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Detection of *Clostridium perfringens* and determination of enterotoxin genes (*cpa* and *cpe*) in traditional turkish chicken doner kebab

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Abstract: The demand for the fast-food industry in the world is increasing day by day. In this sense, chicken doner kebab becomes frequently preferred food source in daily life. At the same time, chicken doner kebab is both a good animal origin protein source and a cheaper option. Therefore, it is prepared and consumed in high amounts in Turkey. In this study, it was aimed to determine the presence of *Clostridium perfringens* and its toxin genes in traditional Turkish chicken doner kebabs purchased from restaurants and modified atmosphere packaged (MAP) samples collected from markets. For this purpose, 100 ready-to-cook and 100 ready-to-eat, totally 200 doner samples have been used as material. As a result, the prevalence of *C. perfringens* has been found 29%, 6% in ready-to-cook and ready-to-eat samples, respectively. The *cpa* gene was detected in all isolates. However, both *cpa* and *cpe* gene was found only in 4% of isolates.
Keywords: *C. perfringens*, *cpa*, *cpe*, doner kebab, ready-to-eat.

Geleneksel türk tavuk döner kebaplarında *Clostridium perfringens*'in tespiti ve enterotoksin genlerinin (*cpa* ve *cpe*) belirlenmesi

Özet: Dünyada fast-food beslenmeye olan talep her geçen gün artmaktadır. Bu anlamda tavuk döner kebap günlük hayatta sıklıkla tercih edilen bir besin kaynağı haline gelmektedir. Aynı zamanda tavuk döner kebap hem iyi bir hayvansal protein kaynağı hem de daha ekonomik bir seçenek olması nedeniyle Türkiye'de yüksek miktarlarda üretilmekte ve tüketilmektedir. Bu çalışmada, restoranlardan satın alınan geleneksel Türk tipi tavuk döner kebapları ve marketten toplanan modifiye atmosfer paketli (MAP) örneklerde *Clostridium* perfringens ve toksin genlerinin varlığının belirlenmesi amaçlanmıştır. Bu amaçla 100 adet pişirilmiş ve paketlenmiş ve 100 adet restoranlardan alınmış ve yenmeye hazır olmak üzere toplam 200 döner örneği materyal olarak kullanılmıştır. Sonuç olarak, pişirilmiş ve paketlenmiş örneklerde *C. perfringens* prevalansı %29 ve restoranlardan alınmış örneklerde ise %6 olarak bulunmuştur. Tüm izolatlarda *cpa* geni tespit edilmiş olmasına rağmen hem *cpa* hem de *cpe* geni izolatların sadece %4'ünde belirlenmiştir.
Anahtar sözcükler: *C. perfringens*, *cpa*, *cpe*, döner kebap, yemeye hazır.

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Introduction

Turkish chicken doner kebab (also called gyro, donair, yeeros, and shawarma) is the traditional and quite common meat product both in Turkey and in countries populated densely by Turks (20, 38). Because of a well-known gastronomic value of our country it is produced and consumed in high amounts in Anatolia (36). Doner kebab's history goes back to the 19th century in Bursa/Turkey. Because of industrialization, urbanization, and globalization, its consumption spreads out to world (2). This product is usually made from breast meat and fat of chicken. Before the chicken meat is impaled on a doner stick, it is marinated with salt, pepper, cumin, onions, tomato paste, and several spices (21). A cone or cylinder shape is given to chicken meat on doner stick before cooking on the fire (34).

In addition, besides its high digestibility, it is also an important protein source in terms of being animal-originated and it contains many nutrients which are necessary for human body such as essential amino acids, fatty acids, and high amounts of minerals (17).

Due to the vertical and surface cooking process, Turkish chicken doner kebab always contains raw materials inside. Because of that, the applied heat treatment penetrates only to a certain depth. Cutting doner meat slices too thick or insufficient cooking time also affects the microbiological quality of the product negatively. This characteristic cooking process may cause the risk of consuming undercooked products. Therefore, it has been reported that it seems probable to encounter food poisoning cases caused by foodborne pathogens such as *Clostridium perfringens*. This is an essential risk that needs attention in terms of public health (19).

