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RESEARCH ARTICLE

Molecular Detection of *Clostridium perfringens* Type D Alpha and Epsilon Toxin Genes from Various Tissues in Lambs

Azam Ali Nasir[1,3], Muhammad Younus[2,*], Muti-ur-Rehman[3], Muhammad Latif[3], Asif Rashid[1], Rashid Ahmad[1] and Muhammad Abbas[1]

[1]Veterinary Research Institute, Zarrar Shaheed Road, Lahore Cantt-54810; [2]Department of Pathobiology, College of Veterinary and Animal Sciences, Jhang (sub-campus of UVAS, Lahore); [3]Department of Pathology, University of Veterinary and Animal Sciences, Lahore, Pakistan
*Corresponding author: younusrana@hotmail.com

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ABSTRACT

Different tissues including duodenal scrapings, liver, lungs and kidneys of each infected (n=6) and control (n=4) lambs were subjected to PCR for the identification of alpha and epsilon toxin genes of *Clostridium perfringens* type D. Alpha and epsilon toxin genes were amplified at annealing temperature 52.2 and 50.2°C with amplicon size about 247bp and 665bp, respectively. A high percentage of alpha toxin genes of *C. perfringens* type D were amplified from duodenal scrapings followed by liver, kidneys lungs in cultured broth while a low percentage was observed when PCR was attempted directly on these tissues without prior culturing in media. It was concluded that distribution of alpha and epsilon toxin genes varies in different organs being the highest in duodenal tissue and PCR is suitable assay for the typing of *C. perfringens* toxin genes.

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INTRODUCTION

Clostridium perfringens type D, the etiological agent of enterotoxemia in sheep and goats, causes great economic losses (Bentancor *et al.,* 1999; Tahir *et al.,* 2013). The disease is caused by *C. perfringens* epsilon toxin and characterized by inconsistent changes in the intestines (Fernandez Miyakawa and Uzal, 2003; Khan *et al*., 2008). Epsilon toxin when absorbed into the general circulation produced systemic effects (Souza *et al.,* 2010). There are several techniques available for the detection of the major toxins of *C. perfringens* in intestinal contents or other body fluids of animals and culture supernatants. Among these are the mouse neutralization tests (Effat *et al.,* 2007), ELISA (Babe *et al.,* 2012) and toxin genes amplification by PCR (Kaneko *et al.,* 2011; Hadimli *et al.,* 2012; Harkness *et al*., 2012). We herewith report the amplification of alpha and epsilon toxin genes from various tissues after optimized PCR conditions along with the new approach for experimental intraduodenal infection of *C. perfringens* type D in lambs. It provides an easy method for the typing of *C. perfringens* type D alpha and epsilon toxin genes and the development of more effective vaccine in different endemic areas.

MATERIALS AND METHODS

Experimental animals and inoculum: Healthy Kajli lambs (n=10), each about 10 week of age and between 10 and 12 kg body weight were procured from a private farm. These lambs were divided into two groups, six in groups A (infected) while four in group B (control). Lambs were not vaccinated nor their dams against enterotoxemia dewormed with Oxfendazole at the recommended dose (Oxafax, GlaxoWellcome) orally and monitored for 10 days before the experiments. Swiss Albino mice (20±5 g BW) procured from Veterinary Research Institute (VRI), Lahore, Pakistan were used for the mouse tests.

The suspected samples of enterotoxemia were collected from the intestinal scrapings, placed in ice bags and transported to Department of Pathology, University of Veterinary and Animal Sciences, Lahore, Pakistan for the isolation and identification of *C. perfringens* type D. Morphological characteristics, biochemical tests, mice inoculation and ELISA were performed from the cultures for the identification of *C. perfringens* type D (Babe *et al*., 2012; Javed *et al.,* 2012). The culture was maintained in reinforced *Clostridium* medium (RCM) by periodical subcultures.

