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Characterization of quinolones resistant *Clostridium perfringens* toxinotype D

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Abstract: *Clostridium perfringens* toxins play role in causing pulpy kidney disease poisoning as well enterotoxaemia. To combat antimicrobial resistance: curbing use, regulations and execution to antimicrobial usage in food along with withdrawal period is necessary. Aim of study was to optimize the toxins production by indigenously characterized *C. perfringens* type D isolates (n=03) under various physicochemical parameters, a lead towards local vaccine production in Pakistan. Indigenous isolates were characterized on the basis of 16S rRNA and MW349974.1, MW341428.1, MW332258.1 accession numbers were received from NCBI GenBank. Isolates were identified as toxinotype D through PCR toxinotyping. Quinolones antibiotic susceptibility testing revealed that isolates observed susceptible to enrofloxacin and resistant to ciprofloxacillin and ofloxacillin. Optimization of toxins production was determined under the influence of physical and chemical parameters. Alpha and epsilon toxin production in reinforced clostridial medium (RCM) broth was observed higher at 37°C after 24h incubation by MW332258.1. Under the influence of 0.2% glucose and 0.3% tween 80 supplementation in RCM, greater production of alpha and epsilon toxin units was observed by MW332258.1. Under optimized physicochemical parameters, maximum toxins units were observed; MW332258.1 isolate is excellent candidate could be used to produce maximum toxin units for vaccine production at industrial scale.

Keywords: Antibiotic resistance, *clostridium perfringens* toxinotype D, glucose, hemolytic units, pulpy kidney disease.

INTRODUCTION

C. perfringens toxinotype D produces alpha and epsilon as major toxins, while enterotoxin (CPE) and lambda λ as minor toxins. *C. perfringens* toxinotype D is the etiological agent of deadly disease of sheep and goats called pulpy kidney disease or overeating disease and a natural commensal as normal microbiota of intestine. The Epsilon toxin of bacterium is an angiotoxin and mostly involved in pathogenesis of this disease and produced in the alimentary tract or absorbed into systemic blood circulation (Pawaiya *et al.,* 2020).

In Pakistan, sheep and goat farming contribute major part of livestock and yields in milk, meat, skin and manure. For last many years, it has been observed that the growth of sheep and goats industry has been decreased. The optimum production of livestock cannot be achieved without protection from infectious diseases particularly enterotoxaemia (Zafar *et al.,* 2022). Among the infectious diseases, enterotoxaemia has proven one of the most atrocious diseases of small ruminants. Incidence of this disease is 2-8% but the case fatality rate may go up to 100 % (Derongs *et al.,* 2020). Vaccination can be graded as one of the best strategies for controlling deadly diseases. Many factors are responsible for the outbreaks of this disease including improper vaccination and poor feeding management (Banwart *et al.,* 2012). Proper vaccination and better feed management is the only way to combat this disease (Alves *et al.,* 2021).

Along with improper vaccination problem, *C. perfringens* emerged as a multi drug resistant (MDR) organism and rapid spread of it poses a serious therapeutic challenge. Due to emerging health crisis antibiotic resistance is an alarming issue of 21st century (Ma *et al.,* 2021). The bacterium has become resistant by adapting against repeated use of antibiotics and by developing chromosomal or plasmid borne resistance genes (Nhung *et al.,* 2017). The present research plan was designed for isolation and molecular typing of the quinolone resistant *C. perfringens* toxinotype D isolates, including optimization of the alpha and epsilon toxins production potential under the effect of various physicochemical parameters. This will be the leading point towards locally characterized vaccine production, which is the lacking domain in previous research history of the Pakistan.

MATERIALS AND METHODS

Isolation and Biochemical characterization

Clostridium perfringens was isolated at Anaerobiology Laboratory, Institute of Microbiology, University of Veterinary and Animal Sciences, Lahore, Pakistan from pulpy kidney diseased fecal samples of sheep (n=25) and goats (n=25) using sterile RCM broth. Cultures were purified on sterile plates of perfringens agar medium supplemented D-cycloserine (400mg/L) and incubated at 37ºC for 24-48h under anaerobic environment. Double

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zone blood hemolysis and lecithinase activity test was used for biochemical characterization (Hwang and Park, 2015).

