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Case Report

Edwardsiella ictaluri in a Colony of Zebrafish (*Danio rerio*) Used in a Teaching Laboratory

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A small colony of zebrafish (*Danio rerio*) experienced 30% acute mortality within a few days after receipt from a commercial source. A few fish presented with small areas of raised scales or tissue necrosis, primarily near the caudal peduncle. *Edwardsiella ictaluri* (*E. ictaluri)* was identified by real-time PCR of pooled zebrafish and swabs of the pre-filter and fine filter pads, with subsequent sequence analysis. *E. ictaluri* is most commonly associated with an enteric septicemia in catfish species and can have significant economic impact on commercial catfish fisheries. However, several references report naturally occurring *E. ictaluri* infection of nonictalurid fishes, including zebrafish. Ours is the first report demonstrating the use of environmental sampling to identify *E. ictaluri* in a zebrafish colony by real-time PCR. Moreover, our report indicates that *E. ictaluri* is a relevant disease for institutions using zebrafish as research species and emphasizes the importance of carefully considering importation and quarantine practices.

Abbreviation: ESC, enteric septicemia of catfish

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Edwardsiella ictaluri (*E. ictaluri)* is a gram-negative facultative intracellular bacterium, known primarily for its economic impact in catfish (*Ictalurus* spp.) aquaculture in the United States. *E. ictaluri* is the causative agent for Enteric Septicemia of Catfish (ESC), or Hole-in-the-Head disease of catfish, and is one of the most commonly reported diseases by US catfish producers.[6,17,22,25] The significant economic impact of ESC has driven ongoing research and development of various vaccines administered through immersion and feeding.[17,22,39] Disease transmission among fish occurs by direct contact through the fecal-oral route, nasal passages, and gills.[6,12,17] In catfish, *E. ictaluri* infection can present as areas of hemorrhage around the base of fins, skin ulceration in various locations, bulging eyes, and a distended abdomen, with mortality of 10 to 50% in populations of pond-raised channel catfish (*Ictalurus punctatus*).[6,12]

Nonictalurid fish that are susceptible to spontaneous infection are phylogenetically diverse. These species of fish include: Ayu (*Plecoglossus altevelis*),[34] Bengal danios (*Devario devario*),[38] green knifefish (*Eigemannia virescens*),[16] a red-bellied piranha (*Pygocentris nattereri*),[19] Nile tilapia (*Oreochromis niloticus*),[37] and hybrid red tilapia (*Oreochromis sp.*).[7] Naturally occurring epizootics have been reported in 3 laboratory zebrafish colonies,[12] and since 2013 IDEXX BioAnalytics has identified *E. ictaluri* as the cause of morbidity and mortality in zebrafish colonies from 6 institutions. Clinical presentation of edwardsiellosis caused by *E. ictaluri* in zebrafish can include tissue necrosis, abdominal distention, general lethargy, raised scales, and skin hemorrhage, although acute mortality without clinical signs is also common.[12,26] The disease is generally systemic. A number of organs can be affected including the kidney, spleen, and brain with large quantities of bacteria present, often located within macrophages. [12]

Experimental *E. ictaluri* infections have also been described in many nonictalurid hosts such as rainbow trout (*Oncorhynchus mykiss*), Chinook salmon (*Oncorhynchus tshawytscha*),[3] and blue tilapia (*Oreochromis aureus*).[28] Zebrafish have been used as an experimental model for ESC.[14,26,33,36]

This article describes an outbreak of *Edwardsiella ictaluri* in zebrafish purchased for use in undergraduate studies. The diagnosis was based on clinical signs, identification of *E. ictaluri* by real-time PCR in both clinically diseased fish and environmental samples from the tank filter, and sequence analysis. To our knowledge, this is the first report demonstrating the use of environmental sampling to identify *Edwardsiella ictaluri* in a colony of zebrafish.

Case Report

An undergraduate laboratory received 24 zebrafish from a commercial vendor in February 2020 and introduced them as a single group into a single unpopulated static 75.7 L glass aquarium. One fish was found dead 2 d after arrival; the death was attributed to shipping and transport stress. However, by 6 d after arrival, 5 fish were observed to have raised scales and small areas of tissue necrosis, mainly near the caudal peduncle. These fish were subsequently isolated into a separate quarantine tank. The fish in the quarantine tank (Batch A) were found dead the next morning and were stored in a –20°C freezer, along with 2 additional fish that were found dead in the primary tank. Batch A were submitted to IDEXX BioAnalytics (Columbia, MO) as a pooled sample for real-time PCR testing for a panel of infectious agents. At that time, the remaining fish did not show any apparent abnormal behaviors or exhibit any clinical signs. However,

