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Research article

Chemotactic factor inducing Interleukin-8 (IL8) gene is transcriptionally elevated in experimental enterotoxaemia in goats caused by *Clostridium perfringens* type D

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ABSTRACT

The current study was designed to analyse the effects of experimental induction of enterotoxaemia through intra-duodenal inoculation of *C. perfringens* type D culture isolated from spontaneous outbreaks in goats. Twenty goats (6–9 month age) were divided into four groups and *C. perfringens* type D culture was inoculated intra-duodenally as per following: Group-I (whole cultures-WC), group-II (culture supernatant-CS), group-III (washed cells-WS), and group-IV (uninfected control-C). The treated animals were sacrificed after 72 h post infection (hpi), and necropsy showed gross changes including haemorrhages and congestion in the ileal and colon mucosa, pulmonary congestion and edema in lung. Kidney, brain and spleen exhibited severe to moderate congestion. Microscopic changes like haemorrhages, degenerative and necrotic changes in the mucosal epithelium of intestine and haemorrhages in kidney parenchyma were observed in the H&E stained sections. Lung alveolar sacs were filled with proteinaceous fluid. Immunohistochemistry revealed positive immunolabelling for *etx* (epsilon toxin) in the mucosa of intestine in WC and CS group. Control animals did not exhibit any significant gross or microscopic changes. PCR amplification of DNA extracted from intestinal tissues of WC and CS groups showed positive for *etx* gene demonstrating the production of epsilon toxin. Transcriptional responses in experimental groups were assessed by quantitative reverse transcription real time PCR (qRT-PCR). Genes including IL-1β and IL2 showed up-regulation in all the experimental groups (WC, CS&WS). Specifically the toxin-based experimental groups (WC&CS) showed up-regulation of the gene responsible for chemotaxis viz. IL-8, while the washed cells group (WS) showed higher transcriptional response to Cathepsin-L (Cat-L) gene denoting the acute inflammatory response due to neutrophil elastase activity. These results take a cue on the evolving nature of the enterotoxaemia in goats due to various strains circulating in the field. The host response and its modulation due to the novel enterotoxaemia strains throws light on the current challenges in efficient control of the disease in goats.

1. Introduction

The major systemic diseases responsible for mortality in goats are the diseases of alimentary system, followed by respiratory and other systemic diseases (Pawaiya et al., 2017). Enterotoxaemia caused by *Clostridium perfringens* inflicts significant mortality in goats and sheep worldwide (Niilo, 1980; Kriek et al., 1994; Veschi et al., 2008). *Clostridium perfringens* type D produces two major toxins, namely alpha and epsilon (Smith and Sherman, 2011), the epsilon toxin being the main virulent factor responsible for disease induction in goats, sheep and mice (Garcia et al., 2013). Enterotoxaemia has been produced experimentally by administering epsilon toxin intravenously in the sheep and goats to study pathology of the disease (Gardner, 1973; Uzal and Kelly, 1998). Necropsy of goats died due to enterotoxaemia showed distinctive characteristic

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lesions including severe enterocolitis (Uzal et al., 1998). Therefore, it has been supposed that the epsilon toxin is mainly responsible for producing enterotoxaemia in goats (Uzal et al., 1997). Sufficient studies have been conducted in the past in sheep, however the disease in caprine is relatively less explored (Uzal and Kelly, 1996; Smith and Sherman, 2011). There is also a need to differentiate the disease process and the associated pathology and manifestation of enterotoxaemia in sheep and goats. To understand the pathobiology of enterotoxaemia in goats, there is a need to assess the molecular changes at the cellular level vis-à-vis the virulent factors in the pathogens. In the past, research has been conducted on the epsilon toxin and its effects on the host (Goswami et al., 1996) and sequencing of epsilon toxin gene components (*etx* B and *etx* D) and its regulation in production of epsilon toxin (Harvard et al., 1992; Hardison, 2000). In related species like *Clostridium difficile*, the key pathogenic component viz. TxA relies on the induction of release of cytokines such as TNF-α and IL-1ß, for the induction of colitis in mice (de AraújoJunqueira et al., 2011).

To understand the key molecular changes during enterotoxaemia in goats, here we report the induction of disease by a novel isolate of *C. perfringens* type D (NK/15 strain). The disease was induced by a surgical laparotomy method by intra-duodenal inoculation of the culture components in goats in comparison with natural outbreaks.

2. Material and methods

2.1. Study plan and methodology

In this experiment, we used a novel isolate of *C. perfringens* type D strain NK/15, which was characterized (PCR-RFLP using *Hind*III) and sequenced for full gene of epsilon toxin (Table S1, Figs. S1, S16 & S17 in Supplementary file) and found mutations at specific positions in the open reading frame (ORF) of the coding region of *etx* gene including the A→G point mutation at nucleotide position (nt) 30, G→A at nt775 (data included in supplementary data; refer to Figs. S2 & S3 in the Supplementary file). This characterized strain was used for the experimental induction of enterotoxaemia in goats using various components of the culture by laparotomy section. The experimental goats were subsequently observed for clinico-pathological changes and they were sacrificed for downstream analysis as described in the subsequent sections.

