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RESEARCH ARTICLE

Molecular Characterization of *Clostridium perfringens* Toxino-types and Type 'D' Multidrug Resistance Profile in Diarrheic Sheep

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ABSTRACT

Clostridium perfringens (*C. perfringens*) causes enterotoxemia in animals. The bacterium is a normal inhabitant in the gut of animals but become pathogenic and proliferate rapidly whenever finds suitable conditions. It produces large amount of exotoxins, which prove lethal locally as well as systemically. This study focused molecular characterization of different *C. perfringens* toxins genes i.e. *cpa*(α), *cpb*(β), *etx*(ε), and *iap*(ι) along with *C. perfringens* type 'D' antimicrobial resistance profile. A total of (n=192) fecal samples from diarrheic sheep were processed and 92.19% (177/192) of the samples were found positive for *C. perfringens*. Elevated bacterial count (>10^7 CFU/g) was found in 37.85% (67/177) of the positive samples, while, 62.15% (110/177) of the positive samples were in the normal range of bacterial count (10^4-10^7 CFU/g). Molecular typing of *C. perfringens* spp was carried out by targeting specific toxin genes using PCR. *C. perfringens* type 'A' was highly prevalent 60.45% (107/177) among positive isolates, followed by type 'D', 'C' and 'B' which were 20.90% (37/177), 13.56% (24/177) and 05.08% (09/177) respectively. None of the samples revealed *C. perfringens* type 'E'. The *in-vitro* antimicrobial resistance profile of *C. perfringens* type 'D' was evaluated. Penicillin, ciprofloxacin and ceftriaxone were found highly sensitive (100%) while, bacitracin, ampicillin and erythromycin were found resistant against *C. perfringens* type 'D'. This study concludes that different toxino-types of *C. perfringens* are present with varying prevalence among sheep in Sargodha division of Pakistan. The bacterium was found resistant to bacitracin, ampicillin and erythromycin while, sensitive to penicillin, ciprofloxacin and ceftriaxone.

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INTRODUCTION

Clostridium perfringens (*C. perfringens*) causes disease, generally, named as enterotoxemia in the animals (Hussain *et al.*, 2017). It is a gram negative bacterium, anaerobic in nature and normally present in the intestinal contents of animals and humans but, whenever, finds suitable environment becomes highly pathogenic and produces large amount of lethal toxins (Li *et al.*, 2013; Nashwa *et al.*, 2016). *C. perfringens* have five major strains (toxino-types) A-E and this classification of bacterial strains depends upon the ability of the bacteria to produce different toxins. All the *C. perfringens* toxino-types produce alpha (α) toxin, so this toxin gene identification is confirmatory for the presence of the *C. perfringens* in the samples. *C. perfringens* type 'A' produces only α toxin, while type 'B' produces three toxins, α, β, and ε. *C. perfringens* type 'C' produces 'α' and 'β' toxins, while, type 'D' produces 'α' and 'ε' toxins. *C. perfringens* type 'E' is a rare form of bacteria which only produces iota toxin along with alpha toxin (Songer, 1996; Petit *et al.*, 1999). Toxino-typing of *C. perfringens* is important as different strains of bacteria pose different pathological conditions to the animals. *C. perfringens* type 'A' involves in the per-acute form of enterotoxemia in goat kids (Tammy, 2004). Yellow lamb disease in sheep is caused by *C. perfringens* type 'A'. It is also involved in the food poisoning and gas gangrene in humans. *C.*

perfringens type 'B' has been involved in the lamb dysentery and hemorrhagic enteritis. Pulpy kidney disease in animals is caused by *C. perfringens* type 'D'. *C. perfringens* type 'C' is involved with the disease named as Struck in the sheep. Diptheric colitis in goat kids is a condition caused by epsilon toxin of *C. perfringens* (Songer, 1996; Petit *et al*., 1999; Uzal and Songer, 2008). Epsilon toxin of *C. perfringens* is ranked third most potent toxin produced by clostridia species (Gil, 1982; Harkness *et al.*, 2012). Enterotoxemia outbreaks are observed in the study area in spite of vaccine program. So, it was need of the time to evaluate the *C. perfringens* types in the animals. *C. perfringens* toxino-typing can be done either by detection of toxin in the intestinal contents or detection of toxin gene in the bacterial genome. Detection of toxin traditionally has been done through toxin neutralization test in mice that is difficult, time consuming and having ethical issues. PCR base detection of toxin gene in the bacteria is more accurate and time efficient method for the typing of *C. perfringens*. Toxin genes are located either on chromosome (*cpa*) or they are plasmid base (*cpb, cpb2, etx, iap*) (Songer, 1996).

