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# Experimental induction of necrotic enteritis in chickens by a *netB*-positive Japanese isolate of *Clostridium perfringens*

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**ABSTRACT.** Necrotic enteritis (NE) is one of the most important bacterial diseases in terms of economic losses. *Clostridium perfringens* necrotic enteritis toxin B, NetB, was recently proposed as a new key virulent factor for the development of NE. The goal of this work was to develop a necrotic enteritis model in chickens by using a Japanese isolate of *C. perfringens*. The Japanese isolate has been found to contain *netB* gene, which had the same nucleotide and deduced amino acid sequences as those of prototype gene characterized in Australian strain EHE-NE18, and also expressed *in vitro* a 33-kDa protein identified as NetB toxin by nano-scale liquid chromatographic tandem mass spectrometry. In the challenge experiment, broiler chickens fed a commercial chicken starter diet for 14 days post-hatch were changed to a high protein feed mixed 50:50 with fishmeal for 6 days. At day 21 of age, feed was withheld for 24 hr, and each chicken was orally challenged twice daily with 2 m*l* each of *C. perfringens* culture ($10^9$ to $10^{10}$ CFU) on 5 consecutive days. The gross necrotic lesions were observed in 90 and 12.5% of challenged and control chickens, respectively. To our knowledge, this is the first study that demonstrated that a *netB*-positive Japanese isolate of *C. perfringens* is able to induce the clinical signs and lesions characteristic of NE in the experimental model, which may be useful for evaluating the pathogenicity of field isolates, the efficacy of a vaccine or a specific drug against NE.

**KEY WORDS:** *C. perfringens*, fishmeal, histopathology, necrotic enteritis, netB toxin

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Necrotic enteritis is most common in 2-to-6-week-old broiler chickens, but has also been reported in 3–6-month-old commercial layers. The disease can be divided into 2 categories, clinical and subclinical. Clinical signs of clinical form include depression, ruffled feathers, diarrhea, huddling, anorexia, sternal recumbency and a sudden rise in flock mortality. Subclinical form is usually associated with reduced feed intake and weight gain and increased feed conversion ratio [3, 21, 27].

NE is caused predominantly by *C. perfringens* type A, and to a lesser extent by type C [8, 10, 21, 27, 34]. Despite decades of research, the virulence factors which lead to the development of NE have yet to be well known [21]. Alpha toxin has long been believed to be the major virulence factor involved in NE [4, 18, 34], but Keyburn and colleagues [8] provide suggestive evidence that alpha-toxin may not be an essential virulence factor in necrotic enteritis in chickens. Recently, a secreted β-pore forming toxin, NetB, has been isolated from a virulent chicken isolate and shown to have an important role in pathogenesis of NE [8, 10].

Vaccines based on alpha or NetB toxins had variable protective success [9, 34]. Vaccines prepared from live attenuated alpha-toxin negative strains of *C. perfringens* offer varying levels of protection, which is suggesting that other antigens of *C. perfringens* may also play an important role in the protective immunity [31]. Recently, some of these protective antigens have been identified [31, 34]. Thus, the development of more effective vaccines against NE is highly desired.

To evaluate the efficacy of a new vaccine, an approach to produce NE lesions in the majority of chicken challenged with a virulent *C. perfringens* strain is very important. Approaches to reproduce the disease have included co-inoculation with *Eimeria* spp. and *C. perfringens*, intraduodenal inoculation with *C. perfringens* broth cultures and intraduodenal inoculation with toxin-containing culture supernatant fluids [3, 23]. Recently, fishmeal and *Eimeria* spp., alone or combined, are the most widely used predisposing factors in experimentally inducible NE models [16, 24, 25, 30]. However, *Eimeria* themselves, depending on the species, may produce severe lesions; and NE lesions produced by a combined infection with *Eimeria* spp. and *C. perfringens* are usually more severe than those did by *C. perfringens* [14, 23, 25, 27].

In Japan, NE due to *C. perfringens* was first found in layer chickens in 1977 [19]. Thereafter, the infection was found in broiler chickens [17]. Recently, NE due to the *netB*-positive *C. perfringens* was noticed by several groups [7]. Data from our laboratory

