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Clostridium perfringens Type A from Broiler Chicken with Necrotic Enteritis in Kashmir Valley, India

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The current study reports occurrence and molecular toxinotyping of *Clostridium perfringens* isolates in Kashmir valley. A total of 217 feacal samples and intestinal contents of broiler chickens suspected for enteritis were collected and screened for *C. perfringens* out of which 66 (30.41%) were found positive using Robertson's cooked meat media and Sulphite Polymixin Sulphadiazine (SPS) agar. The isolates were confirmed by 16S rRNA gene specific PCR and analyzed by multiplex PCR for toxinotyping. All the isolates were found to harbour alpha toxin gene (*cpa*) of *C. perfringens*. However, 13 isolates also carried beta2 toxin gene (*cpb2*). None of the isolates were positive for beta (*cpb*), epsilon (*etx*), iota (*itx*) and enterotoxin (*cpe*) genes. Highest prevalence was found in chicken with age group of 2-6 weeks. Mortality ranged from 2-4 percent per day. Necropsy of chickens revealed that their intestines were dilated with inflammatory exudates and characterized by mucosal necrosis indicating *Clostridium perfringens* type A to be the major causative types for necrotic enteritis of broilers in Kashmir, India with significant role of alpha toxin.

Introduction

Avian necrotic enteritis (NE) caused by *C. perfringens* type A, rod shaped, gram-positive, anaerobic spore forming bacterium is wide spread in broilers imposing a significant economic burden on the poultry industry worldwide (Dahiya *et al.*, 2006). It is an acute, often fatal, disease of chickens characterized by depression, loss of appetite and sudden death. It occurs both as an acute clinical disease and as a subclinical disease with necrosis in the intestines or as *C. perfringens*-associated hepatitic change (CPH) witiohepatitis or fibrinoid necrosis in the liver. In broilers, outbreaks of clinical NE have been reported from all poultry growing areas of the world (Ficken and Wages, 1997; Dahiya *et al.*, 2006). It has also been recorded in wild geese (Wobeser and Rainnie, 1987), wild crows (Asaoka *et al.*, 2004), ostriches (Kwon *et al.*, 2004) and in capercaillies reared in captivity (Hofshagen and Stenwig, 1992). *C. perfringens* type A from soil, dust and contaminated feed and litter has been implicated as a source of infection (Craven *et*

al., 2001). Although, more than one toxin produced by *C. perfringens* might be involved in causing NE, alpha-toxin is the most important (Al-Sheikhly and Truscott, 1977a; Al-Sheikhly and Truscott, 1977b). Later, a novel pore-forming toxin named NetB (necrotic enteritis toxin B-like) was identified in the strains of *C. perfringens* associated with NE.

Under field conditions, NE occurs in two forms: an acute clinical form and a mild subclinical form. The acute form of NE is characterized by visible clinical signs like watery diarrhoea and sudden increase in flock mortality but often without any premonitory signs. Birds that do not succumb to clinical signs, may develop a sub-clinical clostridial infection.

In the sub-clinical form of the disease there is often no peak mortality. Intestinal damage as a consequence of disease leads to a reduction in performance noticeable as reduced weight gain and an increased feed conversion ratio (Kaldhusdal and Hofshagen, 1992; Timbermont *et al.,* 2010). Acute symptoms of the disease are severe depression, decreased appetite, and reluctance to move, ruffled feathers and diarrhoea (Ficken and Wages, 1997). The period of illness is usually short (1-2 hrs) and most of the birds are just found dead (Helmbold and Bryant, 1971). Birds that have died of NE have a foetid odour, are usually dehydrated with dark, dry pectoral musculature and pale kidneys (Long *et al.,* 1974). The unopened intestinal wall is darker in colour than normal and distended due to the presence of large amounts of bile stained contents.

In an affected flock the mortality rate can be anything from 1% to as high as 50% (Craven *et al.,* 2001). Sub-clinical NE does not manifest any clinical signs and mostly under field conditions, only detected at the processing plant(s) by the rejection of carcasses (McDevitt *et al.,* 2006). It is usually associated with hepatitis and cholangiohepatitis.

The environment in the intestinal tract is of vital importance for the growth of *C. perfringens* and there probably has to be a disturbance in the jejunum before *C. perfringens* can start to proliferate in this part of the gut. Important factors are nutrition, pH, oxygen and the microflora in the jejunum (Johansson, 2006). Different types of stress experienced by the birds may also cause disturbances in the gut.

