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EPIDEMIOLOGICAL INVESTIGATION OF ANIMAL DISEASES CAUSED BY *CLOSTRIDIUM PERFRINGENS* STRAINS ISOLATED FROM FEEDINGSTUFFS

EPIDEMIOLOŠKO ISTRAŽIVANJE BOLESTI ŽIVOTINJA UZROKOVANIH SOJEVIMA *CLOSTRIDIUM PERFRINGENS* IZOLIRANIH IZ HRANE

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SUMMARY

C. perfringens is the primary cause of clostridial enteric diseases in domestic animals. Diagnostic difficulties result from common occurrence of these microorganisms in the intestinal tract of healthy vertebrates and laboratory serotyping methods cannot discriminate isolates reliably. *C. perfringens* caused disease may be confirmed by presence of these bacteria in feed and a, high number of these anaerobes in animal intestinal tract by typing methods of comparably high differentiation power.

In this study *C. perfringens* strains isolated from compound feedingstuffs and poultry faeces were enumerated and characterized genotypically for toxin genes presence by mPCR. Presence of *C. perfringens* at the level higher than 10 cfu/g was confirmed in 68% in feed isolates and in 36% in poultry isolates. Dominance of *C. perfringens* strains type A was confirmed among isolated strains from feeds and poultry. Nearly 42% of isolates from feeds and 4% from poultry belonged to subtype β2. There were some positive isolates for *netB* gene. The obtained results of analyses revealed common occurrence status of *C. perfringens* in feeds and clinically healthy birds.

Key words: *Clostridium perfringens*, feedingstuffs, poultry, necrotic enteritis.

INTRODUCTION

As yet, 17 toxins produced by *C. perfringens*, four major toxins (α, β, ε, ı) and additional toxins that have been proposed to be important for the pathogenesis of intestinal disorders were described (Hatheway 1990, Smedley et al. 2004.). The production of the major toxins is a base for classification criterion into one of the five toxotypes (A – E). Expression level of α toxin decides the lethal properties of strains and this feature is linked with opportunistic nature of this species. It is known, that for animals the most important additional toxins are β2 toxin and NetB toxin (Fisher et al. 2005, Herholz et al. 1999, Keyburn et al. 2008). The case of diseases caused by strains with β2 toxin is more

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serious than without the toxin and NetB toxin is a critical virulence factor in the pathogenesis of necrotic enteritis (NE) in chickens. Significance of these anaerobes for food animal morbidity increased following the termination of antimicrobial growth promoters, effectively decreasing the morbidity caused by opportunistic bacteria (Grave et al. 2006).

C. perfringens is the primary cause of clostridial enteric diseases in poultry (Jong 2003, Songer 1996). NE is commonly caused by *C. perfringens* types A and C. Diagnostic difficulties result from common occurrence of these microorganisms both in the environment and the intestinal tract of healthy vertebrates (Cooper and Songer 2009). In view of diagnostic difficulties, the study was undertaken to enumerate and characterize genotypically *C. perfringens* strains isolated from compound feedingstuffs and from poultry faeces.

MATERIALS AND METHODS

Compound feedingstuffs (100 samples) and poultry faeces (100 samples) were analyzed for *C. perfringens* number and isolates from feeds (105 strains) and poultry (225 strains) for presence of toxin genes.

Analyzed compound feedingstuffs were sampled from Polish feed factories, imported feed batches and farms. Examined poultry faeces were sampled from a clinical by healthy chicken broilers farm (70 samples), a turkey broilers farm (20) and a cock farm (10). Chicken broilers were 18, 21, 35, 42, 43, 63 days old and turkey broilers were 105 days old. Size of chicken broiler flocks was 5000, 13000, 17000, 20000, 22000 birds and the turkey broiler flock numbered 12000 birds.

