Research Article

Isolation and Molecular Identification of *Clostridium perfringens* Type D in Goats in District Peshawar

Sidra Shehzadi, Sher Bahadar Khan*, Umar Sadique and Saqib Nawaz

College of Veterinary Sciences, The University of Agriculture, Peshawar, Pakistan.

Abstract | Enterotoxaemia, caused by *Clostridium perfringens* Type D, is a disease of domestic animals particularly sheep and goat widespread in Pakistan due to endemic outbreak in every spring season; therefore the current study was conducted for isolation and molecular identification of new strains of *C. perfringens* Type D for effective diagnosis, treatment and vaccination. A total of 100 fecal samples were collected aseptically from four different zones of district Peshawar during the period of February, 2019 to April, 2019. *C. perfringens* Type D was confirmed through bacterial culturing, Gram staining, biochemical tests and polymerase chain reaction (PCR) . Results revealed that 25 feacal samples collected from suspected goats were positive for *C. perfringens* Type D and the overall prevalence of enterotoxaemia in goats in district Peshawar was 25%. The prevalence of enterotoxaemia was 24%, 44%, 20% and 12% in zone I, II, III and IV respectively. Zone II showed the highest prevalence rate of 44%. However, the colony morphology, microscopy, biochemical tests and polymerase chain reaction of *C. perfringens* type D showed that PCR is an effective diagnostic and confirmatory tool for the toxinogenic typing of *C. perfringens* type D infection.

Received | June 12, 2020; **Accepted** | November 17, 2020; **Published** | February 06, 2021
***Correspondence** | Sher Bahadar Khan, College of Veterinary Sciences, The University of Agriculture, Peshawar, Pakistan; **Email:** sbvetdr@yahoo.com
Citation | Shehzadi, S., S.B. Khan, U. Sadique and S. Nawaz. 2021. Isolation and molecular identification of clostridium perfringens type D in goats in district Peshawar. *Sarhad Journal of Agriculture,* 37(1): 110-114
DOI | http://dx.doi.org/10.17582/journal.sja/2021/37.1.110.114
Keywords | *Clostridium perfringens* Type D; Polymerase chain reaction, Goat

Introduction

Livestock play a major role in alleviating the poverty by uplifting the socioeconomic conditions of small scale farmers. Goats rank first in a list of important farm animals because it serves as a source of meat, milk and income for large number of people. Enterotoxaemia includes in a list of acute and fatal diseases lead to great economic losses in small ruminant caused by *Clostridium perfringens* type D which is gram positive bacilli with round edges, anaerobic, non-motile and toxin producing bacteria. *C. perfringens* is divided into five types A, B, C, D and E on the basis of toxins production. Four major toxins are alpha, beta, epsilon and iota (Rahaman *et al*., 2013).

Alpha toxin is produced by all strains of *C. perfringens* while beta toxins is produced by *C. perfringens* type B and C and epsilon toxin by *C. perfringens* type B and D . Delta, theta, kappa, lambda, mu, nu, gamma eta, neuraminidase and enterotoxin are nine minor toxins which also play an important role in pathogenicity (Nillo, 1986). *C. perfringens* type D is widely distributed in nature commonly present in soil, water and decomposing organic matter. It is normal inhabitant of intestine of different animal species including sheep, goat, cattle and humans but

if there is some alteration in intestine then it became pathogenic and start proliferation and produce large number of copies which are able to show both local and systemic effects (Lewis *et al*., 2000).

Epsilon toxin rank third among the most potent clostridial toxin (after botulinum and tetanus toxins). In the gastrointestinal tract, Epsilon toxin (ETX) is produced as an inactive prototoxin which is activated by photolytic degradation of the C-terminal 14 amino acids. Activated epsilon toxin (ETX) then absorbed through the route of intestine and then transported to their specified target organs i.e. Lungs, brain and kidneys (Tahir *et al*., 2013). Endothelial cells of the brain are affected by these toxins, producing perivascular edema and consequently result in the cerebral necrosis. Systemic changes in goats are pathognomonic lesions i.e. enterocolitis, hydropericardium, brain and lung edema with little and inconsistent intestinal changes.

Outbreak of this disease results in great economic losses so it is necessary to prevent this infectious disease in order to maintain the well-being of small scale farmers by overcoming through effective treatment to prevent measures (Uzal and Songer, 2008). Despite of vaccination, disease is widespread in Pakistan. Therefore, the main objectives of current study are the isolation of *C. perfringens* type D from local goats, comparison of different diagnostic techniques and to provide a base for effective treatment and vaccine production.

