DOI: 10.5937/FFR1801067M
UDK 636.085:579.86

Original research paper

TOXIN GENOTYPES OF *Clostridium perfringens* IN ANIMAL FEED AND THEIR ROLE IN THE ETHIOLOGY OF ENTEROTOXEMIA IN DOMESTIC ANIMALS

Dubravka S. Milanov*[1], Tamaš R. Petrović[1], Dalibor S. Todorović[1], Nevenka R. Aleksić[2], Ivana S. Čabarkapa[3]

[1] Scientific Veterinary Institute „Novi Sad“, 21000 Novi Sad, Rumenački put 20, Serbia
[2] University of Belgrade, Faculty of Veterinary Medicine, 11000 Belgrade, Bulevar oslobođenja 18, Serbia
[3] University of Novi Sad, Institute of Food Technology, 21000 Novi Sad, Bulevar cara Lazara 1, Serbia

*Corresponding author
Tel.: +381 21 4895 346
E-mail: dubravka@niv.ns.ac.rs

ABSTRACT: *Clostridium perfringens* is a Gram-positive, endospore-forming, anaerobic rod, ubiquitous in nature. *C. perfringens* strains can produce about 17 toxins. Many of them can lead to miscellaneous diseases, among which the enteric ailment may be the most common and is of utmost importance. In the present work 34 strains of *C. perfringens* isolated from feed and one from a cow suspected to have died of clostridial infection were subjected to molecular analysis. In order to detect the genotypes, the following genes coding for toxins were targetted: *cpa*, *cpb*, *cpb2, cpe*, *etx* and *iap*. The multiplex PCR assay revealed that all *C. perfringens* isolates from animal feed were of type A and β2-toxinogenic type A strains, possessing only the *cpa* (n=21), or both the *cpa* and the *cpb*2 genes (n=13). The importance of *C. perfringens* toxins α and β-2 in the pathogenesis of enterotoxemia is discussed and the regulation on the detection of this bacteria in animal feed questioned. The use of PCR in practise could enable the toxin-genotyping of *C. perfringens* isolates and, thus, provide a real basis for the establishment of maximum acceptable limits of this bacteria in feed.

Key words: *Clostridium perfringens, toxin genotyping, animal feed, multiplex PCR assay, cpa gen, cpb2 gen*

INTRODUCTION

Clostridium perfringens is a Gram-positive, non-motile, endospore-forming, anaerobic (relatively oxygen-tolerant) bacteria, ubiquitous in nature (found in soil, dust, sewage, and fresh and marine water) and a common inhabitant of the intestinal tract in both humans and homeothermic animals (Songer, 1996; Brynestad and Granum, 2002). The species represents a very heterogeneous group with respect to metabolic bioproducts, toxins and pathogenicity. *C. perfringens* strains can produce about 17 toxins (Uzal et al., 2015; Freedman et al., 2016), four of which (α, β, ε and ι) are major factors of virulence. Based on the capability to produce them, *C. perfringens* is classified into five toxotypes: A, B, C, D and E (Hatheway, 1990; Sawires and Songer, 2006). Two other major toxins are enterotoxin (CPE) and β-2 toxin, both possibly produced by all types of *C. perfringens* (Ata et al., 2013). Each toxotype may cause different diseases in animals, including acute enteritis and fatal

enterotoxemia (Baums et al., 2004; Ata et al., 2013; Lyhs et al., 2013). Some varieties within types A, B and C produce certain combinations of antigens or toxins associated with defined diseases or syndromes (Ata et al., 2013; Uzal et al., 2010, 2014). Their quantities vary greatly between individual strains (Niilo, 1980).

C. perfringens may enter feed via contaminated raw ingredients, or by secondary contamination during processing, storage and dispersal of final products. Feed contamination depends on its composition, the hygiene during production and the storage conditions, and is directly proportional to the levels of soil and faecal contamination (Wojdat et al., 2006).

In the Republic of Serbia, in compliance with regulations (Regulations on the quality of animal feed), feed samples of 50 g must not contain any *C. perfringens*. For food it is stated in The EFSA Journal (EFSA, 2005) that "*C. perfringens* is commonly present in foods and ingredients, occasionally at hundreds per gram, and microbiological testing for *C. perfringens* has limited value in ensuring food safety, because the organism is so common in or on foods that a positive result means little, unless very high numbers are present". In spite of being notorious for extremely toxigenic potential, not all of *C. perfringens* strains are capable of producing illness in neither animals nor humans.

Experimental researh has confirmed that the ingestion of *C. perfringens* will not lead to sickness *per se*, because the majority of bacteria consumed with food are destroyed by hydrochloric acid in the stomach. Moreover, *C. perfringens* does not exhibit adherence and invasive properties towards healthy intestinal mucosa. The development of subclinical or clinical clostridiosis in domestic animals always results from complicated interactions between the virulence of the bacteria (which is highly variable within this species), immune status of the host and the influence of a variety of non-specific factors associated with the farm management.

Gut microbiota disturbances (most frequently resulting from antibiotic therapy), management-related stress, sudden changes in diet, and overeating and/or voraciousness, especially on high-protein and energy-rich foods, are predisposing factors for the development of enteritis and enetrotoxemia (Collier et al., 2008; Timbermont et al., 2011; Uzal et al., 2015).

