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Characterisation of *Clostridium perfringens* isolated from chickens in Vietnam

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Abstract: The objective of this study was isolating and characterising *Clostridium perfringens* from chickens in Vietnam and identifying virulence factors involved with enteritis. Five hundred thirty-one faecal and sixty-eight intestinal samples were collected from healthy and diseased chickens for the *C. perfringens* isolation. The presence of virulence factors was determined by multiplex PCR. The *netB* gene of the selected isolates was sequenced and checked for its expression by SDS-PAGE. Two hundred seventy-two *C. perfringens* isolates were collected. All of them were shown to be positive for the *cpa* gene. The *netB* gene was detected in 26.56% of the *C. perfringens* isolates from the healthy chickens, while 43.45% of the isolates from the faeces and 45% of the isolates from the intestinal samples were positive for this gene in the diseased birds. All eight isolates positive to *netB* from the diseased chickens showed 100% identity in the *netB* sequence and produced the NetB toxin *in vitro*, whereas only two out of eight healthy chicken-derived isolates produced this toxin. Nine out of ten chickens experimentally infected with the *C. perfringens netB*-positive isolate showed typical signs of enteritis. The *cpa* gene was the most prevalent virulence factor identified in the bacteria *C. perfringens*, but the *netB* gene could be a major player responsible for necrotic enteritis progression in chickens.

Keywords: *cpa*; necrosis enteritis; *netB;* poultry; virulence factor

C. perfringens is a Gram-positive, spore-forming anaerobic bacterium that is widely distributed in the environment and commonly found in the gastro-intestinal tract of humans and domestic animals (Kiu and Hall 2018). They were classified into five specific toxinotypes (A–E) (Songer 1996; Petit et al. 1999). More recently, seven *C. perfringens* toxinotypes (A–G) have been proposed, based on the production of six major toxins including α – *cpa*, β – *cpb*, ε – *etx*, ι – *iap*, CPE – *cpe*, and NetB – *netB* (Rood et al. 2018). *C. perfringens* is the key aetiologic agent of necrotic enteritis in poultry (Prescott et al. 2016).

Necrotic enteritis (NE) in poultry was the first described in England in 1961. Since then, outbreaks of necrotic enteritis, caused by *C. perfringens*, have been reported in all poultry producing countries (Prescott et al. 2016). NE is a multi-factorial disease and has significant economic impacts on the poultry industry with billions of dollars lost annually (Wade and Keyburn 2015). In southern-eastern Norway, the incidences of subclinical NE in 2014 were double that in 2013 (Kaldhusdal et al. 2016). The main primary alpha factors involved in this disease progression are documented to be mostly extracellular protein toxins. Clinical NE is thought to occur when *C. perfringens* proliferates to high numbers in the small intestine with the accompanying production of extracellular toxins that damage the intestine (Shojadoost et al. 2012). The num-

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ber of *C. perfringens* in the intestines of healthy birds is normally less than 10^2–10^4 colony-forming units (CFU) per gram of intestinal contents compared to the diseased birds having 10^7–10^9 CFU/g (Shojadoost et al. 2012). In poultry, necrotic enteritis is most commonly caused by type A *C. perfringens*, and more rarely by type C (Shojadoost et al. 2012). Early studies showed that the major toxin responsible for NE was alpha-toxin (Fukata et al. 1988), however, Keyburn et al. (2008) recently reported the NetB toxin as a crucial factor in the development of the disease.

In Vietnam, although necrotic enteritis is believed to be of a significant economic impact for the poultry industry, there are few detailed reports of necrotic enteritis in poultry (Nguyen et al. 2020). In this study, we isolated and characterised the toxicity related factors of *C. perfringens* that were causative agents of the poultry NE disease in Vietnam.