Materials and Methods

Selection of study area

The current study was explored in district Peshawar, Khyber Pakhtunkhwa, Pakistan to investigate the isolation and molecular identification of *C. perfringens* type D in goats in district Peshawar, Khyber Pakhtunkhwa, Pakistan. For this purpose, Peshawar district was divided into four zones comprising zone I, II, III and IV. Zone I consist of Palosi, 512, University dairy farm, Lalazar colony while zone II contains Tajabad, Achenai bala. Zone III consist of charsadda road, khattaku pull, pando and zone IV consist of GT road, haji camp and gulbahar. A total of 100 samples were collected from goats including 25 from each Zone of district Peshawar. Samples were collected aseptically, properly labeled, placed in a sterile sealable plastic bags and transported in a Coleman box at 4°C. The samples were stored at -20°C for further processing.

Bacterial isolation

Fecal samples were first washed with phosphate buffer saline to remove the debris and were then inoculated in thioglycollate broth (HI Media Laboratories Pvt. Ltd., India) and incubated in anaerobic condition for 24 hours at 37°C. After incubation 0.1ml of inoculum showing growth was subculture on tryptose sulphite cycloserine (TSC) agar (HI Media Laboratories Pvt. Ltd., India) to observe the typical blackish colonies of *C. perfringens* type D. These colonies were further confirmed through gram staining, gelatin liquefaction test and indole production test (Khan *et al*., 2018).

PCR protocol

DNA was extracted from pure culture of *C. perfringens* type D through commercially available nucleospin DNA kit (thermo scientific) following the manufacturer protocol. DNA quantity and purity was confirmed by Nanodrop (Thermo scientific Nano drop 2000c spectrophotometer) having wavelength 260/280 and quantity 340ng/µl. All PCR reactions were carried out in a thermo-cycler (Bio Rad T100).

The final volume of PCR product was made 25µl consisting of 10µl of Taq Master Max (thermo scientific), 1.75µl of forward and reverse primer (each), 3.5µl of DNA template and 8µl PCR water. DreamTaq Green PCR Master Mix contains DreamTaq DNA polymerase, 2X DreamTaq Green buffer, dATP, dCTP, dGTP and dTTP, 0.4 mM each, and 4 mM $MgCl_2$. The primer (Macrogen) used for detection of *C. perfringens* type D epsilon toxin/gene was E_etx_F 5'-ATTAAAATCACAATCATTCACTTG-3', E_etx_R 5'-CTTGTGAAGGGACATTAGAG TAA-3'with an amplicon size of 206bp as described by Khan *et al*., 2018. PCR product was run on 1% agarose gel with the addition of 2ul of ethidium bromide dye. A volume of 1ul of 1 kb DNA ladder was added to the first and last well and the remaining well were loaded with PCR product. The voltage and time was adjusted as 110 V (500 MA) for 35 minutes. Visualization of Gel was done through Gel Documentation System and the image was captured with digital camera (CASIO Japan). The data collected in current study was compiled in Microsoft Excel and analyzed through SPSS using percentage.

Results and Discussion

On PCR, A total of 25/100 (25%) samples were positive for *C. perfringens* type D. The prevalence of enterotoxaemia in zone II (44%) was higher among zone I, III and IV in which 24%, 20% and 12% respectively (Table 1). All 100 samples were first propagated in thioglycollate broth and identified by culturing on Tryptose sulphite cycloserine agar (TSC), Gram staining, Motility test and conventional biochemical tests. On TSC agar, *C. perfringens* Type D showed large grayish black colonies while on motility test by Hanging drop method it is non-motile. On gram staining *C. perfringens* Type D is purple colored, straight or slightly curved capsulated single or paired rods. Biochemically the *C. perfringens* Type D give positive result on Gelatin liquefaction test (Figure 1) and Indole test (Figure 2) while negative result on Methyl red and Voges proskaure.

Table 1: *Prevalence of enterotoxaemia on PCR in Goats.*

Areas	Sample size	Positive	Negative
512	6	0	6
Pelosi	10	1	9
University dairy farm	9	0	9
Achiny bala	10	6	4
Taj abad	8	4	4
Board	7	2	5
Charsadda road	11	3	8
Khattaku pull	6	2	4
Pando	8	3	5
GT road	12	2	10
Haji camp	8	1	7
Gulbahar	5	1	4
	100	25 %	75%

Among different confirmatory test such as gelatin liquefaction test, indole test, Gram staining and Polymerase Chain Reaction, PCR is the best diagnostic tool for the diagnosis of enterotoxaemia because it is widely used for identifying the toxinogenic gene of *C. perfringens* type D due to its high sensitivity as shown in (Table 2).

