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Research Article

Molecular Typing of *Clostridium perfringens* Toxins (α, β, ε, ι) and Type 'A' Multidrug Resistance Profile in Diarrheic Goats in Pakistan

Kashif HUSSAIN [1] ✎ Muhammad IJAZ [1] Aneela Zameer DURRANI [1]
Aftab Ahmad ANJUM [2] Shahid Hussain FAROOQI [1] Amjad Islam AQIB [1]
Abdullah Saghir AHMAD [3]

[1] Department of Clinical Medicine and Surgery, University of Veterinary and Animal Sciences, Lahore - PAKISTAN
[2] Department of Microbiology, University of Veterinary and Animal Sciences, Lahore - PAKISTAN
[3] Department of Parasitology, University of Veterinary and Animal Sciences, Lahore - PAKISTAN

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Abstract

Clostridium perfringens (*C. perfringens*) causes disease, generally, named as enterotoxemia in the animals. This bacterium is a normal inhabitant in gastro-intestinal tract (GIT) of animals and become harmful by increasing its colony counts as well as toxin liberation whenever gets an opportunity of favorable conditions. This study focused molecular typing of *C. perfringens* (α, β, ε, ι toxins) and type 'A' multidrug resistance profile in diarrheic goats in Pakistan. Diarrheic fecal samples (n=192) were collected from goats and 80.73% (155/192) of the samples were found positive for *C. perfringens* on the basis of culture growth and PCR. Elevated *C. perfringens* counts (>10^7 CFU/g) were recorded in 33.55% (52/155) of positive samples, while, 66.45% (103/155) of the positive sample appeared in normal range of bacterial counts (10^4-10^7 CFU/g). Molecular detection was carried out by targeting specific toxin genes i.e. *cpa* (α), *cpb* (β), *etx* (ε) and *iap* (ι) of *C. perfringens* using PCR. Among the positive samples, 78.06% of the isolates were found as type 'A', 5.16% isolates were type 'B', 3.23% isolates were type 'C' while, 13.55% of the positive samples were type 'D' *C. perfringens*. None of the isolates was found positive for *iap* toxin gene (type 'E'). *C. perfringens* type 'A' was subjected to in-vitro antibiotic sensitivity test. Penicillin, ciprofloxacin and ceftriaxone were found sensitive while bacitracin, amoxicillin and ampicillin were found least sensitive antibiotics. This study concludes that *C. perfringens* type 'A' is highly prevalent among goats in Pakistan and clinical cases of enterotoxemia can be effectively dealt with penicillin, ciprofloxacin and ceftriaxone antibiotics.

Keywords: *Clostridium perfringens, Toxino-typing, Antibiotic sensitivity, Goat*

Pakistan'da İshalli Keçilerde *Clostridium perfringens* Toksinlerinin (α, β, ε, ι) Moleküler Tiplendirilmesi ve Tip 'A' Çoklu İlaç Direnç Profili

Öz

Clostridium perfringens (C. perfringens) hayvanlarda enterotoksemi olarak adlandırılan hastalığına neden olur. Bu bakteri hayvanların gastrointestinal sisteminin normal florasında yer alır ve uygun şartlar oluştuğunda fırsat bularak koloni sayısını artırarak ve aynı zamanda toksinlerini salarak zararlı hale gelir. Bu çalışma Pakistan'da ishalli keçilerde *Clostridium perfringens*'in (α, β, ε, ι) moleküler tiplendirilmesini ve tip 'A' çoklu ilaç direnç profilinin belirlenmesini amaçlamaktadır. İshalli dışkı örnekleri (n=192) keçilerden toplandı ve örneklerin %80.73'ü (155/192) kültürde üreme ve PCR analizleri ile *C. perfringens* pozitif olarak belirlendi. Pozitif örneklerin %33.55'inde (52/155) artmış *C. perfringens* miktarları (>10^7 CFU/g) tespit edildi. Pozitif örneklerin %66.45'inde (103/155) bakteri miktarları normal aralıklarda (10^4-10^7 CFU/g) gözlemlendi. Moleküler tespit amacıyla PCR kullanılarak *C. perfringens*'in *cpa* (α), *cpb* (β), *etx* (ε) ve *iap* (ι) toksin genleri hedef alındı. Pozitif örnekler arasında izolatların %78.06'sı tip 'A', %5.16'sı tip 'B', %3.23'ü tip 'C' ve %13.55'i tip 'D' *C. perfringens* olarak belirlendi. İzolatların hiçbiri iap toksin geni (tip 'E') için pozitif değildi. *C. perfringens* tip 'A'ya *in-vitro* antibiyotik sensitivite testi uygulandı. Bakterilerin penisilin, siprofloksasin ve seftriaksona karşı duyarlı oldukları, basitrasin, amoksisilin ve ampilisine karşı ise en az duyarlı oldukları tespi edildi. Bu çalışma, Pakistan'da *C. perfringens* tip 'A'nın keçiler arasında oldukça yaygın olduğunu ve klinik enterotoksemi vakalarında penisilin, siprofloksasin ve seftriaksonun etkili bir şekilde kullanılabileceğini göstermiştir.

