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Edwardsiella piscicida-like pathogen in cultured grouper

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ABSTRACT: An *Edwardsiella* sp. was isolated from the kidney of diseased groupers (*Epinephelus aeneus* and *E. marginatus*) cultured in Eilat (Israel, Red Sea). Affected fish presented a severe suppurative nephritis with large abscesses occasionally spreading into the surrounding musculature. Biochemical profiles and phenotypic comparisons failed to provide a clear identification to the species level, and genetic analysis of the 16S subunit failed to discriminate between *Edwardsiella piscicida*, *E. tarda* and *E. ictaluri*. Analysis of the *gyrB* gene, however, placed the grouper isolates into the *E. piscicida*-like group, a newly recognized taxon which also encompasses the non-motile strains previously classified as atypical *E. tarda*. Initial genomic analysis revealed the presence of the *Edwardsiella* type 3 secretion system (T3SS) but also revealed a pathogenicity island encoding a second T3SS with homology to the locus of enterocyte effacement of *Escherichia coli*. Further analysis revealed 3 different type 6 secretion systems that were also present in all sequenced isolates of *Edwardsiella piscicida*-like strains. Based on estimated DNA–DNA hybridization values and the average nucleotide index, the grouper strain fits into the *E. piscicida*-like phylogroup described as *E. anguillarum* sp. nov. The peculiarities associated with this isolate and the association of other conspecific piscine isolates from multiple marine and brackish water species suggest a link of the entire *E. piscicida*-like phylogroup to the marine environment.

KEY WORDS: Edwardsiellosis · Fish · Locus of enterocyte effacement · LEE · Israel · Red Sea · *Epinephelus aeneus* · *Epinephelus marginatus*

INTRODUCTION

The white grouper *Epinephelus aeneus* and the dusky grouper *E. marginatus* are cultured at the Israel Oceanographic and Limnological Research, National Center for Mariculture (IOLR-NCM) in Eilat (Israel; Red Sea). The reproduction, nutrition and physiological needs have been the object of intensive research for the domestication of these important commercial species since the early 1990s. In recent years, the broodstock appeared to be suffering from a chronic nephritis, and a Gram-negative, non-motile, rod-shaped bacterium was repeatedly isolated in pure culture from kidney abscesses in moribund individuals. The isolate presented features similar to those of *Edwardsiella ictaluri* and *E. tarda* but did not fit the biochemical profile of either one.

Edwardsiella piscicida is a newly described species, recently segregated from isolates traditionally identified as *E. tarda* (Abayneh et al. 2013, Griffin et al. 2014). After the original description based on isolates from eels, turbot, and Korean catfish (Abayneh et al. 2013), *E. piscicida* has been associated with mortality events in farmed channel catfish (Griffin et al. 2014), red sea bream (Oguro et al. 2014), largemouth bass (Fogelson et al. 2016) and captive elasmobranchs (Camus et al. 2016). Griffin et al. (2014) phylogenetically analyzed isolates of *Edwardsiella*

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using the *gyrB* gene and determined that *E. ictaluri* and *E. tarda* fall into their own distinct groups, but *E. piscicida* splits into 2 groups, deemed *E. piscicida* and *E. piscicida*-like sp. This was recently supported by Shao et al. (2015), who identified a similar demarcation within the *E. piscicida* group and described strains from this distinct *E. piscicida*-like group as *E. anguillarum* sp. nov. In the present work a new representative of this phylogroup, *E. piscicida*-like isolate EA181011, is phenotypically and genotypically characterized and the host pathology is described.

MATERIALS AND METHODS

Host fish. The 12 fish examined were part of about 50 brood stock specimens of white grouper *Epinephelus aeneus* and dusky grouper *E. marginatus* cultured in two 50 m^3 tanks and one 25 m^3 tank at IOLR-NCM. *E. aeneus* were the offspring of individuals caught several years prior off the Mediterranean coast of Israel, while the *E. marginatus* specimens were caught 2 yr prior, also off the Mediterranean coast. The weight of the diseased fish ranged from 0.920 to 4.200 kg. Persistent mortality occurred from October 2011 to October 2012, resulting in the loss of about 20% of the stock.

