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RESEARCH ARTICLE

Molecular Characterization and Toxins Optimization of Indigenous *Clostridium perfringens* Toxinotype B Isolated from Lamb Dysentery Clinical Cases

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Abstract: *Clostridium perfringens* toxinotype B is causative agent of lamb dysentery in Pakistan. For assessment of gene expression, it is preferred to optimize toxin production potential of indigenous isolates. Current study aimed to confirm the local isolates of *C. perfringens* toxinotype B on molecular basis followed by optimization of major toxins under varied physical and chemical conditions. The isolates were identified using microbiological and biochemical assays and confirmed by PCR followed by nucleotide sequencing of 16S rRNA amplified gene. These nucleotide sequences were submitted to NCBI GenBank® and accession numbers retrieved were MW867097, MW867098, MW867099, MW867100 and MW867101. Confirmation of toxinotype B was done by PCR amplification of alpha beta and epsilon toxin genes. Major toxins were optimized at varied physical (temperature and pH) and chemical (reinforced clostridial media, Robertson's cooked meat media, egg meat media, fluid thioglycolate media and iron milk media) conditions. Higher hemolytic units of alpha toxin (21.45±0.53 HU/mL), epsilon toxin (16.57±0.19 HU/mL) and higher cytotoxic units of beta toxin (18.65±0.34 HU/mL) were produced at 37°C with pre-adjusted pH 6.0 in Robertson cooked meat media. The ELISA percentages of alpha (14.27%, 13.92%, 13.67%, 13.56%, 13.45%), beta (12.43%, 12.81%, 12.39%, 12.61% and 13.07%) and epsilon (13.93%, 14.78%, 14.28%, 14.03% and 13.25%) toxins were also higher at same conditions. These optimized conditions can be used for major toxin gene expression studies of *C. perfringens* toxinotype B.

Keywords: *Alpha, Beta, Clostridium perfringens toxinotype B, Epsilon, Gene expression, Lamb dysentery, Robertson's cooked meat medium*

Kuzu Dizanteri Klinik Olgularından İzole Edilen Lokal *Clostridium perfringens* Toksinotip B'nin Moleküler Karakterizasyonu ve Toksin Optimizasyonu

Öz: *Clostridium perfringens* toksinotip B, Pakistan'da kuzu dizanterisinin yapıcı etkenidir. Gen ekspresyonunun değerlendirilmesi için lokal izolatların toksin üretim potansiyelinin iyileştirilmesi gerekmektedir. Bu çalışma, *C. perfringens* toksinotip B lokal izolatlarını moleküler düzeyde doğrulamayı ve takiben çeşitli fiziksel ve kimyasal koşullar altında majör toksinlerin üretiminin iyileştirilmesini amaçlamıştır. İzolatlar mikrobiyolojik ve biyokimyasal testler ile tanımlanmış ve PCR ile doğrulandıktan sonra ,16S rRNA amplifiye geninin nükleotid dizilimi yapılmıştır. Bu nükleotid dizileri NCBI GenBank®'a gönderilmiş ve MW867097, MW867098, MW867099, MW867100 ve MW867101 erişim numaraları alınmıştır. Toksinotip B'nin doğrulanması, alfa, beta ve epsilon toksin genlerinin PCR ile amplifikasyonu ile gerçekleştirilmiştir. Başlıca toksinler çeşitli fiziksel (sıcaklık ve pH) ve kimyasal (zenginleştirilmiş klostridiyal besiyeri, Robertson pişmiş et besiyeri, yumurtalı et besiyeri, sıvı tiyoglikolat besiyeri ve demir süt besiyeri) koşullarda optimize edilmiştir. Robertson pişmiş et besiyerinde, pH 6.0'da ve 37°C'de daha konsantre hemolitik alfa toksin (21.45±0.53 HU/mL) ve epsilon toksin (16.57±0.19 HU/mL) ve daha konsantre sitotoksik beta toksin (18.65±0.34 HU/mL) üretilmiştir. Aynı koşullarda alfa (%14,27, %13,92, %13,67, %13,56, %13,45), beta (%12,43, %12,81, %12,39, %12,61 ve %13,07) ve epsilon (%13,93, %14,78, %14,28, %14,03 ve %13,25) toksinlerinin ELISA yüzdeleri daha yüksek saptanmıştır. Optimize edilmiş bu koşullar, *C. perfringens* toksinotip B'nin majör toksin gen ekspresyon çalışmalarında kullanılabilir niteliktedir.

Anahtar sözcükler: *Alfa, Beta, Clostridium perfringens toxinotype B, Epsilon, Gen ekspresyonu, Kuzu dizanterisi, Robertson's pişmiş et besiyeri*

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Introduction

Clostridium perfringens type B is causative agent of lamb dysentery. It usually progresses in early days of life. Neonatal lambs catch infection from dam or its environment, then its number increases in the gut especially with heavy lactation by the dam [1]. The result is enterotoxemia complemented by ulceration of small intestine, extensive hemorrhages and enteritis. In per-acute cases, sudden death is primary sign, without premonition. In acute cases, feeding termination and increased abdominal ache are complemented by dysentery followed by recumbence, unconsciousness and fatality in less than 24 hours. Pine is the chronic form of this disease which occurs in older lambs which demonstrates chronic abdominal cramps without diarrhea [2].

C. perfringens virulence is based upon presence of approximately twenty diverse extracellular enzymes and toxins [3]. The organism has been recently classified into seven toxinotypes or strains viz; A to G due to the presence of iota (ι), epsilon (ε), beta (β) and alpha (α) toxins along with recently included necrotic enteritis B-like (NetB) and enterotoxin (CPE) toxins [4]. Additionally, each strain of *C. perfringens* has the ability of production of minor toxins viz; BEC, NetF, PFO, CPB2 etc. There is no single strain or toxinotype known which can produce all the minor toxins [5].

