Isolation and identification of Clostridium perfringens *and its toxins from mutton in Lahore City, Punjab, Pakistan*

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Sheep and goat meat.

Summary
The present study was aimed to detect *C. perfringens* and identify its toxins in mutton samples collected from Lahore City in the Punjab Province of Pakistan. A total of 40 samples of minced and non-minced mutton were collected from local butcher and retail shops representing four areas of the city. The samples were subjected to ELISA for the detection of *C. perfringens* alpha, beta and epsilon toxins. The samples were simultaneously processed for bacterial isolation. The isolates were confirmed both by biochemical testing and a multiplex PCR targeting alpha, beta and epsilon toxin genes of *C. perfringens*. While 10% (4/40) of the samples were positive for *C. perfringens* alpha toxins, 17.5% (7/40) of the samples were positive for the alpha toxin gene. The present study indicated that the samples collected from the local butcher shops were contaminated with *C. perfringens* and its toxins. Interestingly, no such contamination was detected in any of the samples collected from retail meat shops. In conclusion, improper hygienic conditions at butcher shops could lead to the contamination of mutton with *C. perfringens* and its toxins.

Introduction

The meat industry of Pakistan is gradually expanding, contributing a large proportion to the global export of halal meat. The average annual increase in the meat export of Pakistan is estimated at 32% (Magsi *et al.* 2021). Pakistan's meat industry needs research and innovation to compete in the international market, especially for the export of halal meat (Magsi *et al*. 2021). In Pakistan, meat is generally obtained from animals slaughtered using traditional slaughtering practices carried out by non-trained workers which commonly lead to the contamination of meat. *Clostridium perfringens* is one of the microorganisms potentially responsible for foodborne diseases especially posing a risk to the consumers of contaminated meat. The presence of *C. perfringens* or its toxins can pose a serious threat to the export of safe halal meat.

Clostridium perfringens was found associated with the first foodborne outbreak in the world and remains a perpetual source of food poisoning in the industrialized world (Grass *et al.* 2013; Mellou *et al.* 2019; Gohari *et al.* 2021). *Clostridium perfringens* is found in a wide variety of food items, particularly meat and poultry items. Different toxino-types of *C. perfringens* from meat and meat

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products have been reported from various North American, Asian, and some European countries (Brynestad and Granum 2002; Kiu and Hall, 2018). Recently, India has reported the prevalence of 20.8 % *C. perfringens* in foods of animal origin (Yadav *et al. 2022)*.

Clostridium perfringens is ubiquitous in the environment and is a normal inhabitant of animals including sheep and goats. This anaerobic, Gram-positive, and spore-forming bacterium is commonly found within the gastrointestinal tract of animals and humans and causes gas gangrene, enteric disease and enterotoxemia (Gohari *et al.* 2021). The bacterium has not only been found in clinically affected animals but also in healthy animals including sheep and goats (Hamza *et al.* 2018; Yadav *et al.* 2022). The pathogen produces specific toxins, such as alpha, beta, epsilon, iota, enterotoxin, beta-2 and *NetB*, which are used to categorize *C. perfringens* into seven toxinotypes: A through G (Kiu and Hall, 2018). Some of these toxins are responsible for enteric disease. For example, enterotoxin produced by *C. perfringens* type F can cause villus atrophy and mucosal damage in a rabbit's small intestine (Gohari *et al.* 2021). *Clostridium perfringens* types A, C and F are the major cause of human foodborne illness (Rood *et al.* 2018). These Clostridia can produce alpha, beta and enterotoxins (Kiu and Hall, 2018). Type C Clostridia are mainly responsible for enterocyte damage (Gohari *et al.* 2021).