In recent years, it has been reported that the food demand has increased rapidly due to the increasing population. In last five years, Organisation for Economic Co-operation and Development (OECD) has announced that chicken consumption has increased 6.88% in worldwide (29). For this reason, it has been determined that there is an increase in the incidence of foodborne outbreaks (10). It has been reported that chicken meat contains many foodborne pathogens depending on the slaughter process (14, 32). According to the Centers for Disease Control and Prevention (CDC), *C. perfringens* is the fifth pathogen that most frequently cause foodborne poisoning after norovirus, *Salmonella*, Shiga toxin-producing *E. coli* (STEC), and *Campylobacter* in the United States between 2009-2015 (3).

Clostridium perfringens is gram-positive, ubiquitous, anaerobic, not able to motile, rod shaped and subterminal heat resistant spore forming bacterium. Also *C. perfringens* metabolizes gelatin, reduces nitrates to nitrites, and generates black colonies by its ability of sulphite reduction (28). It has been reported that, the bacteria have seven toxigenic types from A to G and it can produce a lot of extracellular toxins such as alpha, beta, epsilon, iota, cpe, and netb according to type of it. The agent is responsible for intoxication-type foodborne poisoning due to toxin consumption and disease occurs when one or more toxins are taken with foods (22). *C. perfringens* type A, B, and F have been found responsible for foodborne intoxication in humans (35). And also *C. perfringens* type A has been determined to be the most frequently identified strain among the other seven types (9, 31). *C. perfringens* toxin-based typing scheme and responsible genes for toxin synthesis is shown in Table 1.

In consequence of consuming improperly cooked or stored foods *C. perfringens* related food poisonings may occur. Due to the consumption of clostridial bacterial toxins, intoxication symptoms such as watery diarrhea and abdominal pain can be observed in humans. It has been reported that symptoms usually disappear within 12-24 hours. Besides, vomiting and fever are not the typical symptoms that can be observed (28).

The objectives of the present study were to investigate the presence of the *C. perfringens* in both MAP and unpacked chicken doner kebab samples and to confirm the presence of cpa and cpe toxin genes by multiplex PCR.

Materials and Methods

Traditional Turkish Chicken Doner Kebab Samples: In this study, between October 2019 – March 2020, 100 ready-to-cook (modified atmosphere packaged) and 100 ready-to-eat, totally 200 traditional Turkish chicken doner kebabs which were collected from restaurants, supermarkets, and butcher shops in Samsun, Turkey were used as material. Both ready-to-cook and ready-to-eat samples were bought at least 350 – 500 grams and samples were transported into the laboratory under cold chain conditions (4°C) as soon as possible after purchased.

Table 1. *C. perfringens* toxin-based typing scheme and genes (shown in parenthesis) (31).

Toxin type	Toxins					
	Alpha (*plc* or *cpa*)	**Beta (*cpb*)**	**Epsilon (*etx*)**	**Iota (*iap* and *ibp*)**	**CPE (*cpe*)**	**NetB (*netB*)**
A	+	-	-	-	-	-
B	+	+	+	-	-	-
C	+	+	-	-	+/-	-
D	+	-	+	-	+/-	-
E	+	-	-	+	+/-	-
F	+	-	-	-	+	-
G	+	-	-	-	-	+

The Isolation and Identification of Clostridium perfringens: Culture based isolation technique was used for isolation and identification. For this purpose, all samples were weighed 10 g into sterile jars under aseptic conditions and diluted with 90 ml Perfingens Enrichment Medium (PEM: Fluid Thioglycolate Medium + Perfringens (TSC) Supplement Oxoid SR 88E). Then, for generation of anaerobic conditions samples were covered with sterile paraffin and incubated at 46°C 20 h (Sanyo, MCO. 18 AIC). After this enrichment, a loopful sample was taken from turbidity and gas production positive enrichment jars and streaked on to Tryptose Sulphite Cycloserine (TSC) agar (Oxoid CM 587). Later, plates were incubated at same anaerobic conditions above. Typical colonies like 2 – 4 mm in diameter and black colored in TSC agar were qualified as suspected. Three or 5 suspected colony were selected and streaked on to TSC agar for biochemical identification test. According to biochemical tests, gram, reverse CAMP, lactose, gelatin, nitrate positive, catalase and motility negative colonies were identified as *C. perfringens* (1). For confirmation, PCR test was performed.

