Toxin genotyping of Clostridium perfringens field strains isolated from healthy and diseased chickens

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ABSTRACT

Clostridium perfringens is well known as the aetiological agent of necrotic enteritis in chicken. Type A and type C are considered the C. perfringens toxin types responsible for this disease. The aim of this study was to determine the presence of genes coding for α, β, ε, ι, β2 and enterotoxin in C. perfringens field strains collected from healthy and diseased chickens. Thirty-seven C. perfringens field strains were toxin typed: all strains resulted to be toxin type A and 3 of these tested positive for the presence of the toxin β2 coding gene. Four isolates showed the cpa gene with the insertion of a group II intron. Our findings confirm the most recent results reported from different countries and the data suggest that the role of C. perfringens type C should be revaluated in the etiopathogenesis of necrotic enteritis.

Key words: Clostridium perfringens, Toxin type, Chicken.

RIASSUNTO

TOSSINOTIPIZZAZIONE DI CEPPI DI CLOSTRIDIUM PERFRINGENS ISOLATI DA POLLI SANI E AFFETTI DA ENTERITE

C. perfringens tipo A e C sono riconosciuti come i principali agenti eziologici dell’enterite necrotica del pollo. Con il presente studio si è voluto indagare la presenza dei geni codificanti per le tossine α, β1, ε, ι, β2 e enterotossina, in ceppi di C. perfringens isolati da polli sani ed affetti da enterite. I 37 ceppi di campo analizzati sono risultati tutti di tipo A e 3 di questi erano positivi anche per il gene codificante la tossina β2. In 4 dei 37 ceppi isolati i primer per la tossina α hanno prodotto un amplificato con un’altezza maggiore di quella attesa. L’analisi della sequenza ha evidenziato l’inserzione di un introne di gruppo II nel gene cpa. I dati ottenuti confermano quanto recentemente riportato anche in altri lavori e suggeriscono che il ruolo dei C. perfringens di tipo C nell’etiopatogenesi dell’enterite necrotica sia da approfondire.

Parole chiave: Clostridium perfringens, Tossinotipo, Pollo.
**Introduction**

_Clostridium perfringens_ (CP) is a Gram positive, spore forming and anaerobic bacterium that is responsible for a wide range of diseases in humans and animals. Its pathogenicity is associated with the production of 17 toxins, of which α, β, ε and ι are the major lethal ones (Meer and Songer, 1997). A commonly used classification scheme divide CP isolates into five types (A-E) on the basis of their capability to produce the major lethal toxins (Meer and Songer, 1997). Some CP strains, in addition to α toxin, produce β2 and enterotoxin: two toxins that have been proposed as being important in the pathogenesis of intestinal disorders in animals and humans respectively (Sarker et al., 1999; Thiede et al., 2001; Manteca et al., 2002). CP is often isolated from the intestinal tract of healthy birds but can also cause outbreaks of disease in poultry, and especially in broiler and turkey flocks. Clostridiosis occurs both as acute or subclinical disease. The acute clinical disease is represented by necrotic enteritis (NE) that causes high mortality rates especially in 2-5 week-old chicks. Intestinal focal necrosis and hepatitis are typical signs frequently associated with subclinical clostridiosis (Engström et al., 2003). The role of CP toxin types in the pathogenesis of NE in poultry is still not clear. Recent studies conducted in Finland, Sweden, Belgium and Denmark demonstrated that CP isolated from chickens affected by NE, belong to toxin type A (Engstrom et al., 2003; Nauerby et al., 2003; Helkinhelmo and Korkeala, 2005; Gholamiandekhordi et al., 2006). Keyburn et al. (2006) demonstrated that α toxin is not essential in causing NE. The aim of this study was to perform toxin genotyping of CP field strains collected from the intestines of diseased and healthy chickens by multiplex PCR for detection of α, β, ε, ι, β2 and enterotoxin genes.

**Material and methods**

Tissue samples were taken from the jejunum and ileum (5 cm back and 3 cm after the Meckel’s diverticulum) of 30 broilers and 7 layers on 37 different commercial farms. Thirty birds were affected by enteric disorders and 5 subjects (4 broilers and 1 layer) showed intestinal lesions compatible with NE. Intestinal samples were introduced in Cooked Meat Medium (Difco) tubes and incubated in anaerobic chamber at 37°C for 24 hours. From each tube, 0.1 ml were plated on Perfringens Agar Base (Oxoid) and plates were incubated in anaerobic conditions at 37°C for 48 hours. CP colonies were identified by using a commercial biochemical panel kit (API 20 A, BioMérieux). CP ATCC 27324 (toxin type E+ enterotoxin), CCUG 2036 (toxin type C), CCUG 2037 (toxin type D), ATCC 10543 (toxin type A+ β2) were used as reference strains. Five colonies of each CP strain included in the study were recovered from the agar plate and the DNA was extracted with GeneElute Bacterial Genomic DNA Kit (Sigma) according to manufacturer’s instructions. Two multiplex PCR were used: one for detection of _cpa_, _cpb1_, _cpetx_ , and _cpi_ genes and one for detection of _cpb2_ and _cpe_ genes. PCR primers and fragment length are listed in Table 1.

