Isolation of *Clostridium Perfringens* type C from a Neonatal Yangtze Finless Porpoise (*Neophocaena Asiaeorientalis Asiaeorientalis*)

Jia Li

Institute of Hydrobiology Chinese Academy of Sciences

Richard William McLaughlin

Gateway Technical College

Yingli Liu

Institute of Hydrobiology Chinese Academy of Sciences

Junying Zhou

Wuhan University Zhongnan Hospital

Xueying Hu

Huazhong Agricultural University

Xiaoling Wan

Institute of Hydrobiology Chinese Academy of Sciences

Haixia Xie

Institute of Hydrobiology Chinese Academy of Sciences

Yujiang Hao

Institute of Hydrobiology Chinese Academy of Sciences

Jinsong Zheng (✉ zhengjinsong@ihb.ac.cn)

Institute of Hydrobiology Chinese Academy of Sciences <https://orcid.org/0000-0002-6541-1594>

Research Article

Keywords: Yangtze finless porpoise, *Neophocaena asiaeorientalis asiaeorientalis*, *Clostridium perfringens* type C, biofilm formation, antibiotic resistance

Posted Date: January 5th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1177769/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

The aim of this study was to culture pathogenic bacteria from the blowhole, lung, stomach and fecal samples of a neonatal crucially endangered Yangtze finless porpoise (*Neophocaena asiaeorientalis asiaeorientalis*) that died 27 days after birth. Bacteria were inoculated and representative isolates were identified through 16S rRNA gene sequence analysis. A total of three *Clostridium perfringens* type C strains from the fecal samples were isolated. Toxin genes, including *cpa*, *cpb* and *cpb2*, were detected by PCR amplification, while the *etx*, *iap* and *cpe* genes were absent. Biofilm formation of the three strains was examined. Only one strain was able to form a biofilm. In addition, isolates showed strong resistance against the antibiotics amikacin (3/3), erythromycin (1/3), gentamicin (3/3), streptomycin (3/3), and trimethoprim (3/3), while sensitivity to ampicillin (3/3), bacitracin (3/3), erythromycin (2/3), penicillin G (3/3), and tetracycline (3/3). The results suggested *C. perfringens* type C could have contributed to the death of this neonatal porpoise.

Introduction

The Yangtze finless porpoise (*Neophocaena asiaeorientalis asiaeorientalis*) is a freshwater subspecies of the narrow-ridged finless porpoise. The porpoise is endemic to the middle and lower reaches of the Yangtze River, as well as Poyang and Dongting Lakes in China (Committee on Taxonomy 2012; Gao and Zhou 1995). The population size of this animal has declined rapidly over several decades. Some of the reasons include illegal fishing, over fishing, pollution, boat transportation, dams and various other human activities in the Yangtze which are detrimental to the porpoise (Wang 2009; Zhao et al. 2008; Mei et al. 2012). As a result, by the end of 2017, the population has declined to approximately 1012 individuals (Huang et al. 2020). The Yangtze finless porpoise was recently upgraded to the First Order of Key Protected Animals in China. It is also currently listed in the International Union for Conservation of Nature Red Data Book as a critically endangered species (Wang et al. 2013).

Clostridium perfringens is an anaerobic bacterium which is capable of endospore formation. In both humans and animals, the bacterium can cause numerous diseases, in part as a result of its ability to secrete several different toxins (Guran and Oksuztepe 2013). *C. perfringens* is divided into five different toxinotypes, which are type A, B, C, D, and E (Siqueira et al. 2012; Milton et al. 2017). This is based on the four major toxins, alpha, beta, epsilon, and iota which different strains of the bacterium can produce. All type C strains must produce both the alpha (CPA) and beta toxins (CPB) (Uzal et al. 2010). In addition, there are some type C isolates that also make enterotoxin (CPE) or beta2 toxin (CPB2) (Sayeed et al. 2008; Uzal et al. 2010). It has been suggested that the beta2 toxin could play a role in caprine enterotoxemia and could cause mortality in kids (Garcia et al. 2013; Dray 2004). Type C can frequently be the cause of enteritis and enterocolitis in neonatal animals, such as sheep, goats, and pigs (Songer 1996; Songer and Uzal 2005; Diab et al. 2012). There are also reports of disease caused by *C. perfringens* in cattle (Milton et al. 2017; Rahman et al. 2012). However, studies on aquatic mammal neonates are very rare.

A male Yangtze finless porpoise was born on May 19, 2018 in the Wuhan Baiji Dolphinarium, Hubei, China. This neonate died on June 14. During this period, the newborn baby was completely artificially feeding. After birth, the head of the calf collided once with the wall of the pool, causing a wound on the right side of the head. Symptoms of obvious decreased appetite appeared before death. In this study, we examined the blowhole, lung, stomach and fecal samples from this neonatal Yangtze finless porpoise to assess the presence of pathogenic bacteria that could have contributed to its death. As a result, a total of three *Clostridium perfringens* type C strains was identified in the fecal samples and then further characterized for biofilm formation and antibiotic susceptibility.

