OCCURRENCE AND EXPRESSION OF TOXIN GENES IN CLOSTRIDIUM PERFRINGENS ISOLATES FROM PIGS

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Received: August 24, 2012 Accepted: December 5, 2012

Abstract

Clostridium perfringens isolates were obtained from pigs of five porcine farms in Poland. The presence of C. perfringens was detected in 92% of faeces samples and its number ranged from 1.0 x 10^5 cfu/g to 1.2 x 10^7 cfu/g. All the isolates belonged to type A and 48.7% of them contained cpb2 gene. The qualitative assessment of toxin genes expression by type A subtype β2 isolates showed expression of cpa gene in 100% of strains and cpb2 gene in 71% of the analysed strains. The isolate from one-day-old piglets demonstrated also the expression of cpa and cpb2 genes.

Key words: pigs, Clostridium perfringens, toxin, gene expression.

One of the essential elements of public health protection is the prevention of food animal diseases. The primary cause of clostridial enteric diseases in animals is Clostridium perfringens (8). Animal morbidity ranges from 15% to 50% and mortality in young animals may come up to 100% (16). The significance of these anaerobes for food animals increased following the termination of antimicrobial growth promoters, effectively decreasing the morbidity caused by opportunistic bacteria (6).

Nowadays, approximately 20 toxins produced by C. perfringens are known. Among them four major toxins (α, β, ε, ι), and a group of additional toxins were described (5, 9, 10, 15). The production of the major toxins is the basis for classification of the C. perfringens species into five toxotypes (A–E). Type A strains produce α toxin, type B – α, β, and ε toxins, type C - α and β toxins, type D - α and ε toxins, and type E - α and ι toxins. C. perfringens type B strains are recognised as frank pathogens, whereas type A strains are commensals in the intestinal tract of warm-blooded animals. For many years it has been thought that the lethal properties of type A strains, is determined by the amount of α toxin produced (15). However, the last two decades has brought new data in the field, confirming the participation of additional toxins (netB and β2 toxin) in the pathogenesis of enteritis caused by C. perfringens type A in various species of animals (4, 5, 7, 9, 18).

Bearing in mind that the confirmation of the presence of the gene encoding the bacterial toxin does not mean the production of the toxin, the aim of the study was to assess the presence and expression of toxin genes of C. perfringens strains isolated from healthy pigs.

Material and Methods

Isolates were obtained from five farms, in which herds comprising from 2 to 150 sows were maintained. Porcine faeces (n=100) were sampled from fatteners, sows, and one-day-old piglets with no evidence of clostridial enteric diseases. In the faeces samples, C. perfringens cells were enumerated according to standard PN-EN 13401:2000 (11) or the presence of the anaerobe was detected by PN-R-64791:1994 (12). Afterwards, ten C. perfringens strains were isolated from positive samples and analysed for the presence of toxin genes. The isolates (n=404) were examined for the presence of cpa (α toxin), cpb (β), cpb2 (β2), ets (ε), iap (ι), and cpe (enterotoxin) toxin genes by multiplex PCR (mPCR) according to Baums et al. (1) with small modifications. Protocol changes regarded the preparation of template DNA, which was obtained from overnight culture of C. perfringens at 37 ±1°C on Willis-Hobbs agar under anaerobic conditions and then resuspended in 2 ml of PBS to obtain a McFarland turbidity standard equal to 3.5. One millilitre 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. Further mPCR conditions were identical to the abovementioned
protocol. PCR products were identified by UV transilluminator, (Chemi-Smart 3000, Vilber Lourmat, France) 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 (Fermentas). Four strains of C. perfringens: 1567/89 (type A) for cpa and cpe genes; 544/84 (type A) for cpa and cpb2 genes; 558/85 (type B) for cpa, cpb, and ets genes; and NCTC 8266 (type E) for cpa and iap genes were used for positive control of the procedure.

Randomly selected isolates (n=31), identified as C. perfringens type A subtype β2 using the mPCR method described above, were tested by reverse transcriptase polymerase chain reaction (RT-PCR) method for the expression of cpa and cpb2 genes. Extraction of total RNA from bacterial strains was performed using a total RNA separation kit (A&A Biotechnology, Poland). Bacterial cells were obtained from fresh overnight brain-heart infusion cultures and centrifuged at 1,500 x g for 15 min at 20°C. Total cellular RNA was extracted according to the manufacturer’s protocol. The obtained RNA extract was resuspended in 100 μl of sterile RNase-free water.

RT-PCR was performed in a 25 μl volume reaction mix containing 10 μl of sterile RNase-free water, 2.5 μl of 10 x TrueStart Taq buffer, 2.5 μl of 25 mM MgCl2, 2.5 μl of 2 mM dNTP mix, 1.25 μl of primers for cpa and cpb2 toxin genes (20 pmol/μl), 0.125 μl of AMV Reverse Transcriptase (20 U/μl), 0.125 μl of RiboLock RNase Inhibitor (40 U/μl), and 0.5 μl of TrueStart Taq DNA Polymerase (5 U/μl). Primer sequences were the same as those described previously for mPCR method. Two microlitres of extracted RNA was then used as template in RT-PCR. After a reverse transcription (50°C, 15 min) and an initial denaturing step of 2 min at 94°C, the samples were subjected to 34 cycles of denaturing (94°C, 30 s), annealing (59°C, 1 min), and extension (72°C, 1 min), followed by a single final extension step of 3 min at 72°C. RT-PCR and mPCR products were separated on a 2% agarose gel and visualised by ethidium bromide staining.