MATERIAL AND METHODS

Sample collection and *C. perfringens* isolates

During the period of January 2016 to January 2019, a total of 531 faecal samples were collected from both clinically normal chickens (n = 266) and chickens showing typical signs of necrotic enteritis (n = 265), including anorexia, ruffled feathers, depression, diarrhoea, and listlessness. In addition, 68 sets of intestinal organs, made up of the small intestine, colon, and caecum with typical NE lesions were collected. The gross lesions were apparent in the duodenum, jejunum, ileum and caecum. The intestinal wall appeared thin and friable. In areas with advanced necrotic lesions, the mucosa was in brownish color. The samples were cultured on sheep blood agar (SBA) plates consisting of an Oxoid Columbia Agar base supplemented with 7% v/v citrated sheep's blood, incubated under anaerobic conditions [an AnaeroGen sachet (AN0025A) was placed in a sealed jar] at 37 °C for 48 h prior to the sub-culture. Colonies which showed characteristic dual haemolytic zones were selected and checked for purity on a Tryptose Sulfite Cycloserine agar (TSC Agar Oxoid, CM0543) before propagation in a fluid thioglycollate (FTG Merk, 108191). The intestinal organs were sampled by scrubbing the inner wall with a cotton swab, then were processed in the similar way to the faecal samples. The isolate identity was established based on the colony morphology, haemolysis pattern, Gram staining morphology and conventional biochemical characteristics including catalase, gelatin hydrolysis, indole, motility, gas, glucose, lactose, maltose, sucrose, mannitol, oxidase and lecithinase activity as described by Quinn et al. (1994). One *C. perfringens* isolate from each bird was selected for further study.

Statement of animal rights

All of the experiments involving the animal care and the sacrifice procedure were approved by the Animal Ethics Committee in Nong Lam University (AEC – NLU), Vietnam, No. 20201606.

Determination of toxigenic *C. perfringens*

The presence of the *cpa*, *cpb*, *iap*, *etx*, *cpe* and *cpb2* genes was determined using the multiplex polymerase chain reaction (PCR) system previously described by Meer and Songer (1997). For the detection of the *netB* gene, the PCR protocol of Keyburn et al. (2008) was used (Table 1).

DNA extraction: A single colony on the SBA plates of each isolate was suspended in 200 µl of distilled water, boiled for 10 min, and centrifuged at 10 000 *g* for 10 minutes. The supernatants were collected, stored at –20 °C, and used as template sources for further PCR reactions.

Characterisation of *netB* sequences

For sequencing, *C. perfringens* was cultured in the fluid thioglycollate (FTG Merk, 108191). The *C. perfringens* DNA was extracted using a Wizard Genomic DNA Purification Kit (A1120; Promega, Madison, WI, USA). The extracted DNA was applied to the PCR reactions to amplify the *netB* gene with the primer set of netB(-100)F/netB(1278)R (Table 1) as described by Abildgaard et al. (2010). The PCR amplicons were purified using a QIAquick Gel Extraction Kit (28706; QIAGEN, Hilden, Germany), then sent to 1st BASE-Singapore for sequencing by the Sanger method. The phylogenetic tree of the *netB* genes was constructed by the maxi-

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Table 1. Primers applied in this study

Toxin genes	Primer	Nucleotide sequence (5'-3')	Amplicon size (bp)	References
cpa	cpa-F	GCTAATGTTACTGCCGTTGA	324	Meer and Songer (1997)
	cpa-R	CCTCTGATACATCGTGTAAG		
cpb	cpb-F	GCGAATATGCTGAATCATCTA	196	
	cpb-R	GCAGGAACATTAGTATATCTTC		
etx	etx-F	GCGGTGATATCCATCTATTC	655	
	etx-R	CCACTTACTTGTCCTACTAAC		
iap	iap-F	ACTACTCTCAGACAAGACAG	446	
	iap-R	CTTTCCTTCTATTACTATACG		
cpe	cpe-F	GGAGATGGTTGGATATTAGG	233	
	cpe-R	GGACCAGCAGTTGTAGATA		
cpb2	cpb2-F	AGATTTTAAATATGATCCTAACC	567	
	cpb2-R	CAATACCCTTCACCAAATACTC		
netB	netB-F	GCTGGTGCTGGAATAAATGC	384	Keyburn et al. (2008)
	netB-R	TCGCCATTGAGTAGTTTCCC		
Sequencing the *netB* gene	netB(-100)F	CCAGTTATGTATAAATTTTGACCAGTT	1 378	Abildgaard et al. (2010)
	netB(1278)R	AAACTTTAGTATTCCTCTCATTTTTTATCCC		