PCRs were performed in 25 µl mixture containing 1.5 mM MgCl2, 250 µM each dNTP, 1.25 U Fast Start DNA polymerase (Roche) and 0.1 µM of Cpa primers, 0.2 µM of Cpb1, Cpetx, Cpi primers for the first multiplex and 0.25 µM of Cpe and Cpb2 primers for the second. The thermal cycling conditions were as follows: initial denaturation for 4 min at 95°C followed by 35 cycles of 1 min at 95°C, 1 min at 60°C for the first multiplex and 56°C for the second, 1 min 20 sec at 72°C and a final step of 2 min at 72°C. PCR products were separated by electrophoresis on 2% agarose gel (Sigma) added with 0.5 µg/ml ethidium bromide (Sigma). Gels were visualized un-
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**Toxin genotyping of C. perfringens**

Table 1. Target toxin gene, oligonucleotide primer sequence and length of amplification product.

| Gene (toxin) | Primers | Sequence (5’-3’) | References | Fragment length |
|--------------|---------|------------------|------------|----------------|
| *cpa* (α toxin) | Cpa_for | AGT CTA CGC TTG GGA TGG AA | Baums *et al.*, 2004 | 900 bp |
|             | Cpa_rev | TTC CCT GGG TTG TCC ATT TC |            |                |
| *cpb1* (β1 toxin) | Cpb1_for | TCC TTT CTG GAG GGA GGA TAAT | Baums *et al.*, 2004 | 611 bp |
|             | Cpb1_rev | TGA ACC TCC TAT TTT GTA TCC CA |            |                |
| *cpetx* (ε toxin) | Cpetx_for | ACT GCA ACT ACT ACT CAT ACT GTG | Yoo *et al.*, 1997 | 541 bp |
|             | Cpetx_rev | CTG GTG CCT TAA TAG AAA GAC TCC |            |                |
| *cpi* (ι toxin) | Cpi_for | AAA CGC ATT AAA GCT CAC ACC | Baums *et al.*, 2004 | 293 bp |
|             | Cpi_rev | GTG CAT AAC CTG GAA TGG CT |            |                |

**Multiplex PCR**

| Gene (toxin) | Primers | Sequence (5’-3’) | References | Fragment length |
|--------------|---------|------------------|------------|----------------|
| *cpe* (Entero-toxin) | Cpe_for | GGG GAA CCC TCA GTA GTT TCA | Baums *et al.*, 2004 | 506 bp |
|             | Cpe_rev | ACC AGC TGG ATT TGA GTT TAA TG |            |                |

**Duplex PCR**

| Gene (toxin) | Primers | Sequence (5’-3’) | References | Fragment length |
|--------------|---------|------------------|------------|----------------|
| *cpb2* (b2 toxin) | Cpb2_for | CAA GCA ATT GGG GGA GTT TA | Baums *et al.*, 2004 | 200 bp |
|             | Cpb2_rev | GCA GAA TCA GGA TTT TGA CCA |            |                |

Under UV transilluminator and the image was captured using GelLogic 100 imaging System (Kodak). PCR product were cleaned with ExoSAP-IT (USB) and sequenced with the ABI-Prism Dye Terminator Cycle Sequencing kit (Applied Biosystems) using ABI PRISM 3130 Genetic Analyzer. PCR product sequences were analysed with the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

**Results and discussion**

Thirty-seven CP field strains were toxin typed. Seven isolates were obtained from chickens without enteric disorders, 30 strains from chickens with gastro-intestinal lesions characterized by catarrhal enteritis, catarrhal-hemorrhagic enteritis or NE. All strains resulted toxin type A and 3/37 (8.1%) were positive for the presence of *cpb2* gene. No strains were positive for the presence of *cpetx* gene. In 4/37 toxin type A (10.8%), a *cpa* amplicon larger than expected (1.5 Kbp) was detected. These amplicons were sequenced and the BLAST analysis showed that the first 162 bp have 100% sequence similarity with CP α toxin gene while the second part of the sequence have 100% sequence similarity with *C. perfringens* *cpa* group II intron.

**Conclusions**

The data highlight that the CP isolates included in the study were of toxin type A and a relatively low percentage of isolates carried the β2 toxin gene, irrespective of enteric lesions. No CP toxin type C were found also in birds affected by NE. Our findings confirm the most recent results reported from different countries and the data suggest that the role of *C. perfringens* type C should be revaluated in the etiopathogen-
thesis of NE. The presence of a group II intron without an open reading frame integrated into a cpa gene of CP was recently demonstrated in one strain isolated from broiler chickens (Ma et al., 2007). The same authors also proved that this type of insertion neither affect α toxin expression or the molecular weight of the α toxin produced. The genotyping results demonstrated that there were no differences in the toxin genotype between sick or healthy chickens or between layer-hens and broilers. These observations suggest that further CP pathogenic mechanisms should be investigated in NE aetiology and studied in association with yet described predisposing factors (nutrition, concomitant diseases) (Kaldhusdal and Skjerve, 1996).

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