Experimental Design: All lambs of infected and control groups were fasted for 24 hours before surgery and were tranquilized by intramuscular injection of xylazine hydrochloride (Xylase, Farvet Laboratory, Netherlands). A new approach was adopted to inoculate the *C. perfringens* type D intraduodenally. Briefly, lambs were placed on dorsal recumbency and a right side para-midline incision was performed about two inches from xiphoid cartilage and about two inches from midline approaching to the abomasum. The pyloric end of the abomasum and first portion of the duodenum were exteriorized. Two hundred milliliters of 20% solution of corn flour in 0.85% saline was injected into the abomasum of all lambs. Then, approximately 150 ml inoculum with 4.6×10^{8}-5.7×10^{8} CFU/ml was administered per lamb of infected group intraduodenally. The abdominal incision was closed by separate muscle layers and skin sutures. After 30 hours of experimental infection, all the lambs were slaughtered and necropsied immediately and morbid tissues were collected for culture and molecular studies. Re-isolation of *C. perfringens* type D was conducted on RCM at 37°C under anaerobic conditions. The cultured broth was centrifuged at 3000 rpm for 15 minutes and supernatant was collected. Swiss Albino mice (n=5) were injected intravenously with 0.4ml culture supernatant and observed for five days (Quinn *et al.,* 2004) while uncultured RCM was injected as control.

Detection of *C. Perfringens* by PCR: DNA was extracted by the method as described by Komoriya *et al.* (2007) with some modification. Briefly, broth culture of *C. Perfringens* was used as template after boiling followed by centrifugation while in preserved tissues, distilled water was added, vortexed and centrifuged. Aliquots of 5µl of the supernatants were used as template for PCR (Burns *et al*., 1997).

Molecular detection of *C. Perfringens* was performed by PCR amplification of the genes (Table 1) as described by different researchers (Songer and Meer, 1996; Greco *et al.,* 2005; Wu *et al.,* 2009). Briefly, PCR assay was performed using a thermal cycler (Eppendorf Master Cycler, Gradient, USA). The reactions were subjected to 35 cycles of amplification consisting of 2 minutes of denaturation, annealing at 52.2°C for 45 seconds for alpha toxin gene and 50.2°C for 45 seconds for epsilon toxin gene with extension for 45 seconds at 72°C. PCR products were visualized by UV illumination. The results were expressed as the percentage of positive genes for *C. perfringens* type D in different tissues. A chi-square test was used to compare the difference between the percentage of genes in A and B groups.

RESULTS

Gram positive and non-motile rods were observed by microscopic examination and a double zone of hemolysis was also seen on blood agar. Turbidity along with abundant gas production was observed on RCM. Colony count was about 4.6×10^{8}-5.7×10^{8} CFU/ml on blood agar. Biochemical identification showed catalase positive. Gas and acid from glucose, fructose, lactose, sucrose and mannitol were also observed. Mouse inoculation test showed the death within 3 days while control mice remained alive. The optical density (OD) of the samples for alpha and epsilon toxin showed a difference greater than 0.150 by indirect ELISA hence, declared positive.

Molecular detection of alpha and epsilon toxin genes was carried on from broth culture and also directly from different tissues. After the extraction, a sufficient amount of DNA was observed by boiling method. Alpha gene was amplified at annealing temperature 52.2°C with amplicon size 247 bp (Fig. 1) but particular gene was not amplified when the primer with expected product size 324 bp was used. The epsilon toxin gene was amplified at annealing temperature 50.2°C with amplicon size 665 bp (Fig. 2) but not with the primer of expected product size 402 bp.

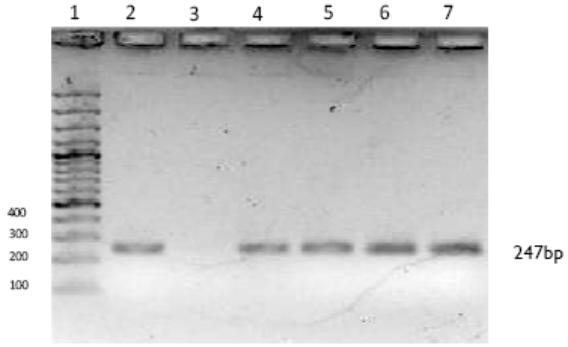

Fig. 1: PCR *Clostridium perfringens* Type D. Lane 1= 100bp DNA molecular marker, Lane 2, 4, 5, 6, 7= Cpa (alpha toxin encoding gene) corresponding in size approximately 247bp can be seen on agarose gel.