Cl. Perfringen type D is a widely distributed microorganism normally present in the gastrointestinal tract of human and most of the animal species. It is known to be the cause of food poisoning in human and enterotoxaemia in Goats. In order to overcome the risk factor, such strategies must be adopted to prevent infected animals to be the part of food chain (Mcdonel, 1986). The suspected samples of *C. perfringens* type D were identified on the basis of their cultural and morphological characteristics. During this study, thioglycollate broth (HI Media Laboratories Pvt. Ltd., India) was used for isolation of *Cl. Perfringen* type D. After incubation for 24 hours at 37°C maximum growth on thioglycollate broth in form of turbidity in comparison with the negative control. For enrichment thioglycollate inoculum were subcultured on selective media Tryptose sulphite cycloserine agar (HI Media Laboratories Pvt. Ltd., India) which produced grayish black colonies. These results are in agreement with the respect to (Cheung *et al*., 2004; Tillotson *et al*., 2002).

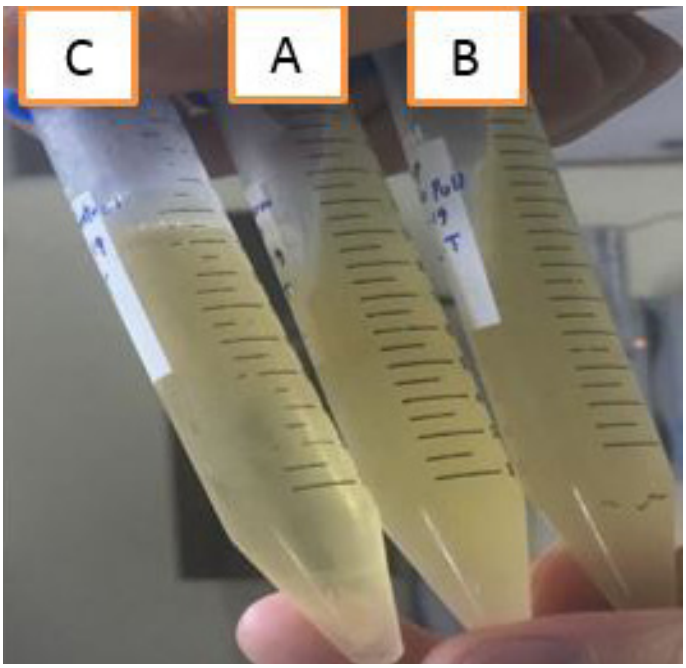

Figure 1: *Tube A and B showed liquefaction due to hydrolysis of gelatin while tube C showed solidification.*

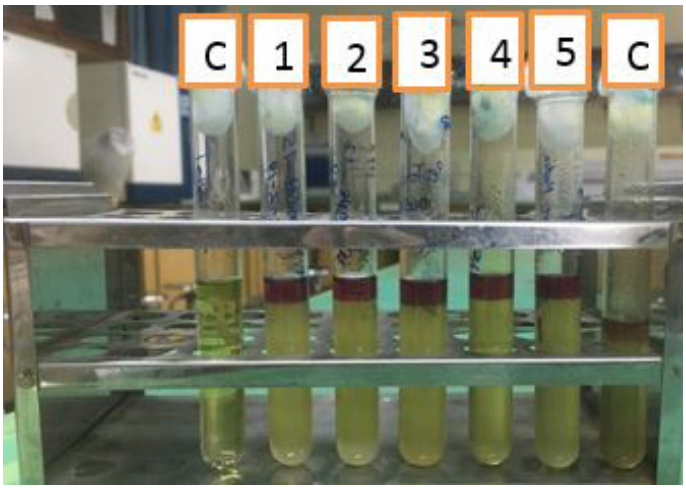

Figure 2: *Positive sample of C. perfringens Type D (tube 1-5) show positive indole by appearing red rose layer to the top while tube C are negative control.*

Table 2: *Comparison of different diagnostic tools used for the diagnosis of enterotoxaemia.*

Zone	Sample size	Polymerase chain reaction	INDOLE test	Gelatin liquefaction test	Gram staining
I	25	6	0	1	03
II	25	11	1	1	04
III	25	5	0	2	02
IV	25	3	1	1	05
Total	100	25	02	05	14