There is irrational practice of antibiotics usage in Pakistan. That is making bacteria resistant against commonly used antibiotics. There are three basics mechanisms bacteria adopt for getting resistance against antibiotics, which are (1) alteration in the cell membrane permeability (2) antibiotics enzymatic degradation (3) bacterial protein modification, which are targets for the antibiotics. Anaerobe has also shown resistance globally against universally active antibiotics (Hedberg and Nord, 2003; Loivukene and Naaber, 2003). Antibiotic sensitivity against 83.7% isolates of *C. perfringens* has shown resistance against three or more antibiotics (Yadav *et al.*, 2017).

Keeping in view the importance of this disease and antibiotic resistance problem, the project had been designed to determine the toxin types of the *C. perfringens* prevailing in the study area and resistance profile of bacteria against commonly used antibiotics in the field in sheep.

MATERIALS AND METHODS

Study design and sampling: The study area was Sargodha division of Punjab province, Pakistan (Fig. 1). A total of n=192 fecal samples from diarrheic sheep were collected irrespective of age, sex, and breed from 2016 to 2017. All the four districts of Sargodha division (Bhakkar, Mianwali, Khushab, Sargodha) were targeted for sampling (n=48 per district). The samples were collected directly from rectum of study animals into a sterile container and transported to the Microbiology laboratory, University of Veterinary and Animals Sciences, Lahore maintaining the cold chain. The animals having diarrhea with no previous treatment were targeted. A data capturing form was designed for samples data collection.

Sample processing and quantification of bacteria: Immediately after transportation, serial dilution of 1g of fecal samples were prepared in the Phosphate Buffer Saline (PBS 1x) and cultured on the Tryptose Sulphite Cycloserine (TSC) agar media (Himedia Labs, Mumbai, India) plates. TSC is a selective media for the culturing of *C. perfringens.* The media plates were then placed in the anaerobic jar along with AnaeroGen™ Sachets (AN35, Oxoid Hampshire, UK) to produce anaerobic conditions in the jar. Cultural plates were incubated at 37°C for 48 hours in the incubator. After incubation, positive samples were observed for typical black colonies on the TSC media plates and colonies were enumerated using colony counter (Fig. 2). The dilution, which produced colonies in the range of 30-300, was considered for the quantification of *C. perfringens.* The bacterial cultures were visualized on 100X oil immersion lens in the light microscope after Gram's staining of the prepared slides (Fig 2B).

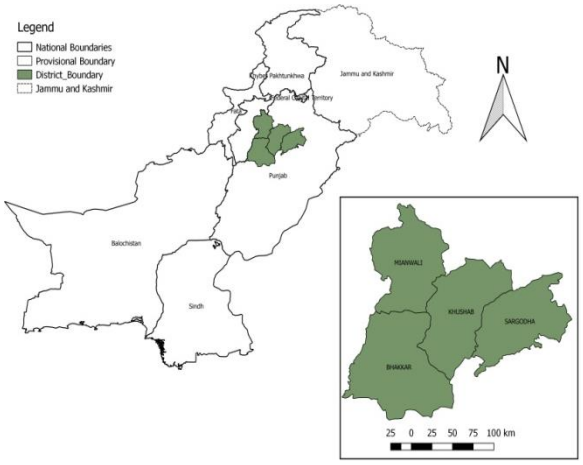

Fig. 1: Map showing the geographical location of the study area.

