DOI:10.22059/IJVM.2024.375486.1005561 Iranian Journal of Veterinary Medicine Original Article

Online ISSN: 2252-0554`

Physicochemical Parameters Affecting the Invitro Toxins Production by Characterized Antibiotic-Resistant *Clostridium perfringens* Toxinotype B Isolates

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Abstract

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

Objective: The research study was planned to characterize the resistant *C. perfringens* type B isolates for *in-vitro* toxin production potential under the influence of various physicochemical parameters.

Materials and method: *C. perfringens* isolates were characterized based on biochemical tests, toxinotyping and 16S rRNA typing. *C. perfringens* toxinotype B indigenously characterized isolates (n=06) were subjected to antibiotic susceptibility profiling through the Kirby-Bauer method. *C. perfringens* type B resistant isolates were subjected for toxin production optimization under physicochemical parameters (Physical: temperature, pH and time of incubation; Chemical: glucose, vitamin mineral mixture, tween 80 and sodium salts in various concentration).

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

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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Introduction

C. perfringens toxinotype B produces alpha, beta and epsilon as major toxins and enterotoxin, lambda or delta as minor toxins. It causes dysentery in newborn lambs, hemorrhagic enteritis in neonatal calves, and enterotoxaemia in sheep (Alves et al., 2021). Enterotoxaemia caused by *C. perfringens* type B, is a disease of great economic importance in sheep and goats

farming worldwide. Disruption of the microbial balance in the gut is the most important factor for initiating the disease. It is due to overeating of carbohydrate rich fodder, which causes proliferation of *C. perfringens* and overproduction of the toxins (Pawaiya et al., 2020). Alpha toxin (CPA) was identified as first bacterial enzyme with lecithinase activity. CPA is a zinc metalophospholipase C with 43 kDa molecular size (Nagahama et al., 2019). CPA is immunogenic, protective immune response is stimulated by its C terminal domain (Wang et al., 2020). Beta toxin (CPB) is a pore forming toxin with molecular weight of 35 kDa (Jin et al., 1996). CPB produces ~ 12-Å channel for monovalent cations selectively (Bhunia, 2018). CPB is virulence factor 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 molecular weight ~33 kDa (Subramanyam et al., 2000).

Enterotoxaemia has proven one of the most horrible diseases of small ruminants. Incidence of this disease is 2-8% but the case fatality rate may go up to 100% (Viana Brandi et al., 2014). To control the enterotoxaemia various vaccines and antimicrobials are used. Many factors are responsible for the outbreaks of this disease including antimicrobial resistance and improper vaccination management. Excess use of antimicrobial agents leads to an increase in

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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 is the only way to combat this disease. To vaccinate the huge population (sheep and goats) 1) cost effective bacterial cell mass and toxin production and 2) large number of vaccine production units are required. Presently only a few public sector veterinary vaccine production units are present in the Pakistan. These existing units are insufficient to meet requirements of the whole country (Tariq et al., 2021).

The present study is designed for genotypic and molecular characterization of *C. perfringens* type B, Antibiogram profiling, production of *C. perfringens* type B toxins (α, β and ε toxin) under influence of various physicochemical parameters for vaccine production at industrial level.

Materials and Methods

Revive and growth of bacteria on specific medium

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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. *C. perfringens* isolates were revived from stock in a reinforced clostridial (RC) broth. Broth medium was supplemented with equal volume mixture of 4 % sodium sulfite and 7 % ferric citrate (0.5 mL/25 mL), along with polymixin B (3mg/L), kanamycin sulfate (12mg/L) and D-cycloserine (400mg/L). Inoculated medium tubes were incubated at 37ºC for 24-48 hours in an anaerobic jar (IndiaMART®) using anaerobic sachet (OXOID®). Isolates were cultured on tryptose sulfite cycloserine (TSC) agar supplemented with components as mentioned above. Microscopic morphology was identified by gram's staining and spore staining.