Alpha (α or CPA) toxin encoding gene *(cpa* or *plc)* is located in the house keeping region of the chromosome which makes it very conserved, stable and produced by *C. perfringens* all toxinotypes [6]. In contrast, the genes which code major toxins resides on large plasmids, hence, in other words, the scheme of *C. perfringens* toxinotyping is based on the presence or absence of plasmids. Actually, among the major toxins, toxinotype B produces ETX, CPB and CPA toxins [7]. Beta toxin is an etiological agent in necrotizing enterocolitis. The reported 50% lethal dose (LD50) of beta toxin is 310 ng/kg [8]. Epsilon (ε-toxin) form pores in infected cells, which results in loss of K^+ and increase in cytoplasmic level of Cl^- and Na^+. Epsilon toxin also causes slightly increase in Ca^+ ions in cytoplasm of infected cells. ETX induced release of K^+, loss of ions activate cell death by necrosis or ATP exhaustion [9].

For gene expression studies, molecularly confirmed *C. perfringens* isolates may be optimized for maximum toxins production under varied physical and chemical parameters. It has been reported that *C. perfringens* has proteolytic as well as saccharolytic activity in thioglycolate medium, reinforced clostridial medium, iron milk medium, egg meat medium and cooked meat medium. In prior studies, differences in the hemolytic activity of toxinotype B and D were reported. There is dire need to quantify the toxin production potential of *C. perfringens* toxinotype B in these culture media. Therefore, current exploration was executed for optimization of chemical and physical conditions to maximize the production of alpha beta and epsilon toxins of *C. perfringens* toxinotype B.

Material and Methods

Ethical Approval

All animal experiments were approved by Institutional Animals Ethical Review Committee of University of Veterinary and Animal Sciences, Lahore, Pakistan and carried out according to the International Ethics law and regulations. All efforts were made to minimize the animal sufferance.

Sample Collection

A total of 35 samples from Lahore and Kasur districts were collected to isolate *C. perfringens* toxinotype B from lambs. Rectal swabs from lambs showing signs of enterotoxaemia were transferred into anaerobic medium by deep insertion and transported to Anaerobiology Section, Quality Operational Laboratory, Institute of Microbiology, University of Veterinary and Animal Sciences, Lahore using cold chain (4°C) for further processing [10].

Isolation of *C. perfringens*

Bacterial enrichment from collected sample was performed as described previously [11]. Peptone water 0.1% (pH 6.8±0.2) supplemented with 0.05% L-cysteine HCl was inoculated with cotton swab fecal samples by cutting the swab stick with a sterile scissor in sterile environment. Bacterial strain isolation and identification were made as described previously but with minor modifications [12]. Briefly, 1:100 dilution of the sample suspension in peptone water (0.1%) was prepared and incubated at 70°C for 30 min. Suspensions were inoculated into reinforced clostridial media (RCM) broth and incubated for 48 h at 37°C in anaerobic atmosphere. Then broth culture (100µL) was spread on perfringens agar supplemented with D-cycloserine and incubated in anaerobic jar at 37°C for 48 h. Colony characteristics were observed carefully and pinpoint black colonies were further subjected to biochemical tests using oxidase, catalase, hemolytic activity on blood agar containing sheep blood, gelatin hydrolysis and lecithinase activity along with fermentation of glucose, maltose, mannitol, salicin, dulcitol, inositol and lactose for identification of *C. perfringens* [13].

Molecular Confirmation of *C. perfringens*

DNA of biochemically confirmed isolates was extracted by using a commercial kit (Exgene™ GeneAll). Extracted DNA was subjected to amplification of 16S rRNA gene for molecular confirmation of *C. perfringens* [14]. Briefly,

Polymerase chain reaction (PCR) reaction mixture of 25 µL was prepared by mixing 12.5 µL (2X) PCR master mix, 1µL of each forward and reverse primers (10 pmol) *(Table 1)*, 2 µL of extracted DNA and 8.5 µL nuclease free water. Amplification of 16S rRNA gene was carried out at 94°C for 10 min (initial denaturation) followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min and final extension at 72°C for 10 min. Gel electrophoresis was performed using 1.8% agarose with ethidium bromide 0.5 µg/mL. Amplified products of 16S rRNA gene were subjected to nucleotide sequencing and FASTA files were retrieved [15]. The nucleotide sequences were submitted to national center for biotechnology information (NCBI) through GenBank® after screening for chimeras by using JUSTbio and accession numbers were retrieved. Phylogenetic analysis of these sequences were performed using molecular evolutionary genetics analysis (MEGA) [16].

Confirmation of *C. perfringens* Toxinotype B

The extracted genome of biochemically confirmed isolates was also processed for amplification of alpha, beta, epsilon and iota toxin genes of *C. perfringens* [17]. Reaction mixtures were prepared as described above. The amplification was carried out at 94°C for 10 min (initial denaturation) followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 45 sec, extension at 72°C for 30 sec and final extension at 72°C for 10 min. Gel electrophoresis was performed using 1.8% agarose with ethidium bromide 0.5µg/mL.

Optimization of Toxin Production ability under Physico-Chemical Conditions

For optimization of toxin production, bacterial inoculum was prepared in normal saline supplemented with L-cysteine HCl (0.05%) as reducing agent following the methods described previously [18]. Fresh growth from perfringens agar was used for this purpose.