Molecular typing and phylogenetic analysis

After DNA extraction through kit (GeneAll Exgene™, lot#10619D22018, Korea), biochemically characterized *C. perfringens* (n=07) isolates were subjected to molecular characterization. Isolates were confirmed through polymerase chain reaction (PCR) thermal cycler (Kyratec, Australia) using 16S rRNA gene specific primers following the method of Asghar *et al.* (2016). FASTA sequences were submitted to National Center for Biotechnology Information (NCBI) GenBank for accession numbers. Phylogenetic analysis was performed using neighbor Joining algorithm and 1000 bootstrap replications through MEGA-X. For molecular toxinotyping, specific primers were used for alpha and epsilon toxin genes following the method of Sattar *et al.* (2023). Amplification was confirmed by agarose gel electrophoresis using 1.5 percent agarose gel.

Physical and chemical parameters optimization for toxins production

Inoculum of bacteria was prepared following the method of Sattar *et al.* (2023). RCM broth with supplements (as mentioned above), was inoculated with 10% of inoculum (v/v). Optimization of toxins production was recorded under the influence of temperature (37, 40, 42°C), time of incubation (24, 48 and 36 h) in incubator (Lab Kits, China), 0.2%: glucose, vitamin mixture (vitamin B-complex and C) and mineral mixture (Iron, Magnesium, Zinc and Copper), tween 80 (0.1, 0.3 and 0.5%), sodium chloride (0.5, 0.75 and 1.0%) and sodium acetate (0.1, 0.3 and 0.5%) in RCM broth (Tariq *et al.,* 2022).

Estimation of alpha and epsilon toxin

Alpha and epsilon toxins were quantified throughs hemolytic assay (Hu *et al.,* 2016). Epsilon toxin containing cell free supernatant (CFS) was activated with 1% trypsin (v/v) at 37°C for 60 min incubation (inactivates alpha toxin) (Sattar *et al.,* 2023). CFS (treated and untreated) was twofold serially diluted in normal saline. Washed 1% sheep red blood cells (RBCs) were used as indicator. Optical density at 595nm was taken and hemolytic units per milli liter (HU/mL) of toxins were calculated (Bkhairia *et al.,* 2016).

Antibiotic sensitivity assay

Antibiotic sensitivity profile of *C. perfringens* type D isolates (n=07) was evaluated against quinolones group of antibiotics such as enrofloxacillin (Oxoid™), ofloxacillin (Oxoid™), ciprofloxacillin (Oxoid™), levofloxacin (Oxoid™) and moxifloxacillin (Oxoid™) antibiotics discs following the Kirby-Bauer method according to the clinical and laboratory standards institute (CLSI) latest 2020 manual.

STATISTICAL ANALYSIS

Data obtained was analyzed through one-way analysis of variance followed by post hoc Duncan's multiple range test using the statistical package for social sciences (SPSS) version 20.0 and p-value 0.05 was used as level of significance for statistical analysis.

RESULTS

Morphological and biochemical characterization

Clostridium perfringens isolates (n=26) were recognized as Gram positive rods and producing oval shaped sub terminal spores. Fifteen isolates were observed positive for double zone blood hemolysis and lecithinase activity on 5% sheep blood and egg yolk emulsion added perfringens agar medium, respectively.

