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ORIGINAL ARTICLE

# Toxinotyping and antimicrobial susceptibility of enterotoxigenic *Clostridium perfringens* isolates from mutton, beef and chicken meat

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**Abstract** A total of 300 meat samples comprising mutton, beef, and chicken meat ($n$=100) collected from either local butcher shops or large meat outlets situated at various areas of Lahore City located in Punjab province of Pakistan were tested for the isolation of *Clostridium perfringens*. Prevalence of the organism was highest in the chicken (6 %) followed by mutton (5 %) and beef (1 %). Contamination level was high (10/150) in the samples collected from local butcher shops in comparison to the samples collected from large meat outlets (2/150). All of the raw meat samples were negative for the presence of alpha, beta and epsilon toxins of *C. perfringens* as detected through ELISA. Out of a total number of 12 isolates only half were capable of producing enterotoxins when cultured in trypticase glucose yeast (TGY) broth. Toxinotyping of the isolates showed that 3 were of type A while one each of the remaining three belonged to type B, C, and D. Antibiotic susceptibility testing of the toxin producing isolates revealed that *C. perfringens* were susceptible to chloramphenicol, ciprofloxacin, metronidazole, and ceftriaxone. All of the other drugs were relatively less effective with a least activity of amoxicillin against the isolates.

**Keywords** Antibiotic susceptibility · *Clostridium perfringens* · Meat · Enterotoxin · Toxinotyping

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## Introduction

*Clostridium perfringens* is a gram positive, rod shaped, spore forming, anaerobic bacteria (Bergey 2009; Quinn et al. 2002) which is a common inhabitant of soil and gastrointestinal tract of humans and animals (García and Heredia 2011; Li et al. 2007). Apart from causing enteric and systemic infections in animals and poultry, *C. perfringens* is responsible for food poisoning cases in humans worldwide (Skariyachan et al. 2010). Strains of *C. perfringens* are grouped into five toxinotypes (A, B, C, D, or E) based upon the ability to synthesize four major toxins named as: alpha, beta, epsilon and iota (Gamboa-Coronado Mdel et al. 2011; Sawires and Songer 2006). *C. perfringens* also produce some minor toxins like enterotoxin, beta-2, and hydrolytic enzyme (Brynestad and Granum 2002). These toxins and enzymes are responsible for the appearance of most of the signs of the diseases (Van Immerseel et al. 2004).

*C. perfringens* is commonly present as a contaminant in various foods, particularly meat and is recognized as significant cause of food poisoning all over the world (Tassew et al. 2010). Food poisoning caused by *C. perfringens* type A is currently ranked as the third most commonly identified foodborne illness in humans (Bos et al. 2005). Spores of contaminating *C. perfringens* might germinate in the food cooked at high temperature (Ryan et al. 2010). Mostly bacteria ingested with the food are killed due to the acidic pH of stomach, but if contamination load is high, some of the vegetative cells might survive and enter small intestine. Anaerobic environment of the intestinal lumen allows the survivors to multiply and sporulate. Production of a specific type of active enterotoxin causes characteristic symptoms of *C. perfringens* food poisoning (García and Heredia 2011). *C. perfringens* presence in meat products is a the major

concern in the preparation of high quality foods (Abostate et al. 2006).

Bacterial contamination of meat might occur either as a result of systemic spread of the organism in carcasses of infected animals or as a result of soiling the meat with intestinal contents during the process of slaughtering. Open slaughtering is commonly practiced in Pakistan and the meat is sold on open meat retail shops. In case of poultry meat onsite slaughtering is particularly preferred by the customers. However, most recently some companies are marketing dressed meat, supplied under cold chain and slaughtered in modern abattoirs. The present study was conducted to test and identify the level of *C. perfringens* contamination in various kinds of meat in Lahore City along with the prevalence of its various toxinotypes.