Molecular characterization

Deoxyribose nucleic acid (DNA) was extracted through DNA extraction kit (WizBio) according to the manufacturer recommendations. For visual confirmation agarose gel electrophoresis was performed using 0.8% agarose gel containing ethidium bromide 0.5µg/mL. *C. perfringens* isolates were characterized through polymerase chain reaction (PCR) using 16S rRNA gene specific primers following the method of Asghar et al. (2016). For molecular toxinotyping, specific primers were used for alpha, beta, epsilon and iota toxin genes following

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the method of Asten et al. (2009). PCR amplicons were observed through agarose gel electrophoresis using 1.5% agarose gel. Ribosomal RNA gene amplicons were subjected to sequencing. These sequences were submitted to NCBI GenBank. Phylogenetic tree for *C. perfringens* was constructed using 36 sequences of 16S rRNA in data base through Neighbor joining algorithm on MEGA X.

Antibiotic susceptibility profiling

Antibiotic susceptibility profile of *C. perfringens* type B isolates (n=03) was evaluated against penicillin, tetracycline, macrolides and of antibiotics such as ampicillin, erythromycin, and tetracycline antibiotics 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 0.5 McFarland inoculum of *C. perfringens* type B. Plates were incubated at 37°C temperature for the duration of 24 hours. Zone of inhibition (ZOI) was recorded in millimeter (mm) and compared with CLSI (2022) standard.

Bacterial toxins optimization

C. perfringens toxinotype B three isolates were subjected to toxins production optimization. Bacterial cell inoculum of 1 McFarland was prepared through adjustment of

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suspension O.D to 0.257 at 630 nm following the methods of Fernandez-Miyakawa et al. (2007a). RC broth was inoculated with 1 McFarland suspension (10% v/v) of *C. perfringens* type B Isolates. Inoculated RCM broth was incubated at 37, 40, 42°C for 24, 36 and 48 hours under anaerobic conditions. Carbohydrates (glucose 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%) and sodium chloride (0.5, 1 and 1.5%) and sodium acetate (0.1, 0.3 and 0.5%) were added in to sterile RC broth tubes (Viana et al., 2014).

Estimation of alpha, beta and epsilon toxin

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

For beta toxin, cytotoxicity assay was performed on Baby Hamster Kidney-21 (BHK21) cell line as described by Nagahama et al. (2003) and stained with 1% crystal violet as described by Almutary & Sanderson (2016). Optical density was taken at 570 nm by ELISA plate reader. Cell survival percentage was calculated according to Equation 1:

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1. $$\text{Cell Survival Percentage} = \frac{\text{O.D of Test} - \text{O.D of Negative Control}}{\text{O.D of Positive Control} - \text{O.D of Negative Control}} \times 100$$

Statistical analysis

Data was analyzed through one-way analysis of variance (ANOVA) followed by post hoc Duncan's multiple range test using statistical package for social sciences (SPSS) version 20.0.

Results

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

Ribosomal 16S phylogenetic analysis

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

For phylogenetic analysis 16S rRNA gene sequences were submitted to NCBI GenBank. From NCBI GenBank n=03 16S sequences were retrieved with following accession numbers: MW332247.1, MW551887.1 and MW332060.1. In phylogenetic tree, accession numbers of *C. perfringens* indigenous isolates were displayed as colored star (Figure 3). MW332060.1 Pak *C.*

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perfringens was 29% evolutionary related to MW332257.1, MW471067.1 and MW556208.1. MW551887.1 was 25% evolutionary connected to LC386311.1 *C. perfringens* D Tokyo. While MW332247.1 Pak *C. perfringens* was 61% evolutionary related to KU836729.1 and these both sequences were 52% evolutionary connected to MW349974.1 Pak *C. perfringens* (Figure 2).

Toxinotyping

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

Antibiotic susceptibility profiling

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

Toxins production optimization

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

Cytopathic effects including shrinking, swelling and clumping of cells of BHK21 cell lines were witnessed. Cytotoxicity of beta toxin was reduced at 40 and 42ºC between 24 to 48 hours of incubation. In comparison with other chemical supplements, at 0.2% concentration of glucose more cytotoxic units ($P>0.05$) production was observed (Table 3).

Epsilon toxin higher hemolytic units were produced at 0.2% concentration of glucose. Among the hemolytic units of epsilon toxin at 0.5% sodium chloride and 0.1 % sodium acetate concentrations insignificant differences were observed ($P>0.05$) (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. Carl Woese and George Fox used 16S sequence data comparisons to set the foundation for intricate phylogenetic research prior to the development of DNA sequencing techniques (Fox et al., 1977). Because there is a dearth of comprehensive research on other highly conserved genes,

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the 16S rRNA gene remains the most widely used marker for bacterial identification and evolutionary genetics. However, accurate identification of many bacterial genera and species frequently requires analyzing multiple 16S regions and/or a longer gene sequence, usually 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 Anju et al. (2021) study that 16S rRNA amplification followed by gene sequencing can serve as an alternative tool for the definitive confirmation of *C. perfringens*.