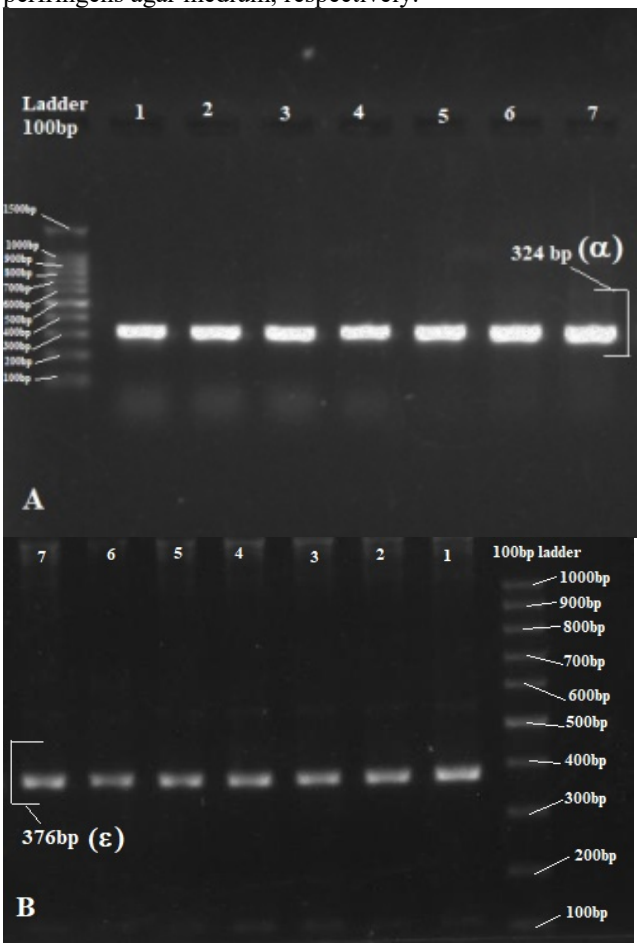

Fig. 1: Molecular typing of *C. perfringens* type D isolates by targeting alpha toxin gene and epsilon toxin gene. (A) Left to right: 100bp ladder, lane 1 to 7: 324bp amplicon of alpha toxin gene. (B) Right to left: 100bp ladder, lane 1 to 7: 376bp amplicon of epsilon toxin gene.

Molecular toxinotyping and phylogenetic analysis

Molecular typing represented that, only seven isolates were containing alpha and epsilon toxin genes with an

amplicon size of 324 and 376bp, respectively (fig. 1). Isolates were identified as *C. perfringens* toxinotype D. For *C. perfringens* toxinotype D isolates (n=07), ribosomal RNA gene amplification has been resulted in 1500bp size bands (fig. 2). Seven accession numbers were retrieved from NCBI. From which three 16S rRNA sequences (MW349974.1, MW341428.1 and MW332258.1) were used for phylogenetic analysis. MW349974.1 Pak *C. perfringens* was 94% evolutionary related to MW341428.1 Pak *C. perfringens* and both sequences 41% related to MW471067.1, MW332060.1 and MW551887.1 (fig. 3).

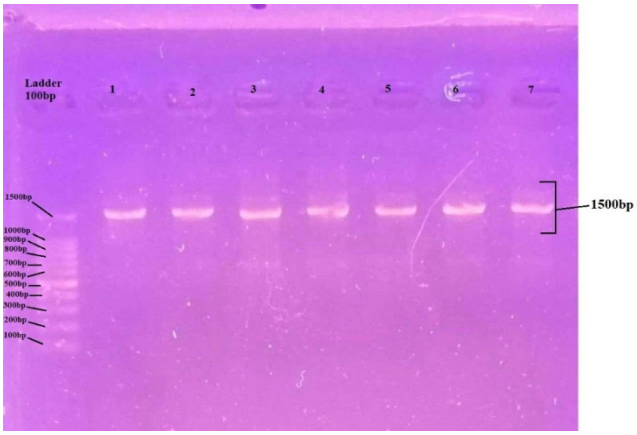

Fig. 2: Molecular typing of *C. perfringens* type D by targeting the 16S rRNA gene, Left to right: 100bp ladder, lane 1 to 7:16SrRNA gene 1500bp amplicon.

Alpha and epsilon toxin production optimization

C. perfringens toxinotype D isolate MW332258.1 has produced higher alpha and epsilon toxin hemolytic units after 24h of incubation at 37ºC. At 0.2, 0.3 and 0.5% concentration of glucose and tween 80 higher hemolytic units of alpha and epsilon toxin were produced by MW332258.1. There were significant differences (P<0.05) observed among the hemolytic units of alpha and epsilon toxin under the influence of glucose (0.2%) and tween 80 (0.3 and 0.5%), respectively (table 1 and 2).

Quinolones resistance profile

C. perfringens toxinotype D isolates were observed resistant against ciprofloxacillin and ofloxacillin. The highest mean zone of inhibition was recorded for enrofloxacillin (11.60±0.60mm) followed by moxifloxacillin (11.31±4.70mm) and levofloxacin (4.51±1.21 mm). The isolates were found resistant against ciprofloxacillin (R≤15) and ofloxacillin (R≤13).