mum likelihood method with 1 000 bootstrap replications (MEGA-X software v7.0). The full-length 969 bp sequences of the *netB* genes determined in the present study were used for the phylogenetic analysis. The reference sequences of the *net*B genes to be included in phylogenetic tree were obtained from the GenBank database.

Characterisation of NetB toxin production of isolated *C. perfringens*

The *C. perfringens* isolates were grown in the FTG media, harvested for the cultured supernatant and purified for the secreted protein as described by Keyburn et al. (2008). Briefly, the culture was centrifuged at 6 000 *g* for 10 min at 4 °C, filtered through a 0.45 µm membrane and concentrated by ultrafiltration through a 10 kDa membrane. The purified protein products were separated on an SDS-PAGE gel for the NetB protein (~33 kDa) analysis.

Experimental infection and gross examination

The isolate used for the experimental infection was cultured from the small intestine of the diseased chicken. This isolate was positive for the *cpa* and *netB* genes by PCR analysis and was shown to produce the toxin *in vitro*. The experimental infection was carried out as described by Olkowski et al. (2006) with a slight modification. A group of ten chickens (10 days old) were fed diets containing 400 g/kg crude protein from fish meal and meat meal. The 10-day-old chickens were orally inoculated daily with 1 ml (3×10^8 CFU/ml) of a 14–15-hour culture of *C. perfringens* for 5 days. The control group (5 chickens) received 1 ml of a sterilised cultured medium as a placebo. All the chickens were monitored twice per day for overt clinical signs. On day 20 (5 days after infection), all the chickens of both groups were euthanised and checked for typical NE symptoms. The intestinal samples of the chickens showing gross pathological changes as well as those of the controlled chickens were collected, preserved in 10% formaldehyde for further examination.

RESULTS

Isolation of *C. perfringens*

Two hundred seventy-two *C. perfringens* isolates were isolated from the chicken faecal and intestinal samples. Of the 232 isolates cultured from the

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Table 2. Prevalence of the toxin genes among the isolated *C. perfringens*

Cases		Number of isolates	Toxin-genes of *Clostridium perfringens*							
			cpa		*cpe*		*netB*		*netB/cpe/cpa*	
			no positive	%	no positive	%	no positive	%	no positive	%
Healthy chickens	faeces	64	64	100	1	1.56	17	26.56	0	0
Diseased chickens	faeces	168	168	100	3	2.23	73	43.45	1	0.59
	intestines	40	40	100	0	0	18	45.00	0	0
Total		272	272	100	4	1.47	108	39.70	1	0.36

faeces, 64 isolates were obtained from the clinically normal birds while 168 isolates came from the diseased chickens (Table 2). Only one isolate from each bird was included for further characterisation. The biochemical testing of all 272 bacterial isolates (data not shown) confirmed the phenotypic characteristics of the isolates were consistent with that described for *C. perfringens* (Quinn et al. 1994).

Detection of toxin genes

The multiplex-PCR results (Figure 1) showed that all the *C. perfringens* isolates in this study, including those from the healthy chickens and chickens with necrotic enteritis possessed the alpha-toxin gene (*cpa*) (Table 2). The *cpe* enterotoxin gene was only found in four of the isolates. According to the recently described typing scheme of Rood et al. (2018), three of these four isolates are type F as they each had the *cpa* and *cpe* gene. A further

