Original Article

Physicochemical Parameters Affecting the In-vitro Toxins Production by Characterized Antibiotic-resistant *Clostridium Perfringens* Toxinotype B Isolates

Madeeha Tariq[1], Aftab Ahmad Anjum[1*], Wajeeha Tariq[2], Tehreem Ali[1], Rabia Manzoor[1]

1. Institute of Microbiology, Faculty of Veterinary Science, University of Veterinary and Animal Sciences, Lahore, Pakistan.
2. Institute of Biochemistry and Biotechnology, Faculty of Bioscience, University of Veterinary and Animal Sciences, Lahore, Pakistan.

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ABSTRACT

Background: Alpha, beta and epsilon toxins of *Clostridium perfringens* toxintype B are the major potent toxins involved in enterotoxemia. To combat this condition, proper vaccination of animals is required with an effective vaccine.

Objectives: This research aimed to characterize the resistant *C. perfringens* type B isolates for in-vitro toxin production potential under the influence of various physicochemical parameters.

Methods: *C. perfringens* isolates were characterized based on biochemical tests, toxinotyping, and 16S rRNA typing. The indigenously characterized *C. perfringens* toxinotype B isolates (n=6) were subjected to antibiotic susceptibility profiling through the Kirby-Bauer method. The resistant *C. perfringens* type B isolates were subjected to toxin production optimization under physicochemical parameters (physical: Temperature, pH and time of incubation; chemical: Glucose, vitamin-mineral mixture, tween 80 and sodium salts at various concentration).

Results: The *C. perfringens* isolates were identified as toxinotype B. Isolates MW551887.1 and MW332247.1 produced higher hemolytic and cytotoxic units of toxins at a 0.2% glucose concentration in the broth after 24 hours at 37 °C, respectively.

Conclusion: To combat disease, controlling antibiotic resistance and ensuring proper vaccination of animals is crucial. These *C. perfringens* isolates may have commercial applications for toxoid vaccine production after further. Conclusion: To combat disease, control of antibiotic resistance and proper vaccination of animals is crucial. These *C. perfringens* isolates may have commercial application for toxoid vaccine production after further characterization and molecular testing of toxins.

Keywords: Alpha toxin, Beta toxin, *Clostridium perfringens* toxinotype B, Epsilon toxin, Glucose

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*** Corresponding Author:**
Aftab Ahmad Anjum, PhD.
Address: *Faculty of Veterinary Science, Institute of Microbiology, University of Veterinary and Animal Sciences, Lahore, Pakistan.*
Phone: *+92 (300) 7259445*
E-mail: *aftab.anjum@uvas.edu.pk*

Introduction

Clostridium perfringens toxinotype B produces alpha, beta and epsilon as major toxins, as well as enterotoxin, lambda, or delta as minor toxins. It causes dysentery in newborn lambs, hemorrhagic enteritis in neonatal calves and enterotoxemia in sheep (Alves et al., 2021). Enterotoxaemia caused by *C. perfringens* type B, is a disease of great economic importance in sheep and goat farming worldwide. Disruption of the microbial balance in the gut is the most important factor for initiating the disease. This disruption is due to overeating of carbohydrate-rich fodder, which causes the proliferation of *C. perfringens* and the overproduction of the toxins (Pawaiya et al., 2020). Alpha toxin (CPA) was identified as the first bacterial enzyme with lecithinase activity. CPA is a zinc metalophospholipase C a molecular size of 43 kDa (Nagahama et al., 2019). CPA is immunogenic, and a protective immune response is stimulated by its C-terminal domain (Wang et al., 2020). Beta toxin (CPB) is a pore-forming toxin with a molecular weight of 35 kDa (Jin et al., 1996). CPB produces a ~ 12-Å channel for monovalent cations selectively (Bhunia, 2018). CPB is a virulence factor responsible for causing necrotic enteritis and enterotoxaemia (Navarro et al., 2018). Epsilon (ε) toxin (ETX) is also a pore-forming toxin. ETX is most potent toxin after botulinum and tetanus toxin of Clostridium bacteria. ETX is produced as an inactive prototoxin (296 amino acids) with a molecular weight of ~33 kDa (Subramanyam et al., 2000).