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**Table 1.** Primers used in this study

| Primer name | Sequence (5′ to 3′) | Target gene | Amplicon size (bp) | Reference or source |
|---|---|---|---|---|
| PLC-F | GCG AGC TCA TGA AAA GAA AGA TTT GTA | *α/cpa* | 1,208 | This study |
| PLC-R | GGG AAG CTT TAT ATT ATA AGT TGA ATT TCC | | | |
| F | GCTAATGTTACTGCCGTTGACC | *α/cpa* | 324 | [37] |
| R | TCTGATACATCGTGTAAG | | | |
| CPB-F | GCG AAT ATG CTG AAT CAT CTA | *β/cpb* | 196 | [37] |
| CPB-R | GCA GGA ACA TTA GTA TAT CTT C | | | |
| ETX-F | GCG GTG ATA TCC ATC TAT TC | *ε* | 655 | [37] |
| ETX-R | CCA CTT ACT TGT CCT ACT AAC | | | |
| IA-F | ACT ACT CTC AGA CAA GAC AG | *iA* | 446 | [15] |
| IA-R | CTT TCC TTC TAT TAC TAT ACG | | | |
| CPE-F | GGA GAT GGT TGG ATA TTA GG | *cpe* | 233 | [15] |
| CPE-R | GGA CCA GCA GTT GTA GAT A | | | |
| 190-F | GCT GGT GCT GGA ATA AAT GCT | *netB* | 384 | [8] |
| 191-R | TCG CCA TTG AGT AGT TTC CC | | | |
| Beta2-F | AGATTTTAAATATGATCCTAACC | *β2* | 567 | [5] |
| Beta2-R | CAATACCCTTCACCAAATACTC | | | |
| 1F | GGGTACCAATTGTAAACATTCCTGATA | Upstream | 1,829 | This study |
| 2R | CGGATTACACTTGTAAGAACTAGTGTA | fragment of *netB* | | |
| 3F | CCTGCAGTAAATGCTTCATATAATGTCC | Downstream | 2,012 | This study |
| 4R | CCTCGAGAAGCATGAACATTATTGCCAG | fragment of *netB* | | |
| 5F | GCGAGCTCATGAAAAGATTAAAAATTA | *netB* | 983 | This study |
| 6R | TAAAATAGAATATTATCTGAAGCTTGC | | | |
| 7F | GAATTAATG TAAAAAGTGCTGATGTAA | internal fragment of *netB* | 536 | This study |
| 8R | TTTGTTTGTTCCTCGCCATT | | | |
| 9F | GCTGGTGCTGGAATAAATGC | internal fragment of *netB* | 384 | This study |
| 10R | TCGCCATTGA GTAGTTTCCC | | | |

revealed that 12 (36.4%) of 33 strains and 2 (40%) of 5 strains isolated from 41 NE and 17 non-NE cases, respectively, contained *netB* gene [7]. The *netB*-positive *C. perfringens* strains were found from various parts of Japan [7]. However, there are no records of pathogenicity of *netB*-harboring Japanese isolates of *C. perfringens*.

The main purpose of this study was to reproduce experimentally NE in broiler chickens without use of coccidia as a predisposing factor, which is considered an important step ahead in development of vaccine against NE in Japan. The work reported here demonstrates some characteristics of the challenge strain of *C. perfringens*, including the carriage of *netB* gene and *in vitro* production of NetB toxin, along with pathological evaluation of NE experimentally produced in broiler chickens orally challenged with the Japanese isolate of *C. perfringens.*

## MATERIALS AND METHODS

### *Bacterial strain and growth conditions*

Strain P-962 isolated in 2008 from a NE outbreak in Japan was chosen as a challenge strain because it was the first identified *netB*-positive isolate in our collection of *C. perfringens*, and some bacteriological characteristics of the strain were determined. *C. perfringens* was grown in tryptone-proteose peptone glucose (TPG) [13], GAM broth (Nissui pharmaceutical Co., Ltd., Tokyo, Japan), Cooked Meat medium (BD, Becton, Dickinson Co., Detroit, MI, U.S.A.), egg-yolk CW agar (EYA) (Nissui Co., Ltd.) and BBL Columbia agar with 5% sheep blood (BD). Agar cultures were grown at 37°C under anaerobic condition.

### *Detection of toxin genes using PCR*

The presence of *cpa*, *cpb*, *cpb2*, *etx* and *netB* genes in *C. perfringens* isolate was examined by using PCR as described previously [5, 10, 15, 33, 37]. The primers used in this study are detailed in Table 1.

### *Presence of netB gene in plasmid*

The presence of *netB* gene in plasmid was investigated using PCR and sequencing. The PCR products containing the nucleotide sequences of interest (the upstream and downstream DNA fragments of *netB* and the full-length of *netB* gene) were cleaned and then cloned into pGEM-T Easy as described previously [32]. DNA sequences from recombinant plasmids were determined by a primer-walking procedure. A homology-based search of the GenBank/EMBL/DDBJ database was done using the BLAST programs (http://www.ddbj.nig.ac.jp).