## Materials and methods

### Sample collection and processing

Meat samples ($n=300$) including mutton, beef, and chicken (100 each) were collected from various localities of Lahore City. Half of the samples were collected from local butcher retail shops where freshly slaughtered meat were sold without cold supply chain while half of the samples were collected from the large meat outlets and grocery stores such as Zenith, Hyperstar, Metro, Mall of Lahore, and Akbari store, where meat were sold under cold supply chain management system. The samples from Zenith and Metro were refrigerated whereas the samples collected from Hyperstar, Mall of Lahore, and Akbari stores were frozen. The meat samples (a 50 g from each point) were collected in sterile zipper bags and transported in an ice box to the Department of Microbiology, University of Veterinary and Animal Sciences, Lahore within few hours of their collection.

Samples were processed immediately after their arrival in the laboratory. For bacterial isolation, 10 g of the meat sample was chopped into very small fine pieces using sterile scissors or scalpel and suspended into 10 ml of sterile 1 % peptone water (Oxoid, Hampshire, UK) (Lin and Labbe 2003). In order to detect bacterial toxins directly from meat, 1 g of the meat sample was suspended in a 9 ml phosphate buffered saline (PBS) containing 0.05 % Tween 20 (Scharlau, Barcelona, Spain) the mixture was centrifuged at 15,000×g for 30 min at 4 °C, the supernatant was passed through a 0.22 μm pore size membrane filter (Orange Scientific, Braine-I Alleud, Belgium) and used for the detection of enterotoxins through enzyme linked immunosorbent assay (ELISA).

### Enrichment and cultivation

Fluid thioglycollate medium (FTM) was used for the enrichment of *C. perfringens*. Diluted meat samples (1 ml) were inoculated into FTM broth and the tubes were incubated for 24 h at 37 °C in an anaerobic jar HP-11 (Oxoid, Hampshire, UK) containing AnaeroGen$^{TM}$ Sacchet AN35 (Oxoid, Hampshire, UK) (Wen and McClane 2004). Enrichment culture (0.1 ml) thus obtained was spread over the tryptose sulfite cycloserine (TSC) agar (Himedia Labs, Mumbai, India) plates using a sterile glass rod and incubated anaerobically for 24 h at 37 °C. The growth of *C. perfringens* was evident by the typical black colored colonies (Abudabos et al. 2013; Kotsanas et al. 2010). Such colonies were selected for further purification by sub-culturing onto TSC agar plates under same conditions.

### Identification of microorganism

Isolates were identified on the basis of their colony characteristics, Gram's staining, morphological features, spore staining and motility testing according to instructions given in Bergey's manual (Bergey 2009). Selected isolates were further characterized through their biochemical profile like double haemolysis on blood agar (García and Heredia 2011), reverse Christie Atkins Munch-Petersen (CAMP) test (Brady et al. 2010), lecithinase and proteolytic activity as well as gelatin liquefaction, nitrate reduction and lactose fermentation tests (Lindstrom et al. 2011; Skariyachan et al. 2010).

### Toxinotyping of *C. perfringens*

Supernatant of the raw meat samples were used for toxinotyping through ELISA. Whereas, biochemically identified *C. perfringens* isolates were inoculated in trypticase glucose yeast (TGY) broth (Himedia Labs, Mumbai, India) and incubated anaerobically at 37 °C overnight (Fernandez-Miyakawa et al. 2007; Miki et al. 2008). Toxinotyping of the TGY broth enrichment cultures were performed through ELISA by using commercially available Bio-X Diagnostics Enterotoxaemia ELISA kit (Bio-X Diagnostics, Jemelle, Belgium) for the detection of alpha, beta, and epsilon toxins according to the manufacturer's instructions.

### Antibiotic susceptibility testing

Selected *C. perfringens* cultures having toxin production ability were subjected to antibiotic sensitivity testing by Kirby Bauer disc diffusion method according to the standards procedures recommended by Clinical Laboratory Standards Institute (CLSI 2012).