Toxinotype B causes lamb dysentery and rarely cased diseases in cattle and horses. Lamb dysentery characterized by necrotic hemorrhagic enteritis and necrosis produced by beta and epsilon toxin respectively. Lamb dysentery is an infection which causes intestinal lesions and enterotoxemia where toxin produced in intestine and also absorbed in circulation and affect distant organs including brain (Munday et al., 2020). Forti et al. (2020) identified *C. perfringens* toxinotypes using PCR toxinotyping. Multiplex PCR was employed for molecular toxinotyping of *C. perfringens*, which produced multiplex results for alpha, beta, epsilon and iota genes

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(Hussain et al., 2017). Similarly Alimolaei and Shamasaddini used multiplex PCR for molecular characterization of *C. perfringens* Type F and G isolated from Diarrhoeic sheep (Alimolaei and Shamsaddini 2023). But in present research singlet PCR was used for *C. perfringens* isolates toxinotyping. *C. perfringens* isolates were found as type B, D and A, respectively. This was found in agreement with previously reported type A, B and D toxinotype isolated from animal samples from Italy (Forti et al., 2020) and 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 temperature range for *C. perfringens* is 37–47ºC, and the ideal incubation period is 18 hours (Guo et al., 2017). For higher alpha, beta, and epsilon toxins production by type B *C. perfringens* at 37°C at 24 to 36 hours of incubation, RC broth (chemically defined media, manufacturer formula) was used. Similar findings were reported for alpha, beta, and epsilon toxin production as chemically defined media brain heart infusion (BHI) and tryptone glucose yeast (TGY) were used. Alpha toxin exhibited toxicity (40 LD50/mL) in BHI medium (Fernandez-Miyakawa et al., 2007a; Fernandez-Miyakawa et al., 2007b). This finding was observed in-contrast to present study that toxin production was observed in RC medium. *C. perfringens* toxinotype D epsilon toxin cytotoxicity was observed in human renal tubular epithelial cells (Fernandez-Miyakawa et al., 2011). In

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contrast to it, in present study animal cell culture (BHK 21 cell line) was used to observed the cytotoxicity of beta toxin. However, alpha and epsilon toxin activities were assessed using a hemolytic assay with 1% sheep red blood cells.

Every kind of bacterium has a pH at which it grows best, and determining this pH in tests is crucial for industrial manufacture. The ideal pH range for *C. perfringens* growth in the bioreactor during batch fermentation was 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 LD50/mL) was observed at a controlled pH of 6.5, compared to pH 7 (102.41 LD50/mL) and pH 7.5 (101.96 LD50/mL) (Kulshrestha, 1974). Toxinotype B, cell-free supernatant (culture broth) lethality without trypsin treatment was attributed only to beta-toxin (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 to this, in RCM broth at pH 6.8 increase toxin production level (alpha: 25.15±0.33HU/mL, beta: 14.04±0.14 CU/mL and epsilon: 34.23±0.15 HU/mL) was observed in present study. Post-trypsin treatment, a decrease in toxin activity was generally observed, though some CFSs

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exhibited increased toxin activity. In agreement to above observations, after trypsin treatment increase in epsilon toxin activity was observed in present study.

Salts, trace elements, sugars, peptone and extracts in growth medium are added to enhance the bacterial growth. Salts act as cofactors and maintain osmolality of medium. Trace elements also act as cofactors. Sugars in culture media for bacteria require as a primary energy source. Peptone and cell/organ extracts provide nitrogen, amino acid and water-soluble Vitamin-B complex, carbohydrates and glutamic acid (Atmanto et al., 2022). Peptone in comparison to artificial source, enhance bacterial growth when used from natural source (Noruzy Moghadam et al., 2023). To enhance the antigen production of *C. perfringens*, glucose, MgSO4, liver extract, and casein-peptone were added in to the medium (Pulotov et al., 2021). Conversely, RC medium was supplemented with 0.2% glucose before inoculation resulted in higher bacterial growth alpha, beta, and epsilon toxins production after 24 hours of incubation. However, the addition of a mineral mixture containing magnesium did not increase toxin production. In 2023 Araghi et al, also used peptone, sugar and salts for *C. perfringens* type B toxin production. In present study RC broth was used, containing yeast extract and peptone as sources of vitamins and amino acids, exhibited low hemolytic and cytotoxicity units production for alpha, beta, and epsilon toxins.