The most important known predisposing factor for NE is damage to the intestinal mucosa, caused by coccidial pathogens. In particular, *Eimeria* species that colonize the small intestine, such as *Eimeria maxima* and *Eimeria acervulina* are known to predispose to necrotic enteritis (Williams *et al.,* 2003). Present study reports the occurrence of fatal necrotic enteritis due to *C. perfringens* in broiler chicken and their virulence determinants.

Materials and Methods

Sample collection

A total of 217 samples (87 fecal materials were from live birds and 130 intestinal samples were from post mortem carcasses) with a history of sudden deaths or diarrhoea were collected from organized farms such as Govt. Poultry Farm, Hariparbath; Instructional Poultry Farm, Faculty of Veterinary Sciences and Animal Husbandry (FVSc & AH), Shuhama, Srinagar; Division of Veterinary Pathology, FVSc & AH, Shuhama and private owners of district Ganderbal and Srinagar with a total flock size of 2672 birds. The samples were collected in sterile vials and transported to the lab on ice.

Isolation and identification of *Clostridium perfringens*

The samples were inoculated in Difco™Cooked meat medium (Becton, Dickinson and Company, Sparks, MD, USA) and incubated anaerobically in 3.5 litre anaerobic jar (Oxoid Limited, Thermo Fisher Scientific Inc., UK) with GasPak™Anaerobe Container System (Becton, Dickinson and Company, Sparks, MD, USA) at 37°C for 24 hrs. Enriched samples were streaked on Sulphite Polymixin Sulphadiazine agar plates (SPS HiVeg™ Agar, Modified; Hi-Media laboratories, Mumbai, India) and the plates were incubated anaerobically as above at 37°C for 24 hrs. Confirmation of the isolates was done by demonstration of the typical cellular morphology in Gram's stained smear, standard biochemical tests and detection of *C. perfringens* by species specific polymerase chain reaction (PCR) using 16S rRNA specific primers.

Extraction of DNA

The DNA was extracted by boiling method. Briefly, suspected isolated colonies from agar plates were suspended in 1.5 ml microcentrifuge tubes containing 100 µl of distilled water by gentle vortexing. The samples were boiled for 5 min, cooled on ice for 10 min and centrifuged at 10,000×g in a table-top microcentrifuge (Cooling Centrifuge, Eppendorf 5418R, Hamburg, Germany) for 10 min. Three microlitres (µl) of the supernatant was used as the template for PCR.

Amplification of 16S rRNA gene of *Clostridium perfringens* by polymerase chain reaction

Isolates of *C. perfringens* were confirmed by amplifying 16S rRNA gene of the *C. perfringens* (Tonooka *et al.*, 2005). The primers used in the experiment have the following sequence: forward-TAACCTGCC TCATAGAGTR and reverse- TTTCACAT CCCACTTAATC. The PCR conditions consisted of initial denaturation at 95ºC for 15 min, followed by 35 cycles of denaturation at 94ºC for 30 sec, annealing at 49ºC for 90 sec and extension at 72ºC for 90 sec. This was followed by final extension at 72ºC for 10 min.

Multiplex Polymerase chain reaction

All the *C. perfringens* isolates were also screened for six different toxin genes using a multiplex PCR for toxinotyping (van Asten *et al.*, 2008). These six toxin genes include α-toxin (*cpa*), β-toxin (*cpb*), ε-toxin (*etx*), ί-toxin (*cpi*), β2-toxin (*cpb-2*) and enterotoxin (*cpe*).

The primers used for the amplification of the genes are shown in Table 1. The PCR conditions consisted of initial denaturation at 95ºC for 15 min, followed by 35 cycles of denaturation at 94ºC for 30 sec, annealing at 53ºC for 90 sec and extension at 72ºC for 90 sec. This was followed by final extension at 72ºC for 10 min. The MTCC culture of *C. perfringens* type B was used as positive control, while sterilized distilled water served as negative controls.

All the PCR assays in this study were performed in 25 µl reaction volume in thermal cycler (Mastercycler Gradient, Eppendorf AG, Hamburg, Germany).

The reaction consisted of template DNA, 2.5 µl of 10X buffer, 0.2 µl of 25mM dNTP mix, 1 U of Taq DNA Polymerase (Fermentas Life Sciences), 0.5µl of each forward and reverse primers and sterile distilled water. The $MgCl_2$ was adjusted at 2.0 mM concentration. The primers were procured from GCC Biotech, Kolkata, India.

