Bacterial count and predisposing factors of *Clostridium perfringens* (targeting *CPA* gene) infection along with antimicrobial sensitivity in diarrheic sheep in Pakistan

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Abstract. *Clostridium perfringens* (*C. perfringens*) is a normal inhabitant in the gut of animals. It may proliferate rapidly in favorable conditions and produces lethal toxins. These toxins may cause lethal effects in the intestines and systemically it may cause enterotoxaemia. In disease conditions, the presence of *C. perfringens* CFU/g in fecal sample can be of diagnostic value. This study aims to determine the bacterial counts and predisposing factors of *C. perfringens* (targeting *CPA* gene) infection in addition to an *in-vitro* antimicrobial trial in entero-toxemic sheep in Pakistan. A total of 192 diarrheic sheep irrespective of age, gender and breed were selected and the CFU/g was determined from the fecal samples. The study showed that 34.9% of the samples had elevated level of bacterial count compared to the normal (10^4-10^7 CFU/g). Out of the total, 7.8% of the samples had subnormal bacterial count (CFU/g), while, 57.3% of the samples showed bacterial counts in the normal ranges. The confirmation of selectively isolated *C. perfringens* was done by amplification of 324bp *CPA* gene fragment using polymerase chain reaction (PCR). The *in-vitro* antimicrobial sensitivity trials showed that penicillin, ciprofloxacin and ceftriaxone are 100% efficacious against *C. perfringens*, while, bacitracin, ampicillin and amoxicillin were found to be least effective. The key determinants in this study which support the *in-vivo* growths of *C. perfringens* were; carbohydrate rich diet and overcrowding with the odds ratios (OR) of 5.44 and 2.26, respectively. This study concludes that *C. perfringens* is highly prevalent in sheep population of Pakistan. The incidence of enterotoxaemia can be minimized by controlling the factors which enhance its *in-vivo* growth. The diseased animal associated with elevated *C. perfringens* levels can be effectively cured using any one of the penicillin, ciprofloxacin and ceftriaxone.

INTRODUCTION

C. perfringens is a toxin producing gram positive bacteria affecting animals as well as humans. It is commonly found in soil and intestinal lumen producing disease in favorable environment (Songer, 1996; Petit *et al.*, 1999; Uzal, 2004). This pathogen causes a disease in small ruminants generally called as enterotoxaemia but named in accordance with different pathological conditions associated with various toxino-types. Type 'A' *C. perfringens* infection named as 'yellow lamb disease' in sheep, while, 'lamb dysentery' and hemorrhagic enteritis are caused by the type 'B' *C. perfringens* in sheep. The disease is mostly observed in young lambs, however, type 'C' *C. perfringens* affects adult sheep and the condition is named as 'Struck'. Pulpy kidney disease is caused by type 'D' *C. perfringens* (Uzal & Songer, 2008). *C. perfringens* produces toxins in intestinal lumen that are lethal both locally as well as systemically

if, it gets access to the systemic circulation. Epsilon toxin has been ranked as the 3rd most potent toxin produced by clostridial species, other 2 are botulism and tetanus toxin (Harkness *et al.*, 2012; Gill, 1982). The type 'A' causes gas gangrene in humans as well as animals. All types of *C. perfringens* produce alpha (α) toxin, while, iota (τ) toxin is produced by the type 'E' *C. perfringens* only. Type 'B' and 'C' *C. perfringens* produce beta (β) toxins while, epsilon (ϵ) toxin is produced only by Type 'B' and 'D' of *C. perfringens*. As 'α' toxin is produced by all types of *C. perfringens*, presence of *CPA* gene in the genome of bacterium is confirmative for the *C. perfringens* (Songer, 1996). Although any toxin gene presence not necessarily associated with the presence of that toxin but phenotype and genotype of bacterial strains match in the 99% of the cases (Meer & Songer, 1997).