*In vitro expression of NetB toxin of C. perfringens strain P-962*

Procedures used for production and purification of NetB were similar to those described previously [8]. Briefly, *C. perfringens* strain P-962 was grown in TPG broth, and culture supernatant was obtained by centrifugation at 18,000 ×g for 15 min at 4°C. The supernatant was concentrated through Centricon Plus-70 centrifugal filter (Merck Millipore, Darmstadt, Germany) followed a second concentration using a Pellicon XL Biomax 10 filter (Merck Millipore). The resulting supernatant was precipitated with 40% (w/v) $(NH_4)_2SO_4$ at 4°C overnight and then centrifuged at 18,000 ×g at 4°C for 2 hr. The resulting pellet was resuspended in PBS and dialyzed against 10 mM Tris-HCl buffer, pH 8.5 at 4°C for 48 hr. The proteins were separated by HiTrap Q FF (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) anion exchange chromatography and submitted to SDS-PAGE coupled with Coomassie brilliant blue (CBB) staining as described previously [32]. A band of approximately 33-kDa on SDS-PAGE was cut out and identified using nano-scale liquid chromatographic tandem mass spectrometry (nano LC-MS/MS, Japan Bio Services Co., Ltd., Saitama, Japan).

*Challenge inoculum and animal trial*

For inoculum preparation, *C. perfringens* strain P-962 was streaked on a plate of blood agar (BBL). After incubation under anaerobic condition (AnaeroPack, Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan) at 37°C, colonies were transferred into 10 m*l* each of cooked meat medium and incubated under aerobic conditions at 37°C for 16 hr. Two hundred $\mu l$ of the resulting cultures was used to inoculate 12 m*l* of GAM broth and incubated at 37°C for 8 or 13 hr.

Eighteen commercial broiler chickens at 14 days of age obtained from a local commercial vendor were divided into the challenge (10 chickens, Nos. 1–10) and control (8 chickens, Nos. 11–18) groups. Chickens of each group were reared in a separate pen and fed an antibiotic-free chicken starter diet mixed 50:50 with fishmeal from 15 to 25 days of age. At day 21 of age, feed was withdrawn, and chickens of the challenge group were administered via oral gavage twice daily (morning and afternoon) with 2 m*l* each of the resulting cultures ($10^9$ to $10^{10}$ colony-forming units, CFU), whereas chickens of the control group were orally administered with 2 m*l* of GAM broth for 5 consecutive days. Numbers of CFU in the inoculum were determined by plating serial 10-fold dilution on EYA. Animal experiment protocol was approved by the Institutional Animal Care and Use Committee of the Nippon Institute for Biological Science.

*Bacterial isolation from pooled chicken feces and characterization*

The pooled fecal samples collected at 15 and 26 days of age were examined for the presence of *C. perfringens* bacteria and their toxin genes by the culture and PCR, respectively, as mentioned above. The fresh fecal samples were carefully collected from each pen (4 corners and the central area of each pen), pooled and suspended in 0.85% NaCl to make a 10% (wt/vol) suspension of solid or semisolid. The pooled fecal suspensions were diluted serially 10-fold and plated onto EYA incubated under anaerobic condition for 24 hr. *C. perfringens* was initially identified based on colony morphology and the presence of an opaque zone around the colonies due to lecithinase activity of alpha toxin. The selected colonies were confirmed and further characterized by PCR for toxin genes.

*Gross pathology*

At day 26 of age, the chickens were euthanized with inhaled carbon dioxide gas, and their digestive tracts were examined for gross necrotic lesions. The gross necrotic lesions in the small intestine (duodenum to ileum) were scored as described previously [10, 35] as follows: 0=no gross lesion; 1=thin or friable walls with roughened mucosa; 2 =focal necrosis or ulceration (1–5 foci); 3=focal necrosis or ulceration (6–15 foci); 4=focal necrosis or ulceration (16 or more foci); 5=patches of necrosis 2–3 cm long; and 6=diffuse necrosis typical of field cases. Chickens with lesion scores of 2 or more were considered as NE positive.

*Histopathology*

Intestinal tissues from infected and control chickens were processed for histopathological examination. Tissue segments of 2 to 3 cm long were collected from 7 areas of small intestine, including two areas from the duodenum and five areas from the jejunoileum (Table 2). The tissues were collected and fixed in 4% phosphate-buffered paraformaldehyde solution, and 4-$\mu$m-thick sections were prepared from paraffin-embedded tissue blocks using standard methods and stained with hematoxylin and eosin (HE). Lesions in the duodenum and jejunoileal segments were examined as described previously [10, 20, 28] and scored from zero to four for each of the following three criteria: (i) villous atrophy (score 1, slight; 2, slight to moderate; 3, moderate to severe ; and 4, severe); (ii) mucosal necrosis (score 1, necrosis or sloughing of the mucosal epithelium; 2, scattering of necrotic foci; 3, multiple necrotic foci; and 4, coalesced or layered necrosis); and (iii) bacterial invasion (including clumps of proliferated rod-shaped bacteria attached to the surface of villi and/or lamina propria; score 1, a small number of bacteria; 2, sporadic clumps of bacteria; 3, multiple clumps of bacteria; and 4, large clumps of bacteria).

The presence of bacteria in and around necrotic lesions was examined by Gram staining. Replicate sections of the intestinal tissue specimens containing multiple Gram-positive long rod-shape bacteria (score ≥3) were subjected to *in situ* hybridization (ISH).

