## DIFFUSION OF CLOSTRIDIUM PERFRINGENS NETB POSITIVE STRAINS IN HEALTHY AND DISEASED CHICKENS

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**Abstract**
For over 30 years α toxin was considered the key virulence factor responsible for the appearance of necrotic enteritis (NE) in chickens but, recently, a new toxin related to the occurrence of NE, called NetB, has been described. The aim of this work was to evaluate the CP toxin-type and the *NetB* gene presence in strains collected from chickens affected or not by enteric diseases. 107 strains were tested: 30 isolated from chickens affected by NE, 54 from subjects affected by other enteric pathologies and 22 from healthy animals. All strains resulted toxin-type A and 26.17% of these was positive also for β2 toxin gene. No strains were positive for *cpe* gene. 27% (29/107) of CP was *NetB* positive and 93% (27/29) of these was isolated from birds affected by intestinal disorders. 16 NetB positive strains were obtained from chickens affected by NE (16/30), 9 from animals affected by other intestinal disorders (9/54) and 4 from healthy animals (4/22). A significant difference between the number of *Net*B positive strains isolated from animals affected by NE and healthy chickens has been observed (p=0.014). However, the finding that the 17.4% of strains isolated from healthy chickens was also positive for *NetB*, confirm that other virulence factors could play an important role on NE appearance.
Key Words: *Clostridium perfringens*, NetB toxin, chickens.

**Diffusione di ceppi di *Clostridium perfringens* NetB positivi in polli sani ed affetti da enterite.**

**Riassunto**
La tossina α prodotta dai ceppi di *Clostridium perfringenes* (CP) è stata considerata per anni come il principale fattore di virulenza dell'enterite necrotica (EN) ma la recente scoperta della tossina NetB ha indotto a riconsiderare l'eziopatogenesi di questa patologia. Lo scopo di questo studio è stato quello di valutare la presenza del gene *NetB* in ceppi di campo di CP isolati da animali sani ed affetti da enterite. A tale scopo 107 ceppi di CP sono stati tossinotipizzati ed è stata valutata la presenza dei geni codificanti le tossine β2, NetB ed enterotossina. 30 di questi ceppi sono stati isolati da polli affetti da EN, 54 da animali affetti da patologie enteriche diverse dalla EN e 22 da animali sani. Tutti i ceppi sono risultati di tossinotipo A ed il 26,2% di questi era anche positivo per il gene *cpb2* (tossina β2). Nessun ceppo è risultato positivo per la presenza del gene codificante l'enterotossina (*cpe*). Il gene *NetB* è stato rilevato nel 27,1% dei ceppi in esame e il 93,1% di questi ceppi postivi era stato isolato da animali affetti da EN. L'analisi statistica dei dati ha messo in evidenza che il numero di ceppi *NetB* postivi è più alto negli animali

affetti da EN rispetto agli animali sani (53,3% verso 16,7%, *p*=0,014); tuttavia, l'isolamento di ceppi positivi a *NetB* anche da animali sani suggerisce che nell'eziopatogenesi dell'enterite necrotica sono coinvolti anche altri fattori di virulenza.
Parole chiave: *Clostridium perfringens*, tossina NetB, pollo.

**Introduction**

*Clostridium perfringens* (CP) is an important enteropathogenic agent in animals and humans. The differential production of the four major toxins (α, β1, ε, and ι) is used to classify strains into five toxin-types. Some CP strains are able to produce two other toxins, β2 and enterotoxin, that have an important role in the pathogenesis of intestinal disorders in animals (Baums *et al.,* 2004). In poultry, CP is well known as the causative agent of both acute and sub clinical necrotic enteritis (NE). Acute NE is characterized by high mortality rates without premonitory signs. In sub-clinical NE the intestinal mucosa damage is limited and this condition is characterized by malabsorption with consequent reduced weight gain and increased feed-conversion ratio (Kaldhusdal *et al.,* 2001). Historically, the α toxin has been recognized as the key virulence factor in this type of pathology but Keybourn and co-workers (2006), using α-toxin knock-out mutant of CP, brought evidences that it is not an essential virulence factor in NE. The same Authors in 2008 described a novel toxin, NetB, that displays a moderate amino acid sequence similarity with CP β1 toxin and that seems to be expressed in most strains isolated in NE outbreaks (Keybourn *et al.,* 2008). However, the role of this new pore forming toxin in NE appearance is still under debate. Recently, Martin *et al.* (2008) demonstrated that *NetB* gene is expressed, although in a low incidence, also in healthy chickens and only in 58.3% of NE affected animals.