Materials And Methods

Sample collection and isolation of *Clostridium perfringens*

Five fecal samples were collected directly from the anus of the neonatal porpoise on days 2, 3, 7, 12 and 23 during artificial feeding. All fecal samples were frozen at -20°C until used. Another fecal (rectal) sample was removed by dissection on day 27. Postmortem was performed within three hours of its death. Amies Media swabs (Copan, Italy) were used to obtain lung and blowhole samples. A sterile soft plastic tube with a diameter of 5 mm was inserted into the forestomach to draw gastric fluid which was then placed in a sterile 1.5 ml tube. All sampling procedures used in this study were approved by the Regulations of the People's Republic of China for the Implementation of Wild Aquatic Animal Protection (promulgated in 1993), adhering to all ethical guidelines and legal requirements in China.

Lung and blowhole samples were inoculated directly onto Blood agar plates (Dijing LS0109, Guangzhou, China) and incubated aerobically at 37°C for 24-48 h. Bacteria from the fecal material and the gastric fluid were inoculated onto both blood and MacConkey agar plates (DijingLS1009, Guangzhou, China). The plates were incubated anaerobically at 37°C for 24-48 h. To obtain a pure culture, bacteria were sub-cultured twice onto Blood agar plates. No growth was observed on the MacConkey agar plates.

Bacteria identification based on 16S rRNA gene sequencing

In order to release the genomic DNA, representative single colonies were randomly selected and added to 10 µL of Lyse and Go PCR Reagent (Thermo Scientific, USA), following the manufacturer's recommendations. The 16S rRNA gene was then amplified by PCR using primers 27F and 1492R (Lane 1991), which amplified nearly the entire 16S rRNA gene. The PCR products were purified and then sequenced using the BigDye terminator cycle sequencing ready reaction kit (Applied Biosystem, USA) on an ABI 3730 automated DNA sequencer. Sequences were assembled using DNAMAN 8.0. For bacterial identification, sequences were compared to the 16S rRNA gene of existing organisms using the NCBI GenBank Nucleotide Database (Madden 2002).

Detection of toxin genes using PCR amplification

All primers (Table 1) and PCR cycling conditions were found in the study by van Asten et al. 2009. All reactions were done as singlets using PowerTaq PCR buffer (Tianyi Huiyuan, Beijing, China). A 10 µl aliquot was subject to electrophoresis through a 2.0% (wt/vol) agarose gel which contained ethidium bromide. PCR products were visualized under UV light.

Biofilm formation and quantification

C. Perfringens strains that had been identified by 16S rRNA gene sequencing were inoculated into Reinforced Medium for Clostridia (RCM; Hopebio, Qingdao, China) and incubated at 37°C for 24 h under static conditions. Then the bacteria were then subcultured at a 1:200 dilution into Dulbecco's modified Eagle Medium (DMEM; Invitrogen) in a 24-well tissue culture plate embedded with cover slips. The plate was then incubated at 35°C under 5% (vol/vol) CO₂ atmosphere conditions without agitation.

Synchronously, *Edwardsiella tarda* PPD130/91 (Ling et al. 2020) and its derived strains were also grown without agitation in tryptic soy broth (TSB; BD Biosciences) at 28°C. For the introduction of type III secretion system (T3SS) proteins, *E. tarda* strains were subcultured in DMEM at 25°C under 5% (vol/vol) CO₂ as a control.

At 24 hours post subculture, the culture supernatant was removed carefully, and the cover slips were gently rinsed three times with prewarmed PBS to remove unattached bacteria. The bacteria that attached to the cover slips were fixed in 4% paraformaldehyde and stained with 0.1% crystal violet for 30 min. Biofilms stained with crystal violet were photographed before being solubilized with a 1% SDS buffer (200 per well) to evaluate the OD₅₉₅ using a microplate reader (SpectraMax, M5).

The data was analyzed statistically with SPSS 18.0 software (SPSS Inc., Chicago, IL, USA) and $P < 0.001$ was considered statistically significant.

Antibiotic susceptibility testing

Single colonies of *C. Perfringens* from a pure culture plate were selected and then suspended in 0.9% NaCl to a 1 McFarland standard (Clinical and Laboratory Standards Institute 2015). To examine antibiotic resistance, bacteria growing logarithmically (1 McFarland standard) were streaked on Brucella blood agar plates (Pangtong Medical, Chongqing, China) using a sterile cotton swab and E-test strips (Liofilchem, Italia) were then added using sterile forceps. Plates were incubated at 37°C under anaerobic conditions for 48 h.

The minimum inhibitory concentration (MIC) value was read from the scale in terms of µg mL⁻¹ where the relevant inhibition ellipse edge intersected with the strip, using *Clostridium difficile* ATCC 700057 as the quality control strain (Clinical and Laboratory Standards Institute 2015). The antibiotics tested were ampicillin, amikacin, bacitracin, erythromycin, gentamicin, penicillin G, streptomycin, tetracycline, and trimethoprim.

