The Pharma Innovation Journal 2022; SP-11(7): 1248-1251

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2022; SP-11(7): 1248-1251

www.thepharmajournal.com
Received: 16-04-2022
Accepted: 24-05-2022

Renu
IIVER, Bahu Akbarpur, Rohtak, Haryana, India

H Dadhich
Department of Veterinary Pathology, CVAS, Bikaner, Rajasthan, India

M Mathur
Department of Veterinary Pathology, CVAS, Bikaner, Rajasthan, India

PK Boyal
Help in Suffering, Maharani Farm, Durgapura, Jaipur, Rajasthan, India

M Mehra
Department of Veterinary Pathology, CVAS, Bikaner, Rajasthan, India

S Rani
Department of Veterinary Pathology, CVAS, Bikaner, Rajasthan, India

Prakash
Department of Animal Genetics & Breeding, CVAS, Bikaner, Rajasthan, India

Corresponding Author
Renu
IIVER, Bahu Akbarpur, Rohtak, Haryana, India

Molecular identification of species specific and epsilon gene of *Clostridium perfringens* type D in sheep

Renu, H Dadhich, M Mathur, PK Boyal, M Mehra, S Rani and Prakash

Abstract
Clostridium perfringens type D secrets epsilon toxin which causes enterotoxemia in sheep. Enterotoxemia can cause acute or superacute disease, with sudden death of the affected animal. It induces huge economic losses when large numbers of livestock are affected. The present study was conducted to molecular identification of the bacterial species specific and toxigenic gene from 16s rRNA with product size 481bp and epsilon (etx) with product size 655bp. A detailed necropsy was performed on 362 sheep irrespective of age, sex and breeds. Out of these, 123 sheep suspected for enterotoxemia were processed for molecular confirmation of *Clostridium perfringens* type D from intestinal contents by using PCR. After conducting PCR, 66 sheep found positive for *Clostridium perfringens* type D.

Keywords: *Clostridium perfringens*, epsilon, PCR, sheep

Introduction
Clostridium perfringens is a rod-shaped, gram-positive, sporulating and anaerobic bacillus, which produces the largest number of toxins. The genus clostridium includes various significant human and animal pathogens. The genus clostridium is categorized into several species on the basis of mode and site of action of their potent exotoxin. Among these, *Clostridium perfringens* is an important pathogenic organism in sheep. *Clostridium perfringens* presents normally in the G.I. tract of animals and human (Cato *et al*. 1986) [2]. According to the presence of four major lethal toxins (alpha, beta, epsilon and iota), *Clostridium perfringens* is classified into 5 toxigenic types (A to E). Type A produces alpha toxin, type B produces alpha, beta and epsilon toxins, type C produces alpha and beta toxins, type D produces alpha and epsilon and type E produces alpha and iota toxins (Jolivet *et al*. 1988) [8] (Hatheway, 1990) [5]. Toxinotype D is responsible for enterotoxemia which is a fatal, economically important disease of sheep found worldwide. Enterotoxemia affects all ages of sheep but lambs under 10 weeks of age are most susceptible as they are nursed by heavy-lactating ewes and the weaned lambs on lush pasture or in feedlots (Songer, J.G. 1999) [13] (Jemal *et al*. 2016) [7]. ETX is the third most potent clostridial toxin after botulinum and tetanus toxins. It is produced as an inactive prototoxin in the gastrointestinal tract of animals and then activated by proteolytic removal of the C-termial 14 amino acids (Minami *et al*. 1997) [10]. Activated ETX is absorbed through the intestinal tract and transported to various target organs, including the brain, lungs, and kidneys (Uzal and Songer 2008) [14]. The present study was conducted to identify *Clostridium perfringens* in sheep by PCR. PCR revealed the fragment length at 481bp species specific and), 655 bp for (epsilon) toxins.

Material and methods
Collection of samples for molecular identification of *Clostridium perfringens* type D: Intestinal contents were collected from enterotoxemia suspected necropsied sheep. Samples were collected in sterilized biohazard polybags and transport to laboratory in icebox filled with ice, and stored at 4°C until used.

