

***Vibrio cholerae* non-O1/non-O139 infection in fish in the Czech Republic**

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ABSTRACT: Pathogenic *Vibrio cholerae* non-O1/non-O139 was isolated from the fry of the Cardinal tetra, *Paracheirodon axelrodi* and in adult Raphael catfish, *Platydoras costatus* kept in aquarium conditions. Further, an outbreak of vibriosis occurred in the wild populations of common nase, *Chondrostoma nasus*; chub, *Squalius cephalus*; gudgeon, *Gobio gobio*; stone loach, *Barbatula barbatula*; barbel, *Barbus barbus*; European grayling, *Thymallus thymallus*; schneider, *Alburnoides bipunctatus*; and brown trout, *Salmo trutta* morpho *trutta*. Mortality was not observed in the Eurasian minnow, *Phoxinus phoxinus*. During 14 days the acute rather than the sub-acute-to-chronic process of the disease prevailed with severe gross lesions in common nase and chub (diffused and focal haemorrhages, erythema and hyperaemia located especially in the abdominal region, within the mouth and at the base of fins). Only in the infection of the common nase was the whole eye affected, causing rupture of the globe and destruction of ocular structures. Gross pathological lesions in experimentally infected fish manifested themselves as gasping, erratic swimming, congested capillaries on the wall of the air bladder and fluid and blood accumulation in the abdominal cavity. Injection of 2×10^4 bacteria (common carp, rainbow trout) and injection of 2×10^8 bacteria (common nase) via *i.p.* route resulted in mortalities within 120 hrs and 16 h, respectively. Factors underlying the rise of infection are discussed, including an extraordinary increase in water temperature (20 °C to 23 °C), fluctuating oxygen content and low water level which contributed to increasing the concentration of the faecal contamination of water: these factors could stimulate the virulence of the vibrios, which survive in aquatic flora and fauna and in the biofilm on the surface of sediments.

Keywords: ornamental fish; freshwater fish; common nase; chub; pathogenicity; experimental infection

The genus *Vibrio* includes, among others, the most significant marine fish bacterial pathogens. Species of this genus have also been isolated from freshwater environments and have been found to be responsible for fish disease outbreaks. As with aeromonads in fresh water, isolation rates increase with elevated environmental temperature and where organic loads are high. Austin and Austin (2007) described the isolation of 16 *Vibrio* species from fish. Pathogenicity was represented by septicemic lesions in acute cases. In sub-acute or chronic cases, ulcers may also be found.

An outbreak of vibriosis with *V. cholerae* non-O1 in ayu sweetfish, *Plecoglossus altivelis* (Temminck and

Schlegel 1846) in Japan was described by Yamanoi et al. (1980), Kiiyukia et al. (1992), Nakajima et al. (1992) and Yashima and Odajima (1992), and *Vibrio* was isolated from skin lesions of sea mullet, *Mugil cephalus* L., with red spot disease in Australia (Callinan and Keep 1989) and from a number of fish species with ulcerative disease syndrome in America (McGarey et al. 1990). Mortalities of goldfish, *Carassius auratus* (Linnaeus, 1758) associated with *V. cholerae* non-O1 infection were reported by Reddacliff et al. (1993). Du Preez et al. (2010) conducted a study to determine whether the estuarine and freshwater environment in Beira, Mozambique, serves as a reservoir of *Vibrio cholerae* O1 and

This work was financially supported by the Ministry of Culture of the Czech Republic by institutional financing of long-term conceptual development of the research institution (the Silesian Museum, No. MK 000100595), and by an Internal Grant of the Silesian Museum (No. IGS 201402/2014).

doi: 10.17221/7921-VETMED

O139. Senderovich et al. (2010) found that fish of various species and habitats contain *V. cholerae* non-O1/ non-O139 in their digestive tract. They suggested that fish serve as intermediate vectors of *V. cholerae* since they create a link in the food chain between chironomidae and copepods on the one hand and waterbirds on the other (Halpern et al. 2008). As such, they are likely to pose a health risk to humans who consume them. Booth et al. (1990) described wound infection caused by *V. cholerae* non-O1 in a farmworker. The same organism was isolated from his tropical fish tank. Manfrin et al. (1999) and Manfrin et al. (2001) pointed out that importation of tropical fishes from non-EU countries, principally Asian, could be a potential risk with regard to the introduction of pathogens such as *V. cholerae* either to fish or humans.

