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Prevalence and molecular typing of *Clostridium perfringens* isolates from edible offal of broiler

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Summary: The aim of the study was to determine the prevalance and molecular characterization of *Clostridium perfringens* in edible offal of broiler. For this purpose a total of 90 samples, including 45 heart-livers and 45 gizzards were obtained from different supermarkets and transported to the laboratory in an ice box and tested at the same day. Culture technique was used for the isolation and selected biochemical tests, including acid phosphatase and reverse-CAMP tests was used for the identification of *C. perfringens* from the samples. Twenty one (46.7%) of 45 heart-liver and eight (17.7%) of 45 gizzards were found to be contaminated with *C. perfringens*. Isolates were molecular characterized by using multiplex PCR with alpha (*cpa*), beta (*cpb*), beta 2 (*cpb2*), epsilon (*etx*), iota (*iA*) and enterotoxin (*cpe*) genes. Results showed that all isolates were carrying *cpa* gene and named as type A but none of them were positive for *cpe* gene. Only two gizzard isolates were positive for *cpb2* gene. Our results indicate that edible offal of broiler are highly contaminated with *C. perfringens* type A which is mostly responsible for the foodborne poisoning worldwide. According to the high contamination rate edible offal should be considered as one of the important causes of *C. perfringens* type A foodborne disease.

Key words: Broiler edible offal, *Clostridium perfringens*, molecular typing, multiplex PCR.

Yenilebilir piliç iç organlarında *Clostridium perfringens* prevalansı ve moleküler tiplendirilmesi

Özet: Bu çalışmada yenilebilir piliç iç organlarında *Clostridium perfringens* prevalansı ve elde edilen izolatların moleküler karakterizasyonlarının belirlenmesi amaçlanmıştır. Çalışmada farklı süpermarketlerde satışa sunulan 45 kalp-karaciğer ve 45 taşlık olmak üzere toplam 90 yenilebilir iç organ soğuk zincir altında laboratuvara getirilerek aynı gün içerisinde analize alınmıştır. Örneklerden *C. perfringens* izolasyon ve identifikasyonunda klasik kültür tekniği ile asit fosfataz ve reverse-CAMP gibi biyokimyasal testler kullanılmıştır. Kırk beş kalp-karaciğer örneğinin 21'inin (% 46.7) ve 45 taşlık örneğinin sekizinin (% 17.7) *C. perfringens* ile kontamine olduğu saptanmıştır. Elde edilen izolatların alfa (*cpa*), beta (*cpb*), beta 2 (*cpb2*), epsilon (*etx*), iota (*iA*) ve enterotoksin (*cpe*) genlerine ait primer dizilimleri kullanılarak multipleks PCR ile moleküler tiplendirilmeleri yapılmıştır. Sonuçlar incelendiğinde bütün izolatların *cpa* geni taşıdığı belirlenmiş ve tip A olarak sınıflandırılmıştır, bununla beraber hiçbir izolatın *cpe* genine sahip olmadığı saptanmıştır. İki farklı taşlık örneğinden elde edilen izolatların *cpa* yanında *cpb2* genini de taşıdıkları belirlenmiştir. Sonuç olarak yenilebilir piliç iç organlarının dünya genelinde gıda zehirlenmelerinde en sıklıkla rastlanan *C. perfringens* tip A ile yüksek oranda kontamine olduğu belirlenmiştir. Yüksek kontaminasyona bağlı olarak yenilebilir piliç iç organlarının *C. perfringens* tip A zehirlenmeleri açısından önemli birer kaynak oldukları saptanmıştır.

Anahtar sözcükler: *Clostridium perfringens*, moleküler tiplendirme, multipleks PCR, yenilebilir piliç iç organ.

Introduction

Clostridium perfringens is a Gram-positive, spore-forming, non-motile, rod-shaped bacterial pathogen which resides in environment and intestinal tracts of human and animals (20). It is mostly responsible for two different foodborne diseases, Type A and C, and gas gangrene in humans as well as necrotic enteritis and enterotoxemia in poultry (5, 17). It causes estimated one million cases each year in the United States that makes it second most common bacterial cause of foodborne disease following up *Salmonella* spp.. *C. perfringens* is also commonly reported as a foodborne pathogen and it also causes outbreaks in worldwide, including Japan, Australia, Israel, Denmark, Finland, Turkey, England and Wales (8, 9, 13, 14, 19). The pathogenicity of the organism is associated with several toxins which are also used for toxin typing of the bacteria, within them all strains of the bacterium produce alpha (α) toxin encoded by *cpa* gene. The other major lethal toxins produced by the organism are beta (β), epsilon (ε) and iota (ι) that are closely related with the virulence of bacteria (16, 30). In addition to these major lethal toxins, some strains, with a ratio of 0 to 5 %, have a capability of producing *C. perfringens* enterotoxin encoded by *cpe* gene that is the main cause of common *C. perfringens* type A food poisoning (18, 25).

