The Haryana Veterinarian (December, 2024) **63(2),** 201-205 ISSN 0033-4359 (P) **Research Article**

MOLECULAR DETECTION OF ANTIBIOTIC RESISTANT GENES AND ENUMERATION OF *CLOSTRIDIUM PERFRINGENS* FROM ENTEROTOAXAEMIA SUSPECTED CASES OF SHEEP

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SUMMARY

The present study was envisaged with an aim to study the antibiotic resistance genes of *Clostridium perfringens* and to enumerate the bacterial count among healthy and diseased sheep. A total of 50 samples from enterotoxaemia suspected flocks and 25 healthy flocks were collected from different regions of western Maharashtra. The recovered isolates were phenotypically identified as presumptive *C. perfringens* and further genotypically confirmed with 16SrRNA PCR. Antibiotic sensitivity test was performed on all 56 isolates of *C. perfringens*, followed by processing for extended spectrum beta-lactamases (ESBL) and tetracyline resistant gene PCR. Also, enumeration of *C. perfringens* was done by colony forming unit (CFU). Antibiotic sensitivity test for the *C. perfringens* isolates revealed the highest sensitivity towards Enrofloxacin (46.4%) followed by Amikacin (26.7%). The isolates were found resistant to Oxytetracycline (100%) followed by Ceftriaxone Tazobactam (91%). The PCR detection of beta -lactamases resistance genes revealed *bla*TEM (92.8%), *bla*CTX-M (28.6%) and *bla*SHV (10.7%) genes while tetracycline resistance genes showed *tet*B (48.21%) and *tet*A (27%). Enumeration of *C. perfringens* count from enterotoxemia suspected sheep revealed a higher bacterial count (35.8%) as compared to the healthy animal. The majority of the isolates in the present study had multidrug resistance patterns with beta -lactamases and tetracycline resistant genes posing threat to animal and public health.

Keyword: Antibiotic resistance, *Clostridium perfringens*, Enterotoxaemia, ESBL, Tetracyline resistant genes

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Sheep are the predominant domestic ruminants, widely distributed in the rural part of India and are one of the primary contributors to the livestock industry. Among the various infectious diseases of sheep, enterotoxaemia plays a significant role with high case fatality rates leading to considerable economic losses to the farmers. Enterotoxaemia in sheep is caused by different toxinotypes of *Clostridium perfringens* that produce different toxins. The bacterium *C. perfringens* produces the main toxins alpha (α), beta (β), epsilon (ε), iota (ι), enterotoxin (*cpe*) and *net*B, which categorize this into seven different types, from A to G (Rood *et al.*, 2018).

Clostridium perfringens is a spore-forming, Gram-positive, rod that causes a variety of toxic-specific lesions and gastrointestinal (GI) diseases in sheep which is highly toxigenic bacteria in nature due to its ability to produce spores and large number of toxins in unfavourable conditions leading to enterotoxaemia (Van Asten *et al.*, 2010).

Stressful conditions and prolonged use of antimicrobial agents have led to the problem of development of resistance among *C. perfringens* and increase in the pathogenic power of the bacteria worldwide (Anju *et al.*, 2021). For the treatment of bacterial infections, mostly β-Lactam antibiotics are widely used which is leading to the development of antibiotic-resistant bacteria, causing serious untreatable diseases. To overcome this, simple and rapid methods for the screening of antibiotics that show β-lactamase resistance are required. Some of the studies, detected the presence of β-lactamase and tetracycline resistant genes of *C. perfringens* in chicken and pigs (Ali and Islam, 2021 and Li *et al.*, 2021). In this study, genotypic characterization of the antibiotic-resistant gene is studied to understand the resistant pattern of *C. perfringens* in small ruminants. *Clostridium perfringens* is a normal inhabitant of the gastrointestinal tract, which whenever find favourable conditions, multiply rapidly, producing lethal toxins leading to an increase in its colony counts as well as toxin liberation causing disease conditions. So, to rule out the load of *C. perfringens* in diseased conditions, enumeration of *C. perfringens* from both diseased and healthy animals was done.

