Research Article

The prevalence of *Clostridium perfringens* in retail meat of Mardan, Pakistan

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Abstract: The most prevailing bacteria in distribution is *Clostridium perfringens* (*C. perfringens*) which is ranked 1st among fatal diseases in small ruminants. This rapidly growing food borne pathogen is present in water, food, soil, air, and humans and animals intestinal tract. In the present study, a cross-sectional study was performed to sort out the prevalence of *C. perfringens* types estimation in various retail meats and areas of Mardan district, Pakistan. 600 fresh meat samples (equally from cows, sheep and goats) were collected from retail shops. *C. perfringens* was diagnosed in various types of meat however highest prevalence was found in goats 174/200 (87%) followed by sheep 103/200 (51.5%) while the least prevalence was in cow meat 89/200 (44.5%). All obtained isolates were further genotyped through multiplex PCR indicating 93/174 (53.45%) *C. perfringens* type A, 16/174 (9.19%) type B, 3/174 (1.72%) type C and 62/174 (35.63%) type D in goat isolates. 53/103 (51.46%) *C. perfringens* type A, 11/103 (10.68%) type B, 3/103 (2.91%) type C and 36/103 (34.95%) type D in sheep isolates. 73/89 (82.02%) *C. perfringens* type A, 9/89 (10.11%) type D and 7/89 (7.86%) type E in cow isolates. Out of 89 cow meat isolates 4 (4.49%) carried *cpe* gene, 16 (17.98%) contained *cpb2* gene and 1 (1.12%) carried both *cpe* and *cpb2* genes. In 103 sheep meat isolates, 2 (1.94%) carried *cpe* gene, 8 (7.77%) carried *cpb2* gene and 3 (2.91%) carried both *cpe* and *cpb2* genes. In 174 goat's meat isolates, 3 (1.72%) carried *cpe* gene, 18 (10.34%) carried *cpb2* gene while (1.72%) carried both *cpe* and *cpb2* genes. While analyzing prevalence in months and area wise, no significance was observed through chi-square test. It indicates that this pathogen is prevalent in various meat sources in Mardan and could be a threat to public health.

Keywords: Anaerobic, *Clostridium perfringens,* multiplex PCR, toxin genes

1. Introduction

The meat industry of Pakistan is gradually expending, contributing a big proportion to the global export of halal red meat. The average annual increase in the meat export is recorded as 30%. The Pakistani meat industry need more improvisation to potentially compete in international export of halal meat as well as to meet local demands [1]. In Pakistan, meat is generally obtained from food animals slaughtered through traditional way, which pose a biggest source of contamination from intestinal microflora. Other deficits are lack of modern slaughtering techniques, low education and lack of awareness about food safety and food hygiene. *C. perfringens* constitute a common cause of food borne diseases. *C. perfringens* was found first associated with the first food borne outbreak in the world and still remains a source of food poisoning in the industrialized world. Different toxino-types of *C. perfringens* from meat and meat products have been reported by different countries including USA, Canada, Asian and some European countries [2–4]. It is anaerobic, gram-positive spore forming bacteria, which is frequently present in the gastro intestinal tract of animals and humans where it causes enteric problems [5,6]. The pathogen produces different types of toxins, such as alpha, beta, epsilon, and iota, which make a basis to divide *C. perfringens* in to five subtypes (A to E) [6,7]. *C. perfringens* type A produces alpha toxin, type B produces alpha, beta and epsilon toxins, type C produces alpha and beta toxins, type D produces alpha and epsilon toxins and type E produces alpha and iota toxins [5]. Types A and C are the major cause of human food poisoning [8]. Besides the major toxins, minor toxins i.e. enterotoxin and b2 are also

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produced from *C. perfringens* which make another reason of human food poisoning [9]. The pathogen encoded by *cpe* is mainly involved in food poisoning, but it is present only in 5% of all the strains. This 319 amino acids containing polypeptide is produced in the small intestine [10]. The *cpb2* gene expression causes the production of b2 toxin which is responsible for antibiotic associated diarrhea [3]. *C. perfringens* type A constitutes a major part of microflora causing gas gangrene [8,11].