Different types of foods have been involved in the outbreaks of *C. perfringens* in meat, particularly poultry meat and meat products (5, 14, 16). As *C. perfringens* is an ubiquitous pathogen and common intestinal inhabitant of poultry, different stages of poultry production line can be evaluated as a contamination source even starting from the hatchery. Chicken carcass and meat parts may also be contaminated with *C. perfringens* by intestinal contents during slaughterhouse process especially on evisceration (6, 7, 17, 27, 31).

Although *C. perfringens* in poultry meat has been intensively studied and well determined worldwide, there is limited scientific data on isolation and molecular characterization of *C. pefringens* in edible offal of broiler. Therefore, the study aimed to determine the presence of *C. perfringens* in heart-liver and gizzard of broiler and to determine the toxin profile [alpha (*cpa*), beta (*cpb*), beta 2 (*cpb2*), epsilon (*etx*), iota (*iA*) and enterotoxin (*cpe*)] of the isolates using multiplex PCR.

Materials and Methods

Sample collection: Forty five heart-liver and 45 gizzard samples of healthy broilers were obtained after evisceration of carcasses from a broiler slaughterhouse near Ankara between April and June 2011. Samples were taken to the laboratory in an ice box and analyzed within 2 hours.

Microbiological analysis: The techniques described by Baumgart et al. (1990), Schalch et al. (1996) and Baumgart (1997) were used to isolate and identify *C. perfringens.* For enrichment of *C. perfringens*, a 25 g portion of each sample was aseptically placed in a sterile plastic bag containing 225 ml Perfringens Enrichment Medium [PEM; Fluid Thioglycollate Medium, supplemented with Perfringens (TSC) supplement, Oxoid SR 88, Oxoid, Hampshire,UK] and homogenized by a stomacher (AESAP 1068-Easy Mix; AES Laboratories, Combourg, France) for approx. 2 min and then incubated at 46°C for 20 h in an anaerobic condition (Gas generating kit, B 36, Oxoid). After the samples were enriched in PEM, one loopful of enrichment was streaked onto TSC agar (Tryptose Sulphite Cycloserine agar, Oxoid CM 857; Oxoid) and the plates were further incubated at 46°C for 20 h in a jar with Gas Pak system (Gas generating kit, B 36, Oxoid) anaerobically. In order to confirm *C. perfringens*, up to five suspect black colonies from each positive TSC agar plates were purified and identified biochemically by using Gram staining, catalase test, lactose fermentation, gelatinase production, nitrate reduction, motility test, acid phosphatase reaction, haemolysis test and the reverse CAMP testing.

DNA extraction: Extraction of DNA from all of the isolates was performed using a boiling technique. All isolates, stored at 80°C in cryovials, were incubated in cooked meat broth (Oxoid CM0081) at 37°C for 24 h in anaerobic conditions (top of tubes were covered by sterile liquid paraffin). One milliliter of each enrichment culture was separately transferred to a microcentrifuge tube. All tubes were centrifuged (Eppendorf Centrifuge 5417R) for 3 min at 12 000 *g*. The pellets were resuspended in 200 µl sterile distilled water. The suspensions were mixed and heated at 95°C for 20 min in a water bath (Memmert WB⁄OB 7-45, WBU 45, Schwabagh, Germany) and centrifuged for 3 min at 12 000 *g* and cooled on ice. A volume of 10 µl was used as a template in the PCR (11).

Bacterial strains: *Clostridium perfringens* ATCC 13124 (for *cpa* gene), NCTC 8239 (*cpa*, *cpe* genes), ATCC 3626 (*etx*, *cpb* genes) and CCUG 44727 (*iA* gene) were used as positive controls in this study.