DISCUSSION

For bacterial identification, phenotypic methods are categorized into Gram staining, colony morphology, growth requirements, enzymatic reactions, metabolic activities. Identification through biochemical test results becomes compromised due to in experiences and technological biases. Ribosomal RNA 16S gene sequence based genotypic identification emerged as accurate, objective and reliable method for bacterial identification with defining taxonomic relationships among bacteria (Franco-Duarte *et al.,* 2019). Thomas *et al.* (2014) publicized that 16S rRNA gene sequencing following amplification can be selected as alternative tool for conclusive confirmation of *Clostridium perfringens.* In current study isolates were identified as *C. perfringens* on the basis of 16S rRNA gene sequence blast analysis (95-98% sequence identity) and phylogeny. DNA-based PCR and hybridization techniques proved as reliable alternative methods to testing in laboratory (Babaie *et al.,* 2021). Though, due to presence of variability in *in vitro* production of toxins of *C. perfringens,* so there is problem in using immunological tests. Genotyping, based on PCR have become the standard test for toxin typing of *C. perfringens* (Kiu and Hall, 2018). In current study, *C. perfringens* isolates identified as toxinotype D on the basis of presence of *cpa* and *etx* toxin genes PCR.

Among the economically important diseases which affect the ruminants, enterotoxaemia (pulpy kidney) holds first rank. *C. perfringens* growth was increased after 4h of incubation at 35°C temperature (Gibson and Robert, 1986). RCM was used to grow the *C. perfringens* toxinotype D and growth was enhanced at 37°C after 48h of incubation. Length of lag phase is inversely related to maximum enzymatic activities (post lag phase) (Bertrand, 2019). In a fermenter and stationary culture *C. perfringens* active growth was started between 2-3h (Rai *et al.,* 2017). After 24h of growth at 37°C in RC medium toxinotype D alpha toxin and epsilon toxin (after trypsin treatment) hemolytic units were 4224 and 8448 HU/mL, respectively (Javed *et al.,* 2019). These observations were found in contrast to present study because after 24h of incubation at 37°C in RCM broth alpha and epsilon toxin units were observed lower. Temperature and stirring rotation per minute also affect the toxin production *in-vitro*. Higher epsilon toxin production was observed at 37°C at 100-200rpm (Fernandez Miyakawa *et al.,* 2011). It is difficult to maintain the constant pH of growth medium during cultivation, so it strictly effected the microbial growth and enzymatic activity. Lower alpha and epsilon toxin production was observed under the influence of vitamin mineral mixture, tween 80, sodium chloride and sodium acetate supplemented in RCM broth. Under the influence of 0.2% glucose supplementation, higher alpha and epsilon toxin hemolytic units were observed. These observations were in agreement to findings of Tariq *et al.* (2022) that at 0.2% glucose supplementation maximum toxin have produced.

Antibiotic resistance due the use of antibiotics in feeds as growth promotor is a sweltering issue in subcontinent (Ahmed *et al.,* 2019). Prime focus of animal feed manufacturers is to control the coccidiosis and *C. perfringens* diseases (M'Sadeq *et al.,* 2015).

Table 1: Optimization of Alpha toxin hemolytic units (HU/mL) under physicochemical parameters

Temperature (°C)	Time (h)	Alpha toxin Hemolytic Units		
		MW332258.1	MW349974.1	MW341428.1
37	24	4.53±0.04 [b]	4.26±0.20[a]	4.52±0.02[b]
	36	2.40±0.00 [a,b]	2.14±0.22[a]	2.50±0.08[b]
	48	2.38±0.04[a]	2.32±0.01[a]	2.37±0.01[a]
40	24	1.81±0.02[a]	1.74±0.05[a]	1.80±0.03[a]
	36	1.80±0.03[a,b]	1.77±0.00[a]	1.83±0.03[b]
	48	1.60±0.01[b]	1.50±0.07[a]	1.61±0.01[b]
42	24	1.28±0.06[a]	1.29±0.05[a]	1.31±0.01[a]
	36	1.08±0.01[a]	1.04±0.05[a]	1.05±0.03[a]
	48	1.01±0.01[b]	1.02±0.01[b]	0.99±0.01[a]
Chemicals	Concentration (%)			
Glucose	0.2	20.81±0.00[a]	20.41±0.36[a]	19.67±01.33[a]
Vitamin Mix	0.2	2.56±0.01[a]	2.52±0.05[a]	2.50±0.08[a]
Mineral Mix	0.2	2.58±0.02[b]	2.46±0.06[a,b]	2.42±0.11[a]
Tween 80	0.1	11.78±0.00[b]	10.81±0.05[a]	10.45±0.57[a]
	0.3	17.09±0.10[b]	16.24±0.08[a]	15.54±0.59[a]
	0.5	16.36±0.03[b]	15.56±0.73[a,b]	14.55±0.68[a]
NaCl	0.5	3.22±0.00[a]	3.08±0.12[a]	3.21±0.01[a]
	0.75	3.03±0.05[a]	2.96±0.08[a]	2.88±0.20[a]
	1	5.11±0.01[a]	4.51±0.52[a]	5.05±0.06[a]
CH_3COONa	0.1	1.76±0.00 [c]	1.72±0.00 [b]	1.66±0.02 [a]
	0.3	3.32±0.03 [a, b]	3.17±0.15 [a]	3.41±0.05 [b]
	0.5	1.67±0.00[a]	1.66±0.00[a]	1.66±0.01[a]