Fig. 1. Number of *C. perfringens* in compound feedingstuffs (cfu/g)
Graf. 1 Broj *C. perfringensa* u smjesama

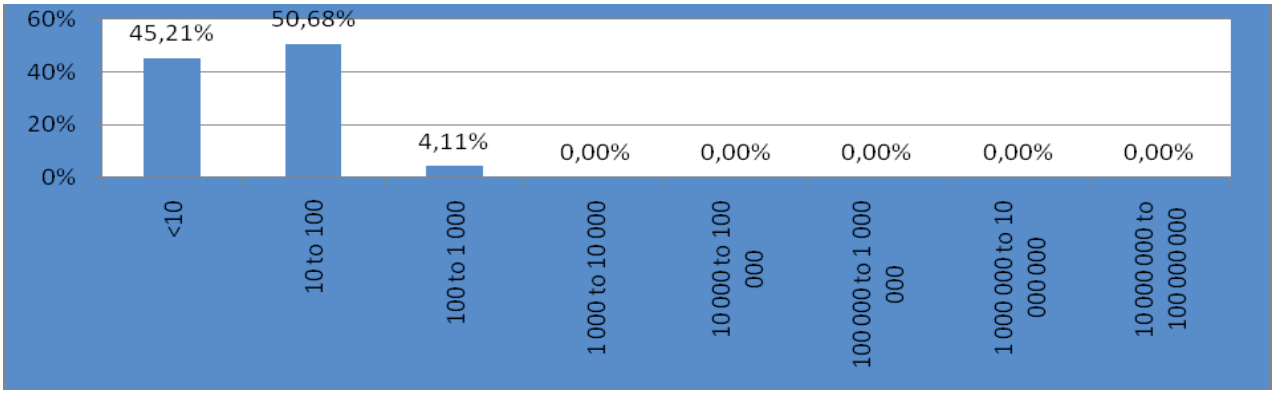


Fig. 2. Number of *C. perfringens* in poultry faeces (cfu/g)
Graf. 2 Broj *C. perfringensa* u fecesu peradi

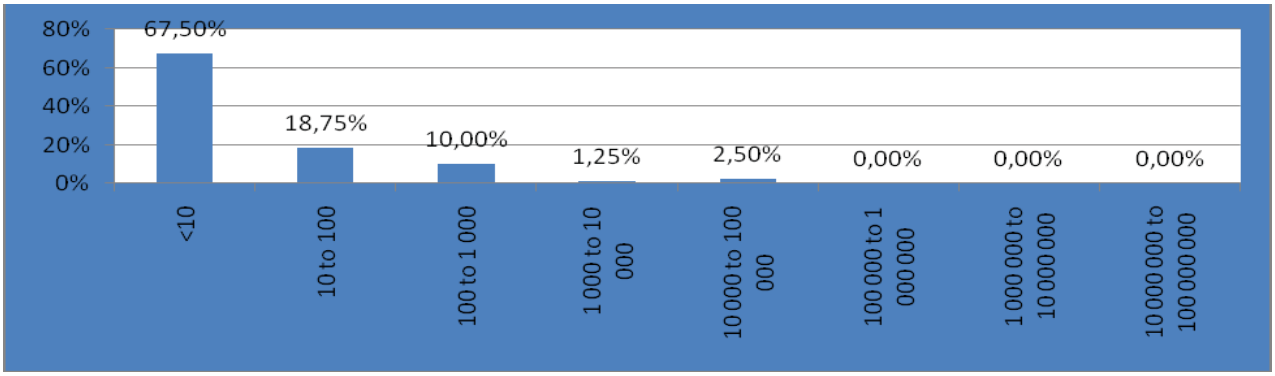


Fig. 3. ***C. perfringens*** **toxotype occurrence in compound feedingstuffs**
Graf. 3 **Pojava toksotipa *C. perfringens* u smjesama**