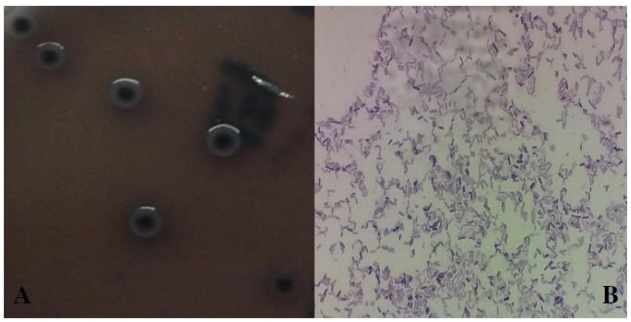

Fig. 2: (A) Typical black colonies of *C. perfringens* on TSC media showing lecithinase activity with egg yolk supplement in the media. (B) Showing microscopic slide of *C. perfringens* 100X oil immersion lens.

PCR for molecular typing of *C. perfringens*: The DNA extraction was performed using bacterial DNA extraction kit TIANGEN® (TIANamp Genomic DNA Kit, Catalogue no. DP302). The bacterial colonies were further sub cultured on the TSC media for purification. After purification, loop full black colonies of *C. perfringens* were picked for the extraction of DNA. The extracted DNA was checked for the purification and quantification using Nano-drop at 260/280nm wavelength. The DNAs were then immediately shifted to freezer for storage at -20ºC till analysis by PCR.

Specific primers used in this study have been shown in Table 1; optimized conditions were used for the PCR of the four toxin genes as reported by Van *et al.* (2009). DNAs from all the samples were individually checked for each of the four toxin genes through PCR. Reaction mixture for PCR was constituted with 12.5 µl master mix

(2X Amp Maste^{TM}Taq, Gene All Biotechnology®, Catalogue No. 541-002), 2 µl DNA sample, 1 µl each of the forward and reverse primer and 8.5 µl double distilled water. Thermocycler programming for PCR was set on 94ºC (10 min) initial denaturation, followed by 40 cycles of 94°C (1 min) denaturation, annealing at 53°C (45 s), and extension at 72°C (1 min). Final extension was done at 72°C (10 min). After removing PCR tubes from thermocycler, PCR products were electrophoresed on 2% agarose gel (Ultrapure^{Tm} agarose, Invitrogen®, Cat. No 16500-500). Ethidium bromide was added in the agarose as a stain before solidification of gel. DNA ladder (100bp molecular weight) was used in the gel along with PCR product for determining the molecular weight of the DNA products (Fig. 3).

Antibiotic sensitivity: *In-vitro* antibiotic sensitivity of *C. perfringens* type 'D' was tested against ten different antibiotics using Kirby-Bauer antibiotic sensitivity test method following CLSI (2012) guidelines. *C. perfringens* type 'D' was selected because epsilon toxin of the type 'D' is most lethal toxin in all *C. perfringens* strains. Antibiotics selected for this study were tetracycline (30µg), metronidazole (5µg), penicillin (10U), ampicillin (10µg), amoxicillin (30µg), erythromycin (15µg), vancomycin (30µg), ciprofloxacin (10U), bacitracin (10µg) and ceftriaxone (30µg).

RESULTS

Identification and quantification of *C. perfringens*: Typical black colonies of *C. perfringens* appeared on TSC media and 92.19% (177/192) of the samples were found positive for the *C. perfringens* (Table 2). *C. perfringens* is normal inhabitant of sheep gastro-intestinal tract (GIT) and bacterial load (10^4-10^7 CFU/g) in feces is considered normal range of bacterial count. Only 37.85% (67/177) of the positive samples had bacterial count higher than normal range. The samples having bacterial count (CFU/g) in the normal ranges were 62.15% (110/177). Alpha (α) toxin is produced by all toxino-types of *C. perfringens*. The gene *cpa* codes for the alpha toxin in the *C. perfringens*. Thus, identification of *cpa* gene through PCR confirms the presence of *C. perfringens*. All the isolates from samples were positive for the *cpa* gene.