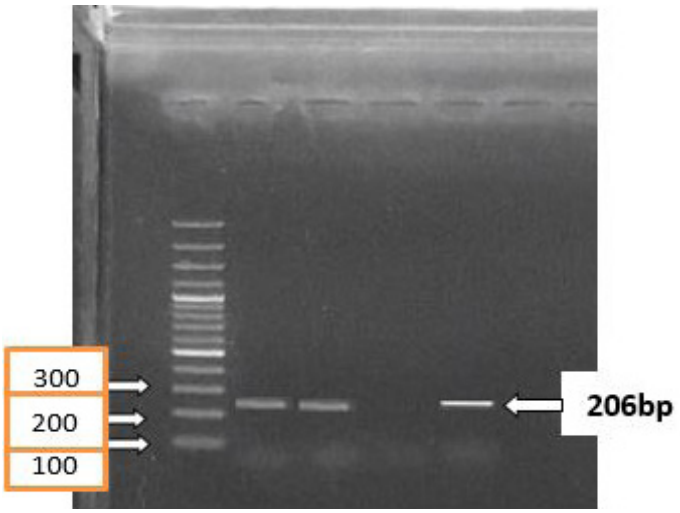

Figure 3: *C. perfringens type D on PCR, Lane 1: 1kb gene marker. Lane 2, 3 showing positive results of epsilon Toxin Gene with 206 bp clear bands. Lane 4: Show negative control while lane 5 shows positive control.*

During current study Peshawar district was divided into four zones. The Prevalence of positive isolates in zone I, II, III and IV were 24%, 44%, 20% and 12% respectively. Zone II showed highest prevalence among other zones. Overall prevalence of enterotoxaemia in goats in district Peshawar was 25%. These results are in contrast with Bachhil and Jaiswal (1989). This high prevalence in the present study could be attributed to unawareness of farmers about disease structure, poor management, nomadic system, lack of vaccination and treatment.

Gram staining from TSC agar revealed *C. perfringens* type D as some gram positive purple colored bacilli arranged in a single or paired. These isolates were found to be non-motile by hanging drop method when observed under microscope. In the present study some specific and standard biochemical tests were used for identification of *Cl. Perfringen* type D. All samples showing blackish colony and purple colored bacilli were subjected to these tests specified for *Cl. Perfringen* type D and give positive results on indole test by appearing red rose layer to the top and gelatin liquefaction test by partial or complete liquefaction of gelatin in nutrient gelatin media while negative to methyl red test. These results are similar with report of (Galizzi *et al*., 2001). Epsilon toxin (ETX) is one of the most potent bacterial toxin responsible for *C. perfringens* type D infection. It is produced as an inactive prototoxin and get activated when cleaved by proteases from the host or from *C. perfringens* type D. In the current study, extraction of DNA was carried through kit method because of fragility of cell wall of *C. perfringens* type D. A same protocol was reported by Warren *et al*. (1999); Effat *et al*. (2007) and Komoriya *et al*. (2007).

Polymerase Chain Reaction is widely used for identifying the toxinogenic gene of *C. perfringens* type D due to its high sensitivity. During this study, epsilon toxin with expected size of 206bp (Figure 3) were used which revealed that PCR is the best tool for toxinogenic typing of *C. perfringens* type D from bacterial cultures. These results are in agreement with the report of (Fach and Guillon, 1993). Out of 100 suspected fecal samples, 25 sample gave positive result by appearing bands at 206bp for epsilon toxin which revealed that *C. perfringens* type D is the most predominant type of *C. perfringens* in goats. These results are in agreement with the report of (Songer and Meer (1996).

Conclusions and Recommendations

The overall prevalence of enterotoxaemia in goat's population of study area was 25%. The Zone wise prevalence of disease in goats were 24%, 44%, 20% and 12% in the four zones respectively. Zone II presents the highest disease occurrence rate of 44%. On PCR analysis the toxigenic typing of *C. perfringens* type D revealed an amplicon size of 206bp confirmed the presence of an epsilon toxin. The comparative analysis of various diagnostic tests revealed that PCR is more sensitive as compared to biochemical tests and confirms that *C. perfringens* type D is widely spread in all the four zones of district Peshawar. The study should be proceeding further for genomic sequencing of *C. perfringens* type D, Local vaccine and Diagnostic kit should be prepared. Chemotherapeutic trial needed for effective treatment of this disease.

