlığı açısından önem taşımaktadır.

Anahtar Sözcükler: Tavuk, *Clostridium perfringens*, izolasyon, PZR, alfa toksin

Introduction

Clostridium perfringens is a Gram positive, spored and anaerobic bacteria that causes serious infections in humans and animals by toxins. *C. perfringens* is divided into five types, A, B, C, D and E, based on the synthesis of four major lethal toxins, alpha, beta, epsilon and iota. Alpha toxin is produced by all types. Alpha toxin has lecithinase activity and hydrolyzes lecithin into

phosphorylcholine and diglyceride and causes pathologic changes in tissues (1-3).

C. perfringens types A, C and D have been shown to cause necrotic enteritis in chickens (4-6). It is reported that coccidiosis plays a role in the occurrence of necrotic enteritis (7,8). *C. perfringens* is a member of normal intestinal flora that reproduces at high rates and produces toxins (9). *C. perfringens* type A, which causes infection

in chickens, has been reported to cause food poisoning in humans as well (10).

Routine typing procedure consumes a lot of antisera and experimental animals. Additionally it is labor. In order to avoid high cost and the use of experimental animals, several researchers have been working on a PCR method for detection of genes encoding *C. perfringens* toxins (2,3,11-13).

There have been a large number of studies conducted to isolate *C. perfringens* from chickens. Tschirdewahn et al. (14) have reported that they isolated *C. perfringens* in 80% of feces samples of chickens. Latinovic (6) examined the intestinal contents of 312 chickens with necrotic enteritis and isolated *C. perfringens* type A in 12, type C in 3 and type D in 3. Awad et al. (4) analyzed samples of 245 diseased and 232 healthy chickens and isolated *C. perfringens* type A in 138, type C in 41 and type D in 1. Long (15) reported that 855 of 11,076 (7.7%) broiler chickens had necrotic enteritis and isolated *C. perfringens* in 80 samples and found that 50% of 80 isolates were *C. perfringens* type A. In another study, intestinal contents of 100 slaughtered broilers were analyzed and *C. perfringens* was isolated in 41 samples and 21% of isolates were found to be type A (5). Craven et al. (16) reported that *C. perfringens* was recovered from broiler carcasses after chilling in 13 (81%) of 16 flocks.

Among studies conducted in Turkey, Demirözü (9) isolated *C. perfringens* type A in the intestinal contents of 58 of 500 (11.60%) chickens that were obtained from a slaughterhouse. Nadas et al. (17) looked at sera samples of 101 broiler and 103 laying chickens for the presence of alpha toxin and found 31 and 29 positive sera respectively. Alp (18) examined 127 feed and feed ingredient and isolated *C. perfringens* in 47, 27 of which were type A, and the rest could not be typed.

The aim of this study was to isolate *C. perfringens* from chickens and to detect the presence of the alpha toxin gene in the isolates.

Materials and Methods

Samples

Intestinal contents were taken from slaughtered apparently healthy broiler chickens aged 45 days from 8 farms at a local abattoir in Elaziğ, Turkey. The samples were obtained from animals that consumed feed containing antibiotic and coccidiostat.

Isolation and Identification of *C. perfringens*

Samples were cultured on Perfringens agar (TSC Agar, Oxoid) plates and incubated in an anaerobic chamber at 37 °C for 24 h. Black colonies, presumed to be *C. perfringens*, were tested for Gram staining. The suspected isolates were identified by biochemical tests and semi-antitoxin petri method using *C. perfringens* alpha toxin antisera and agar containing egg yolk (18,19).

Typing

Typing of *C. perfringens* was performed by the mouse neutralization test using antisera of alpha, beta and epsilon toxin (1).

PCR

Primers

A pair of primers (5'-TGCTAATGTTACTGCCGTTGAT AG-3' and 5'-ATAATCCCAATCATCCCACTATG-3') was used for detection of the gene for *C. perfringens* alpha toxin.

