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Pathogenicity markers of *Clostridium* spp. in commercial turkeys

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ABSTRACT

Since growth promoters ban in Europe, enteritis of different aetiologies (virus, bacteria and protozoa) are increasingly becoming the main cause of economic loss in commercial turkeys production. This study is focused on typing of *Clostridium* spp. isolated from samples of jejunum and ileum of 82 birds out of 17 turkeys flocks. The birds were 6-day to 104-day old, both male and female, with enteric disorders. The presence of toxin NetB was investigated. Multiplex PCR to detect cpa, cpb1, cpetx, cp1, cpb2 and cpe toxin genes were used for *Clostridium* typing. No lesions of necrotic enteritis were observed. *Clostridium perfringens* type A was isolated from 25 enteric samples, *Clostridium difficile* was found in 4 cases and *Clostridium sordelli* in one case. *Clostridium perfringens* was present from 6 to 104 days of age indicating its possible role in the enteric disorders of commercial turkeys. NetB toxin was found in no sample. 3 out of 4 isolates of *Clostridium difficile* were characterized by the presence of toxin genes.

Key words: Turkey, Clostridiosis, Toxins.

RIASSUNTO

MARKER DI PATOGENICITÀ PRESENTI IN *CLOSTRIDIUM* SPP. ISOLATI DA TACCHINI COMMERCIALI

In seguito al bando europeo degli antibiotici promotori della crescita, le enteriti di differente eziologia (virus, batteri, protozoi) stanno diventando la principale causa di perdita economica nell'allevamento del tacchino commerciale. Lo scopo del presente lavoro è la tipizzazione di Clostridium *spp. isolati da campioni di digiuno ed ileo di 82 tacchini provenienti da 17 allevamenti. Gli uccelli, sia maschi che femmine, avevano un' età compresa tra i 6 e i 104 giorni ed avevano problemi enterici. E' stata ricercata la presenza della tossina NetB. Sono inoltre state effettuate Multiplex PCR per rilevare la presenza dei geni tossigeni cpa, cpb1, cpetx, cp1, cpb2 and cpe. Non sono state riscontrate lesioni ascrivibili ad enterite necrotica.* Clostridium perfringens *tipo A è stato isolato da 25 campioni enterici,* Clostridium difficile *è stato isolato in 4 casi e* Clostridium sordelli *in 1 caso.* Clostridium perfringens *era presente dai 6 ai 104 giorni di età indicando un suo possible ruolo nei problemi enterici del tacchino. Non è stata riscontrata la presenza della tossina NetB. Tre dei 4 isolati di* Clostridium difficile *erano caratterizzati dalla presenza di geni tossigeni.*

Parole chiave: *Tacchini, Clostridiosi, Tossine.*

Introduction

The main *clostridia* responsible for a wide range of diseases in avian species are: *Clostridium colinum, C. botulinum, C. septicum and C. perfringens, C. fallax, C. novyi, C. sporogenes* and *C. difficile* (Barnes *et al*., 1980).

Pathological signs are caused by the different toxins but in many cases cofactors such as dietary ingredients or changes, severe stress, coccidiosis and other protozoal diseases of the intestinal tract or immunosuppressive infections can enhance the disease (Barnes *et al*., 1980). *Clostridium perfringens* (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. CP is a Gram-positive, spore forming and anaerobic bacterium 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 divides 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). Clostridiosis occurs as acute or subclinical disease. The acute clinical disease is characterized by necrotic enteritis (NE). 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. Studies conducted in Finland, Sweden, Belgium and Denmark demonstrated that CP isolated from chickens affected by NE, belong to toxin type A (Engström *et al.*, 2003; Nauerby *et al*., 2003; Heikinheimo and Korkeala, 2005; Gholamiandekhordi *et al*., 2006; Keyburn *et al*., 2006) and demonstrated that α toxin is not essential in causing NE in broilers. Very few studies are focused on turkeys although, since the growth promoters ban in Europe in 2006, it has become a pathology of major concern. Recently, NetB, a novel toxin that is associated with broiler NE, has been described (Keyburn *et al*., 2006). The toxin was identified using screens for proteins from the supernatant of *C. perfringens* cultures that were cytotoxyc for chicken hepatocellular carcinoma cells (LMH) in vitro. The aim of this study was to perform toxin genotyping of CP field strains collected from the intestines of diseased turkeys by multiplex PCR for detection of α, β, ε, ι, β2, NetB and enterotoxin genes.

Material and methods

Birds

Eighty-two birds from 17 commercial turkeys flocks showing enteric disorders were humanly euthanized and necropsied. The turkeys were 6-day to 104-day old, both male and female. In average 5 birds were taken from each farm. 11.6% of the sample was less than one week old, 16.8% was in the second week of life, 48% in the third, 6.4% in the fourth, 3.8% in the fifth, 6.4% in the sixth and 6.4% was older than twelve weeks.