The data were statistically analyzed by Chi-Square using Statistical Packaging for Social Science (SPSS) software version 13 (IBM Corporation, NY, United States of America).

## Results

### Prevalence of *C. perfringens* in various kinds of meat

Out of 100 samples from each meat type, the highest prevalence of 6 % was found in chicken meat followed by 5 % in the mutton and 1 % in the beef. Complete distribution of *C. perfringens* in the chicken, mutton and beef collected from local butcher shops and larger meat outlets is given in the Table 1. Overall 10/150 samples from local butcher shops and 2/150 from large meat outlets were positive for the *C. perfringens*. Statistical analysis showed that no significant difference exist between the isolation of *C. perfringens* within various meat types ($P>0.05$) while meat samples from local butcher shops had a significantly higher level of *C. perfringens* contamination in comparison to the meat from large meat outlets ($P<0.05$).

### Toxinotyping of the isolates

All the raw meat samples were negative for the alpha, beta and epsilon toxin as detected through ELISA. Six out of twelve *C. perfringens* isolates were able to produce various enterotoxins after culturing in the TGY broth (Table 2). Toxin profiling clarified that three of the isolates were of type A while one of the each was confirmed as type B, C, and D.

### Antibiotic susceptibility profile

Six bacterial isolates capable of producing toxins were selected for testing their antibiotic susceptibility profile against 13 antibiotics. It is not clear from Table 3 that all of the six isolates were susceptible to chloramphenicol, ciprofloxacin, metronidazole and ceftriaxone. Five out of the six isolates were susceptible whereas one was classified as intermediate to tetracycline, lincomycin and cefotaxime. Only one of the six isolates was resistant and five were susceptible to erythromycin. Amoxicillin was proved to be least effective as five of the isolates were resistant and only one was susceptible to it.

## Discussion

In order to study the prevalence of *C. perfringens* in various kinds of meat, the samples were collected from different localities of Lahore City. Within various kinds of meat, prevalence of the organism was relatively higher in the chicken meat (6 %) as compared to mutton (5 %) and beef (1 %). A similar trend was also recorded by Miwa et al. (1998) where a relatively high prevalence (82–84 %) of *C. perfringens* was recorded in the poultry meat in comparison to the beef (16 %) and pork (10 %). However, Singh et al. (2005) demonstrated that the contamination level of *C. perfringens* is relatively higher in goat meat followed by chicken and buffalo meat. In that study more reports of enterotoxemia in the study area were supposed to be linked with the high incidence of the *C. perfringens* isolations in the mutton samples. While comparing the results of meat samples taken from various sources, the samples collected from local butcher shops had a significantly higher level of contamination in comparison to the samples collected from the grocery stores. Such difference could be attributed to the improper hygienic conditions at the local butcher shops in comparison to strict hygienic measures adopted at large meat outlets/grocery stores. Another study also showed that, level of *C. perfringens* contamination was higher in the meat samples collected from small butcher shops (15/80) when compared to the samples obtained from the local market (2/16) (Kamber et al. 2007). Environmental conditions at local butcher shops favor bacterial contamination. It is a common observation that at local butcher shops, animals are slaughtered under poor hygienic conditions where separate knives and slaughtering instruments are not used at different stages of slaughtering. Use of same instruments for the removal of intestines and cutting of meat specially increases the chances of carcass contamination by fecal material. Series wise slaughtering process at large slaughter houses followed by washing after each step improves the meat quality

**Table 1** Occurrence of *Clostridium perfringens* in chicken, mutton and beef meat samples collected from various meat outlets of Lahore City

| Serial No. | Meat type | Source of sample | Positive sample / Total samples | Percent prevalence | Total samples positive |
|---|---|---|---|---|---|
| 1 | Chicken | Local butcher shops | 5/50 | 10 | 6 |
| | | Large meat outlets | 1/50 | 2 | |
| 2 | Mutton | Local butcher shops | 4/50 | 8 | 5 |
| | | Large meat outlets | 1/50 | 2 | |
| 3 | Beef | Local butcher shops | 1/50 | 2 | 1 |
| | | Large meat outlets | 0/50 | 0 | |