*Values with same superscripts (a, b, c) in rows differ non-significantly and with different superscripts differ significantly.

Table 2: Optimization of Epsilon toxin hemolytic units (HU/mL) under physicochemical parameters

Temperature (°C)	Time (h)	Epsilon toxin Hemolytic units		
		MW332258.1	MW349974.1	MW341428.1
37	24	2.73±0.02[a]	2.69±0.01[a]	2.67±0.04[a]
	36	2.60±0.01[b]	2.48±0.08[a]	2.61±0.03[b]
	48	2.46±0.03[a]	2.40±0.01[a]	2.44±0.03[a]
40	24	1.98±0.00[a]	1.99±0.00[a]	1.87±0.11[a]
	36	1.80±0.00[a]	1.76±0.04[a]	1.73±0.07[a]
	48	1.68±0.02[a]	1.69±0.02[a]	1.65±0.03[a]
42	24	1.32±0.00[a]	1.25±0.06[a]	1.20±0.10[a]
	36	1.27±0.00[a]	1.25±0.01[a]	1.25±0.01[a]
	48	1.17±0.00[a]	1.14±0.03[a]	1.16±0.00[a]
Chemicals	Concentration (%)			
Glucose	0.2	34.05±0.00[c]	33.37±0.59[b]	33.09±0.97[a]
Vitamin Mix	0.2	8.58±0.01[b]	8.48±0.08[b]	7.48±0.14[a]
Mineral Mix	0.2	8.58±0.03[a]	8.57±0.03[a]	8.43±0.11[a]
Tween 80	0.1	6.33±0.03[a]	6.23±0.12[a]	6.29±0.08[a]
	0.3	12.21±0.22[b]	11.55±0.39[a]	12.17±0.24[b]
	0.5	12.94±0.00[a]	12.43±0.48[a]	12.72±0.25[a]
NaCl	0.5	10.64±0.01[a]	9.91±0.65[a]	10.38±0.27[a]
	0.75	10.29±0.00[b]	9.82±0.40[b]	8.82±0.40[a]
	1	9.79±0.00[a]	9.33±0.40[a]	9.37±0.36[a]
CH_3COONa	0.1	4.88±0.09[a]	4.86±0.10[a]	4.84±0.04[a]
	0.3	7.40±0.00[c]	7.37±0.00[a]	7.38±0.00[b]
	0.5	5.43±0.00[a]	5.30±0.18[a]	5.43±0.00[a]

*Values with same superscripts (a, b, c) within rows not differ significantly and with different superscripts differ significantly.