For all these reasons, without the identification of toxin genotypes and the levels of feed contamination it is difficult to assess the health risk *C. perfringens* in feed poses to animals. Nonetheless, toxin genotyping of *C. perfringens* strains isolated from food stuffs and animal feed is not part of routine laboratory practice in Serbia.

In this work *C. perfringens* isolates (n=34) from food intended for animals were subjected to multiplex PCR essay in order to detect genes: *cpa* (α toxin), *cpb* (β), *cpb2* (β2), *etx* (ε), *iap* (ι) and *cpe* (enterotoxin). Further, the importance of those findings are discussed from the standpoint of health risk to food-producing animals and, indirectly, to humans, consumers of animal products. The results of such investigations can be the basis for risk assessment and determination of critical limits of the presence of *C. perfringens* in animal feed.

MATERIALS AND METHODS

***C. perfringens* isolates**. *C. perfringens* was isolated from feed samples according to the standard SRPS ISO 7937:2010. The species identification was based on: (1) the characteristics of the colonies grown on agar with the addition of 5% sheep blood and incubated in anaerobic conditions (GasPak EZ, Becton Dickinson and Company, USA), (2) synergistic haemolysis with *Streptococcus agalactiae* (reverse CAMP test), and (3) biochemical properties: catalase test (negative), fermentation of glucose and lactose and production of lecithinase. Thirty-four isolates of *C. perfringens* were subjects of genotyping. Their origin is presented in Table 2. In addition, one isolate from the organs of a cow which was suspected to have died of enterotoxemia was assessed: the organs were sent to the laboratory with the feed that was allegedly the source of the infection.

DNA extraction and PCR amplification

From *C. perfringens* isolates incubated overnight on blood agar suspensions in PBS were made and turbidity adjusted to 3.5 McFarland. One milliliter was transferred to an Eppendorf tube and boiled in water for 15 minutes. The tubes were cooled on ice and centrifuged for 8 min at 11,000 x g. The supernatant obtained (100 μL) was preserved in a freezer at -20 °C until being processed further. The multiplex PCR assay (mPCR) was performed according to the protocol described by Baums et al. (2004). Six sets of oligonucleotide primers were used (Table 1). The mPCR method was carried out using the "HotStar Taq Master Mix Kit" (Qiagen, Hilden, Germany), with small modification of manufacturer's instructions. Briefly, the amplification reaction was carried out in a volume of 25 µl containing 3 µl of DNA sample, 12.5 µl of master mix and 25 pmol of each primer. *C. perfringens* ATCC 13124 was used as the positive and *C. sporogenes* ATCC 19404 as the negative control.

The cycling conditions (Thermocycler Gradient, Eppendorf, Germany) were the following: initial denaturation at 95 °C for 2 min 30s, 40 cycles of 1 min at 95 °C, 1 min at 55 °C and 1 min at 72 °C, which was followed by the final extension for 10 min at 72 °C. Ten microlitres of the PCR products were separated by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide (0.25 g/ml) and documented with a gel documentation system (GelDoc-It^2 Imager, UVP, Cambridge, UK).

RESULTS

All of the *C. perfringens* isolates formed small translucent colonies surrounded by the zone of double haemolysis on blood agar (Figure 1A). Clear zone of haemolysis results from the theta (θ) toxin, whilst the α-toxin is responsible for incomplete haemolysis. The production of α-toxin in all isolates was confirmed with the positive reverse CAMP test (Figure 1B).

The multiplex PCR assay revealed the presence of specific amplicons: 900-bp long, which are characteristic of *cpa* gene, 396 bp of *etx*, and 200 bp of the *cpb*2 gene encoding for *C. perfringens* toxins (Figures 2, 3 and Table 2). All *C. perfringens* isolates derived from feed were identified as type A and β2-toxinogenic type A strains, since they possessed only the *cpa* (n=21), or the *cpa* and the *cpb*2 genes (n=13). In neither isolate from feed genes coding for beta (*cpb*), epsilon (*etx*) and iota toxin (*iap*) were detected.