*Preparation of probe targeting netB gene*

The primers 7F, 8R and 9F, 10R (Table 1) that flank 536- and 384-bp regions, respectively, of *netB* gene were used for preparation of probe. Briefly, genomic DNA was extracted from pure culture of *C. perfringens* using the High pure PCR template

**Table 2.** Histological necrotic enteritis lesion scores in broiler chickens inoculated with *Clostridium perfringens* and uninoculaed controls

| Group | Number of Chicken | Lesions[a)] | Duodenum | | Jejunoileum | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Upper part | Lower part | Upper part | | Middle part | | Lower part |
| | | | | | Anterior | Posterior | Anterior | Posterior | |
| Challenged | 10 | Villous atrophy | 1.50 | 1.30 | 2.40 | 2.00 | 1.60 | 1.60 | 1.00 |
| | | Mucosal necrosis | 2.00 | 2.00 | 2.30 | 2.80 | 2.30 | 1.80 | 1.30 |
| | | Bacteria invasion | 1.70 | 1.60 | 2.80 | 2.50 | 1.80 | 1.30 | 1.20 |
| Control | 8 | Villous atrophy | 0.25 | 0.13 | 0.25 | 0.25 | 0.13 | 0.13 | 0.13 |
| | | Mucosal necrosis | 0.25 | 0.25 | 0.38 | 0.25 | 0.25 | 0.13 | 0.13 |
| | | Bacteria invasion | 0.25 | 0.13 | 0.38 | 0.25 | 0.13 | 0.13 | 0.13 |

a) Two tissue sections each from two areas of duodenum and five areas of jejunoileum were scored, and a higher score, if any, was assigned as the lesion score of that tissue area. The numbers represent the average lesion score in each group (10 and 8 birds in challenged and control groups, respectively).

preparation kit (Roche Diagnostics GmbH, Mannheim, Germany). The first PCR was performed using the PCR kit (Takara Bio Inc., Otsu, Japan) with the primers 7F and 8R and the purified DNA template prepared from *C. perfringens* strain P-962. The PCR products were purified using the QIAGEN Gel Purification kit (QIAGEN Sciences, Germantown, MD, U.S.A.) and sequence-confirmed. Finally, a second PCR that incorporates digoxigenin-11-dUTP (Roche Diagnostics GmbH) was done using the purified PCR products as template and primers 9F and 10R.

### *In situ hybridization*

The presence of *C. perfringens* harboring *netB* gene in tissue sections was detected by ISH. The ISH was performed as described previously [6] with some modifications. Briefly, deparaffinized sections were treated with 0.2 N HCl at room temperature for 20 min and then digested with proteinase K (Sigma-Aldrich Japan Inc., Tokyo, Japan) at 37°C for 15 min. The sections were then incubated overnight at 42°C in standard hybridization buffer together with the *netB*-specific probe. After the washing and blocking steps, the sections were incubated with anti-DIG antibody conjugated with alkaline phosphatase diluted 1/200 in blocking reagent (Boehringer Mannheim, Co., Ltd., Tokyo, Japan) at room temperate for 1 hr and then detected by BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/p-nitroblue tetrazolium chloride). Color was allowed to develop for 10 min in the dark. The sections were counterstained with Nuclear Fast Red (Roche Diagnostics GmbH) and systematically viewed under a light microscope. No background hybridization was seen when replicate tissue sections were incubated with an unrelated digoxigenin-labeled probe (PCV2-specific probe) [6] or when matched tissues from unaffected chickens were incubated with a *netB* gene-specific probe.

## RESULTS

Using primers specific for the toxin genes, isolate P-962 of *C. perfringens* was found to be type A and positive for *cpa* and *netB* genes. Sequence analysis showed that *netB* is 969 bp in size encoding a 322 amino acid protein, including a 30 amino acid secretion signal sequence. Then, the isolate was tested for *in vitro* expression of NetB in batch culture. Purified product from the culture supernatant of *C. perfringens* isolate was analyzed by SDS-PAGE, and a band of approximately 33 kDa was observed (Fig. 1). The nano LC-MS/MS analysis identified the first 26 amino acids of the protein as NLSGEIIKENGKEAIKYT SSDTASHK. Mascot search results showed that the protein has a molecular mass of 46,491 Da and is NetB toxin.

Additionally, sequence analysis and the homology-based search showed that the upstream and downstream DNA fragments of *netB* gene are approximately 1,829 and 2,012 bp in size and encode the internalin A (LPXTG motif) and ricin-type beta-trefoil domain proteins (Fig. 2), respectively, located on an approximately 85 kb plasmid [22].