Enterotoxaemia has proven to be one of the most horrible diseases of small ruminants. The incidence of this disease is 2-8% but the case fatality rate may reach up to 100% (Viana Brandi et al., 2014). To control enterotoxaemia, various vaccines and antimicrobials are used. Many factors contribute to the outbreaks of this disease, including antimicrobial resistance and improper vaccination management. Excess use of antimicrobial agents leads to an increase in antimicrobial resistance and the spread of antimicrobial resistance among pathogens and commensal organisms (Khan et al., 2019). There are only a few reports about the antibiogram of *C. perfringens* isolated from sheep and goats. Proper vaccination and better feed management are the only effective means to combat this disease. To vaccinate the large population of sheep and goats, 1) Cost-effective bacterial cell mass and toxin production, and 2) A large number of vaccine production units are required. Currently, only a few public-sector veterinary vaccine production units exist in Pakistan. These existing units are insufficient to meet the requirements of the entire country (Tariq et al., 2021).

The present study aimed at genotypic and molecular characterization of *C. perfringens* type B, antibiogram profiling, and the production of *C. perfringens* type B toxins (α, β and ETX) under the influence of various physicochemical parameters for vaccine production at an industrial level.

Materials and Methods

Revival and growth of bacteria on specific media

Previously, biochemically characterized *C. perfringens* (n=10) isolates from sheep and goats were procured from the Institute of Microbiology, project TDF02-028, UVAS, Lahore, Pakistan. The *C. perfringens* isolates were revived from stock in a reinforced clostridial (RC) broth. The broth medium was supplemented with an equal volume mixture of 4% sodium sulfite and 7% ferric citrate (0.5 mL/25 mL), along with polymixin B (3 mg/L), kanamycin sulfate (12 mg/L) and D-cycloserine (400 mg/L). Inoculated medium tubes were incubated at 37 ˚C for 24-48 hours in an anaerobic jar (IndiaMART®) using anaerobic sachets (OXOID®). The isolates were cultured on tryptose sulfite cycloserine (TSC) agar supplemented with components as mentioned above. Microscopic morphology was identified by gram staining and spore staining.

Molecular characterization

Deoxyribose nucleic acid (DNA) was extracted through a DNA extraction kit (WizBio) according to the manufacturer's recommendations. For visual confirmation, agarose gel electrophoresis was performed using 0.8% agarose gel containing ethidium bromide at 0.5 µg/mL. *C. perfringens* isolates were characterized through polymerase chain reaction (PCR) using *16S rRNA* gene-specific primers following the method by Asghar et al. (Asghar et al., 2016). For molecular toxinotyping, specific primers were used for alpha, beta, epsilon, and iota toxin genes, following the method by Asten et al. (Asten et al., 2009). PCR amplicons were observed through agarose gel electrophoresis using a 1.5% agarose gel. Ribosomal RNA gene amplicons were subjected to sequencing and these sequences were submitted to NCBI GenBank. A phylogenetic tree for *C. perfringens* was constructed using 36 sequences of 16S rRNA in the database through the Neighbor-Joining algorithm on MEGA X.

Antibiotic susceptibility profiling

The antibiotic susceptibility profile of *C. perfringens* type B isolates (n=03) was evaluated against penicillin, tetracycline, macrolides, and antibiotics, such as ampicillin, erythromycin, and tetracycline, following the Kirby-Bauer method according to the Clinical And Laboratory Standards Institute CLSI (2022) manual. Antibiotic discs were dispensed on TSC media plates inoculated with a 0.5 McFarland inoculum of *C. perfringens* type B. The plates were incubated at 37 ˚C for 24 hours. The zone of inhibition (ZOI) was recorded in millimeters (mm) and compared with the CLSI (2022) standard.

Bacterial toxin optimization

Three *C. perfringens* toxinotype B isolates were subjected to toxin production optimization. A bacterial cell inoculum of 1 McFarland was prepared by adjusting the suspension optical density (OD) to 0.257 at 630 nm, following the methods of Fernandez-Miyakawa et al. (Fernandez-Miyakawa et al., 2007a). RC broth was inoculated with a 1 McFarland suspension (10% v/v) of *C. perfringens* type B isolates. The inoculated RCM broth was incubated at 37 ˚C, 40 ˚C and 42 ˚C for 24, 36 and 48 hours under anaerobic conditions. Carbohydrates (glucose at 0.2%), vitamin mixture (0.2%-vitamin B-complex and C), mineral mixture (0.2%-iron, magnesium, zinc and copper), Tween-80 (0.1%, 0.3% and 0.5%), sodium chloride (0.5%, 1% and 1.5%), and sodium acetate (0.1%, 0.3% and 0.5%) were added to sterile RC broth tubes (Viana et al., 2014).