To analyze the impact of physical and chemical changes on *C. perfringens* type B major toxins, confirmed isolates (n=5) were cultured on reinforced clostridial (RC) media and incubated at 32°C, 35°C, 37°C, 39°C and 42°C to know the optimum temperature for maximum toxin production. Likewise, the same isolates (n=5) were cultured on reinforced clostridial (RC) media with pre-adjusted pH at 6.0, 7.0, 8.0 respectively to know the optimum pH for maximum toxin production. Later on, same isolates (n=5) were cultured on Robertson's cooked meat, egg meat, fluid thioglycolate and iron milk media and incubated at pre-optimized temperature and pH [19].

Quantification of Alpha Beta and Epsilon Toxins

Cell-free supernatant from broth culture was obtained by centrifugation at 8000 rpm for 5 min. Supernatant were poured into small aliquots of 1.5 mL and stored at 4°C for future use. For detection of alpha, beta and epsilon toxins, Bio-X Diagnostics MULTISCREEN Ag-ELISA kit was used and sandwich ELISA assay was performed for *C. perfringens* type B isolates as per manufacturer's recommendations [20]. Optical density of Ag-ELISA plate was recorded at 450nm wavelength. Percentage absorbance of the corresponding toxin was calculated as:

$$Prcentage\ Absorbannce = \frac{Optical\ density\ of\ the\ test\ sample}{Positive\ control\ value\ of\ the\ respective\ toxin} \times 100$$

For quantification of alpha toxin cell-free supernatant was used untreated but for epsilon toxin, cell-free supernatant was activated with 1% trypsin solution and mixed in a 9:1 ratio and incubated at 37°C for 30 min. Hemolytic activity of alpha and epsilon toxins were assessed by micro titer plate assay by the methods described previously [21,22]. Toxins in supernatant was two folds diluted (v/v) serially in phosphate buffer saline using 96 well flat bottom microtiteration plates. Washed sheep red blood cells (1%) suspension in sterile phosphate buffer saline was prepared. In each well, 100 µL of 1% suspension of sheep red blood cells were added. These micro titer plates were incubated

Table 1. *Primers used for molecular confirmation of C. perfringens toxinotype B*

Genes	Primers	Primer Sequences	Product Size
16S rRNA	8FLP(F)	5`AGTTTGATCCTGGCTCAG-3`	1500 bp
	XB4(R)	5`-GTGTGTACAAGGCCCGGGAAC-3`	
Alpha *(cpa)*	CPAlphaF	5'-GCTAATGTTACTGCCGTTGA-3'	324 bp
	CPAlphaR	5'-CCTCTGATACATCGTGTAAG-3'	
Beta *(cpb)*	CPBetaF3	5'-GCGAATATGCTGAATCATCTA-3'	195 bp
	CPBetaR3	5'-GCAGGAACATTAGTATATCTTC-3'	
Epsilon *(etx)*	CPEpsilonF	5'-TGGGAACTTCGATACAAGCA-3'	376 bp
	CPEpsilonR2	5'-AACTGCACTATAATTTCCTTTTCC-3'	
Iota *(iap)*	CPIotaF2	5'-AATGGTCCTTTAAATAATCC-3'	272 bp
	CPIotaR	5'-TTAGCAAATGCACTCATATT-3'	

with shaking for 60 min at 37°C. Optical densities were measured at 595 nm by spectrophotometer and 50% hemolytic units (HU/mL) were documented.

The cytotoxicity of baby Hamster kidney 21 (BHK 21) cell line was used for quantification of beta toxin. Sterile Glasgow minimal essential medium (GMEM) containing 8-10% fetal calf serum in 96 well flat bottom microtiteration plates were used to culture BHK 21 cells. Each well of microtiteration plate was inoculated with $1x10^5$ cells. After 24 h of incubation, two fold serially diluted beta toxin was added in the wells containing cell growth and incubated at 37°C with supply of 5% CO_2. Subsequently, the cells were washed with sterile PBS and stained as described previously but with minor modifications [23]. Optical densities were recorded by ELISA plate reader at 570 nm. Cell survival percentage was valued by the formula given below.

$$\text{Cell Survival percentage} = \frac{\text{Optical Density of Test} - \text{Optical density of Negative Control}}{\text{Optical density of Possitive Control} - \text{Optical density of Negative Control}} \times 100$$

Statistical Analysis

All the experimental data was analyzed by using SPSS software and represented as mean±SE. Statistical significance was determined using the analysis of repeated measurements variance followed by post hoc, Duncan's multiple range (DMR) test.

Results

Biochemical Characterization of *C. perfringens*

Out of 35 samples collected from clinical cases of lamb dysentery, 11 were positive for anaerobic growth as cultured on reinforced clostridial media and perfringens agar. The isolates (n=10) were characterized as *C. perfringens* based on colonial morphology, microscopic appearance and biochemical profile. All isolates (n=10) were negative for catalase and oxidase test whereas six (6) isolates showed double hemolysis, two (2) showed beta hemolysis and two (2) depicted partial hemolysis. Moreover, all isolates (n=10) were positive for lecithinase and gelatin hydrolysis test. Furthermore, isolates (n=10) fermented sugars viz., fructose, mannitol, maltose, lactose, sucrose and glucose with gas production.