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

Conflict of interest

The authors declare that they have no conflict of interests.

Authors' contribution

Project 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.

Funding

The Higher Education Commission (HEC) of Pakistan has funded this research through a technology development fund (TDF-02-028).

Acknowledgments

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The authors would like to acknowledge Institute of Microbiology, University of Veterinary and Animal Sciences, Lahore, Pakistan and supervisor for their eminence contribution in this research work.

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Table 1: *Clostridium perfringens* type B antibiogram.

Sr. No	Antibiotics	Zone of inhibition (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^{b}	4.41 ± 0.68^{a}	5.63 ± 0.54^{b}	8.23 ± 0.50^{e}	$6.22\pm1.15^{b,c}$	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}

a,b,c,d,e Values with different superscripts in rows differ significantly ($P<0.05$)

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

Temperature (ºC)	**Time (h)**	**Hemolytic units (HU/mL)**		
		MW332247.1	**MW332060.1**	**MW551887.1**
37	24	2.73±0.04[a]	2.69±0.00[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±0.00[a]	1.99±0.01[a]	1.91±0.11[a]
40	24	1.80±0.02[a]	1.79±0.00[a]	1.80±0.04[a]
	36	1.61±0.16[a,b]	1.69±0.00[b]	1.43±0.00[a]
	48	1.60±0.06[a]	1.67±0.02[a]	1.60±0.01[a]
42	24	1.51±0.00[a]	1.54±0.07[a]	1.53±0.01[a]
	36	1.50±0.01[a]	1.52±0.02[a]	1.49±0.00[a]

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	48	1.40±0.01[a]	1.34±0.08[a]	1.45±0.08[a]
Chemicals	**Concentration (%)**			
Glucose	0.2	24.92±0.00[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±01.10[a]
	0.3	21.86±0.00[a,b]	21.95±0.08[b]	20.62±01.07[a]
	0.5	21.17±0.61[b]	19.62±01.30[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]	10.40±0.41[a]
	1.5	6.10±0.00[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±0.00[a]	1.37±0.09[a]

a,b,c,d,e Values with different superscripts in rows differ significantly (P<0.05)

Table 3. Optimization Beta toxin of *C. perfringens* toxinotype B under physicochemical parameters

Temperature (ºC)	**Time (h)**	**Cytotoxic Units (CU/mL)**		
		MW332247.1	**MW332060.1**	**MW551887.1**
37	24	2.78±0.01[a]	2.77±0.10[a]	2.78±0.02[a]
	36	3.89±02.16[a,b]	2.65±0.10[a]	6.40±0.09[b]
	48	2.58±0.00[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±0.00[a]	2.36±0.03[a]	2.33±0.00[a]
	48	2.30±0.00[a]	2.52±0.37[a]	2.31±0.01[a]
42	24	2.18±0.00[a,b]	2.17±0.00[a]	2.19±0.00[b]
	36	2.16±0.03[a,b]	2.19±0.00[b]	2.13±0.00[a]
	48	2.03±0.00[a]	2.03±0.00[a]	2.02±0.00[a]

Chemicals	Concentration (%)			
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±0.00[a]
Tween 80	0.1	7.33±0.00[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±0.00[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±0.10[a]
Sodium Acetate	0.1	4.58±0.39[a]	4.40±0.13[a]	4.10±0.10[a]
	0.3	7.42±0.00[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]

a,b,c,d,e Values with different superscripts in rows differ significantly (P<0.05)

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

Temperature (ºC)	**Time (h)**	**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]	6.00±0.17[a]
	48	4.43±0.04[a,b]	4.39±0.00[a]	4.48±0.01[b]
40	24	2.56±0.01[a]	2.70±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]	2.30±0.00[a]	2.35±0.01[b]
42	24	2.31±0.00[a,b]	2.32±0.00[b]	2.31±0.00[a]
	36	2.12±0.00[a,b]	2.10±0.01[a]	2.13±0.01[b]
	48	1.73±0.02[a,b]	1.70±0.00[a]	1.76±0.02[b]
Chemicals	**Concentration (%)**			
Glucose	0.2	34.05±0.00[a,b]	33.41±0.55[a]	34.23±0.15[b]