Bacterial isolation and identification. Isolations were carried out from kidney of either moribund fish or fish that had died overnight. Tryptic soy agar (TSA) and brain heart infusion (BHI) agar (Acumedia) prepared with 25% aged sea water (3 wk at 4°C) were used for culture. The inoculated media were incubated at 24 ± 1°C. Several subcultures were made to ensure colony purity. The isolates were cryopreserved in 8–15% glycerol at −80°C until further use. To obtain a comprehensive and comparable biochemical profile, the isolates were characterized using 2 miniaturized commercial kits, API E and API 50 CH, as well as the Vitek 2 System version 05.04 (BioMérieux). Motility was tested at 25 and 37°C by stab-inoculating a colony into semisolid TSA (0.4% agar) medium. Hemolysis was tested on both commercial sheep blood agar and TSA prepared with 25% aged sea water and supplemented with 5% outdated human blood-bank blood.

Confirmation of the preliminary identification based on phenotypic characteristics was attempted by partial sequencing of the 16S ribosomal RNA gene. PCR products from template DNA of needle-touched bacterial colonies (3 replicates) were amplified using universal primers fD1 (27F) (Weisburg et al. 1991) and 1100R (Turner et al. 1999). The GoTaq Green Master Mix (Promega) was used in a total volume of 50 µl, and PCR assays were performed with the Eppendorf Mastercycler gradient (Eppendorf). Cycling parameters consisted of an initial 4 min denaturation at 94°C, followed by 30 cycles of 1 min denaturation at 94°C, 1 min annealing at 50°C, and 1.5 min extension at 72°C, with a final 10 min extension step at 72°C. The initial denaturation step was extended to 4 min, and the final extension step was extended to 10 min. PCR products were analyzed in 1.2% agarose gels containing ethidium bromide and visualized under UV light. Amplified products were purified using a PCR purification kit (QIAGEN), and the quantity and purity (260/280 ratio) of the double-stranded PCR products were estimated in a microplate spectrophotometer (PowerWave^{TM} XS, BioTek). For the sequencing analysis, internal universal primers designed by us were used: 537F (5′-CAG CCG CGG TAA TAC G-3′) and 550R (5′-CGC TCG AGA CCT ACG TAT TAC C-3′). DNA sequencing was conducted at Hy Laboratories Ltd. (Hylabs). The 16S sequences of all the isolates used in this study were compared with isolates available in the National Center for Biotechnology Information GenBank database (NCBI/BLAST).

Consensus PCR primers for detection of *Edwardsiella* spp., EDWF1 (5′-CTT TCA GTA GGG AGG AAG GTG TGA-3′) and EDWR1 (5′-CCG TAT CTC TAC AGG ATT CGC TG-3′), were designed to target the 16S rRNA of *Edwardsiella* spp. Genomic DNA was extracted from the spleen of infected grouper according to Ucko et al. (2002). As control for DNA quality, the 18S rRNA gene was also amplified using the universal primers KuF1/KuR1 (Diamant et al. 2005). The *Edwardsiella* spp.-specific primer set was validated against other piscine bacterial isolates frequently affecting Israeli mariculture, including *Vibrio* sp., *Photobacterium damselae* ssp. *piscicida*, *Mycobacterium marinum* and *Streptococcus iniae*, as well as other clinically healthy fish species, including sea bass *Dicentrarchus labrax*, sea bream *Sparus aurata*, white grouper *Epinephelus aeneus*, and mullet *Mugil cephalus* cultured at IOLR-NCM facilities.

Genome sequencing. High through-put sequencing of the EA181011 genome was conducted using an Ion Torrent Proton Semiconductor Sequencer (Life Technologies). The sequence was analyzed using the Ion Torrent de novo assembler, open reading frame (ORF) finder and BLAST at NCBI, and the YASS genomic similarity search tool (http://bioinfo.lifl.fr/yass/) to make phylogenetic comparisons to other *Edwardsiella* spp., including the *gyrB* gene. In addition, the completed genome was further ana-

lyzed to evaluate potential factors that could contribute to virulence of E181011 and provide an indication of its pathogenic potential compared to other *Edwardsiella* spp.

The draft genome was then closed using Pacific Biosciences (Pac-Bio) sequences, mapping the Ion Torrent reads to the Pac-Bio assembled chromosome (Reichley et al. 2015). DNA–DNA hybridization (DDH) estimate values were determined for the closed EA181011 (Reichley et al. 2015) and the *E. anguillarum* type strain ET080813 (GenBank acc. no. CP006664; Shao et al. 2015).

Plasmid preparation. Isolation of plasmid DNA was attempted from each of 5 Israeli isolates using both a Qiagen miniprep kit (Qiagen Sciences) and a Qiagen large-construct kit. Plasmid preps were analysed on 0.6% agarose gels. To further evaluate the possible presence of plasmids, the PlasmidFinder program at the Center for Genomic Epidemiology was used to analyze the genome sequencing data and the recently released complete genome sequence (GenBank acc. no. CP011364; Reichley et al. 2015).

