# GENOTYPING OF *CLOSTRIDIUM PERFRINGENS* ISOLATED FROM CATTLE AND PIGS WITH DIARRHEA IN HANOI AND SURROUNDING AREAS, VIETNAM

**Huỳnh Thị Mỹ Lệ[1]*, Đỗ Ngọc Thúy[2], Nguyễn Bá Hiên[1]**

*[1]Faculty of Verterinary Medicine, Hanoi University of Agriculture; [2]National Veterinary Institute*

**Email: huynhtmle@hua.edu.vn*

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ABSTRACT

Diarrhea is a major cause of economic loss in cattle and pig farming. The aim of this study was epidemiological survey of prevalence and molecular typing of *C. perfringens* isolates associated with diarrhea in cattle and pigs. The study was carried out from 2007 to 2010 in Hanoi and surrounding areas (including Bac Ninh and Vinh Phuc provinces). The biochemical properties of isolated *C. perfringens* were tested. The results showed that: all isolates of *C. perfringens* had biochemical properties as described before. PCR typing of isolates was carried out by multiplex PCR. *C. perfringens* isolated from fecal samples of diarrheic cattle were type A (57.34%), type D (41.33%) and type C (1.33%); whereas all *C. perfringens* isolated from fecal samples of healthy cattle, diarrheic and healthy pigs were type A. The prevalence of *cpe* and *cpb2* varied with isolated genotypes. There was a significant association between *cpb2* positive *C. perfringens* isolates and diarrhea in pigs. Of the 304 isolates from pigs with diarrhea examined, 138 (45.39%) were positive for the *cpb2* gene and 52 (17.11%) were positive for the *cpe* and *cpb2* genes, whereas none of the isolates from healthy pigs were positive for the *cpb2* gene.

Keywords: *Clostridium perfringens*, cattle, pigs, diarrhea, multiplex PCR.

## Xác định genotyp của vi khuẩn *Clostridium perfringens* phân lập được từ bò và lợn mắc hội chứng tiêu chảy nuôi tại Hà Nội và vùng phụ cận

TÓM TẮT

Hiện nay hội chứng tiêu chảy là nguyên nhân gây thiệt hại kinh tế cho ngành chăn nuôi bò và lợn. Nghiên cứu này được tiến hành nhằm mục đích điều tra tỷ lệ lưu hành, xác định genotype vi khuẩn *C. perfringens* ở đàn bò và lợn bị tiêu chảy nuôi tại Hà Nội và vùng phụ cận (Bắc Ninh, Vĩnh Phúc). Kết quả giám định đặc tính sinh hóa cho thấy vi khuẩn *C. perfringens* phân lập được mang đầy đủ đặc tính như các tài liệu kinh điển đã mô tả. Sử dụng phản ứng multiplex PCR để xác định định typ và xác định gen mã hóa độc tố của vi khuẩn phân lập được. Kết quả cho thấy vi khuẩn *C. perfringens* phân lập được từ bò bị tiêu chảy thuộc ba typ với tỷ lệ lần lượt là typ A (57,34%), typ D (41,33%) và typ C (1,33%); trong khi đó toàn bộ các chủng phân lập được từ bò khỏe mạnh, từ lợn bị tiêu chảy và khỏe mạnh đều thuộc typ A. Tỷ lệ mang gen độc tố *cpe* và *cpb2* là khác nhau giữa các typ vi khuẩn phân lập được. Đặc biệt chỉ các chủng phân lập được từ lợn bị tiêu chảy mới mang gen *cpb2*, còn các chủng phân lập được từ lợn khỏe mạnh đều âm tính với gen này; trong số 304 chủng phân lập từ lợn bị tiêu chảy có 138 chủng (45,39%) dương tính với gen *cpb2* và 52 chủng (17,11%) mang cả hai gen mã hóa độc tố *cpe* và *cpb2*.

Từ khóa: *Clostridium perfringens*, cattle, pigs, diarrhea, multiplex PCR.