Histopathological examinations

Pathological tissues from the lung, liver, kidney, stomach and intestine were sampled and fixed in 10% neutral-buffered formalin. Subsequently embedded in paraffin wax, cut into 5-7 µm sections and stained with hematoxylin and eosin (HE) for histopathological examination.

Results

Isolation and identification of *C. perfringens*

Table 2 lists all the bacteria which were isolated from the blowhole, lungs, stomach and fecal samples from the neonatal Yangtze finless porpoise.

Only two fecal samples, collected on days 2 and 27, contained *C. perfringens*. The colony from the fecal (rectal) sample collected by dissection on day 27 was numbered strain F9#1, and the two colonies obtained from the sample collected on day 2 were numbered strains F9#2 and F9#3.

Pairwise sequence comparisons to sequences deposited in the GenBank nucleotide database using the BLAST algorithm indicated that all the three sequences showed 99% identity to *C. perfringens* partial 16S rRNA gene, strain 18115 (KP944158) as well as 99% identity to the 16S rRNA gene, partial sequence, of several other *C. perfringens* strains. These similarity values are above the proposed cutoff value of 98.7% routinely used for the delineation of novel species of bacteria (Stackebrandt and Ebers 2006; Yarza et al. 2014). The 16S rRNA gene sequences from our study have been deposited in the GenBank nucleotide database under the accession number MH569435 to MH569437.

Toxin genotyping of *C. perfringens* isolates

All isolates were classified as *C. perfringens* type C based on the presence of the *cpa* and *cpb* genes. As well, all isolates contained the *cpb2* gene. No *etx*, *iap* and *cpe* genes were detected (Fig. 1).

Biofilm formation profile

Previous studies have demonstrated that EseB mediate wild-type *E. tarda* PPD130/91 is capable of biofilm formation when cultured in DMEM, whereas the mutation strain Δ eseB cannot (Tan et al. 2005; Gao et al. 2015). In this study, we examined *C. perfringens* strains F9#1, F9#2 and F9#3 for their ability to form a biofilm. As shown in Fig. 2, *C. perfringens* strains F9#1 and F9#2 did not adhere to the cover slips (OD₅₉₅ 0.07 and 0.11, respectively). This was similar to the negative control strain Δ eseB (OD₅₉₅ of 0.10). Whereas, strain F9#3 showed adherence to the coverslip (OD₅₉₅ 1.06), but at a decreased value compared to the positive control, strain *E. tarda* wild-type (OD₅₉₅ 2.48). This demonstrated that strain F9#3 can form biofilm, whereas strains F9#1 and F9#2 cannot.

Antimicrobial susceptibility patterns

The resistance profile of the three *C. perfringens* isolates to nine antibiotics used to treat infections caused by this bacterium is shown in Table 3. Using the E-test method, antibiotic resistance against

amikacin (3/3), erythromycin (1/3), gentamicin (3/3), streptomycin (3/3), and trimethoprim (3/3) was observed for the three *C. perfringens* isolates, while isolates were sensitive to ampicillin (3/3), bacitracin (3/3), erythromycin (2/3), penicillin G (3/3), and tetracycline (3/3). However, strain F9#1 was resistance to erythromycin, while the other two strains F9#2 and F9#3 were sensitive.

Histopathological examinations

Histopathological diagnosis showed that there was mild inflammation in the left lung of the neonatal porpoise (Fig S1A), the heart had mild focal degeneration and necrosis (Fig S1B), while other internal organs, such as the liver, kidney and intestine, showed no obvious lesions. Although the lung and heart had some mild lesions, this did not contribute directly to the death of the animal.

Discussion

As shown in Table 2 no bacteria were cultured from the gastric fluid collected on day 2, while *Enterococcus sp.* and *Clostridium bifermentans* were cultured from the gastric fluid collected on day 27. *Aeromonas hydrophila* and *Citrobacter freundii* were cultured from the lung swabs. Five different types of bacteria were cultured from the blowhole swabs. It is not known if these bacteria permanently resided in the blowhole or if the bacteria were only present in the water and contaminated the tissue. Although there were some mild lesions in the lungs, as shown by histology (Fig. S1A), this did not directly contribute to the death of this animal. Furthermore, aquatic mammals such as porpoises, in the process of choking can develop a lung infection. No obvious evidences of lung or other internal organ damage caused by other *Clostridium spp.* or pathogenic bacteria, such as *Staphylococcus spp.*, was found at necropsy.