Histopathology. Tissue samples of kidney, spleen, heart and liver were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 6 µm and stained with hematoxilin-eosin. In addition, the Kinyoun acid-fast cold stain was performed to rule out an association of the granulomatous-like lesions with possible acid-fast bacteria (Sheehan & Hrapchak 1980).

Antibiogram. Florfenicol, oxytetracycline dihydrate and sulfadimethoxine/ormetoprim were selected since they are the only 3 antibacterial drugs approved in the USA by the FDA (Food and Drug Administration) and allowed in Israel for veterinary use in food-fish aquaculture. Sensitivity of the isolates was established using the disk-diffusion method of antibiotic impregnated disks (Oxoid) on BHI agar. Diameters of inhibition zones were measured, and interpretation of sensitivity was determined by comparing the zone of inhibition with the zone diameter breakpoints recommended by the manufacturer and the British Society for Antimicrobial Chemotherapy (BSAC 1998).

RESULTS

Gross pathology

Behavioral changes and clinical signs were relatively non-specific. The affected fish displayed loss of equilibrium and were found either lying on one side at the bottom of the tank or floating at the surface as a result of swim bladder hyperinflation. Skin ecchymoses and ulcerations, fin and tail erosion and, occasionally, exophthalmia and cataracts were also observed. The gills were pale and inflamed. Ascitic fluid collected in the abdominal cavity. Kidney, heart, spleen and liver appeared congested, haemorrhagic and oedematous. Whitish watery abscesses were often visible on the surface of these organs. The kidney, however, appeared to be most severely affected, swollen and sparsely mottled. Occasionally, large purulent abscesses spread into the surrounding musculature.

Histopathology

Histopathology analyses confirmed the severe suppurative nephritis observed at gross pathology examination, with bacteria conspicuously visible in masses of degenerate neutrophils in the necrotic, liquefied tissue (Fig. 1). Abscesses were also occasionally observed in the liver (Fig. 2), spleen and heart. The larger ones were generally irregular in shape, bounded by an extensive inflammatory cell (macrophages and neutrophils) response, fibrin coating, and filled with fluids and hollow areas. In at least 2 individuals the myocarditis was particularly severe with conspicuous thickening of the epicardium (Fig. 3).

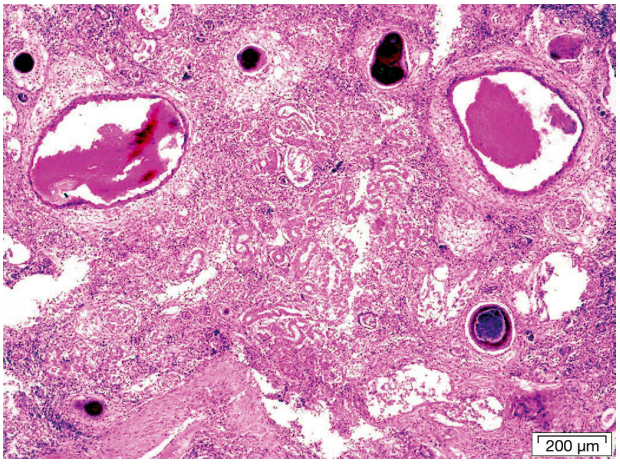

Fig. 1. Suppurative nephritis in the white grouper *Epinephelus aeneus*. Interstitial abscesses with *Edwardsiella piscicida*-like (EA181011) bacteria in conspicuous masses of degenerate macrophages and neutrophils are visible. Hollow areas within the larger abscesses were probably occupied by gas, most likely H_2S, produced by the bacteria metabolic activity

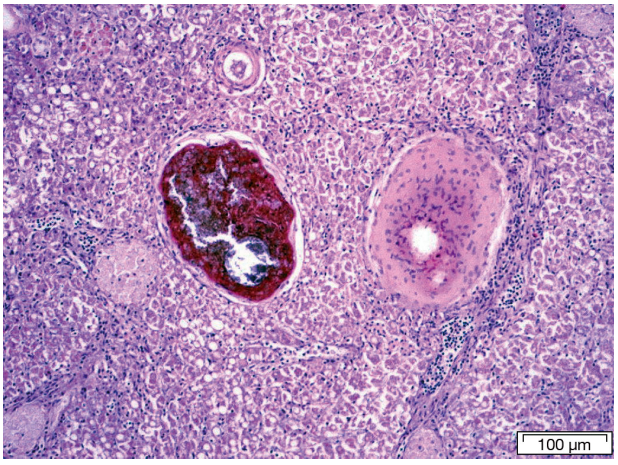

Fig. 2. Suppurative abscesses by *Edwardsiella piscicida*-like (EA181011) in more (left) and less (right) advanced stage of development, in white grouper *Epinephelus aeneus* liver (H&E stain)