Estimation of alpha, beta, and epsilon toxins

For the quantification of CPA units, the cell-free supernatant (toxins) was not activated with 1% trypsin. However, the cell-free supernatant containing epsilon toxin was activated with a 1% trypsin solution, mixed in a 9:1 ratio, and incubated at 37 ˚C for 30 minutes. A hemolytic assay was performed for both alpha and epsilon toxins (Hu et al., 2016).

For CPB, a cytotoxicity assay was performed on the baby hamster kidney-21 (BHK21) cell line, as described by Nagahama et al. (2003) and stained with 1% crystal violet (Almutary & Sanderson, 2016). The OD was measured at 570 nm using an ELISA plate reader. Cell survival percentage was calculated according to Equation 1:

1. Cell Survival Percentage=(OD of Test-OD of NC/ OD of Positive Control-OD of NC)×100

, were: OD=Optical density, and NC: Negative control.

Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) followed by post hoc Duncan's multiple range test, using the SPSS software, version 20.

Results

The isolates of *C. perfringens* were identified as gram-positive rods that produced an oval-shaped sub-terminal spore. The bacterium produced black colonies with a diameter of 2-3 mm on TSC agar.

Ribosomal 16S phylogenetic analysis

From the molecular typing of *C. perfringens*, 1500 bp size bands of the *16S rRNA* gene were obtained (Figure 1a).

For phylogenetic analysis, *16S rRNA* gene sequences were submitted to NCBI GenBank. From NCBI GenBank, three 16S sequences were retrieved with the following accession numbers: MW332247.1, MW551887.1 and MW332060.1. In the phylogenetic tree, the accession numbers of the *C. perfringens* indig-

Table 1. *C. perfringens* type B antibiogram

Sr. No	Antibiotics	Mean±SD					
		ZOI (mm)					
		MW332247.1	MW551887.1	MW332060.1	ZR06B	ZR08	CPB03
1	Ampicillin	19.45±1.49^{d}	18.65±1.48^{c}	17.98±1.22^{b}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}
2	Tetracycline	5.51±0.76^{d}	4.41±0.68^{a}	5.63±0.54^{b}	8.23±0.50^{e}	6.22±1.15^{b,c}	7.1±0.55^{d}
3	Erythromycin	4.0±1.5^{c}	5.43±0.23^{d}	1.76±1.20^{b}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}

Note: Values with different superscripts in rows differ significantly (P<0.05)

Table 2. Optimization of alpha toxin production of *C. perfringens* toxinotype B under physicochemical parameters

Temperature (˚C)	Time (h)	Mean±SD		
		Hemolytic Units (HU/mL)		
		MW332247.1	MW332060.1	MW551887.1
37	24	2.73±0.04[a]	2.69±0.0[a]	2.72±0.09[a]
	36	2.41±0.02[a,b]	2.33±0.05[a]	2.44±0.05[b]
	48	1.96±0.0[a]	1.99±0.01[a]	1.91±0.11[a]
40	24	1.8±0.02[a]	1.79±0.0[a]	1.8±0.04[a]
	36	1.61±0.16[a,b]	1.69±0.0[b]	1.43±0.0[a]
	48	1.6±0.06[a]	1.67±0.02[a]	1.6±0.01[a]
42	24	1.51±0.0[a]	1.54±0.07[a]	1.53±0.01[a]
	36	1.5±0.01[a]	1.52±0.02[a]	1.49±0.0[a]
	48	1.4±0.01[a]	1.34±0.08[a]	1.45±0.08[a]
Chemicals	**Concentration (%)**	**Hemolytic Units (HU/mL)**		
		MW332247.1	**MW332060.1**	**MW551887.1**
Glucose	0.2	24.92±0.0[a,b]	25.15±0.33[b]	23.78±0.98[a]
Vitamin mixture	0.2	1.54±0.04[a]	1.51±0.06[a]	1.85±0.23[b]
Mineral mixture	0.2	1.54±0.00[a,b]	2.06±0.45[b]	1.51±0.05[a]
Tween 80	0.1	11.85±0.12[b]	11.61±0.49[b]	10.14±01.1[a]
	0.3	21.86±0.0[a,b]	21.95±0.08[b]	20.62±01.07[a]
	0.5	21.17±0.61[b]	19.62±01.3[a,b]	19.02±0.83[a]
Sodium Chloride	0.5	5.96±0.01[c]	4.65±0.32[b]	3.65±0.56[a]
	1.0	11.98±0.01[b]	11.32±0.56[b]	10.4±0.41[a]
	1.5	6.1±0.0[b]	5.39±0.41[a]	6.36±0.34[b]
Sodium Acetate	0.1	6.26±0.03[a]	6.11±0.12[a]	6.33±0.14[a]
	0.3	11.91±0.01[a]	10.93±0.95[a]	10.37±01.38[a]
	0.5	1.48±0.01[a]	1.48±0.0[a]	1.37±0.09[a]