Procedure

A few colonies from suspected isolates grown on selective agar were suspended in 300 µl of distilled water, and the mixture was incubated at 56 °C for 30 min. The samples were treated with 300 µl of TNES buffer (20 mM Tris pH 8.0, 150 mM NaCl, 10 mM EDTA, 0.2% SDS) and proteinase K (200 µg/ml). After incubation at 37 °C for 2 h the mixture was boiled for 10 min. To that suspension, the same volume of phenol (saturated with Tris-HCl) was added, the suspension was shaken vigorously by hand and centrifuged at 11,600 g for 10 min. The upper phase was transferred into another tube and sodium acetate (0.1 volume) and ethanol (2.5 volume) were added. The suspension was kept at -20 °C for 1.5 h and then centrifuged at 11,600 g for 10 min. The pellet was washed with 95% and 70% ethanol, each step followed by 5 min centrifugation. Finally the pellet was dried and resuspended in 50 µl of distilled water (20).

The PCR was performed in a touchdown thermocycler (Hybaid) in a total reaction volume of 50 µl containing 5 µl of 10xPCR buffer (10mM Tris-HCl, pH 9.0, 50mM KCl, 0.1% Triton X-100), 5 µl of 25 mM MgCl₂, 250 µM of each deoxynucleotide triphosphate, 2 U of Taq DNA polymerase, 1 µM of each primer and 5 µl of template DNA. Amplification was obtained with 30 cycles following an initial denaturing step at 94 °C for 5 min. Each cycle

involved denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min, and synthesis at 72 °C for 2 min. Then 10 µl of the amplified product was electrophoresed in a 1.5% agarose gel and stained with ethidium bromide. Amplified bands were visualized and photographed under UV illumination (20).

Results

In this study, 160 intestinal contents were analyzed and *C. perfringens* was isolated from 8 (5%) samples. All of the 8 suspected isolates grown on selective agar were PCR positive for the alpha toxin gene of *C. perfringens*. Typing procedures revealed that 6 of these isolates were type A and 2 could not be typed. PCR products for the alpha toxin gene (247 bp) of *C. perfringens* are shown in the Figure.

Discussion

Different types of *C. perfringens* cause enteric infections in chickens. Although generally types C and D produce necrotic infections, type A is also reported to be a causative agent of the infection too (9,18).

The isolation rate (5%) of *C. perfringens* in this study is lower than the rate previously reported in Turkey (9)

or in some other countries (4-6,14,15). This low rate could be due to the use of antibiotic and coccidiostatic drugs in broiler farms. Antibiotic additives can change the intestinal flora of animals. Coccidia are known to have a role in necrotic enteritis, which causes an increase in the number of *C. perfringens* in the gut (7,8).

PCR has been widely used in identifying the toxin genes of *C. perfringens* because of its high sensitivity. In this study, a pair of primers derived from the alpha toxin gene, which is present in all the strains of *C. perfringens*, was used. The alpha toxin gene was detected in all suspected isolates. In the present study, we have shown that PCR can be used for identification of *C. perfringens*. It has previously been shown that *C. perfringens* could be identified by PCR in feces samples after enrichment (21,22).

It is always possible to isolate *C. perfringens* from intestinal contents as it is a normal habitant of the intestine. Therefore, the detection of toxin produced by bacteria is a more convenient method (1). A routine diagnosis method for *C. perfringens* infection is the toxin neutralization test applied in mice in which intestinal content is centrifuged and supernatant is injected to mice with antitoxins and the type of toxin is evaluated by the protection of mice from death by antitoxin. Due to easy

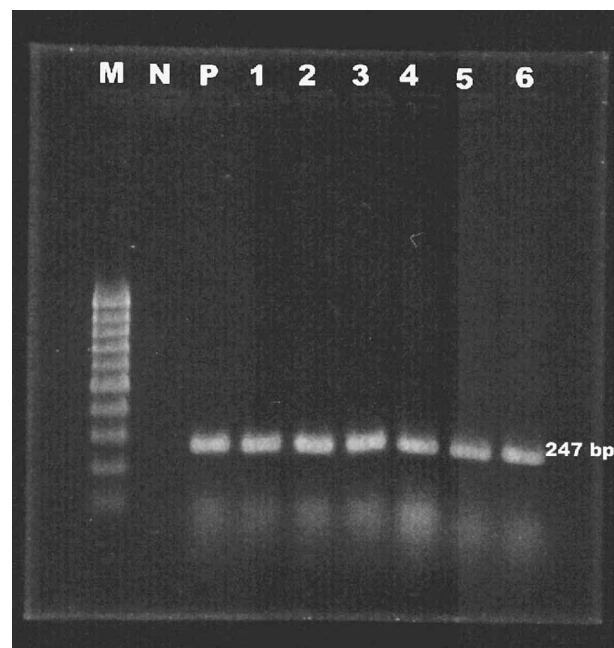


Figure. Agarose gel electrophoresis of PCR products of *C. perfringens* isolates. M: Marker, N: Negative control, P: Positive control, 1-6: Suspected isolates.