Clostridium perfringens can grow quickly in protein-rich foods such as animal and poultry meat or meat/poultry products. Intestinal contents containing *C. perfringens* are the main source of contamination for meat and meat products. However, a large number of *C. perfringens,* up to 10^8 vegetative toxin-producing cells per gram, acquired from improperly stored food, that can colonize the intestine and sporulate are required to induce enteric disease in humans (European Food and Safety Authority, 2005). Human consumption of contaminated or undercooked meat and meat products characterized by improper cooling, improper temperature maintenance during storage and inadequate reheating before consumption should be considered for *C. perfringens*-related food poisoning (Leung *et al.* 2017). *Clostridium perfringens*-related food poisoning is exacerbated by the development of multi-drug resistant strains of *C. perfringens* (Bendary *et al.* 2022). In most cases, the cause of poisoning by *C. perfringens* is the unstable storage temperature of foods prepared for catering in institutions such as hospitals, educational institutions, nursing homes and jails, where large quantities of food is prepared many hours before consumption (Kamber *et al.* 2007). Since a small number of the organism is frequently present after cooking, subsequent improper storage could lead to bacterial growth sufficient to pose a food safety risk. There could be myriad factors leading to *C. perfringens* associated food poisoning from meat: animal carcasses stored unrefrigerated before retail, meat products cooled slowly after cooking or stored unrefrigerated and, in addition, improperly cooked meat products ingested without proper re-heating. These conditions allow the replication of vegetative forms of *C. perfringens* that, once ingested, can cross the gastric acid barrier and reach the intestinal lumen, where they can sporulate releasing an important toxin, the enterotoxin, responsible for the enteric disease. However, properly reheating the food before eating can inactivate heat-labile clostridial toxins, such as alpha toxin (Leung *et al.* 2017).

Most published studies have centered on the isolation of *C. perfringens* or the detection of its genes from meat and meat products (Wen and McClane, 2004; Khan *et al.* 2020), but only limited published literature reports the detection of its toxins in meat. Some studies have reported the isolation of *C. perfringens* from meat collected from markets and butcher shops but only limited data are available on the detection of *C. perfringens* toxins in meat from Lahore, Punjab, Pakistan. Recently *C. perfringens* was detected in various types of meat in the Mardan District of Khyber Pakhtunkhwa Province of Pakistan. The highest prevalence of the bacterium was noted in goat (87%) and sheep (51.5%) meats (Khan *et al.* 2020). Owing to the food safety risks associated with meat, the screening of mutton sold in local butcher and retail shops for *C. perfringens* and its toxins warrants investigation. Therefore, the present study was designed to detect *C. perfringens* and its toxins from mutton samples collected from the local butcher and retail shops in Lahore City of the Punjab Province of Pakistan.

Materials and methods

Sample collection

The samples of fresh minced and non-minced mutton were collected from local butcher shops and retail meat shops. Ten samples were collected from each of the four sampling areas of Lahore City: Islampura, Shadman, Ichhra and Gulberg, for a total of 40 samples (Figure 1).

In Pakistan fresh meat is sold in two types of shops: butcher shops and retail meat shops. A butcher shop is an open shop run by one or two butchers who cut and sell the meat on the spot. The carcasses are usually hung outside the shop in open, exposed to the outside environment. On the other hand, a retail meat shop is commonly run by a company that has hired trained staff to sell meat stored in meat chillers and packaged in clean disposable containers.

The present study's sampling was done twice, once in the last week of June 2021 and again in the second week of July 2021. Twenty samples were collected on each sampling day. Ten of the study samples originated from retail shops and the rest 30 came from butcher shops. Twenty of the samples collected were minced and the rest of the samples were non-minced mutton. Each sample was collected in a clean Ziplock bag and transported maintaining a cold chain process to the Anaerobiology Section, Quality Operations Laboratory, Institute of Microbiology, University of Veterinary and Animal Sciences, Lahore, Pakistan and was processed within 2-4 hours of collection.

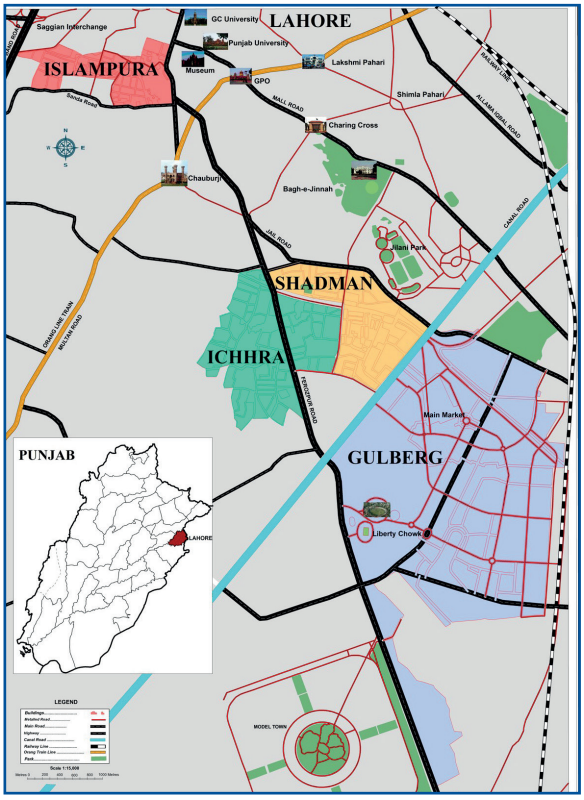

Figure 1. *Sampling sites in Lahore City: Islampura, Shadman, Ichhra and Gulberg.*