MATERIAL AND METHODS

A total of 75 samples were collected from different parts of Satara, Sangli and Pune of Western Maharashtra comprising of 44 intestine and six faecal samples from enterotoxaemia suspected sheep and 25 faecal samples from healthy sheep flocks. Clinical representation for

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enterotoxaemia were based on signs of sheep showing symptoms of severe and profuse diarrhoea or sudden death within 9-12 hours after onset of the disease with less intake of feed. The samples of intestine from enterotoxaemia suspected cases of necropsied sheep were collected from Department of Veterinary pathology, Shirwal, and from Omega Laboratory Lonand, Maharashtra.

Isolation of *Clostridium perfringens*

All the samples were transported to the laboratory and were inoculated in Robertson's cooked meat medium (RCCM) for initial enrichment for 18 hrs described in Bergey's manual of systemic Bacteriology, 1986. After incubation, loopful of inoculum from RCMM were streaked on selective agar plates. Perfringens agar base with TSC (Tryptose Sulphite Cycloserine) supplement (Himedia, India) was used as selective agar and incubated for 24 hours at 37º C anaerobically with an anaerogas pack (Himedia, India) and observed for black colonies.

Identification of *Clostridium perfringens*

The confirmation of the isolates was done morphologically by Schaeffer and Fulton staining method and by standard biochemical test such as Oxidase test, Catalase test, Voges-Proskauer test, and Methyl Red test (Cruickshank *et al.*, 1975). Detection of *C. perfringens* was confirmed by species specific polymerase chain reaction (PCR) using 16SrRNA gene primers.

Molecular detection of *C. perfringens* by 16SrRNAgene

Further, presumptive *C. perfringens* were confirmed by amplifying the 16SrRNA species specific gene of the *C. Perfringens* as reported by Nazki *et al.* (2017). The PCR conditions consisted of initial denaturation at 94º C for 2 min, following by 35 cycles of denaturation at 94º C for 30 sec, annealing at 56º C for 30 sec and extension at 72º C for 1 min. The final extension at 72º C for 2 min. The reaction mixture comprises of 12.5 µl of PCR master mix containing dNTPs, Taq DNA polymerase, buffer, $MgCl_2$ (HiMedia Pvt. Ltd. Mumbai), 8.5 µl of nuclease-free water, 1 µl of each reverse and forward primer and 2 µl of DNA template and subjected to thermal cycling.

Antibiogram pattern of *C. perfringens*

Genotypically confirmed isolates were subjected to antimicrobial susceptibility testing (AST) using 11 different antibiotic discs. The commercially available discs and zone interpretation charts were procured from HiMedia, India. The disk diffusion method was used for AST as per the method described by Bauer *et al.* (1966). The antibiotics and its concentrations used for this test were Amoxycillin-clavulanic acid (30 mcg), Amoxycillin-Sulbactam (30/15 mcg), Ceftriaxone-Tazobactam (30/10 mcg), Enrofloxacin (10 mcg), Penicillin-G (2 units), Ciprofloxacin (10 mcg), Amikacin (30 mcg), Metronidazole (10 mcg), Oxytetracycline (30 mcg) and Gentamicin (10 mcg).

Molecular detection of beta-lactamase and tetracycline-resistant genes of *C. perfringens*

Preparation of DNA template:

DNA of bacteria was extracted by carrying out boiling and snap chilling methods. For DNA extraction one pure colony of bacteria was suspended in 50 µl of sterile water. After suspension it was boiled for 5 min at 95° C in a thermal cycler. This was rapidly followed by snap chilling in crushed ice for 10 min thereafter, the suspension was centrifuged at 10,000 rpm for 1 min at 4° C in a refrigerated centrifuge. The 2 µl of supernatant was used as the DNA template for PCR. The extracted DNA was stored at -20º C for further use.