Molecular Confirmation

Biochemically characterized isolates were further confirmed through 16S rRNA gene amplification by polymerase chain reaction (PCR) and amplicons of 1500 bp were subjected to nucleotide sequencing. The FASTA files received were analyzed through BLAST (mega blast) for similarity. BLAST represented that these isolates were 100% identical to aligned sequences (data base) with 95 to 97% query coverage and E value was <0.0. Out of 10 sequenced isolates, 09 isolates were considered as *C. perfringens* on the basis of 16S rRNA gene. The accession numbers of nucleotide sequences of *C. perfringens* isolates (n=5) received were MW867097, MW867098, MW867099, MW867100 and MW867101. Confirmed isolates were targeted for toxinotype identification by amplification of alpha, beta and epsilon toxin genes using toxin specific primers by conventional PCR and amplified DNA bands were visualized on agarose gel (1.8%) under UV. All isolates revealed alpha toxin gene nucleic acid band of 324 bp whereas, seven (7) isolates revealed beta toxin gene amplicon of 197 bp and nine (9) isolates were positive for epsilon gene (376 bp) *(Fig. 1)*. On the basis of molecular characterization, seven (7) isolates were confirmed as toxinotype B and two (2) isolates were confirmed as toxinotype D.

Phylogenetic Analysis of *C. perfringens* Based on 16S rRNA Gene Sequences

Phylogenetic analysis of submitted 16S rRNA gene sequences was carried out using MEGA. Dendrogram for *C. perfringens* was completed using 16S rRNA sequences, bootstrap as phylogeny method and 2000 bootstrap replication in neighbor joining algorithm. In dendrogram, current *C. perfringens* sequences were represented with accession numbers in stained boxes. MW867101.1, sequence 46% evolutionary related to the MW867097.1 which is 35% evolutionary related to MW867098.1 and FJ978611.1 kept as out group in evolutionary tree. MW867099.1 displayed 41% evolutionary relation to MW8697100.1. Phylogenetic tree represented that MW332247.1 distantly related to MT463463.1 and MT464449.1 *(Fig. 2)*.

Optimization of Toxin Production by *C. perfringens* Toxinotype B

A higher hemolytic units (11.03±0.16 HU/mL) of alpha toxin of *C. perfringens* toxinotype B were produced at 37°C incubation temperature. The alpha toxin of *C. perfringens* type B isolates produced higher hemolytic units (11.03±0.16 HU/mL) at 37°C temperature of incubation. Similarly, ELISA percentages for alpha toxin were higher (8.92, 9.65, 9.42, 9.34 and 9.19%) at 37°C temperature. Whereas, least hemolytic units of alpha toxin (02.72±0.53 HU/mL) and least ELISA percentages for alpha toxin (1.50,1.25, 1.21, 1.71 and 0.98%) were produced at 42°C incubation temperature.

Higher hemolytic units (16.72±0.15 HU/mL) of alpha toxin of *C. perfringens* toxinotype B were produced at 37°C on pre-adjusted pH 6.0 and ELISA percentages for alpha toxin were also higher (10.32, 10.29, 10.08, 10.65 and 10.23%) on the same temperature and pre-adjusted pH. Meanwhile, least hemolytic units of alpha toxin (09.39±0.31 HU/mL)

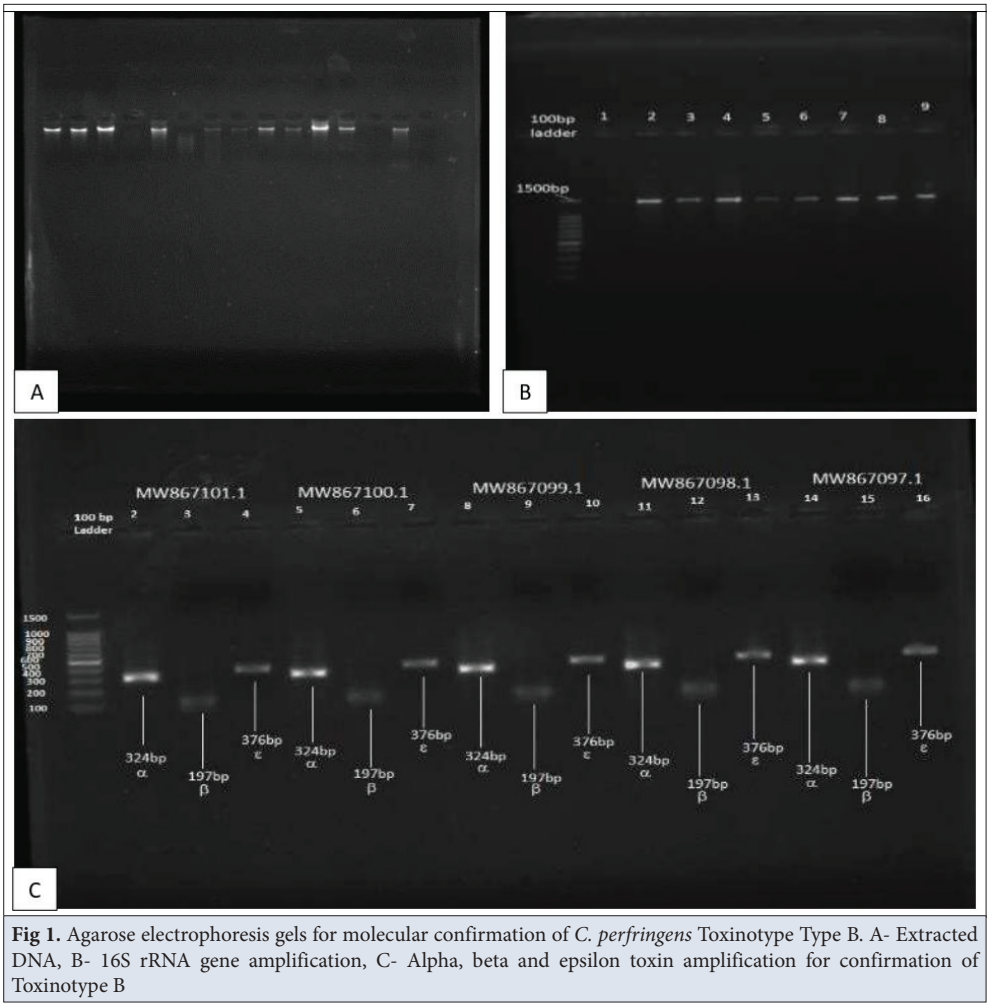

Fig 1. Agarose electrophoresis gels for molecular confirmation of *C. perfringens* Toxinotype Type B. A- Extracted DNA, B- 16S rRNA gene amplification, C- Alpha, beta and epsilon toxin amplification for confirmation of Toxinotype B