2.2. Preparation of inoculums

Culture inoculum (*C. perfringens* type D NK/15 strain) was prepared in bulk of Robertson's cooked meat medium (RCM) under anaerobic conditions as described previously (Cowan and Steel's, 1993). The purity of the inoculum was confirmed by Gram's staining and subculture on Clostridial-supplemented Brucella blood agar (CLS-BBA) and Egg yolk agar (EYA), and further confirmed by, toxinotyping multiplex PCR (Tm-PCR) and sequencing studies (supplementary data) as described previously (Babe et al., 2012; Nasir et al., 2015).

Various culture components were prepared including whole culture (WC-Group-I) by decanting the clear broth supernatant, followed by Culture supernatant (CS-Group-II), by centrifugation at 10000 rpm for 25 min at 4 °C and the supernatant thus collected was activated by Trypsin following the protocol as described elsewhere (Habeeb, 1969). Further, trypsinized culture supernatant toxin was titrated in mouse (20–25g body weight) to find out the MLD50 (Sterne and Batty, 1975; Uzal and Kelly, 1998). For experimentation in mice (IAEC) Permission No. 105/IAEC/16 dated: 03-02-2016 was obtained. Besides, the washed cells (WS-Group-III) inoculum was prepared from the bacterial cell pellets obtained from previous component (CS) were washed two times with sterile PBS (pH 7.2) and suspended in the same in sterile phosphate buffered saline (PBS). The sterile RCM media supernatant were used for uninfected control animals (C-Group-IV). The experimental doses for WC and WS groups were calculated as CFU/ml by standard technique cultured on CLS-BBA with 5% sheep de-fibrinated sheep blood after incubation anaerobically for 24 h. The doses were adjusted to 3×10^7 CFU/ml for WC and WS groups for final inoculation.

2.3. Experimental studies on post weaned goats (Animals)

Twenty goats of either sex, 5–9 months age weighing 12–16 kg were used for experimental induction of enterotoxaemia. They were divided into four groups-I (WC), II (CS), III (WS) and IV (uninfected Control), with 5 animals in each group.

Ethical Approval for animal experiment: For experimentation in goats, permission was obtained from the Institutional animal ethical committee (IAEC) vide letter No. 02/IAEC of ICAR-CIRG dated 29.12.2015.

2.4. Experimental procedures and sample collection

All the animals of group-I, II, III and IV were subjected to laparotomy section. Prior to giving CS, WC, WS and sterile RCM media (Control group), 200 ml of potato starch (20% Arrow root) was infused directly in abomasum followed by infusion of inoculums (3×10^7 CFU/ml) into duodenum with a dose 300 ml/animal in all the animals with the total dose amounting to 9×10^9 CFU/animal. After administration of culture inoculum, suturing and post-operative care was done. The treated animals were kept under observation for 24–72 h. Those animals remained clinically healthy during that period or those showed intermittent diarrhoea and depression were also sacrificed 72 h post-inoculation (hpi) (Figure 1). Out of 05 animals, one animal died within 20 hpi and two animals showed depression, anorexia and developed diarrhoea after 24 hpi from WC and CS groups. All the animals were euthanized (according to standard protocols committed in IAEC) to observe the gross pathological changes and samples were collected for bacteriological isolation, identification (Babe et al., 2012; Van Asten et al., 2009), whereas distal ileal loop was collected aseptically for RNA extraction and qRT-PCR based gene expression study. Sample of duodenum, ileum, colon, spleen, kidney, heart, liver, lung and brain were collected and preserved in 10% neutral buffered formalin and processed for histopathology (Luna, 1972).

2.5. Toxinotyping multiplex -polymerase chain reaction (TmPCR)

RCM culture and intestinal contents were subjected to DNA extraction using commercially available kit (QIAamp DNA Mini Kit. catalogue No-51304) following manufacturers protocol. The sample DNA prepared was amplified using specific published primers for toxinotypes mainly *Cpa*, *Cpb*, *Etx*, *Iap*, *Cpe* and *Cpb*2 (Table 1) (Van Asten et al., 2009) by

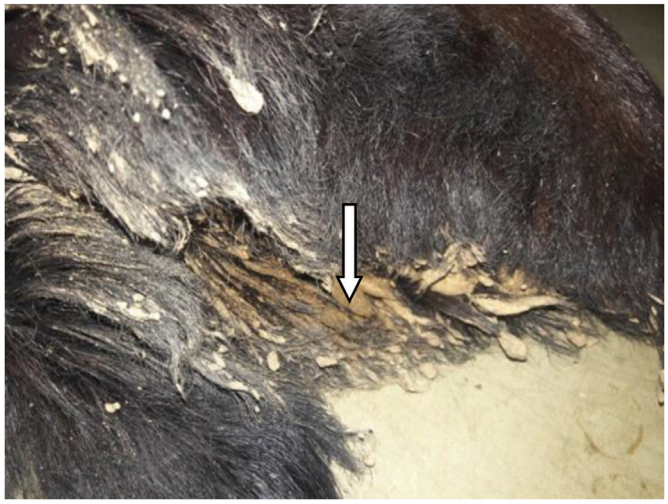

Figure 1. Goat showing perineal region soiled with feces.

Table 1. Toxinotyping primers for TmPCR (Van Asten et al., 2009).

