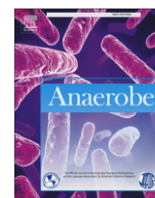


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Note

Isolation of *Robinsoniella peoriensis* from the fecal material of the endangered Yangtze finless porpoise, *Neophocaena asiaorientalis asiaorientalis*

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ABSTRACT

The aim of this study was to determine the causative agent of diarrhea in an endangered Yangtze finless porpoise (*Neophocaena asiaorientalis asiaorientalis*). From the fecal material collected from this porpoise *Robinsoniella peoriensis* was isolated.

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Beginning in Spring 2010, a captive killer whale (*Orcinus orca*) in an aquarium in Hong Kong and several beluga whales (*Delphinapterus leucas*) in aquariums across China, suffered from serious diarrhea and then died. In all of these animals, the diarrhea persisted for approximately 5 days and was difficult to cure. Veterinarians thought the diarrhea could be caused by infection with *Clostridium difficile*, however, this pathogen was not identified in any fecal samples examined (Zhao et al., personal communication).

The Yangtze finless porpoise (*Neophocaena asiaorientalis asiaorientalis*) is the only freshwater subspecies of the narrow-ridged finless porpoise (*Neophocaena asiaorientalis*), which is endemic to the middle and lower reaches of the Yangtze River and its adjoining Poyang and Dongting Lakes, China [1,2]. As a result of illegal fishing, pollution, transportation, dam construction and other detrimental human activities, the population size of the

porpoise has been declining rapidly in the last three decades [3–5]. For these reasons, the Yangtze finless porpoise is listed in the Second Order of Key Protected Animals in China, and in the International Union for Conservation of Nature Red Data Book as an endangered population [6].

On February 16, 2012, a Yangtze finless porpoise was found tangled in gill net at the Honghu section of the Yangtze River main channel. It was rescued and transported to the Wuhan Baiji Dolphinarium, where it received medical treatment and rehabilitation care. It was a male calf, 95 cm in length and approximately 4 months old. Many wounds were distributed over the entire body, especially in the mouth and head, and pectoral and caudal flippers. External wounds were disinfected and then treated with the Chinese traditional medicine Yunnan Baiyao. In addition, the antibiotic amikacin was given by injection. The calf was bottle fed with formula milk consisting of fresh carp mash, infant milk powder, acidophilus milk, carp oil, lecithin, taurine, multivitamin and warm water for the first 2 days. The animal also began eating freshly defrosted carp on the third day. During this 3-day period, amikacin was still given parentally and levofloxacin was given orally to treat

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the infected wounds. On February 19, the porpoise vomited once and diarrhea began the next afternoon. Thereafter, he reduced his intake of solid food. Amikacin injections continued to be given and levofloxacin, omeprazole, colloidal bismuth, amoxicillin, and clarithromycin were administered orally. The porpoise was also gavaged with normal saline once every day, however, the diarrhea continued. Beginning February 24, the porpoise ceased ingesting solid food and became dependent on a liquid supplement consisting of fresh carp mash, salmon oil, cream, animal fat, plant fat, glucose, lecithin, taurine, NaCl, dicalcium phosphate, marine mammal vitamins and warm water. The porpoise was also given enrofloxacin, Nystatin and Sporanox from February 24 to 27, however, the diarrhea persisted. The animal vomited once on February 27 and again on February 28 and, as a result, was given metoclopramide orally whilst continuing the enrofloxacin. On February 28, the rectal temperature began decreasing and dropped below 35 °C in the afternoon. The next morning the rectal temperature was only 32.5 °C and, several hours later, the porpoise died. The entire rescue and rehabilitation effort lasted for 2 weeks.

A second neonatal porpoise was found dead at the Jianli section of the Yangtze River main channel, on May 28 2012. She had a body length of 68 cm and she was about 1 month old. The body was moderately decomposed, however, no obvious external wounds were seen and the body weight was only 4.3 kg. The porpoise was necropsied. The stomach was empty and almost nothing was found in the intestines. Dark green thick fecal material was present in the rectum. It was concluded the porpoise died of starvation, probably after losing her mother. As a neonate, she was incapable of locating prey alone. A map depicting the location the porpoises lived is shown in Fig. 1.