Table 1. Primers for multiplex PCR detection of *Clostridium perfringens* toxin genes (24).
Tablo 1. *Clostridium perfringens*'e ait toksin genlerin belirlenmesi için multiplex PCR aşamasında kullanılan primer dizilimleri (24).

Primers	Primer sequence (5´-3´)	Size (bp)	Gene
CPA F	GCTAATGTTACTGCCGTTGA	324	*cpa*
CPA R	CCTCTGATACATCGTGTAAG		
CPB F	GCGAATATGCTGAATCATCTA	196	*cpb*
CPB R	GCAGGAACATTAGTATATCTTC		
ETX F	GCGGTGATATCCATCTATTC	655	*etx*
ETX R	CCACTTACTTGTCCTACTAAC		
IA F	ACTACTCTCAGACAAGACAG	446	*iA*
IA R	CTTTCCTTCTATTACTATACG		
CPE F	GGAGATGGTTGGATATTAGG	233	*cpe*
CPE R	GGACCAGCAGTTGTAGATA		
CPB2 F	AGATTTTAAATATGATCCTAACC	567	*cpb2*
CPB2 R	CAATACCCTTCACCAAATACTC		

Multiplex PCR: Molecular typing of *C. perfringens* was performed by multiplex PCR (24). In the PCR assay CPA, CPB, ETX, IA, CPE and CPB2 primer pairs (Promega, Madison, Wisconsin, USA) were used as shown in Table 1. The multiplex PCR was performed in a total volume of 50 µl. Reaction mixture contains 1 x Reaction buffer (Promega), 1.5 mmol l^{-1} $MgCl_2$, 0.12 mmol l^{-1} dNTPs, 0.34 µmol l^{-1} of each *cpe* primers, 0.36 µmol l^{-1} of each *cpb* primers, 0.36 µmol l^{-1} of each *cpb2* primers, 0.44 µmol l^{-1} of each *etx* primers, 0.5 µmol l^{-1} of each *cpa* primers, 0.52 µmol l^{-1} of each *iA* primers, 5 units of Taq DNA polymerase (Promega, Madison) and 10 µl template DNA. Thermal cycling (Biometra Personel Cycler, Goettingen, Germany) was carried out with 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. A 10 µl aliquot of each PCR product was subjected to 1.5% agarose gel electrophoresis containing 0.1 µg ml^{-1}

ethidium bromide for 1 h at 100 V. Amplicon visualization and documentation were performed using gel documentation and analysis system (Syngene Ingenius, Cambridge, UK).

Results

A total of 90 heart-liver (45) and gizzard (45) samples were collected from broiler slaughterhouse near Ankara and analyzed for isolation of *C. perfringens* by conventional cultivation technique. All isolates were confirmed with PCR using *cpa* gene. Then *cpa* gene carrying isolates were analyzed by multiplex PCR in order to determine the toxin genes for the molecular typing. According to the results 21 of 45 heart-liver (46.7%) and eight of 45 gizzard (17.7%) samples were found to be contaminated with *C. perfringens* and from a total of 29 positive samples 77 colonies were analyzed for toxin genes. In all 77 *C. perfringens cpa* gene was detected (Fig 1). However only two gizzard samples were carrying *cpb2* gene and the *cpb*, *etx*, *iA* and *cpe*