and ELISA percentages (5.63, 5.21, 5.72, 5.93 and 5.72%) were produced at pre- adjusted pH 8.0. Higher hemolytic units (21.45±0.53 HU/mL) of alpha toxin of *C. perfringens* toxinotype B were produced in Robertson cooked meat medium (RCMM) at 37°C temperature of incubation with pre-adjusted pH 6.0. Likewise, ELISA percentages for alpha toxin were also higher (14.27, 13.92, 13.67, 13.56 and 13.45%) in Robertson cooked meat medium at 37°C temperature of incubation and pre-adjusted pH 6.0. On the other side, least hemolytic units (06.21±0.36HU/mL) and ELISA percentages (3.45, 3.29, 3.75, 3.98 and 3.69%) of alpha toxin were recorded in egg meat medium. Significant differences were observed among the alpha toxin hemolytic units produced by *C. perfringens* toxinotype B under the influence of varied temperature, pH and culture media *(Table 2)*.

The higher cytotoxic units (9.32±0.19CU/mL) of beta toxin of *C. perfringens* toxinotype B were produced at 37°C temperature of incubation and the ELISA percentages for beta toxin were also higher (6.38, 6.21, 6.87, 5.98 and 6.47%) at 37°C incubation temperature. Whereas, least cytotoxic units (01.73±0.19 CU/mL) and ELISA percentages (0.79, 0.86, 0.94, 0.91 and 0.65%) of beta toxin were observed at 32°C temperature for incubation. The beta toxin of *C. perfringens* toxinotype B isolates produced higher cytotoxic units (14.63±0.28 CU/mL) at 37°C on pre-adjusted pH at 6.0. Similarly, ELISA percentages for beta toxin were also higher (8.84, 8.98, 9.13, 9.29 and 8.79%) on the same temperature and pre-adjusted pH. While, least cytotoxic units (05.84±0.28 CU/mL) and ELISA percentages (4.23, 4.15, 3.98, 3.95 and 4.07%) of beta toxin were observed at pre-adjusted pH 8.0. The cytotoxic units of beta toxin produced by *C. perfringens* type B were higher (18.65±0.34 CU/mL) in Roberson cooked meat medium upon 37°C incubation temperature with pre- adjusted pH 6.0. It was also observed that ELISA percentages for beta toxin were higher (12.43, 12.81, 12.39, 12.61 and 13.07%) at 37°C incubation temperature with pre-adjusted pH 6.0 in Roberson cooked meat medium. It was also reported that least cytotoxic unit (04.76±0.29 CU/mL) and ELISA percentages (4.72, 4.34, 4.23, 4.07 and 4.83%) were verified in iron milk medium. Significant differences were observed among the cytotoxic units of beta toxin produced by *C. perfringens* type B under the