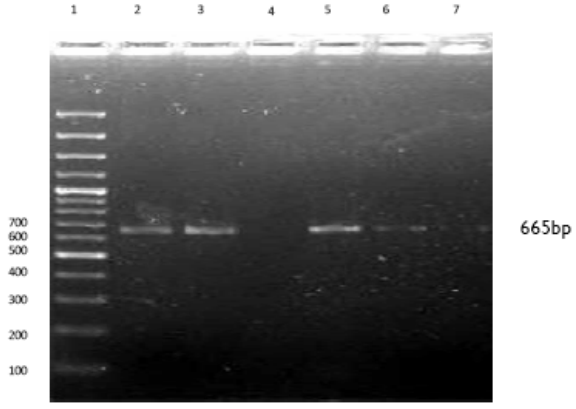

Fig. 2: PCR *Clostridium perfringens* Type D. Lane 1= 100bp DNA molecular marker, Lane 2, 3,5,6,7= PCR amplified band corresponding in size to the Epsilon (etx) toxin encoding gene about 665bp can be seen on agarose gel.

In infected group, 100% amplification for alpha toxin of *Clostridium perfringens* type D was observed in duodenal scrapings followed by liver, kidneys and lungs while from tissues directly, almost same sequence was found but at lower percentage. Epsilon toxin genes were amplified a low percentage from alpha when tissues were taken directly (Table 2).

DISCUSSION

Alpha and epsilon toxins of the *C. perfringens* type D are considered to be the major toxins, involved in the disease pathogenesis in lambs. The activity of alpha toxin

Table 1: Oligonucleotide primers for amplification of *C. perfringens* type D alpha and epsilon toxin genes

Gene	Target Primer sequence (5 to 3)	TM °C	Expected	Reference
Cpa (F)	TGCTAATGTTACTGCCGTTGATAG	55.4	247	(Greco *et al.,* 2005)
Cpa(R)	ATAATCCCAATCATCCCAACTATG	52.5		
Etx(F)	GCGGTGATATCCATCTATTC	50.7	665	(Wu *et al.,* 2009)
Etx (R)	CCACTTACTTGTCCTACTAAC	50.2		

Table: 2: Amplification of alpha and epsilon toxin genes from broth culture and tissues in lambs of group A and B

Sample	Toxins	Group	Duodenum			P value	Liver			P value	Kidney			Lungs			P value
			No. of positive	%	Chi^2 Value		No. of positive	%	Chi^2 Value		No. of positive	%	Chi^2 Value	No. of positive	%	Chi^2 Value	
Broth	Alpha	A*	6	100	28.57	0.00	5	83	141.88	0.00	5	83	141.88	4	66	98.50	0.00
		B	3	75			0	0			0	0		0	0		
	Epsilon	A*	6	100	120	0.00	4	66	98.50	0.00	4	66	98.50	4	66	98.50	0.00
		B	1	25			0	0			0	0		0	0		
Tissue	Alpha	A*	6	100	66.66	0.00	3	50	66.66	0.00	3	50	66.66	2	33	100.75	0.00
		B	2	50			0	0			0	0		0	0		
	Epsilon	A*	4	66	98.5	0.00	2	33	100.75	0.00	2	33	100.75	1	17	18.75	0.00
		B	0	0			0	0			0	0		0	0		

A=Infected group (n=6), B=Control group (n=4); * Significant difference between the groups; df = 1

is lethal, necrotizing and hemolytic where as epsilon is considered as lethal and necrotizing (Quinn *et al.,* 2004).

DNA extraction of *C. perfringens* by boiling method was found suitable during the current study. This might be due to fragility of cell wall of *C. perfringens* type D which easily extracted the DNA from bacteria. A similar procedure was reported by Warren *et al*. (1999) while extracting the DNA from formalin-paraffin embedded tissue. Songer and Meer (1996) used boiling method for the DNA extraction of *C. perfringens* from field samples. Similar procedures were adopted by Effat *et al.* (2007) and Komoriya *et al*. (2007), who extracted the genomic DNA of *C. perfringens* by boiling method. It is suggested that boiling method is an effective, cheap and less time consuming but should be used in fresh culture about 18 hours because with the passage of time, it is difficult to break the bacterial cell wall. In this study, the alpha primers with expected amplicon size 324bp of Wu *et al.* (2009) and primers for epsilon toxin with expected size 402bp of Songer and Meer, (1996) did not amplify any products from extracted DNA even after repeated optimization attempts. A same situation was observed by Wang *et al.* (2011) who unable to amplify the *C. perfringens* β_2 genes from the primers used by Gurjar *et al.* (2007). This inconsistency may be due to melting temperature of forward and reverse primer, lack sufficient sensitivity of primer for the detection of the genes and different optimized conditions. The amplified epsilon toxin gene with product size 667bp as described by Wu *et al*. (2009) correlates with current study.