## 1. INTRODUCTION

Diarrhea is a major source of economic loss in cattle and pig farming, causing livestock growth retardation and even high mortality. In Vietnam, there were many studies on diarrhea in cattle and pigs with emphasis on the role of *Escherichia coli* (*E. coli*) and *Salmonella*;

nevertheless, there are few studies on *C. perfringens*. Although *C. perfringens* enterotoxaemia in cattle has emerged in Northern provinces since 1997, no effective prevention program has been put in place. A study on the role of *C. perfringens* in gastrointestinal diseases in domestic animals is therefore necessary.

*C. perfringens* is a Gram-positive, spore-forming, anaerobic bacterium that has long been recognized as a significant cause of both histotoxic and gastrointestinal (GI) diseases in humans and domestic animals (Songer 1996). *C. perfringens* strains are classified into five toxinotypes (A, B, C, D, and E), according to the production of four major toxins: alpha, beta, epsilon and iota. Each toxinotype is associated with a particular disease. Some *C. perfringens* isolates (mostly belong to type A) produce *C. perfringens* enterotoxin (CPE) and some type produce the beta2 toxin (CPB2). Several worker have noted an association of cpb2-positive strains of *C. perfringens* type A and the occurrence of enteric disease in domestic animals, particular piglets (Klaasen et al., 1999; Garmory et al., 2000).

The main objective of this study was epidemiological survey of prevalence and molecular typing of *C. perfringens* isolates associated with diarrhea in cattle and pigs in Hanoi and surrounding areas. The results could lead to optimal disease control strategies.

## 2. MATERIAL AND METHODS

### 2.1. Fecal samples

Fecal samples from all aged cattle (diarrheic, *n = 128*; healthy, *n = 42*) and 1 - 90 days old pigs (diarrheic, *n = 522*; healthy, *n = 82*). Clinical signs of diarrheic animals were depression, yellowish or grayish diarrhea, possibly bloody diarrhea, and had a stinking smell. Fecal samples were collected directly from the rectum in sterile plastic bags and transported to the laboratory within 2 - 8 hours after collection.

### 2.2. Isolation and confirmation of *C. perfringens*

Samples were cultured on Thioglycollate (TGC) (Oxoid) and incubated anaerobically at $37^0$C for 24 hours. A loop full from overnight TGC was subsequently cultured onto Clostridium welchii agar (CW) plates with 4% egg yolk emulsion (Nissui Ltd.) and incubated anaerobically at $37^0$C. The plates were read after 24 to 48 hours from growth of *C. perfringens*. Typical colonies were identified by characteristic colony morphology, lecithinase activity on CW, hemolysis on blood agar, Gram staining, reverse CAMP reaction and other biochemical tests. Toxicity of *C. perfringens* isolated was evaluated for the presence of lethal toxin by intravenous injection in mice. Typing of *C. perfringens* isolates were determined by multiplex PCR.

### 2.3. DNA extraction

Four to five colonies of *C. perfringens* grown on a blood agar plate were suspended in 200 µl of distilled water and the mixture then placed in boiling water bath for 15 min for cell lyses, following by 10 min in ice. The pellets were removed by centrifugation at 12.000 × g for 10 min, and the supernatant was used as the DNA template for PCR.

### 2.4. Primer and multiplex PCR

Specific primers design were based upon the sequence of each target genes as published by Songer and Bueschel (1999) and were synthesized commercially (Invitrogen) (Table 1).

PCR amplification: the multiplex PCR was performed in a MasterCycler Thermalcycler (Eppendorf). Total reaction volume of 25 µl containing 5 µl of 10 × PCR buffer (Advanced Biotechnologies), with 750 mM Tris - HCl (pH = 8,) 200 mM $(NH_4)_2SO_4$; 0,1% (v/v) Tween 20, dNTPs, 2 mM $MgCl_2$ (Fermentas); 1 µl of each primer (10 pmol/µl); 0,1 µl (500 UI/ µl) of *Tag* DNA polymerase (Advanced Biotechnologies) and 2 µl of DNA template. Amplification was obtained with a program composed of 5 min at $94^0$C, 40 cycles consisting of 1 min at $94^0$C, 1 min at $50^0$C, 1 min at $72^0$C, and a final incubation for 7 min at $72^0$C.