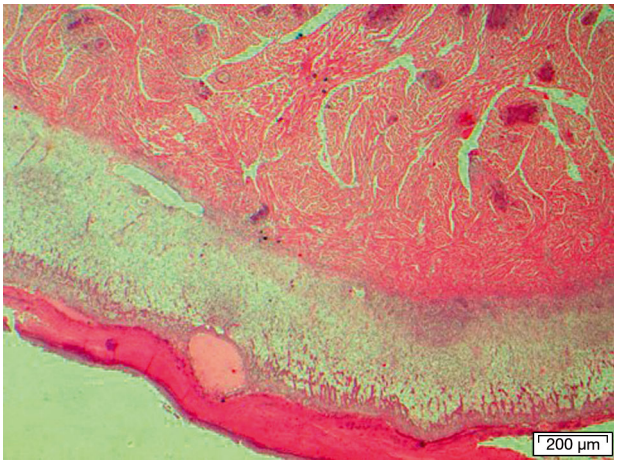

Fig. 3. Severe myocarditis and epicarditis in *Edwardsiella piscicida*-like (EA181011) infected white grouper *Epinephelus aeneus*. A thick amorphous fibrotic membrane envelops the myocardium

Biochemical profile

The phenotypical characteristics of the Eilat isolates were identical in all 12 groupers examined. They are summarized and compared to *Edwardsiella tarda*, *E. piscicida*, *E. ictaluri*, and *E. anguillarum* sp. nov. in Table 1. Our strain was susceptible to all 3 antibiotics tested.

Molecular analyses

None of the grouper isolates showed any evidence of plasmids in either the mini-prep or large construct kits. The absence of plasmids was further confirmed by using PlasmidFinder on the complete genome sequence of EA18101 (GenBank acc. no. CP011364).

Comparison of the partial sequence of the 16S ribosomal RNA gene of EA181011 (1099 bp) with the whole genome sequences of isolates available in the GenBank database showed 100% alignment of the grouper strains to *E. piscicida*-like strain LADL05-105 (GenBank acc. no. CPO11516.1) and *E. ictaluri* 93-146 (GenBank acc. no. CP001600), 99.9% identity to *E. anguillarum* sp. nov. 080813 (GenBank acc. no. CP006664), 99.8% identity to *E. piscicida* isolates C07-087 (GenBank acc. no. CP004141), EIB202 (GenBank acc. no. CP00001135), FL6-60 (GenBank acc. no. CP002154) and 99.3% identity to *E. tarda* FL95-01 (GenBank acc. no. CP011359). The partial sequence of isolate EA181011 was selected as representative of 5 identical grouper isolates and is available as GenBank acc. no. KP876579.

The primer set EDWF1/EDWR1 amplified a unique product of the expected size (580 bp) only in *Edwardsiella* isolates but not in other bacterial species, including *Vibrio* sp., *Photobacterium damselae* ssp. *piscicida*, *Mycobacterium marinum* and *Streptococcus iniae*, nor in clinically healthy fish, including sea bass *Dicentrarchus labrax*, sea bream *Sparus aurata*, white grouper *Epinephelus aeneus* and mullet *Mugil cephalus*. This procedure permitted rapid confirmatory diagnosis of an *Edwardsiella* infection in subsequent disease outbreaks.

Initial high through-put sequencing of the genome of one of the grouper strain EA181011 yielded 3 917 714 bp in 304 contigs ranging from 4874 to 164 212 bp with an average depth of coverage of 140. The available contigs are accessible at NCBI as a whole genome shotgun sequencing project WGS JUBF01000000 (GenBank acc. nos. JUBF01000000–JUBF01000304). These Ion Torrent sequencing reads were later mapped (16× genome coverage) to a Pac-Bio assembled chromosome to correct variations in homopolymer lengths in the consensus Pac-Bio data and produce a single consensus contiguous sequence (GenBank acc. no. CP011364; Reichley et al. 2015). Comparison of the *gyrB* gene obtained from this sequencing showed that EA181011 was most similar to *gyrB* in the *E. piscicida*-like group (as identified by Griffin et al. 2014).