The purpose of our study was to determine if *C. difficile* was associated with diarrhea in the male Yangtze finless porpoise which subsequently died. Diarrheic fecal material was collected from this animal and, as a control, solid fecal material from the second neonatal female porpoise which appeared to die of starvation was tested.

To detect the presence of *C. difficile*, DNA was extracted from the fecal samples of the two porpoises using the ZR Fecal DNA kit (Zymo Research Inc., CA, USA) according to the manufacturer's instructions. Three sets of PCR primers: tpiF/tpiR, NK2/NK3 and tcdBF/tcdR were then used. These primers allow for the PCR detection of an internal fragment of the triose phosphate isomerase housekeeping gene (*tpi*), the non-repeating portion of the *C. difficile*

toxin A gene (*tcdA*) and an internal fragment of the toxin B gene (*tcdB*), respectively. The PCR cycling conditions are found in Ref. [7].

In an attempt to culture *C. difficile*, equal amounts of diarrheic fecal material from the male porpoise and solid feces from the female porpoise were subjected to ethanol shock treatment [8]. After treatment, 100 µl from each fecal sample was plated on Tryptic Soy Agar with 5% Sheep Blood (Qingdao Hope Bio-Technology Co. Ltd., China) and incubated anaerobically at 37 °C for 48 h. No colonies were visible on the plate onto which solid feces was inoculated. On the plate onto which diarrheic feces was inoculated, three different colony morphologies were seen. To obtain a pure culture, one representative colony of each colony type was sub-cultured, and the three agar plates incubated at 37 °C anaerobically for 48 h. Pure cultures of the three isolates, designated YRC1, YRC2 and YRC3, were obtained. To identify the three different organisms, PCR amplification of the 16S rRNA gene was undertaken using the universal primers fD1 and rP2 [9]. A partial sequence of approximately 1400 bp was obtained. For identification, sequences were compared to the 16S rRNA gene of existing organisms using the NCBI database and the Ribosomal Database Project Classifier.

Using *C. difficile* specific PCR primers, *C. difficile* could not be detected in the diarrheic and non-diarrheic porpoise. This was confirmed based on the analyses of the 16S rRNA gene from the three organisms isolated from the diarrheic fecal material. None of the organisms was identified as *C. difficile*. Interestingly, one of the isolates, YRC3 (GenBank ID: JX424580), showed 99% similarity to *Robinsoniella peoriensis* strain PPC44 (GenBank ID: AF445283) and strain CCUG 52336 (GenBank ID: DQ681227). After sub-culturing this isolate onto sheep blood agar plates and incubating anaerobically for 48 h, smooth, grayish, non-hemolytic colonies were observed (Fig. 2A). A Gram stain showed a rod-shaped Gram-positive bacterium (Fig. 2B). The phenotypic characteristics of this isolate were in agreement with those previously reported for *R. peoriensis* [10].

To our knowledge, this is the first time *R. peoriensis* has been recovered from a Yangtze finless porpoise with diarrhea. In humans, there have been seven reported cases of *R. peoriensis* infection [10–14]. In all cases, a health care associated infection was suggested [14]. In animals, this organism has been found naturally in swine manure [15] and it can contaminate local water supplies [16]. In China, most manure is applied to farmland, but some does end up in waterways [17]. It is possible that the main channel of the Yangtze



Fig. 1. A map depicting the location the porpoises lived.

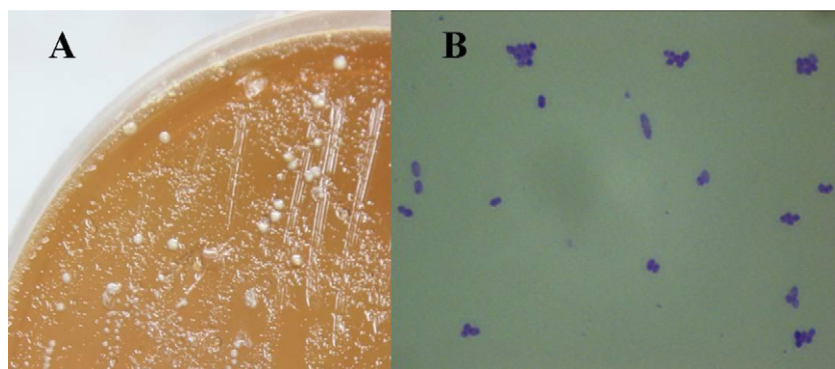


Fig. 2. Gray circular colonies of different sizes, having an entire margin and a smooth, shiny texture (A). The morphology of the organism as seen under the light microscope by Gram stain (B).