Anahtar sözcükler: *Clostridium perfringens, Toksin tiplendirmesi, Antibiyotik sensitivitesi, Keçi*

✎ **İletişim (Correspondence)**
☏ +92 344 7491900
✉ hkashifdvm@gmail.com

INTRODUCTION

Clostridium perfringens (C. perfringens) is a Gram's positive anaerobe bacterium. It is normally present in animals and humans intestinal contents but sometimes causes infection and proves highly pathogenic regarding intestinal diseases [1,2]. Different toxino-types of *C. perfringens* cause different intestinal pathologic conditions in animals that's why typing of this bacteria have gained importance. *C. perfringens* has 5 major types (A-E) based on the types of toxins it produces. Alpha (α) toxin is produced by all toxino-types of *C. perfringens*; beta (β) toxin is produce by 'B' and 'C' types, epsilon (ε) toxin is produced by 'B' and 'D' type of *C. perfringens,* while, iota (ι) toxin is produced only by type 'E' along with alpha (α) toxin [3,4]. *C. perfringens* can also produce some minor toxins like β2 and enterotoxins but their presence is not associated with the 'typing' of bacteria as these toxins are not linked permanently with some specific types, all the times [5,6]. There is a new toxin named as 'perfingolysin O' (PFO) also identified in the *C. perfringens* that is considered as having potential of causing disease [7]. Enterotoxemia is the name generally used for the disease, caused by all toxino-types of *C. perfringens*. This disease is sometimes named in accordance with pathological conditions or with involvement of some specific strains (toxino-type) of *C. perfringens*. *C. perfringens* type 'A' causes food poisoning and gas gangrene in humans. In a recent report, *C. perfringens* is found with the necrotic pancreatitis in the human beings [8]. Type 'D' is associated with 'pulpy kidney disease' and dysentery in sheep, while, type 'C' causes necrotic enteritis in animals and a condition named as struck [3,4]. *C. perfringens* type 'A' is also involved in the acute enterotoxemia in the goat kids [9]. Epsilon toxin present in the type B and D of *C. perfringens* is mostly involved in the disease pathogenesis. Epsilon toxin in goats causes enterocolitis, but in sheep it is also seen in systemic effects on the brain and lungs. The difference in the pathogenesis in two species is lies in the fact that epsilon toxin modify water and ion transport in the intestines of sheep and goats with different mechanism [10].

In spite of vaccination, some sporadic outbreaks of entero-toxemia are observed over the study area in recent years that are usually not reported. Hence, it is important to know about the types of *C. perfringens* prevailing in the animals that will give assistance for improving vaccines. *C. perfringens* toxino-typing can be done either by detection of toxins itself or its gene. Traditionally, typing has been done by toxin neutralization test in the mice that is difficult, time consuming and having ethical issues. PCR is reliable and convenient method for the typing of *C. perfringens,* as each of the toxins has a specific toxin gene in the genome. Alpha (α) toxin gene *(cpa)* is located on the bacterial chromosome, while, β *(cpb),* β2 *(cpb2)*, ε *(etx)*, and ι *(iap)* toxin genes are considered to be plasmid based. Enterotoxin *(cpe)* gene can be either chromosomal or plasmid borne [4]. Presence of toxin's gene does not give guarantee for the presence of toxin but in 99% of the cases, genotype matches with the phenotype of bacteria [11].

In the recent years, anaerobe bacteria have shown resistance against antimicrobial, globally, even against universally active antibiotics like carbapenems and imidazole [12,13]. In Pakistan, there is irrational practice regarding use of antibiotics in animal and it is important to know about the current resistance profile of *C. perfringens* against commonly available antibiotics in the field conditions. Keeping in view the importance of this disease, this project was designed with the following objectives; assessment of *C. perfringens* load in the fecal samples of diarrheic goats, toxino-typing of *C. perfringens* local isolates and antimicrobial resistance profile of *C. perfringens*.

