Incidence of *Clostridium perfringens* in Broiler Chickens in the Czech Republic

I. SVOBODOVÁ, I. STEINHAUSEROVÁ, M. NEBOLA

Department of Meat Hygiene and Technology, Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic

Received June 4, 2007
Accepted October 2, 2007

Abstract

Svobodová I., I. Steinhauserová, M. Nebola: Incidence of *Clostridium perfringens* in Broiler Chicken in the Czech Republic. Acta Vet. Brno 2007, 76: S25-S30.

Clostridium perfringens is a causative agent of human and animal foodborne diseases. It is known as a normal inhabitant of the intestinal tract of chickens as well as a potential pathogen causing necrotic enteritis. The aim of the present study was to detect the incidence of *C. perfringens* in healthy broiler chickens. From May 2005 to September 2006, 609 samples of caecal content from broilers from 23 intensive poultry farms were analyzed. The samples were cultivated on TSC and blood agar, typical colonies were identified and biochemically confirmed. The total number of positive samples was 112 (18.39%). The samples were processed by the multiplex polymerase chain reaction method (PCR) for toxin genotyping. The presence of alpha, beta, beta2 and enterotoxin gene was detected. All *C. perfringens* isolates were classified as type A, four isolates had the *cpb2* gene. In conclusion the prevalence of *C. perfringens*-positive farms is approximately 74% and the amount ranges about 10^4 cfu/g of caecal content.

Clostridium, necrotic enteritis, PCR, occurrence

Clostridium perfringens is a widespread spore-forming, Gram-positive, anaerobic, non-motile rod. It is recognized as an enteritic bacterial pathogen in humans, poultry, other farm animals and wildlife worldwide (Songer 1996; Craven et al. 2003). It is often found in the intestinal tract of healthy birds but it can cause necrotic enteritis (NE) in many species of poultry and especially in broiler and turkey flocks (Engström et al. 2003). At low population levels (< 10^4 cfu) the organism is non-pathogenic. The pathogenicity of the organism is associated with several toxins. *C. perfringens* is usually classified into five types (A - E) on the basis of its ability to produce the major lethal toxins α, β, ε and ι (Yoo et al. 1997) and according to specific animal hosts. Only *C. perfringens* types A and C are pathogenic for poultry. However, the presence of *C. perfringens* does not lead directly to the disease. Although 75 - 95% of birds are colonised by *C. perfringens*, only a small proportion of these ever shows symptoms of the disease (McDevitt et al. 2006). The disease in poultry is called necrotic enteritis and is presented in an acute or subclinical form. The acute form leads to increased mortality in the last weeks of the rearing period. Typical clinical signs include depression, dehydration, somnolence, ruffled feathers, diarrhoea and decreased feed consumption. The subclinical form is characterized by damage to the intestinal mucosa that decreases digestion, absorption and reduces weight gains. Subclinical *C. perfringens* infection is also associated with hepatitis and cholangiohepatitis. The gut epithelial damage associated with NE coincides with infection by the coccidian genus *Eimeria*, and so NE and coccidiosis are often linked as one set of similar symptoms (Williams 2005).

Toxins: The major lethal effects associated with α-toxin are necrotic enteritis and enterotoxemia in animals (Siragusa et al. 2006). α-toxin (phospholipase C) is commonly produced by all 5 types. It can hydrolyze lecithin into phosphorylcholine and diglyceride, which leads to tissue damage. β-toxin is produced by *C. perfringens* type B and type C strains and it causes a necrotic enteritis characterized by haemorrhagic mucosal ulceration

Address for correspondence:
I. Svobodová
Department of Meat Hygiene and Technology
University of Veterinary and Pharmaceutical Sciences
Palackého 1-3, 612 42 Brno, Czech Republic

Phone: + 420 541 562 746
Fax: +420 541 321 230
E-mail: svobodovai@vfu
http://www.vfu.cz/acta-vet/actavet.htm

or superficial mucosal necrosis of the small intestine in animals (types B and C) (Smedley III. et al. 2004). Toxins ε and ι play no role in pathogenesis in poultry diseases. *C. perfringens* produces many minor toxins, but the most important are β2-toxin and enterotoxin. The β2-toxin was first cloned and sequenced by Gibert et al. in 1997 (Gibert et al. 1997; Schotte et al. 2004). Despite its name, β2-toxin has no significant amino acid similarity with β-toxin. Detailed studies of the mechanism of action of β2-toxin have not been performed and published yet (Smedley III. et al. 2004). Its biological activity is comparable with that of the β-toxin (Schotte et al. 2004). The β2-toxin has also been demonstrated in avian *C. perfringens* type A strains, but its exact role in pathogenesis needs to be further elucidated. All types of *C. perfringens* can produce enterotoxin that is responsible for provoking disease in both man and animals (Van Immerseel et al. 2004). Enterotoxin relates to food poisoning and is produced during sporulation in the infected host intestine, where it binds to the intestinal epithelium, forms pores and causes diarrhoea (Nakamura et al. 2004).