C. perfringens is among the most common cause of foodborne illnesses in humans (Centers for Disease Control and Prevention 2015) and this bacterium can cause disease in several different animals (Diab et al. 2012; Minamoto et al. 2014). In many cases, diseases caused by this bacterium are life threatening or even fatal (Awad et al. 1995). Although adult animals can contract the bacterium and become ill, disease most often occurs in neonates (Timoney et al. 1988). Piglets are highly susceptible to type C infections (Fitzgerald et al. 1988; Johnson et al. 1992). Infections occur in many different neonatal terrestrial mammals, such as calves (Griner and Bracken 1953), lambs (Griner and Johnson 1954), goat kids (Garcia et al. 2012) and foals (Drolet et al. 1990). Sometimes necro-hemorrhagic enteritis results from an infection. This is caused by the beta-toxin being absorbed by the intestine and then going into general circulation (Songer 1996). Death of the animal can result. In marine mammals, such as captive killer whales (*Orcinus orca*), false killer whales (*Pseudorca crassidens*), dwarf sperm whales (*Kogia sima*), and bottlenose dolphins (*Tursiops truncatus*), *C. perfringens* is a part of the normal intestinal flora (Walsh et al. 1994). However, the bacterium can still have the ability to cause disease which ranges from mild to severe and in some cases even resulting in death (Sawires and Songer 2006). For example, it caused death of a captive Atlantic bottlenose dolphin (Buck et al. 1987).

In this study, the head of the calf once collided with the wall of the pool after birth, and a wound appeared on the right side of the head. It is assumed that *C. perfringens* entered the animal through the external

wound, and subsequently vegetative cell developed under anaerobic conditions and produced toxins. *C. perfringens* infections spread rapidly because of the destructive effects of the toxin. It was similar to the death of a captive bottlenose dolphin living in Mystic Marinelife Aquarium (Mystic, Connecticut) in Florida (Buck et al. 1987). Currently, nothing is known about the epidemiology of *C. perfringens* in the Yangtze finless porpoise. Here, we investigated the presence and toxin diversity of *C. perfringens* isolated from a neonatal Yangtze finless porpoise that died 27 days after birth.

A total of three *C. perfringens* strains were isolated in this study (Table 2). The toxin genes *cpa*, *cpb*, and *cpb2*, were detected in this study, whereas the *etx*, *iap* and *cpe* genes were absent (Fig. 1). The *cpa* gene can be present in all *C. perfringens* strains. However, if this gene is not present the bacterium is unable to cause disease (Niilo 1980). All type C strains contain both the *cpa* and *cpb* genes. The beta2 toxin, which is encoded by the plasmid-borne *cpb2* gene (Gibert et al. 1997), is the cause of most of the clinical symptoms and it is considered the main virulence factor (Sayeed et al. 2008). This toxin has also been reported to cause many enteric diseases in animals (Petit et al. 1999; Garmory et al. 2000). Das et al. 2009 found that *C. perfringens* containing the *cpa* gene was present in both healthy piglet controls and diarrheic animals, while isolates carrying the additional *cpb2* gene was found only in diarrheic pigs and piglets, but absent in healthy animals. This evidence strongly suggests that the *cpb2* gene is implicated in pig enteritis (Das et al. 2009). The toxin produced is a necrotizing and lethal toxin which has cytotoxic effects on intestinal cells (Guran et al. 2014).

A biofilm is a bacterial community which is formed on a surface. A polymeric matrix is developed which allows the bacteria to stick to each other (Hall-Stoodley et al. 2004). Biofilm formation has been shown to have an important role in gastrointestinal infections (Hu et al. 2018). Biofilm formations also helps bacteria to survive and it may play a role in virulence (Charlebois et al. 2014). In a recent study, all isolates from beef, chicken, duck and pork were able to form a robust biofilm. Biofilm formation was also observed in most poultry and swine isolates (83%), as well as in most clinical and commensal isolates (96.7%) (Hu et al. 2018). In this study, one of the three *C. perfringens* type C strains was able to form a biofilm (Fig. 2). It was reported that the ability of *C. perfringens* to form a biofilm depends on the environmental conditions (Pantaléon et al. 2014).

Studies have shown signs of acquired antibiotic resistance in *C. perfringens* indicating that antibiotic resistance is emerging (Lyras et al. 2009; Soge et al. 2009; Charlebois et al. 2002). In a recent study, multidrug resistant (MDR) *C. perfringens* strains were isolated from dogs in Korea (Chon et al. 2018). Other studies have reported MDR strains of *C. perfringens* in other animals (Dutta and Devriese 1981; Marks and Kather 2003; Rood et al. 1978). A recent study in South Korea, in which *C. perfringens* isolates collected from diseased animals in poultry farms showed susceptibility to ampicillin, penicillin, bacitracin, and erythromycin, but high resistance to apramycin, gentamicin, and streptomycin (Park et al. 2015). A study in Belgium found that *C. perfringens* resistance to penicillin was very rare (Osman and Elhariri 2013). These findings are similar to our results. Overuse or the misuse of antibiotics in both human and veterinary medicine, without proper antibiotic susceptibility testing, may be a major factor in increasing antimicrobial resistance (Wellington et al. 2013).

Conclusions

In this study, *C. perfringens* type C was isolated and characterized from the fecal materials of a neonatal Yangtze finless porpoise that died 27 days after birth. The toxin profile suggests that the bacterium could have contributed to the death of this porpoise, since some neonatal animals are affected by the toxins produced during a type C infection. In addition, antibiotic resistance poses therapeutic challenges. Future studies will potentially expand our insight into the role *C. perfringens* plays in the overall health and disease of both adult and neonatal Yangtze finless porpoises.