Toxin	Name of Primer	Primer Sequence (5′-3′)	Size of PCR products (bp)	Reference
Alpha	*cpa*	F - GCTAATGTTACTGCCGTTGA R- CCTCTGATACATCGTGTAAG	324 bp	(Van Asten et al., 2009)
Beta	*cpb*	F- GCGAATATGCTGAATCATCTA R- GCAGGAACATTAGTATATCTTC	195 bp	
Beta 2	*cpb2*	F-AAATATGATCCTAACCAAMAAA R- CCAAATACTYBTAATYGATGC	548 bp	
Epsilon	*etx*	F-TGGGAACTTCGATACAAGCA R-AACTGCACTATAATTTCCTTTTCC	376 bp	
Iota	*iap*	F- AATGGTCCTTTAAATAATCC R- TTAGCAAATGCACTCATATT	272 bp	

(F) = Forward primer; (R) = Reverse primer 2.7.

using TmPCR (Eppendorf, PCR Sys, USA). Multiplex PCR master mix was prepared using 12.5μl of 2X master mix (Qiagen Multiplex PCR kit, Cat# 206143), 0.2mM of each primers (*cpb*, *etx*, *iap*, *cpe*) except the beta2 toxin primers used at 0.4 mM followed by PCR-grade water and 1 μl of DNA template to make up the final volume of the reaction to exactly 25μl. The PCR program consisted of 2 min of initial denaturation at 98 °C followed by 30 cycles of denaturation at 98 °C for 30 s, annealing at 55 °C for 30 s followed by extension at 72 °C for 40 s and final extension at 72 °C for 10 min. PCR amplified DNA products were subjected to submarine gel electrophoresis using 1.8% TAE-EtBr agarose gel. At the end of electrophoresis, the gel was visualized under the UV transilluminator (Alpha Innotech) and documented.

2.6. Gene expression studies

Gene expression studies were done to capture the transcriptional response of key inflammatory genes in the hosts during the pathogenesis of enterotoxaemia. To broaden the horizon, we also compared the experimental groups with the naturally enterotoxaemia affected animals. Distal ileal loop collected was subjected to RNA extraction using TRIzol™ method (cat# 15596026, Thermo scientific) following the manufacturer's instructions. The RNA was reverse transcribed to copy DNA using PrimeScript 1st strand cDNA Synthesis Kit (Cat# 6110A, TaKaRa Bio, Japan). The candidate genes selected for this study includes IL-1β, IL-2, IL-8 and Cathepsin-L along with normalization of endogenous gene (GAPDH).

2X TB Green Premix Ex Taq II(Cat# RR820B, TaKaRa, Japan) was prepared in a 20μl master mix with the respective primers used at 5pmol final concentration, 1.0 μl of cDNA template equivalent to 50ng of total RNA and assayed as duplicates per sample per gene in the CFX96™Realtime PCR system®, BioRad. The thermal conditions have been followed as described by the manufacturer with the annealing temperature at 55 °C. All the genes used in the study were previously standardized and calculated for regression co-efficient and efficiency using the standard curve generated by absolute quantification. The amplified products were further confirmed by additionally adding a step of melting curve analysis for each genes studied. The primers used for gene expression are given in Table 2.

2.7. Statistical analysis

The data analysis for gene expression studies was done using the CFX-96 manager (CFX Real-time PCR system, Biorad®, USA) using the $2^{-\Delta\Delta CT}$ algorithm. The data were further analysed by One Way ANOVA followed by Tukey's Post-hoc test using Graph Pad Prism V4.0 software.

3. Results

3.1. Clinical examination

All the animals recovered from anaesthesia after 1–2 h post laparotomy. One animal died in CS infused group after 20 hpi. Three replicates each from CS and WC groups showed diarrhoea at 20–24 hpi. Other animals showed only dullness and anorexia at 20–24 hpi. In WS group, 2 animals showed depression, anorexia and developed diarrhoea at 24–36 hpi. The diarrhoea was fluidy and foul smelling with presence of mucous in the faeces. None of the animals from the control group showed any overt clinical signs.

3.2. Post mortem findings

3.2.1. Gross findings

All the animals of CS and WC group showed congestion of serosal blood vessels and hemorrhages at the mucosa of distal portion of ileum and colon (Figure 2). The wall of affected part of ileum and colon was thickened. Lungs showed emphysema with severe edema of the airways

Table 2. Gene expression primers for qRTPCR.

S. No	Gene of interest	Oligo sequences 5′→3′	Remarks
1	Interleukin 1β (IL1β)	F: CCTTGGGTATCAGGGACAA R: GGGTATGGCTTTCTTTAGG	Baron et al. (2014)
2	Interleukin 2 (IL2)	F: TCCAAGCAAAAACCTGAACC R:CAGCGTTTACTGTTGCATCATC	Sarre et al. (2015)
3	Interleukin 8 (IL8)	F: ATGAGTACAGAACTTCGA R: TCATGGATCTTGCTTCTC	Smeed et al., 2007
4	Cathepsin-L (Cat-L)	F: ATGCACATTGGCACCAGTGGA R: AGGCATTCATTGCCATGCTG	Designed in-house
Endogenous gene-GAPDH			
5	Glyceraldehyde-3 phosphate dehydrogenase (GAPDH)	F: GGTGATGCTGGTGCTGAGTA R:TCATAAGTCCCTCCACGATG	Sarre et al. (2015)