C. perfringens is a normal inhabitant in the intestines of ruminants. It proliferates rapidly and produces potent toxins by following abrupt changes in micro (intestinal) and macro environment. There are some risk factors which favor the disease conditions. Some of the distinct risk factors are; sudden change in diet, carbohydrate rich diet, season, humidity, and overcrowding etc. Diagnosis of the disease is based on history, clinical symptoms and postmortem lesions. Presence of *C. perfringens* in the intestine or fecal samples of diseased or dead animals are not merely diagnostic because *C. perfringens* is a normal inhabitant of the intestinal tract. In the disease conditions, bacterial count of *C. perfringens* has diagnostic value and bacterial counts are usually found elevated i.e. higher than 10^4–10^7 CFU/g (Lewis, 2000; Uzal, 2004). Irrational use of antibiotics is causing *C. perfringens* to become resistant against different antimicrobial classes (Hedberg & Nord, 2003; Loivukene & Naaber, 2003). Due to the importance of this disease, this study was carried out with the aim of (1) estimating *C. perfringens* counts in diarrheic fecal samples of sheep, (2) risk factors assessment and (3) *in-vitro* antimicrobial trials against *C. perfringens*.

MATERIAL AND METHODS

Study Design
This study was conducted in small sheep herds located in Sargodha division of Punjab province, Pakistan during the year 2016-2017. Diarrheic sheep from the small holder herds were targeted for the estimation of elevated *C. perfringens* levels (CFU/g) along with associated risk factors. For this purpose, a total of 192 diarrheic sheep were selected from four districts of Sargodha division i.e. Bhakkar, Mianwali, Khushab and Sargodha, respectively. All the study districts contributed equal number of samples, from each district (48 diarrheic animals selected per district). Same number of samples were collected randomly from each district. Inclusion criteria for the sampled animals were, animal should have clinical signs, history of diarrhea and should not have received any prior treatment. Data capture form regarding hypothesized risk factors accompanied each sample collection.

Sample Culturing & Quantification of bacteria
Fecal samples were collected from diarrheic sheep (n=192; 48/district) and were kept at 4ºC during collection period. The samples were transported to Medicine laboratory maintaining the cold chain. After pooling of samples for first sampling phase, the samples were shifted to Microbiology laboratory for processing. The samples were preceded to selection and enrichment techniques anaerobically using anaerobic gas packs. Tryptose Sulphite Cycloserine (TSC) (Catalogue # M837I, Himedia Labs®, Mumbai, India) selective media for *C. perfringens* was used for the cultural growth. Serial dilutions (10 fold) of fecal samples in the Phosphate Buffer Saline (PBS 1x) were made and used for the quantification of bacterial load in the study sample. The samples were incubated anaerobically at 37°C for 48 hours. Afterwards, bacterial colonies were enumerated using colony counter and the dilution which produced 30-300 colonies on agar plates were considered significant for CFU count.

PCR for CPA gene

The typical black colonies of *C. perfringens* appeared on the culture plates were subjected to DNA extraction using TIANGEN® TIANamp, Bacterial DNA Kit, (Catalogue no. DP302) following the manufacturer's instructions. The extracted DNAs were checked for concentration and purity using Nano-drop at 260/280nm wavelength. The DNAs were further preceded to PCR targeting CPA gene fragment of *C. perfringens*. Previously reported primers (Van *et al.*, 2009) (forward primer: CPAlphaF: GCTAATGTTACTGCCGTTGA and reverse primer: CPAlphaR: CCTCTGATACATCGTG TAAG) against *CPA* gene were utilized to amplify a 324bp fragment of *C. perfringens CPA* gene. PCR reaction mixture was prepared in a final volume of 25µl consisting of 12.5µl of TOPreal™ qPCR 2x PreMIX (containing 0.2 U of Taq/µl), 2µl of DNA sample and 1µl of each primer (5pmol) and 8.5µl double distilled water. Reaction was cycled 40 times after initial denaturation at 95°C for 10 minutes. Thermocycler programing for 40 cycles of PCR was denaturation at 95°C for 1 minute followed by annealing at 53°C for 45seconds and finally an extension step at 72°C for 1 minute, a final elongation step at 72°C for 10 min was performed. The PCR products were electrophoresed at the end of reaction on ethidium bromide stained 2% agarose gel to identify the amplified 324bp fragment of *CPA* gene against 100bp molecular weight marker.