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4 more zebrafish were found dead over the following week. On 19 d after arrival, the remaining 12 fish were euthanized; none of them showed abnormal behaviors or physical clinical signs at euthanasia. DLAR obtained 4 of these zebrafish; 2 of them (Batch B) were submitted to IDEXX BioAnalytics (Columbia, MO) for microbiologic culture and real-time PCR analysis, along with swabs of the feed and the pre-filter and fine filter pads. The other 2 zebrafish (Batch C) were submitted to the DLAR in house diagnostic laboratory for necropsy and histologic evaluation.

After euthanasia of this group of fish, the tank and related equipment were sanitized, and a new group of zebrafish was obtained from another investigator at our institution as replacements. These fish showed no signs of disease throughout the study and were euthanized after laboratory use at the end of the semester as originally planned.

Materials and Methods

Animals. Upon receipt, male zebrafish (n = 24) were placed into a single 75.7 L (20-gallon) static glass aquarium with a gravel floor substrate. The water source was municipal city water treated by reverse osmosis and deionization and then adjusted to appropriate salinity and conductivity with 60 mg/L of Instant Ocean (Blacksburg, VA). Fish were maintained on a 14:10 light:dark cycle with 10% to 15% water exchanged weekly. Water temperature and pH were monitored daily, with ammonia, nitrate, and nitrite levels monitored on a weekly basis. Accepted ranges for those parameters were 26 to 30 °C, 7.2 to 7.4, less than 0.02 mg/L, less than 35 mg/L, and less than 0.2 mg/L respectively. The aquarium was equipped with air stone aeration and a canister filter (EHIEM Aquatics Group, Germany) with media to provide adequate surface area for nitrifying bacteria performing biologic filtration. Fish were fed dry fish flakes (Tetra, Blacksburg VA) once per day.

The affected zebrafish were purchased from a commercial vendor that provides biological supplies to high school and undergraduate classes and sells zebrafish to research laboratories. Zebrafish were housed in a classroom setting, as they were approved for use in teaching and research laboratories in an undergraduate biology class. A total of 4 tanks were used in the classroom, and tank populations were separated by source and day of arrival. The colonies were intended for short-term use, as the studies on this protocol were initiated and completed within a single semester by the biology students. All procedures were performed in accordance with the guidelines set forth by the Guide for the Care, and Use of Laboratory Animals,[15] All animal use at our facility is covered by protocols approved by the Duke University Medical Center (DUMC) Institutional Animal Care and Use Committee (IACUC). DUMC is fully AAALAC-accredited and maintains a Public Health Service Animal Welfare Assurance.

Necropsy and Histopathology. Live zebrafish were euthanized just prior to necropsy by immersion in MS-222 (Tricaine methanosulfonate, 500 mg/kg in the water, Syndel, Fernandale, WA) buffered to a pH of 7.0-7.5 in accordance with the American Veterinary Medical Association 2020 euthanasia guidelines[18] and Duke IACUC euthanasia policies. Two fish (Batch C) were submitted to the Duke Division of Laboratory Animal Resources (DLAR) for assessment. A gross necropsy was performed on 2 of the zebrafish; this included internal and external examination of fish and collection of gill and fin clippings for wet mount preparations. Gill arches and lamellae were snipped on one side of the fish using iris tenotomy scissors. Samples were placed on a clean histology slide and covered with a coverslip. Slides were examined under light microscopy for the presence of bacteria, fungi, protozoa and other parasites.

For the wet mount, a drop of fish tank water was placed on a clean histology slide. The skin behind the fins, on the caudal peduncle and under the mandible were gently scraped with a scalpel blade and sample placed on the slide. A coverslip was added, and the slide was examined under light microscopy for the presence of bacteria, fungi, protozoa and other parasites. A similar technique was used for wet mount preparation of the fins.

For fixed tissue, a lengthwise incision was made through the body wall from the anal fin along the belly to the gill chamber, exposing the coelomic cavity and viscera to fixation. Zebrafish were fixed in 10% buffered formalin (VWR International, PA) for 3 d, then cut in half longitudinally and embedded in paraffin as 2 whole body longitudinal sections. Serial, 5 µm thick whole body longitudinal sections were performed 150 µm apart and stained with hematoxylin and eosin for microscopic evaluation by a veterinary pathologist.