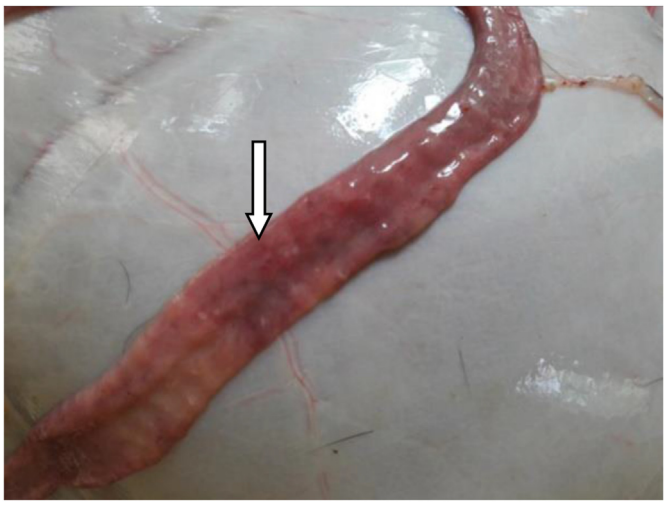

Figure 2. Intestine showing thickening of intestinal mucosa with congestion of mucosa.

(Figure 3). The apical lobe and bronchioles were filled with frothy fluid. The mesenteric lymph nodes were also enlarged. Two animals of WAS group also showed congestion and thickening of mucosa of colon. In brain, only congestion of meningeal blood vessels was observed in all the three treatment groups (Figure 4) (Table 3).

3.2.2. Microscopic examinations

The Histopathological examination of intestine showed congestion of serosal and sub mucosal blood vessels. Haemorrhages in mucosal epithelium with denudation of crypts in the ileal and colonic mucosa were also observed (Figure 5). The number of goblet cells was increased in the affected part of intestine. The mucosa was infiltrated with neutrophils with degeneration and necrosis of villous epithelium. In lungs, oedematous fluid was filled in alveoli with congestion of alveolar capillaries. Liver showed congestion of central vein with coagulative necrosis of hepatocytes. Brain revealed congestion of blood vessels in cerebral cortex (Figure 6).

3.2.3. Culture and isolation

The intestinal contents when anaerobically cultured showed gas production on RCM. Subsequent anaerobic culture on CLS-BBA (with 5% de-fibrinated sheep blood) and EYA showed double haemolytic colonies and lecithinase activity (Figures 7 and 8) respectively in WC and WS but not in CS group. In CS group, only toxin was present without bacterial cell culture. RCM culture when stained by Gram's technique, showed gram positive rod with truncated ends, and it was further confirmed by TmPCR, while the intestinal contents were also toxinotyped by TmPCR to corroborate with the results of the isolation done in RCM for all the treatment and control groups (Figure 9).

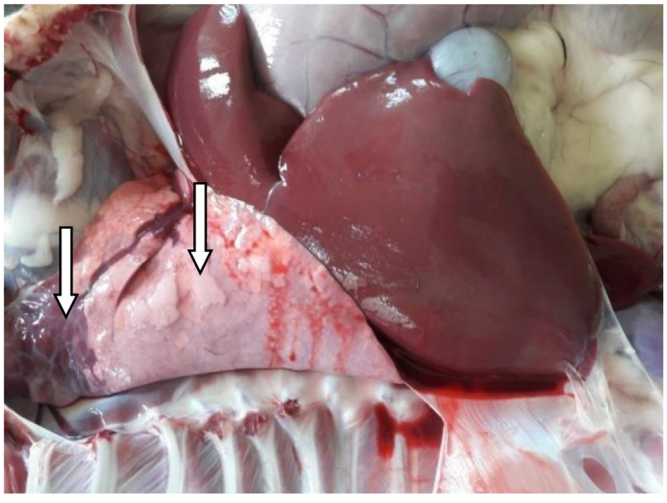

Figure 3. Lung showing congestion and emphysema with edema in the right lobe of lung.

3.3. Gene expression

The candidate genes viz., IL-1β, IL-2, IL-8 and Cat-L were analysed in the study due to their significant involvement in toxaemia and inflammation in the ileal tissues. The highest expression of IL-1β and IL-2 gene was observed in spontaneous outbreaks of enterotoxaemia (ET) followed by experimental groups- CS, WS and WC (Figures 10A and B). The Expression of IL-8 was highest in CS group followed by spontaneous cases of ET, WS and WC (Figure 10D) in that order. The expression of Cat-L was highest in spontaneous group followed by WC, WS and CS (Figure 10C) (Also refer to Table S2 and Figs. S4 to S15 in the supplementary file).

4. Discussion

The present study was performed to understand the molecular pathogenesis of enterotoxaemia caused by *C. perfringens* type D in goats, especially the unique CIRG NK/15 strain. Hence, experimental studies were simulated to resemble the natural cases of enterotoxaemia by direct inoculation of various types of culture inoculums (WC, CS and WS) by artificially creating anaerobic environment in guts using starch as reported elsewhere (Uzal and Kelly, 1998; Uzal et al., 2004). Except CS group, the culture inoculum was standardized to 3×10^7 CFU/ml for intra-duodenal inoculation, since it has been studied earlier that the severity of disease was depending upon dose and time of infusion of toxin of *C. perfringens* type D (Uzal and Kelly, 1998). For CS group, the dose of the toxin supernatant was already titrated in the mouse as per the standard protocol. Experimentally, the disease produced severe lesions in WC than those received the CS and WS components. This indicates that the bacterial component including their secretory components (toxin) contributes to the changes in the predilection site.