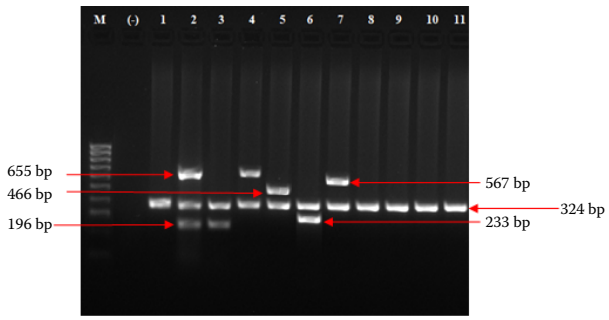

Figure 1. Representative result of the *C. perfringens* toxin typing by multiplex PCR

(–) = negative control; M = 100 bp DNA ladder maker

Lane 1: positive control for type A; lane 2: positive control for type B; lane 3: positive control for type C; lane 4: positive control for type D; lane 5: positive control for type E; lane 6: positive control for type A (cpa^+; cpe^+); lane 7 positive control for type A (cpa^+; $cpb2^+$); lane 8–11: samples

104 isolates carried the *cpa* and *netB* genes and are, therefore, type G. The PCR for the detection of the *netB* gene (Figure 2), showed that 17/64 (26.56%) *C. perfringens* isolated from the healthy chickens carried this gene while, in the isolates from the faecal and intestinal samples of the diseased birds, 73/168 (43.45%) and 18/40 (45%) were positive for the isolates, respectively. One isolate from a diseased chicken was positive for all three toxin genes including *cpa*, *cpe*, and *netB*.

Analysis of the *netB* sequences

From 91 of the *netB*-positive isolates from the diseased chickens, we selected eight isolates for the *netB* sequence analysis.

The analysed sequences with 1 230 bp in length covered both the coding sequence (969 bp) and the upstream promoter region (60 bp). All these sequences (registered accession number on GenBank: MT032252–MT032259) were completely similar to each other and the *netB* sequences previously submitted to the GenBank. Both the coding sequence

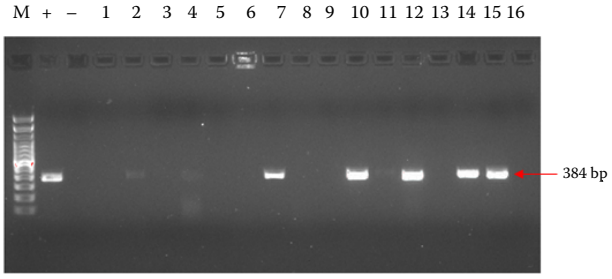

Figure 2. Representative result of the *netB* gene detection by PCR reaction

(+) = positive control; (–) = negative control; M = 100 bp DNA ladder

Lane 1–16: samples (lane 7, 10, 12, 14, 15: positive for *netB* gene; lane 1, 2, 3, 4, 5, 6, 8 and 9: negative for *netB* gene)

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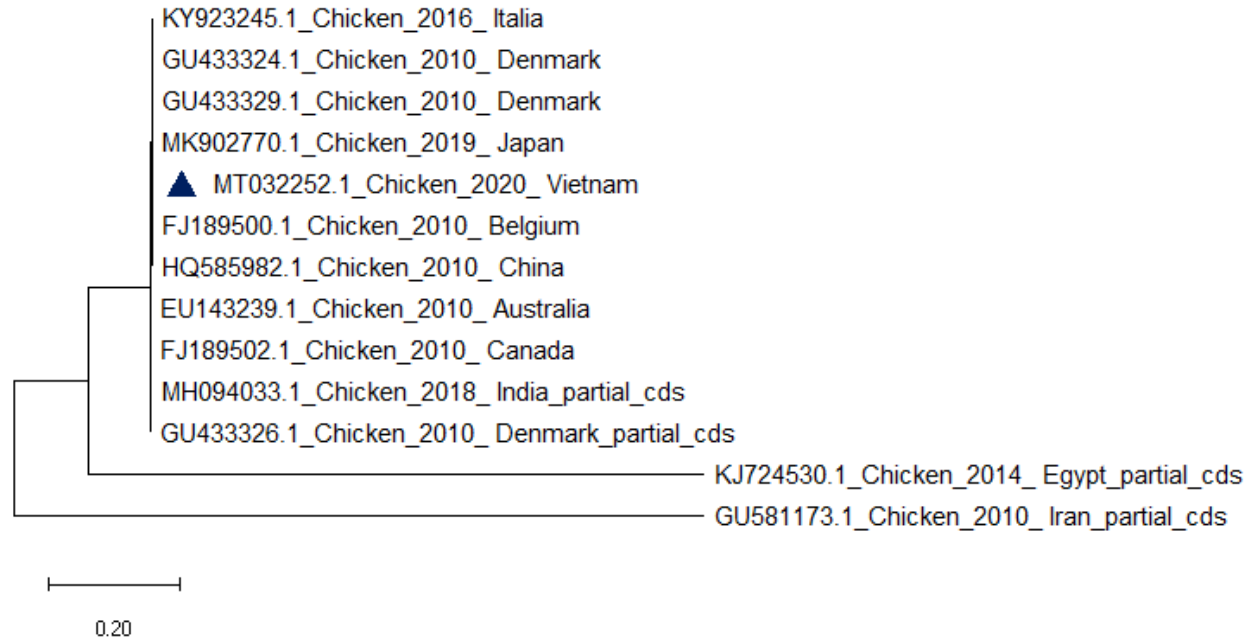