Microbiologic analysis. Skin and trunk kidney were cultured from euthanized zebrafish (Batch B) at IDEXX BioAnalytics and streaked for culture and isolation onto BBL Trypticase Soy Agar with 5% sheep blood (TSA II; Becton Dickinson), BBL Chocolate II Agar (Becton Dickinson), BBL Hektoen Enteric Agar (Becton Dickinson), and Tryptone Yeast Extract Salts (TYES) Agar, which was prepared inhouse according to a published formulation.[13] Cultures were incubated aerobically at 22 °C for 5 to 7 d. Representative bacterial colonies were harvested for proteomic analysis using a previously described direct transfer method.[27] Bacteria were overlaid with 1 µL of a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, and 2.5% trifluoroacetic acid (Matrix HCCA, Bruker Daltronics, Billerica, MA). The matrix was subsequently allowed to dry, and bacteria were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) using a mass spectrometer (Microflex, Bruker Daltronics) and flexControl software (Bruker Daltronics). Bacterial identification was based on automated analysis by MALDI BioTyper software (Bruker Daltronics), which compared the spectra for each isolate with an integrated reference spectral database.

Molecular analysis. Total nucleic acids were extracted from 2 pools of whole zebrafish (Batch A and Batch B) and separately for filter swabs and feed samples for real-time PCR analysis to detect a wide array of infectious agents including *Edwardsiella ictaluri*, *Flavobacterium columnare*, *Ichthyophthirius multifiliis*, Infectious spleen and kidney necrosis virus (ISKNV), *Mycobacterium abscessus*, *M. chelonae*, *M. fortuitum*, *M. gordonae*, *M. haemophilum*, *M. marinum*, *M. peregrinum*, *M. saopaulense*, *Myxidium streisingeri*, *Piscinoodinium pillulare*, *Pleistophora hyphessobryconis*, *Pseudocapillaria tomentosa*, *Pseudoloma neurophilia*, and a recently described virus, Zebrafish picornavirus.[1]

Batch A comprised 5 frozen fish from the acute, early mortalities, and Batch B included 2 fish from the final colony cull. The canister filter of the original tank was opened to allow access to both the prefilter and fine filter pads, which were swabbed and and analyzed separately. The interior of the feed bottle was also swabbed. Nucleic acid extractions were performed using a commercially available platform according to the manufacturer's protocol (NucleoMag VET Kit; Macherey-Nagel GmbH and KG, Düren, Germany). The Tetro cDNA Synthesis Kit (Bioline, London, United Kingdom) was used to synthesize cDNA. Real-time PCR assay hydrolysis probe and primers were designed using PrimerExpress version 3.0 software (Applied BioAnalytics; Waltham, MA) based on the IDEXX Laboratories proprietary

service platform using the genome sequences available in GenBank. Analysis was performed at IDEXX BioAnalytics (Columbia, MO) using standard primer and probe concentrations and the master mix LightCycler 480 Probes Master (Roche Applied Science, Indianapolis, IN) in a commercially available instrument (LightCycler 480; Roche Applied Science). Real-time PCR assay-specific positive and negative controls were included in all runs of the assays. Hydrolysis-probe–based real-time PCR assays targeting a eukaryotic gene (18S rRNA) or bacterial gene (16S rRNA) were used to ensure nucleic acid recovery and the absence of PCR inhibition. Positive real-time PCR results were confirmed by sequence analysis of the amplicon. Sequence analysis was also performed using previously published *Edwardsiella* sequencing primers and cycling conditions.[9] Primers targeting DNA gyrase subunit A (*gyrA*) were GyrAF (5′-AGCGCCTTGTACTCATCCAG-3′) and GyrAR (5′-TGGTGCATGAGATCCCCTAT-3′), and primers targeting DNA gyrase subunit B (*gyrB*) were GyrBF (5′-CCCTGTCTGAAAAGCTGGAG-3′) and GyrBR (5′-CTCGTTCATCAGCGACTCAA-3′).[9] The resulting amplicons were sequenced in both directions using Sanger methodology (GENEWIZ, South Plainfield, NJ), assembled into contigs using Sequencher software (Gene Codes Corporation), and compared with GenBank sequences using BLAST software (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/).

Results

Necropsy findings. Gill, skin, and fin clippings from the 2 zebrafish submitted to DLAR from the final cull (Batch C) were examined by wet mount and were negative for bacteria, fungi, protozoa, and other external parasites. External examination of Batch B and Batch C fish revealed a small, irregular, 1 to 2 mm diameter, slightly gelatinous and hemorrhagic area at the base of the tail and rare small hemorrhages caudal to the operculum (Figure 1). The remainder of the gross necropsy observations were unremarkable.