Initially, *C. perfringens* were identified and toxino-typed with toxin neutralization tests. Besides this,, mice and pig inoculation methods were used for toxino-typing of *C. perfringens* which are now obsolete as ethical issues, and were also involved with testing animals. However, this time consuming and expensive techniques has now been replaced by the most reliable alternatives like PCR and multiplex PCR, real time PCR and microarray where various protocols are adopted for genotyping of the *C. perfringens* on the basis of toxins produced by them. Among molecular techniques multiplex PCR is the rapid, easy and continuous one step detection method of detection of multiple genes at reasonable cost. Because of these qualities multiplex PCR is the most preferred method for detection of *C. perfringens* [6,9]. Keeping in view the pathogenicity of *C. perfringens,* the food items available at the open market should be screened for the presence of types of *C. perfringens* strains. The present study is made to estimate the load of different strains of *C. perfringens* in the most consumable meat sources in district Mardan, Pakistan.

2. Materials and methods

2.1. Sampling
A total of 600 samples of fresh meat (200 each from cow, sheep and goat) were randomly purchased from butcher's retail shops between Septembers 2017 to February 2018 in Mardan district of Khyber Pakhtunkhwa Province, Pakistan, through convenient purposive sampling technique. The whole district was further divided into ten most populated towns and from each town 20 meat samples (equally from cow, sheep and goat) were collected. The samples were packed twice in air tight sterile cupped bottles and transported to the laboratory under cold chain conditions.

2.2. Processing of samples and identification of *C. perfringens*
The whole meat samples were grounded and homogenized for 2 minutes in a stomacher and enriched 1:10 in Perfringens Enrichment Medium (PEM) (Merck KGaA, Darmstadt, Germany) under anaerobic conditions. Tryptose sulphite cycloserine agar media (TSC) added with D-cycloserine (200 mg/500 mL) (Himedia Laboratories Pvt. Ltd., Mumbai, India) was smeared with PEM samples

and were incubated in CO_2 incubator on 38 ± 1.0 °C for 24 h. The colonies were confirmed through morphology, gram staining and biochemical tests (Remel RapID ANA II System Test Kit, Thermo Fisher Scientific Inc., Waltham, MA, USA) [5].

2.3. Extraction of DNA
For DNA extraction, *C. perfringens* isolates were first incubated for conservation in Robertson Cooked Meat (RCM) medium. DNA extraction kit (GeneAll Biotechnology Co., Seoul, South Korea) was used to extract DNA. Briefly, the cultured broth taken in 1.5 mL micro centrifuge tubes and centrifuged at $20000 \times g$ to obtain pellets. Lysozyme (LYS702, Bioshop Canada Inc., Ontario, Canada) 180 µL and Proteinase K 20 µL (20 mg/mL) was mixed with each pellet and incubated at 56 °C and 70 °C for half an hour. Buffer BL 200 µL was added and incubated at 56 °C for 30 min and again for 30 min at 70 °C. Buffer BL 200 µL and ethanol absolute 200 µL was added and centrifuged in SV column at $6000 \times g$ for 1 min. Washed with BW 600 µL then buffer TW 700 µL and added 200 µL buffer AE to elute DNA. Its concentration was measured with NanoDrop 2000 (Thermo Fisher Scientific Inc., Waltham, MA, USA) and preserved at –20 °C before using in PCR.

2.4. Polymerase chain reaction
A reaction mixture of 50 µL was prepared by adding 10 X PCR buffer (10 mM Tris-HCl, pH = 9.0, 50 mM KCL), 2 µL of 50 mM of $MgCl_2$, 250 M of each deoxy-nucleotide triphosphate, 5 U of Taq DNA polymerase, DNA 5 uL, multiplex primers (Table 1) 100 p moles in themocycler (BIO RAD T 100 USA). PCR tubes containing reaction mixture were placed in a thermocycler. The DNA samples were denatured at 95 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 60 min. DNA strands were then annealed at 55 °C for 30 seconds followed by extension for 60 min and final extension at 72 °C for 5 min. 1.5% agarose gel was used to separate DNA bands and visualized under UV light [5–12].

2.5. Statistical analysis
Statistical analysis was performed using SPSS version 17.0 (SPSS Chicago, IL, USA). The distribution of virulent genes was compared using chi-square test. P value less than 0.05 was considered significant.