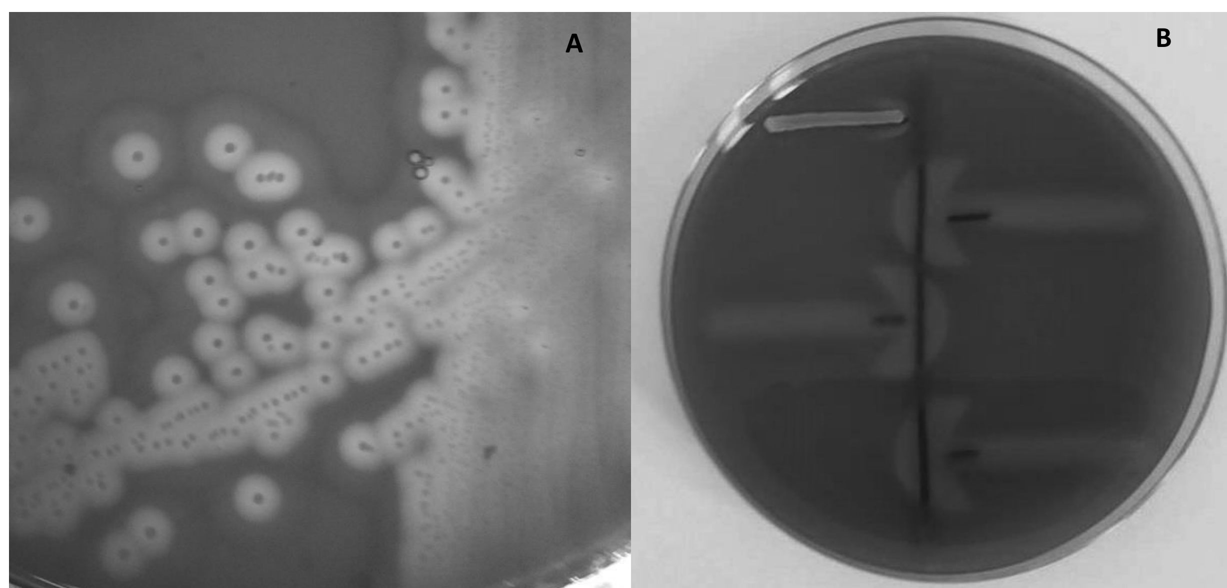

Figure 1. Characteristic colonies of *C. perfringens* on blood agar surrounded by zone of double haemolysis (A); Positive reverse CAMP test: *Str. agalactiae* ATCC 13813 (vertical line), *Staphylococcus aureus* ATCC 25923 (positive control, upper left horizontal line) and *C. perfringens* isolates (B)

Table 1.
Target toxin genes, primer sequences and length of amplification products of *C. perfringens* in multiplex PCR (Baums et al., 2004).

Toxin gene	Primers	Sequence (5`-3`)	Length of amplification products (bp)
cpa	CPA5L CPA5R	AGTCTACGCTTGGGATGGAA TTTCCTGGGTTGTCCATTTC	900
cpb	CPBL CPBR	TCCTTTCTTG,AGGGAGGATAAA TGAACCTCCTATTTTGTATCCCA	611
cpe	CPEL CPER	GGGGAACCCTCAGTAGTTTCA ACCAGCTGGATTTGAGTTTAATG	506
etx	CPETXL CPETXR	TGGGAACTTCGATACAAGCA TTAACTCATCTCCCATAACTGCAC	396
iap	CPIL CPIR	AAACGCATTAAAGCTCACACC CTGCATAACCTGGAATGGCT	293
*cpb*2	CPB2L CPB2R	CAAGCAATTGGGGGAGTTTA GCAGAATCAGGATTTTGACCA	200

Table 2.
The sources of *C. perfringens* isolates and the results of their toxo-typing

Sources and sample numbers	Genotype			Phenotype
	***cpa*+**	***cpa*+, *cpb*2+**	***cpa*+, *cpe*+**	
Poultry feed mixture (13)	10	4	-	A
Pig feed mixture (8)	5	4	-	A
Cow feed mixture (7)	4	4	-	A
Sunflower meal (2)	2	-	-	A
Premix (1)	-	1	-	A
Cow's organs (n=1)	-	-	1	A
Total	21	13	1	

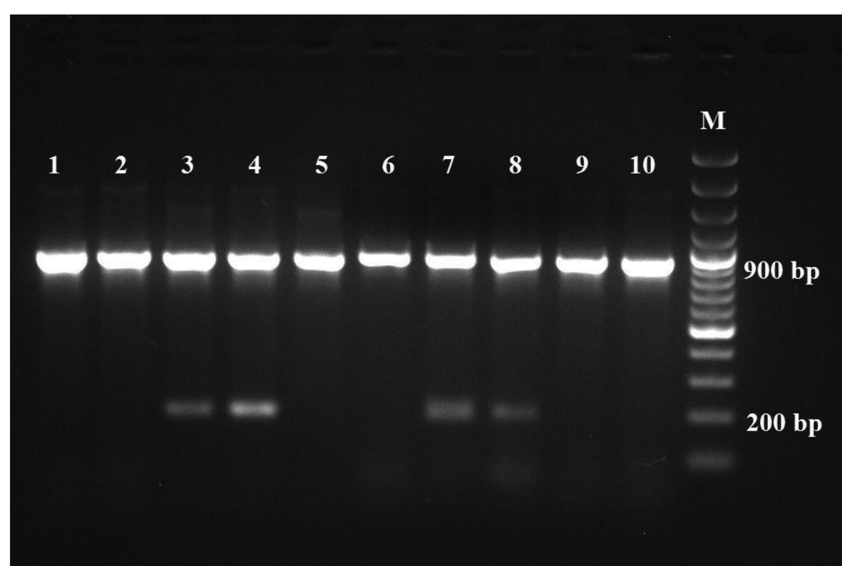

Figure 2. Lane 1 *C. perfringens* ATCC 13124; lanes 2, 5, 6, 9 and 10: *C. perfringens* isolates type A strains with α toxin (*cpa*); lanes 3, 4, 7 and 8: *C. perfringens* type A with α (cpa) and β2 toxins (*cpb*2)

Figure 3. Lane 1 *C. perfringens* ATCC 13124; lanes 6 and 12: *C. perfringens* isolates type A strains with α toxin (*cpa*); lanes 2, 3, 5, 7, 8, 9 and 11: *C. perfringens* type A with α (cpa) and β2 toxins (*cpb*2); lane 4 *C. perfringens* type A with enterotoxin (*cpe*); lane 10 negative control (*Clostridium sporogenes* ATCC 19404)

Gene for the enterotoxin (*cpe*) was detected in only one strain, which was isolated from the cow's organs.