Figure 3. Phylogenetic tree based on the complete 969 bp *netB* gene comparison, including the *netB* complete sequences obtained from the chickens in Vietnam named MT032252

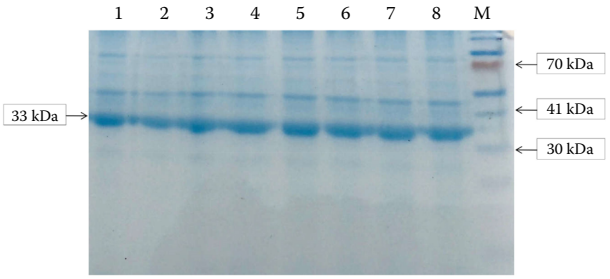

Figure 4. NetB toxin produced by the *netB*-positive *C. perfringens* isolated from the diseased chickens by SDS-PAGE

M = pre-stained marker

Lanes 1–8: protein samples purified from the overnight cultured media of eight *C. perfringens* isolates from the diseased chickens

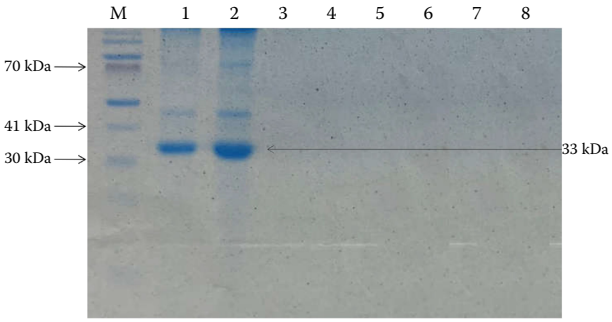

Figure 5. NetB toxin produced by eight *netB*-positive *C. perfringens* isolates from the healthy chickens by SDS-PAGE

M = pre-stained marker

Lanes 1–2: expressed NetB toxin; lanes 3–8: non-expressed NetB toxin

and the promoter region did not show any nucleotide variation. Twelve *netB* sequences were available at the GenBank from several countries (Italia, Denmark, Japan, Belgium, China and Canada) showing completely identical sequences to ours (Figure 3).

Production of the NetB toxin

Eight of the 91 *netB*-positive isolates from the diseased chickens and eight of seventeen isolates in the healthy birds were selected for the SDS-PAGE screening for the NetB toxin production *in vitro*. The SDS-PAGE analysis of the overnight cultured supernatant of eight diseased chicken-derived isolates showed prominent bands at the position appropriate to the molecular weight of the NetB toxin, 33 kDa (Figure 4), whereas only 2/8 *netB*-positive ones from the healthy birds produced this protein band (Figure 5).