The aim of our study was to evaluate the presence of genes coding for α (*cpa*), β1 (*cpb1*), ε *(etx*), ι (*cpi*), β2 (*cpb2*), enterotoxin (*cpe*) and *NetB* toxins in CP field strains collected from healthy chickens and from subjects affected by enteric diseases.

**Material and methods**

*Strains and growth conditions*. 107 CP field strains were analyzed, 83 obtained from broilers and 24 from layers. 30/107 strains were isolated from birds affected by NE, 54/107 from animals with intestinal diseases not ascribable to NE and 22/107 from healthy ones. All strains were obtained streaking on Perfringens Agar Base (Oxoid) 0.1 ml of 24 h broth (Cooked Meat medium, Difco) previously inoculated with intestinal samples. 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. All strains were incubated in anaerobic conditions at 37 °C for 48 hours.

DNA *extraction*. Five colonies of each CP strain included in the study 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 other PCR for *cpb2* , *cpe* and *NetB* genes detection were used (Yoo *et al.*, 1997; 1997; Baums *et al.,* 2004; Keyburn *et al.*, 2006). The sequencing of the *NetB* amplified product confirmed that the targeted gene was correctly 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 helminthes.

*Statistical analysis*. Fisher's exact test was used to estimate the association between *NetB* gene positivity and NE appearance.

**Results and discussion**

All CP field strains were positive for α toxin (toxin-type A) and the 26.2 % (28/107) of these was positive also for the *cpb2* gene without significant differences between healthy and sick animals. None of the strains carried the *cpe* gene. 27% (29/107) CP were *NetB* positive and 93% (27/29) of these was isolated from birds affected by intestinal disorders, 16 from birds affected by NE and 9 from animals affected by other enteric pathologies. Only 4/22 isolates obtained by healthy animals carried *NetB* gene. The statistical analysis demonstrated that the number of *NetB* positive strains is higher (p=0.014) in chickens affected by NE than in healthy ones, and the difference was more evident (p=0.0079) when only CP isolated from animals affected by NE and tested negative at the parasitological examination were examined. Similar results were obtained when we analyzed separately the strains isolated from broilers where the disease has a greater economic impact compared with layers. 23/83 CP were positive for *NetB* gene, 14 were obtained from broilers affected by NE but only 1 from healthy birds. The data analysis underlined that the CP *NetB* positive strains are also in this case more frequent in animals affected by NE with a *p* value of 0.027. The *p* value reached the 0.0019 when only strains isolated from animals affected by NE and tested negative at parasitological examination were taken into consideration (table 1).

**Table 1.** Origin and number of *Clostridium perfringens* field strains tested for major toxin coding genes and for *NetB* gene. (EN=chickens affected by necrotic enteritis; E=chickens affected by other enteric diseases; H= healthy animals)

| | | N° | Toxin-type A (%) | Toxin-type A+β2 (%) | *NetB* positive (%) |
|---|---|---|---|---|---|
| **Broilers and layers** | | 107 | 79 (73.8) | 28 (26.2) | 29 (27.1) |
| **107** | **NE** | 30 | 26 (86.7) | 4 (13.3) | 16 (53.3) |
| | **E** | 54 | 41 (75.9) | 13 (24.0) | 9 (16.7) |
| | **H** | 22 | 12 (52.2) | 11 (47.8) | 4 (17.4) |
| **Broilers** | | 83 | 66 (79.5) | 17 (20.5) | 23 (27.7) |
| **83** | **NE** | 28 | 24 (85.7) | 4 (14.3) | 14 (50.0) |
| | **E** | 44 | 36 (81.8) | 8 (18.2) | 8 (18.2) |
| | **H** | 11 | 6 (54.5) | 5 (45.4) | 1 (9.1) |

**Conclusion**

In conclusion, all CP isolates from Italian poultry flocks, as previously reported, belong to toxin-type A (Drigo *et al.*, 2008). A relatively high percentage of isolates carry the β2 toxin gene with no significant differences between healthy and sick animals. The absence of *cpe* gene lead to suppose that chickens products do not represent an important risk factor for transmission of enteropathogenic CP to humans. A difference between the number of *NetB* positive strains isolated from animals affected by NE

and healthy chickens has been found. However, the percentage of positive strains in NE isolates was only 53.3% and, in addition, even if in low percentage (17.4%), CP that carry *NetB* gene were isolated also in healthy animals. Our results are in agreement with the data obtained by Martin *et al.* (2008) and, though they confirm the involvement of *NetB* toxin in the pathogenesis of NE, prompt to consider that also other pathogenic mechanisms could play an important role on NE appearance.

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