Results and Discussion

All the broiler chickens showed severe depression, decreased appetite, diarrhea, reluctance to move and ruffled feathers prior to death. Post mortem examination showed that birds were dehydrated and had fetid odor. Intestines of affected birds revealed congestion with catarrhal exudate (Fig. 1). Focal necrosis and hemorrhages on the upper surface of liver was also noticed in few chicken. These findings correlate with the reports of detection of *C. perfringens* in poultry (Kerry *et al.,* 2013)

Out of 217 samples of chicken, 66 (19 from live birds and 47 from postmortem carcass) harboured the *C. perfringens.* The fecal and morbid samples after overnight incubation in cooked meat medium, showed turbidity and pink colouration of the meat pieces. On Sulphite Polymixin Sulphadiazine (SPS) agar *C. perfringens* colonies were typically black pigmented or creamish in colour (Fig. 2). On Gram's staining the black colonies isolated on SPS agar appeared typically Gram-positive rods forming subterminal endospores suggestive of *C. perfringens* (Fig. 3). All the isolates produced stormy fermentation, coagulation in litmus milk, showed lecithinase activity on egg yolk agar plates and fermented glucose and lactose. On sheep blood bacterial colonies were found small dew drop like colonies surrounded by an inner zone of complete haemolysis and an outer zone of incomplete haemolysis (Fig. 4). All the 66 isolates which were morphologically and biochemically identified as *C. perfringens,* were also confirmed by 16S rRNA gene specific PCR (Fig. 5). Among 66 isolates, 29 were from organised sector and 37 from unorganised sector (Table 2). All the 66 isolates were found to possess *cpa* gene alone as a major toxin gene, thus were designated as toxinotype A. Thirteen of the isolates also carried beta2 toxin gene (*cpb2*) (Fig. 6). None of the isolates carried *cpb*, *etx* or *cpi* genes indicating the absence of *C. perfringens* toxinotype B, C, D or E in chicken samples. The findings are in agreement with other workers from different parts of the world who reported isolation of *C. perfringens* type A from chicken (Nauerby *et al.,* 2003; Baums *et al.,* 2004; Malmarugan *et al.,* 2012; Park *et al.,* 2015). Gholamiandekhordi *et al.,* (2006) detected β2 toxin gene in five out of 63 isolates from poultry flocks with different disease conditions and suggested that the β2 toxin is not an important or essential virulence factor in the development of disease in poultry. Similarly, *cpb2* gene was detected in *C. perfringens* type A isolates from commercial broiler chickens and parent broiler breeder hens in Ezypt (Osman *et al.,* 2012) and from native fowl suffering from necrotic enteritis in India (Thomas *et al.,* 2014). In the present study, none of the isolates carried *cpe*-gene which is in agreement with the finding of other workers (Engstrom *et al.,* 2003, Heikinheimo and Korkeala, 2005). Recently, it was speculated that *cpb2* positive *C. perfringens* may cause focal duodenal necrosis in egg-laying chickens (França *et al.,* 2016). For a long time, alpha toxin was proposed to be the main virulence factor for necrotic enteritis in poultry, although the normal broiler microbiota and outbreak strains of *C. perfringens* were type A. (Nauerby *et al.,* 2003; Gholamiandehkordi *et al.,* 2006). In the recent past, a novel toxin called necrotic enteritis B-like toxin (NetB) was described that were associated with necrotic enteritis in broilers (Keyburn *et al.,* 2008). However, we did not detect the toxin in our study. On the other hand, *netB*-negative isolates from birds suffering from necrotic enteritis may indicate that other, yet to be determined, virulence factors are produced by these strains. However, *netB*-negative strains do not induce necrotic enteritis in experimental bird models.