**Results**

The presence of C. perfringens at the level higher than 1.0 x 10^3 cfu/g was detected in 92% of pig faecal samples and the anaerobe number ranged from 1.0 x 10^3 cfu/g to 1.2 x 10^7 cfu/g (Fig. 1). However, nearly 40% of faecal samples contained no more than 10^2 cfu/g. The number of C. perfringens in faeces was positively correlated with herd size and age of the animals. The anaerobes were detected also in 80% of one-day-old piglets, but their level did not exceed 10 cfu/g. It should be mentioned that the limit of detection for C. perfringens enumeration method was 10 cfu/g, which means that in samples classified as negative, anaerobes may occur on the level less than 10 cfu/g. The obtained results for C. perfringens number were analysed according to the distributive series. Fig. 1 presents bacterial number in logarithmic scale (e.g. \( \log_{10} = 1 \) means range from 10^1 to 999 cfu/g; \( \log_{10} = 2 \) means range from 10^2 to 9999 cfu/g; \( \log_{10} = 3 \) means range from 10^3 to 999,999 cfu/g; \( \log_{10} = 4 \) means range from 10^4 to 99,999,999 cfu/g, etc.).

Identification of toxin type and its subtypes revealed that all the isolates belonged to type A and 48.69% of them contained cpb2 gene (subtype β2) (Fig. 2). The strains isolated from one-day-old piglets had also genes encoding α and β toxins. The presence of enterotoxin gene was demonstrated only in two isolates (0.66%) from among 404 studied strains. Additionally, both cpe- positive isolates contained cpb2 gene. There were no isolates classified to type B, C, D or E.

![Fig. 1. C. perfringens number in swine faeces presented in logarithmic scale (more details see in chapter Results).](image1)

![Fig. 2. Occurrence of C. perfringens toxotypes in pig faeces.](image2)
The qualitative assessment of toxin genes expression by C. perfringens type A subtype β2 isolates from pigs showed the expression of cpa gene in 100% of the strains and cpb2 gene in 71% of the analysed strains. The strain isolated from one-day-old piglets also demonstrated the expression of cpa and cpb2 genes. Cpb2 toxin gene expression was not only observed in nine tested isolates.

Discussion

Types A and C of C. perfringens are aetiological agents of the clostridial enteric diseases in pigs. Type C strains are found in extremely low numbers in the gastrointestinal tract of healthy animals and they are detected in high numbers in animals with C. perfringens-associated disease. C. perfringens type A is a natural inhabitant of the pig’s intestine and the pathogenesis of the disease is still not well comprehended. Type A strains produces α toxin, as do all types of C. perfringens, but the results of recent investigations seems to suggest that the α toxin alone does not cause enteritis. Numerous studies indicate the involvement of additional toxins in the pathogenesis of enteric disorders caused by C. perfringens type A and among them β2 toxin is often mentioned (2, 4, 9, 18).

In the study, the occurrence of toxin genes in C. perfringens isolates was assessed by PCR method and expression of the genes - by RT-PCR. All studied porcine isolates were C. perfringens type A and nearly half of them were cpb2-positive. The observed dominance of type A isolates was also noted by other authors, who analysed isolates from soil and warm-blooded animal intestinal tract (2, 13-14). Higher percentage of cpb2-positive strains (75.8) was demonstrated by Bueschel et al. (2), who analysed the isolates from pigs with enteritis, as well as, healthy animals. Lower percentage of cpb2-positive strains (11.1) was noted by these authors in healthy swine. In turn, in another Polish study by Wasinski et al. (17), all isolates obtained from suckling 1-10-day-old piglets with diarrhoea were toxotype A, and 41% of them were subtype β2. These authors also observed α toxin expression in all 36 C. perfringens isolates. It is worth noting that in the study the strain derived from the one-day-old piglets was also cpa- and cpb2-positive and both toxin genes were expressed. The percentage of cpb2-positive strains was also relatively high (71). The β2 toxin production was also confirmed by other authors with the use of Western blotting. The presence of β2 toxin protein was detected in 96.9% of isolates from healthy, as well as, sick swine (2). Besides, expression of β2 toxin was observed among avian (from 40% to 90%) and bovine (50%) isolates (2-3). Furthermore, the presence of cpb2 gene in C. perfringens enterotoxic strains (cpe-positive) was observed, which previously was demonstrated in bovine, caprine, equine, and ovine isolates (2).

Gene expression is the process by which information from a gene is used in the synthesis of a functional gene product. The expression of bacterial toxin genes is the evidence of the genes’ activity. The study confirmed the occurrence of active genes encoding α and β2 toxins in C. perfringens strains isolated from healthy pigs.

Acknowledgments: This work was supported by the Polish Ministry of Science and Higher Education research programme (No. R1202302).

References

11. PN-EN 13401:2000 “Microbiology of food and animal feeding stuffs. Horizontal method for enumeration of Clostridium perfringens”.
12. PN-R-64791:1994 “Feeding stuffs - Requirements and microbiological examination”.
14. Sipos W., Fischer L., Schindler M., Schmoll F.: Genotyping of Clostridium perfringens isolated from