Note: Values with different superscripts in rows differ significantly (P<0.05).

enous isolates were displayed as colored stars (Figure 2). MW332060.1, representing Pak *C. perfringens*, was 29% evolutionarily related to MW332257.1, MW471067.1, and MW556208.1. MW551887.1 was 25% evolutionarily connected to LC386311.1 *C. perfringens* D Tokyo. Meanwhile, MW332247.1, representing Pak *C. perfringens*, was 61% evolutionarily related to KU836729.1, and these two sequences were 52% evolutionarily connected to MW349974.1 Pak *C. perfringens* (Figure 2).

Toxinotyping

Molecular toxinotyping indicated that the amplicon sizes of alpha, beta, and epsilon toxin genes were 324, 197 and 374 bp, respectively (Figures 1b, 1c and 1d). According to PCR toxinotyping results, one, six, and ` isolates were identified as *C. perfringens* type A, B, and D, respectively.

Table 3. Optimization of CPB production of *C. perfringens* toxinotype B under physicochemical parameters

Temperature (°C)	Time (h)	Mean±SD		
		Cytotoxic Units (CU/mL)		
		MW332247.1	MW332060.1	MW551887.1
37	24	2.78±0.01[a]	2.77±0.1[a]	2.78±0.02[a]
	36	3.89±02.16[a,b]	2.65±0.1[a]	6.4±0.09[b]
	48	2.58±0.0[a]	2.74±0.22[a]	2.53±0.09[a]
40	24	2.29±0.09[a,b]	2.21±0.08[a]	2.44±0.05[b]
	36	2.34±0.0[a]	2.36±0.03[a]	2.33±0.0[a]
	48	2.3±0.0[a]	2.52±0.37[a]	2.31±0.01[a]
42	24	2.18±0.0[a,b]	2.17±0.0[a]	2.19±0.00[b]
	36	2.16±0.03[a,b]	2.19±0.0[b]	2.13±0.0[a]
	48	2.03±0.0[a]	2.03±0.0[a]	2.02±0.0[a]
Chemicals	**Concentration (%)**	**Cytotoxic Units (CU/mL)**		
		MW332247.1	**MW332060.1**	**MW551887.1**
Glucose	0.2	13.93±0.01[a]	14.04±0.14[a]	13.93±0.05[a]
Vitamin mixture	0.2	2.19±0.02[a]	2.16±0.05[a]	2.17±0.01[a]
Mineral mixture	0.2	2.18±0.01[a]	2.12±0.04[a]	2.16±0.0[a]
Tween 80	0.1	7.33±0.0[a]	6.88±0.59[a]	7.47±0.15[a]
	0.3	12.39±0.04[a]	12.46±0.50[a]	12.25±0.13[a]
	0.5	11.86±0.00[a]	12.09±0.69[a]	11.13±0.77[a]
Sodium Chloride	0.5	4.41±0.01[c]	4.31±0.0[b]	4.24±0.02[a]
	1.0	7.44±0.01[b]	7.29±0.05[a]	7.37±0.06[a,b]
	1.5	4.54±0.04[a]	4.28±0.24[a]	4.32±0.1[a]
Sodium Acetate	0.1	4.58±0.39[a]	4.4±0.13[a]	4.1±0.1[a]
	0.3	7.42±0.0[b]	7.33±0.04[a]	7.32±0.02[a]
	0.5	2.19±0.02[b]	2.09±0.05[a]	2.14±0.02[a,b]

Note: Values with different superscripts in rows differ significantly (P<0.05)

Antibiotic susceptibility profiling

One isolate out of six was found to be completely resistant to erythromycin and tetracycline. Two Isolates were found to be resistant to erythromycin, neomycin, spectinomycin, and colistin. The highest mean ZOI of 19.45±1.49 mm was recorded for ampicillin against the MW332247.1 isolate (Table 1).