Sample processing

Twenty-five grams of each sample were weighed aseptically and homogenized in a Turrax T-25 (IKA® - Werke GmbH & Co. KG, Staufen in Breisgau, Germany) homogenizer with 25 ml sterile distilled water (Kamber *et al.* 2007). Ten milliliters of each homogenized sample were transferred to sterile falcon tubes and centrifuged at 1500 rpm for 10 minutes. A 1 ml of cell-free supernatant was collected in a separate tube and refrigerated for performing enzyme-linked immunosorbent assay (ELISA) on the same day.

Enzyme-linked immunosorbent assay

A direct sandwich ELISA was used to identify alpha, beta, or epsilon toxins of *Clostridium perfringens* following Kamber *et al.* (2007).

Briefly, 25 grams of a mutton sample were homogenized with 25 ml of sterilized water.

Ten milliliters of the homogenate were centrifuged at 1500 rpm for 10 minutes to clarify the sample. One milliliter of each supernatant was added to an ELISA kit (BIO K 270 Multiscreen AgELISA Enterotoxaemia, BIO X Diagnostics, Rochefort, Belgium) and incubated at -20°C for one day, following the manufacturer's instructions. Rows A, C, E and G of the test kit were sensitized with antibodies specific for alpha, beta and epsilon toxins, whereas rows B, D, F and H were sensitized with non-specific antibodies.

If the antigen was present in mutton samples, specific antibodies were able to capture it.

To distinguish between specific and non-specific binding, rows of non-specific antibodies were included.

To identify the toxins produced by *C. perfringens*, each sample was analyzed. After adding Stop Solution (1M phosphoric acid), optical densities (OD) were measured using an ELISA microplate reader (Biobase, Jinan, China) at 450 nm.

The OD value of each sample well was subtracted from the respective negative control before calculating its net OD. For the alpha, beta and epsilon toxins according to the QC data sheet from the manufacturer, the OD positive limit was set at 0.150.

As a result, each sample with an OD difference greater than or equal to 0.150 was interpreted as "Positive" for the toxins being examined. However, any sample with a difference in OD of less than 0.150 was regarded as a "Negative".

Bacterial culture:

The supernatant from processed samples was swabbed on Perfringens Agar Base media plates and incubated in an anaerobic jar at 37°C for 72 hours. The colonies were identified through morphology, Gram staining and biochemical tests.

The confirmed *C. perfringens* colonies were further purified by four-way streaking on Perfringens Agar Base plates followed by incubation at 37°C for 48 hours in anaerobic conditions.

The bacterial isolates were refrigerated at 4°C.

Biochemical testing:

The isolates were tested by catalase, oxidase, lecithinase and sugar fermentation tests.

Toxin gene detection:

DNA extraction

DNA was extracted from freshly cultured *C. perfringens* colonies that were identified by microscopy and colony morphology using a commercial kit (Gene All, Seoul, South Korea) following the manufacturer's instructions.

Polymerase chain reaction

A PCR targeting alpha, beta and epsilon genes of *C. perfringens* was performed in a multiplex reaction using already published primer information (van Asten *et al.* 2009). The reaction mixture was prepared by adding 12.5 µL of master mix, 2 µL of template DNA, 0.5 µL each of forward and reverse primers (Table 1), and 7.5 µL of nuclease-free water. PCR tubes containing the reaction mixture were placed in a thermocycler (Bio-Rad, Hercules, CA, USA) using a protocol described previously (Khan *et al.* 2020). Agarose gel 1.5% (HiMedia Laboratories, Mumbai, India) was used to separate DNA bands that were visualized under UV light using a Gel Documentation System (Bio-Rad, Hercules, CA, USA).