Fig. 2. and 3. show agarose gel electrophoresis of the amplicons obtained by multiplex PCR. Lane M – O'Gene Ruler 100 bp DNA marker (Fermentas).

DISCUSSION

C. perfringens is notorious for being one of the common contaminants of various foodstuffs, which was also proven in this research conducted on several types of animal feed (Kanakaraj et al., 1998; Casagrande et al., 2013; Kukier et al., 2013; Tessary et al., 2014; Udhayavel et al., 2017). In this study, all *C. perfringens* isolates (n=34) were identified as type A and β2-toxinogenic type A strains, possessing only the *cpa* (n=21), or both the *cpa* and the *cpb*2 genes (n=13), respectively. There are numerous data of the widespread presence of these. Type A was the most prevalent toxo-type in feed samples, domestic animal intestines and environmental samples (Wojdat 2006; Kanakaraj et al., 1998; Kukier et al., 2010; Mueller-Spitz et al., 2010).

For example, 174 *C. perfringens* isolates from environ-mental sources and sewage were identified as type A (Mueller-Spitz et al., 2010) and 53.3% of isolates from poultry feed belonged to type A and 42% to type A subtype β-2 (Kukier et al., 2010). In 96% faecal samples from poultry fed on these mixtures *C. perfringens* type A was isolated and in 4% β-2 type A; all samples were taken from chickens and poultry which did not show any signs of diseases.

In Poland 334 *C. perfringens* isolates from animal feed stuffs were processed with multiplex PCR to detect six genes coding for toxins. Most of the isolates, 50%, possessed genes coding for both α and β2 toxins, whilst the gene responsible for α toxin only was detected in 46.4% isolates, which means that the vast majority of the strains were C. *perfringens* type A. *C. perfringens* types E, C, (including the one with a β2 toxin gene) and type D capable of producing β2 toxin made up for less than 4% of the isolates in total (each present in less than 1% of the isolates), whilst *C. perfringens* type B was not detected (Wojdat et al., 2006).

C. perfringens type A is the most common of all *C. perfringens* types, and is part of the normal gut microbiome in humans and animals. Of all major lethal toxins, type A produces only the α-toxin, common to all

C. perfringens toxotypes (Uzal et al., 2010). The α-toxin is an enzyme, a phospholipase which hydrolyzes lecithin (Niilo, 1980; Yoo et al., 1997). Because the membranes of the majority mammalian cells are composed of lipoproteins containing lecithin, its destruction leads to necrosis, hemolysis, or even death, depending on the tissue(s) involved. The α-toxin is essential for the pathogenesis of gas gangrene and its production is considered a housekeeping capacity of all *C. perfringens* strains. However, the role of this microorganism and its major α-toxin in natural intestinal disease remains controversial and poorly documented (Uzal et al., 2010). *C. perfringens* type A is even considered to be the most confusing organism in respect to its pathogenicity (Niilo, 1980; Ata et al., 2013).

C. perfringens type A has been reported to cause enterotoxemia in sheep and calves, sometimes even leading to sudden deaths. However, most of the data were not proven and based mainly on bacterial isolation and clinical signs, which were unspecific. Alpha toxin is present in the gut content of many clinically healthy animals. Thus, its detection in the intestinal content of affected animals is irrelevant for the diagnosis of the disease (Uzal et al., 2010). In addition, *C. perfringens* type A can be readily isolated from samples taken soon after death, can be grown easily and may mask other bacteria, implying that it had been the only causative agent. Researchers failed to produce acute enterotoxemia in calves and sheep by intraduodenal administration of *C. perfringens* type A. Administered intravenously, the toxin led to death only in relatively large doses applied within a short time, whilst slow application resulted in milder effects, probably due to rapid elimination. In sum, it seems unlikely that α-toxin can be produced in the intestines in high quantities and absorbed rapidly enough to result in fatal toxemia (Niilo, 1980).

Alfa-toxin production was believed to be an essential virulence factor in the pathogenesis of both clinical and subclinical necrotic eneteritis (NE) in laying hens and turkeys (Lyhs et al., 2013; Tessari et al., 2014). Recently it was established that only strains producing NetB toxin, a β-poreforming toxin, are capable of inducing NE in broiler chickens under specific predisposing conditions (Keyburn et al., 2008; Timbermont et al., 2011; Antonissen et al., 2014; Uzal et al., 2015). High numbers of *C. perfringens* present in the intestinal tract or inoculated to chickens do not produce NE (Baba et al., 1997; Kukier et al., 2010; Lyhs et al., 2013¸ Antonissen et al., 2014). Given that *C. perfringens* does not produce enzymes for the synthesis of 13 aminoacids, its growth is limited in the environment with restricted aminoacid contents (Cooper and Songer, 2009; Brynestad and Granum, 2002; Antonissen et al., 2014). The outbreak of the disease is influenced by various non-specific factors contributing to intestinal environment, which favours the growth of *C. perfringens,* leading to mucosal damage, increased gut permeability and decreased peristaltic movements. The best-known predisposing factor is mucosal damage caused by coccidia (Baba et al., 1997; Williams, 2005; Collier et al., 2008). A variety of factors can contribute to the outbreak of the disease, such as high wheat, barley or fish meal contents in feed (Lyhs et al., 2013), high levels of indigestible, water-soluble, non-starch polysaccharides (Jia et al., 2009), high concentrations of animal proteins (Gholamiandehkordi et al., 2007), animal fat (Knarreborg et al., 2002), programmed alterations in the feeding regime and immune-suppressive agents such as the viruses of chick anaemia, Gumboro disease or Marek's disease (Timbermont et al., 2011), and the Fusarium mycotoxin deoxynivalenol (Antonissen et al., 2014).