3. Results

3.1. Identification of bacteria
C. perfringens colonies were identified through their characteristic black color. The colonies were observed in 369 out of 600 samples on TSC selective media. The identified colonies were further confirmed through biochemical test kit (Remel RapID ANA II System Test Kit, Thermo Fisher Scientific Inc., Waltham, MA, USA).

Table 1. List of primers related to toxins (Meer and Songer 1997; Garmory et al. 2000).

Toxin/gene	Primers	Sequences (50–30)	Length of fragment (bp)
Alpha/*cpa*	CPALPHATOX-F CPALPHATOX-R	GCTAATGTTACTGCCGTTGA CCTCTGATACATCGTGTAAG	324
Beta/*cpb*	CPBETATOX-F CPBETATOX-R	GCGAATATGCTGAATCATCTA GCAGGAACATTAGTATATCTTC	196
Epsilon/*etx*	CPETOXIN-F CPETOXIN-R	GCGGTGATATCCATCTATTC CCACTTACTTGTCCTACTAAC	655
Iota/*iA*	CPIOTA-F CPIOTA-R	ACTACTCTCAGACAAGACAG CTTTCCTTCTATTACTATACG	446
Enterotoxin/*cpe*	CPENTEROTOK-F CPENTEROTOK-R	GGAGATGGTTGGATATTAGG GGACCAGCAGTTGTAGATA	233
b2/*cpb2*	CPBETA2TOK-F CPBETA2TOK-R	AGATTTTAAATATGATCCTAACC CAATACCCTTCACCAAATACTC	567

Toxin = Type of gene, F =Forward primer, R = Reverse primer.

3.2. Prevalence of *C. perfringens* serotypes in meat samples

Among 369 isolates, 366 isolates confirmed as *C. perfringens* by multiplex PCR as 59.84% (219/366) *C. perfringens* type A, 7.38% (27/366) type B, 1.64% (6/366) type C, 29.23% (107/366) type D and 1.91% (7/266) type E. The general prevalence of *C. perfringens* was noted as 44.33% in all 3 meat types. *C. perfringens* were most frequently present in goat meat (87%), followed by sheep (51.5%), while the least isolation occurred in cow meat (44.5%). The prevalence in goats was significantly higher than the cow and sheep. No significance was observed between cow and sheep prevalence (P > 0.05).

3.3. Serotype distribution of *C. perfringens* in species specific meat

Among the 89 isolates from cow meat, 73/89 (82.02%) were *C. perfringens* type A, 9/89 (10.11%) type D and 7/89 (7.86%) type E. In case of 103 isolates from sheep meat, 53/103 (51.46%) were *C. perfringens* type A, 11/103 (10.68%) type B, 3/103 (2.91%) type C and 36/103 (34.95%) type D. Among 174 isolates from goat meat, 93/174 (53.45%) were *C. perfringens* type A, 16/174 (9.19%) type B, 3/174 (1.72%) type C and 62/174 (35.63%) type D (Table 2, P < 0.05) (Figures 1 and 2).

It was further noted in cow meat isolates that 3 (3.37%) of type A, 1 (1.12%) of type D carried *cpe* gene and 9 (10.11%) of type A, 7 (7.86%) of type D carried *cpb2* gene while 1 (1.12%) of type A carried *cpe* gene as well as *cpb2* gene (Table 2, P < 0.05).

In sheep meat isolates, 2 (1.94%) of type A carried *cpe* gene and 6 (5.83%) of type A, 2 (1.94%) of type D carried *cpb*2 gene while 1 (0.97%) of type B, 1 (0.57%) of type C, 1 (0.57%) of type D carried both *cpe* and *cpb*2 genes (Table 2, P < 0.05).

In goats, meat isolates 3 (1.72%) of type A carried *cpe* gene and 14 (8.05%) of type A, 4 (2.30%) of type D carried *cpb*2 gene while 1 (0.57%) of type B, 1 (0.57%) of type C, 1 (0.57%) of type D carried both *cpe* and *cpb*2 genes (Table 2, P < 0.05).

3.4. Area and month wise distribution of different serotypes of *C. perfringens*

Comparing all ten towns for the prevalence of *C. perfringens*, no major difference was observed (Table 2). The highest level of prevalence was observed in Old Bazar Mardan 55/366 (15.03%) followed by Takht Bhai 45/366 (12.30%), Lund Khwar 39/366 (10.66%), Takar 34/366 (9.29%), Manga and Sawal Dher 34/366 (9.28%), Katlang 32/366 (8.74%), Shergarh and Rustam 31/366 (8.47%) while the lowest was in Sheikh Maltun Town 29/366 (7.92%).