Histopathology. Two zebrafish from Batch C were examined histologically. No evidence of edwardsiellosis or other significant lesions was observed, except that several digenetic trematode metacercaria were encysted in the muscles and other tissues, including the esophagus, the base of the heart, and tail, with various levels of inflammatory infiltrate composed of macrophages and lymphocytes (Figure 2).

Figure 1. Gross image of a zebrafish displaying a small, irregular, 1-2 mm diameter, slightly gelatinous and hemorrhagic area at the base of the tail (black arrow) and rare small hemorrhages caudal to the operculum (white arrow).

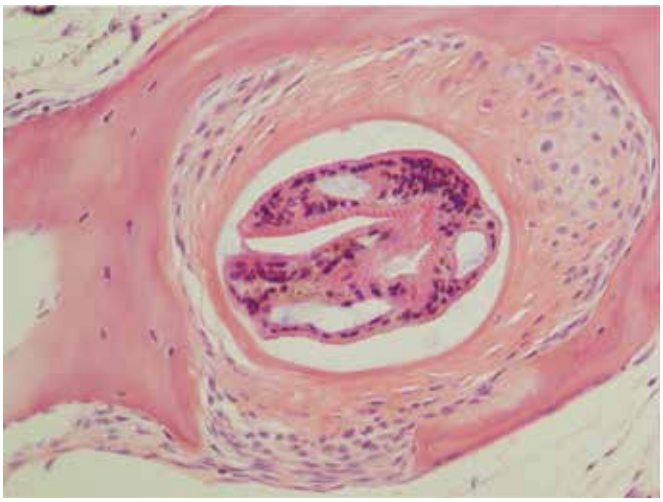

Figure 2. Photomicrograph of encysted metacercaria in zebrafish tissue.

Table 1. Molecular analysis

Sample	Date	*E ictaluri* PCR result
Batch A (5 fish)	February 25	+
Pre-Filter pad	March 23	+
Fine Filter pad	March 23	+
Feed	March 23	—
Batch B (2 fish)	March 23	—

Molecular analysis and microbiology (Table 1)**.** The pooled Batch A zebrafish sample tested strongly positive for *E. ictaluri* by real-time PCR, and the positive result was confirmed by sequence analysis of the amplicon. Other than the positive *E. ictaluri* result, the Batch A samples were negative for all the other infectious agents included in the test panel. The second set of zebrafish (Batch B) was negative for *E. ictaluri* by microbial culture. Both the Batch B zebrafish and feed samples were also negative for *E. ictaluri* by real-time PCR; however, the filter swabs tested positive for *E. ictaluri* by real-time PCR (assays performed by IDEXX Laboratories are validated to detect 10 or fewer template copies per reaction). Additional confirmation was obtained by *Edwardsiella*-specific conventional PCR and sequence analysis of 2 genes: *gyrA* and *gyrB*. Sequence analysis of *gyrA* from the pooled Batch A zebrafish sample displayed 100% identity to *E. ictaluri* over 593 bases. Similarly, sequence analysis of *gyrB* from the pooled Batch A zebrafish sample displayed 100% identity to *E. ictaluri* over 615 bases. *Edwardsiella*-specific sequence analysis of *gyrB* from the filter swabs displayed 100% identity to *E. ictaluri* over 623 bases. Sequence analysis of *gyrA* from the filter swabs was unsuccessful.

Discussion

Zebrafish are the most common finfish used in the research setting and are susceptible to infection with *E. ictaluri*. Here, we report an outbreak of *E. ictaluri* in an aquarium housing a shipment of 24 zebrafish purchased from a company that supplies biologic materials and organisms primarily to high school and undergraduate classrooms for teaching purposes, as well as to research institutions. Biologic supply companies have been described as high-risk vendors for laboratory zebrafish along with wholesalers, pet stores, and multispecies aquaculture facilities

that do not practice colony health monitoring and lack adequate biosecurity practices.[4] A surprising number of investigators continue to use zebrafish from high-risk vendors for biomedical research, even though several reports link high-risk vendors to introduction of zebrafish pathogens.[2,12,29,35]

The educational use and management of the zebrafish described in this report is unique for our institution. The fish were purchased for short-term use by undergraduate biology students during a one-semester class. The fish were not being bred, and the shipments of fish were managed with an "all-in all-out" approach. The allocated space for aquaria was limited to four 75.7 L tanks located on a classroom table, and the total zebrafish population was generally less than 100 fish. Although new batches of zebrafish did undergo basic quarantine and acclimation after receipt, they did not undergo the standard, more rigorous quarantine practices used for our large, long-term, zebrafish colonies. Fortunately, because the tanks were populated in an "all-in all-out" system with no transfers among tanks, none of the other tanks in the room experienced any morbidity or mortality.