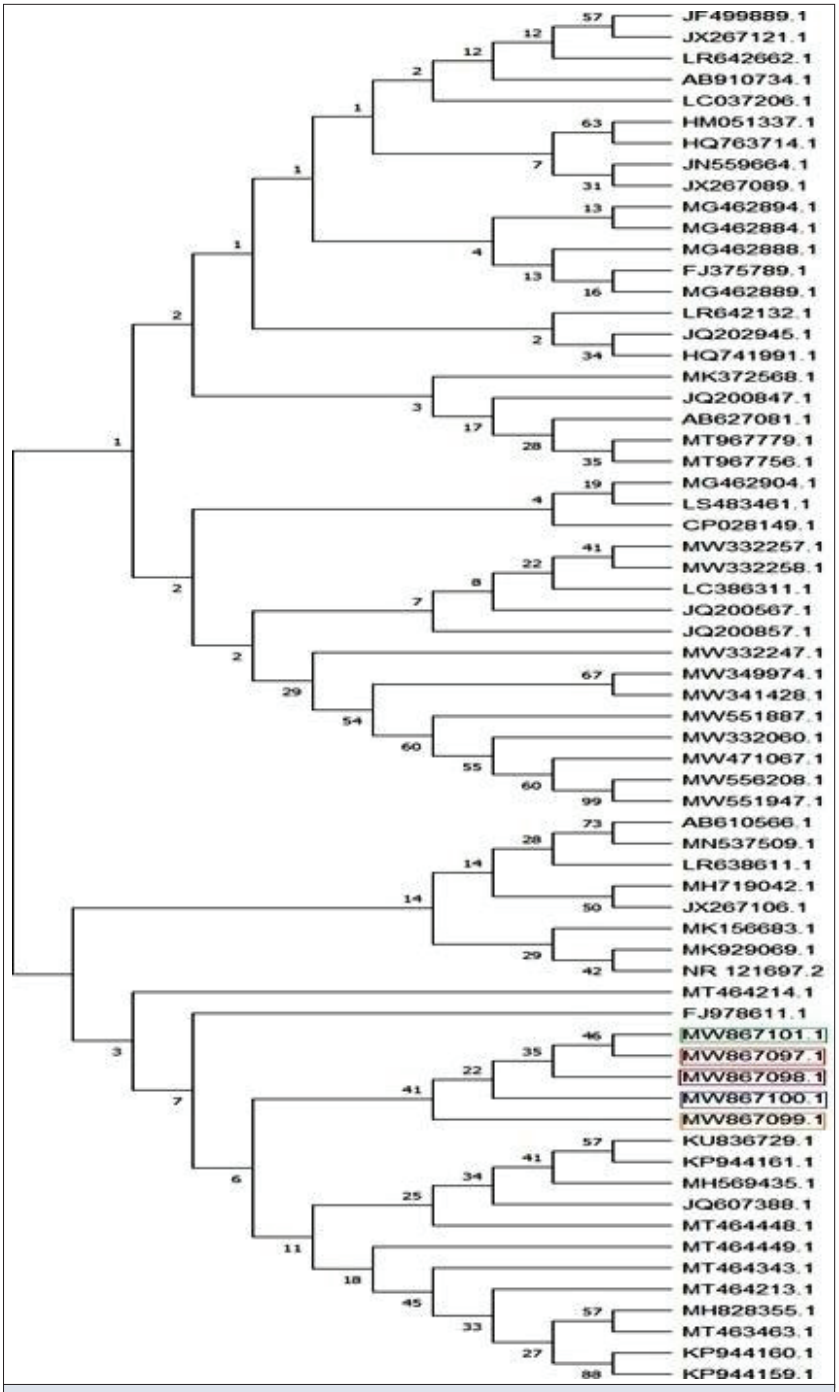

Fig 2. Phylogenetic analysis of *C. perfringens* on the basis of 16S rRNA gene

influence of varied temperature, pH and culture media *(Table 3)*.

The epsilon toxin of *C. perfringens* type B isolates produced higher hemolytic units (08.52±0.29 HU/mL) when incubated at 37°C temperature. Similarly, the ELISA percentages of epsilon toxin were also higher (7.14, 7.02, 6.94, 6.49 and 6.87%) at 37°C of incubation temperature. The least hemolytic units (01.36±0.08 HU/mL) and ELISA percentages (0.89, 0.97, 1.20, 1.18 and 1.31%) of epsilon toxin were recorded at 32°C temperature. The epsilon toxin of *C. perfringens* type B isolates produced higher hemolytic units (11.96±0.45 HU/mL) at 37°C on pre-adjusted pH at 6.0. Likewise, ELISA percentages for epsilon toxin were also higher (9.49, 9.32, 9.73, 9.56 and 9.18%) on the same temperature and pre adjusted pH. The least reported hemolytic units (06.97±0.31 HU/mL) and ELISA percentages (3.94, 3.82, 3.91, 3.71 and 3.62%) of epsilon toxin were observed at pre- adjusted pH 8.0. The hemolytic units of epsilon toxin produced

Table 2. Optimization of alpha toxin of C. perfringens type B under different physico-chemical conditions (hemolytic units and ELISA)

Parameters	Hemolytic Units (HU/mL)	ELISA (%)				
	(Mean±SE)	MW867097.1	MW867098.1	MW867099.1	MW867100.1	MW867101.1
Temperature						
32°C	02.96±0.27^{c}	2.12	2.65	2.42	2.84	2.34
35°C	05.97±0.37^{b}	3.24	3.13	3.71	3.19	3.29
37°C	11.03±0.16^{a}	8.92	9.65	9.42	9.34	9.19
39°C	06.75±0.48^{b}	4.34	4.18	4.63	4.21	4.71
42°C	02.72±0.53^{c}	1.50	1.25	1.21	1.71	0.98
pH						
6.0	16.72±0.15^{a}	10.32	10.29	10.08	10.65	10.23
7.0	11.03±0.16^{b}	8.92	9.65	9.42	9.34	9.19
8.0	09.39±0.31^{b}	5.63	5.21	5.72	5.93	5.72
Culture Media						
Reinforced Clostridial Medium	16.72±0.15^{b}	10.32	10.29	10.08	10.65	10.23
Egg Meat Medium	06.21±0.36^{c}	3.45	3.29	3.75	3.98	3.69
Iron Milk Medium	07.89±0.32^{c}	4.41	4.67	4.83	4.29	4.81
Robertson Cooked Meat Medium	21.45±0.53^{a}	14.27	13.92	13.67	13.56	13.45
Fluid Thioglycolate Medium	14.34±0.27^{b}	9.23	9.24	9.52	9.17	9.06

^{a,b,c} *Values with in column with different superscript differ significantly at P<0.05*

Table 3. Optimization of beta toxin of C. perfringens type B under different physico-chemical conditions (cytotoxic units and ELISA)

Parameters	Cytotoxic Units (CU/mL)	ELISA (%)				
	(Mean±SE)	MW867097.1	MW867098.1	MW867099.1	MW867100.1	MW867101.1
Temperature						
32°C	01.73±0.19^{c}	0.79	0.86	0.94	0.91	0.65
35°C	04.74±0.57^{b}	1.71	1.65	1.81	1.39	1.56
37°C	09.32±0.19^{a}	6.38	6.21	6.87	5.98	6.47
39°C	04.03±0.37^{b}	2.10	2.03	1.91	1.87	1.90
42°C	01.92±0.32^{c}	1.30	0.98	1.18	1.03	0.87
pH						
6.0	14.63±0.28^{a}	8.84	8.98	9.13	9.29	8.79
7.0	09.32±0.19^{b}	6.38	6.21	6.87	5.98	6.47
8.0	05.84±0.28^{c}	4.23	4.15	3.98	3.95	4.07
Culture Media						
Reinforced Clostridial Medium	14.63±0.28^{b}	8.84	8.98	9.13	9.29	8.79
Egg Meat Medium	05.48±0.76^{c}	5.68	5.74	5.86	5.43	5.21
Iron Milk Medium	04.76±0.29^{c}	4.72	4.34	4.23	4.07	4.83
Robertson Cooked Meat Medium	18.65±0.34^{a}	12.43	12.81	12.39	12.61	13.07
Fluid Thioglycolate Medium	12.49±0.71^{b}	10.30	09.89	10.28	10.78	10.24