EA181011 encodes 2 type 3 secretion systems (T3SS). One encompasses 31 051 bp encoding 31 ORFs with homology to the locus of enterocyte effacement (LEE) T3SS encoded by enteropathogenic *Escherichia coli* (see Jerse et al. 1990). The sequence of the entire LEE-like pathogenicity island

Table 1. Biochemical profiles of the grouper isolates compared to other fish-pathogenic *Edwardsiella* species (*E. tarda*, *E. piscida*: Griffin et al. 2013; *E. ictaluri*: Waltman et al. 1986, Plumb & Vinitnantharat 1989; *E. anguillarum*: Shao et al. 2015, their supplementary material). TSI: triple sugar iron; OF glucose: oxidation/fermentation of glucose; VP: Voges-Proskauer acetoin test; ONPG: ortho-nitrophenyl-β-galactoside; RBC: red blood cell. K/A: alkaline slant and acid butt (only glucose fermented; peptone utilized); nd: not determined; Fg: glucose fermented; d: reaction delayed

	E. tarda	*E. piscicida*	*E. ictaluri*	*E. anguillarum* (ET080813T)	*E. piscicida*-like (EA181011)
Gram stain	–	–	–	–	–
Morphology	Rod	Rod	Rod	nd	Rod
CyOx	–	–	–	–	–
TSI	K/A	K/A	K/A	nd	K/A
H_2S on TSI	+	+	–	+	+
OF glucose	Fg	Fg	Fg	nd	Fg
Motility					
at 25°C	+	+	+	nd	–
at 37°C	+	+	–	nd	–
Gelatin	–	–	–	–	–
Indole	+	+	–	+	–
Urease	–	–	–	–	–
Simmon's citrate	–	–	–	+	+
Phenylalanine	–	–	–	nd	nd
Nitrate reduction	+	+	+	nd	+
Methyl red	+	+	+	+	nd
VP	–	–	–	+	–
ONPG	–	–	–	nd	–
Malonate	–	–	–	nd	–
Catalase	+	+	+	+	+
Arginine	–	–	–	–	–
Lysine	+	+	+	+	+
Ornithine	+	+	+	+	–
Temperature effects on growth					
20°C	+	+	+	nd	+
25°C	+	+	+	nd	+
30°C	+	+	–	+	+
40°C	+	+	–	+ (37–42°C)	+
RBC-hemolysis	Beta	Beta	Alpha	nd	Gamma
Metabolism using					
Glucose	+	+	+	+	+
Arabinose	–	–	–	+	–
Adonitol	–	–	–	nd	–
Cellebiose	–	–	–	nd	–
Dulcitol	–	–	–	nd	–
Erythrytol	–	–	–	nd	nd
Galactose	+	+	+	+	+
Inositol	–	–	–	–	–
Lactose	–	–	–	–	–
Maltose	+	+	+	+	+d
Melebiose	–	–	–	–	–
Mannitol	–	–	–	+	+d
Mannose	+	+	+	–	+
Rhamnose	–	–	–	–	–
Salicin	–	–	–	nd	–
Sorbitol	–	–	–	–	–
Sucrose	–	–	–	–	–
Trehalose	–	–	–	–	–
Xylose	–	–	–	nd	–
Growth in NaCl					
1%	+	+	+	+	+
2%	+	+	–	+	+
3%	+	+	–	+	+

(PAI) in EA181011 is available as GenBank acc. no. KP234023. Further analysis showed the LEE-like T3SS in EA181011 is only present in the *Edwardsiella piscicida*-like isolates ET080813, RSB1309 and LADL05-105, not in either *E. ictaluri* or *E. piscicida*. Alignment of the grouper isolate with other *Edwardsiella* ssp. revealed EA181011 also carries the T3SS identified in the other fish pathogenic *Edwardsiella* spp., with 100% query coverage (QC) and 99% nucleotide identity (I) for *E. piscicida*-like (*E. anguillarum* sp. nov.) isolate ET080813, and 95% QC with 89% I for both *E. ictaluri* 93-146 and *E. piscicida* C07-087.