Confirmation of Clostridium perfringens

DNA Extraction: For the aim of procuring template DNA from *C. perfringens* isolates, GENESpin DNA Isolation Kit (eurofins, genescan 5224400605) was used in this study. For this purpose, isolates which were stored in – 20°C were revived in PEM anaerobically for 24 h at 37°C. Fresh cultures were used for DNA extraction process as mentioned in the manual of kit.

Primers Used for Detection: For this purpose, the presence of *cpa* and *cpe* genes were investigated in biochemically identified isolates. As recommended by Meer and Songer (26) *cpa* (400 bp) (5'- GCTAAT GTTACTGCCGTTGA 3' / 5'-CCTCTGATACATCGT GTAAG 3') and Mahamat Abdelrahim, Radomski (20) *cpe* (178 bp) (5'- ATAGATAAAGGAGATGGTTGGA 3' / 5'- CCATATTCTACAGATGCTTGTA 3') primer pairs were used for confirmation and enterotoxin characterization.

Multiplex PCR Conditions: *Clostridium perfringens* NCTC 8239 was applied as positive control in our study because of harboring both *cpa* and *cpe* genes.

The amplification was performed in thermal cycler (Bio-Rad MJ Mini) and conditions were initial denaturation 95 °C for 10 min, followed by 45 cycles 95 °C for 10 sec, annealing at 55 °C for 10 sec, extension at 72 °C for 20 sec, and for cooling step 40 °C for 30 sec. Electrophoresis of amplicons were separated at 2% agarose gel at 80 volts (Bio-Rad Power Pac-Basic & Bio-Rad electrophoresis tank). The PCR products were visualized under UV light (Wise-UV-Wuv-L50, Korea).

Results

In this study, a total of 200 chicken doner kebab samples, 100 ready-to-cook (MAP) and 100 ready-to-eat were analyzed. As a result, the prevalence of *C. perfringens* was 29%, 6% in ready-to-cook and ready-to-eat samples, respectively. We totally obtained 50 *C. perfringens* isolates, 12 from ready-to-cook and 38 from ready-to-eat samples (Table 2). The *cpa* gene was detected by PCR in all 50 isolates (100%). However, *cpe* gene was detected only in 2/50 isolates (4%) which were only ready-to-cook sample originated (Figure 1).

Table 2. The Prevalence and Toxin Gene Presence of *C. perfringens* in Samples.

Chicken Doner Kebab	Sample (n)	*C. perfringens* positive samples (%)	*C. perfringens* isolates	*cpa* positive isolates	*cpe* positive isolates
Ready-to-cook	100	29 (29%)	12	12	2 (5.2%)
Ready-to-eat	100	6 (6%)	38	38	-
Total	**200**	**35 (17.5%)**	**50**	**50**	**2 (4%)**

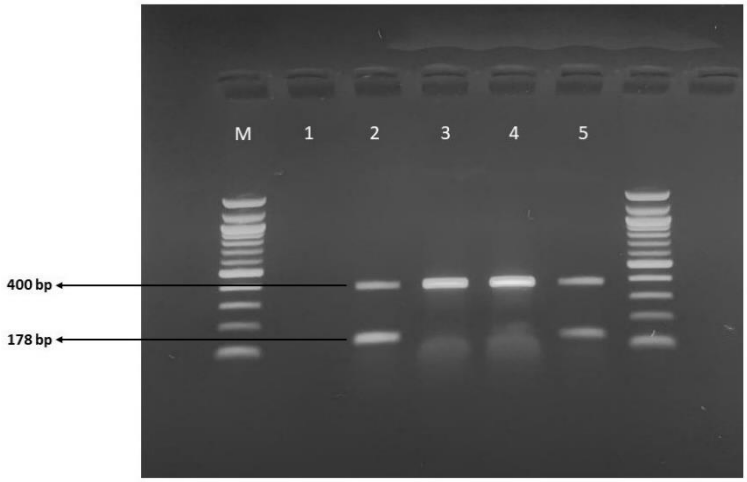

Figure 1. M: 100 bp DNA Marker, 1 distillated water, 2 *Clostridium perfringens* NCTC 8239, 3-4 *cpa* gene positive isolates, 5 *cpa* and *cpe* genes positive isolates.

Discussion and Conclusion

In our study, we investigated the traditional Turkish chicken doner kebab, which is immensely popular street food in our country. Our results indicate that 17.5% of our samples were contaminated with *C. perfringens*. This contamination level was 29%, and 6% in ready-to-cook (MAP) and ready-to-eat, respectively. The higher contamination level in modified atmosphere packed samples was attributed to the fact that the agent has anaerobic metabolism.