Molecular typing of *C. perfringens* toxins: Typing of *C. perfringens* is based upon the presence of major toxins produced by the bacteria which are involved in the disease pathology. These toxins are coded by specific genes and all four major toxin genes are identified in each of the isolates of *C. perfringens* through PCR. *C. perfringens* type 'A' which contains only *cpa* gene (α-toxin) was highly prevalent as compared to other types of *C. perfringens*. Type 'A' was found 60.45% (107/177) of all positive isolates. *C. perfringens* type 'B' which contains all three major toxins (α, β, ε) genes; it was the least dominant strains in the sheep samples and only 05.08% (09/177) samples positive for it. *C. perfringens* type 'C' which contains (α, β) toxin genes was only found in the 13.56% (24/177) of the isolates. *C. perfringens* type 'D' having (α, ε) toxins genes found 20.90% (37/177) of all the *C. perfringens* isolates. Epsilon is considered as the most potent toxin in the *C. perfringens*. None of the isolates was found positive for the iota toxin (ι) gene. So the type 'E' was not present in any of the samples.

Antibiotic sensitivity of *C. perfringens* type 'D': *C. perfringens* type 'D' was subjected to *in-vitro* antibiotics resistance profile. *C. perfringens* type 'D' six positive isolates were selected and antibiotic discs diffusion method was applied by following Kirby Bauer antibiotics sensitivity test method. Ten different antibiotics were tested in this study as shown in Table 3, it was found that penicillin, ciprofloxacin and ceftriaxone were the most sensitive antibiotics showing 100% sensitivity followed by metronidazole, vancomycin, tetracycline and amoxicillin presenting 88.83, 66.67, 50.00 and 33.33% sensitivity respectively while bacitracin, erythromycin and ampicillin were found resistant against *C. perfringens* type 'D'. Hence, it is concluded from the results of the present study that the most effective antibiotics against field isolates of *C. perfringens* type 'D' are penicillin, ciprofloxacin and ceftriaxone with 100% sensitivity.

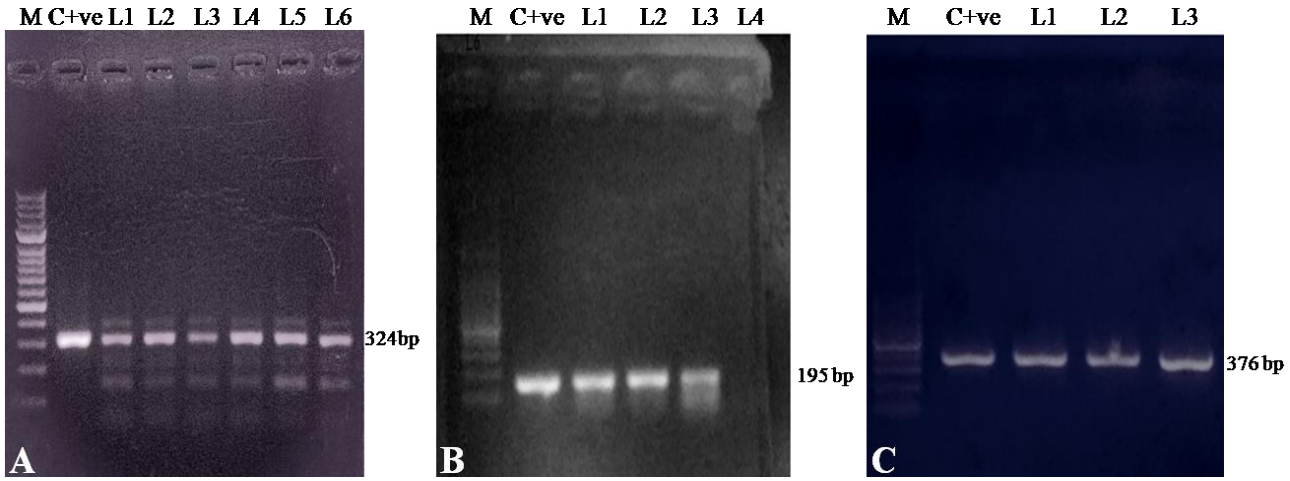

Fig. 3: PCR products of genes on 2% agarose gel stained with ethidium bromide along with 100bp DNA Ladder. M: Marker, C+ve: control positive, **(A)** 324bp L1-L6 *cpa* gene fragments **(B)** 195bp L1-L4 *cpb* gene fragments **(C)** 376 bp L1-L3 *etx* gene fragments.