Declarations

Statements & Declarations

Funding This work was supported by grants from the National Natural Science Foundation of China (No. 31870372), and the Bureau of Science & Technology for Development, Chinese Academy of Sciences (No. ZSSD-004), to Jinsong Zheng. Xiaoling Wan has received research support from China Postdoctoral Science Foundation (No. 2020M682530).

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Author contributions Jinsong Zheng and Richard William McLaughlin contributed to the study conception and design. Material preparation, data collection and analysis were performed by Jia Li, Richard William McLaughlin, Yingli Liu, Junying Zhou, Xueying Hu, Haixia Xie, Yujiang Hao, Xiaoling Wan and Jinsong Zheng. The first draft of the manuscript was written by Jia Li, Richard William McLaughlin and Jinsong Zheng, all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data availability The 16S rRNA gene sequences have been deposited in the GenBank nucleotide database under the accession numbers MH569435 to MH569437.

Acknowledgments We thank the veterinarian and the trainers of the Wuhan Baiji Dolphinarium, especially Zhiyuan Wang, Chaoqun Wang, Zhengyu Deng and Zhangbing Kou, for their hard work of taking care the neonate Yangtze finless porpoise till its death, and for their help of collecting samples during this period. This work was supported by grants from the National Natural Science Foundation of China (No. 31870372), the Bureau of Science & Technology for Development, Chinese Academy of Sciences (No. ZSSD-004), and China Postdoctoral Science Foundation (No. 2020M682530).

References

Awad MM, Bryant AE, Stevens (1995) Virulence studies on chromosomal alpha-toxin and theta-toxin mutants constructed by allelic exchange provide genetic evidence for the essential role of alpha-toxin in *Clostridium perfringens*-mediated gas gangrene. Mol Microbiol 15:191-202.

Buck JD, Shepard LL, Spotte S (1987) *Clostridium perfringens* as the cause of death of a captive Atlantic bottlenosed dolphin (*Tursiops truncatus*). J Wildl Dis 23:488-91.

Centers for Disease Control and Prevention (2015) *Clostridium perfringens*. Available at: <http://www.cdc.gov/foodsafety/diseases/clostridium-perfringens.html>. Accessed 8 October 2015.

Charlebois A, Jacques M, Archambault M (2014) Biofilm formation of *Clostridium perfringens* and its exposure to low-dose antimicrobials. Front Microbiol 5:83.

Charlebois A, Jalbert LA, Harel J, Masson L, Archambault M (2002) Characterization of genes encoding for acquired bacitracin resistance in *Clostridium perfringens*. PLoS One 7:e44449.

Chon JW, Seo KH, Bae D, Park JH, Khan S, Sung K (2018) Prevalence, toxin gene profile, antibiotic resistance, and molecular characterization of *Clostridium perfringens* from diarrheic and non-diarrheic dogs in Korea. J Vet Sci 9:368-374.

Clinical and Laboratory Standards Institute (2015) Performance standards for antimicrobial susceptibility testing. In: 25th informational supplement. M100-S25. Clinical and Laboratory Standards Institute, Wayne, PA.

Committee on Taxonomy (2012) List of Marine Mammal Species and Subspecies. Society for Marine Mammalogy. www.marinemammalscience.org. Accessed 25.07.2012.

Das A, Mazumder Y, Dutta BK, Kumar A, Selvi S (2009) Diagnosis of acute diarrhea in pigs and piglets in Meghalaya, India. Malaysian J Microbiol 5:38-44.

Diab SS, Kinde H, Moore J, Shahriar MF, Odani J, Anthenill L, Songer G, Uzal FA (2012) Pathology of *Clostridium perfringens* type C enterotoxemia in horses. Vet Pathol 49:255-263.

Dray T (2004) *Clostridium perfringens* Type A and Beta 2 toxin associated with enterotoxemia in a 5-week-old goat. Can Vet J 45:251–253.

Drolet R, Higgins R, Cécyre A (1990) Necrohemorrhagic enterocolitis caused by *Clostridium perfringens* type C in a foal. Can Vet J 31:449-450.

Dutta GN, Devriese LA (1981) Macrolide-lincosamide-streptogramin resistance patterns in *Clostridium perfringens* from animals. Antimicrob Agents Chemother 19:274-278.

Fisher DJ, Fernandez-Miyakawa ME, Sayeed S, Poon R, Adams V, Rood JI, Uzal FA, McClane BA (2006) Dissecting the contributions of *Clostridium perfringens* type C toxins to lethality in the mouse intravenous injection model. Infect Immun 74:5200-5210.

Fitzgerald GR, Barker T, Welter MW, Welter CJ (1988) Diarrhea in young pigs: Comparing the incidence of the five most common infectious agents. Vet Med 83:80-86.