Fig.1 Congestion with catarrhal exudate in small intestines

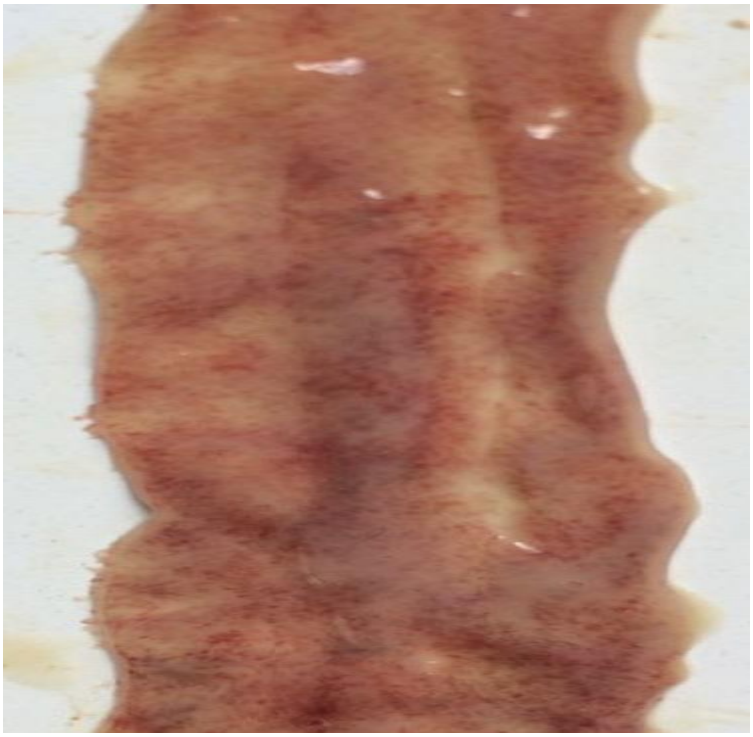

Fig.2 Culture of *Clostridium perfringens* on Sulphite Polmixin Sulphadiazine (SPS) agar medium showing black and cream colonies

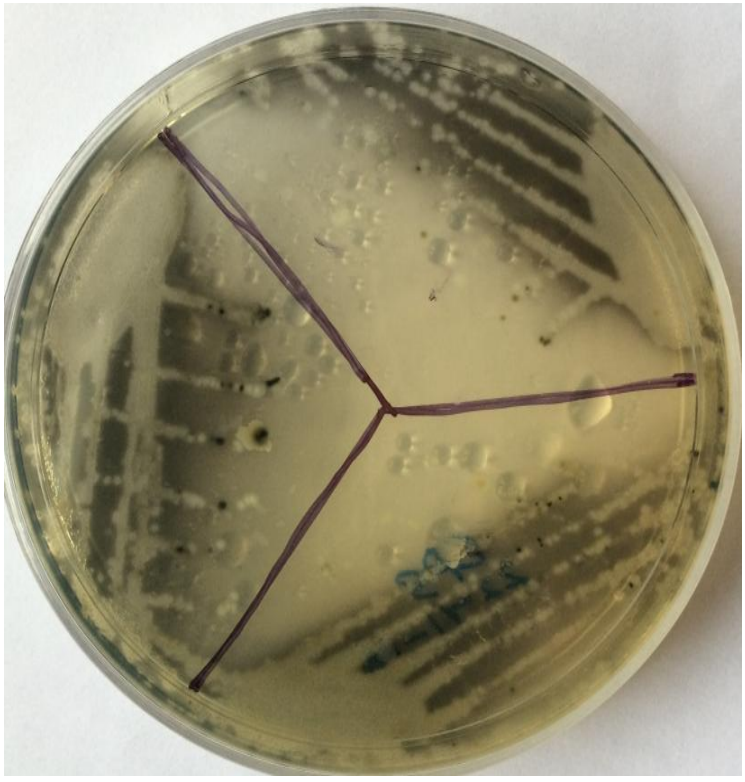

Fig.3 Gram stained smear of *Clostridium perfringens* showing Gram positive violet rods under oil immersion