Detection ofbeta-lactamasegenes of *Clostridiumperfringens*

Multiplex PCR was carried out for *bla*SHV, *bla*TEM, *bla*CTX-M and *bla*OXA genes encoding for beta-lactamase. PCR was performed consisting of 12.5 µl master mix supplied with Taq DNA polymerase, $MgCl_2$ and dNTPs, 1µl each of forward and reverse primers (total 04 gene), 2 µl template DNA and 2.5 µl nuclease free water making the final volume 25 µl. Samples were subjected to 30 PCR cycles, consisting of initial denaturation of 15 min at 95º C; 30 sec of denaturation at 94° C; 90 sec of annealing at 62° C, 60 sec of extension at 72° C and final extension at 72° C for 10 min (Fang *et al.*, 2008).

Detection of tetracycline-resistant genes *tet*A and *tet*B of *Clostridium perfringens*

The multiplex PCR was performed for the detection of *tet*A and *tet*B genes in a total reaction volume of 25 µl containing 12.5 µl PCR Mastermix containing dNTPs, Taq DNA polymerase, buffer, $MgCl_2$ (M/s. HiMedia Pvt. Ltd. Mumbai), 5.5 µl of nuclease free water, 1 µl of each reverse and forward primers (total 02 gene), 3 µl of DNA template. Amplification was obtained with 35 cycles following an initial denaturation step at 95º C for 10 min. Each cycle involved denaturation at 94º C for 1 min, annealing at 62º C for 90 sec, synthesis at 72º C for 1 min and the final extension step at 72º C for 10 min (Randall *et al.*, 2004).

Enumeration of *C. perfringens*

Ten faecal samples each from both healthy and enterotoxaemia suspected cases of sheep were collected for enumeration of the pathogen. Serial dilution of 1g of faecal samples was freshly prepared in the BHI broth from 101 to 10 10 for each sample. Perfringens base agar with TSC supplement was used for the culturing of *C.*

Table 1. Oligonucleotide sequences used in multiplex PCR for detection of beta-lactamases

Target Gene	Oligonucleotide sequences	Amplicon size (bp)
blaSHV	F- CTT TATCGG CCC TCA CTC AA	237 bp
	R-AGG TGC TCATGG GAAAG	
blaTEM	F-CGC CGC ATA CAC TAT TCT CAG AAT GA	445 bp
	R ACG CTC ACC GGC TCC AGATTT AT	
blaCTX-M	F- ATG TGC AGY ACC AGT AAR GTK ATG GC	593 bp
	R- TGG GTR AAR TAR GTS ACC AGA AYC AGC GG	
blaOXA	F-ACA CAA TAC ATA TCAACT TCG C	813 bp
	R- AGT GTG TTT AGA ATG GTG ATC	

Table 2. Oligonucleotide sequences of primers for detection of tetracycline-resistant genes *tet*A and *tet*B

Target Gene	Oligonucleotide sequences	Amplicon size (bp)
tetA	F- GGTTCACTCGAACGACGTCA	557 bp
	R- CTGTCCGACAAGTTGCATGA	
tetB	F- CCTCAGCTTCTCAACGCGTG	634 bp
	R -GCACCTTGCTGATGACTCIT	

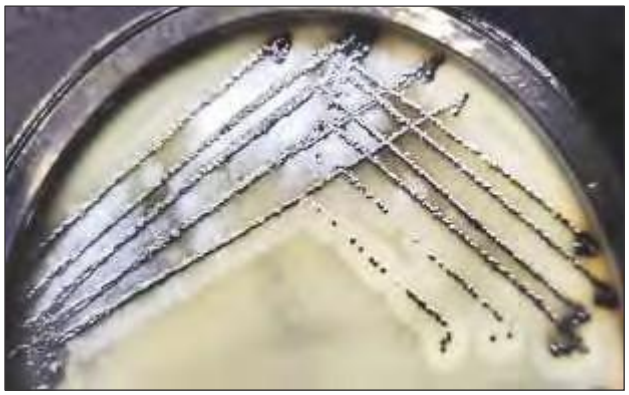

Fig. 1. Black colour colonies of *Clostridium perfringens* on Perfringens agar base with Tryptose Sulphite Cycloserine (TSC) supplements