Toxin production optimization

Type B isolates produced 24.92±0.00, 25.15±0.33, and 23.78±0.98 HU/mL of CPAs at a 0.2% concentration of glucose (P<0.05) (Table 2).

Cytopathic effects, including shrinking, swelling, and clumping of BHK21 cell lines, were witnessed. The cy-

Table 4. Optimization of epsilon toxin production of *C. perfringens* toxinotype B under physicochemical parameters

Temperature (°C)	Time (h)	Mean±SD		
		Hemolytic Units (HU/mL)		
		MW332247.1	MW332060.1	MW551887.1
37	24	6.31±0.02[a]	6.31±0.02[a]	6.36±0.06[a]
	36	5.87±0.03[a]	5.89±0.09[a]	6±0.17[a]
	48	4.43±0.04[a,b]	4.39±0.0[a]	4.48±0.01[b]
40	24	2.56±0.01[a]	2.7±0.25[a]	2.58±0.01[a]
	36	2.42±0.02[a]	2.41±0.02[a]	2.46±0.02[a]
	48	2.33±0.02[b]	2.3±0.0[a]	2.35±0.01[b]
42	24	2.31±0.0[a,b]	2.32±0.0[b]	2.31±0.0[a]
	36	2.12±0.0[a,b]	2.1±0.01[a]	2.13±0.01[b]
	48	1.73±0.02[a,b]	1.7±0.0[a]	1.76±0.02[b]
Chemicals	**Concentration (%)**	**Hemolytic Units (HU/mL)**		
		MW332247.1	**MW332060.1**	**MW551887.1**
Glucose	0.2	34.05±0.0[a,b]	33.41±0.55[a]	34.23±0.15[b]
Vitamin mixture	0.2	10.89±0.0[a]	10.43±0.39[a]	9.69±01.03[a]
Mineral mixture	0.2	10.54±0.02[a]	9.73±0.77[a]	10±0.7[a]
Tween 80	0.1	9.88±0.0[a,b]	10.51±0.55[b]	9.79±0.08[a]
	0.3	16.17±0.05[a]	16.11±0.96[a]	15.61±0.51[a]
	0.5	18.46±0.03[a,b]	17.88±0.70[a]	18.98±0.47[b]
Sodium Chloride	0.5	7.66±0.0[b]	7.54±0.09[a]	7.61±0.0[a,b]
	1.0	7.08±0.10[b]	6.59±0.62[b]	5.28±0.7[a]
	1.5	6.81±0.02[a]	6.71±0.09[a]	6.72±0.1[a]
Sodium Acetate	0.1	7.6±0.0[b]	6.55±0.86[a]	7.5±0.0[a,b]
	0.3	5.57±0.02[b]	5.43±0.03[b]	4.54±0.5[a]
	0.5	2.11±0.11[b]	1.95±0.0[a]	1.87±0.05[a]

Note: Values with different superscripts in rows differ significantly (P<0.05).

totoxicity of CPB was reduced at 40 °C and 42 °C during the 24 to 48 hours of incubation. In comparison with other chemical supplements, greater production of cytotoxic units (P>0.05) was observed at a 0.2% concentration of glucose (Table 3).

Higher hemolytic units of epsilon toxin were produced at a 0.2% concentration of glucose. Among the hemolytic units of epsilon toxin, insignificant differences were observed at 0.5% sodium chloride and 0.1% sodium acetate concentrations (P>0.05) (Table 4).

Discussion

For many years, phylogenetic connections and the identification of clinical and environmental isolates have been established in bacteria by the sequencing of the 16S rRNA gene. Fox et al. used 16S sequence data com-