For simulating the favourable conditions, anaerobiasis was created using starch solution in the abomasum causing a decrease in thepH and subsequently aiding in the rapid proliferation of bacteria leading to production of toxins. The clinical signs produced in CS group were anorexia and diarrhoea after 24 hpi. WS treated animals did not exhibit prominent gross lesions in intestine which may be attributed to extended time-frame taken for recovery of anaerobic bacteria from lag phase to log

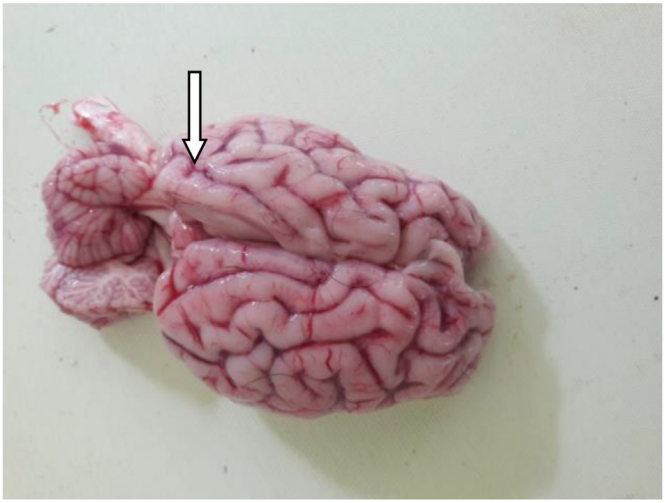

Figure 4. Brain showing meningeal congestion in cerebrum and cerebellum.

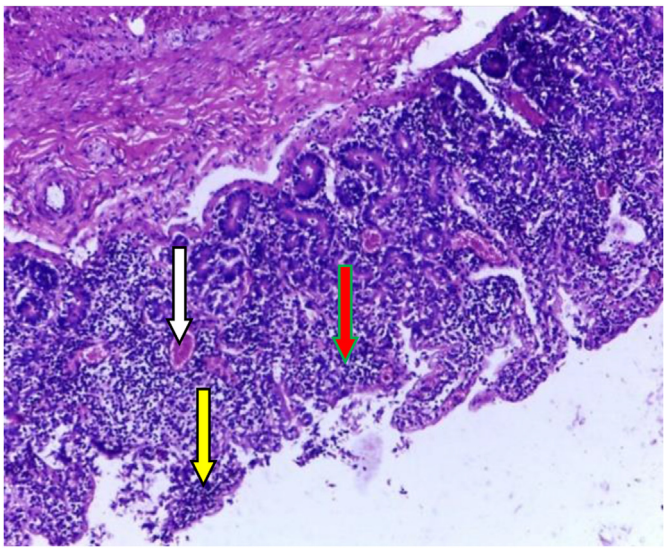

Figure 5. Intestine mucosal lining epithelium showing congestion of mucosal blood vessels with degeneration of villous epithelium and infiltration of neutrophils.H&E 100x.

phase followed by production of toxin to produce lesions. The findings also suggested that toxin groups (CS and WC) produced overt lesions in the ileum and colon leading to secretory diarrhoea while manifesting mild systemic lesions as compared to sheep (Uzal and Kelly, 1998; Uzal et al., 2004). Our clinical findings are corroborated to the findings observed by other workers where clinical signs are compared between goats and sheep (Uzal and Kelly, 1998; Uzal et al., 2004) using intra-duodenal infusion of *C. perfringens* type D. In this study, enterocolitis was a predominant lesion observed in treatment groups, while mild changes were observed in the brain (Blackwell and Butler, 1992; Smith and Sherman, 2011). The experimental animals showed diarrhoea as a predominant clinical sign, but on necropsy, enterocolitis, and pulmonary congestion and edema was the most consistent finding in goats which are similar to the findings described elsewhere (Smith and Sherman, 2011).

The results showed that, in goat, *C. perfringens* type D has specific affinity to intestinal tract, particularly distal ileum, causing enterocolitis, which is not a common lesion/sign observed in ovine enterotoxaemia (Barker et al., 1993). The reason that toxin causes selective damage to distal ileal mucosa in goats is unknown, but many explanations can be

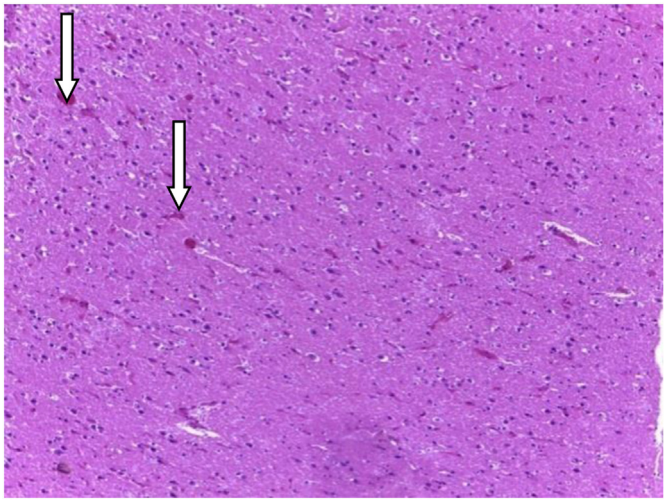

Figure 6. Brain section showing mild congestion of cerebral blood vessels.H&E 100x.