The history, clinical signs, pathological lesions and bacteriological findings of an outbreak of vibriosis in aquarium fish and freshwater wild fish are described in this report.

MATERIAL AND METHODS

Fish. In the course of our laboratory's diagnostic activities, strains of *Vibrio* species were isolated from aquarium fish obtained from two aquarium fish breeders/exporters/importers.

Samples of moribund wild fish were submitted to the laboratory in water in 20-litre plastic bags, on two occasions-July 11 and July 13 – during an outbreak of vibriosis.

Bacteriology. Bacterial procedures consisted of tissue smear preparing and staining, inoculation of growth media and evaluation of microbial cultures. Blood agar plates (Columbia Agar Base MERCK) incubated at room temperature (approximately 24 °C) were used for primary isolation from selected tissues of affected aquarium fish and from the liver, kidney, spleen, eye and blood of affected wild fish.

Phenotypic identification of the bacterial strains. The commercial API 20 E kit and the apiweb software (BioMerieux, Marcy-l'Etoile, France), and ENTEROtest 24 and TNW Pro 6.0 Win software (Erba Lachema, Brno, Czech Republic) employed according to the manufacturer's instructions, were used for identification of the strains of the Gram-negative oxidase-positive rods. Complementary conventional tests described

previously by Farmer et al. (2003) were also used. Strains were inoculated on TCBS-selective medium for vibrios (thiosulphate-citrate-bile-sucrose agar). The cultures were serotyped using slide agglutination in *V. cholerae* antisera O1 and O139 (Denka Seiken, Tokyo, Japan). The production of *Vibrio cholerae* enterotoxin was tested by reverse passive latex agglutination using the VET-RPLA kit (Denka Seiken, Tokyo, Japan).

MALDI-TOF mass spectrometry identification. For the analysis, cultures were grown on Columbia blood agar (Oxoid) and transferred with a bamboo toothpick onto an MSP 96 polished steel target (Bruker Daltonics). The samples were covered with 1.0 µl matrix solution: a saturated solution of α-cyano-4-hydroxy-cinnamic acid (Sigma) in 50% acetonitrile and 2.5% trifluoroacetic acid. The samples were analysed using a MALDI-TOF mass spectrometer (Microflex LT, Bruker Daltonics, Germany). The recorded spectra were processed using the Flex Analysis (version 3.3) and BioTyper (version 3.0) software with the version 3.2.1.0 database and Security Relevant Library.

The *Vibrio* strains were deposited in the collection of the cultures of the NRL for *E. coli* and Shigella (NRL/ECS), NIPH, Prague. One strain, *V. cholerae* No. 215, was deposited in the Czech National Collection of Type Cultures NIPH, Prague (No.CNCTC 5593).

Sampling procedure for histopathology. Tissue samples of liver, hepatopancreas, spleen and kidney were fixed in Davidson's alcohol formaldehyde acetic acid solution, embedded in paraffin and sectioned at 5 µm. Histological sections were stained by haematoxylin and eosin. Special stains for specific substances included Gram, the PAS method (periodic acid Schiff's reagent), Gomori's method, and Van Gieson stains.

Blood chemistry. Blood samples were taken by puncturing the caudal vessels. Sodium heparin was used as anticoagulant. Plasma was obtained by centrifuging the blood at 4100 × g for 10 min followed by subsequent separation into plastic syringes. A Hitachi 717 (Tokyo, Japan) instrument was used for determination of all indices (total protein, blood urea nitrogen, uric acid, glucose, triacylglycerol, inorganic phosphate, total calcium, sodium, potassium, and the enzymes aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, lactate dehydrogenase and creatine kinase). Kits produced by PLIVA-Lachema, a.s. Brno, Czech

Republic, and DIALAB Wien, Austria and Prague, Czech Republic, were used for the determination.