Antibiotic Sensitivity

Antibiotic sensitivity was conducted on 7 locally isolated *C. perfringens* following Kirby-Bauer antibiotic sensitivity test method. The local isolates were subjected to 10 different antibiotics salts i.e. Tetracycline (30µg), Metronidazole (5µg), Penicillin (10U), Ampicillin (10µg), Amoxicillin (30µg), Erythromycin (15µg), Vancomycin (30µg), Ciprofloxacin (10 U), Bacitracin (10µg) and Ceftriaxone (30µg) (Cat. No. DE018 Himedia Labs®,Mumbai, India).

Statistical analysis

Percentage was calculated for the antibiotic sensitivity test. Odds ratio was determined for the risk factors association with elevated bacterial count. For the determination of significance level, Chi-square test was applied. All the analyses were carried out using SPSS version 16. The *p*-value less than '0.05' and odds ratio greater than '1' were considered significant.
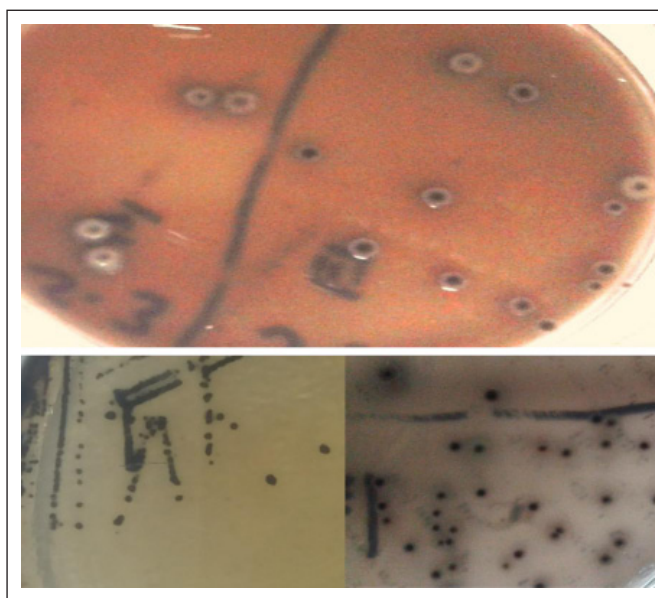

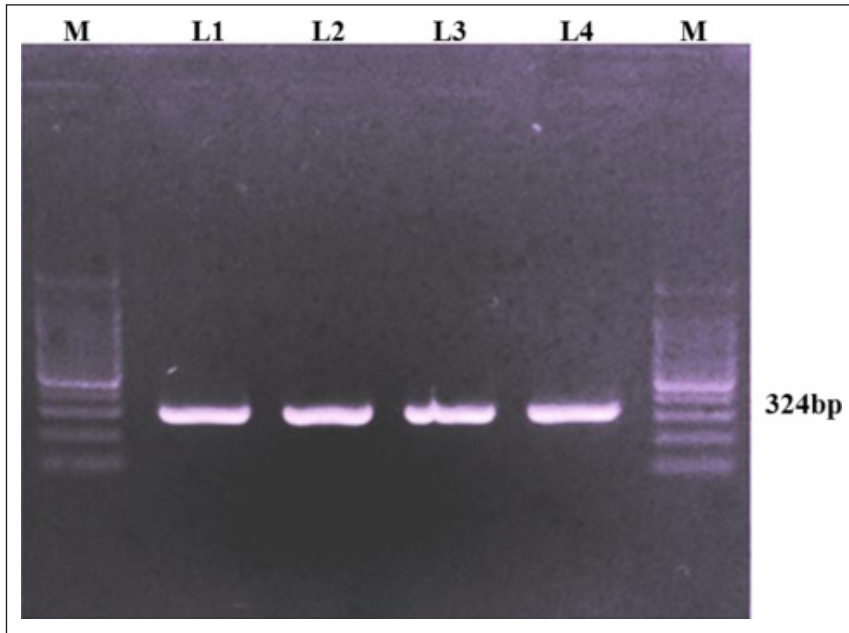Figure 1. Showing typical black colonies of *C. perfringens* on TSC media.