In the Czech Republic no monitoring of *C. perfringens* incidence in poultry is conducted. The aim of the present study was to detect the incidence of *C. perfringens* in healthy broiler chickens from farms with intensive rearing technology and partial zoohygiene monitoring. The next aim was to find out whether the incidence of *C. perfringens* in the alimentary tract increased comparing years 2005 and 2006.

Materials and Methods

Profiles of farms

In the Czech Republic there is a preponderance of farms with intensive rearing technology. The length of rearing periode between 34 - 37 days, deep litter of straw, commercial pelleted feed mix and all-in-all-out technology are typical for these farms. The feed mix contained coccidiostats. Production unit number ranges from 35 000 to 275 000 birds in farm. In all farms were used only two broiler lines: Cobb 500 and Ross 308.

Sample collection

Samples were collected from 23 intensive poultry farms in the period from June 2005 to September 2006. Eight farms were investigated in 2005 and 15 farms in 2006. Samples from farms coded 5 - 42 (Table 1) were collected in the slaughterhouse after veterinary inspection. Contents of gastrointestinal tracts were placed into sterile plastic bags and immediately transported to the laboratory. Samples from farms coded 43, 44 and 45 were obtained directly at the farm, where broilers were slaughtered there and then transported to the laboratory. Poultry carcasses were eviscerated to obtain the gastrointestinal tract. On each farm, a sample of litter, feed from the feeding trough and water from the drinking trough were collected for environmental and zoohygienic parameters monitoring purposes.

Preparation

Five grams of sterile caecal intestinal content obtained were added to 45 ml of saline solution and thoroughly mixed by Stomacher. The samples were then diluted with saline solution to the final concentration of 1 : 1000. All samples of litter, feed and water were processed likewise, water samples were left undiluted, while litter and feed samples were diluted to the final concentration of 1 : 100.

Microbiological methods

Samples of 1 ml of the final suspension (1ml of water) were placed into the Petri dishes and overlaid with tryptose-sulphite-cycloserine (TSC) agar (Oxoid, Basingstoke, UK), with Perfringens Selective Supplement (Oxoid, Basingstoke, UK) and without egg yolk emulsion. The samples were incubated for 22 ± 2 h at 37 ºC. After incubation, typical black colonies were counted and selected for cultivation on blood agar (Oxoid, Basingstoke, UK) with 5% defibrinated sheep blood (Bioveta, Ivanovice na H., CZ). All incubations were conducted under anaerobic conditions using the AnaeroGen Atmosphere Generation System (Oxoid, Basingstoke, UK). Isolated colonies with a typical double zone of haemolysis were then biochemically tested. The commercial available test kit ANAERO test 23® (Pliva-Lachema, Brno, CZ) was used for the tests. All isolated strains were stored in a cooked meat medium (Oxoid, Basingstoke, UK) at –70 °C for subsequent toxin genotyping.

DNA isolation

DNA was isolated using DNeasy® Tissue kit (Qiagen, Germany) according to the manufacturer's instructions.

Multiplex PCR

The type (A or C) and presence of the enterotoxin gene (*cpe*) and the β2-toxin (*cpb2*) gene of each *C. perfringens* isolate were determined by a modified version of the multiplex PCR assay (Baums et al. 2004). 2 µl template DNA was added to a 25 µl reaction mixture with the following reagents: 10 µl of PPP master mix (PPP Master

Table 1. Incidence of *C. perfringens* in caecal content and the results of toxinotyping