MATERIAL and METHODS

Study Design

This study was conducted in Sargodha division of Punjab province in Pakistan. Small goat farms and household animals were targeted and a total of n=192 fecal samples were collected from diarrheic goats irrespective of age, sex and breed. Samples were collected from all 04 districts of Sargodha division and from each district, 48 diarrheic animals were selected.

Sample Culturing and Quantification of Bacteria

Collected samples were transported to the Microbiology laboratory maintaining the cold chain. The samples were cultured anaerobically on Tryptose Sulphite Cycloserine (TSC) media (Himedia Labs, Mumbai, India) using AnaeroGen™ Sachets (AN35, Oxoid®, Hampshire, UK) to produce anaerobic conditions. TSC is a selective media for the growth of *C. perfringens.* Serial dilutions of fecal samples were made by taking 1 g of fecal material in the Phosphate Buffer Saline (PBS) and then it was cultured on TSC media plates. After 48 hours of incubation at 37°C typical black colonies suspected for the *C. perfringens* appeared on the media plates. Colonies were enumerated using colony counter and the dilution which produced 30-300 colonies was considered for the CFU count of *C. perfringens*.

PCR for Toxin Genes

After purification of primary culture by sub-culturing on the TSC media, 4-5 black colonies were picked for the DNA extraction using DNA extraction kit TIANGEN® (TIANamp Genomic DNA Kit, Catalogue no. DP302). The extracted DNA quantity and purification were checked using Nano-drop 260/280nm wavelength and DNA was stored at -20°C. PCR was performed for the amplification of 04 toxin genes of *C. perfringens*, α *(cpa)*, β *(cpb)*, ε *(etx)*, and ι *(iap)* using specific primers [14] *(Table 1)*.

Concentration of each forward and reverse primer was 5 pmoles. PCR reaction mixture was consisting of 12.5 µL of

Table 1. *C. perfringens toxin genes with respective primer sequences*

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	

master mix (2X Ampmaster™Aq, GeneAll®), 2 µL of DNA sample, 1 µL of each primer (5 pmol) and 8.5 µL of distilled water. PCR programming on the thermocycler for all the toxin genes was initial denaturation at 94°C (10 min), then 40 cycles of denaturation at 94°C (1 min), annealing at 53°C (45 s), and extension at 72°C (1 min). Final extension was done at 72°C (10 min). PCR products were electrophoresed on ethidium bromide stained 2% agarose gel (Invitrogen®). A 100bp molecular weight marker was used as a ladder for the determination of the sizes of PCR products *(Fig. 1)*.

Antibiotic Sensitivity

In-vitro antibiotic sensitivity was checked for 06 different *C. perfringens* type 'A' isolates using Kirby-Bauer antibiotic sensitivity test method. Ten antibiotics selected for sensitivity test were tetracycline (30 µg), metronidazole (5 µg), penicillin (10U), ampicillin (10 µg), amoxicillin (30 µg), erythromycin (15 µg), vancomycin (30 µg), ciprofloxacin (10U), bacitracin (10 µg), and ceftriaxone (30 µg).

RESULTS

Isolation and Identification of C. perfringens Toxinotypes

This study revealed 80.73% (155/192) fecal samples positive for *C. perfringens,* appeared as typical black colonies on TSC selective media. The normal ranges (10^4-10^7) CFU/g of bacterial count was recorded in 66.45% (103/155) of positive samples, while, the rest of the positive samples 33.54% (52/155) had shown elevated level of CFU/g (>10^7) for the *C. perfringens.* As the *C. perfringens* is normal inhabitant of intestinal tract of animals, 10^4-10^7 CFU/g is considered normal range of bacterial concentration in fecal samples [12]. PCR results showed that all the *C. perfringens* isolates were positive for alpha (α) toxin gene *(cpa)*. Presence of *cpa* gene in the genome of bacteria is confirmatory for the *C. perfringens* presence. None of the isolates was positive for the iota *(ι)* toxin gene *(iap). C. perfringens* type 'A' which contains only *cpa* gene was dominant strain in all the toxinotype and it was found 78.06% (121/155) of all positive samples. As the type 'A' was the most prevalent toxino-type of *C. perfringens* in the samples, it was selected for the antibiotic sensitivity. *C. perfringens* type 'B' containing *cpa, cpb* and *etx* gene was only 5.16% (8/155) of all positive isolates. Type 'C' *C. perfringens* contributed 3.23% (5/155) having *cpa* and *cpb* genes, while, *C. perfringens* type 'D' was found 13.55% (21/155) with *cpa* and *etx* gene combination. None of the isolates was positive for type 'E' *(iap gene) (Table 2).*