Figure 2. PCR Products (324bp) of *cpa* gene on ethidium bromide stained 2% Agarose gel. M: Marker, 100bp DNA Ladder. L1, L2, L3, *cpa* Gene fragments from field samples. L4: Positive Control.
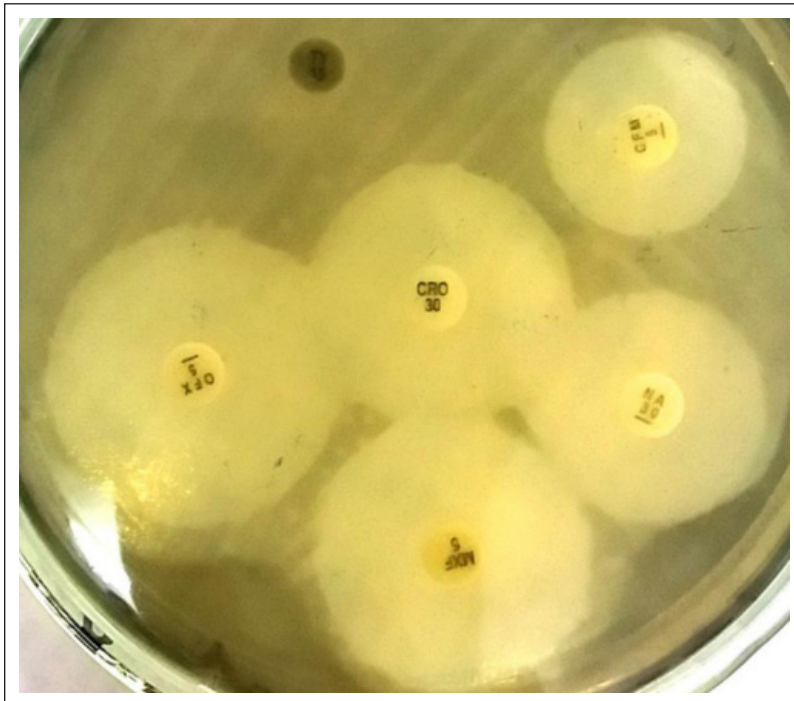

Figure 3. Antibiotic sensitivity of *C. perfringens* isolated from sheep fecal samples.

Table 1. *In-vitro* antibiotic susceptibility against *C. perfringens* isolates from sheep fecal samples

Antibiotics used	No. of isolates tested	Antibiotic susceptibility		
		Resistantn (%)	Intermediaten (%)	Susceptiblen (%)
Tetracycline (30µg)	7	3 (42.86)	4 (57.14)	0 (00.00)
Metronidazole (5µg)	7	0 (00.00)	2 (28.57)	5 (71.43)
Penicillin (10 U)	7	0 (00.00)	0 (00.00)	7 (100.0)
Ampicillin (10µg)	7	6 (85.71)	1 (14.29)	0 (00.00)
Amoxicillin (30µg)	7	5 (71.43)	2 (28.57)	0 (00.00)
Erythromycin (15µg)	7	1 (14.29)	4 (57.14)	2 (28.57)
Vancomycin (30µg)	7	0 (00.00)	2 (28.57)	5 (71.43)
Ciprofloxacin (10 U)	7	0 (00.00)	0 (00.00)	7 (100.0)
Bacitracin (10µg)	7	7 (100.0)	0 (00.00)	0 (00.00)
Ceftriaxone (30µg)	7	0 (00.00)	0 (00.00)	7 (100.0)

Table 2. Associated risk factors with bacterial count of *C. perfringens* in diarrheic sheep

Factors	Status	>10^7 CFU/g	<10^7 CFU/g	OR	RR	% Risk	*P-value*
Age	Young (65)	24	41	1.11	1.09	36.92	0.67
	Adult (127)	43	84			33.86	
Deworming	Yes (53)	24	29	1.84	1.46	45.28	0.06
	No (139)	43	96			30.94	
Season	Summer (96)	34	62	1.05	1.03	35.42	0.88
	Winter (96)	33	63			34.38	
Overcrowding	Yes (114)	48	66	2.26	1.73	42.10	0.01
	No (78)	19	59			24.36	
Carbohydrate rich diet	Rich (58)	36	22	5.44	2.68	62.07	0.00
	Poor (134)	31	103			23.13	

OR = odds ratio, RR = Relative Risk.