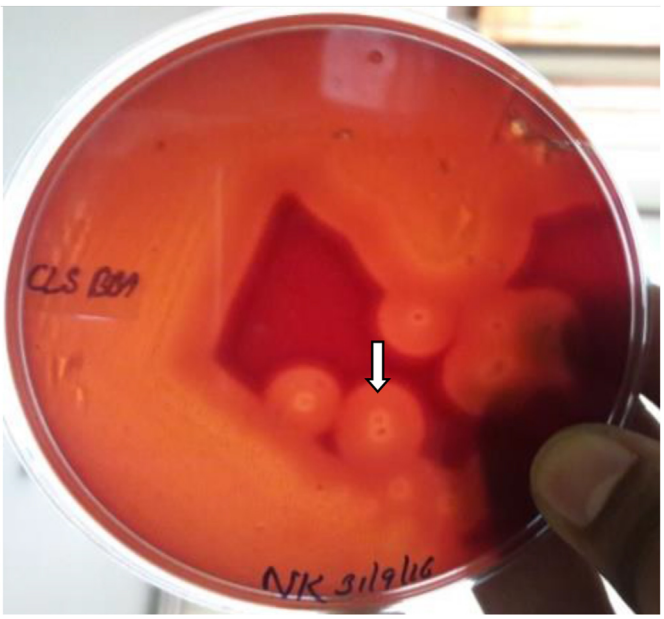

Figure 7. *C. perfringens* isolate from diarrhoeic fecal sample with double zone of hemolysis in clostridium supplemented blood agar with smooth, round, flat, white spreading colony.

hypothesized. In goats the transit speed of the intestinal content is about 3 h for the small intestine and 18 h for large intestine (Smith and Sherman, 2011). This could have accounted for longer duration of exposure time taken by epsilon toxin in the large bowel than that of the small intestine leading to enterocolitis in goats. The other provable justification for the selective damage of large bowel could have been that epsilon toxin activated in the presence of trypsin secreted in the small intestine and then it goes to large bowel and causes damage to colon.

We also did not find any lesion in brain like the observations of Uzal and Kelly (1998), which may be due to scanty systemic changes coupled with lower retention time of toxin in small intestine and its fast clearance

Figure 8. *C. perfringens* isolate from diarrhoeic fecal sample on egg yolk agar showing lecithinase activity (opalescence around the growth).

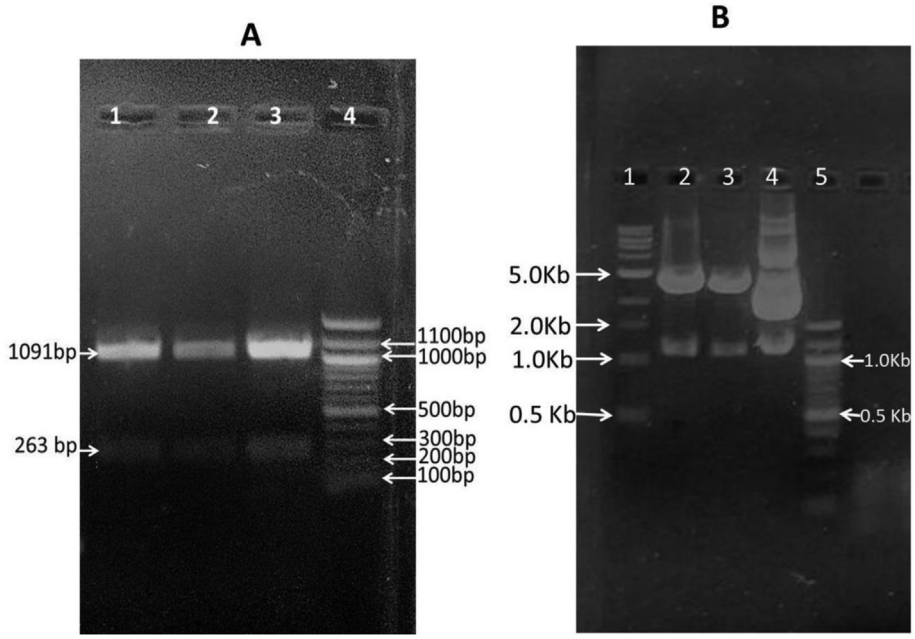

Figure 9. Gel electrophoresis showing PCR cloning of CIRG-NK isolate from goat in TA vector of ETX full length gene. (A) Etx full gene digested with Hind III cut into 1091bp and 263bp (B) Linearized vector (Lane 2 and 3) and supercoiled vector (Lane 4); Lane 5 showing 1.0kb ladder (For original uncropped images please refer to Figs. S16 & S17 in the supplementary file).

due to enterocolitis induced secretory diarrhoea in goats (Smith and Sherman, 2011).