Isolation of genomic DNA: DNA from the intestinal contents samples of sheep was using QIAamp Fast DNA stool mini kit (cat. No.51604) as per the following protocols and quality and quantity of genomic isolated DNA was done by agarose gel electrophoresis and UV-Spectrophotometer method.
16S rRNA gene based species specific identification of *Clostridium perfringens* type D was done as per the method described by (Hart *et al*. 2015) [4] (Nazki *et al*. 2017) [11].

The sequence of the primer pair for 16S rRNA gene used was as follows:
Forward primer: 5' TAA CCT GCC TCA TAG AGT 3'
Reverse Primer: 5' TTT CAC ATC CCA CTT AAT C 3'

Amplification of DNA for 16S rRNA gene species specific identification- The reaction mixture (total 25µl) was prepared using Thermo Fisher Scientific (USA) gene amplification master mix and PCR reaction was prepared as summarized below.
The expected product size was 481bp

Table 1: Showing composition of master mix for PCR reaction for species specific Identification of 16S rRNA

No.	PCR components	Quantity
1.	Master mix	12.5 µl
2.	Primer-F (10 pM/µl)	1 µl
3.	Primer-R (10 pM/µl)	1 µl
4.	Template DNA	3 µl
5.	MiliQ water	7.5µl
Total		25µl

The PCR was performed in applied biosystem using following cycling parameters:

Table 2: Showing steps in species specific PCR reaction for 16S rRNA

Step No.	Temperature	Time
1.	Initial denaturation (95°C)	15 min
2.	Denaturation (94°C)	30 sec
3.	Annealing (49.5°C)	1 min
4.	Extension (72°C)	1.30 min
5.	Cycles	35
6.	Final extension (72°C)	10 min
7.	Hold (4°C)	-

Amplification of DNA for epsilon (ε) gene amplification for *Clostridium perfringens* type D: Epsilon (ε) gene amplification was done as per method described by (Kumar *et al*. 2014) [9] (Nazki *et al.* 2017) [11].
The sequence of the primer pair for epsilon gene was used as follows:

Table 3: The sequence of primers with the respective product sizes

Type of gene	Primer sequence 5'-3'	Product size (bp)
Epsilon *Etx*	F-GCG GTG ATA TCC ATC TAT TC	655
	R-CCA CTT ACT TGT CCT ACT AAC	

Amplification of DNA for gene identification
The reaction mixture (total 25µl) was prepared using ThermoFisher Scientific (USA) gene amplification master mix and PCR reaction was prepared as summarized below.

Table 4: Composition of master mix for PCR reaction for epsilon gene identification

No.	PCR components	Quantity
1.	Master mix	12.5 µl
2.	Primer-F (10 pM/µl)	1 µl
3.	Primer-R(10 pM/µl)	1 µl
4.	Template DNA	3 µl
5.	MiliQ water	7.5µl
Total		25µl

The PCR was performed in applied biosystem using following cycling parameters:

Table 5: Steps in gene specific PCR reaction

Step No.	Temperature	Time
1.	Initial denaturation (94°C)	3 min
2.	Denaturation (94°C)	1 min
3.	Annealing (53°C)	1 min
4.	Extension (72°C)	1 min
5.	Cycles	35
6.	Final extension (72°C)	10 min
7.	Hold (4°C)	-

Scoring of the PCR products: The PCR products (for species specific PCR) were subjected to electrophoresis at 100 V/cm for 1-2 hr (depending upon length of the gel or till the dye migrated more than half of the length of the gel) in 1.2% agarose gel prepared in 1x TBE buffer containing 0.5 ng/ml of ethidium bromide. The PCR products (5µl) were run along with 100 bp DNA ladder (Thermoscientific) and amplicons were visualized under UVP Gel Doc Bioimaging System.
PCR bands were designated on the basis of their molecular size (length of DNA fragment amplified). The molecular size was estimated by comparing with molecular weight marker loaded simultaneously with each primer product in gel. The distance run by amplified fragments from the well was translated to molecular size with reference molecular weight marker.