Table 2. *Distribution of C. perfringens different toxin genes in goats diarrheic samples*

Distribution *of C. perfringens* Toxin Gene	Number of Animals	Type of *C. perfringens*
cpa (α)	121	A (78.06%)
cpa (α), *cpb* (β), *etx* (ε)	08	B (05.16%)
cpa (α), *cpb* (β)	05	C (03.23%)
cpa (α), *etx* (ε)	21	D (13.55%)
cpa (α), iap (ι)	00	E (00.00%)
Total samples positive for *C. perfringens*	155	
Total negative	037	
Total animal tested	192	

Table 3. *Antibiotic Sensitivity against Type A, C. perfringens*

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

Antibiotic Sensitivity of C. perfringens Type 'A'

Antibiotic sensitivity test was applied on 06 different isolates of *C. perfringens* type 'A'. Ten antibiotics *(Table 3)* were tested by Kirby Bauer antibiotic sensitivity test method against *C. perfringens* type 'A'. Penicillin, ciprofloxacin and ceftriaxone were the most sensitive antibiotic according to the results based on the zone of inhibitions they produced.

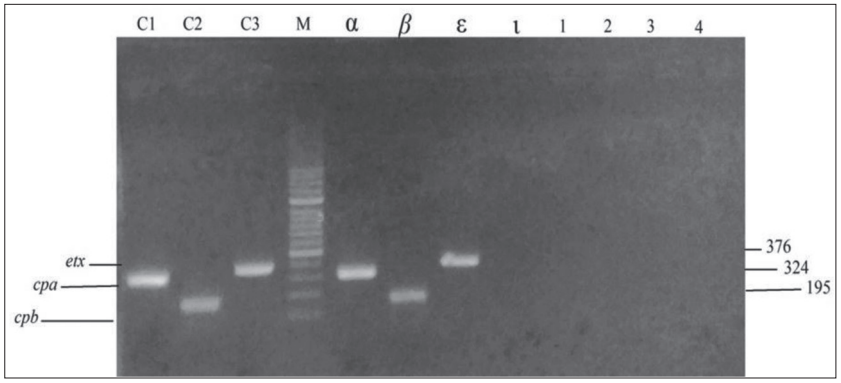

Fig 1. Showing 2% agarose gel PCR products. C1, C2, C3: positive control of *cpa*, *cpb* and *etx* gene of *C. perfringens*. M; (marker 100 bp) DNA Ladder. α, β, and ε are gene products from sample isolated *C. perfringens.1,2,3,4* are the negative control

Ampicillin, amoxicillin and bacitracin were found least sensitive against *C. perfringens* type 'A'.

DISCUSSION

C. perfringens is a normal inhabitant of GIT of animals and 10^4-10^7 CFU/g level in feces is considered as normal range of bacterial count in sheep and goats. In the disease conditions bacterial count elevates $>10^7$ CFU/g [15]. In this study, (n=192) diarrheic goats fecal samples were processed to determine *C. perfringens* bacterial count on TSC selective media. It was found that 80.72% (155/192) of the samples were positive for *C. perfringens.* Out of 155 positive samples, 33.54% (52/155) samples had elevated bacterial count, while, 66.45% (103/155) of the samples had normal range of bacterial load for *C. perfringens*. Kumar *et al.*[16] studied the prevalence of *C. perfringens* in enterotoxemia suspected sheep and found 69.29% of the sheep positive for the *C. perfringens.* In the current study, prevalence was found 80.73% in the diarrheic goats. Vaikosen & Ikhatua [17] found 91 samples positive for the lecithinase enzyme of the *C. perfringens* out of 342 fecal samples of the sheep and goats in Nigeria. Goekce *et al.*[18] declared prevalence of *C. perfringens* 84.61% in a study based on ELISA, while on IAT test bases, it was 58.46% in the sheep. Presence and bacterial count of *C. perfringens* fluctuate in the intestinal contents. Its presence or absence in animal fecal content cannot be declared absolute. In Iran, Ahsania *et al.*[19] found prevalence of *C. perfringens* 2.2% and 54.0% in vaccinated and non-vaccinated local sheep breeds, respectively. A variation pattern in the prevalence of *C. perfringens* is reviewed in different regions of the world. It varies even from herd to herd in the same region. Such variations are obvious from the fact that enterotoxemia is a risk factors oriented disease. Micro and macro environmental changes (determinants) like crowding status, carbohydrate richness in diet, sudden change in diet, deworming status and even season can affect the growth rate of bacteria in the intestine.