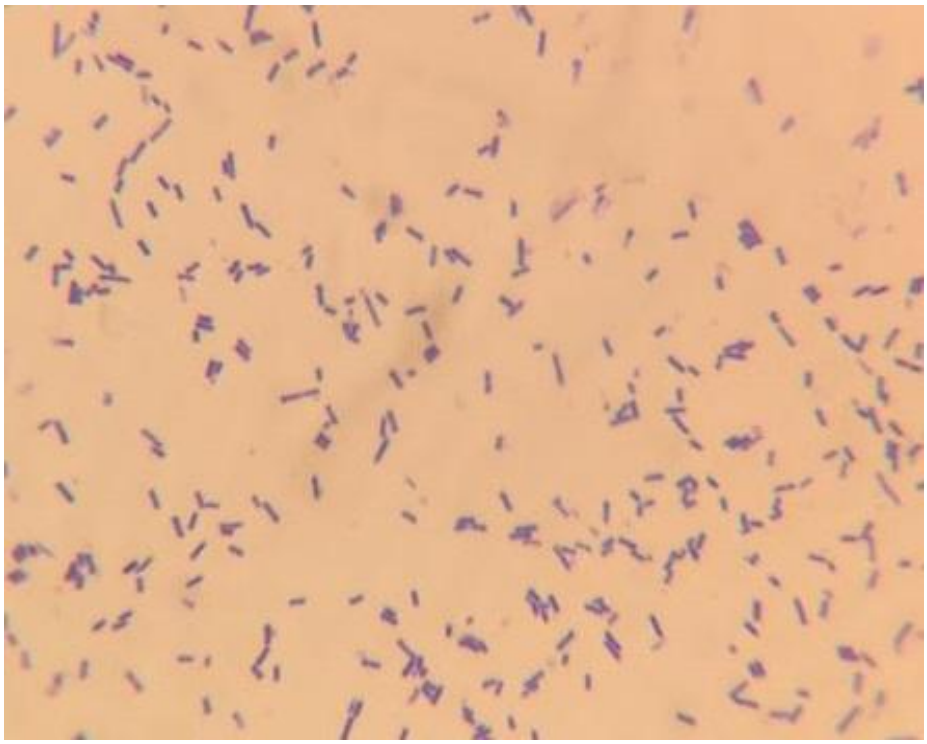

Fig.4 Double zone of hemolysis produced by *Clostridium perfringens* on sheep blood agar

Fig.5 Agarose gel electrophoresis showing 481 bp amplicon of 16SrRNA gene of *Clostridium perfringens* Lane M : 100 bp ladder, Lane 1: Negative control, Lane 2: Positive control, Lane 3 & 4: *C. perfringens* with amplified 16SrRNA gene

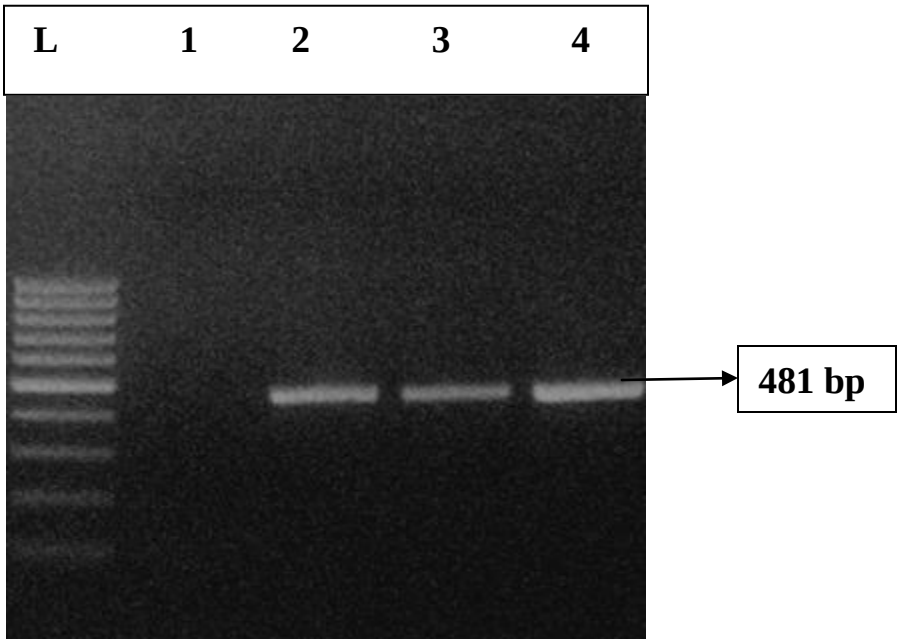

Fig.6 Agarose gel electrophoresis of multiplex PCR amplicons of different virulence genes of *Clostridium perfringens*. Lane M: 100 bp ladder, Lane 1: Positive control, Lane 2: Negative control, Lane 3: *C. perfringens* Type A with amplified *cpa* (324 bp) and *beta* 2 (548 bp) genes, Lane 4: *C. perfringens* Type A with amplified *cpa* (324 bp) gene