One of the difficulties in the understanding of the pathogenesis and diagnosis of enteric disease caused by *C. perfringens* is based on the fact that most of its types can frequently be found in the intestines of healthy animals. Simple isolation of *C. perfringens* is therefore of no use in diagnostic of enteric infections (Uzal *et al*., 2015).The intestines of birds suffering NE contain large numbers of *C. perfringens*, up to 10^6 or 10^8 CFU/g of intestinal contents (Cooper and Songer, 2009), whereas in healthy broilers the counts usually range

up to 10^2, or 10^3 CFU/g (Baba et al., 1997; Kukier et al., 2010). For epizootical analysis of animal diseases caused by *C. perfringens*, strains isolated from affected birds and the feed they had consumed should be compared. First, the toxotype and subtype is to be detected (with PCR), which is followed by the confirmation of identical DNA profiles (with pulsed-field gel electrophoresis) (Kukier et al., 2010). However, additional research may detect some other anaerobes in feed, which may also conduce to diseases. PCR analysis may detect that healthy birds can carry 2-5 genotypes of *C. perfringens* type A, contrary to those affected, which are colonized by a single genetic type other than that found in diseased birds (Cooper and Songer, 2009).

The answer to the question whether *C. perfringens* is a general foodborne pathogen is immensely complicated. In its toxigenic behaviour type A can be subdivided into two varieties. The "classical" variety, characterized mainly by α-toxin production, is associated with gas gangrene, traumatic infections and the normal intestinal tract. In the beginning, α-toxin was considered to play the main role in human food poisoning and enteritis, but another variety of type A, which is producing the enterotoxin (CPE), was later determined. The enterotoxin was first isolated in 1970 (Brynestad and Granum, 2002). About 1-5% of all *C. perfringens* type A strains can produce it (Miyamoto et al., 2004; Lindström et al., 2011; Mueller-Spitz et al., 2010).

Foodborne diseases caused by *C. perfringens* can only result from strains carrying the *cpe* gene, which encods for the enterotoxin (Niilo, 1980; Goldner et al., 1986; Xiao et al., 2012) and is located in the bacterial chromosome or on large plasmids (Brynestad and Granum, 2002; Smedley et al., 2004; Miyamoto et al., 2004; Lindström et al., 2011; Kukier et al., 2010). The enterotoxin is a cytotoxic polypeptide which causes fluid and electrolyte loss from the intestinal mucosa. Type A CPE-positive strains also cause several non-foodborne human gastrointestinal diseases, including about 5–10% of all cases of antibiotic-associated diarrhea (Freedman et al., 2016). CPE is produced in the small intestine after ingestion of at least 10^7 C. *perfringens* cells (Brynestad and Granum, 2002).

Molecular detection of genes encoding for enterotoxins is extremely important in the inspection of animal-derived foodstuffs and is more reliable than conventional enterotoxin detection; they are all intended for the prevention of the entrance of these strains into the food chain (Kanakaraj et al., 1998). In this research, the gene coding for enterotoxin was detected only in one *C. perfringens* strain isolated from a dead cow, but not in the feed mixture which the animal had been fed on. This result implies that animal feed is irrelevant to the entrance of *cpe*-positive strains of *C. perfringens* to the food chain. Previously, the use of toxin genotyping of *C. perfringens* from swine feed sampled on different farms (n=60) found no isolates positive for *cpe* (Kanakaraj et al., 1998). In addition, molecular typing of isolates obtained from swine denied that this species was a source of *cpe*-positive strains of *C. perfringens* (Kanakaraj et al., 1998).

Gene coding for β-2 toxin (*cpb*2) was confirmed in 13 *C. perfringens* isolates: in those originating from feed intended for poultry (n=4), pigs (n=4) and cows (n=4), and from a praemix (n=1). This toxotype might be important for the development of enteric diseases in pigs and cattle. A significant correlation (82%) between *C. perfringens* possessing the β2-toxin gene and diarrhoea in piglets was identified, suggesting that β2-toxin may play a key role in the pathogenesis of the disease (Garmory et al., 2000). More than 85% of isolates from cases of porcine enteritis and 91.8% isolates of newborn pig enetiritis were positive for the *cpb*2 gene (Bueschel et al., 2003). However, *cpb*2-positive strains can be found in both healthy and diseased chickens and its presence does not correlate with disease (Keyburn et al., 2008). Pulsed-field gelelectrophoresis performed on *C. perfringens* isolated from turkeys with NE and from healthy birds, in all of them detected α-toxin, and in one both α and β2 toxins, which indicates that the β2 toxin is not an important virulence factor in the development of NE (Lyhs et

al., 2013). Beta-2 toxin has been associated with porcine, equine and bovine gastroenteritis (Bueschel et al., 2003; Van Immerseel et al., 2004).

In type A strains isolated from various animals, food products and environment the prevalence of the *cpb*2 gene is high (Bueschel et al., 2003; Garmory, et al. 2000; Mueller-Spitz et al., 2010). The gene is plasmid-borne, at least in some strains (Shimizu et al., 2002), which implies its mobility and the possibility of transfer among strains of *C. perfringens*.