Experimental infection. Experimental infection was carried out under laboratory conditions at a water temperature of 18 °C. An isolate from common nase (strain No. 1161) was used in this experiment. Infection assays were conducted using five fish held in a 200-litre aquarium. Three species of fish, rainbow trout, *Oncorhynchus mykiss* (Walbaum) [average standard length (L_s) 163 mm and weight (W) 65 g], common carp, *Cyprinus carpio* (average L_s 85 mm and W 22 g) and one common nase, *Chondrostoma nasus* (L_s 252 mm and W 260 g) were tested to assess the infectivity of vibriosis and to determine the susceptibility of fish to the bacteria. Rainbow trout and common carp had been kept for experimental purposes for four months, while the common nase had adapted for 10 days before the experiment under laboratory conditions. The organism was grown on nutrient agar at 24 °C and suspended in sterile 0.85% physiological saline water (PSW). The fish were anaesthetised with Menocaine (3-aminobenzoic acid ethyl ester sodium hydrogen sulphate) at a concentration of 0.06 g/l (Král 1988) and were injected intraperitoneally with 100 µl PSW containing 2×10^4 bacteria (common carp, rainbow trout) or 2×10^8 bacteria (common nase). Control fish were similarly injected with PSW. The continuously aerated and filtered water had the following physical and chemical characteristics: pH 7.1, water hardness 9°N, water temperature 20 °C, dissolved oxygen content 9.5 mg/l, oxygen saturation 102%, COD_{Mn} 1.9 mg O_2 /l, NH_4^+ 0.03 mg/l, NO_2^- 0.03 mg/l and NO_3^- 6.4 mg/l.

RESULTS

Fish

Pathogenic *Vibrio cholerae* non-O1/non-O139 was isolated from the fry of the Cardinal tetra, *Paracheirodon axelrodi* (L.P.Schultz, 1956) (strain No. 215) and from adult Raphael catfish, *Platydoras costatus* (Linnaeus, 1758) (strain No. 433) and common nase, *Chondrostoma nasus* (Linnaeus, 1758) (strain No. 1161) under aquarium conditions. In these species the disease had an acute course and primarily affected young fish.

An outbreak occurred in wild populations of common nase (strain No. 1161); chub, *Squalius*

cephalus (Linnaeus, 1758); gudgeon, *Gobio gobio* (Linnaeus, 1758); stone loach, *Barbatula barbatula* (Linnaeus, 1758); barbel, *Barbus barbus* Linnaeus, 1758; European grayling, *Thymallus thymallus* (Linnaeus, 1758); schneider, *Alburnoides bipunctatus* (Bloch, 1782); and brown trout, *Salmo trutta* morpha *trutta* Linnaeus, 1758. Mortality was not observed in the Eurasian minnow, *Phoxinus phoxinus* (Linnaeus, 1758). In a 5 km river section (watershed of the River Morava, Czech Republic) downstream of the supposed source of municipal pollution, water temperature was increased during a two-week period at the end of July/beginning of August (20 °C to 23 °C, unusual under the existing conditions) and the water flow was slowed down. In this period, mortality occurred in the following order: schneider, stone loach, gudgeon and the younger European grayling and brown trout; followed by adult brown trout, barbel, common nase and chub. An acute course of the disease with a minimum presence or complete absence of clinical signs prevailed in the first seven species/categories, whereas in the common nase and chub the disease had a sub-acute-to-chronic course. The gross lesions observed in the common nase and chub manifested themselves as diffused and focal haemorrhages, erythema and hyperaemia located especially in the abdominal region, within the mouth and at the base of fins (Figures 1 to 4). Gill congestion was frequently observed in the common nase (Figure 5), and it was only in common nase infection that the whole eye was infected, causing rupture of the globe and destruction of ocular structures (Figure 6). Most of the common nase had a pale liver with hyperaemic areas (Figures 7 and 8). Histologically, both the common nase and chub exhibited aneurysm of the secondary lamellae, with a more frequent occurrence in the common nase (Figure 9), and with extensive lamellar hypertrophy and hyperplasia of the secondary lamellae in the chub.

Bacteriology

Three strains [No. 215 (NRL ECS 13/232), No. 433 (NRL ECS 4/Vib 03) and No. 1161 (NRL ECS 12/563)] were identified as *Vibrio cholerae* non-O1/non-O139. MALDI-TOF MS identification, using Security Relevant Library, confirmed these strains as *Vibrio cholerae*. MALDI-TOF MS analysis with the application of the standard Bruker taxonomy database gave the result of *Vibrio albensis*.