inactivation of the toxin, this test might not give true results in nonfresh samples. The toxin neutralization test is also used for typing *C. perfringens* strains. Some strains may not be able to produce toxin enough to kill mice under laboratory conditions and this causes an obstacle for typing by the test. In two different studies (18,23) conducted in Turkey 42% and 37%, respectively, of strains could not be typed. Similarly, in the present study 6 isolates (75%) were found to be type A and 2 (25%) could not be typed. Several studies reported that the most predominant type in chickens is type A (4,6,9,15,24). The enterotoxins of type A have been reported to cause food-borne infections in humans (21,25).

In conclusion, isolation of *C. perfringens* type A in chickens slaughtered for human consumption has a crucial impact on public health. Cross contamination with

C. perfringens occurring during slaughter and meat processing and unsuitable storage conditions could be an important threat to public health. As classical identification methods are expensive and time consuming and also because of their low sensitivity, PCR can be used to determine the presence of toxin genes and for typing *C. perfringens*. This technique gives the opportunity to type isolates that could not be typed by toxin neutralization test. Detailed epidemiological studies are needed to give a certain idea on chicken enterotoxemia.

Acknowledgments

The authors wish to thank the laboratory workers of the Veterinary Control and Research Institute, Elazığ, for their help.

References

- Arda, M., Minbay, A., Leloğlu, N., Aydın, N., Kahraman, M., Akay, Ö., Ilgaz, A., İzgür, M., Diker, K.S.: Özel Mikrobiyoloji. Epidemiyoloji, Bakteriyel ve Mikotik İnfeksiyonlar. Medisan Yayın Serisi No: 26. Medisan Yayınevi. Ankara, 1997
- Meer, R. R., Songer, J.G.: Multiplex polymerase chain reaction assay for genotyping *Clostridium perfringens*. Am. J. Vet. Res., 1997; 58: 702-705.
- Yoo, H.S., Lee, S.U., Park, K.Y., Park, Y.H.: Molecular typing and epidemiological survey of prevalence of *Clostridium perfringens* types by multiplex PCR. J. Clin. Microbiol., 1997; 35: 228-232.
- Awad, F.I., Bassicunni, A.A., Gadalla, M.S., Elsis, M.A., Hussein, A.Z.: Studies of poultry anaerobes in Egypt. 1. An attempt to isolate anaerobic bacteria from the intestinal tract of normal and dead chickens. 2. The effect of alpha and beta toxins of *Clostridium perfringens* Type A and C introduced by different routes. 3. The effect of ration on chickens infected with *Clostridium perfringens* type C. Egypt J. Vet. Sci., 1977; 13: 1-22.
- Götze, U.: Vorkomen von *Clostridium perfringens* bei lebenden masthähnchen und miglichkeiten der kontamination beim schlachten. Fleischwirtschaft, 1976; 56: 231-235.
- Latinovic, V.: Study of characteristics of *Clostridium perfringens* strains isolated from broilers with enteritis. Veterinaria Yugoslavia, 1983; 32: 267-275.
- Al-Sheikhly, F., Al-Saieg, A.: Role of coccidia in occurrence of necrotic enteritis of chickens. Avian Dis., 1980; 24: 324-333.
- Baba, E., Ikemeto, T., Fukata, T., Sasai, K., Arakawa, A., McDonald, L.R.: Clostridial population and the intestinal lesions in chickens infected with *Clostridium perfringens* and *Eimeria necatrix*. Vet. Microbiol., 1997; 54: 301-308.
- Demiröz, K.: Sağlıklı tavukların ince bağırsak içeriklerinde *Clostridium welchii* mikroorganizmalarının aranması ve bunun tavuk enterotoksemileri bakımından öneminin saptanması. Pendik Vet. Kont. Araşt. Enst., 1989; 20: 15-34.
- Fach, P., Guillou, J.P.: Detection by in vitro amplification of the alpha-toxin (phospholipase C) gene from *Clostridium perfringens*. J. Appl. Bacteriol., 1993; 74: 61-66.
- Engström, B.E., Fermer, C., Lindberg, A., Saarinen, E., Baverud, V., Gunnarsson, A.: Molecular typing of isolates of *Clostridium perfringens* from healthy and diseased poultry. Vet. Microbiol., 2003; 9: 225-235.
- Yamagashi, T., Sugitani, K., Tanishima, K., Nakamura, S.: Polymerase chain reaction test for differentiation of five toxin types of *Clostridium perfringens*. Microbiol. Immunol., 1997; 41: 295-299.
- Baums, C.G., Schotte, U., Amtsberg, G., Goethe, R.: Diagnostic multiplex PCR for toxin genotyping of *Clostridium perfringens* isolates. Vet. Microbiol., 2004; 20: 11-16
- Tschirdewahn, B., Notermans, S., Wernars, K., Untermann, F.: The presence of enterotoxigenic *Clostridium perfringens* strains in faeces of various animals. Int. J. Food Microbiol., 1991; 14: 175-178.
- Long, J. R.: Studies on necrotic enteritis in broiler chickens with emphasis on the role of *Clostridium perfringens*. Dissert. Abs. Int., 1974; 35: 2503.
- Craven, S.E., Stern, N.J., Barley, J.S., Cox, N.A.: Incidence of *Clostridium perfringens* in broiler chickens and their environment during production and processing. Avian Dis., 2001; 45: 887-896.