The challenged chickens displayed clinical signs of the disease, which include diarrhea and ruffled feather in 8 and 9 of 10 (80 and 90%) chickens at days 2 and 3 post-challenge (23 and 24 days of age), respectively; depression and reluctance to move in 2 and 3 of 10 (20 and 30%) chickens at day 5 post-challenge. Whereas, the control chickens orally administered with GAM broth did not display any clinical signs of the disease. Nine of 10 chickens challenged with the *C. perfringens* (90%) developed gross NE lesions with scores ranged from 2 to 5, whereas one of 8 control chickens developed the gross lesions with a lesion score of 3.

The gross lesions included focal, multifocal to coalescing reddish to brownish-grey areas with or without pale yellowish pseudomembranes (Fig. 3a and 3b), and they were more common in jejunoileum than duodenum.

Characteristic microscopic lesions of NE in the small intestine were found in all samples from the challenged chickens and samples from one control chicken (Table 2). They consisted of focal, multifocal to coalescing necrosis of enterocytes or in severe cases, coagulative necrosis of the entire superficial mucosa separating underlying viable lamina propria with infiltration of mild to moderate inflammatory cells (Fig. 3c and 3d). The inflammatory cell infiltrates consisted of lymphocytes, plasma cells and heterophils in varied degrees admixed with clumps of long rod-shaped bacteria and sloughed degenerated cells (Fig. 3d, inset). Gram-staining revealed a few to numerous Gram-positive and long rod-shaped bacteria attached to the epithelium and/or lamina propria (Fig. 3e). These microscopic lesions were largely consistent with those seen in field cases of NE.

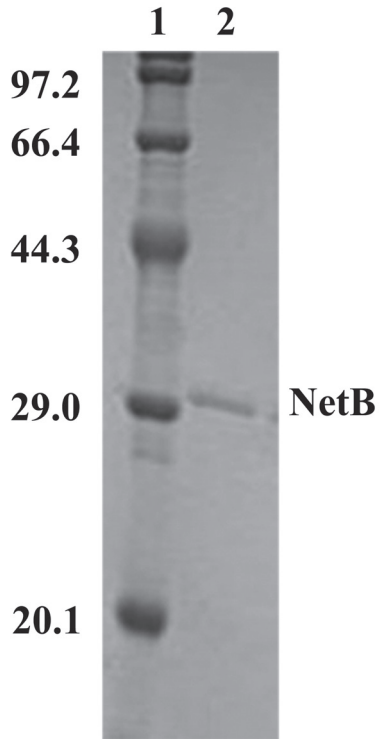


**Fig. 1.** SDS-PAGE of purified native NetB (approximately 33 kDa) stained with CBB: 1, molecular masses in kilodaltons; 2, native NetB.

*C. perfringens* bacteria were isolated from the pooled fecal samples collected at 26 days of age, but not from the pooled fecal samples collected at 15 days of age just before feeding them with a high protein feed. Five colonies derived from the fecal sample of the challenged chickens were positive for *cpa* and *netB*, whereas five colonies derived from the fecal sample of the control chickens were positive for *cpa* and negative for *netB* as determined by PCR. Sequences of *netB* gene from *C. perfringens* re-isolated were 100% identical to those of strain P-962 used for challenge, suggesting that the recovered strain was the same as the inoculating strain. ISH assay revealed that seven examined specimens of jejunoileum from the challenged chickens having bacterial scores 3–4 were *netB* positive (Fig. 3f), whereas jejunoileal specimens from one control chicken having score of 3 was *netB* negative. Replicate of the examined specimens had no any signal when hybridized with PCV2-specific probe.

In our preliminary infection experiments, *C. perfringens* strain P-962 could not induce any clinical sign(s) and lesions characteristic of NE in specific-pathogen-free (SPF) chickens, and therefore, the SPF chickens were not used for challenge experiment.

## DISCUSSION

*C. perfringens* strains producing NetB toxin have been considered as the definitive cause of NE in chickens [10, 16, 26]. However, the simple infection is not sufficient to precipitate disease; predisposing factors are necessary to facilitate the proliferation of *C. perfringens* by either providing nutrients or creating a favorable niche [36] both in disease outbreaks in the field and in models for experimental induction of the disease. At present, animal protein (fishmeal) and/or coccidia (*Eimeria* spp.) have been considered to be the important predisposing factors in reproducing the disease experimentally. Recently published studies showed that lesions of NE were observed when either *Eimeria* spp., fishmeal or the combination of both was administered as predisposing factors [25, 30, 35]. NE lesions induced by *Eimeria* spp. combined with *C. perfringens* are usually more severe than those did by fishmeal combined with *C. perfringens* [25, 27]. It has been shown that the damage caused by the *Eimeria* infection results in release from the damaged intestine of the essential amino acids that *C. perfringens* requires which in a "bacterium only model" can be supplied by fish meal [16, 23, 25]. Rodgers *et al*. [25] and Shojadoost *et al*. [27] suggested that when the purpose of the study is to test a vaccine or a specific drug against NE, it is probably better to produce the NE without the help of *Eimeria* challenge. Fishmeal predisposes chickens to NE and especially results in more severe duodenal lesions [2, 25, 27]. The present results showed that the *netB*-harboring Japanese isolate of *C. perfringens* is able to induce NE in the chicken experimental model without use of coccidia as a predisposing factor.