- Gao A, Zhou K (1995) Geographical variation of external measurements and three subspecies of *Neophocaena phocaenoides* in Chinese waters. *Acta Theriol Sinica* 15:81-92.
- Gao ZP, Nie P, Lu JF, Liu LY, Xiao TY, Liu W, Liu JS, Xie HX (2015) Type III secretion system translocon component EseB forms filaments on and mediates autoaggregation of and biofilm formation by *Edwardsiella tarda*. *Appl Environ Microbiol* 81:6078-6087.
- Garcia JP, Adams V, Beingesser J, Hughes ML, Poon R, Lyras D, Hill A, McClane BA, Rood JI, Uzal FA (2013) Epsilon toxin is essential for the virulence of *Clostridium perfringens* Type D infection in sheep, goats, and mice. *Infect Immun* 81:2405-2414.
- Garcia JP, Beingesser J, Fisher DJ, Sayeed S, McClane BA, Posthaus H, Uzal FA (2012) The effect of *Clostridium perfringens* type C strain CN3685 and its isogenic beta toxin null mutant in goats. *Vet Microbiol* 157:412-419.
- Garmory HS, Chanter N, French NP, Bueschel D, Songer JG, Titball RW (2000) Occurrence of *Clostridium perfringens* β 2-toxin amongst animals, determined using genotyping and subtyping PCR assays. *Epidemiol Infect* 124:61-67.
- Gibert M, Jolivet-Renaud C, Popoff MR (1997) Beta2 toxin, a novel toxin produced by *Clostridium perfringens*. *Gene* 203:65-73.
- Griner LA, Bracken KF (1953) *Clostridium perfringens* (type C) in acute hemorrhagic enteritis in calves. *J Am Vet Med Assoc* 122:99-102.
- Griner LA, Johnson HW (1954) *Clostridium perfringens* type C in hemorrhagic enterotoxemia of lambs. *J Am Vet Med Assoc* 125:125-127.
- Guran HS, Oksuztepe G (2013) Detection and typing of *Clostridium perfringens* from retail chicken meat parts. *Lett Appl Microbiol* 57:77-82.
- Guran HS, Vural A, Erkan ME (2014) The prevalence and molecular typing of *Clostridium perfringens* in ground beef and sheep meats. *J Verbr Lebensm* 9:121-128.
- Hall-Stoodley L, Costerton JW, Stoodley P (2004) Bacterial biofilms: from the natural environment to infectious diseases. *Nat Rev Microbiol* 2:95-108.
- Hu WS, Kim H, Koo OK (2018) Molecular genotyping, biofilm formation and antibiotic resistance of enterotoxigenic *Clostridium perfringens* isolated from meat supplied to school cafeterias in South Korea. *Anaerobe* 52:115-121.
- Huang J, Mei ZG, Chen M, Han Y, Zhang XQ, Moore J, Zhao XJ, Hao YJ, Wang KX, Wang D (2020) Population survey showing hope for population recovery of the critically endangered Yangtze finless porpoise. *Biol Conserv* 241:108315.

Johnson MW, Fitzgerald GR, Welter MW, Welter CJ (1992) The six most common pathogens responsible for diarrhea in newborn pigs. *Vet Med* 87:382-386.

Lane DJ (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds) *Nucleic acid techniques in bacterial systematics*. John Wiley & Sons, Inc., Chichester, pp 115–175.

Ling SHM, Wang XH, Xie L, Lim TM, Leung KY (2020) Use of green fluorescent protein (GFP) to study the invasion pathways of *Edwardsiella tarda* in *in vivo* and *in vitro* fish models. *Microbiology* 146:7-19.

Lyras D, Adams V, Ballard SA, Teng WL, Howarth PM, Crellin PK, Bannam TL, Songer JG, Rood JI (2009) ISCpe8, an IS1595-family lincomycin resistance element located on a conjugative plasmid in *Clostridium perfringens*. *J Bacteriol* 191:6345-6351.

Madden TL (2002) The BLAST sequence analysis tool. In: McEntyre J (ed) *The NCBI handbook*. National Library of Medicine (US), National Center for Biotechnology Information, Bethesda, MD.

Marks SL, Kather EJ (2003) Antimicrobial susceptibilities of canine *Clostridium difficile* and *Clostridium perfringens* isolates to commonly utilized antimicrobial drugs. *Vet Microbiol* 94:39-45.

Mei ZG, Huang SL, Hao YJ, Turvey ST, Gong WM, Wang D (2012) Accelerating population decline of Yangtze finless porpoise (*Neophocaena asiaeorientalis asiaeorientalis*). *Biol Conserv* 153:192-200.

Milton AAP, Agarwal RK, Bhuvana Priya G, Saminathan M, Aravind M, Reddy A, Athira CK, Ramees T, Sharma AK, Kumar A (2017) Prevalence and molecular typing of *Clostridium perfringens* in captive wildlife in India. *Anaerobe* 44:55-57.