**Table 2** Toxinotypes of the *Clostridium perfringens* cultures isolated from various types of meat on the basis of their toxin production potential

| Culture ID | Alpha toxin | Beta toxin | Epsilon toxin | *Clostridium perfringens* | Toxinotype |
|---|---|---|---|---|---|
| CPC 01 | − | − | − | + | NTP |
| CPC 02 | + | − | − | + | A |
| CPC 03 | + | − | − | + | A |
| CPC 04 | − | − | − | + | NTP |
| CPC 05 | + | + | − | + | C |
| CPM 06 | − | − | − | + | NTP |
| CPM 07 | + | − | − | + | A |
| CPM 08 | + | + | + | + | B |
| CPM 09 | + | − | + | + | D |
| CPB 10 | − | − | − | + | NTP |
| CPC 11 | − | − | − | + | NTP |
| CPM 12 | − | − | − | + | NTP |

Key: *CPC Clostridium perfringens* chicken, *CPM Clostridium perfringens* mutton, *CPB Clostridium perfringens* beef, *NTP* No toxin produced

by removing the soiling material from meat surface (Haileselassie et al. 2013). Hygienic issues during the transportation and marketing of mutton or beef in Lahore City might also lead to high level of contamination at local butcher shops.

Most of the meat products are cooked to high temperatures which are adequate to inactivate the vegetative cells of *C. perfringens*, while their spores can withstand the cooking process. Even a small numbers of *C. perfringens* spores can then germinate, multiply and lead to the production of toxins. Therefore, meat can be a health hazard if improperly cooked, processed under improper hygienic conditions or if the cold storage chain is not maintained before and after cooking (Kamber et al. 2007). Changing food habits of people, like preference of more ready to eat foods where chances of contamination are high, predispose the community to more *C. perfringens* food poisoning cases.

In the present study five different meat outlets were selected for the collection of chilled meat and no significant difference was observed among the occurrence of the *C. perfringens* from these meat outlets. Meat samples collected from larger grocery stores are usually kept at temperatures below 10 °C. Refrigeration or frozen temperatures are detrimental for the survival of *C. perfringens* (de Jong et al. 2004) and might be responsible for low isolation rates in such types of meat samples. Within all samples collected from five large meat outlets, the samples from Zenith and Metro were refrigerated while the ones collected from Hyperstar, Mall of Lahore and Akbari stores were frozen. The organism was not isolated from any of the frozen meat and two isolations were made from the refrigerated meat samples only. This finding supports the assumption that food samples tested for the presence of *C. perfringens* vegetative cells should be analyzed immediately without being frozen (García and Heredia 2011).

An overall prevalence of *C. perfringens* in the meat samples was 4 % (12 out of 300 samples) for all types of meat. Some previous studies show a variable prevalence of *C. perfringens* that ranges from 30 to 80 % in USA (Lin and Labbe 2003; Wen and McClane 2004), 8 to 71 % in Japan (Miki et al. 2008; Miwa et al. 1998), 18 to 65 % in Turkey (Cadmak 2001; Kamber et al. 2007) and 11 to 76 % in India (Gurmu et al. 2013; Singh et al. 2005). Most of previous studies used a range of specialized media (modified Duncan strong medium, motility nitrate medium and lactose gelatin medium) for the detection of *C. perfringens* which might have contributed to the higher isolation rates while we used only TSC agar for the bacterial culture. Additionally, some of the studies used molecular (PCR) or serological (ELISA)