Table 1: Primer sets used for the toxino-typing of *C. perfringens*

Toxin gene	Primer	Sequence (5′-3′)	Product size
cpa (α-toxin)	CPAlphaF	GCTAATGTTACTGCCGTTGA	324bp
	CPAlphaR	CCTCTGATACATCGTGTAAG	
cpb (β-toxin)	CPBetaF3	GCGAATATGCTGAATCATCTA	195bp
	CPBetaR3	GCAGGAACATTAGTATATCTTC	
etx (ε-toxin)	CPEpsilonF	TGGGAACTTCGATACAAGCA	376bp
	CPEpsilonR2	AACTGCACTATAATTTCCTTTTCC	
iap (ι-toxin)	CPIotaF2	AATGGTCCTTTAAATAATCC	272bp
	CplotaR	TTAGCAAATGCACTCATATT	

Table 2: Distribution of *C. perfringens* different toxin genes in diarrheic fecal samples of sheep

Toxin gene *of* *C. perfringens*	Type of *C. perfringens*	Positive cases n(%)	Toxino-types of *C. perfringens*			
			Bhakkar	Khushab	Sargodha	Mianwali
cpa(α)	A	107 (60.45)	31	12	27	37
cpa(α), *cpb (β)*, *etx*(ε)	B	09(05.08)	00	09	00	00
cpa(α), *cpb (β)*	C	24(13.56)	03	11	08	02
cpa(α), *etx*(ε)	D	37(20.90)	05	14	12	06
cpa(α), iap (ι)	E	00 (00.00)	00	00	00	00

Table 3: Antibiotic sensitivity against *C. perfringens* Type 'D'

Antibiotic used	No. of isolates	Antibiotic susceptibility		
		Resistant (%)	Intermediate (%)	Sensitive (%)
Tetracycline (30μg)	6	0 (0.00)	3 (50.00)	3 (50.00)
Metronidazole(5μg)	6	0 (0.00)	1 (16.67)	5 (83.33)
Penicillin(10U)	6	0 (0.00)	0 (00.00)	6 (100.0)
Ampicillin(10μg)	6	3 (50.0)	3 (50.00)	0 (0.00)
Amoxicillin(30μg)	6	0 (0.00)	4 (66.67)	2 (33.33)
Erythromycin(15μg)	6	0 (0.00)	6 (100.0)	0 (0.00)
Vancomycin(30μg)	6	0 (0.00)	2 (33.33)	4 (66.67)
Ciprofloxacin(10U)	6	0 (0.00)	0 (00.00)	6 (100.0)
Bacitracin(10μg)	6	4 (66.67)	2 (33.33)	0 (0.00)
Ceftriaxone(30μg)	6	0 (0.00)	0 (00.00)	6 (100.0)

DISCUSSION

C. perfringens is considered normal micro flora in the intestinal contents of animals. A range of (10^4-10^7 CFU/g) of fecal material is considered normal bacterial count of *C. perfringens* in sheep. There are some factors (determinants) which render the growth of *C. perfringens* in the GIT of ruminants. *C. perfringens* proliferates rapidly and produces lethal toxins whenever finds favorable environment. In disease conditions, bacterial count in fecal samples elevates than normal range >10^4-10^7 CFU/g (Uzal, 2004). That is why, enterotoxemia is considered as risk factors oriented disease. There are five (A-E) major types (strains) of *C. perfringens* that are also called as toxino-types because their division based upon ability of the strain to produce any of the four (α, β, ε and ι) specific toxins. Detection of these toxin genes through PCR is simple and convenient method for the typing of *C. perfringens.*

In this study (n=192) fecal samples from diarrheic sheep were collected from Sargodha division of Punjab province, Pakistan. Fecal samples were subjected to cultural growth for *C. perfringens* on a selective media (TSC media). Results showed 92.19% (177/192) of the samples were positive for the *C. perfringens.* Out of positive samples, 37.85% (67/177) of the samples had elevated bacterial count than normal range (>10^4-10^7 CFU/g) of fecal material, while, 62.15% (110/177) of the samples had normal bacterial count. As it is normal inhabitant of GIT in animals then simple presence of bacteria in the samples do not have diagnostic value until bacterial count is done. In a previous study (Kumar *et al.*, 2014) prevalence of *C. perfringens* was found 69.29% (97/140) in sheep suspected for the enterotoxemia, while, it was 39.71% (27/68) in the healthy sheep. His study design was different from the current study as the samples were divided in healthy and suspected cases, while, in the current study, sampling criteria was diarrheic animals. Goekce *et al.* (2007) found 84.61% prevalence of *C. perfringens* based on ELISA in his study, while, 58.46% prevalence was found based on the IAT test in sheep. The ELISA based results coincide with the current study in which prevalence was 92.19% based upon PCR. *C. perfringens* presence and bacterial count is highly variable in the intestinal contents of sheep. This is because of many risk factors like; carbohydrate rich diet; overcrowding and seasonal effect on its growth in the intestines. In Iran, prevalence was found very low in the vaccinated sheep (2.2%) while, in the non-vaccinated sheep it was 54.0% (Ahsani *et al.*, 2011).