Minamoto Y, Dhanani N, Markel ME, Steiner JM, Suchodolski JS (2014) Prevalence of *Clostridium perfringens*, *Clostridium perfringens* enterotoxin and dysbiosis in fecal samples of dogs with diarrhea. *Vet Microbiol* 174:463-473.

Niilo L (1980) *Clostridium perfringens* in animal disease: a review of current knowledge. *Can Vet J* 21:141-148.

Osman KM, Elhariri M (2013) Antibiotic resistance of *Clostridium perfringens* isolates from broiler chickens in Egypt. *Rev Sci Tech* 32:841-850.

Pantaléon V, Bouttier S, Soavelomandroso AP, Janoir C, Candela T (2014) Biofilms of *Clostridium* species. *Anaerobe* 30:193-198.

Park JY, Kim S, Oh JY, Kim HR, Jang I, Lee HS, Kwon YK (2015) Characterization of *Clostridium perfringens* isolates obtained from 2010 to 2012 from chickens with necrotic enteritis in Korea. *Poultry Sci* 94:1158-1164.

- Petit L, Gibert M, Popoff MR (1999) *Clostridium perfringens*: toxinotype and genotype. Trends Microbiol 7:104-110.
- Rahman MS, Sharma RK, Borah P, Chakraborty A, Mandakini RKD, Longjam N (2012) Characterization of *Clostridium perfringens* isolated from mammals and birds from Guwahati city, India. J Venom Anim Toxins 18:83-87.
- Rood JI, Maher EA, Somers EB, Campos E, Duncan CL (1978) Isolation and characterization of multiply antibiotic-resistant *Clostridium perfringens* strains from porcine feces. Antimicrob Agents Chemother 13:871-880.
- Sawires YS, Songer JG (2006) *Clostridium perfringens*: insight into virulence evolution and population structure. Anaerobe 12:23-43.
- Sayeed S, Uzal FA, Fisher DJ, Saputo J, Vidal JE, Chen Y, Gupta P, Rood JI, McClane BA (2008) Beta toxin is essential for the intestinal virulence of *Clostridium perfringens* type C disease isolate CN3685 in a rabbit ileal loop model. Mol Microbiol 67:15-30.
- Siqueira FF, Almeida MO, Barroca TM, Horta CC, Carmo AO, Silva RO, Pires PS, Lobato FC, Kalapothakis E (2012) Characterization of polymorphisms and isoforms of the *Clostridium perfringens* phospholipase C gene (*plc*) reveals high genetic diversity, Vet Microbiol 159:397-405.
- Soge OO, Tivoli LD, Meschke JS, Roberts MC (2009) A conjugative macrolide resistance gene, *mef(A)*, in environmental *Clostridium perfringens* carrying multiple macrolide and/or tetracycline resistance genes. J Appl Microbiol 106:34-40.
- Songer JG (1996) Clostridial enteric diseases of domestic animals. Clin Microbiol Rev 9:216-234.
- Songer JG, Uzal FA (2005) Clostridial enteric infections in pigs. J Vet Diagn Invest. 17:528-36.
- Stackebrandt, Ebers J (2006) Taxonomic parameters revisited: Tarnished gold standards. Microbiol Today 33:152–155.
- Tan YP, Zheng J, Tung SL, Rosenshine I, Leung KY (2005) Role of type III secretion in *Edwardsiella tarda* virulence. Microbiology 151:2301-2313.
- Timoney JF, Gillespie JH, Scott FW, Barlough JE (1988) Hagan and bruners microbiology and infectious diseases of domestic animals. Cornell University Press, Ithaca.
- Uzal FA, Vidal JE, McClane BA, Gurjar AA (2010) *Clostridium Perfringens* toxins involved in mammalian veterinary diseases. Open Toxinology J 2:24-42.
- van Asten AJ, van der Wiel CW, Nikolaou G, Houwers DJ, Gröne A (2009) A multiplex PCR for toxin typing of *Clostridium perfringens* isolates. Vet Microbiol 136:411-412.

Walsh MT, Thomas LA, Songer JG, Campbell TW, Tucker LS (1994) *Clostridium perfringens* isolates from cetaceans. Proc 25th annual workshop of the International Association for Aquatic Animal Medicine, Vallejo, CA p 95.

Wang D (2009) Population status, threats and conservation of the Yangtze finless porpoise. Chinese Sci Bull 54:3473-3484.

Wang D, Turve ST, Zhao XJ, Mei ZG (2013) *Neophocaena asiaeorientalis ssp. asiaeorientalis*. The IUCN Red List of Threatened Species e.T43205774A45893487.

Wellington EM, Boxall AB, Cross P, Feil EJ, Gaze WH, Hawkey PM, Johnson-Rollings AS, Jones DL, Lee NM, Otten W, Thomas CM, Williams AP (2013) The role of the natural environment in the emergence of antibiotic resistance in gram negative bacteria. Lancet Infect Dis 13:155-165.