Vitamin Mixture	0.2	10.89±0.00[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]	10.00±0.70[a]
Tween 80	0.1	9.88±0.00[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±0.00[b]	7.54±0.09[a]	7.61±0.00[a,b]
	1.0	7.08±0.10[b]	6.59±0.62[b]	5.28±0.70[a]
	1.5	6.81±0.02[a]	6.71±0.09[a]	6.72±0.10[a]
Sodium Acetate	0.1	7.60±0.00[b]	6.55±0.86[a]	7.50±0.00[a,b]
	0.3	5.57±0.02[b]	5.43±0.03[b]	4.54±0.50[a]
	0.5	2.11±0.11[b]	1.95±0.00[a]	1.87±0.05[a]

a,b,c,d,e Values with different superscripts in rows differ significantly (P<0.05)

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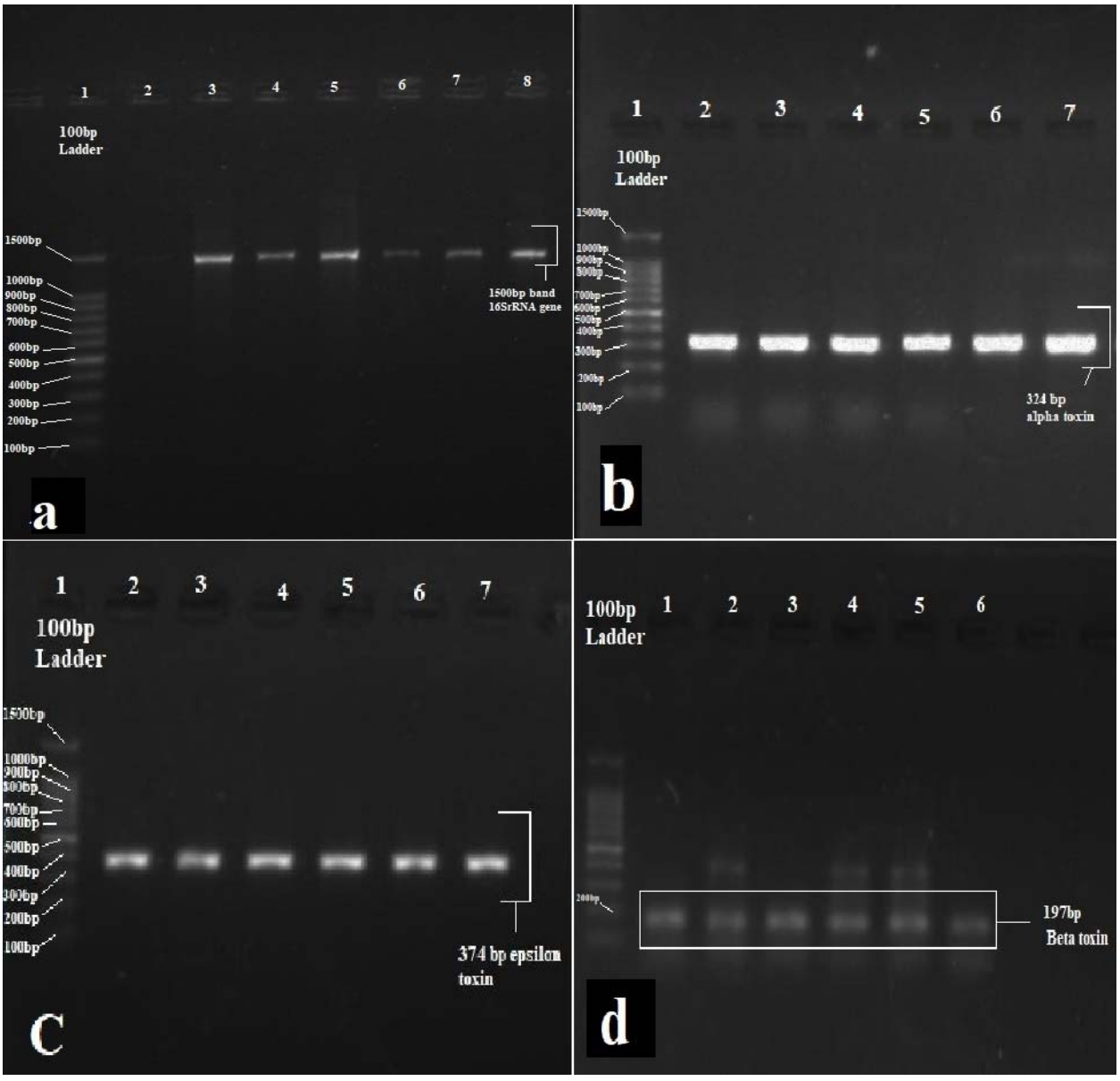
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800bp
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600bp
500bp
400bp
300bp
200bp
100bp
1500bp band 16SrRNA gene
a
1 2 3 4 5 6 7
100bp Ladder
1500bp
1000bp
900bp
800bp
700bp
600bp
500bp
400bp
300bp
200bp
100bp
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b
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1500bp
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900bp
800bp
700bp
600bp
500bp
400bp
300bp
200bp
100bp
374 bp epsilon toxin
C
100bp Ladder 1 2 3 4 5 6
200bp
197bp Beta toxin
d