Strains and growth conditions

All strains were obtained streaking on Perfringens Agar Base (Oxoid) 0.1 ml of 24 h broth (Cooked Meat Medium, Difco) culture of jejunum and ileum fragments (5 cm back and 3 cm after the Merkel's diverticulum) collected from sick commercial turkeys. CP ATCC 27324 (toxin-type E+enterotoxin), CCUG 2036 (toxin-type C), CCUG 2037 (toxin-type D), ATCC 10543 (toxin-type

Table 1. Primers used to detect *C. perfringens* toxin coding genes.

Gene	Primers	Sequence (5'-3')	Fragment length
cpa	cpa_F	GTT GAT AGC GCA GGA CAT GTT AAG	402
	cpa_R	CAT GTA GTC ATC TGT TCC AGC ATC	
cpb	cpb_F	ACT ATA CAG ACA GAT CAT TCA ACC	236
	cpb_R	TTA GGA GCA GTT AGA ACT ACA GAC	
cpetx	etx_F	ACT GCA ACT ACT ACT CAT ACT GTG	541
	etx_R	CTG GTG CCT TAA TAG AAA GAC TCC	
cpi	cpi_F	GCG ATG AAA AGC CTA CAC CAC TAC	317
	cpi_R	GCG ATG AAA AGC CTA CAC CAC TAC	
cpe	cpe_F	GGG GAA CCC TCA GTA GTT TCA	506
	cpe_R	ACC AGC TGG ATT TGA GTT TAA TG	
cpb2	cpb2_F	AGA TTT TAA ATA TGA TCC TAA CC	567
	cpb2_R	CAA TAC CCT TCA CCA AAT ACT C	
NetB	AKP78_F	GCT GGT GCT GGA ATA AAT GC	384
	AKP79_R	TCG CCA TTG AGT AGT TTC CC	

A+b2) were used as reference strains. All strains were incubated in anaerobic conditions at 37°C for 48 hours.

DNA extraction

Colonies of each CP strain were recovered from the agar plate and the DNA was extracted with DNeasy Blood & Tissue Kit (Qiagen) according to manufacturer's instructions.

Toxin coding gene detection

One multiplex PCR for cpa, cpb1, cpetx, and cpi genes and three single PCR for cpb2 (Meer and Songer, 1997), cpe (Baums *et al.,* 2004) and NetB (Keyburn *et al*., 2006) genes detection were used. PCR primers and fragment length are listed in Table 1. The sequencing of the amplified product confirmed that the targeted netB gene was indeed amplified with the PCR assay.

Parasitological examination

Intestinal mucosa of all chickens was scraped in different districts and observed by optic microscope searching for protozoa and helminths (eggs and worms).

Results and discussion

At necropsy, all 82 turkeys showed enteric lesions. In younger subjects (1 to 3 weeks) intestinal lesions were consistent with viral enteritis, a common finding in Italian flocks. In older turkeys (3 to 6 weeks of age) coccidiosis was diagnosed. Twenty-five (30.48%) out of 82, aged from 6 to 104 days old, were positive for *C. perfringens* type A. All strains resulted positive for a α toxin gene (toxin-type A) and only 1(1.2%) of these was positive also for ß2 toxin (toxin-type A+ß2). No CP cpe-positive or NetB positive strains were detected. Four (4.8%) turkeys were positive for *C. difficile*. Among these, 1 was negative for both toxin genes while 2 were positive for TcdA and TcdB and 1 was positive only for TcdB. 1 (1.2%) was positive for *C. sordelli.*

The data highlight that the CP isolates included in the study were type A toxin positive and a relatively low percentage of isolates carried the ß2 toxin gene, irrespective of enteric lesions. No CP toxin type C was found also in our sample. The data suggest that the role of CP type C should be revaluated in the pathogenesis of NE. The presence of *Clostridium* spp. was often associated with other pathogens, such as viral enteritis in the first 3 weeks, coccidiosis between 3 and 5 weeks and hemorrhagic enteritis between 6 and 12 weeks of age.

Conclusions

These observations underline the importance of predisposing factors (nutrition, drug treatments, concomitant diseases) in poultry clostridiosis. It must be underlined the presence of CP type A already in 6 days old turkeys. The role of this pathogen at such young age must be clearly understood but surely it could play an important role in developing enteric disorders. After the growth promoters ban in 2006, enteric imbalances are a main concern. The lack of NetB positive findings, which seems to play a major role in NE of chickens, is an important result as there is no data available for this toxin in turkeys. Moreover, the presence of *C. difficile* in 4 samples, 3 of them toxin genes positive, is quite interesting because of its potential zoonotic role.

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