**Table 1. Nucleotide sequences of primers**

| Primers | Nucleotide sequences (5' - 3') | Size (bp) |
|---|---|---|
| *cpa* (alpha toxin) | 5'-GCTAATGTTACTGCCGTTGA-3'<br>5'-CCTCTGATACATCGTGTAAG-3' | 324 bp |
| *cpb* (beta toxin) | 5'-GCGAATATGCTGAATCATCTA-3'<br>5'-GCAGGAACATTAGTATATCTTC-3' | 196 bp |
| *etx* (epsilon toxin) | 5'-GCGGTGATATCCATCTATTC-3'<br>5'-CCACTTACTTGTCCTACTAAC-3' | 655 bp |
| *iA* (iota toxin) | 5'-ACTACTCTCAGACAAGACAG-3'<br>5'-CTTTCCTTCTATTACTATACG-3' | 446 bp |
| *cpe* (enterotoxin) | 5'-GGAGATGGTTGGATATTAGG-3'<br>5'-GGACCAGCAGTTGTAGATA-3' | 233 bp |
| *cpb2* (beta2 toxin) | 5'-AGATTTTAAATATGATCCTAACC-3'<br>5'-CAATACCCTTCACCAAATACTC-3' | 567 bp |

The results were examined by electrophoresis in a 2% agarose gel (Seakem GTG) for 30 min at 50V and straining with ethidium bromide. PCR marker was 100 bp DNA Ladder (Invirogen). Amplified bands were visualized and photographed by Gel Doc 2000 (BioRad). Positive strains were *C. perfringens* NCTC 8239 (type A,) *C. perfringens* NCTC 6121 (type B,) and *C. perfringens* NCTC 8346 (type C.) Negative strain was *C. difficle* ATCC 43593.

Data were analyzed by Chi-square test (Minitab 14.0 software) and Fisher Exact Test (SAS 8.1 software).

## 3. RESULTS AND DISCUSSION

### 3.1. Prevalence of *C. perfringens*

Prevalence of *C. perfringens* isolated from fecal samples were shown in Table 2.

Prevalence rates of identified *C. perfringens* in fecal samples of diarrheic animals were significantly higher than of samples from healthy ones ($P < 0.05$). There were no differences among the prevalence of identified *C. perfringens* in samples collected from studied regions ($P > 0.05$).

The characteristic of the isolates were positive fermentation of glucose, lactose, saccharose, maltose, and mannose; a double-zone hemolysis around the colonies on blood agar; hydrolysis of gelatin; production of lecithinase; and a positive reverse CAMP test result. Also, $H_2S$ production properties of isolates from cattle and pigs with and without diarrhea were 85.33%, 86.67%, 82.89%, and 66.67%, respectively.

**Table 2. Prevalence of *C. perfringens***

| Species | Clinical signs | No of examined samples | Positive *(n, %)* | Negative *(n, %)* |
|---|---|---|---|---|
| Cattle | Diarrhea | 128 | 75 (*58.59*) | 53 (*41.41*) |
| | Healthy | 42 | 15 (*35.71*) | 27 (*64.29*) |
| Pigs | Diarrhea | 522 | 304 (*58.24*) | 218 (*41.76*) |
| | Healthy | 82 | 21 (*25.61*) | 61 (*74.39*) |

*No = number*

### 3.2. Genotyping of *C. perfringens*

The PCR assay was performed on all *C. perfringens* isolates. Of the 75 *C. perfringens* isolates from diarrheic cattle, 57.34%, 41.33%, and 1.33% belonged to type A, type D, and type C, respectively; whereas all *C. perfringens* isolated from fecal samples of healthy cattle, diarrheic and healthy pigs were type A.

As reported in the previous studies, all *C. perfringens* isolates from cattle belonged to type A (Le Lap et al., 2007; Nguyen Quang Tinh, 2008). This was the first time *C. perfringens* type D and type C being isolated from cattle in Vietnam. Because the distribution of *C. perfringens* toxinotypes varied in different geographical areas (Yoo et al., 1997), this result would be very useful for epidemiological studies, prophylaxis programs, and the design of strategies for correct use of *C. perfringens* vaccines in Vietnam.

### 3.3. Prevalence of cpe and cpb2 positive isolates

In this study, all isolates *C. perfringens* were analysed by multiplex PCR to determine the toxicity of *C. perfringens* isolates and the correlation between diarrhea in animals and the presence of *cpe* and *cpb2* genes positive *C. perfringens*. The results were shown in Table 3.