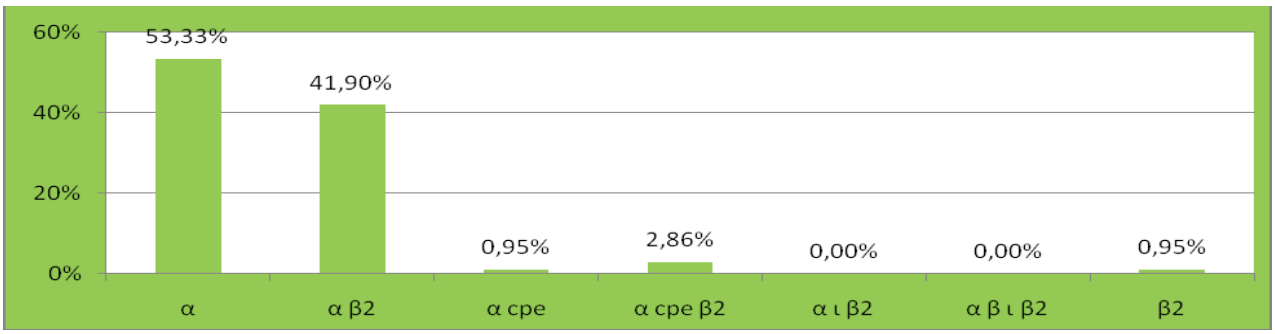
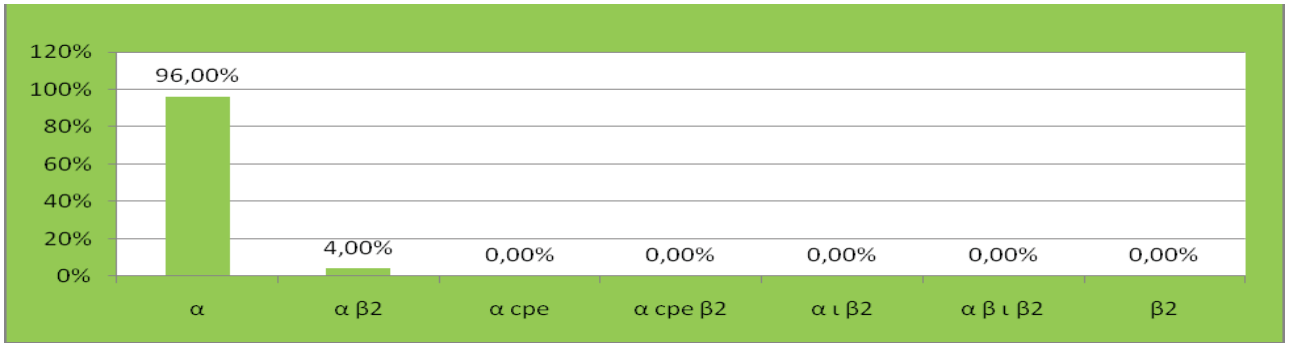


Fig. 4. ***C. perfringens*** **toxotype occurrence in poultry faeces**
Graf. 4 **Pojava toksotipa *C. perfringens* u fecesu peradi**



All samples were analyzed for number *C. perfringens* according to standard PN-EN 13401:2000 "Microbiology of food and animal feeding stuffs. Horizontal method for enumeration of *Clostridium perfringens*". From positive samples 1 to 13 strains suspected for belonging to *C. perfringens* species, were isolated and for toxotypes.

Obtained results for *C. perfringens* number were analyzed according to distributive series. Figures 1 – 6 present occurred bacterial number in logarithm scale (e.g. $\log_{10} = 3$ means range from 10^3 to 9 999 cfu/g; $\log_{10} = 4$ means range from 10^4 to 99 999 cfu/g, etc.)

C. perfringens isolates were examined for the presence of *cpa* (α toxin), *cpb* (β), *cpb2* (β2), *etx* (ε), *iap* (ι) and *cpe* (enterotoxin) toxin genes by multiplex PCR with slight modifications (Baums et al. 2004) and by PCR for the presence of *netB* (NetB) toxin gene (Martin and Smyth 2009). Template DNA was obtained from overnight culture of *C. perfringens* at 37 ± 1°C on Willis-Hobbs agar under anaerobic conditions, resuspended in 2 ml of physiological saline solution to obtain a McFarland turbidity standard equal to 3.5. One milli of this solution was transferred to Eppendorf tube and boiled in water for 15 min. After heat lysis, the tube was cooled on ice and centrifuged at 11 000 x *g* for 8 min. The obtained supernatant (10 µl) was added to master mix as a source of target DNA. DNA amplification during mPCR was performed using 10 x concentrated *Taq* buffer, 250 µM of dNTP, 2.5 mM of $MgCl_2$ (Fermentas), primers (200 nM CPA5L, 200 nM CPA5R, 138 nM CPBL, 138 nM CPBR, 67 nM CPEL, 67 nM CPER, 46 nM CPETXL, 46 nM CPETXR, 83 nM CPIL, 83 nM CPIR, 117 nM CPB2L i 117 nM CPB2R), and 2.5 U of *Taq* polymerase. After an initial denaturing step of 2 min 30 s at 95°C, the samples were subjected to 35 cycles of denaturing (95°C, 1 min), annealing (55°C, 1 min)

and extension (72°C, 1 min 20 s), followed by a single final extension step of 2 min at 72°C. PCR products were identified by UV transilluminator, following electrophoresis through a 2% agarose gel containing 1 µg/ml of ethidium bromide. The size of amplicons was compared with O'Gene Ruler 100 bp DNA Ladder.