River became contaminated with *R. peoriensis* through swine manure and this particular porpoise became infected and developed an illness as a result of exposure. However, there are no studies published on the pathogenesis of infection with this bacterium so this conclusion remains quite speculative. There is little known about possible virulence factors in this organism and so much more work is required. As a first step, the genome could be sequenced and putative virulence factors identified. Currently, work is underway to determine if the organism is present in river water. Water samples are being collected from different sections of the Yangtze main channel, where they will be used to try to culture the bacterium.

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References

- [1] Committee on Taxonomy. List of marine mammal species and subspecies. Society for Marine Mammalogy, www.marinemammalscience.org; 2012 [accessed 25.07.2012].
- [2] Gao A, Zhou K. Geographical variation of external measurements and three subspecies of *Neophocaena phocaenoides* in Chinese waters. *Acta Theriol Sinica* 1995;15:81–92.
- [3] Wang D. Population status, threats and conservation of the Yangtze finless porpoise. *Chin Sci Bull* 2009;54:3473–84.
- [4] Zhao XJ, Barlow J, Taylor BL, Pitman RL, Wang KX, Wei Z, et al. Abundance and conservation status of the Yangtze finless porpoise in the Yangtze River, China. *Biol Conserv* 2008;141:306–18.
- [5] Mei ZG, Huang SL, Hao YJ, Turvey ST, Gong WM, Wang D. Accelerating population decline of Yangtze finless porpoise (*Neophocaena asiaorientalis asiaorientalis*). *Biol Conserv* 2012;153:192–200.
- [6] Hilton-Taylor C. 2000 IUCN red list of threatened species. Gland, Switzerland: IUCN–The World Conservation Union; 2000.
- [7] Alvarez-Perez S, Alba P, Blanco JL, Garcia ME. Detection of toxigenic *Clostridium difficile* in pig feces by PCR. *Vet Med* 2009;54:360–6.
- [8] Marler LM, Siders JA, Wolters LC, Pettigrew Y, Skitt BL, Allen SD. Comparison of five cultural procedures for isolation of *Clostridium difficile* from stools. *J Clin Microbiol* 1992;30:514–6.
- [9] Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* 1991;173:697–703.
- [10] Shen D, Chen R, Ye L, Luo Y, Tang YW. *Robinsoniella peoriensis* bacteremia in a patient with pancreatic cancer. *J Clin Microbiol* 2010;48:3448–50.
- [11] Lopez P, Belda S, Garcia M, Royo G. Infection of a spontaneous muscular hematoma due to *Robinsoniella peoriensis*, in a patient with alcoholic liver cirrhosis. *Enferm Infecc Microbiol Clin* 2010;28:565–7.
- [12] Gomez E, Gustafson DR, Colgrove R, Ly T, Santana R, Rosenblatt JE, et al. Isolation of *Robinsoniella peoriensis* from four human specimens. *J Clin Microbiol* 2011;49:458–60.
- [13] Ferraris L, Aires J, Butel MJ. Isolation of *Robinsoniella peoriensis* from the feces of premature neonates. *Anaerobe* 2012;18:172–3.
- [14] Cassir N, Laget L, Renvoisé A, Gennari JM, Drancourt M. *Robinsoniella peoriensis* infection following surgery for scoliosis: a case report. *J Med Case Rep* 2012;6:174.
- [15] Cotta MA, Whitehead TR, Falsen E, Moore E, Lawson PA. *Robinsoniella peoriensis* gen. nov., sp. nov., isolated from a swine-manure storage pit and a human clinical source. *Int J Syst Evol Microbiol* 2009;59:150–5.
- [16] Cotta MA, Whitehead TR, Zeltwanger RL. Isolation, characterization and comparison of bacteria from swine faeces and manure storage pits. *Environ Microbiol* 2003;5:737–45.
- [17] Ma X, Wang Z, Yin Z, Koenig A. Nitrogen flow analysis in Huizhou, South China. *Environ Manage* 2008;41:378–88.