Novelty Statement

Molecular identification of *C. perfringens* was first

time conducted in KP province. PCR is an effective and sensitive technique for the diagnosis of C. perfringens type D as compared to conventional methods.

Author's Contribution

Sidra Shehzadi and Saqib Nawaz: Collected samples and did experiments.
Sher Bahadar Khan and Umar Sadique: Designed the study and wrote manuscript

Conflict of interest
The authors have declared no conflict of interest.

References

Bachhil, N.N. and T.N. Jaiswal. 1989. Occurrence of *Clostridium perfringens* in buffalo meat. Indian Vet. Med. J., 13: 229–233.

Cheung, J.K., M.M. Awad, S. McGowan and J.I. Rood. 2004. Functional analysis of the Vir SR phosphorelay from *Clostridium perfringens*. J. Vet. Microbiol., 105: 130-136.

Effat, M.M., Y.A. Abdallah, M.F. Soheir and M.M. Rady. 2007. Characterization of *Clostridium perfringens* field isolates, implicated in necrotic enteritis outbreaks on private broiler farms in Cairo by multiplex PCR. Afr. J. Microbial. Res., 1: 29-32.

Fach, P. and J.P. Guillon. 1993. Detection by *in vitro* amplification of the alpha-toxin gene from *Clostridium perfringens*. J. App. Bacteriol., 74: 61-66. https://doi.org/10.1111/j.1365-2672.1993.tb02997.x

Galizzi, A., F. Scoffone, G. Milanesi and A.M. Albertini. 2001. Integration and excision of a plasmid in *Bacillus subtilis*. Mol. Microbiol., 182: 99-105. https://doi.org/10.1007/BF00422774

Khan, M.A., A.Z. Durrani, S.B. Khan, S.G. Bokhari, I. Haq, I.U. Khan, N. Ullah, N.U. Khan, K. Hussain and A.U. Khan. 2018. Development and Evaluation of *Clostridium perfringens* Type D Toxoid Vaccines. Pakistan J. Zool., 50(5): 1857-1862. https://doi.org/10.17582/journal.pjz/2018.50.5.1857.1862

Komoriya, T., A. Hashimoto, A. Shinozaki, M. Inoue and H. Kohno. 2007. Study on the partial purification of produced from obligate anaerobe *Clostridium perfringens*. Rep. Res. Inst. Ind. Technol., Nihon University, Japan, pp. 1-13.

Lewis, C.J., W.B. Martin and I.D. Aitken. 2000. *Clostridial* diseases of sheep. Blackwell Sci., 97: 76-90.

Mcdonel, J.L., 1986. Toxins of *Clostridium perfringens* types A, B, C, D and E in sheep. Pharm. Bacterial Toxins, pp. 477-517.

Nillo, L., 1986. Experimental production of hemorrhagic enterotoxemia by *Clostridium perfringens* type C in maturing lambs. Can. J. Vet. Res., 50: 32–35.

Rahaman, M., M. Akhter, M. Abdullah, S. Khan, M., Jahan and M. ZiaulHaque. 2013. Isolation, identification and characterization of Clostridium perfringens from lamb dysentery in Dinajpur district of Bangladesh. Sci. J. Microbiol., 2(4): 83-88.

Songer, J.G. and R.R. Meer. 1996. Genotyping of *Clostridium perfringens* by Polymerase Chain Reaction is a useful adjunct to diagnosis of Clostridial enteric disease in animals. Anaerobe, 2: 197-203. https://doi.org/10.1006/anae.1996.0027

Tahir, M.F., M.S. Mahmood and I. Hussain. 2013. Preparation and comparative evaluation of different adjuvanted toxoid vaccines against enterotoxaemia. Pak. J. Agric. Sci., 50: 293-297.

Tillotson, N., S. Wessler and S. Backert. 2002. Role of the cag-pathogenicity island encoded type IV secretion system in Helicobacter pylori pathogenesis. FEBS J., 278: 1190-1202.

Uzal, F.A. and J.G. Songer. 2008. Diagnosis of *Clostridium perfringens* intestinal infections in sheep and goats. J. Vet. Diagn. Invest., 20:253–265. https://doi.org/10.1177/104063870802000301

Warren, A.L., F.A. Uzal, L.L. Blackall and W.R. Kelly. 1999. Polymerase Chain reaction detection of *Clostridium perfringens* type D in formalin fixed paraffin embedded tissues of goat and sheep. Lett. App. Microbial., 29: 15-19. https://doi.org/10.1046/j.1365-2672.1999.00567.x