^{a,b,c} *Values with in column with different superscript differ significantly at P<0.05*

Table 4. *Optimization of epsilon toxin of C. perfringens type B under different physico-chemical conditions (hemolytic units and ELISA)*

Parameters	Hemolytic Units (HU/mL)	ELISA (%)				
	(Mean±SE)	MW867097.1	MW867098.1	MW867099.1	MW867100.1	MW867101.1
Temperature						
32°C	01.36±0.08[c]	0.89	0.97	1.20	1.18	1.31
35°C	03.54±0.28[b]	2.87	3.03	3.24	3.23	2.98
37°C	08.52±0.29[a]	7.14	7.02	6.94	6.49	6.87
39°C	03.82±0.17[b]	3.82	3.67	3.73	3.85	3.29
42°C	01.68±0.09[c]	1.48	1.67	1.73	1.82	1.38
pH						
6.0	11.96±0.45[a]	9.49	9.32	9.73	9.56	9.18
7.0	08.05±0.29[b]	7.14	7.02	6.94	6.49	6.87
8.0	06.97±0.31[b]	3.94	3.82	3.91	3.71	3.62
Culture Media						
Reinforced Clostridial Medium	11.96±0.45[b]	9.49	9.32	9.73	9.56	9.18
Egg Meat Medium	03.17±0.29[c]	3.94	4.63	4.25	4.16	4.72
Iron Milk Medium	04.01±0.18[c]	6.54	5.96	6.07	5.84	6.23
Robertson Cooked Meat Medium	16.57±0.19[a]	13.93	14.78	14.28	14.03	13.25
Fluid Thioglycolate Medium	09.62±0.29[b]	9.78	9.76	9.45	9.30	9.89

[a,b,c] *Values with in column with different superscript differ significantly at P<0.05*

by *C. perfringens* type B were higher (16.57±0.19HU/mL) in Robertson's cooked meat medium upon 37°C incubation temperature with pre- adjusted pH 6.0. Additionally, ELISA percentages for epsilon toxin were higher (13.93, 14.78, 14.28, 14.03 and 13.25%) at 37°C incubation temperature with pre- adjusted pH 6.0 in the Robertson cooked meat medium. Furthermore, least hemolytic units of epsilon toxin (03.17±0.29HU/mL) and ELISA percentages (3.94, 4.63, 4.25, 4.16 and 4.72%) were recorded in egg meat medium. Significant differences were observed among the hemolytic units of epsilon toxin produced by *C. perfringens* type B under the influence of varied temperature, pH and culture media *(Table 4)*.

Discussion

C. perfringens is a rod shaped, gram positive, spore forming, non-motile, anaerobic pathogenic bacteria of domestic animals and human [24]. *C. perfringens* divided in to five types A, B, C, D and E on the basis of four types of major toxins produced by these types [24,25]. In many earlier studies, a range of specified media (Motility nitrate medium, reinforced clostridial medium, modified ducan strong medium, lactose gelatin medium and tryptose sulphite cycloserine medium) provided higher isolation rates. In this study we used two types of media, reinforced clostridial medium and tryptose sulphite cycloserine medium to get wide range of results. All of the positive samples gave straw color colonies on TSC agar plates as per previous report [26]. Typical black color colonies were sign of specificity for *C. perfringens* positive isolates on the surface of TSC agar plates [27].

To isolate *C. perfringens* form lambs, fecal samples / rectal swabs (n=35) were collected from Lahore and Kasur districts of Punjab province and processed based on microscopic studies, biochemical profile and molecular characteristics. On the basis of biochemical profile, 10 (28.57%), samples were characterized as having *C. perfringens*. In a study of the prevalence of *C. perfringens*, out of 177 samples (25 from goats and 152 from sheep) collected from diarrheic animals were screened for *C. perfringens* toxinotypes. Out of these 177 samples, 125 (70.62%) were found positive for *C. perfringens*, of which 15 (60%) were from goats and 110 (72.36%) were from sheep [28].

In current study, on the basis of 16S rRNA gene amplification it was revealed that 9/35 were confirmed as *C. perfringens*. Similarly it was reported that PCR for 16S rRNA gene is reliable method for detection of *C. perfringens* from a group of individual showing similar type of biochemical profile [29]. In present study, the toxinotypes confirmed as type B were 7/35 (20%) while type D were 2/35 (5.71%). Whereas, a team of researchers conducted a study in Nigeria on 245 samples, which were tested by toxin-antitoxin neutralizing tests, of which 127

were type A, 17 were type B, 14 were type C, 44 were type D, 19 were type E. According to that study 90.20% isolates were *C. perfringens* [30].

Out of 41 isolates which were positive for *C. perfringens* on biochemical basis, it was observed that 38 isolates were positive after PCR amplification of 16S rRNA gene and sequencing as *C. perfringens*. It was revealed that 16S rRNA followed by gene sequencing can be opted as alternative tool for definitive confirmation of *C. perfringens* [31]. Earlier it was elaborated that diverse clostridial species were identified with the help 16S rRNA gene amplification and sequencing [32]. In 1994, Wang and coworkers identified *C. perfringens* on the basis of 16S rRNA gene amplification by using species specific primers [33]. DNA-based techniques (PCR and hybridization) have been developed for *C. perfringens* typing and are a reliable alternative method to testing in laboratory animals [6]. However, there is variability to in-vitro production of toxins of *C. perfringens*, so it is problem in using immunological tests. Molecular methods (genotyping), which are mainly based on polymerase chain reaction (PCR) have become the standard for toxin typing of *C. perfringens* [34]. In current study, out of 38 isolates 16 were positive for *cpa* toxin gene while 14 isolates contained both *cpa* and *etx* toxin genes and 8 isolates were positive for *cpa*, *cpb* and *etx* toxin genes.