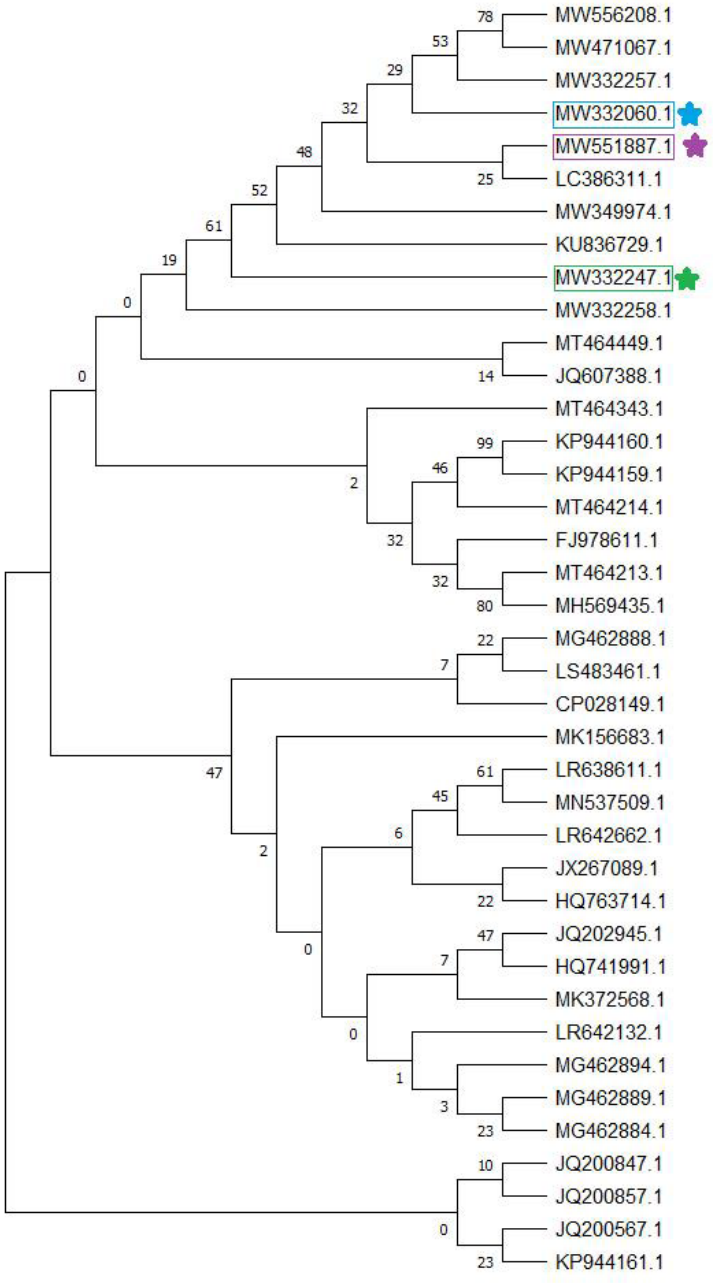

Figure 1. Molecular typing

a) Lane 1: Ladder, lane 3- 8:16S r RNA gene amplicon (1500bp); b) Lane 1: ladder, lane 2- 7: Alpha toxin gene amplicon (324bp); c) Lane 1: Ladder, lane 2- 7: Epsilon toxin gene amplicon (374bp) d) Left: Ladder, lane 1- 6: CPB gene amplicon (197bp).

parisons to lay the foundation for intricate phylogenetic research prior to the development of DNA sequencing techniques (Fox et al., 1977). Due to a lack of comprehensive research on other highly conserved genes, the *16S rRNA* gene remains the most widely used marker for bacterial identification and evolutionary genetics. However, the accurate identification of many bacterial genera and species often requires the analysis of multiple 16S regions and/or a longer gene sequence, typically up to 1,060 base pairs (CLSI, 2018). Genotype identification using *16S rRNA* gene sequence has proven to be an accurate, objective, and reliable method for identifying bacteria and defining their taxonomic relationships (Clarridge et al., 2004). In this study, bacterial isolates were identified as *C. perfringens* through *16S rRNA* gene sequence BLAST analysis and phylogenetic analysis. This was found in agreement with the study by Anju et al. (Anju et al., 2021), which indicated that 16S rRNA amplification