Albini *et al.*[20] used simple conventional PCR and Real time multiplex PCR technique for the evaluation of different toxin genes of *C. perfringens* in ten different animal's fecal samples. He found both techniques in agreement for the detection of toxin genes of *C. perfringens.* Aras & Hadimli [21] found 95 samples positive for *C. perfringens* from 300 meat samples of beef, chicken and turkeys. Confirmation was done through detection of alpha toxin gene in all isolates. *C. perfringens* type A, B, C, and D were found 88.33%, 0.0%, 6.4%, and 3.2% in all the meat samples, respectively. In two of the isolates of turkey meat samples type 'E' was also found. These results percentages of *C. perfringens* types come in accordance with the current study in which type 'A' (78.06%), 'B' (5.16%), 'C' (3.23%), 'D' (13.55) and 'E' (0.0%) prevalence was found. Most of the studies showed type 'A' as dominant strain of the *C. perfringens* that was also found in this study. Hashimoto *et al.*[22] studied 804 *C. perfringens* different strains isolated from different sewage water system of humans and animals (chicken, pig, cattle). It was revealed in the study that the *C. perfringens* isolated from human's sewage water had enterotoxin gene, while, animals sewage *C. perfringens* isolates did not have enterotoxin gene. So it was concluded that if the *C. perfringens* enterotoxin gene found positive in aquatic system, it might be considered that it was polluted with human fecal contents. Interestingly, Gkioutzidis *et al*.[23] found 5.13% prevalence of *C. perfringens* type 'A' (*cpa*) in diseased lambs, while, prevalence of type 'B' *(cpa, cpb, etx)*, type 'C' *(cpa, cpb)* and type 'D' *(cpa, etx)* was 46.15%, 20.51%, and 28.20%, respectively (minor toxins detail not present here). In a recent study, the prevalence of *C. perfringens* was studied age wise in the lambs. The prevalence was highest (100%) in the lambs up to 1 month of age, then it decreased to (67%) in the age of two months. Older than this age, the prevalence was found in between 7 to 36%. Even *C. perfringens* was found in one day old lamb fecal sample. Based upon multiplex PCR, *C. perfringens* type A was present in highest ratio. Type C and D were also present but in low prevalence [24].

C. perfringens type A was the predominant strain (78.06%) in all the toxinotypes, so this strain was selected for the *in-vitro* antibiotic sensitivity test. Un-judicial use of antimicrobial on the animals is making the bacteria resistant against commonly available antibiotics. Anaerobes are also getting resistance against antimicrobials. In a hospital study of antimicrobial against clostridial species, penicillin was found 100% sensitive, while, Clindamycin was only

50% sensitive against clostridia isolates [25]. In the current study, penicillin, ciprofloxacin, and ceftriaxone appeared 100% sensitive against *C. perfringens* type 'A' strain, while bacitracin, amoxicillin and ampicillin were the least sensitive antibiotics. It is observed that ampicillin and amoxicillin are commonly being used in the animals in field conditions. Khan *et al*.[26] in Pakistan tested different antibiotics for resistance profile of *C. perfringens* and found amoxicillin resistant against *C. perfringens,* isolated from different meat samples. Bacteria use three fundamental mechanisms for developing resistance against antibiotics. These are (1) enzymatic degradation of antibiotics (2) alteration in the proteins structure which is target for antibiotics (3) changes in the permeability of cell member. These adaptions are directed by specific genes located either on plasmid or chromosomes [27].

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Authors' Contributions

KH, MI, AAA, designed and executed the study, KI, SHF did study sampling, KH, ASA processed the samples, SHF, ASA, AIA arranged and analyzed the statistical data, KH wrote the manuscript. AZD, MI reviewed and approved the manuscript for submission.

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