Results and Discussions
In the present investigation, a detailed necropsy was performed on 362 sheep irrespective of age, sex and breeds. Out of these, 123 sheep suspected for enterotoxemia were processed for molecular confirmation of species specific and one toxigenic gene epsilon (etx) of *Clostridium perfringens* type D from intestinal contents by using PCR.
On gel electrophoresis analysis, an amplicon yielding 481 bp for species specific (Fig.1) and 655 bp for epsilon (etx) (Fig.2) were procured in 66 samples. Similar to the present study, Ahsani *et al*. (2010) [1] identified the toxin genes yielding amplified products at the same regions viz. 655bp (e/ etx) using multilplex PCR. Earlier reports also described the identification of this toxigenic genes using various primers that amplified different areas of toxin genes with a product size of 206 bp (etx) by Elsify *et al.* (2016) [3], 376 bp (etx) by Rasool *et al*. (2017) [12], 376 bp (etx) by Hussain *et al.* (2018) [6] respectively using multiplex PCR.

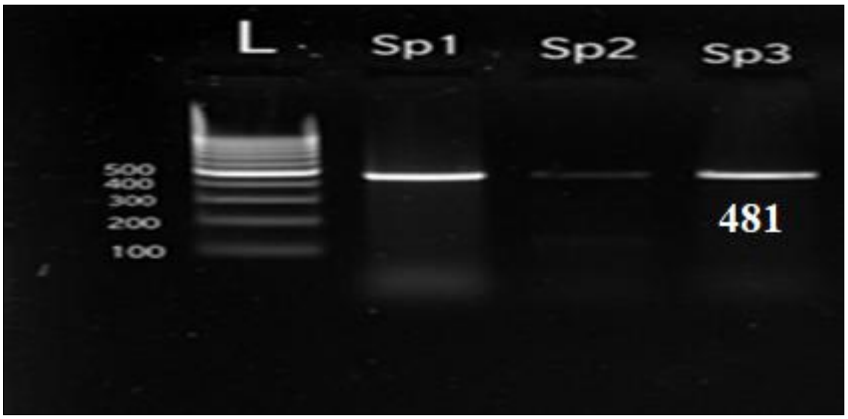

Fig 1: 16S rRNA based (PCR) confirmation of *Clostridium perfringens* type D showing 481 bp amplified products with the reference of 100bp DNA ladder.

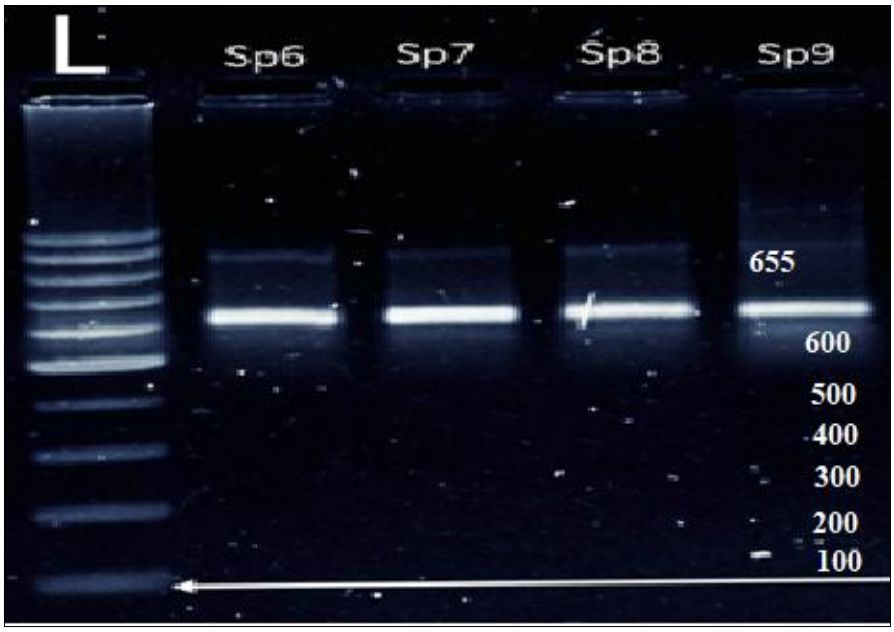

Fig 2: PCR amplicon 655bp with the reference of 100bp DNA ladder of epsilon gene of *Clostridium perfringens* type D

Conclusion

It is concluded that *Clostridium perfringens* type D is mainly responsible for enterotoxemia in sheep which is fatal disease that causes economically losses. It is confirmatory diagnosed by PCR.