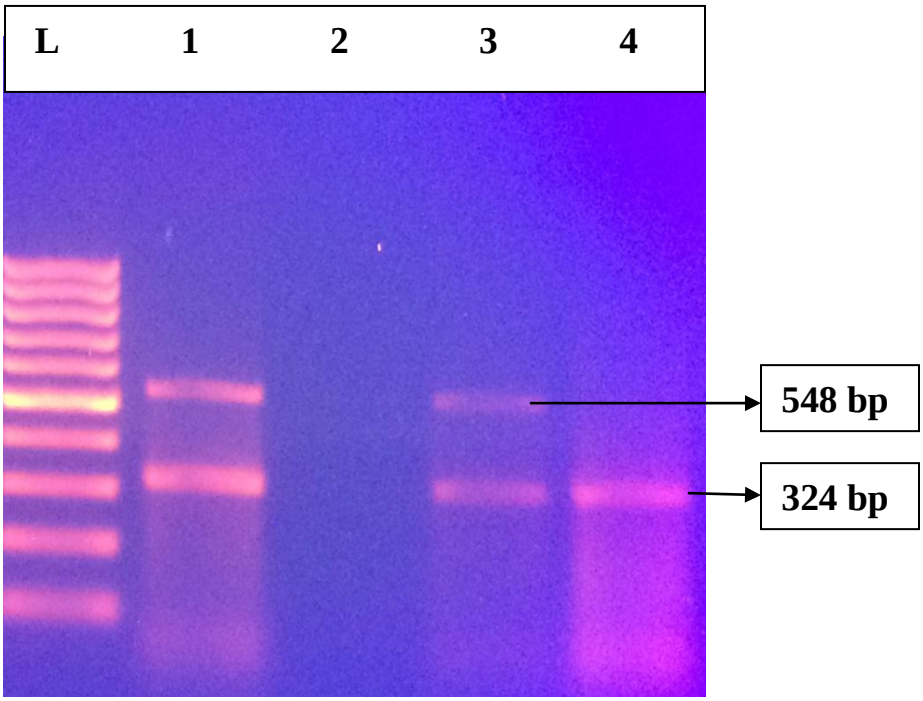

Table.1 List of primers used for toxinotyping

Primers	Primer Sequences (5′-3′)	Target gene	Primer conc. (µM each)	Product size (bp)	Reference
Cpa-F	GCTAATGTTACTGCCGTTGA	*Cpa*	0.4	324	van Asten *et al.*, (2008)
Cpa-R	CCTCTGATACATCGTGTAAG				
Cpb-F	GCGAATATGCTGAATCATCA	*Cpb*	0.4	195	
Cpb-R	GCAGGAACATTAGTATATCTTC				
Etx-F	TGGGAACTTCGATACAAGCA	*Etx*	0.4	376	
Etx-R	AACTGCACTATAATTTCCTTTTCC				
Cpi-F	AATGGTCCTTTAAATAATCC	*Cpi*	0.4	272	
Cpi-R	TTAGCAAATGCACTCATATT				
cpb2-F	AAATATGATCCTAACCAACAA	*cpb2*	0.4	548	
cpb2-R	CCAAATACTCTAATYGATGC				
Cpe-F	TTCAGTTGGATTTACTTCTG	*Cpe*	0.4	485	
Cpe-R	TGTCCAGTAGCTGTAATTGT				

Table.2 Occurrence of *Clostridium perfringens* toxinotypes in chicken under organized and unorganized rearing condition

S No.	**Source**	**No of samples tested**	**No of samples positive for *C. perfringens***	**Toxinotype**	**Occurrence of *C. perfringens* (%)**
1	Private sector	131	37	A	28.24
2	Govt. sector	86	29	A	33.72
	Total	217	66		30.41

Table.3 Age wise distribution of *Clostridium perfringens* in chickens

Age group	No. of samples	No. Positive
2-6 weeks	141	47(33.33%)
> 6 weeks	76	19(25%)

The rate of isolation was more (33.33%) from broilers of 2 to 6 wk of age than older birds (25%) (Table 3). This is in agreement with Lovland and Kaldhusdal (2001), who reported detection of C. *perfringens* in 2-6 wk broiler chickens. Furthermore, *C. perfringens* is auxotrophic for 13 amino acids (Myers *et al.*, 2006) and an increase in available nutrients would thus allow *C. perfringens* to proliferate extensively, leading to increased intestinal toxin levels. Thus, predisposing factors are important, but it is also essential that poultry pathogenic strains should be present to induce the disease (Timbermont *et al.*, 2011).

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