Results of the experimental infection

Five days post-infection, nine out of ten birds in the test group infected with *C. perfringens* iso-

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lated from the disease birds showed typical signs of the disease, including anorexia, ruffled feathers, depression, diarrhoea, and listlessness. This is in contrast to the test control group fed a sterile medium, which showed no signs of the disease and appeared clinically normal. The gross internal examination of the challenge group showed lesions under the surface of the stomach which were black in colour (Figure 6A), the small intestines had occasional surface haemorrhages (Figure 6C), the mucosa was lined with yellow areas, and the intestinal wall appeared thin (Figure 6D). In some cases, there were ulcers on the surface of the small intestines and the liver had focal white areas of necrosis (Figure 6B). The control group did not show any observed lesions. The bacterial isolation and detection of toxin genes of *C. perfringens* (by PCR) were carried out in all the experimentally infected chickens. Toxin genes were present in all *C. perfringens* strains which were reisolated from infected chicken (data not shown). In contrast, none of the typical *C. perfringens* strains with the toxin genes were recovered from the control chickens (data not showed).

DISCUSSION

In this study, we isolated and analysed the toxinotypes of *C. perfringens* from chickens in Vietnam. Identification of isolates was based on established morphological criteria, conventional biochemical testing and the presence or absence of toxin genes (identified using PCR results).

The cultural analysis showed that *C. perfringens* was present in 24.06% (64/266) faecal samples from the healthy chickens and 63.39% (168/265) from the diseased birds. The percentage of *C. perfringens* isolated from the healthy chickens in this study was lower than the result of Shahdadnejad et al. (2016) in Iran, where 50/122 samples were reported to be positive for *C. perfringens*.

However, our results were higher than those of Kalender and Ertas (2005) in Turkey where *C. perfringens* was isolated from only 5% (8/160) of the samples.

The discrepancies between our results and others may be due to the differences in the isolation methods or the different predisposing factors including nutritional components (Baba et al. 1997), effects