Acknowledgement

Dr. Vishnu Sharma Former Hon'ble Vice Chancellor of RAJUVAS, Bikaner and Dr. R. K. Singh Dean, C.V.A.S. Bikaner for providing all facilities in this institution required during present investigation. Mr. Om Prakash gave me technical help during this research, Mangal Chand, MrSaleem and Jitendra also helped me, providing material for this research.

References

1. Ahsani MR, Mohammadabadi MR, Shamsaddini MB. *Clostridium perfringens* isolate typing by multiplex PCR. Journal of Venomous Animals and Toxins. 2010;16(4):573-578.
2. Cato EP, George WL, Finegold SM Genus *Clostridium prazmowski* 1880, 23 AL. In: Sneath, P.H.A., Mair, N.S., Sharpe, M.E. and Holt, J.G., editors. Bergey's Manual of Systematic Bacteriology. The Williams & Wilkins Co., Baltimore. 1986;1:1141-1200.
3. Elsify A, Tarabess R, Nayel M, Salama A, Allaam M, El-Gaber AM. Bacteriological and molecular studies on *Clostridium perfringens* isolated from sheep in three Egyptian provinces. African Journal of Microbiology Research. 2016;10(20):725-732.
4. Hart ML, Meyer A, Johnson PJ, Ericsson AC. Comparative Evaluation of DNA Extraction Methods from Feces of Multiple Host Species for Downstream Next-Generation Sequencing. PLoS One. 2015;10(11):1-16.
5. Hatheway CL. Toxigenic clostridia. Clinical Microbiology Reviews. 1990;3:66-98.
6. Hussain K, Ijaz M, Farooqi SH, Rizvi SNB, Ali A, Ghaffar A, *et al*. Molecular Characterization of *Clostridium perfringens* Toxino-types and Type 'D' Multidrug Resistance Profile in Diarrheic Sheep. Pakistan Veterinary Journal. 2018;38(3):271-275.
7. Jemal D, Shifa M, Kebede B. Review on pulpy kidney disease. Journal of Veterinary Science & Technology. 2016;7(5):361.
8. Jolivert-Reynaud C, Moreau H, Alouf JE. Purification of alpha toxin from *Clostridium perfringens*; phospholipase. Methods in Enzymology. 1988;165:91-94.

9. Kumar VN, Sreenivasulu D, Reddy YN. Prevalence of *Clostridium perfringens* toxin genotypes in enterotoxemia suspected sheep flocks of Andhra Pradesh, Veterinary World. 2014;7(12):1132-1136.
10. Minami J, Katayama S, Matsushita O, Matsushita C, Okabe A. Lambda-toxin of *Clostridium perfringens* activates the precursor of epsilon-toxin by releasing its N- and C-terminal peptides. Microbiology and Immunology. 1997;41:527-535.
11. Nazki S, Wani SA, Parveen R, Ahangar SA, Kashoo ZA, Hamid S, *et al*. Isolation, molecular characterization and prevalence of *Clostridium perfringens* in sheep and goats of Kashmir Himalayas, India. Veterinary World. 2017;10(12):1501-1507.
12. Rasool S, Hussain I, Wani SA, Kashoo ZA, Beigh Q, Nyrah, Q. Molecular Typing of *Clostridium perfringens* Isolates from Faecal Samples of Healthy and Diarrhoeic Sheep and Goats in Kashmir. International Journal of Current Microbiology and Applied Sciences. 2017;6(10):3174-3184.
13. Songer JG. Clostridial enteric diseases of domestic animals. Clinical Microbiology Reviews. 1999;9(2):216-234.
14. Uzal FA, Songer JG. Diagnosis of *Clostridium perfringens* intestinal infections in sheep and goats. Journal of Veterinary Diagnostic Investigation. 2008;20:253-265.