C. perfringens type B bacteria produced higher hemolytic units (21.45±0.53) and ELISA % (14.27, 13.92, 13.67, 13.56 and 13.45) of alpha toxin when cultured in Robertson Cooked Meat Medium (RCMM) under anaerobic conditions at 37°C with pre adjusted pH 6.0. It was reported that after inoculation active growth started within ~3-4 hours and gas production in broth cultures was also observed as bubbling. In fermenter and stationary culture, *C. perfringens* active growth started within 2 and 3 h [35]. In broth culture, there is decreased production of alpha toxin units in present study. Previously, *C. perfringens* type B hemolytic titer were observed in culture supernatant of reinforced clostridial (RC), thioglycolate (TG),egg meat (EM) and iron milk (IM) medium [19]. After 4 hours of growth in RC medium alpha toxin hemolytic units of type B were 66 HU/mL. In Present study results were in contrast to this study, because type B produced low hemolytic units of alpha toxin.

It was reported that *C. perfringens* produces various acids viz; propionic acid, butyric acid and acetic acid in routine culture media due to multiple metabolic activities resulting in decreased pH [36]. It was established that highest hemolytic units by *C. perfringens* were reported at pH 4.2 to 5.8 [19]. In present research hemolytic activity of alpha and epsilon toxin was observed at pH 6. Brandi and colleagues recorded that the *C. perfringens* type B growth dropped the pH from 7 to 5.2 due to production of organic acid production. It was claimed that from pH 5.5 to 8.00 *C. perfringens* grow well. It is difficult to maintain the constant pH during cultivation, so it severely affected the microbial and enzyme activity of *C. perfringens* [37].

C. perfringens toxinotype B bacteria produce higher cytotoxic units (18.65±0.34 CU/mL) and ELISA % (12.43, 12.81, 12.39, 12.61 and 13.07) of beta toxin when cultured in Robertson Cooked Meat Medium (RCMM) under anaerobic conditions at 37°C with pre-adjusted pH 6.0. It was reported that type B cell free supernatant lethality without trypsin treatment was found to be of beta toxin. But after trypsin pre-treatment both beta and epsilon toxins are important and a part of beta toxin still remains active. *C. perfringens* Type B was grown in TGY medium at 37°C and beta toxin level was 16.5±2.3 µg/mL [18]. It was found that beta toxin variant was having increase trypsin sensitivity and more cytotoxicity. This increase cytotoxicity was attributable and compensates for increase trypsin sensitivity of beta toxin variant CN 3685. Beta toxin in monomeric form liable to trypsin activity but after oligomer formation on the host cell the complex becomes trypsin resistant. Beta toxin produced pore in cell membrane. In present research beta toxin was cytotoxic for BHK21 cell line. After treatment with toxin for 24 hours swelling, round and clumping of cells was observed.

C. perfringens type B bacteria produced higher hemolytic units (16.57±0.19 HU/mL) and ELISA % (13.93, 14.78, 14.28, 14.03 and 13.25) of epsilon toxin when cultured in Robertson Cooked Meat Medium (RCMM) under anaerobic conditions at 37°C with pre adjusted pH 6.0. In a previous study, *C. perfringens* type B epsilon toxin produced 1040 HU/mL hemolytic units. After treatment with 1% trypsin, epsilon toxin activated and there were increased hemolytic units observed. In thioglycolate and RC medium, after trypsin treatment hemolytic units of epsilon toxin were 2056 HU/mL [19]. Hemolytic units of epsilon toxin of type B did not increase after 1% trypsin treatment in present study. During epsilon toxin production in synthetic medium fresh caprine heart and liver tissue used and minimum lethal dose (MLD) was 4000/mL. For toxin production temperature, pH, stirring rotation per min were 37°C, 7.0 and 100-200rpm. Glucose (0.2%) was supplemented in intervals of every 2 hours for toxin maximum production [35]. But there low hemolytic and $TCID_{50}$ units of alpha, beta and epsilon toxins were observed. But in RCMM maximum production of alpha, beta and epsilon toxins was observed.

In conclusion, as per results of current study, toxin production potential of *C. perfringens* toxinotype B isolated from lamb dysentery cases can be maximized in robertson's cooked meat medium with pre-adjusted pH 6.0 at 37°C of incubation temperature. Gene expression

studies under these conditions may result in higher expression of alpha, beta and epsilon toxins genes.

Availability of data and materials

Given data in this paper is to be submitted accurately and come from the University of Veterinary and Animal Sciences, Lahore, Pakistan. Data that support the findings of this study are available on request from the corresponding author (A.A. Anjum). The data are not publically available due to privacy and ethical restrictions.

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Conflict of Interest

It is certified that there is no conflict of interest in any part of the manuscript or among the authors.

Author Contributions

MMKS and AAA performed experiments regarding isolation of *C. perfringens* from clinical cases along with molecular toxinotyping for confirmation of *C. perfringens* and written the initial draft while YFC performed phylogenetic analysis and proofread the final draft of the manuscript whereas, TY and AA performed the experiments regarding optimization of toxin production and edited the final version of the manuscript.

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