In this research all animal feed samples from which *C. perfringens* was isolated the spore numbers were less than 100 CFU/g. In similar trials the numbers in feeds did not exceed 10^2 CFU/g (Kukier et al., 2010) or 10^3 CFU/g (Wojdat et al., 2006). Thus, it is reasonable to raise the question why the numbers of *C. perfringens* in feed are to be assessed given that the Regulation orders that feed be condemned if this species is even detected. Estimating the numbers would be sound if maximum allowed numbers per gram had been established. The PCR test ensures the detection of all genes considered to be important virulence factors in *C. perfringens*-mediated enteritis or enterotoxaemia and thus provides a useful and reliable tool for *C. perfringens* genotyping in routine veterinary diagnostics (Baums et al., 2004). The mPCR assay is recommended for risk assessment of *C. perfringens* toxotypes isolated from animal feed. The obtained results can help to establish microbiological criteria for critical limits of the presence of *C. perfringens* in feed (Wojdat et al., 2006).

ACKNOWLEDGEMENTS

This work was supported by the grant provided by the Ministry of Education, Science and Technological Development of the Republic of Serbia for the Projects Nos. TR 31071 and III 46002.

REFERENCES

1. Antonissen, G., Van Immerseel, F., Pasmans, F., Ducatelle, R., Haesebrouck, F., Timbermont L., Verlinden, M., Paul, G., Janssens, J., Eeckhaut, V., Eeckhout, M., De Saeger, S., Hessenberger,S., Martel, A., Croubels, S. (2014). The mycotoxin deoxynivalenol predisposes for the development of *Clostridium perfringens*-induced necrotic enteritis in broiler chickens. *PLoS ONE, 9* (9), e108775. DOI:10.1371/journal.pone.0108775.
2. Ata, N., Khairy, E.A., Dorgham, S.M., Zaki, M.S. (2013). *Clostridium perfringens* disease. *Life Science Journal, 10* (1), 1599-1602.
3. Baba, E., Ikemoto, T., Fukata, T., Sasai, K., Arakawa, A., McDougald, L.R. (1997). Clostridial population and the intestinal lesions in chickens infected with *Clostridium perfringens* and *Eimeria necatrix*. *Veterinary Microbiology, 54* (3-4), 301-308.
4. Baums, C.G., Schotte, U., Amtsberg, G., Goethe, R. (2004). Diagnostic multiplex PCR for toxin genotyping of *Clostridium perfringens* isolates. *Veterinary Microbiology*, *100* (1-2), 11–16.
5. Brynestad, S., Granum, P.E. (2002). *Clostridium perfringens* and foodborne infections. *International Journal of Food Microbiology, 74* (3), 195-202.
6. Bueschel, D.M., Jost, B.H., Billington, S.J., Trinh, H.T., Songer, J.G. (2003). Prevalence of *cpb*2, encoding beta2 toxin, in *Clostridium perfringens* field isolates: correlation of genotype with phenotype. *Veterinary Microbiology, 94* (2), 121–129.
7. Casagrande, M.F., Cardozo, M.V., Beraldo-Massoli, M.C., Boarini, L., Longo, F.A., Paulilo, A.C., Schocken-Iturrino, R.P. (2013). *Clostridium perfringens* in ingredients of poultry feed and control of contamination by chemical treatments. *The Journal of Applied Poultry Research, 22* (4), 771–777.
8. Collier, C.T., Hofacre, C.L., Payne, A.M., Anderson, D.B., Kaiser, P., Mackie, R.I., Gaskins, H.R. (2008). Coccidia-induced mucogenesis promotes the onset of necrotic enteritis by supporting *Clostridium perfringens* growth. *Veterinary Immunology and Immunopathology, 122* (1-2), 104–115.
9. Cooper, K.K., and Songer, J.G. (2009). Necrotic enteritis in chickens: A paradigm of enteric infection by *Clostridium perfringens* type A. *Anaerobe, 15* (1-2), 55–60.
10. EFSA (2005). Opinion of the Scientific Panel on Biological Hazards on a request from the Commission related to *Clostridium* spp in foodstuffs.*The EFSA Journal, 199,* 1-65. Adopted on 9-10 March 2005. (https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2005.199).
11. Freedman J.C., Shrestha A., McClane B.A. (2016). *Clostridium perfringens* enterotoxin: Action, genetics, and translational applications. *Toxins, 8* (3), 73, DOI:10.3390/toxins8030073.
12. Garmory, H.S., Chanter, N., French, N.P., Bueschel, D., Songer J.G., Titball, R.W. (2000). Occurrence of *Clostridium perfringens* beta 2-toxin amongst animals, determined using genotyping and subtyping PCR assays. *Epidemiology and Infection, 124* (1), 61–67.