Comparative sequence analysis of *netB* gene revealed that nucleotide and amino acid sequences of *netB* of Japanese isolate of *C. perfringens* were 100% identical to those of the prototype gene characterized in Australian *C. perfringens* strain EHE-NE18 [8]. In addition, sequence data of the upstream and downstream of *netB* gene suggest that the *netB* gene was located on a large plasmid of

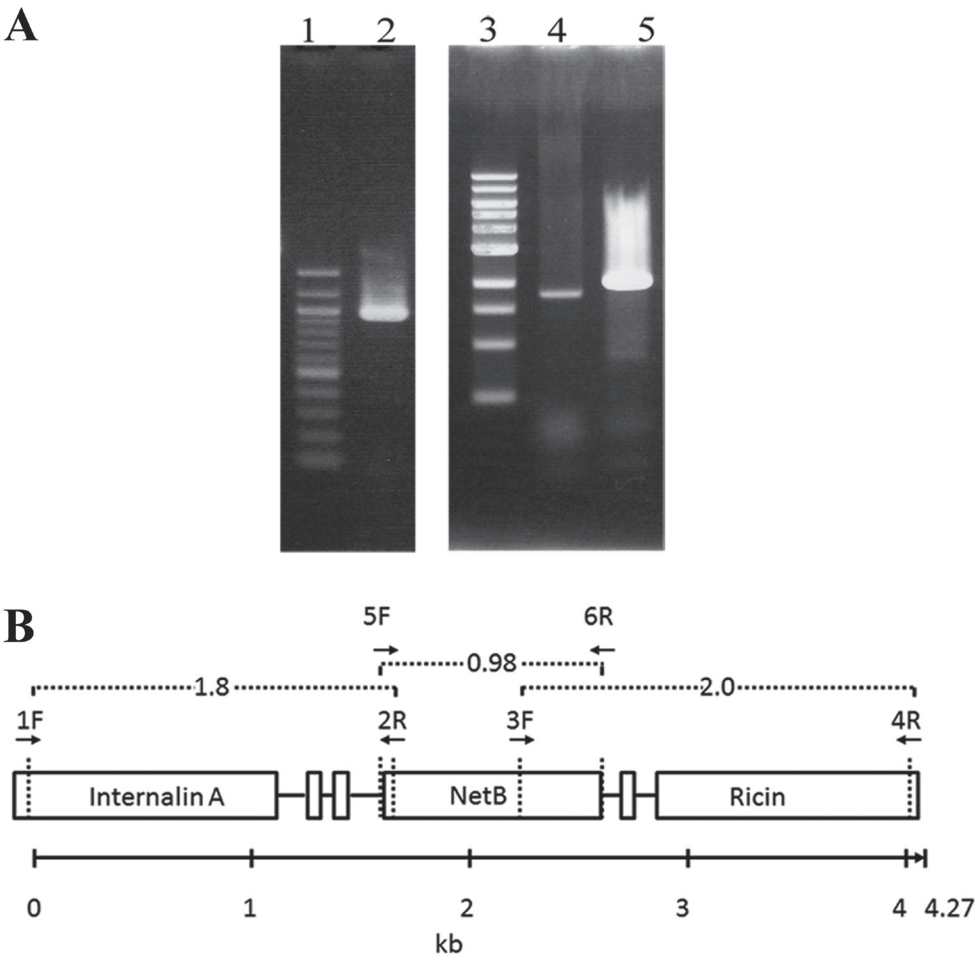


**Fig. 2.** PCR analysis of *netB* region of *Clostridium perfringens* isolate P-962. (A) result of a PCR assay with primers designed to amplify 963-, 1,829- and 2,012-bp PCR products corresponding to *netB* (2), the upstream (4) and downstream (5) fragments of *netB* gene. 100-bp ladder (1) and 1-kb (3) DNA markers are shown on the left of each gel. (B) Schematic diagram showing the location of each primer in P-962 DNA. The numbers with broken line indicate the length (kb) of PCR product amplified by the corresponding primers. The numbers with an arrow indicate kb.

85 kb [11, 12, 22]. Recently published data show that the plasmid contained additional virulence-associated genes, supporting the role of the plasmid in NE. The *C. perfringens* strain tested produced NetB toxin *in vitro*. The *in vitro* toxin production is regularly used as a measure for evaluating virulence of pathogens, including *C. perfringens* strains [1]. A recent report revealed that nearly all (12 out of 13)*netB*-positive isolates from NE chickens produced the NetB toxin *in vitro,* whereas only 4 out of 14 *netB*-positive isolates from healthy chickens produced NetB toxin *in vitro* [1]. It appears that expression of NetB toxin involves to virulence of strains.