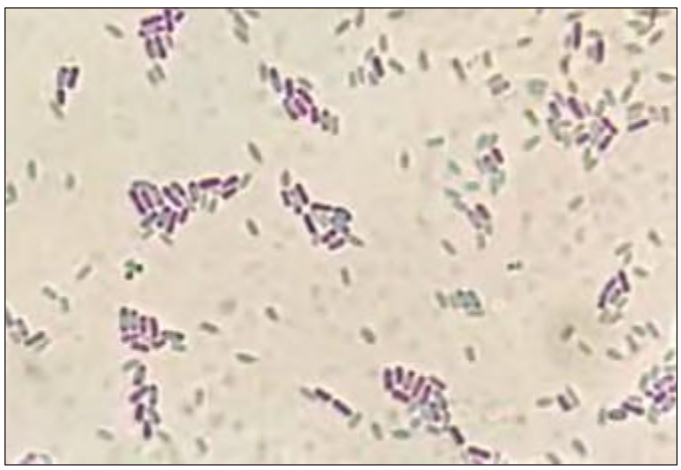

Fig. 2. Spore of *Clostridium perfringens* in green colour by Schaeffer and Fulton staining method

perfringens by overlaid technique described by Sengupta *et al.* (2011). Sets of triplicate plates for each dilution were incubated anaerobically at 37º C for 24 hours and observed for black colored colonies on the TSC media plates. The

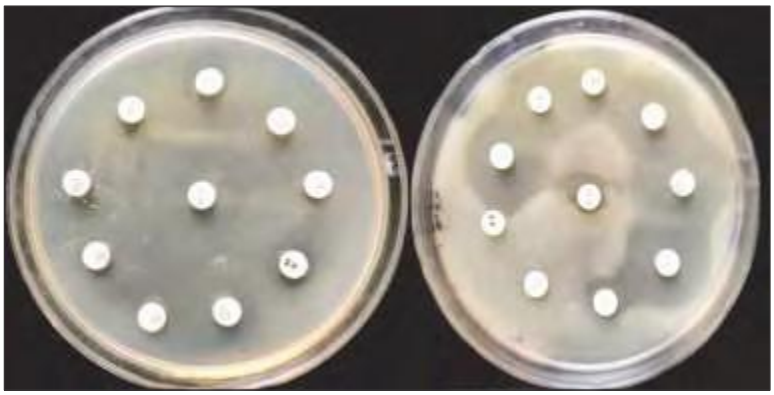

Fig. 3. Antibiotic Sensitivity pattern of *Clostridium perfringenson* Mueller Hinton agar (MHA) plates

colonies in the range of 30-300 were enumerated using the colony counter.

RESULTS AND DISCUSSION

Clostridium perfringens were isolated and identified based on cultural, morphological and biochemical characteristics from the enterotoxaemia suspected cases of sheep. All 75 samples were inoculated into RCMM. The RCMM showed heavy turbidity with gas production turning the meat particle pink in colour and proved RCMM as the suitable medium for the initial enrichment of *C. perfringens* in anaerobic conditions. Further, streaking on Perfringens agar base with TSC supplements showed black colour colony with sulphite reduction after incubation shown in Fig 1. Similar findings of black coloured colonies was observed by Shoukat *et al.* (2018) and Elsify *et al.* (2016) on perfringens agar base with TSC supplement from healthy and enterotoxaemia suspected cases of sheep.

Morphological identification by spore staining

revealed the presence of green coloured subterminal spores with red vegetative bodies (Fig. 2). The biochemical test such as Oxidase, Catalase, Indole test, Methyl red, and Voges Proskauer, showed negative results. Similar findings were recorded by Verma *et al.* (2014) and Miah *et al.* (2011) on biochemical analysis of their isolates.