Code of farm	Year of sampling/ broiler line	No. of samples (N)	No. of positive samples (N+)	No. of positive samples (N+)%	CFU/G	Type
5	2006 / Ross 308	30	2	6.67	$4.5 . 10^3$	2A *cpb2*
6	2006 / Ross 308	20	0	0	-	-
7	2005 / Cobb 500	36	0	0	-	-
8	2005 / Cobb 500	25	14	56.00	$< 10^4$	14 A
9	2005 / Cobb 500	29	2	6.90	$< 10^4$	2 A
11	2005 / Ross 308	30	0	0	-	-
13	2005 / Cobb 500	30	0	0	-	-
14	2006 / Cobb 500	30	11	36.67	$2.1 . 10^5$	11 A
15	2006 / Ross 308	31	15	48.39	$2.6 . 10^5$	15 A
16	2006 / Cobb 500	15	6	40.00	$2.8 . 10^4$	6 A
18	2006 / Cobb 500	20	1	5.00	$1 . 10^3$	1 A
19	2005 / Cobb 500	29	18	62.07	$< 10^4$	18 A
20	2006 / Ross 308	30	8	26.67	$7.8 . 10^4$	8 A
21	2006 / Cobb 500	30	9	30.00	$2.4 . 10^4$	9 A
22	2006 / Cobb 500	30	2	6.67	$3 . 10^5$	2 A
33	2006 / Ross 308	15	1	6.67	$1 . 10^3$	1 A
36	2006 / Cobb 500	29	15	51.72	$1.2 . 10^4$	15 A
40	2005 / Ross 308	30	0	0	-	-
41	2005 / Ross 500	30	0	0	-	-
42	2006 / Cobb 500	30	2	6.67	$1.5 . 10^5$	2 A
43	2006 / Ross 308	20	1	5.00	$3 . 10^4$	1 A
44	2006 / Ross 308	20	2	10.00	$1.5 . 10^4$	1 A; 1 A *cpb2*
45	2006 / Ross 308	20	3	15.00	$3 . 10^4$	2 A; 1 A *cpb2*
23 farms	-	609	112	18.39	-	108 A; 4 A *cpb2*

mix, Top-Bio, CZ), 2 µl of the mixture of primers (200 nM CPA5L, 200 nM CPA5R; 138 nM CPBL, 138 nM CPBR; 67 nM CPEL, 67 nM CPER; 117 nM CPB2L, 117 nM CPB2R) and 11 µl distilled water. Primers for ε- and ι-toxins were not used, because these toxins belong to the types B, D and E (Table 2).

Table 2. Oligonucleotide sequences of the primers used

Toxin/gene	Primers	Oligonucleotide sequence (5‘-3‘)	Fragment lenght (bp)
α/*cpa*	CPA5L	AGTCTACGCTTGGGATGGAA	900
	CPA5R	TTTCCTGGGTTGTCCATTTC	
β/*cpb*	CPBL	TCCTTTCTTGAGGGAGGATAAA	611
	CPBR	TGAACCTCCTATTTTGTATCCCA	
β2/*cpb2*	CPB2L	CAAGCAATTGGGGGAGTTTA	200
	CPB2R	GCAGAATCAGGATTTTGACCA	
Enterotoxin/*cpe*	CPEL	GGGGAACCCTCAGTAGTTTCA	506
	CPER	ACCAGCTGGATTTGAGTTTAATG	

The multiplex PCR program was slightly modified. The reaction was performed in a Palm-cycler (Corbett Research, Australia) under the following conditions: initial denaturation at 95 ºC for 3 min; 35 cycles at 95 ºC for 1 min; 55 ºC for 1 min; 72 ºC for 1 min 30 s; final extension at 72 ºC for 2 min. 5 µl of the amplicons were separated on 2% agarose gel (SERVA, Germany) and stained with ethidium bromide (SERVA, Germany). Standard DNA molecular weight marker (GeneRuler™ 100bp DNA Ladder, Fermentas, Canada) was used to indicate the size of amplification products.

Bacterial strains

The *C. perfringens* reference strains CIP 106640 (type C; *cpa*, *cpb*, *cpb2* genes; Collection de I’Institut Pasteur,

Paris, France), NCTC 8237 (type A; cpa gene; Czech Collection of Microorganisms, Brno, CZ) NCTC 8238 (type A; cpa, cpe genes; Czech Collection of Microorganisms, Brno, CZ) CNCTC 57/70 (type C; *cpa*, *cpb* genes; SZU, Praha, CZ) were used as an internal control.

Results and Discussion

Between June 2005 and September 2006, a total of 609 caecum content samples from healthy broiler chickens from 23 farms were examined for the presence of *C. perfringens*. With 18.39% samples (n = 112) the cultivation was successful (Table 1). In six of the 23 farms investigated, no *C. perfringens* in healthy poultry was found, which corresponds to the 73.91% *C. perfringens* prevalence on the farms. The monitoring of *C. perfringens* incidence on intensive and extensive broiler farms was also conducted in Italy (Manfreda et al. 2006). In his study 22 intensive farms (total of 99 samples examined) and 11 extensive broiler farms (total of 50 samples), were tested and the authors reported an overall prevalence of over 90%. The pathogen was detected in 87 of the total of 149 samples (58.40%). No statistically significant differences between intensive and extensive broiler farms were shown. The incidence in Italy seems to be higher than in the Czech Republic (> 90 vs. 73.91%). The cultivation methods are similar, so the results are comparable.

