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Molecular identification of *Clostridium perfringens* from intestine of sheep

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Abstract
Clostridium perfringens is a gram-positive, rods shaped, non-motile, and toxin-producing anaerobe responsible for producing several diseases in animals. It is widely spread in the environment and is normally found in the gastrointestinal tract of animals. The pathogenicity of this organism is associated with exotoxins. *Clostridium perfringens* is normally present in the intestine of animals and humans. The present study was conducted to molecular identification of the bacterial species specific gene from 16s rRNA with product size 481bp. 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* from intestinal contents by using PCR. After conducting PCR, 66 sheep found positive for *Clostridium perfringens*.

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

Introduction
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. It is gram positive, rod shaped, spore forming and anaerobic bacillus. *Clostridium perfringens* reside normally in the G.I. tract of animals and human (Cato *et al.* 1986) [1]. 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) [5], (Hatheway, 1990) [4]. Moreover, *Clostridium perfringens* type F and G has been discovered (Rood *et al.*, 2018). More than 15 different toxins secreted by *Clostridium perfringens* are responsible for its virulence, several of which have lethal properties (McDonel, 1986) [6] (Hatheway, 1990) [4]. It is responsible for causing a wide range of diseases, such as food poisoning, enterotoxemia, gas gangrene, lamb dysentery, hemorrhagic enteritis in humans and many severe enterotoxemic diseases in domestic animals. It normally presents in quiescent state in intestine of sheep that multiplies and produces toxins during overeating and abrupt change in diet (Fernandez-Miyakawa *et al.* 2007) [2]. This toxin then passes through the intestinal barrier and spreads into several organs, causing general edema, toxic shock and death.

Material and Methods
The affected tissue samples of intestine from carcasses of sheep were collected for proposed investigation irrespective of sex, age groups and breeds from various Veterinary hospitals, rural areas in and around Bikaner district of Rajasthan. The samples received from field veterinarians in the Department of Veterinary Pathology were also included in this study.

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) [3] (Nazki *et al*. 2017) [7].

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 bio system 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)	-

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 *Clostridium perfringens* were processed for molecular confirmation of species specific *Clostridium perfringens* from intestinal contents by using PCR. After conducting PCR, 66 sheep found positive for *Clostridium perfringens*. On gel electrophoresis analysis, an amplicon yielding 481bp for species specific gene was procured in 66 samples (fig. 1). Similar to the present study (Hart *et al*. 2015) [3] and (Nazki *et al*. 2017) [7] identified the species specific gene yielding amplified products at the same regions.

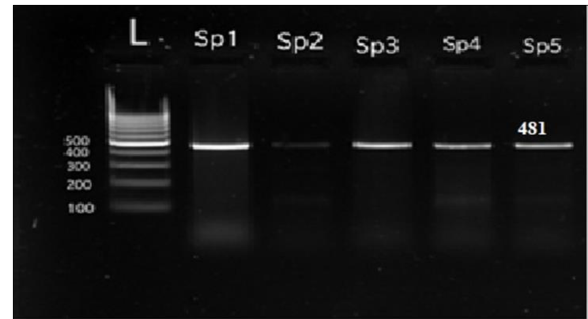

Fig 1: 16S rRNA based (PCR) confirmation of *Clostridium perfringens* type D showing 481 bp amplified products with reference of 100bp DNA ladder

Conclusion
It is concluded that *Clostridium perfringens* in sheep produces several diseases but it mainly causes enterotoxemia which is responsible for heavy economic losses to sheep industry

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