13. Gholamiandehkordi, A.R., Timbermont, L., Lanckriet, A., Van Den Broeck, W., Pedersen, K., Dewulf J., Pasmans, F., Haesebrouck, F., Ducatelle, R.,Van Immerseel, F. (2007). Quantification of gut lesions in a subclinical necrotic enteritis model. *Avian Pathology, 36* (5), 375-82.
14. Goldner, S.B., Solberg, M., Jones, S., Post, L.S. (1986). Enterotoxin synthesis by nonsporulating cultures of *Clostridium perfringens*. *Applied and Environmental Microbiology, 52* (3), 407-412.
15. Hatheway, C.L. (1990). Toxigenic Clostridia. *Clinical Microbiology Reviews, 3* (1), 66–98.
16. Jia, W., Slominski, B.A., Bruce, H.L., Blank, G., Crow, G., Jones, O. (2009). Effect of diet type and enzyme addition on growth performance and gut health of broiler chickens during subclinical *Clostridium perfringens* challenge. *Poultry Science*, *88* (1), 132-140.
17. Kanakaraj, R., Harris, D.L., Songer, J.G., Bosworth, B. (1998). Multiplex PCR assay for detection of *Clostridium perfringens* in feces and intestinal contents of pigs and in swine feed. *Veterinary Microbiology, 63* (1), 29-38.
18. Keyburn, A.L., Boyce, J.D., Vaz, P., Bannam, T.L., Ford, M.E., Parker, D., Di Rubbo, A., Rood, J.I., Moore, R.J. (2008). NetB, a new toxin that is associated with avian necrotic enteritis caused by *Clostridium perfringens*. *PLoS Pathogens, 4*, e26, DOI:10.1371/journal.ppat.0040026.
19. Knarreborg, A., Simon, M.A., Engberg, R.M., Jensen, B.B., Tannock, G.W. (2002). Effects of dietary fat source and subtherapeutic levels of antibiotic on the bacterial community in the ileum of broiler chickens at various ages. *Applied and Environmental Microbiology, 68* (12), 5918-5924.
20. Kukier, E., Goldsztejn, M., Kwiatek, K. (2010). Epidemiological investigation of animal diseases caused by *Clostridium perfringens* strains isolated from feedingstuffs. *Krmiva, 52* (6), 339-343.
21. Kukier, E., Goldsztejn, M., Grenda, T., Kwiatek, K., Bocian, L. (2013). Microbiological quality of feed materials used between 2009 and 2012 in Poland. *Bulletin of the Veterinary Institute in Pulawy, 57* (4), 535-543.
22. Lindström, M., Heikinheimo, A., Lahti, P., Korkeala, H. (2011). Novel insights into the epidemiology of *Clostridium perfringens* type A food poisoning. *Food Microbiology, 28* (2), 192–198.
23. Lyhs, U., Perko-Mäkelä, P., Kallio, H., Brockmann, A., Heinikainen, S., Tuuri, H., Pedersen, K. (2013). Characterization of *Clostridium perfringens* isolates from healthy turkeys and from turkeys with necrotic enteritis. *Poultry Science*, *92* (7), 1750–1757.
24. Miyamoto, K., Qiyi Wen, Q., Bruce, A. McClane, B.A. (2004). Multiplex PCR genotyping assay that distinguishes between isolates of *Clostridium perfringens* type A carrying a chromosomal enterotoxin gene (*cpe*) locus, a plasmid *cpe* locus with an IS*1470*-like sequence, or a plasmid *cpe* locus with an IS*1151* sequence. *Journal of Clinical Microbiology, 42* (4), 1552-1558.
25. Mueller-Spitz, S.R., Stewart, L.B., Klump, J.V., McLellan, S.L. (2010). Freshwater suspended sediments and sewage are reservoirs for enterotoxin-positive *Clostridium perfringens*. *Applied and Environmental Microbiology, 76* (16), 5556-5562.
26. Niilo, L. (1980). *Clostridium perfringens* in animal disease: a review of current knowledge. *The Canadian Veterinary Journal, 21* (5), 141-148.
27. Sawires, Y.S., Songer, J.G. (2006). *Clostridium perfringens*: Insight into virulence evolution and population structure. *Anaerobe, 12* (1), 23-43.
28. Shimizu, T., Ohtani, K., Hirakawa, H., Ohshima, K., Yamashita, A., Shiba, T., Ogasawara, N., Hattori, M., Kuhara, S., Hayashi, H. (2002). Complete genome sequence of *Clostridium perfringens*, an anaerobic flesh-eater. *Proceedings of the National Academy of Sciences of the United States of America, 99* (2), 996-1001.
29. Smedley, J.G., Fisher, D.J., Sayeed, S., Chakrabarti, G., McClane, B.A. (2004). The enteric toxins of *Clostridium perfringens*. *Reviews of Physiology, Biochemistry and Pharmacology, 152*, 183-204.
30. Songer, J.G. (1996). Clostridial enteric diseases of domestic animals. *Clinical Microbiology Reviews, 9* (2), 216-234.
31. SRPS ISO 7937: Horizontalna metoda za određivanje broja *Clostridium perfrin*gens – Tehnika brojanja kolonija (Horizontal method for enumeration of *Clostridium perfringens*-Coloniy-count technique), Institut za standardizaciju Srbije, 2010.
32. Tessari, E.N.C., Cardoso, A.L.P., Kanashiro, A.M.I., Stoppa, G.F.Z., Luciano, R.L., deCastro, A.G.M. (2014). Analysis of the presence of *Clostridium perfringens* in feed and raw material used in poultry production. *Food and Nutrition Sciences, 5* (7), 614-617.
33. Timbermont, L., Haesebrouck, F., Ducatelle, R., Van Immerseel, F. (2011). Necrotic enteritis in broilers: an updated review on the pathogenesis. *Avian Pathology, 40* (4), 341–347.
34. Udhayavel, S., Ramasamy, G.T., Gowthaman, V., Malmarugan, S., Senthilvel K. (2017). Occurrence of *Clostridium perfringens* contamination in poultry feed ingredients: Isolation, identification and its antibiotic sensitivity pattern. *Animal Nutrition, 3* (3), 309-312.
35. Uzal, F.A., McClane, B.A., Cheung, J.K., Theoret, J., Garcia, J.P., Moore, R.J., Rood, J.I. (2015). Animal models to study the pathogenesis of human and animal *Clostridium perfringens* infections. *Veterinary Microbiology, 179* (1-2), 23-33.
36. Uzal, F.A., Freedman, J.C., Shrestha, A., Theoret, J.R., Garcia, J., Awad M.M., Adams, V., Moore, R.J., Rood, J.I., McClane, B.A. (2014). Towards an understanding of the role of *Clos-*