86.66% out of 15 *C. perfringens* isolates from healthy cattle were *cpe*- and *cpb2*-. This prevalence was significantly higher than that of isolates from diarrheic cattle ($P < 0.05$.) All *cpe* gene positive *C. perfringens* isolates were originated from diarrheic cattle. The percentage of *cpb2* and both *cpe* / *cpb2* genes positive *C. perfringens* isolated from diarrheic pigs were 45.39% and 17.11%, respectively. There were no *cpb2* positive isolates from healthy pigs. Along with the major toxin, enterotoxin and beta2 play the major role in several diseases (Songer, 1996; Gibert et al., 1997; Petit et al., 1999.) The beta2 toxin was first purified from *C. perfringens* type C strain CWC245, which was isolated from a piglet that died of necrotizing enterocolitis (Gibert et al., 1997) and has been associated with enteric diseases in domestic animals (Gurjar et al., 2008.) Enterotoxin is considered a virulence attribution in animal strains of *C. perfringens* (Meer and Songer, 1997.)

**Table 3. Prevalence of cpe and cpb2 positive *C. perfringens* types isolated from fecal samples**

| Species | Type | Isolate source | *cpe*$^+$ *(n, %)* | *cpb2*$^+$ *(n, %)* | *cpe*$^+$ and *cpb2*$^+$ *(n, %)* | *cpe*$^-$ and *cpb2*$^-$ *(n, %)* |
|---|---|---|---|---|---|---|
| Cattle | A | Diarrhea *(n = 43)* | 9 (*20.93*) | 15 (*34.88*) | 6 (*13.95*) | 13 (*30.23*) |
| | | Healthy *(n = 15)* | | 1 (*6.67*) | 1 (*6.67*) | 13 (*86.66*) |
| | D | Diarrhea *(n = 31)* | 12 (*38.71*) | | 1 (*3.23*) | 18 (*58.06*) |
| | C | Diarrhea *(n = 1)* | | | 1 (*100*) | |
| Pigs | A | Diarrhea *(n = 304)* | 67 (*22.04*) | 138 (*45.39*) | 52 (*17.11*) | 47 (*15.46*) |
| | | Healthy *(n = 21)* | 5 (*23.81*) | | | 16 (*76.19*) |

*+ : positive; - : negative*

The enterotoxigenic strains of *C. perfringens* were found in cattle and horse isolates (Tschirdewahn et al., 1991.) Enterotoxin is most often produced by type A, but it may be produced by all of other *C. perfringens* types. Enterotoxigenic *C. perfringens* type A strains cause outbreaks of food poisoning in humans (Kalender et al., 2005).

The prevalence of *cpe* and *cpb2* genes negative isolates out of *C. perfringens* isolates originated from healthy cattle was significantly higher than that of isolates from diarrheic cattle ($P < 0.05$), meaning *C. perfringens* had changed in toxicity and would become one of the hazardous agents causing diarrhea.

Although in this study, the role of enterotoxin was not confirmed in *C. perfringens* infections of cattle, the study result may reveal a warning of the risk of source of CPE+ *C. perfringens*, which can lead to outbreaks of food poisoning in the studied areas.

The most important finding in this study is the detection of *cpb2* positive *C. perfringens* isolates in cases of diarrhea only, and not in healthy pigs, corroborating the results of others (Bueschel et al, 2003; Das et al, 2009; Klaasen et al, 1999.) This finding suggested that *C. perfringens* type A isolates carrying an additional *cpb2* gene might play an important role in causing diarrhea in pigs in Hanoi, Vietnam.

## 4. CONCLUSION

In conclusion, prevalence rates of identified *C. perfringens* in fecal samples of diarrheic animals were significantly higher than of samples from healthy ones ($P < 0.05$.) We demonstrated for the first time that *C. perfringens* type A, C and D isolated from diarrheic cattle in Vietnam. In addition, the finding that *cpb2* gene positive *C. perfringens* type A might play a role in causing diarrhea in pigs could help the development of vaccines to protect against the effects of the β2 toxin in pigs in Hanoi, Vietnam.

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