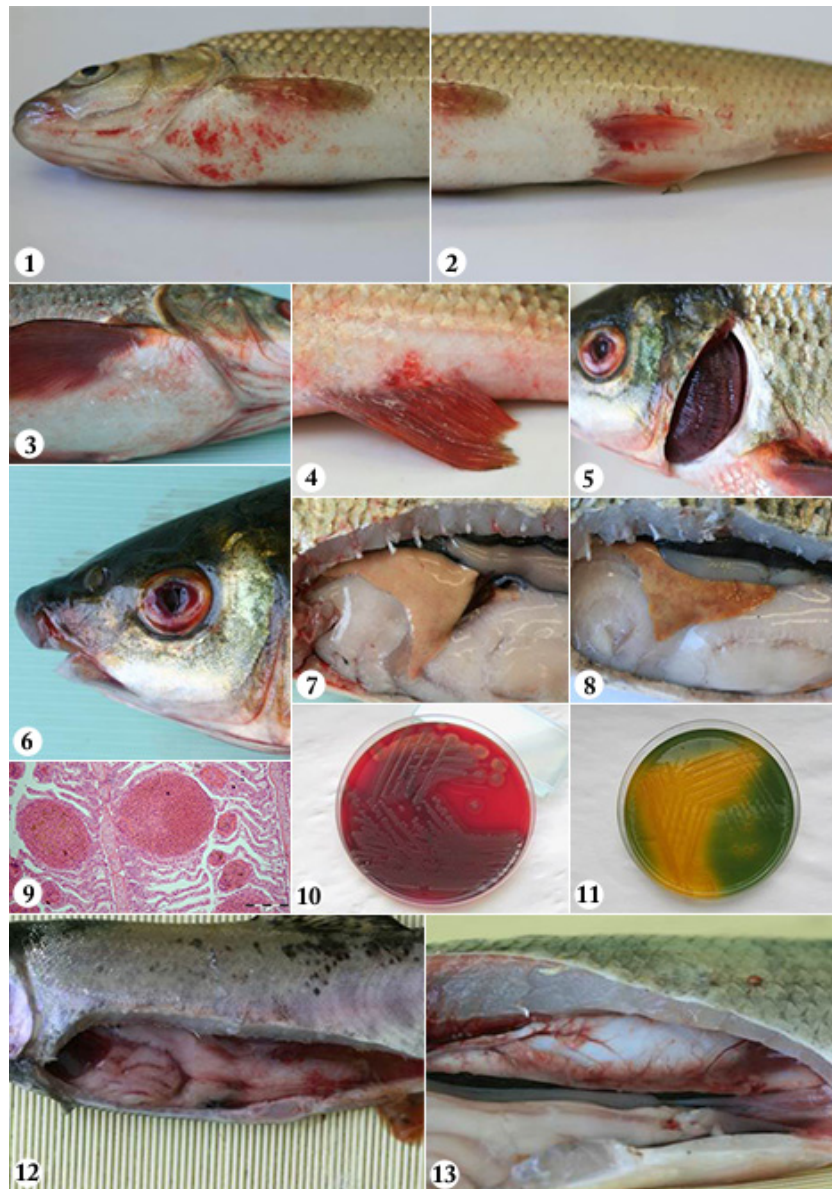


Plate 1. Figures 1–8. Gross pathological lesions in affected fish. Haemorrhages and hyperaemia in the abdominal region, at the base of fins and between fin rays in chub (1,2) and common nase (3,4); gill congestion in common nase (5); severe acute or sub-acute intraocular bacterial haemorrhage causing rupture of the globe and destruction of ocular structures (6); pale liver with mild (7) and severe (8) hyperaemic areas in common nase. Figure 9. Aneurysm of the secondary lamellae in common nase. Figures 10 and 11. 16-hour culture of *Vibrio cholerae* non-O1/non-O139 (37 °C aerobic) on blood agar (10) and on selective TCBS medium for vibrios (thiosulphate-citrate-bile-sucrose agar) (11). Figures 12 and 13. Experimental infection of fish. Venous congestion on the wall of the air bladder in rainbow trout (12) and common nase (13)

All phenotypic characteristics of the three *V. cholerae* strains were in accordance with literature data (Farmer et al. 2003): gram-negative, slightly curved rods, oxidase-positive, and robust growth on the TCBS. Acid was produced from sucrose and glucose (without gas), but not from lactose. Lysine decarboxylase, β -galactosidase, indole and catalase were produced, but not arginine dihydrolase, H_2S ,

ornithine decarboxylase or phenylalanine deaminase. Nitrates were reduced, Voges-Proskauer reaction and methyl red test were positive. All strains were sensitive to the vibriostatic compound O/129 and string test was positive.