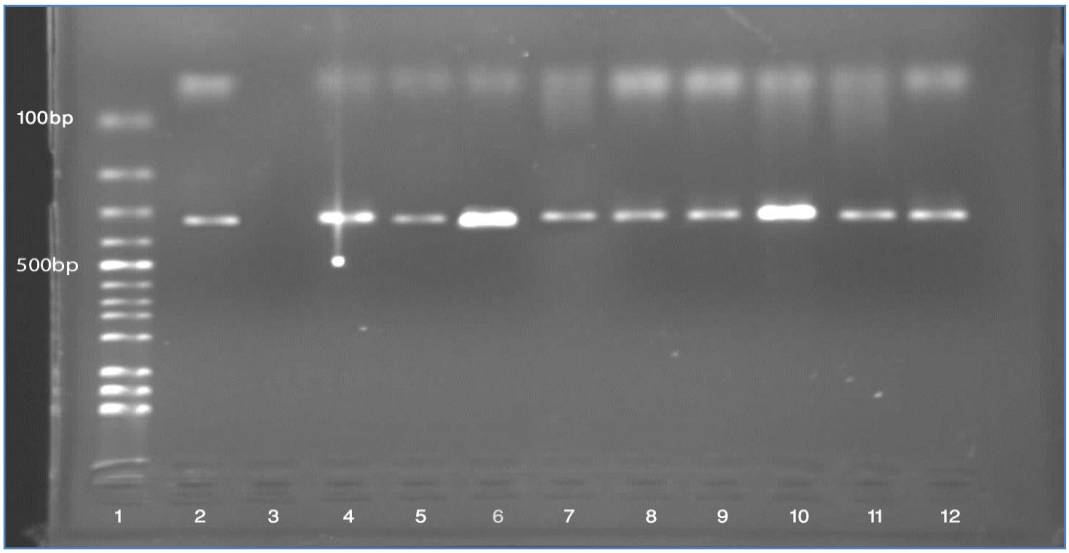

Figure 1. *Cpa* gene (324 bp) detected *C. perfringens* isolates. (1: 100 bp DNA marker, 2: Positive control (*C. perfringens* ATCC 13124), 3: Negative control (PCR mix without template DNA), 4-12: *cpa* positive *C. perfringens* isolates).
Şekil 1. *Cpa* geni bulunan *C. perfringens* izolatlarının görünümü. (1: 100 bp DNA marker, 2: Pozitif kontrol (*C. perfringens* ATCC 13124), 3: Negatif kontrol (DNA içermeyen PCR karışımı), 4-12: *cpa* pozitif *C. perfringens* izolatları).

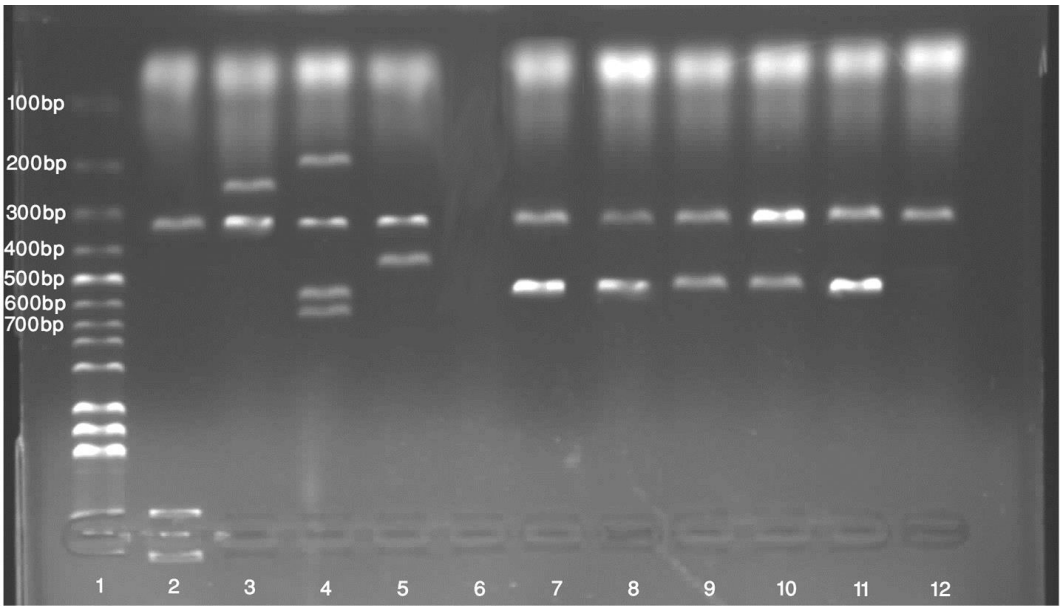

Figure 2. Agarose gel electrophoresis of multiplex PCR products of *cpa* (324bp), *cpb* (196 bp), *etx* (655bp), *ia* (446bp), *cpb2* (567bp), *cpe* (233bp) genes. (1: 100 bp DNA marker, 2-5: Positive control group: 2; *C. perfringens* ATCC 13124 (*cpa* gene), 3; *C. perfringens* NCTC 8239 (*cpa*, *cpe* genes), 4; *C. perfringens* ATCC 3626 (*etx*,*cpb* genes), 5; *C. perfringens* CCUG 44727 (*iA* gene), 6; Negative control (PCR mix without template DNA), 7-12; *C. perfringens* isolates *(cpa* and *cpb2* genes).
Şekil 2. Multipleks PCR ile *cpa* (324bp), *cpb* (196 bp), *etx* (655bp), *ia* (446bp), *cpb2* (567bp), *cpe* (233bp) genlerinin görünümü. (1: 100 bp DNA marker, 2-5: Pozitif kontrol grubu: 2; *C. perfringens* ATCC 13124 (*cpa* geni), 3; *C. perfringens* NCTC 8239 (*cpa*, *cpe* genleri), 4; *C.perfringens* ATCC 3626 (*etx*,*cpb* genleri), 5; *C. perfringens* CCUG 44727 (*iA* geni), 6; Negatif kontrol (DNA içermeyen PCR karışımı), 7-12; *C. perfringens* izolatları *(cpa* ve *cpb2* genleri).