The gross lesions in the small intestine (duodenum to ileum) were observed in nine of 10 challenged chickens and one of 8 control chickens. The *netB*-positive *C. perfringens* bacteria were only found in the samples tested from the challenged chickens, but not from the control chicken. Disease rate (approximately 90%) and gross lesion scores (ranging from 2 to 5) in our study were similar to those reported by Keyburn *et al.* [8–10]. And, characteristic microscopic lesions of NE as well as the presence of *C. perfringens* were found in 10 of 10 challenged chickens and 1 of 8 control chickens. The samples with characteristic lesions had areas of coagulative necrosis extending from the superficial villi into the submucosa and muscular layers of intestine. The presence of *C. perfringens* was frequently detected on the luminal surface of necrotic villi and apices of exposed lamina propria. The bacteria in the section of jejunoileum were confirmed by Gram staining, and *netB* gene was determined by either ISH or PCR. In field cases, lesions are most common in jejunum, followed by ileum, duodenum and cecum [3]. In our experimentally infected chickens, jejunoileal lesions were most common, and these findings supported partially results reported by Copper and Songer [3], who showed that jejunal lesions were most common in their challenged chickens. In contrast, the experimentally infected SPF chickens lacked gross or histological lesions in their intestinal tissues (results obtained from our preliminary infection experiments). This may be due to differences in the intestinal microbiota composition between the commercial and SPF chickens, which may help explain why ones have not used SPF chickens in an experimental induction model of NE.

Bacteriologic examination was carried out primarily to recover *C. perfringens* for determining whether the re-isolated bacteria