Monthly distributions of *C. perfringens* were 19.13% (70/366) in September 2017, 16.94% (62/366) in October 2017, 16.12% (59/366) in November 2017, 13.94% (51/366) December 2017, January 2018, 13.94% (51/366) and February 2018 19.95% (73/366).

4. Discussion

Food of animal origin plays an important role in food-borne zoonosis including collibacilliosis, food poisoning associated with *C. perfringens*, salmonellosis, shigellosis and other fatal diseases including tuberculosis, listeriosis and hemorrhagic and toxic shock syndrome. Meat and

Table 2. Isolates of *Clostridium perfringens* in cow, sheep and goat fresh meats.

S.No	Toxin types	Toxin gene	Isolates percentage			Total	P value
			Cow	Sheep	Goat		
1	A	*Cpa*	60 (67.41)	45 (43.69)	76 (43.68)	181 (30.17)	0.050
2		*cpa, cpb*2	9 (10.11)	6 (5.83)	14 (8.05)	29 (4.83)	0.003
3		*cpa, cpe*	3 (3.37)	2 (1.94)	3 (1.72)	8 (1.33)	0.001
4		*cpa, cpe, cpb*2	1 (1.12)	---	---	1 (0.17)	0.000
5	B	*Cpa, cpb, etx*	---	10 (9.71)	15 (8.62)	25 (4.17)	0.003
6		*Cpa, cpb, etx, cpb*2		1 (0.97)	1 (0.57)	2 (0.33)	0.001
7	C	*Cpa, cpb*	----	2 (1.94)	2 (1.15)	4 (0.67)	0.0015
8		*Cpa, cpb, cpb*2*, cpe*		1 (0.97)	1 (0.57)	2 (0.33)	0.001
9	D	*Cpa, etx*	7 (7.86)	33 (32.04)	57 (32.76)	97 (16.17)	0.004
10		*Cpa, etx, cpe*	1 (1.12)	2 (1.94)	4 (2.30)	7 (1.17)	0.003
11		*Cpa, etx, cpb*2	1 (1.12)	---	---	1 (0.17)	0.001
12		*Cpa, etx, cpb*2*, cpe*	---	1 (0.97)	1 (0.57)	2 (0.33)	0.001
13	E	*Cpa, iA*	7 (7.86)	---	---	7 (1.17)	0.002
Total			89/200 (44.5)	103/200 (51.5)	174/200 (87)	366/600 (61)	-

Cpa = α (Alfa toxin), cpb = β (Beta toxin), cpe = ε (Entero toxin), iA = Iota toxin, etx = Epsilon toxin.
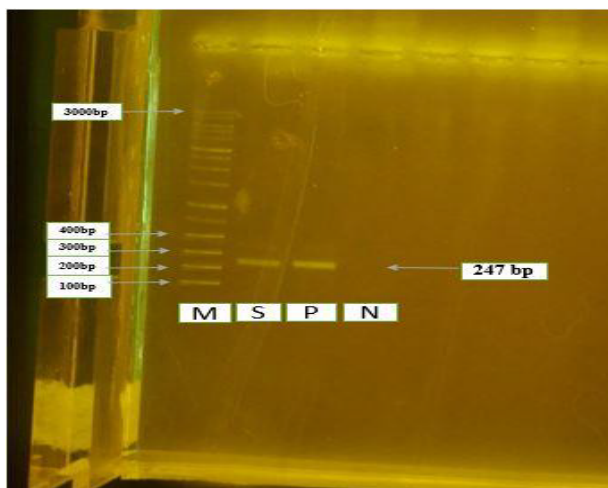Significant at 0.05 level of significance.