genes were not detected in any of the isolates (Fig 2). No abnormalities were observed in any of edible offal samples. Our results showed that all isolates were *C. perfringens* type A and *cpe* negative.

Discussion and Conclusion

Many studies have focused on the *C. perfringens* isolated from poultry meat and products by the detection of toxin genes using PCR and almost all of them reported that type A is the predominant type in poultry. The enterotoxins of type A have been reported to cause food-borne infections in humans. Epidemiological studies showed that more than 90% of *C. perfringens* outbreaks were associated with only meat and poultry products. (14, 25, 27, 32). In various studies type A was reported to be the dominant type of *C. perfringens* isolated in poultry diseases (especially in necrotic enteritis) and from industry, processing line, products and environment worldwide (6, 17, 21, 32).

Our results suggested that toxin type A of *C. perfringens* was the most prevalent causative agent in broiler edible offal samples. *C. perfringens* types prevalent in poultry meat and meat parts conducted in Turkey were similar to those found in our study (8, 11, 15). Although there are no published data on detection of *C. perfringens* in poultry edible offal samples, previous studies in Turkey showed that edible offal of broiler can be contaminated with different foodborne pathogens mainly including *Salmonella* spp. (1, 10, 12, 28).

According to our results 29 of 90 (32.2%) edible offal samples were found to be contaminated with *C. perfringens* type A. Similar to our study, Craven et al. (2001a) reported that *C. perfringens* incidence of broiler carcasses were ranged between 8% and 68% with a mean value of 30%. In a different study, Lin and Labbe (2003) found out that 30% of retail food samples in the USA were contaminated with *C. perfringens* at different level and none of them was positive for *cpe* gene. Also meat, poultry and fish were detected as the common food. In another study, 37% of the meat and poultry samples were detected as positive for *C. perfringens* but different from our results 17% of the isolates were carrying *cpe* gene (27). Similarly Wen and McClane (2004) reported that 56 of 147 (38%) chicken meat were contaminated with *C. perfringens* and only one isolate was positive for *cpe*.

In contrast to our results, Cakmak et al. (2006) isolated *C. perfringens* from the 70% of the frozen ground poultry samples in Turkey. Also Guran and Oksuztepe (2013) reported that 154 of 200 (77%) chicken meat part samples were found to be contaminated with *C. perfringens* and one isolate was carrying *cpe* gene. On the other hand, similar to our results, 5% of the isolates were *cpb2* positive in that study. Miwa et al. (1998) detected *C. perfringens* in 42 of 50 (84%) of chicken samples in Japan. Likewise, in another study conducted in Japan, 70% of the Japanese retail raw meat samples were found to be contaminated with different numbers (MPN/g) of *C. perfringens* and 4% of the isolates were carrying *cpe*. The higher prevalence could be resulting from different sampling regimes and differences in the hygienic conditions of the process and products (26).

This study showed that the contamination rate of *C. perfringens* was higher in heart-liver samples (46.7%) then gizzard samples (17.7%). According to the studies on *C. perfringens* associated diseases in poultry it is being recognized as a cause of hepatitis in chickens. Results of these studies indicated that liver lesions are remarkably varied and in some cases appeared only with small focal lesions (2, 23). The high incidence in heart-liver samples in our study may be explained with these scientific facts.

In conclusion, our results indicate that edible offal of broiler were highly contaminated with *C. perfringens* and type A is the most common type within these isolates. Only two samples carried both *cpa* and *cpβ2* toxin genes. According to the high contamination rate edible offal should be considered as one of the important cause of *C. perfringens* type A foodborne disease. As this is the first report on *C. perfringens* contamination of edible offal of broiler it could provide a useful data for future studies.

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