A total of 46 presumptive *C. perfringens* were recovered from 50 enterotoxaemia suspected samples showing the occurrence of 92%. From healthy sheep, total 25 faecal samples were collected, out of which 10 (40%) presumptive *C. perfringens* were recovered (Figs. 1 and 2). From our findings, the samples from the enterotoxaemia suspected cases of sheep showed a high occurrence of 92% as compared to the healthy 40% in healthy animals the load of *C. perfringens* present in the normal intestinal flora is less and is mainly excreted by normal peristaltic movement. Moreover, sudden change in feed produces favourable conditions leading to an increase in the population of bacteria leading to diseased condition.

Further, all the 56 presumptive *C. perfringens* amplified 481bp product16SrRNA species-specific PCR corresponding to *C. perfringens*. These present findings are in close association with Shoukat *et al.* (2018) and Rasool *et al.* (2017) who reported amplicon of 481bp by the species-specific 16SrRNA PCR from all the isolates which were culturally identified as C. perfringens from the samples of sheep. For the accurate identification of Clostridium species, 16SrRNA PCR works as a good confirmatory tool for further study of *C. perfringens* (Tonooka *et al.*, 2005).

Then, all 56 positive isolates were tested for *in vitro* antibiotic susceptibility profiles (Fig. 3). The *C. perfringens* showed sensitivity to Enrofloxacin (46.4%) followed by Amikacin (26.7%) and Metronidazole (23.2%). All the isolates were resistant to Oxytetracycline (100%), followed by Ceftriaxone-Tazobactam (91%), Ampicillin-Sulbactum (89.2%), Amoxyclav (87.5%). Also, Gentamicin (82.1%), Ciprofloxacin (80.3%) and penicillin G (78.5 %) showed resistance to *C. perfringens* isolates. In the present study, most of the isolates showed resistance to more than three classes of antibiotics showing multidrug resistance (MDR) patterns which were also observed by Anju *et al.* (2021) and Bendary *et al.* (2022) in their study. Due to the repeated use of antibiotics, the bacteria becomes adapted causing resistance, defining the urgent need for solid guidelines for the use of antibiotics and for the safe production of food products of animal origin in India.

A total of six isolates were found to be positive for the ESBLs gene *bla*SHV, 52 for *bla*TEM, and 16 for *bla*CTX-M, whereas none of the isolates tested *bla*OXA-positive. Additionally, the 56 isolates were subjected for the tetracycline resistance genes by using PCR and targeting *tet*A and *tet*B. Out of 56 isolates, 15 (26.78%) and 27 (48.21%) were *tet*A and *tet*B positive, respectively. In contrary to the present study, Anju *et al.* (2021) reported a higher number of *tet*A compared to *tet*B from the isolates of different animals from Tamilnadu.

Since β-lactamase-encoding genes such as *bla*TEM, *bla*SHV and *bla*OXA can be released into the environment, and resistance in animals used for food received less attention, they pose a serious risk to the health of both animals and people as a whole.

The total viable count of *C. perfringens* was carried out in faeces samples collected from healthy and enterotoxaemia suspected cases of sheep and statistical analysis of the data from the faeces revealed a significant result. Healthy animals had a considerably lower bacterial count ($p< 0.01$) than enterotoxaemia suspected cases of sheep. There was a 35.8% rise in bacterial count in comparision to healthy animal bacterial count. Moreover, the current study's findings revealed that sheep with suspected enterotoxaemia had a greater bacterial load (10^4 -10^7 CFU/g) than healthy sheep. These findings are consistent with research by Hussain *et al.* (2018), who found higher bacterial counts (>107 CFU/g) in 37.85% (67/177) of sheep with diarrheal symptoms and 33.55% (52/155) of goats with diarrheal symptoms.

CONCLUSION

In the present study, most of the isolates showed multidrug-resistant pattern with the presence of ESBL and tetracycline resistant genes. The results of the study raise the concern over the use of antibiotic in food animals. The study shows enterotoxaemia as one of the fatal diseases, hence treating it may involve deliberately using the most sensitive antibiotic. Further studies can be carried, out on more animals, involving different geographical location. This will allow to study the different antibiotic pattern circulating. The study of the resistant genes will help to overcome this problem and will help in improving animal and human health.

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