tridium perfringens toxins in human and animal disease. *Future Microbiology*, *9* (3), 361-377.

37. Uzal, F.A., Vidal, J.E., McClane, B.A., Gurjar, A.A. (2010). *Clostridium perfringens* toxins involved in Mammalian veterinary diseases. *The Open Toxinology Journal, 2*, 24–42.
38. Xiao, Y., Wagendorp, A., Moezelaar, R., Abee T., Wells-Bennik M.H. (2012). A wide variety of *Clostridium perfringens* type A food-borne isolates that carry a chromosomal *cpe* gene belong to one multilocus sequence typing cluster. *Applied and Environmental Microbiology, 78* (19), 7060–7068.
39. Van Immerseel, F., De Buck, J., Pasmans, F., Huyghebaert, G., Haesebrouck, F., Ducatelle, R. (2004). *Clostridium perfringens* in poultry: an emerging threat for animal and public health. *Avian Pathology, 33* (6), 537-549.
40. Williams, R.B. (2005). Intercurrent coccidiosis and necrotic enteritis of chickens: rational, integrated disease management by maintenance of gut integrity. *Avian Pathology, 34* (3), 159-180.
41. Wojdat, E., Kwiatek, K., Kozak, M. (2006). Occurrence and characterization of some *Clostridium* species isolated from animal feedingstuffs. *Bulletin of the Veterinary Institute in Pulawy, 50* (1), 63-67.
42. Yoo, H.S., Lee, S.U., Park, K.Y., Park, Y.H. (1997). Molecular typing and epidemiological sur-vey of prevalence of *Clostridium perfringens* types by multiplex PCR. *Journal of Clinical Microbiology, 35* (1), 228-232.

ТОКСИН ГЕНОТИПИЗАЦИЈА СОЈЕВА *Clostridium perfringens* ИЗОЛОВАНИХ ИЗ ХРАНЕ ЗА ЖИВОТИЊЕ И ЊИХОВ ЗНАЧАЈ У ЕТИОЛОГИЈИ ЕНТЕРОТОКСЕМИЈА ДОМАЋИХ ЖИВОТИЊА

Дубравка С. Миланов*[1], Тамаш Р. Петровић[1], Далибор С. Тодоровић[1], Невенка Р. Алексић[2], Ивана С. Чабаркапа[3]

[1] Научни институт за ветеринарство „Нови Сад“, 21000 Нови Сад, Руменачки пут 20, Србија
[2] Универзитет у Београду, Факултет ветеринарске медицине, 11000 Београд, Булевар ослобођења 18, Србија
[3] Универзитет у Новом Саду, Научни институт за прехрамбене технологије у Новом Саду, 21000 Нови Сад, Булевар цара Лазара 1, Србија

Сажетак: *Clostridium perfringens* је Грам-позитивна, анаеробна, спорулишућа, штапићаста бактерија, убиквитарно распрострањена у природи. Код различитих сојева *C. Perfringens,* до данас, идентификовано је око 17 врста токсина. *C. perfringens* је узрочни агенс различитих обољења (синдрома), али су цревне инфекције/интоксикације најчешће и од највећег значаја за здравље фармски гајених животиња. У овом раду приказујемо резултате испитивања сојева *C. perfringens* пореклом из хране за животиње (n=34) и једног изолата из органа краве угинуле са знацима ентеротоксемије на присуство гена: *cpa*, *cpb*, *cpb2, cpe*, *etx* и *iap* применом мултиплекс PCR технике. Сви сојеви *C. perfringens* изоловани из хране за животиње, идентификовани су као тип А који поседује само *cpa* ген (n=21) или тип А који продукује β2-токсин, односно има *cpa* и *cpb*2 гене (n=13). У раду дискутујемо о улози алфа (α) и бета-2 (β2) токсина у патогенези ентеротоксемија домаћих животиња, као и актуелном законском пропису по којем ова врста бактерије не сме бити присутна у храни за животиње. Примена PCR технике у свакодневној пракси омогућила би токсин-генотипизацију сојева *C. perfringens,* а тиме и реалне основе за успостављање граничних дозвољених вредности за ову врсту бактерије у храни за животиње.

Кључне речи: *Clostridium perfringens, токсин генотипизација, храна за животиње, мултиплекс PCR техника, cpa ген, cpb2 ген*

Received: 17 May 2018

Received in revised form: 6 June 2018

Accepted: 9 June 2018