It was found that *C. perfringens* type D has tissue tropism in duodenum followed by liver, kidney and lungs. Our studies suggested that identification of epsilon toxin genes from any organ other than duodenum may be indicative of *C. perfringens* infection as not from a single case epsilon gene was amplified in lambs of the control group. The distribution of *C. perfringens* varies in different organs within a species.

Fach and Guillou (1993) tried to find out the reasons for the poor efficacy by PCR amplification from tissue sections directly and suggested that the presence of mediators in intestinal contents which may inhibit DNA polymerase and thus hamper the PCR. Similarly, Miller *et al*. (1997) reported that apart from tissue inhibitors, amount of tissue used for DNA recovery is small as compared to bacterial culture. A low rate of positivity was also recorded by Ferrarezi *et al*. (2008) which may reflect a problem with template preparation or viability. The current study revealed that *C. perfringens* type D is not a common inhabitant because in animals of control groups, epsilon toxin gene was amplified only from duodenum of one animal only. These findings are closely matched with the observations of Miyashiro *et al*. (2007) who reported that *C. perfringens* type D is best known pathogen type that cause disease in sheep and goats and appear to have cosmopolitan in distribution but not a normal inhabitant. Hadimli *et al*. (2012) recorded the highest percentage of alpha toxin genes from lambs suspected for enterotoxemia which support our findings. Wang *et al.* (2011) collected fecal samples from healthy cattle and performed duplex PCR which detected only 8.5% epsilon toxin genes which support our findings. Similarly, Radostitis *et al.* (2006) reported that about 61% of healthy goats carried any type of enteric clostridia but only 3% was *C. perfringens* type D. The role of clostridia was assessed by Greco *et al.* (2005) in neonatal diseases of lambs and kids and found the most prevalent toxin-type of C. *perfringens* was type A about 84% followed by *C. perfringens* type D (16%).

Conclusion: PCR is a good tool for the toxinogenic typing of *C. perfringens* type D in cultures and from tissues and the distribution of genes vary in different organs. The amplification of epsilon toxin gene is of great significance in establishing enterotoxemia and differentiating from *Salmonella, E. coli* and *Cryptosporidium* infections. Although the efficiency of the assay is not very good from direct tissues but can be improved by other DNA extraction methods and samples from different sites as bacteria are unevenly distributed in the tissues.

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REFERENCES

Babe T, B Jafari and S Bahmanpour, 2012. Typing of toxigenic isolates of *Clostridium perfringens* by ELISA in ostrich. Afr J Micobiol Res, 6: 1766-1769.

Bentancor AB, MR Fermepín, LD Bentancor and RA de Torres, 1999. Detection of the etx gene (O-toxin inducer) in plasmids of high

molecular weight in *Clostridium perfringens* type D. FEMS Immunol Med Microbiol, 24: 373-377.

Burns WC, YS Liu, C Dow, RJ Thomas and WA Phillips, 1997. Direct PCR from paraffin-embedded tissues. Biotechniques, 22: 638-640.

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

Fach P and JP Guillou, 1993. Detection by in vitro amplification of the alpha-toxin (phospholipase C) gene from *Clostridium perfringens.* J App Bacteriol, 74: 61-66.

Fernandez Miyakawa ME and FA Uzal, 2003. The early effects of *Clostridium perfringens* type D epsilon toxin in ligated intestinal loops of goats and sheep. Vet Res Commun, 27: 231-241.

Ferrarezi MC, TC Cardoso and IS Dutra, 2008. Genotyping of *Clostridium perfringens* isolated from calves with neonatal diarrhea. Anaerobe, 14: 328-331.