Table I. *Primers for multiplex PCR for* Clostridium perfringens *alpha, beta, and epsilon toxins (van Asten et al. 2009).*

Toxin gene	Primer	Sequence (5'-3')	Product size (bp)
Alpha / *cpa*	CPAlphaF	GCTAATGTTACTGCCGTTGA	324
	CPAlphaR	CCTCTGATACATCGTGTAAG	
Beta / *cpb*	CPBetaF3	GCGAATATGCTGAATCATCTA	195
	CPBetaR3	GCAGGAACATTAGTATATCTTC	
Epsilon / *etx*	CPEpsilonF	TGGGAACTTCGATACAAGCA	376
	CPEpsilonR2	AACTGCACTATAATTTCCTTTTCC	

Results and discussion

Clostridium perfringens is the leading cause of food poisoning worldwide and is abundantly present in the environment. A total of 40 mutton samples were collected from the local butcher and retail shops in Lahore, Punjab, Pakistan. Four out of 40 (10%) samples tested positive for the alpha toxin of *C. perfringens* by ELISA but none of the samples was positive for beta or epsilon toxins. When bacterial isolation was attempted, a total of 35% (14) mutton samples were culture-positive for *C. perfringens*. These were identified as *C. perfringens* by colony morphology (Figure 2), Gram staining and biochemical testing. All 14 samples were Gram-positive and demonstrated lecithinase activity. These samples tested negative for catalase and oxidase tests. Multiplex PCR was performed on 8 randomly selected positive samples, including those (n=4) that were positive for *C. perfringens* alpha toxin by ELISA (Table 2). Seven out of 8 samples demonstrated clear 324 bp bands for *C. perfringens* alpha toxin gene (Figure 3). PCR successfully amplified alpha toxin gene in the four ELISA positive samples. No samples were PCR positive for beta and epsilon toxin genes of *C. perfringens*. Moreover, only samples collected from butcher shops were positive suggesting lapses in the implementation of food safety standards at the local butcher shops in Lahore City.

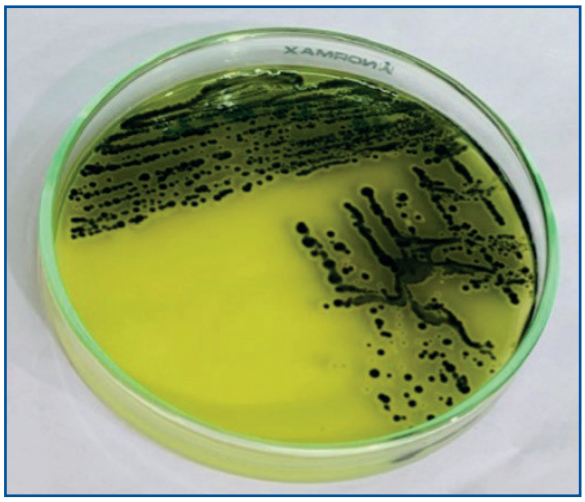

Figure 2. *Colonies of* Clostridium perfringens *isolated from mutton samples on Perfringens Agar Base.*

Table II. Clostridium perfringens *samples positive by microscopy, ELISA and PCR for alpha, beta and epsilon toxins and their genes.*

Sr. No.	Method	No. of positive samples
1	Microscopy	14
2	ELISA	4
3	PCR	7

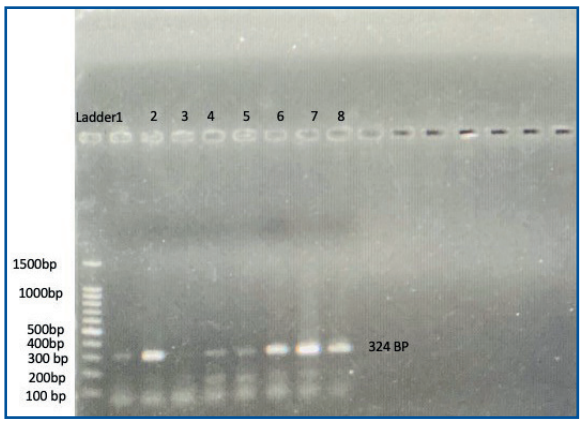

Figure 3. *Gel image of PCR products of mutton samples (lanes 1, 2, 4, 5, 6, 7, 8) showing* Clostridium perfringens *alpha toxin gene band at 324 bp.*

The mutton samples can be contaminated with *C. perfringens* during slaughter, transport and/or storage. One reason for the contamination of mutton in the present study could be that the animals might have been slaughtered in unsanitary conditions using traditional practices. *Clostridium perfringens* present in the intestinal contents of animals can be a source of contamination during slaughter (Miwa *et al.* 1997). Carcasses can be contaminated with animal gut contents, feces or soil. Cross-contamination with contaminated surfaces, butcher's hands, or slaughtering equipment could also occur. The butchers commonly do not use hygienic methods for slaughtering animals. Since the tools used in local butcher shops are usually not sanitized properly on a regular basis, those could be a source of meat contamination. In the present study, the carcasses were transported to local markets or to small butcher shops from slaughterhouses.