Similarly, in several studies, the presence of *C. perfringens* was investigated in chicken doner samples. Kayisoglu et al. (18), Elmali et al. (5), Katsurayama et al. (16), and Vazgecer et al. (37) isolated *C. perfringens* in traditional Turkish chicken doner kebab 60%, 32%, 12%, and 7%, respectively. The differences between the outcomes of the studies were thought to result from the regional discrepancy, different cooking procedures, microbiological quality, and the number of food samples taken. Moreover, the difference between results can be attributed to the formula, quality, and possible antibacterial effect of spices which were used during marination of doner. However, the number of studies in which MAP samples were studied is quite limited. This situation also contributes to the originality of our study.

Contrary to this, Haskaraca and Kolsarici (12), Lopašovský et al. (24), Öksüztepe and Beyazgül (30), Bostan et al. (2), Hampikyan et al. (11), Küpeli Gençer and Kaya (23) were used chicken doner kebab as material and the researchers reported that *C. perfringens* was found below the detection limit in their studies. These results can be linked to the cleaning and disinfection of equipment and hygienic conditions of food establishments as well.

In the present study, all our isolates were confirmed by mPCR due to housing the *cpa* gene. It has been reported that all toxigenic types of *C. perfringens* contain the *cpa* gene as well (31). In addition to this, *C. perfringens* type A is the predominant type in most of investigations and also it is the first toxigenic type in food intoxications related to *C. perfringens* in the US, Europe, and Japan (27). In parallel to our results, in Finland, Heikinheimo and Korkeala (13) detected *cpa* gene in all isolates as well. However, none of their isolates possessed *cpe* gene. Correlatively, Jang et al. (15) investigated *C. perfringens* in meat products and they got 33 isolates from chicken meat and *cpa* gene was also found in all isolates. In another study, Gholamiandehkordi et al. (8) analyzed *C. perfringens* in broilers in Belgium and they identified *C. perfringens* type A in all 71 isolates due to presence of *cpa* gene.

C. perfringens enterotoxin (CPE) is critical for human food intoxication and CPE is encoded by *cpe* gene as well (31). In our study, the *cpe* gene was detected only 2/50 (4%) of our isolates. In many studies, parallel to our results, *cpe* was rarely detected. Our results are also in agreement with Zhang et al. (40) as they detected *cpe* gene 3% as well. In India, Dar et al. (4) isolated 51 *C. perfringens* isolates from 184 chicken samples and all the isolates were identified as *C. perfringens* type A by a multiplex PCR. Accordingly, none of them carried *cpe* gene. Egyptian scientists have reported several data recently. In one of their investigation, the *cpe* gene was also carried by 1/10 (10%) of chicken isolates by genotyping analysis. However, all isolates contained *cpa* gene and were identified as *C. perfringens* type A (7). Also in another study, Shaltout et al. (33) reported that *C. perfringens* type A was detected 8/27 (29.6%) but *cpe* gene was not appointed by multiplex PCR assay. In our country, *cpa* gene was detected in all 22 isolates from turkey meat, according to Erol et al. (6). Nevertheless, *cpe* gene was not determined in their study.

Contrary to this, in Bursa-Turkey Yibar et al. (39) detected *cpe* gene 7/22 (31.8%) higher than our results in their investigation. This difference may be related to micro floral variation and microbiological quality of samples.

In conclusion, *C. perfringens* is an important food-borne pathogen which contains wide spectrum of toxins. In our study, it was isolated from ready-to-eat and ready-to-cooked (MAP) samples. The detection of *C. perfringens* in ready-to-eat foods has been the most important and critical outcome of our investigation in terms of public health.

Based on all results of our investigation, we would like to emphasize that precautionary warnings such as good hygiene and good manufacturing practice are dramatically essential for public health. Also, we highly recommend effective disinfection of equipment in the phase of preparation of foods. Further investigations are needed for the existence of *C. perfringens* in traditional Turkish chicken doner kebab samples. In addition, the detection of an important food pathogen such as *C. perfringens* in ready-to-eat foods is examined as a critical situation that should be remarkably underlined in terms of public health.

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Ethical Statement

This study does not present any ethical concerns.

Conflict of interest

The authors declared that there is no conflict of interest.

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