Figure 1. PCR results for C.P type D alpha toxin (247 bp) on Agrose gel. M indicates 1kb molecular weight marker, S indicates sample, P indicates control positive, and N indicates control negative.
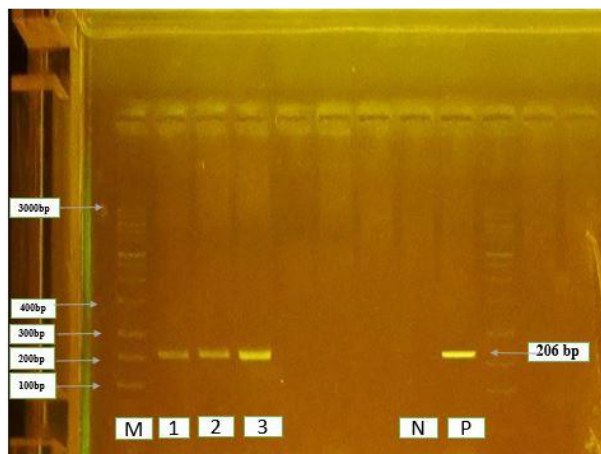

Figure 2: PCR results for C.P type D epsilon toxin (206bp) on Agrose gel. M indicates 1kb molecular weight marker, Lane 1-3 indicates samples, N indicates control negative, and P indicates control positive.

meat products of food animals play a crucial role in the spread of *C. perfringens* associated foodborne diseases. Meat and meat products are passing through a series of different process including slaughtering, evisceration, washing, cutting and packing before ready for sale. These processes might contribute to contamination of meat and meat products with *C. perfringens* in addition to intestinal and fecal contamination of the animals themselves. Alpha toxin is produced by all types of *C. perfringens*. *C. perfringens* type A is frequently found in intestine and feces of different mammalian species such as cattle, sheep and goats [13,14]. Previous studies have reported the prevalence of *C. perfringens* type A as 54.3% in Japan

(Miki et al. 2008), 20.4 % in beef samples and 16.6 % in lamb meat samples in USA [15]. Similarly, our results also indicated highest distribution i.e. 82.02%, 51.46% and 53.45% of *C. perfringens* type A in cow, sheep and goat meat isolates respectively. The difference in the results could be stemmed from the difference in t samples, slaughtering and packing process, and environmental and geographical locations.

C. perfringens types B, C, D and E are widely present in the humans and animal's gastrointestinal tract where they cause enteric diseases [6,11]. In our results 7.38% (27/366) were type B, 1.64% (6/366) were type C, 29.23% (107/366) were type D and the remaining 1.91% (7/266) were type E. Similarly, another study demonstrated that 262 *C. perfringens* sheep meat isolates were genotyped and found type B as 1.53%, type C as 3.82% and type D as 28% while type E was not found [12]. Other researchers identified *C. perfringens* type A, B, C and D in enterotoxaemic sheep, lambs, cattle, and calves respectively [13,16,17]. The highest contamination noticed after *C. perfringens* type A was type D, B and C [14,18]. *C. perfringens* type A, D and E were isolated from cow meat which was same with the study conducted by Miyashiro et al. [19]. We found that *C. perfringens* type A containing *cpe* gene was isolated from 3 (3.37%) of cow meat, 2 (1.94%) of sheep meat and 3 (1.72%) of goat meat isolates. This contamination rate in meat and meat products is similar to the previous studies conducted in Turkey [5,12,20]. The pathogens also carry both the *cpe* and *cpb*2 genes which are responsible for antibiotic associated diarrhea [21]. Similarly, in our results, 9 (10.11%) of cow meat, 6 (5.83%) of sheep meat and 14 (8.05%) of goat meat isolates contain *cpb*2 gene. The highest contamination of *C. perfringens* in Old bazaar Mardan followed Takht Bhai

and Lund Khwar town might be due to over population, unhygienic and low affording power of people in these areas compared to the lowest prevalence in Sheikh Malttun town. The difference in each location could be stemmed from the difference in slaughtering and handling practices of butchers and workers as well as the role of regulatory and law enforcement agencies because big cities are under strict and vigilant observations.

It is concluded that *C. perfringens* is highly prevalent in different high consumable fresh meat market of Mardan district, of Khyber Pakhtunkhwa, and necessary measures need to be adopted to decrease its prevalence. Extensive surveillance is required throughout the province to report this important contaminant, as it is implicated and causing serious food poisoning and gas gangrene in human population. Furthermore, mass vaccination and quarantine practices should be started in animal population to deal with this threat and avoid further complications. Moreover, a public awareness campaign should be initiated to educate the public regarding the pathogenicity of *Clostridium* species.

Conflict of interest
The authors declared no conflict of interest.

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