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: beta toxin gene amplicon 197bp.

Uncorrected Proof

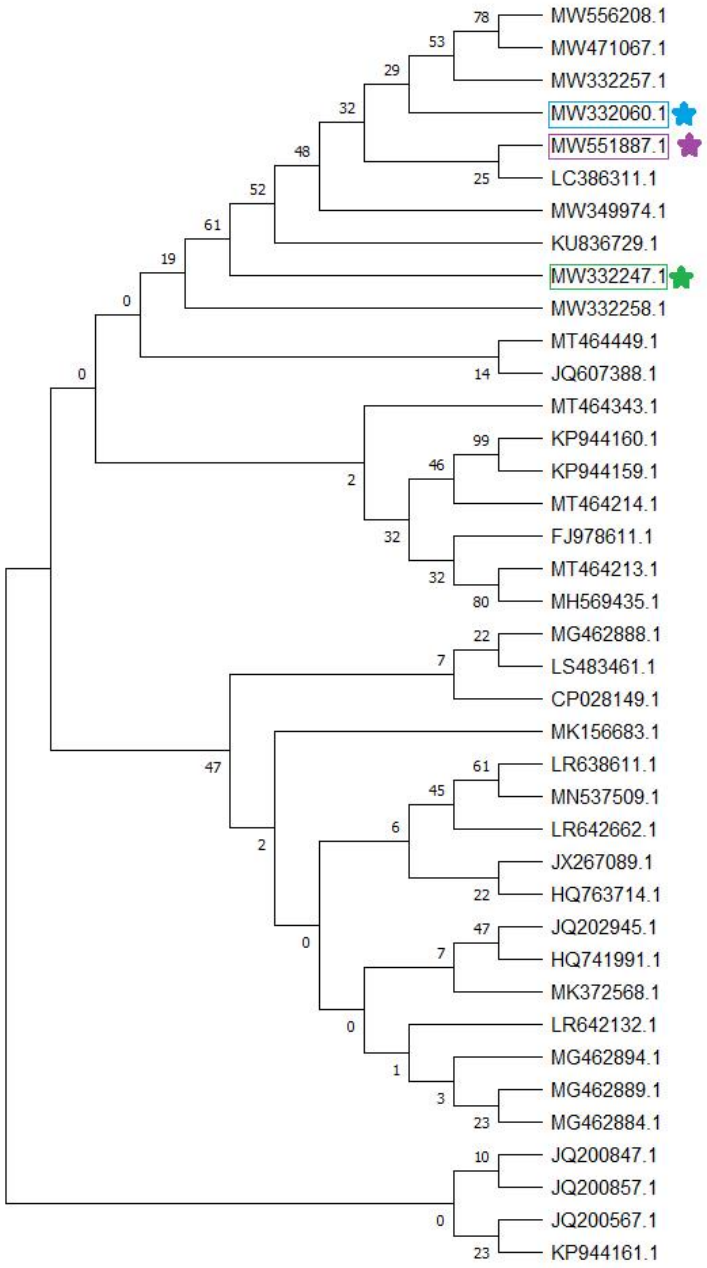
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Figure 2. Phylogenetic analysis of *C. perfringens* isolates on the basis of 16S rRNA gene sequences.

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