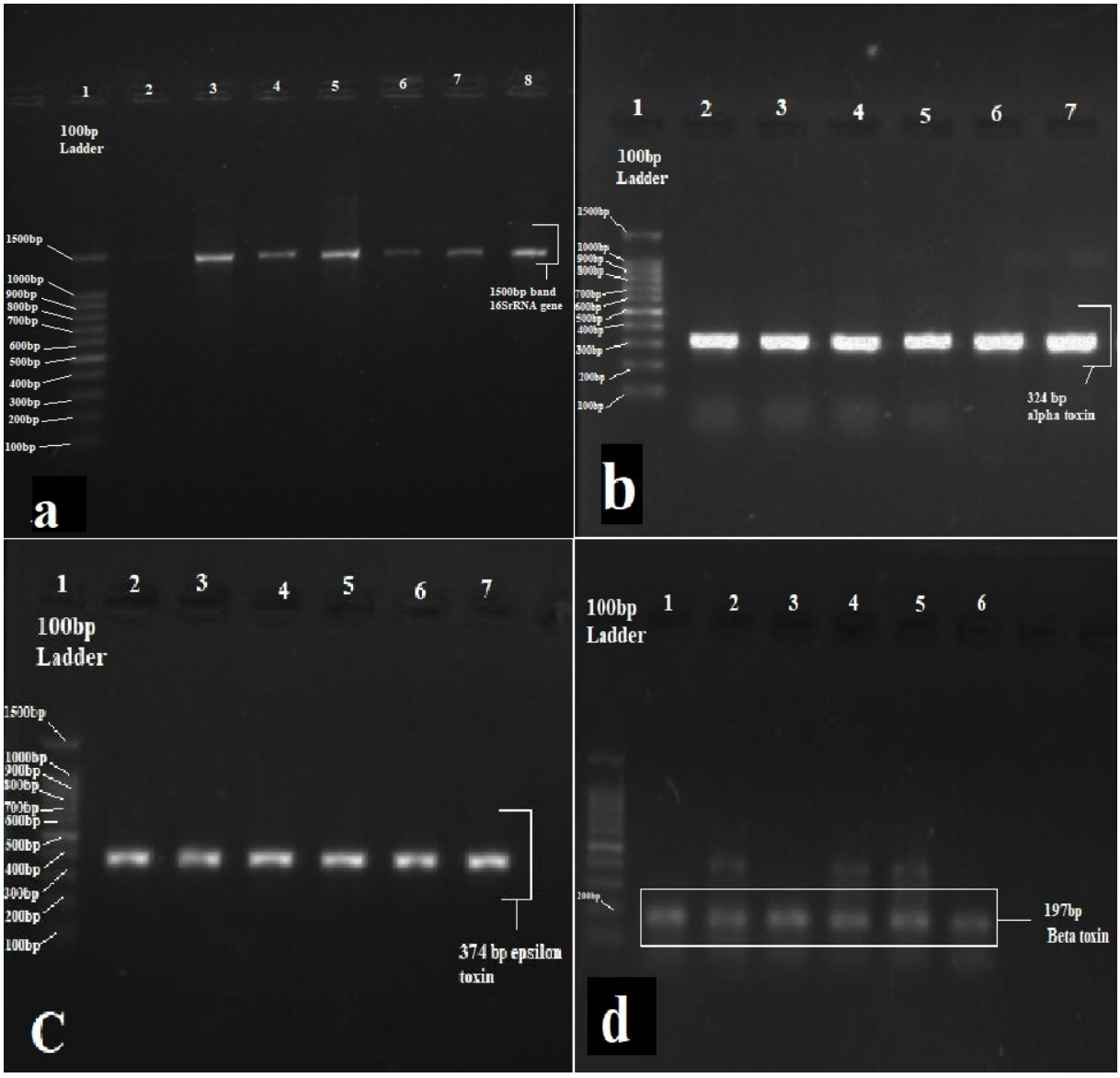

Figure 2. Phylogenetic analysis of *C. perfringens* isolates based on *16S rRNA* gene sequences

followed by gene sequencing can serve as an alternative tool for the definitive confirmation of *C. perfringens*.

Toxinotype B causes lamb dysentery and rarely causes diseases in cattle and horses. Lamb dysentery is characterized by necrotic hemorrhagic enteritis and necrosis produced by beta and epsilon toxins, respectively. It is an infection that causes intestinal lesions and enterotoxemia, where toxins produced in the intestine are absorbed into the circulation and affect distant organs, including the brain (Munday et al., 2020). Forti et al. identified *C. perfringens* toxinotypes using PCR toxinotyping (Forti et al., 2020). Multiplex PCR was employed for the molecular toxinotyping of *C. perfringens*, which produced multiplex results for the alpha, beta, epsilon and iota genes (Hussain et al., 2017). Similarly Alimolaei and Shamasaddini used multiplex PCR for the molecular characterization of *C. perfringens* types F and G isolated from Diarrhoeic sheep (Alimolaei & Shamsaddini 2023). However, in the present research, a single PCR was used for the toxinotyping of *C. perfringens* isolates. The *C. perfringens* isolates were identified as type B, D and A, respectively. This finding is consistent with previously reported toxinotypes A, B and D isolated from animal samples in Italy (Forti et al., 2020) and the Punjab province of Pakistan (Mohiuddin et al., 2020).