RESULTS AND DISCUSSION

It should be emphasized, that the lower limit of method for *C. perfringens* enumeration was 10 cfu/g. When the number of bacteria was under than 10 cfu/g *C. perfringens* may or may not occurred in low numbers.

Among 100 analyzed feed samples, presence of *C. perfringens* was confirmed in 68%. The level of contamination ranged from under 10 cfu/g to 9.5 x 10^2 cfu/g. Toxin type and its subtype identification revealed that 53.3% of the isolates belonged to type A and 42% to type A subtype beta2. It was observed that 0.95% enterotoxic strains belonged to type A and 2.9% enterotoxic strains belonged to type A subtype beta2. Presence of *C. perfringens* in poultry faeces was confirmed in 36 samples among 100 analyzed. Number of these anaerobes ranged from under 10 cfu/g to 3.6 x 10^4 cfu/g. All isolated strains from poultry classified as type A (96%) or type A subtype beta2 (4%). There were some positive isolates for *netB* gene.

C. perfringens strains were often isolated from feeds and this matrix is considered to be a source of these anaerobes in food chain (Dosoky 1989, Xylouri et al. 1997). Our previous study confirmed occurrence of these bacteria in nearly 50% sample of compound feeds which is convergent with other authors (Kanakaraj et al. 1998, Wojdat 2006). Detected levels of *C. perfringens* in analysed feeds did not exceed 10^3 cfu/g, which is 10-fold less than in years 2003 – 2006 (Wojdat 2006). Number of *C. perfringens* in poultry strongly depended on hygiene in farms (positive correlation). Not less important was the way of sampling. Faeces should be sampled from live birds and transported as quick by as possible to laboratory in cool temperature. Faeces may be frozen before analysis, but it may slightly change the number of anaerobes. Intestine sampling of dead animals showed high numbers of *C. perfringens* (10^6 to 10^8 cfu/g) which is typical of NE, although high numbers of these anaerobes in the intestinal tract are not sufficient to cause the disease.

CONCLUSIONS

Analysed feed samples originated from official control sampling and poultry faeces samples derived from clinically healthy birds. However, for epizootical investigation of animal disease caused by *C. perfringens*, strains isolated from NE-affected birds and used feeds may be compared. The first step in the procedure is toxotype and subtype conformity (by PCR) and the second one is confirmation of identical DNA profile (e.g. by pulsed field gel electrophoresis - PFGE). *C. perfringens* in healthy birds intestine should not exceed 10^4 cfu/g. Additional data may provide anaerobes designation in feed because they may cause conduce disease occurrence. Genetic analysis may be useful, showing that healthy birds may carry from two to five genotypes when birds with NE are colonized by a single genetic type (Cooper and Songer 2009).

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SAŽETAK

Clostridium perfringens je primarni uzrok klostridialnih enteričkih bolesti domaćih životinja. Poteškoće dijagnosticiranja rezultat su česte pojave mikroorganizama u intestinalnom traktu zdravih kralježnjaka pa laboratorijske metode ne mogu pouzdano razlučiti izolate. Bolest koju je prouzročila *C. perfringens* može se potvrditi prisutnošću ovih bakterija u hrani i velikim brojem anaeroba u intestinalnom traktu životinja metodom tipiziranja razmjerno visoke sposobnosti diferenciranja.

U ovom su radu sojevi *C. perfringens* izolirani iz krmnih smjesa i fecesa peradi nabrojani te genotipski opisana prisutnost gena toksina pomoću mPCR. Prisutnost *C. perfringensa* na razini višoj od 10 cfu/g potvrđena je u 68% izolata hrane i 36% izolata peradi. Među izoliranim sojevima u hrani i peradi potvrđeno je prevladavanje sojeva *C. perfringens* tipa A. Gotovo 42% izolata hrane i 4% peradi pripadaju podtipu B2. Bilo je nešto pozitivnih izolata za gen netB. Dobiveni rezultati analiza otkrivaju čestu pojavu *C. perfringensa* u hrani i klinički zdravoj peradi.

Ključne riječi: *Clostridium perfringens*, hrana, perad, nekrotični enteritis