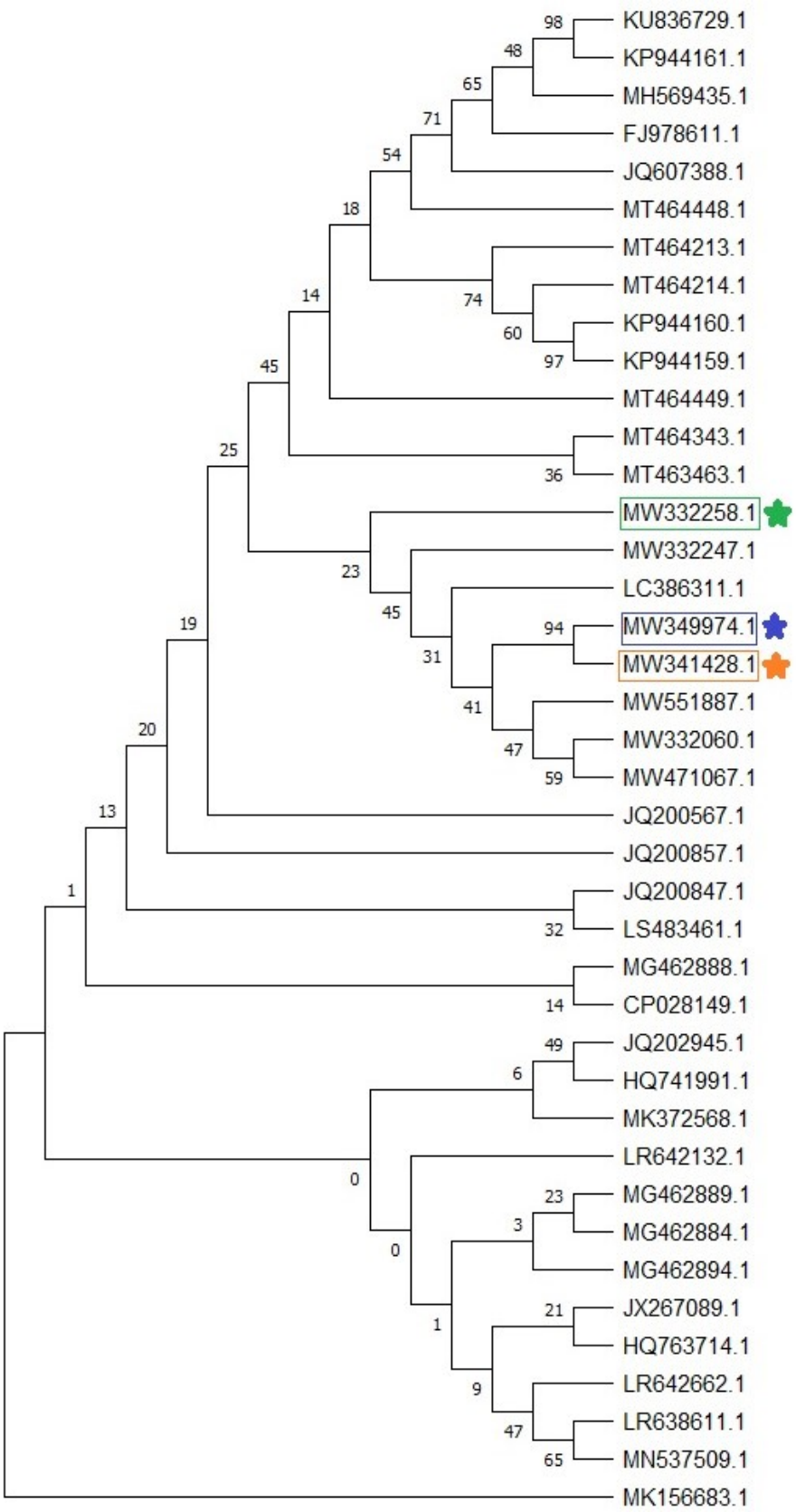

Fig. 3: Phylogenetic analysis based on 16S rRNA gene sequence (*C. perfringens* toxinotype D 16S sequences represented as colored star).

Present study revealed that *C. perfringens* toxinotype D isolates were found resistant against ciprofloxacillin (Oxoid^{TM}) and ofloxacillin (Oxoid^{TM}). The highest mean zone of inhibition was recorded for enrofloxacillin (Oxoid^{TM}) followed by moxifloxacillin (Oxoid^{TM}) and levofloxacin (Oxoid^{TM}). Results of Ali and Islam (2021) research were observed similar to findings of present study that *C. perfringens* isolates were found resistant to ciprofloxacin and norfloxacin. This is foreshadowing to veterinary and public health aspects because of variation of usage of human antibiotics in veterinary sector.

CONCLUSION

Ciprofloxacillin (Oxoid^{TM}) and ofloxacillin (Oxoid^{TM}) resistance was observed against *C. perfringens* toxinotype D isolates. To combat antimicrobial resistance: curbing use, regulations and execution to antimicrobial usage in food along with withdrawal period is necessary. *C. perfringens* toxinotype D MW332258.1 is an excellent candidate may be used to produce alpha and epsilon toxin in higher amount under optimized physicochemical parameters (0.2% glucose and 0.3% tween-80 in RCM broth at 37°C incubation for 24h). MW332258.1 may be use at large industrial scale for toxoid vaccine preparation against antibacterial resistant *C. perfringens* toxinotype D.

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