The optimization of bacterial growth and metabolism is dependent on several factors, including temperature, incubation duration, and pH. The ideal growth tempera-

ture range for *C. perfringens* is 37-47 ˚C and the ideal incubation period is 18 hours (Guo et al., 2017). To achieve higher production of alpha, beta, and epsilon toxins by type B *C. perfringens* at 37 ˚C during 24 to 36 hours of incubation, RC broth (chemically defined media, manufacturer formula) was used. Similar findings were reported for the production of alpha, beta and epsilon toxins when chemically defined media brain heart infusion (BHI) and tryptone glucose yeast (TGY) were used. CPA exhibited toxicity (40 LD_{50}/mL) in the BHI medium (Fernandez-Miyakawa et al., 2007a; Fernandez-Miyakawa et al., 2007b). This finding was observed in contrast to the present study where toxin production was observed in an RC medium. The cytotoxicity of *C. perfringens* toxinotype D epsilon toxin was observed in human renal tubular epithelial cells (Fernandez-Miyakawa et al., 2011). In contrast, in the present study, an animal cell culture (BHK 21 cell line) was used to observe the cytotoxicity of CPB. However, the activities of alpha and epsilon toxins were assessed using a hemolytic assay with 1% sheep red blood cells.

Every type of bacterium has a pH, at which it grows best, and determining this pH in tests is crucial for industrial manufacturing. The ideal pH range for *C. perfringens* growth in the bioreactor during batch fermentation is 6.5–7.0 (Guo et al., 2017). Maintaining a constant pH during cultivation is challenging and significantly affects microbial and enzyme activity. Maximum toxin production was observed at pH levels between 6.00 and 8.00. The highest toxin level (102.56 LD_{50}/mL) was observed at a controlled pH of 6.5, compared to pH 7 (102.41 LD_{50}/mL) and pH 7.5 (101.96 LD_{50}/mL) (Kulshrestha, 1974). Toxinotype B's cell-free supernatant (culture broth) lethality without trypsin treatment was attributed solely to CPB. Type B *C. perfringens* grown in TGY medium at 37 ˚C showed toxin levels of 6.5±1.2 mg/mL (epsilon) and 16.5±2.3 mg/mL (beta) (Fernandez-Miyakawa et al., 2007b). In contrast, in RCM broth at pH 6.8, an increase in toxin production levels (alpha: 25.15±0.33HU/mL, beta: 14.04±0.14 CU/mL and epsilon: 34.23±0.15 HU/mL) was observed in the present study. Post-trypsin treatment, a decrease in toxin activity was generally observed, although some CFSs exhibited increased toxin activity. In agreement with the above observations, after trypsin treatment, an increase in epsilon toxin activity was observed in the present study.

Salts, trace elements, sugars, peptone and extracts in the growth medium are added to enhance bacterial growth. Salts act as cofactors and maintain the osmolality of the medium. Trace elements also act as cofactors. Sugars in culture media serve as a primary energy source for bacteria. Peptone and cell/organ extracts provide nitrogen, amino acid, water-soluble vitamin B complex, carbohydrates, and glutamic acid (Atmanto et al., 2022). Compared to artificial sources, peptone enhances bacterial growth when derived from natural sources (Noruzy Moghadam et al., 2023). To enhance the antigen production of *C. perfringens*, glucose, $MgSO_4$, liver extract, and casein-peptone were added to the medium (Pulotov et al., 2021). Conversely, supplementing the RC medium with 0.2% glucose before inoculation resulted in higher bacterial growth and production of alpha, beta, and epsilon toxins after 24 hours of incubation. However, the addition of a mineral mixture containing magnesium did not increase toxin production. In 2023, Araghi et al. (2023) also used peptone, sugars, and salts for *C. perfringens* type B toxin production. In the present study, RC broth containing yeast extract and peptone as sources of vitamins and amino acids exhibited low hemolytic and cytotoxicity unit production for alpha, beta, and epsilon toxins.

Conclusion

The results obtained suggest a potential application of *Syzygium cumini* leaves to treat infections caused by antibiotic-resistant *C. perfringens* toxinotype B. Type B isolates may be used to produce alpha, beta and epsilon toxins in the highest amounts at a large industrial scale for toxoid-containing vaccine production.

Ethical Considerations

Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

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Authors' contributions

Supervision: Aftab Ahmad Anjum; Analytical testing: Madeeha Tariq, Wajeeha Tariq, Tehreem Ali, and Rabia Manzoor; Writing: Madeeha Tariq and Rabia Manzoor; Final approval: All Authors.

Conflict of interest

The authors declared no conflict of interests.

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