**Table 3** Antibiotic susceptibility profile of the toxin producing *Clostridium perfringens* isolates ($n$=6)

| S # | Antibiotic Disc (concentration/disc) | Resistant (%) | Intermediate (%) | Susceptible (%) |
|---|---|---|---|---|
| 1 | Tetracycline (30 mcg) | 0 (0) | 1 (17) | 5 (83) |
| 2 | Chloramphenicol (25 mcg) | 0 (0) | 0 (0) | 6 (100) |
| 3 | Metronidazole (5 mcg) | 0 (0) | 0 (0) | 6 (100) |
| 4 | Penicillin (10 U) | 1 (17) | 1 (17) | 4 (77) |
| 5 | Ampicillin (10 mcg) | 2 (33) | 2 (33) | 2 (33) |
| 6 | Amoxicillin (30 mcg) | 5 (83) | 0 (0) | 1 (17) |
| 7 | Erythromycin (15 mcg) | 1 (17) | 0 (0) | 5 (83) |
| 8 | Vancomycin (30 mcg) | 2 (33) | 0 (0) | 4 (77) |
| 9 | Ciprofloxacin (30 mcg) | 0 (0) | 0 (0) | 6 (100) |
| 10 | Bacitracin (10 U) | 0 (0) | 2 (33) | 4 (77) |
| 11 | Lincomycin (10 mcg) | 0 (0) | 1 (17) | 5 (83) |
| 12 | Ceftriaxone (30 mcg) | 0 (0) | 0 (0) | 6 (100) |
| 13 | Cefotaxime (30 mcg) | 0 (0) | 1 (17) | 5 (83) |

techniques for the detection of organism in the samples which are more sensitive than the isolation procedures adopted in the present study hence this high sensitivity of the test might resulted into increased number of positive samples.

Toxinotyping of the isolates is significant to group the isolates and to determine its disease association (McClane 2007). Six out of twelve isolates were able to produce single or multiple toxins whereas the rest of the isolates did not produce any kind of toxins. All the *C. perfringens* isolates are not equally capable of producing toxins in vitro conditions (Wen and McClane 2004). Similar findings were recorded by Kamber et al. (2007) where 13 out of 17 *C. perfringens* isolates were found positive for toxin production and Singh et al. (2005) who reported 22 out of 116 isolates were positive for the toxin production. In the present study antigen detection ELISA assay using commercial kit was employed for the toxinotyping of the isolates. Such ELISA kits have a detection limit of 10 ng/ml for alpha, beta and epsilon toxins (Kamber et al. 2007). All the meat samples directly processed for toxinotyping might have toxin concentration below the detectable limit of the assay and hence regarded as negative.

Toxin producing isolates were selected for testing their antibiotic susceptibility profile against 13 commonly used antibiotics to evaluate the most suitable antibiotic for *C. perfringens* infection. It was observed that *C. perfringens* was susceptible to most of the antibiotics. Ceftriaxone, ciprofloxacin, metronidazole, and chloramphenicol were proved to be most effective as all of the isolates were susceptible to these agents followed by cefotaxime, tetracycline, and lincomycin where 5 out of six isolates were sensitive. Similar observations were recorded by Skariyachan et al. (2010) where *C. perfringens* isolated from meat showed susceptibility to ampicillin, chloramphenicol, and tetracycline while the organisms were partially susceptible to erythromycin and vancomycin. Singh et al. (2005) screened toxin producing isolates of *C. perfringens* against various antibiotics and found ciprofloxacin and tetracycline as most effective agents. We found amoxicillin as least effective antibiotic as five out of six isolates were resistant to this drug. Development of antibiotic resistance following the excessive of amoxicillin in the food of animals could be a possible reason for least effectiveness of this drug.

## Conclusion

Meat sold at local butcher shops in Lahore City can be potentially contaminated with *C. perfringens* especially with Type A toxinotype of the organism. Most of the toxin producing isolates of *C perfringens* are susceptible to chloramphenicol, ciprofloxacin, metronidazole and ceftriaxone while resistant to amoxicillin.

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