RESULTS

Isolation and identification of *C. perfringens*

In this study, 92.2% (177/192) of the samples showed growth of *C. perfringens* on TSC media which was equal or more than 10^4 CFU/g. On the basis of bacterial count growth results were divided into three groups. Elevated bacterial count (CFU/g) was recorded in 34.9% (67/192) of the samples in comparison with standard normal range 10^4–10^7 CFU/g (Uzal, 2004). The samples with bacterial count in normal ranges was 57.3% (110/192), while, samples with very low or zero growth (10^0–10^3 CFU/g) was 7.8% (15/192). The confirmation of isolates for *C. perfringens* was made by amplification of 324bp fragment of *CPA* gene using PCR.

Antibiotic Sensitivity

Study on antibiotic sensitivity against 7 *C. perfringens* local isolates was carried out on ten different antibiotics discs against *C. perfringens* for *in-vitro* sensitivity test. The results revealed that penicillin, ciprofloxacin and ceftriaxone were 100% efficacious against *C. perfringens* isolated from diarrheic sheep fecal samples. Bacitracin showed no inhibition to *C. perfringens* growth while, amoxicillin, ampicillin and tetracycline were least effective (Table 1).

Association of Risk Factors

Enterotoxaemia is a risk factors oriented disease, the etiological bacterium proliferates rapidly and produces large amount of deadly toxins when condition in micro (intestinal) and macro (ambient) environment are suitable. In this study, factors like; age, deworming, season, overcrowding and carbohydrate rich diet were studied for their association to enterotoxemia. The p-values based on chi-square test and odds ratios based on logistic regression for the association of different risk factors with growth of bacteria (CFU/g) were determined. On the basis of chi-square test, 'carbohydrate rich diet' and 'overcrowding' were recorded significantly associated ($p<0.05$) with elevated bacterial count of $C.$ $perfringens$ in fecal samples of sheep, while, on the bases of logistic regression, among the studied hypothesized determinants carbohydrate rich diet was highly associated (OR=5.44) with elevated bacterial count (CFU/g) of $C.$ $perfringens$ than normal range in sheep, diets rich in carbohydrates were enhancing the growth of $C.$ $perfringens$ more than the diets poor in carbohydrates. Another factor found as key determinant was the crowding factor (OR=2.26), animals kept in overcrowded environment were affected more than those kept in less crowded environment. Deworming status of herds was also studied which was associated significantly (OR=1.84) with occurrence of disease, herds, where deworming was practiced were affected more as compared to those with no practice of deworming. Age factor also showed positive association (OR=1.11) with disease dynamics, young aged animals were affected more than the adult ones. The last factor studied was the season which was associated (OR=1.05) in occurrence of elevated $C.$ $perfringens$ count; the occurrence was recorded higher in summer as compared to winter season.