Slide agglutination with antisera O1 and O139 was negative; none of the three strains produced cholera enterotoxin.

A 16-hour culture of *Vibrio cholerae* non-O1/non-O139 (37 °C aerobic) on blood agar is shown in Figure 10 and a culture on the TCBS is shown in Figure 11.

Experimental infection of fish

In common carp and rainbow trout, the first signs of clinical infection (gasping and erratic swimming) occurred already after 24 h and all the fish died within 120 h. Post-mortem examination showed the presence of fluid with blood accumulation in the abdominal cavity, and in rainbow trout revealed venous congestion on the wall of the air bladder (Figure 12). The infection had a very dramatic course with the same signs in the common nase: the fish died after 16 h following one hour of agony. The post-mortem finding in the air bladder was the same as in rainbow trout (Figure 13). The organism was re-isolated from the visceral organs of all fish. Control fish injected with 0.3 ml of sterile PSW did not become ill and bacteria were not isolated from these fish when examined after 144 h. Blood was sampled from the caudal vein of the common nase immediately after death and the biochemical examination of blood plasma revealed an increased catalytic concentration of aspartate aminotransferase (30.85 µkat/l).

DISCUSSION

Strains of *V. cholerae* non-O1/non-O139 can on rare occasion cause a severe cholera-like disease, but they are usually isolated from patients with mild diarrhoea and extra-intestinal infections, from seafood and from the environment. These strains usually do not produce cholera enterotoxin. The NRL/ECS has a laboratory collection of *V. cholerae* strains, including two strains of *V. cholerae* O1 (Petráš 2002), isolated from patients with diarrhoea, wounds, and ear infections.

The described case of *Vibrio* infection in aquarium fish is the second such report in the Czech Republic; the first was a finding of a medium-intensity occurrence of *Vibrio cholerae* organisms in samples of water from aquarium fish culture tanks and in samples of live aquarium fish (Plesnik and Prochazkova 2006). However, this was not a very comprehensive article, and did not include charac-

teristics of bacterial strains. Moreover, the authors did not describe the diseased fish, as they primarily focused on discussing the relevance of the finding for the possible occurrence and transmission of cholera between aquarists in countries outside the endemic areas of the disease. Decisions were subsequently made to take adequate veterinary measures to prevent the spreading of the causal agent of the infection. According to our estimate, the occurrence of cholera infections in aquarium fish culture is more widespread than stated in the relevant literature, owing to the increasing import and export of fish, often with insufficient veterinary inspection. Manfrin et al. (2001) drew attention to the risk of the spread of cholera infection: they warned that the import of tropical fish from countries outside the European Union or “third countries”, principally in Asia, constitutes a potential risk of introducing both human and fish pathogens. So far no case of cholera in aquarists has been reported in connection with their hobby but Manfrin et al. (2001) warned that although *Vibrio cholerae* non-O1 does not cause epidemic cholera, its enterotoxins could be responsible for gastroenteritis in humans. This is considered as a potential risk for aquarium operators and tropical fish hobbyists, as well as being potentially responsible for some disease outbreaks in fish.

From the zoogeographical point of view, an epizootic of *Vibrio* non-O1/non-O139 in freshwater fish in Central Europe can be treated as an isolated and rare case, with respect to the history of the occurrence of this vibriosis primarily in the southern parts of Europe. The outbreak described by us under the conditions of abnormally changed abiotic environmental factors, including, in particular, a higher water temperature, is in keeping with the results published by Yamanoi et al. (1980), who recorded a higher mortality of ayu at water temperatures of 21 °C and 26 °C, while no death occurred if the water temperature was at 16 °C. The mortality of eel also increased with increasing temperatures during a three- to seven-day period. From this it follows that water temperatures within the indicated range, as recorded at our site, could stimulate the virulence of cholera vibrios or contribute to “waking up” their dormant forms, which live in aquatic plants and fauna and in the biofilm on the surface of sediments (Plesnik and Prochazkova 2006; Du Preez et al. 2010).