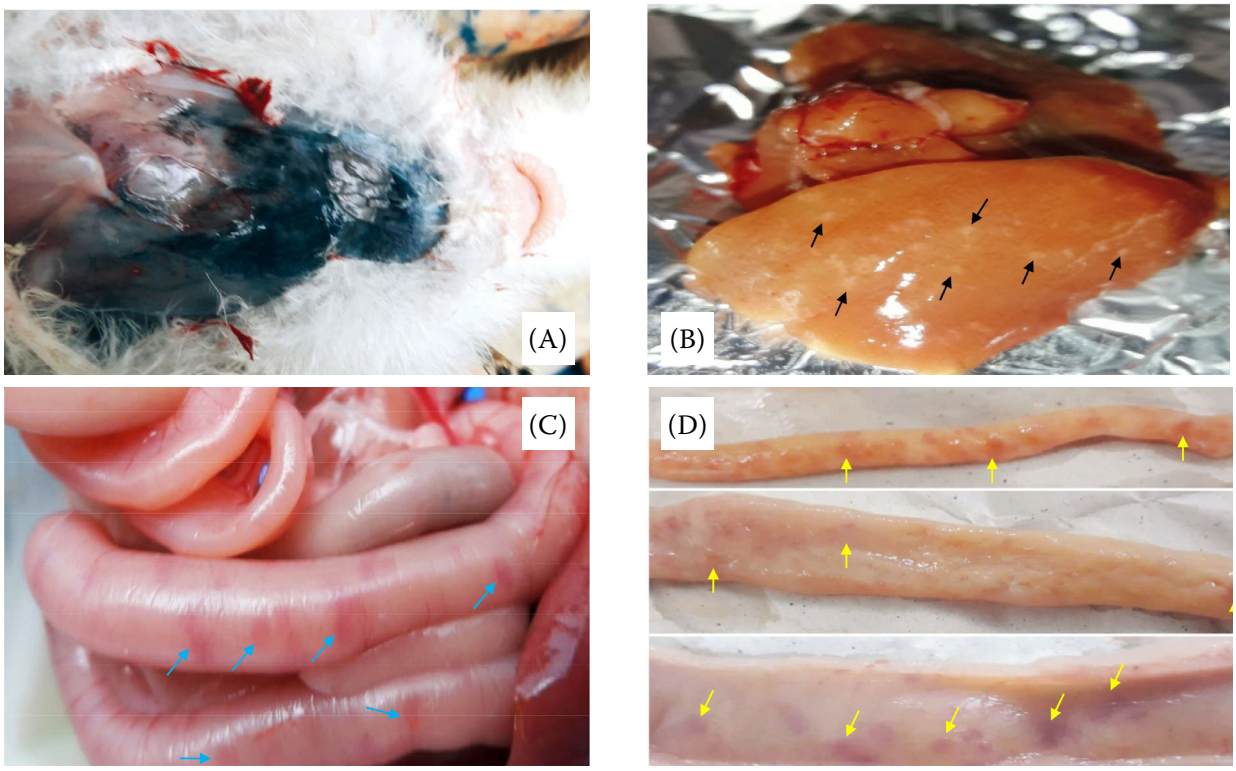

Figure 6. Lesions caused by *C. perfringens* in the chickens

A black colour on the surface of the stomach (A). The liver has many white necrotic spots (black arrows) (B). Small intestine (caecum) has many bleeding points (blue arrows) (C). Small intestinal mucosa (caecum) has several focal haemorrhages and shows necrosis (yellow arrows) (D)

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of probiotics and prebiotics (Hofacre et al. 1998), and antimicrobial drugs (Lanckriet et al. 2010).

C. perfringens type A has proven to be the most toxic type that initiates NE in poultry and causes food poisoning in humans (Petit et al. 1999). Even the alpha-toxin was considered a key virulence factor for a long time, Timbermont et al. (2011) reported that this toxin may not be essential for the development of NE diseases. In this study, we found all 272/272 *C. perfringens* isolates carried the *cpa* gene. This result was consistent with previous reports in China (Zhang et al. 2018), Korea (Park et al. 2015), Japan (Matsuda et al. 2019), the USA (Li et al. 2017; Yang et al. 2018; Gu et al. 2019; Mwangi et al. 2019), and in Iran (Shahdadnejad et al. 2016). The prevalence of the *cpe* gene in a poultry-derived *C. perfringens* population has been documented as being quite different among studies. While it is reported to be 34.2% in the research of Matsuda et al. (2019) in Japan, no prevalence was found in the study of Yang et al. (2018) in the USA or only 3% (4/130) of the isolates were positive in the research of Zhang et al. (2018) in China. In the study, we found only 4/272 *C. perfringens* isolates possessed the *cpe* gene.