DISCUSSION

Fecal samples from diarrheic sheep (n=192) were collected for the evaluation of bacterial ($C.$ $perfringens$) count. Samples were cultured under anaerobic conditions on TSC media. Bacterial counts in case of $C.$ $perfringens$ are important as it is normally present in the intestine of the animals. In disease conditions, CFU/g of $C.$ $perfringens$ increases in the intestinal contents. Fecal bacterial count 10^7 CFU/g can be considered as a differentiating point between healthy and diseased sheep but it can't be declared as ultimate diagnostic point (Uzal, 2004). A study in India reported 69.3% prevalence of $C.$ $perfringens$ in sheep (Kumar et $al.$, 2014), while, in current study overall prevalence was found to be 92.2% (177/192) from which only 34.9% (67/192) of the samples had bacterial count $>10^7$ CFU/g. The number of samples having bacterial count in the normal ranges (10^4–10^7 CFU/g) were; 57.3% (110/192). The samples that were either negative or with less than 10^4 CFU/g were; 7.8% (15/192). Goekce et $al.$ (2007) also studied prevalence of $C.$ $perfringens$ and found it in 84.6% of the sheep. In Iran the prevalence of $C.$ $perfringens$ was found to be 2.2% and 54.0% in fecal samples of vaccinated and non-vaccinated sheep, respectively (Ahsania et $al.$, 2011). In Nigeria, Vaikosen & Ikhatua, (2005) investigated the presence of $C.$ $perfringens$ toxins in fecal samples of sheep and goats by ELISA. They recorded 26.9% (92/342) of the samples to be positive for the lecithinase enzyme of the $C.$ $perfringens$. Variation in the prevalence of $C.$ $perfringens$ in different regions was observed. The variations in prevalence can be attributed to management and environmental determinants.

All the isolates 177/192 in the current study were found to be positive for the alpha toxin (CPA) gene. Aras & Hadimli, (2015) also found all isolates of $C.$ $perfringens$ from meat samples to be positive for the CPA gene. Albini et $al.$ (2008) found CPA gene in all isolates of $C.$ $perfringens$ from 10 different animal fecal samples. It can be concluded that CPA gene to be the universal gene in the $C.$ $perfringens$. This bacterium is developing resistants against antibiotics that are used in field conditions in Pakistan. Penicillin, ciprofloxacin and ceftriaxone are the only antibiotics found to be 100% sensitive against $C.$ $perfringens$ in the current study.

Bacitracin was 100% ineffective against *C. perfringens*, while, amoxicillin, ampicillin and tetracycline were less effective against *C. perfringens*. Novak *et al.* (2015) reported penicillin to be 100% sensitive against clostridial species in humans, based on records published from a hospital. Similarly, Skariyachan *et al.* (2010) observed ampicillin, tetracycline and chloramphenicolto be sensitive against *C. perfringens*. Khan *et al.* (2014) in Pakistan found *C. perfringens* to be sensitive against ciprofloxacin, chloramphenicol, metronidazole, and ceftriaxone, while, amoxicillin showed resistants.

C. perfringens resides in intestine as a normal microflora and causes disease only when, there is a suitable environment for growth and produce toxins. Risk factors such as suitable environment for bacterial growth can contribute to growth. Some of the risk factors were determined in this project. Carbohydrate rich diet, overcrowding, age, season, and deworming were found to be positively associated for the growth of bacteria in the intestine. In terms of odds ratio, carbohydrate rich diet gave the highest association with elevated bacterial count i.e. OR=5.44 ($p<0.05$) that shows it as a major factor in this disease. Overcrowding (odds ratio = 2.26) was the 2nd major risk factor according to this study. Other factors positively associated but not significant based on chi-square test are deworming as a risk factor but shows contradictions in its role in prevention of the disease.

Authors' contributions:
KH, MI, AAA, designed and executed the study, KI, AAN, SHF did study sampling, KI, AAN processed the samples, SHF, ASA, AIA arranged and analyzed the statistical data, KI wrote the manuscript, AZD, MI reviewed and approved the manuscript for submission.

REFERENCES

Ahsania, M.R., Shamsaddini, B.M., Esmailizadeha, A.K. & Mohammadabadi, M.R. (2011). Genotyping of isolates of *Clostridium perfringens* from vaccinated and unvaccinated sheep. *Small Ruminant Research* **95**(1): 65-69.

Albini, S., Brodard, I., Jaussi, A., Wollschläger, N., Frey, J., Miserez, R. & Abril, C. (2008). Real-time multiplex PCR assays for reliable detection of *Clostridium perfringens* toxin genes in animal isolates. *Veterinary Microbiology* **127**(1): 179-185.