Taking into account that man is a natural host of *Vibrio cholerae*, an incidental or continuous faecal

doi: 10.17221/7921-VETMED

pollution of water can be a source of vibrios. As the vibrios were isolated from the internal organs of diseased fish, the infection could be of an endogenous nature, associated with how the fish populations, primarily consisting of benthophagous fishes, sought their food. The common nase, which was the most severely affected species, is not a typical benthophage, but its pathological relevance is linked to the fact that while scraping algae off hard surfaces it also eats, besides the biofilm, the organisms present in the benthic fauna. This feeding habit is not uncommon in the nase, as observed on many occasions at other sites and the hypothesis is in keeping with the findings of Senderovich et al. (2010) and Halpern et al. (2008), referred to above. They suggested that fish serve as an intermediate vector of *V. cholerae* since they create a link in the food chain between chironomids and copepods on the one hand and water birds on the other. The absence of gross lesions in some fish testifies to an acute course of the infection, as observed mainly in young or smaller-size fishes (schneider, gudgeon, stone loach). The gross external lesions described in the common nase (L_s 407 to 437 mm, W 670 to 760 g) and chub (L_s 97 to 158 mm, W 15.7 to 85.7 g) corresponded to the description of similar lesions in other fishes infected by other *Vibrio* species. For infections with *V. anguillarum*, this was documented by Anderson and Conroy (1970) (erythema at the base of the fins, around the vent and within the mouth), for infections with *V. pelagius* (haemorrhages at the base of the fins) by Angulo et al. (1992), for infection with *V. salmonicida* (haemorrhages evident around the abdomen) by Holm et al. (1985), for infection with *V. splendidus* (haemorrhages in the mouth and base of the fins) by Lupiani et al. (1989) and Angulo et al. (1994), and for infection with *V. harveyi* (haemorrhages in the vicinity of the mouth and fins) these were described by Zorrilla et al. (2003). Eye lesions (exophthalmia, corneal opaqueness) were described in cases of vibriosis caused by *V. alginolyticus* (Lee 1995), *V. harveyi* (Hispano et al. 1997) and *V. anguillarum* (Anderson and Conroy 1970).

Histological changes in the gills (lamellar aneurysm or lamellar telangiectasis, lamellar hypertrophy, and hyperplasia) in the common nase and chub cannot be clearly linked to the infection because these pathological changes are often a response to changed abiotic environmental factors, i.e. those associated with physical or chemical trauma. Gross pathological lesions in experimentally infected fish (congested capillaries on

the wall of the air bladder) were similar to the description of vibriosis caused by *V. alginolyticus* (Colorni et al. 1981; Austin et al. 1993). Pale liver in turbot, *Scophthalmus maximus* (L.) is described by Lupiani et al. (1989) and Angulo et al. (1994). Compared to the data published by Luskova (1995), who studied the physiological values of blood plasma metabolites in wild common nase, the catalytic concentration of aspartate aminotransferase determined by us can be considered as several times higher (30.85 vs 0.7–9). This suggests that both the isoenzymes (cytoplasmic and mitochondrial fractions) were released into circulation, which might be indicative of severe damage to the liver.

The occurrence of *Vibrio* infection caused by *V. cholerae* in wild fish in a river ecosystem highlights the importance of thorough veterinary inspection and of close cooperation between angling clubs, their fishing guards and the environmental health authorities. This is essential for minimising the risk of disease in farmed fish or in fish kept in rearing facilities located in river basins exposed to similar threats, as well as for keeping water clean and safe. We cannot satisfactorily explain the factors underlying the absence of disease in the Eurasian minnow. This motivates us to make further experimental efforts to examine the pathogenicity of, and susceptibility to, the isolated strain No. 1161 – the more so that this fish species is used as an indicator of chemical pollution of water in some raw drinking water treatment facilities.

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Received: 2014–09–08

Accepted after corrections: 2014–12–10

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