Natural cases of ET showed higher transcriptional response of IL-1β followed by CS group. This is attributed to receptor IL-1R an integral TIR domain of TLR, involved in recognition of bacterial components or PAMPs. Since, there are no reports on the group of genes studied in response to ET infection or its specific host transcriptional response; we tried to explain with similar infections or conditions to corroborate the mechanisms behind the disease. A closer parameter related to our current study was reported by de AraújoJunqueira et al. (2011), where there was an effect of *Clostridium difficile* Toxin A (TxA) on the gene expression of adenosine receptors of mouse ileal loops. TxA is a potent inducer of inflammatory reaction and release of cytokine such as IL1ß and TNF-α has a role in TxA induced colitis in mice causes induction of mucosal disruption via neutrophil infiltration and Nitric oxide (NO). It can be correlated with our study in which the epsilon toxin induces expression of inflammatory cytokines during natural as well as in experimental ET. In another study in naïve goats, an increased expression of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-10 and interferon (IFN)-γ mRNA upon *C. burnetii* stimulation was observed (Ammerdorffer et al., 2014). Probably, it reflects a common language in comparison to the aforesaid reports, where severe inflammatory process evinced during the process of disease leads to up-regulation of IL-1β. Similarly, in an *in vitro* study on PPR virus, Danasekaran et al. (2014) observed weak response of

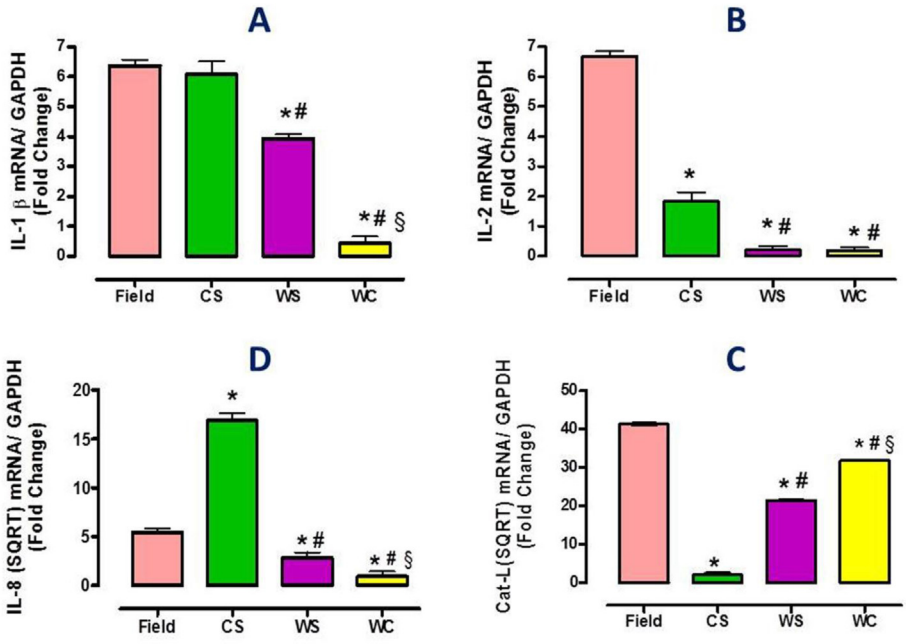

Figure 10. Differential gene expression in various experimentally infected ET groups. Clockwise from top left.A. IL-1β gene expression in different treatment groups of post weaned goats with enterotoxaemia by SYBR green qRT-PCR assay. B. IL-2 gene expression in different treatment groups of post weaned goats with enterotoxaemia by SYBR green qRT-PCR assay. C. Cat-L gene expression in different treatment groups of post weaned goats with enterotoxaemia by SYBR green qRT-PCR assay. D. IL-8 gene expression in different treatment groups of post weaned goats with enterotoxaemia by SYBR green qRT-PCR assay.

Table 3. Results of experimental animals inoculated intraduodenally with *Clostridium perfringens type D* inoculums containing whole culture, culture supernatant, washed cells and culture media.

Treatment group	No. of animals necropsied	Duration of set up of diarrhea	External appearance	Intestine gross lesions	Lung Gross lesions	Isolation of *C. perfringens* and TmPCR
Group-I (CS)	05	20–24 h	One died after 20 hpi and 02 showed soiled perineal region with diarrhoeic faecal material	Distal portion of ileum mild congested	Emphysema and edema	Isolation- No TmPCR- Yes
Group-II (WC)	05	20–24 h	02 showed soiled perineal region with diarrhoeic faecal material	Distal portion of ileum mild congested	Emphysema and edema	Yes
Group-III (WS)	05	30–36 h	02 showed depression and anorexia	Distal portion of ileum mild congested	Mild congestion	Yes
Group-IV (Control)	03	Apparently normal		Apparently normal		No