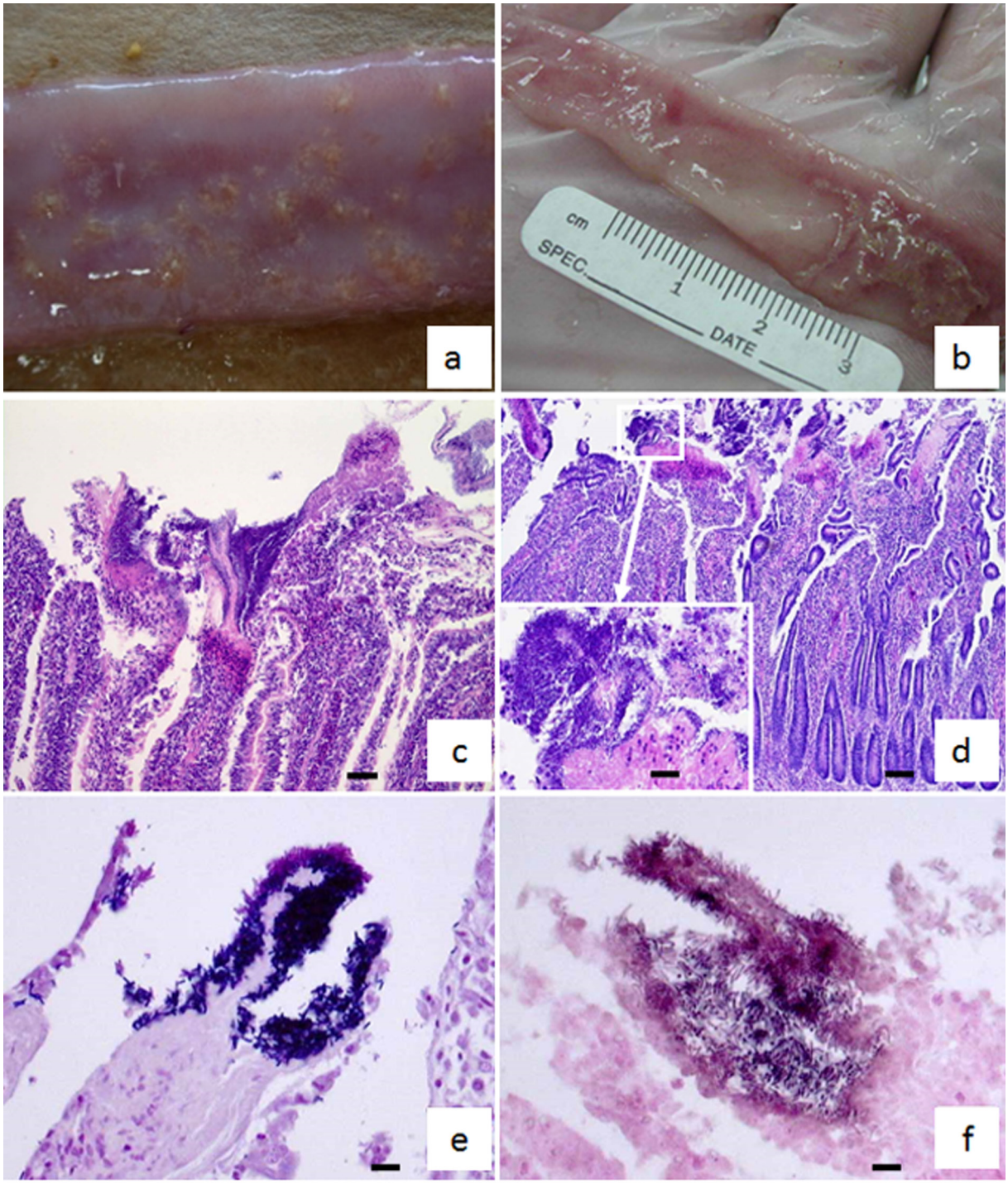


**Fig. 3.** Gross and histologic lesions of NE in broiler chickens orally inoculated with *Clostridium perfringens* (Cp). (a) Gross pathology of the lower part of the small intestine of chicken No. 8, showing multiple foci of mucosal necrosis (score 4). (b) Gross pathology of the lower part of the small intestine of chicken No. 5 with advanced lesions, showing a large area of mucosal erosion (center) and pseudomembrane covering necrotic villi of the surrounding mucosa (score 5). (c) Histopathology of the upper jejunoileal mucosa of chicken No. 8 (score 3), showing necrosis of some villous tips and infiltration of inflammatory cells in the underlying lamina propria (HE), scale bar=40 $\mu$m. (d) Histopathology of the upper jejunoileal mucosa of chicken No. 5 (score 4), showing necrosis of many villous tips and marked infiltration of inflammatory cells in the underlying lamina propria due to extensive bacterial growth on their villous tips (inset) (HE), scale bars=20 $\mu$m. (e) A large number of Gram-positive bacteria attaching to the surface of necrotic region of a villus of chicken No. 5 (Gram staining), scale bar=10 $\mu$m. (f) A large number of *netB*-positive bacteria in the tip of necrotic villus of chicken No. 5 (ISH), scale bar=10 $\mu$m.

were the same as the inoculating *C. perfringens*. Low level carriage in chickens examined at 15 days of age (<100 CFU/g of feces) together with results of toxin gene typing and sequence analysis of *netB* of *C. perfringens* recovered at 26 days of age suggested that the NE lesions in the challenged chickens were produced by *netB*-positive *C. perfringens* strain P-962.

The fact that *netB*-positive *C. perfringens* isolates recovered from diseased or normal chickens produced NE in challenged chickens has provided suggestive evidence that NetB is critical to the development of NE in chicken [28, 29]. The role of NetB in pathogenesis of NE in chickens was not defined in the present study. Nevertheless, data from previous reports by Keyburn *et al*. [8, 10] showed that *netB* knockout mutants failed to produce NE, whereas the *netB* mutants complemented with the wild-type *netB* gene caused significant level of NE in chickens. A few recent studies have also found that there are some isolates from diseased chickens that do not carry *netB*, suggesting that NetB may not be a critical virulence factor or that other factors can cause virulence in its absence [26]. The validity of this conclusion cannot be ruled out in all cases, however, *netB*-negative *C. perfringens* isolates reproduced disease at very low rates or no disease, whereas *netB*-positive isolates could readily induce disease in a standard disease induction model [26, 28, 29]. These findings are consistent across a range of strains using different disease induction models. It is likely that the *netB*-negative strains isolated from diseased birds are either pathogenic strains that have lost the *netB* plasmid during isolation and culturing or there may be other virulence factors yet to be determined that are produced by the *netB*-negative strains.

One of 8 control chickens treated with only GAM medium had lesion score of 3. ISH analysis showed that bacteria in lesions were negative to *netB*. The occurrence and severity of NE in this case are still not fully understood. One plausible explanation could be due to the immunosuppression, disorders in gut microbiota by supplemented fishmeal and/or yet unrecognized virulent factors. A low NE rate of control chickens in an experimental induction model is reported previously [25]. The *netB*-negative strains reportedly produced the disease, but rates of the infection were low in broiler chickens [3].

Recent epidemiological surveys of *C. perfringens*-associated NE revealed that *netB*-possitive *C. perfringens* isolates have been found in various parts of Japan [7], posing an emerging threat to poultry industry. Therefore, further studies on epidemiology of the disease and characterization of isolated strains, along with the development of an effective vaccine against NE should be carried out in the future.

In conclusion, we showed that a *netB*-positive *C. perfringens* isolate from chicken suffering from a NE outbreak in Japan was able to produce the clinical disease with lesions characteristic of NE in broiler chickens. The isolate was able to express NetB toxin *in vitro* that closely resembles the NetB found in Australian strain EHE-NE18. The present findings support the suggestion by many other investigators that NetB is a critical factor in NE development and that combination of fishmeal and *netB*-positive *C. perfringens* is enough to induce lesions similar to NE in the field. The current study may be useful for evaluating the pathogenicity of field isolates, the efficacy of a vaccine or a specific drug against NE.

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