Yarza P, Yilmaz P, Pruesse E, Glöckner FO, Ludwig W, Schleifer KH, Whitman WB, Euzéby J, Amann R, Rosselló-Móra R (2014) Uniting the classification of cultured and uncultured bacteria and archaea using 16S rRNA gene sequences. Nat Rev Microbiol 12:635–645.

Zhao XJ, Barlow J, Taylor BL, Pitman RL, Wang KX, Wei Z, Stewart BS, Turvey ST, Akamatsu T, Reeves RR, Wang D (2008) Abundance and conservation status of the Yangtze finless porpoise in the Yangtze River, China. Biol Conserv 141:3006-3018.

Tables

Table 1 PCR primers used to amplify toxin genes.

Toxin gene	Primers	Sequence (5'-3')	Product
<i>cpa</i> (α -toxin)	CPAlphaF	GCTAATGTTACTGCCGTTGA	324 bp
	CPAlphaR	CCTCTGATACATCGTGTAAG	
<i>cpb</i> (β -toxin)	CPBetaF3	GCGAATATGCTGAATCATCTA	195 bp
	CPBetaR3	GCAGGAACATTAGTATATCTTC	
<i>cpb2</i> (β 2-toxin)	CPBeta2totalF2	AAATATGATCCTAACCAAA(C)AA	548 bp
	CPBeta2totalR	CCAAATACTYC(T)TAATYGATGC	
<i>etx</i> (ϵ -toxin)	CPEpsilonF	TGGGAACTTCGATACAAGCA	376 bp
	CPEpsilonR2	AACTGCACTATAATTTCTTTTCC	
<i>iap</i> (ι -toxin)	CPlotaF2	AATGGTCCTTTAAATAATCC	272 bp
	CplotaR	TTAGCAAATGCACTCATATT	
<i>cpe</i> (enterotoxin)	CPEnteroF	TTCAGTTGGATTTACTTCTG	485 bp
	CPEnteroR	TGTCCAGTAGCTGTAATTGT	

Table 2 Bacteria recovered from different samples in the neonatal Yangtze finless porpoise.

Samples	Date Collected	Bacteria	Strain number
Feces 1	2018.5.20	<i>Clostridium perfringens</i>	Strain F9#2–Strain F9#3
Feces 2	2018.5.21	<i>Clostridium disporicum</i>	
Feces 3	2018.5.25	/	
Feces 4	2018.5.30	<i>Clostridium bifermentans</i>	
Feces 5	2018.6.10	<i>Clostridium bifermentans</i>	
Feces 6	2018.6.14	<i>Clostridium perfringens</i>	Strain F9#1
Gastric fluid 1	2018.5.20	/	
Gastric fluid 2	2018.6.14	<i>Enterococcus sp.</i>	
		<i>Clostridium bifermentans</i>	
Lung swabs	2018.6.14	<i>Aeromonas hydrophila</i>	
		<i>Citrobacter freundii</i>	
Blowhole swabs	2018.6.14	<i>Stenotrophomonas sp</i>	
		<i>Citrobacter sp.</i>	
		<i>Aeromonas hydrophila</i>	
		<i>Pseudomonas aeruginosa</i>	
		<i>Acinetobacter calcoaceticus</i>	

Table 3 MIC values for the three *C. perfringens* strains.

Antibiotic ($\mu\text{g mL}^{-1}$)	MIC ($\mu\text{g mL}^{-1}$)		
	Strain F9#1	Strain F9#2	Strain F9#3
Ampicillin (0.016-256)	0.094 (S)	0.016 (S)	<0.016 (S)
Amikacin (0.016-256)	>256 (R)	>256 (R)	>256 (R)
Bacitracin (0.016-256)	0.75 (S)	0.38 (S)	0.45 (S)
Erythromycin (0.016-256)	>256 (R)	1.5 (S)	2 (S)
Gentamicin (0.064-1024)	128 (R)	96 (R)	96 (R)
Penicillin G (0.016-256)	0.25 (S)	0.032 (S)	0.023 (S)
Streptomycin (0.064-1024)	256 (R)	384 (R)	384 (R)
Trimethoprim (0.002-32)	>32 (R)	>32 (R)	>32 (R)
Tetracycline (0.016-256)	1.5 (S)	1.5 (S)	1.5 (S)

MIC: Minimum Inhibitory Concentration; *S* susceptible; *R* resistant.

Figures

Fig. 1

Figure 1

2.0% agarose gel (wt/vol) electrophoresis of PCR products obtained with six *C. perfringens* toxin types.

M1: Marker 1 (600 bp DNA Ladder, Dongsheng Biotech); M2: BM2000 DNA Marker (2000 bp DNA Ladder, Biomed). Lanes 1-6: strain F9 #1; lanes 7-12: strain F9 #2; lanes 13-18: strain F9 #3. *cpa*: 1, 7, 13; *cpb*: 2,

Fig. 2

Figure 2

Biofilm formation ability of *C. perfringens* isolates.

***Significance ($P < 0.001$) was determined by SPSS.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryInformationrevision.docx](#)