IL-1β in caprine cells infected with PPR virus when stimulated by TLR3 and TLR7 ligands, which suggested a greater role of the cytokine gene in acute infections. In other words it can be said that acute inflammatory reactions leads to up-regulation of the marker gene viz., IL-1β which is a potent pro-inflammatory cytokine synthesized predominantly by epithelial cells (Owen et al., 2013). The mRNA expression of IL-2 gene was highest in field outbreaks followed by the CS groups, which can be due to the rapid influence of activated toxin in natural cases as well as inoculation of high doses of toxin in CS group compared to WC. The lower transcriptional response of IL-2 in WC (lower toxin dose in inactivated form) and WS (without toxin supernatant) can be explained by the fact that bacterial cells requires more time to adjust to the environment and produce toxin to cause the inflammatory effects. This is attributed to receptor IL-1R, an integral TIR domain of TLR, involved in recognition of bacterial components or PAMPs. That can be the reason why the Field cases, CS and WS showed higher expression of IL-2 in that order compared to WC group. IL2 being a pro-inflammatory cytokine helps in T cell proliferation and B-cell differentiation and leads to increased immune response in candidates that contain significant levels of toxin or PAMPs of *C. perfringens*, which is obviously abundant in natural cases followed by WC and then WS group. Higher serum IL-2 levels are coupled with progression of autoimmune conditions such as scleroderma and rheumatoid arthritis (Kahaleh and LeRoy, 1989) and IL-2 levels are also elevated in chronic hepatitis B infection (Bozkaya et al., 2010). On contrary to the above interleukin genes, the higher fold change of IL8 observed in CS group compared to natural cases can be attributed to its chemotactic function directly influenced by the abundant toxic components in the former. IL-8 is a chemokine for neutrophils secreted by activated leucocytes and plays a role in inflammation and angiogenesis. This explains the fact that the CS which has more toxin component has phenomenal expression of IL-8. IL-8 is an important mediator for innate immunity in mammals and involved in recruitment and activation of different leukocytes through modulating cytokine production (Min et al., 2001). Similar findings were reported in another study, where IL8 was up-regulated significantly ($P < 0.0159$) to 26.6 fold in uterine tissue of buffaloes with endometritis when compared to the healthy uterine tissue (Mossallam et al., 2015). The current study showed higher expression of IL-8 mRNA in CS group compared to all other groups studied, which may be due to the presence of bacterial secretory components in excess that increases the possibility of IL-8 signalling to attract neutrophils to the area of infection. The natural ET groups showed a phenomenal fold change of 1700.59 for the Cat-L gene compared to the experimental ET infected groups. This indicated that along with the epsilon toxin, bacterial component is also important for pathogenesis of ET. The major portion of lysosomal enzymes contains Cathepsins and they are involved in inflammation, tumour progression and metastasis via bulk proteolysis in the lysosomes, processing of proteins and matrix degradation and they also play role in release of cytokines, phagocytosis and endothelial adhesion of monocytes (Ishidoh and Kominami, 1995; Gerber et al., 2002). Effect of exposure of bacterial exotoxins could have elicited the Cat-L response culminating to cell death and associated with the pathogenesis of enterotoxaemia. Effect of exposure of bacterial exotoxins in the perspectives of cell death has been studied using the potent pore-forming cytotoxin viz. alpha-toxin of *Clostridium septicum* in the murine myoblast cells (C2C12 cells) (Kennedy et al., 2009). The Nile tilapia injected with *S. agalactiae* showed up-regulated expression of Cat-L in all the tissues (Liang et al., 2017). It can be speculated that bacterial component may not be present in CS group and the phagosome-lysosome probably plays a minor role in soluble antigens like epsilon toxin. Most of the cathepsins have a major role via lysosomal hydrolases and proteases. Cat-L are the major group of cysteine proteases that play a role in degrading intracellular proteins and endocytosed proteins. Cathepsin L has a potent elastinolytic and collagenolytic activities, causes major factor in tissue degradation (Chapman et al., 1994). To control the disease, the infiltration of neutrophils at inflammatory sites, releases polypeptide causes inhibition of proteinase may have significance in prevention of tissue damage.

5. Conclusion

The current study aimed at deciphering the pathogenesis of local strains of *C. perfringens* which showed unique changes in the coding region of the virulence associated gene '*etx*'. Subsequently, we identified certain mutations which were reported elsewhere outside India in the CIRG strains especially the *C. perfringens* type D strain NK/15. Due to this mutation, it can be speculated that why the disease occurs despite vaccination in certain organized goat herds, which were regularly vaccinated for enterotoxaemia. Although here we report one such isolate from a group of isolates that were responsible for E.T. outbreak in the western Uttar Pradesh, India, but these mutations needs to be confirmed based on more isolates obtained from a larger geographical area and the Indian subcontinent. The disease symptoms and other lesions require correlation at the microscopic as well as molecular level, and hence in this current study a detailed pathological and molecular investigation was done. Host transcriptional response to the current isolate 'CIRG NK-15' was studied to corroborate with the clinical and pathological picture obtained during the experimental induction of enterotoxaemia. This explains the fact that why certain biomolecules like IL-1ß, IL2 and Cat-L were transcriptionally elevated in natural cases of ET; while IL8 and IL2 genes up-regulated significantly in CS experimental groups suggesting the interplay of cytokine molecules. This study suggests that the species-wise protectivity of the current enterotoxaemia vaccine, which should include more strains of goat origin to expand the horizons in controlling the enterotoxaemia.

Declarations

Author contribution statement

Gangwar, N.K., Sharma, D.K.: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Pawaiya, R.V.S.: Analyzed and interpreted the data.

Gururaj, K.: Conceived and designed the experiments; Wrote the paper.

Singh, D.D.: Performed the experiments.

Andani, D.: Performed the experiments; Wrote the paper.

Kumar, A.: Performed the experiments; Analyzed and interpreted the data.

Rao, A. R: Analyzed and interpreted the data; Wrote the paper.

Rai, A.: Conceived and designed the experiments; Analyzed and interpreted the data.

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Data availability statement

Data associated with this study has been deposited at the Open Science Framework.

Competing interest statement

The authors declare no conflict of interest.

Additional information

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