Novak, A., Rubic, Z., Dogas, V., Goic-Barisic, I., Radic, M. & Tonkic, M. (2015). Antimicrobial susceptibility of clinically isolated anaerobic bacteria in a University Hospital Centre Split, Croatia in 2013. *Anaerobe* **31**: 31-36.

Gill, D.M. (1982). Bacterial toxins: a table of lethal amounts. *Microbiological Reviews* **46**(1): 86.

Goekce, H.I., Genç, O., Soezmen, M. & Gökçe, G. (2007). Determination of *Clostridium perfringens* toxin-types in sheep with suspected enterotoxemia in Kars Province, Turkey. *Turkish Journal of Veterinary and Animal Sciences* **31**(5): 355-360.

Harkness, J.M., Li, J. & McClane, B.A. (2012). Identification of a lambda toxin-negative *Clostridium perfringens* strain that processes and activates epsilon protoxin intracellularly. *Anaerobe* **18**(5): 546-552.

Hedberg, M. & Nord, C.E. (2003). Antimicrobial susceptibility of Bacteroides fragilis group isolates in Europe. *Clinical Microbiology and Infection* **9**(6): 475-488.

Khan, M., Nazir, J., Anjum, A.A., Nawaz, M. & Shabbir, M.Z. (2015). Toxinotyping and antimicrobial susceptibility of enterotoxigenic *Clostridium perfringens* isolates from mutton, beef and chicken meat. *Journal of Food Science and Technology* **52**(8): 5323-5328.

Kumar, N.V., Sreenivasulu, D. & Reddy, Y.N. (2014). Prevalence of *Clostridium perfringens* toxin genotypes in entero-

toxemia suspected sheep flocks of Andhra Pradesh. *Veterinary World* **7**(12): 1132-1136.

Lewis, C.J. Clostridial diseases. In: Martin WB, Aitken ID, editors. (2000) Diseases of sheep. Oxford: Blackwell Science.

Loivukene, K. & Naaber, P. (2003). Antibiotic susceptibility of clinically relevant anaerobes in Estonia from 1999 to 2001. *Anaerobe* **9**(2): 57-61.

Meer, R.R. & Songer, J.G. (1997). Multiplex polymerase chain reaction assay for genotyping *Clostridium perfringens*. *American Journal of Veterinary Research* **58**(7): 702-705.

Petit, L., Gibert, M. & Popoff, M.R. (1999). *Clostridium perfringens*: toxinotype and genotype. *Trends in Microbiology* **7**(3): 104-110.

Skariyachan, S., Mahajanakatti, A.B., Biradar, U.B., Sharma, N. & Abhilash, M. (2010). Isolation, identification and characterization of *Clostridium perfringens* from cooked meat-poultry samples and in silico biomodeling of its delta enterotoxin. *International Journal of Pharmaceutical Sciences Review and Research* **4**: 164-172.

Songer, J.G. (1996). Clostridial enteric diseases of domestic animals. *Clinical Microbiology Reviews* **9**(2): 216.

Uzal, F.A. & Songer, J.G. (2008). Diagnosis of *Clostridium perfringens* intestinal infections in sheep and goats. *Journal of Veterinary Diagnostic Investigation* **20**(3): 253-265.

Uzal, F.A. (2004). Diagnosis of *Clostridium perfringens* intestinal infections in sheep and goats. *Anaerobe* **10**(2): 135-143.

Vaikosen, E.S. & Ikhatua, U.J. (2005). Detection of high level of enterotoxin of Clostridum perfingens types C and D in small ruminants in Nigeria. *Small Ruminant Research* **58**(3): 287-290.

Van Asten, A.J., van der Wiel, C.W., Nikolaou, G., Houwers, D.J. & Gröne, A. (2009). A multiplex PCR for toxin typing of *Clostridium perfringens* isolates. *Veterinary Microbiology* **136**(3-4): 411-412.

Aras, Z. & Hadimli, H.H. (2015). Detection and molecular typing of *Clostridium perfringens* isolates from beef, chicken and turkey meats. *Anaerobe* **32**: 15-17.