The NetB toxin has been reported to be a major player in the NE progression in chickens (Keyburn et al. 2008). It was proven that *netB* mutant *C. perfringens* lost their pathogenic characteristics that cause NE symptoms in chickens, while such mutants complemented with the wild type *netB* gene recovered their toxicity triggering NE (Keyburn et al. 2008). Per our results, the prevalence of *C. perfringens* isolates positive to *netB* were determined to be 43.45% and 45% of the isolates from the faecal and intestinal samples of the diseased chickens, respectively. While only 26.56% of the isolates from the healthy chickens carried this gene. This result is lower than investigations carried out in USA, where the prevalence of the *netB* gene in *C. perfringens* isolates from healthy and diseased chickens were 68% and 81% (Mwangi et al. 2019), were 61% and 58% in Denmark (Abildgaard et al. 2010), and were 4% and 47% in Korea (Park et al. 2015), respectively. The difference observed between this study and others may be due to the differences in the status of the samples as, in other studies, the samples were taken from dead chickens or chickens with clear symptoms of NE, whereas we collected samples from suspected NE chickens. In this study, there was a difference between the prevalence of the *netB*-positive *C. perfringens* isolates in the healthy and diseased chickens. In the investigation in Denmark, the number of isolates carrying the *netB* gene from healthy chickens were higher (60%) than from healthy birds (50%) (Abildgaard et al. 2010). In fact, *netB*-positive *C. perfringens* isolated from healthy chickens with a high prevalence are often apathogenic (Johansson et al. 2009; Martin and Smyth 2009). In addition, although there were a high number of isolates from healthy poultry that carried the *netB* gene, not all of them could produce the toxin (Abildgaard et al. 2010). An *in vitro* study reported that the NetB toxin was only produced in 4/14 healthy chickens derived with *C. perfringens* isolates, positive to the *netB* gene, whereas most of the *netB*-positive isolates from NE chickens (12/13) expressed and secreted this toxin (Abildgaard et al. 2010). In this study, we also found that the capacity of the toxin production of the *netB*-positive isolates from the diseased chickens was higher than those from the healthy ones. The overnight cultured media of all eight diseased chicken-derived isolates positive to *netB* contained a substantial amount of the NetB toxin that was observed on the SDS-PAGE gel (Figure 4), but only two of eight healthy chicken-derived isolates expressed this toxin (Figure 5). Similar results were also found in our previous study as we found five *netB*-positive *C. perfringens* isolates from diseased ostriches producing the NetB toxin *in vitro* while none of the isolates from the healthy ones could produce this toxin (Nguyen et al. 2020). The use of a western-blot with a specific antibody (Keyburn et al. 2010) is necessary to confirm the presence of the NetB toxin and this needs to be undertaken in future studies. In order to confirm the pathogenesis of the *netB*-positive *C. perfringens* isolate, we infected 10-day-old chickens at a rate of 3×10^8 CFU per chicken. Unlike the results of Olkowski et al. (2006), we observed clinical signs of the disease (anorexia, ruffled feathers, depression, diarrhoea, and listlessness) one day post-infection. The disagreement between our results and Olkowski et al. (2006) may be due to the variation in the infection dose used or differences in the virulence of the isolates used in the two studies. It was interesting to find that the skin under the stomach of all the infected chickens turned black in colour which has not been described previously. Olkowski et al. (2006) found that pathological changes were clearly observed within 24 h post the *C. perfringens* challenges at a dose of 3×10^{10}. Consistently, we also observed pathological changes in the intestinal or-

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gans of 9/10 infected chickens. The results from this experiment indicated the roles of NetB in initiating NE diseases. However, the induction of NE diseases might be triggered by other factors such as nutritional factors, the coccidia infection status or immune suppression (Shojadoost et al. 2012)

The genetic analysis indicated that all of the sequences derived from the eight *netB*-positive isolates showed absolute identity (100%). There were no variations observed in the nucleotide sequence in both the coding sequence and the promoter region, suggesting that the *netB* sequence of the *C. perfringens* isolates from the poultry raised in Vietnam is thus conservative. The phylogenetic analysis of the *netB* gene of the present *C. perfringens* isolate (MT032252) with the ten selected and published sequences of *C. perfringens* isolates from difference countries showed an extremely high sequence identity, and the functionally critical motifs, structures and amino acids for the toxic activity in the NetB toxin were absolutely conserved. Similar results were reported by Profeta et al. (2020), these authors analysed 960 sequences of the *netB* genes of strains isolated from poultry in Italy and found a strong conservation in the *netB* gene sequences in their countries and in other countries. We further checked twenty other *netB* gene sequences available in the GenBank in order to figure out which mutated points occurred in the *netB* genes of the *C. perfringens* isolated from the Vietnamese chickens. We found there was a single nucleotide deletion at position –58 bp in our *netB* gene sequence compared to the other *netB* gene sequences available in the GenBank. Further studies should be performed to determine the effect this deletion may have on the expression of NetB in *C. perfringens*.

This study is the first report of the prevalence of *C. perfringens* in chickens raised in Vietnam. The results of this study confirmed that the *cpa* gene was present in both apparently healthy and diseased chickens. However, the *netB* gene might be the major virulent factor responsible for the development of NE in chickens as only the *C. perfringens* isolates expressing the NetB toxin were able to produce NE diseases in chickens with the experimental infection.

Conflict of interest

The authors declare no conflict of interest.

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