Greco G, A Madio, D Buonavoglia, M Totaro, M Corrente, V Martella and C Buonavoglia, 2005. *Clostridium perfringens* toxin-types in lambs and kids affected with gastroenteric pathologies in Italy. Vet J, 170: 346-350.

Gurjar AA, NV Hegde, BC love and BM Jayarao, 2007. Real-time multiplex PCR assay for rapid detection and toxin typing of *Clostridium perfringens* toxin producing strains in feces of dairy cattle. Mol Cellular Probes, 22: 90-95.

Hadimli HH, O Erganiş, Z Sayin and Z Aras, 2012. Toxinotyping of *C. perfringens* isolates by ELISA and PCR from lambs suspected of enterotoxemia. Turk J Vet Sci, 36: 409-415.

Harkness JM, J Li and BA McClane, 2012. Identification of a lambda toxin-negative *Clostridium perfringens* strain that processes and activates epsilon prototoxin intracellularly. Anaerobe, 18: 546-552.

Javed S, M Rafeeq, MM Tariq, MA Awan, N Rashid and M Ali, 2012. Study on in vitro biochemical growth characterization and assessment of hemolytic toxin of *Clostridium perfringens* type B and D. Pak J Zool, 44: 1575-1580.

Kaneko I, K Miyamto, K Mimura, N Yumine, H Utsunomiya, S Akimoto and BA McClane, 2011. Detection of enterotoxigenic *Clostridium perfringens* in meat samples by using molecular methods. Appl Environ Microbiol, 77: 7526-7532.

Khan A, I Ali, I Hussain and N Ahmad, 2008. *Clostridium perfringens* type D enterotoxaemia in the Chinkara Deer (*Gazella bennettii*). Turk J Vet Anim Sci, 32: 225-228.

Komoriya T, A Hashimoto, A Shinozaki, M Inoue and H Kohno, 2007. Study on the partial purification of produced from obligate anaerobe *Clostridium perfringens.* Report of the Research Institute of Industrial Technology, Nihon University, Japan, pp: 1-13.

Miller J, A Jenny, J Rhyan, D Saari and D Suarez, 1997. Detection of *Mycobacterium bovis* in formalin-fixed paraffin-embedded tissues of cattle and elk by PCR amplification of an IS6110 sequence specific for *Mycobacterium tuberculosis* complex organisms. J Vet Diagn Invest, 9: 244-249.

Miyashiro S, AFC Nassar, C Del Feva, AD Cabral and M Silva, 2007. *Clostridium perfringens* types A and D associated with enterotoxemia in an 18- month-old goat. J Venom Anim Toxins Trop Dis, 13: 885-893.

Quinn PJ, MA Carter, B Markey and GR Carter, 2004.Clinical Veterinary Microbiology. 2Ed: Elsevier - Health Sciences Division. USA, pp: 191-208.

Radostitis OM, CC Gay, KW Hinchcliff and PD Constable, 2006. Diseases associated with *Clostridium* species. In: Veterinary Medicine. 10 Ed. WB Saunderss Company Ltd. London, UK, pp: 841-843.

Songer JG and RR 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.

Souza AM, JK Reis, RA Assis, CC Horta, FF Siqueira, S Facchin, ER Alvarenga, CS Castro, FM Salvarani, RO Silva, PS Pires, C Contigli, FC Lobato and E Kalapothakis, 2010. Molecular cloning and expression of epsilon toxin from *Clostridium perfringens* type D and tests of animal immunization. Gene Mol Res, 18: 266-276.

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

Wang G, J Zhou, F Zheng, G Lin, X Cao, X Gong and Q Changqing, 2011. Detection of different genotypes of *Clostridium perfringens* in feces of healthy dairy cattle from China using Real-Time Duplex PCR assay. Pak Vet J, 31: 120-124.

Warren AL, FA Uzal, LL Blackall and WR Kelly, 1999. PCR detection of *Clostridium perfringens* type D in formalin fixed, paraffin-embedded tissues of goats and sheep. Lett Appl Microbiol, 29: 15-19.

Wu J, W Zhang, B Xie, M Wu, X Tong, J Kalpoe and D Zhang, 2009. Detection and toxin typing of *Clostridium perfringens* in formalin-fixed, paraffin-embedded tissue samples by PCR. J Clin Microbiol, 47: 807-810.