By the PCR method all 112 isolated strains from our study were determined as type A and in four of them the presence of *cpb2* gene was demonstrated. The enterotoxin gene was detected in none of the samples tested (Table 1). Manfreda et al. (2006) reported that all strains belong to the type A, too. The author does not mention any enterotoxin gene or *cpb2* gene detection. Similar results have also been published by authors from other European countries. Heikinheimo and Korkeala (2005) from Finland reported the results of their tests of 118 poultry isolates using the multiplex PCR method: all the strains were classified as type A strains and, like in isolates obtained in the Czech Republic no enterotoxin-positive strain was found. The material was not tested for the presence of *cpb2* gene. Engström et al. (2003) from Sweden reported the presence of *cpb2* gene in only 2 of the total of 53 strains tested. All of the strains were classified as type A with no enterotoxin gene present. The most recent study from Belgium (Gholamiandekhordi et al. 2006) reports the presence of *cpb2* gene in 4 strains, and the presence of the enterotoxin gene in 2 strains of a total of 27 healthy broiler chickens examined. A comparison with our study shows an agreement in type classification. The above authors either used isolated strains or they do not give the numbers of tested samples and positive samples, thus making total prevalence comparisons impossible.

The quality of zoohygiene is attested by data given in Table 3. The incidence *of C. perfringens* in litter and water, which act as vectors for the spread of *C. perfringens* among individual animals was only demonstrated on one of the farms investigated. All the farms sanitize fattening premises between individual all-in-all-out production cycles, and the results obtained are therefore comparable.

Table 3. The incidence of *C. perfringens* in enviroment of farm coded 43, 44 and 45

	Farm code, amount and type of *C. perfringens*		
Sample type	43	44	45
Water	-	-	type A; *cpb2* gene; $1,2 . 10^2$ cfu/ml
Litter	-	-	type A; $1,5 . 10^4$ cfu/g
Feed	-	-	-

Monitoring during two years enables comparison of incidence in a longer time period, too. Thirty-four strains (14.2%) of *C. perfringens* were isolated from a total of 239 intestines examined in the Czech Republic in 2005. In 2006, 370 intestines were examined and 78 strains (21.1%) were isolated. The samples were negative from 5 farms in 2005, while

in 2006 samples were negative only from one farm. This finding enables to say, that the prevalence on farms is rising. This fact can apparently relate to the phasing out of grow-promoting antibiotics (GPA). Since January 2006 the use of previously approved growth-promoting antibiotics like flavophospholipol, monensin, salinomycin and avilamycin has been banned in chicken feed. The end to use of growth-promoting antibiotics in the Czech Republic manifested itself by deteriorated intestinal integrity, increased morbidity and it affected the overall production efficiency (Cviková et al. 2006). Unfortunately, there are no specific data about using GPA on the investigated farms, but it is known, that on all farms are still used ionophore coccidiostats.

For better illustration of the situation it would be better to monitor the number of cfu in the samples, but this monitoring was not the main target in this study. On the basis of the used dilution it is possible to estimate the number of *C. perfringens* like colonies per gram (Table 1). Only in four cases the value is higher than 10^5 cfu/g, in the other cases ranges the value between 10^3 to 10^4 cfu/g Manfreda (2006) reported the average value $4{,}5.10^4$ cfu/g, with no significant differences between intensive and extensive broiler farm types.

It follows from the research that *C. perfringens* occurrence found in the Czech Republic is similar to the other European countries. Maintenance of intestinal integrity is a critical component of modern poultry management. An outbreak of a disease would entail serious economic losses for farmers and the incidence should therefore be closely monitored and great attention should be paid to zoohygiene and other preventive measures.

Výskyt *Clostridium perfringens* u brojlerových kuřat v České republice

Clostridium perfringens patří mezi známé původce onemocnění lidí i zvířat. U drůbeže způsobuje nekrotickou enteritidu představující vážný ekonomický problém. V období od května 2005 do konce září 2006 bylo vyšetřeno celkem 609 vzorků obsahu slepých střev brojlerových kuřat z 23 farem s intenzivním způsobem výkrmu. Vzorky byly kultivovány na TCS agaru a krevním agaru, typické kolnie byly dále konfirmovány biochemicky. Stanovení toxinových genů bylo provedeno multiplexní polymerázovou reakcí (multiplex-PCR). Celkový počet pozitivních vzorků byl 112, což je asi 18%. Všechny izolované kmeny byly přiřazeny k typu A, u dvou z nich byl detekován gen pro β2 toxin.

Acknowledgement

We thank the members of PoultryFlorGut team for providing their results. Financial support was provided by PoultryFlorGut FOOD-CT-200X-007076 and the Ministry of Education, Youth and Sports of the Czech Republic No. 6215712402.

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