17. Nadas, Ü.G., Berkoğlu, A., Alp, R.: Sağlıklı tavukların kan serumlarında *Clostridium welchii* alfa antitoksininin aranması ve bunun tavuk enterotoksemileri yönünden değerlendirilmesi. Pendik Hay. Hast. Araşt. Enst. Derg., 1991; 22: 94-106.
18. Alp, R.: Kanatlı yemlerinde ve yem ham maddelerinde *Clostridium welchii* aranması ve bunun tavuk enterotoksemileri yönünden önemi. Pendik Vet. Kont. Araşt. Enst. Derg., 1992; 23: 147-154.
19. Russo, T.A., Gorbach, S.L.: Anaerobic Infections: Sporofomers, Diagnostic Procedures for Bacterial Infections. Ed: Wentworth, B.B. Seventh Edition. American Public Health Association. Inc., Washington, 1987
20. Çetinkaya, B., Karahan, M., Atil, E., Kalin, R., De Baere, T., Vanechoutte, M: Study of prevalence of caseous lymphadenitis in sheep and goats in the east of Turkey. Vet. Microbiol., 2002; 88: 75-83.
21. Kanakaraj, R., Haris, D.C., Soger, J.G., Bosworth, B.: Multiplex PCR assay for detection of *Clostridium perfringens* in feces and intestinal contents of pigs and swine feed. Vet. Microbiol., 1998; 63: 29-38.
22. Miwa, N., Nishina, T., Kubo, S., Atsumi, M.: Most probable number method combined with nested polymerase chain reaction for detection and enumeration of enterotoxigenic *Clostridium perfringens* in intestinal contents of cattle, pigs and chickens. J. Vet. Med. Sci., 1997; 59: 89-92.
23. Arslan, N., Öztürk, G., Kalender, H., Katı, M.N.: Elazığ bölgesindeki ishalleri koyun ve kuzularda *Escherichia coli*, *Campylobacter* ve *Clostridium perfringens*'in izolasyon ve identifikasyonu üzerine çalışmalar. Pendik Vet. Kont. Araşt. Enst. Derg., 1996; 27: 43-53.
24. Holfshagen, M., Stenwig, H.: Toxin production by *Clostridium perfringens* isolated from broiler chickens and capercaillies (*Tetrao urugalls*) with and without necrotizing enteritis. Avian Dis., 1992; 36: 837-843.
25. Lukinmaa, S., Takkunen, E., Siitonen, A.: Molecular epidemiology of *Clostridium perfringens* related to foodborne outbreaks of disease in Finland from 1984 to 1999. Appl. Environ. Microbiol., 2002; 68: 3744-3749.