The carcasses were delivered to the markets daily but they might have been stored for more than 24 hours before retail purchase at the butcher shops. In the butcher's shop, the carcasses are commonly kept for days or till they are sold out. In the local market, the butchers hang the carcasses out in the open from morning till evening, where these get exposed to ambient temperature, dust, and other environmental factors, including flies. Moreover, *C. perfringens* optimal growth takes place at 43°C-47°C (Taormina and Dorsa 2004). In the summer season, the ambient temperature in Lahore ranges from 30°C to 48°C, suggesting the ambient temperature of the city may support *C. perfringens* growth if the meat is stored at this temperature (Hall and Angelotti, 1965).

Toxin production varies among *C. perfringens* isolates which may explain why some of the *C. perfringens*-positive samples in this study tested negative for toxins (McClane *et al.* 2013). The limit of detection for alpha, beta and epsilon toxins for test kits used in this study was 10 ng/ml. The inability of test kits to detect beta and epsilon toxins suggests either the absence of both these toxins from the tested samples or the presence of these toxins at levels below the detection limit of the test kit. However, no detection of beta and epsilon genes in the same set of samples confirms the absence of beta and epsilon toxins-producing *C. perfringens*.

The multiplex PCR allows the simultaneous detection of toxin genes of *C. perfringens*. The results of the present study indicated that even though the targets and the results of both ELISA and PCR differed, both tests are important in detecting toxigenic *C. perfringens*. Moreover, PCR proved more sensitive in detecting toxigenic *C. perfringens*. Tests that detect toxins in mutton as well as those that detect toxin genes can be useful in identifying meat samples posing a food safety risk.

The results of the present study indicate that some mutton samples were contaminated with *C. perfringens* and its toxins at the time of purchase corroborating Wen and McClane (2004) who reported the presence of *C. perfringens* in American foods at the time of retail purchase (Wen and McClane 2004). *Clostridium perfringens* and/or its toxins have also been identified in meat sold in Turkey, Japan and Iran's markets (Hassani *et al.* 2022; Miki *et al.* 2008; Yibar *et al.* 2017). In Japan, 3.3–64% of beef samples and 0–54% of minced-meat samples were contaminated with *C. perfringens* (Fukushima *et al.* 1987). In Pakistan, one study has reported the detection of *C. perfringens* and its toxins in mutton in the Mardan City of Khyber Pakhtunkhwa Province of Pakistan (Khan *et al.* 2020). Another study done in Lahore City reported *C. perfringens* in only 4% of mutton samples but no toxin was detected (Khan *et al.* 2015). The present study reports the presence of *C. perfringens* and its alpha toxin in mutton samples from Lahore, Pakistan. The present study raises food safety concerns as it suggests that *C. perfringens* contamination can take place due to improper hygiene practices. This is compounded by the fact that an expanding population of the city has pushed many untrained workers in the animal slaughtering business to meet increased demand for meat. These untrained workers are unaware of proper slaughtering and food safety principles required therein leading to the contamination of carcasses.

The present study although comprised of a limited number of samples and four areas of Lahore City yet provides baseline data on the presence of *C. perfringens* and its toxins in locally sold mutton. However, the small sample size does not allow conclusions to be drawn regarding the prevalence of *C. perfringens* in the mutton being sold in the local market. Further studies covering other areas of Lahore and other cities and provinces of Pakistan are warranted.

Future research should evaluate the bacterial load by both classical (isolation) and biomolecular methods (quantitative PCR) at different stages of the food chain, from slaughtering to retail. Bacterial counts and quantitative PCR performed on samples collected separately can detect quantitative differences. These comparisons could allow focusing on improving hygienic practices at each stage of the food chain. In this way, scientific data on the exact site of improper handling of mutton could be generated and made available to local food authorities, thus helping the government to find strategies to train workers and educate people on safe handling and proper storage of foodstuff.

Clostridium perfringens is present in the fresh meat market of Lahore City of Punjab, Pakistan. The present study highlights the need to

raise hygienic standards for handling mutton and maintaining better conditions for its storage to minimize food safety risks, especially at local butcher shops. The staff at slaughterhouses and butcher shops should be trained to avoid contamination of meat. The present study's results should be taken into consideration by the relevant food authorities when developing guidelines to minimize mutton-related food safety risks. The use of standard practices by properly training staff for slaughtering animals in designated slaughterhouses should be made strictly mandatory. Developing and implementing standard operating practices for handling and storage of meat at the butcher shops along with regular monitoring to improve the hygiene quality of meat and meat products available in the market for public consumption is likely to enhance food safety.

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