The standard quarantine procedures for the long-term colonies at our institution include the following steps: 1) all incoming embryos or adult fish immediately enter a quarantine room; 2) only progeny from embryos produced in quarantine by the imported adult fish and surface disinfected using both sodium hypochlorite and iodine can enter the main zebrafish colonies; 3) once subsequent generations or required data from the imported fish have been obtained, the original imported fish are euthanized according to approved protocol methods; and 4) the quarantine room has a dedicated supply of tanks, nets, food, filters, etc.; these items are not removed from the room for any reason. The fourth requirement is enforced because equipment that has been in contact with quarantined fish or systems can act as a fomite and thereby introduce pathogens into the main holding areas. The Zebrafish International Resource Center (ZIRC) uses similar quarantine practices, in addition to receiving or cryopreserving sperm and performing in vitro fertilization with colony eggs.[21] With regard to the latter practices, a recent study found that *E. ictaluri* and other zebrafish pathogens survived various cryopreservation and freezing protocols, although the study did not evaluate the infectivity and transmission of the surviving organisms.[24]

The acute morbidity and mortality described in this case study were consistent with the previously described epizootic course of edwardsiellosis in zebrafish.[12,26] Strongly positive real-time PCR-positive results from the homogenized pool of Batch A fish identified *Edwardsiella ictaluri*, which was confirmed by sequence analysis of the amplicon and by sequence analysis of 2 additional bacterial genes, *gyrA* and *gyrB*. We could not perform histopathology on the fish from Batch A because they died acutely and were frozen by the lab. By week 4, only 12 fish remained in the 75.7 L tank. These fish were clinically normal and continued to be free of any physical or behavioral abnormalities. The 2 fish that we examined histologically were obtained from these late surviving, clinically normal fish; these 2 fish had no histologic evidence of *E. ictaluri* infection. The surviving fish in this case report may not have been exposed to a minimal infectious dose, as fewer than one zebrafish per liter remained in the tank after the initial acute episode of mortality; these fish were quickly removed to avoid cannibalism. In contrast, laboratory zebrafish are commonly housed at densities ranging from 5 to 10 fish/L. A previous report found that lower density of catfish after harvest reduced the odds of having an ESC event.[6] Furthermore, one or more of the surviving fish may have arrived as asymptomatic carriers from the population of origin. Molecular analysis of various types of environmental samples has been used to detect *E. ictaluri* in commercial aquaculture[8] and to detect other pathogens in laboratory zebrafish.[5,20,23] However, ours is the first report demonstrating the use of environmental sampling to detect *E. ictaluri* in zebrafish. In our case study, the environmental sample provided corroboration of the diagnosis. In other situations, environmental testing may provide evidence that counters an absence of histopathology findings or low numbers of affected animals in a population.

We recognize that some residual *E. ictaluri* material may have been present in the filters from previous tank populations. However, the combination of clinical disease, acute deaths, the timing of the findings, and positive PCR from fish samples leads us to conclude that all of our findings arose from a single related event. When questioned, the biological supply company stated that they had not experienced any unexpected mortality.

E. ictaluri was probably present in a few of the initial 24 fish received and, after shipping stress, resulted in an outbreak and acute mortality. As in mammals, acute or chronic husbandry-related stress in zebrafish gives rise to elevated cortisol levels, resulting in a stress leukogram and increased susceptibility to pathogens.[11,30-32] The presence of encysted metacercaria in some of the same zebrafish that were affected by *E. ictaluri* suggests that they were reared in outdoor ponds with exposure to snails, birds, and/or other hosts that would allow continuation of the trematode life cycle. The *Edwardsiella* outbreak further indicates that this commercial source of zebrafish lacks adequate biosecurity practices for laboratory zebrafish. As a result of this experience, the teaching lab now obtains fish from biosecure research colonies in our own institution. These inhouse facilities employ stringent quarantine and bioexclusion measures, and no other outbreaks have occurred since this case. The implementation of standard practices in rodent research, including approved vendor lists, diligent adherence to proven quarantine practices, routine health surveillance, and work and materials flow that protect the colony health status are all essential to the biosecurity of research zebrafish colonies, allowing protection of zebrafish health, human health, and scientific validity and reproducibility.

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