In the present study, toxino-typing was done through PCR by detection of four toxin genes, *cpa, cpb, etx,* and *iap* coded for (α, β, ε, ι toxins, respectively). Simple conventional PCR or multiplex PCR both can be used for the typing of *C. perfringens* as both give results in agreement (Albini *et al.*, 2008). This study results shows that *C. perfringens* type 'A' is the most prevalent 60.45% (107/177) strain in sheep herds in the Sargodha region of Pakistan. In another study of goats, *C. perfringens* type 'A' was found 78.06% while prevalence of type 'B', 'C' and 'D' were 05.16, 03.23, and 13.55% respectively. None of the isolate was found positive against type 'E' (Hussain *et al.*, 2017). In this study, *C. perfringens* 'B' 'C' and 'D' were found 05.08% 13.56% and 20.90%, respectively. Results of goats study and current study reveals close percentages of *C. perfringens* toxino-types. In the current study none of the isolates was found positive for *C. perfringens* type 'E' which had *iap* gene along with *cpa* gene simultaneously in the genome. In most of the studies, *C. perfringens* type 'A' was found as dominant strain but interestingly in another study, prevalence of *C. perfringens* type 'A' was found 5.13% in the lambs suspected for the disease that is very low as compared to current study. Highest prevalence was found for type 'B' 46.15%. *C. perfringens* type 'C' and 'D' prevalence was also high as compared to current study (Gkiourtzidis *et al.*, 2001).

C. perfringens type 'D' causes enterotoxemia that is specifically named as 'pulpy kidney disease'. Epsilon toxin of type D is considered third most potent toxin in the

clostridial species. Most of the times type 'D' out breaks in animals are most lethal in pathological terms. Thus, *C. perfringens* type 'D' was selected for the antibiotic sensitivity test against ten commonly available antibiotics in the field. There is an irrational use of antibiotics in animals in Pakistan that is affecting sensitivity of antibiotics against bacteria. Anaerobes are also getting resistant against antibiotics globally (Hedberg and Nord, 2003; Loivukene and Naaber, 2003). The resistance profile of the *C. perfringens* was determined and it was found that; penicillin, ciprofloxacin, and ceftriaxone appeared to be 100 % sensitive against *C. perfringens* type 'D' isolates. Bacitracin, ampicillin and erythromycin were found resistant against field isolates of *C. perfringens* type 'D'.

Shanmugasundaram *et al*. (2017) studied the antibiotic sensitivity pattern of *C. perfringens* and found gentamicin 100% sensitive, while, ciprofloxacin and ofloxacin were both 86.67% sensitive. In another study, penicillin was found 100% sensitive against clostridia species (Novak *et al.,* 2015). In Pakistan, Khan *et al.* (2014) studied the susceptibility of *C. perfringens* against antibiotics and results resembled the current study as they declared penicillin, ciprofloxacin and ceftriaxone as sensitive antibiotics while amoxicillin as resistant. Recently, Yadav *et al.* (2017) studied different antibiotics sensitivities against *C. perfringens* and found 83.7% isolates resistant against three or more antibiotics. There is a need to regulate the use of antibiotics in the animals and farmers should be given awareness about the possible issues with misuse of antibiotics and their future losses.

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Authors contribution: KH, MI, SHF, designed and executed the study, KH, SHF, AIA performed sampling, KH, processed the samples, MKI, AA, AG arranged and analyzed the statistical data, KH, SNBR wrote the manuscript. MI reviewed and approved the manuscript for submission.

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