Finally, BLAST analysis of the 3 type 6 secretion systems (T6SS1, T6SS2, and T6SS3) described for *E. piscicida*-like (*E. anguillarum* sp. nov.) isolate ET080813 (see Shao et al. 2015) indicated all 3 were present in EA181011, as well as *E. piscicida*-like isolate LADL05-105, with 93–100% QC and 99–100% I for each of the 3 systems. Further analysis showed T6SS1 was present in all of the *E. piscicida*-like, *E. piscicida*, and *E. ictaluri* isolates that have completely sequenced genomes. T6SS2 and T6SS3, however, were only present in the *E. piscicida*-like isolates, although partial matches of only 1–17% QC were found in the complete genomes of the other *Edwardsiella* species.

DISCUSSION

Histopathology was typical of the severe suppurative nephritis described for *Edwardsiella tarda* in tilapia (Kubota et al. 1981) and turbot (Padrós et al. 2006). The disease described here for our *E. piscicida*-like EA181011 grouper strain has an evident chronic course. The abscesses' hollow areas are consistent with hydrogen sulphide produced by the metabolic activity of this strain.

The biochemical profile (Table 1) and classification based on phenotypic characteristics indicated closest similarity of the grouper isolates to *E. tarda*/*E. piscicida.* The grouper strain differs from *E. ictaluri* based on the production of H_2S on TSI, being non-motile at 25°C, having positive growth at 30, 35 and 40°C and in 2 and 3% salt, and lacking the characteristic pEI1 and pEI2 plasmids of *E. ictaluri* (Fernandez et al. 2001). The grouper strain, however, also differs from *E. tarda* and *E. piscicida* in being non-motile at both 25 and 35°C, indole negative, citrate positive, ornithine negative, and mannitol positive, and differ from *E. anguillarum* sp. nov. in being negative for arabinose and ornithine, but positive for mannose.

From a practical viewpoint, there is little latitude for antibiotic therapy for food fish, since only the 3 antibacterial drugs we tested are legal and commonly in use in Israel by either the food fish or ornamental fish industry. *Edwardsiella* spp. are generally reported as sensitive to oxytetracycline (Stock & Wiedemann 2001), although the effectiveness of this antibiotic declines in sea water (Herwig 1979, Lunestad & Goksøyr 1990). The fish were treated with Florocol (Schering-Plough Animal Health, florfenicol 50% active ingredient, 1 g kg^{-1} feed) for 13 d, and no mortalities were observed in a subsequent 3 mo period following the end of the treatment. Occasional mortalities, however, resumed afterward, and the same pathology was observed again. The development of PCR for detection and identification of *Edwardsiella* spp. directly from tissues based on 16S rRNA gene sequences allowed for a rapid diagnosis (or ruling out) of the infection.

By 16S rRNA gene sequence analysis alone, the 3 species *E. piscicida*, *E. tarda* and *E. ictaluri* might be all recognized as one. Analysis of the *gyrB* gene, however, placed EA181011 firmly into the *E. piscicida*-like group described by Abayneh et al. (2013) and Griffin et al. (2014), which also encompasses the non-motile, atypical *E. tarda* strains described by Matsuyama et al. (2005) and Sakai et al. (2009), as well as the isolate from tilapia (LADL05-105) where the fish were being raised in re-circulating systems ranging from 28–35 ppt NaCl (Greg Lutz, LSU Aquaculture Research facility, Baton Rouge, Louisiana, pers. comm.). Interestingly, all of the described isolates came from fish in a saline environment. In addition, when isolates from a freshwater environment (previously classified as *E. tarda*) were examined by a discriminatory PCR, 44 of 44 proved to be *E. piscicida*. None of the archived isolates were *E. piscicida*-like sp. or *E. tarda* (Griffin et al. 2014).

The halophilic nature of the reported isolates and the lack of *E. piscicida*-like strains in freshwater cases, coupled with the ability to grow in salt concentrations up to 3%, suggest a strong preference by *E. piscicida*-like strains for the marine or brackish water environment.

Reports on *E. piscicida*-like spp. pathogenesis are limited. Matsuyama et al. (2005) found that strains were virulent in red sea bream following challenge by intraperitoneal injection, virulent to a lesser extent in yellowtail, and were avirulent in Japanese flounder. Yang et al. (2012) showed that zebrafish injected intramuscularly with 5×10^5 colony-forming units (CFU) of *E. piscicida*-like (*E. anguillarum* sp. nov.) ET080813 had 100% mortality after 3 d and

showed clinical signs that included hemorrhage at the site of injection and ulceration and necrosis with a high bacterial load in the internal organs. Although the pathology is more acute due to the delivery by injection, the ulcerative and necrotic lesions are as experienced in the grouper. More recently, Shao et al. (2015), also using strain ET080813, showed a low 50% lethal dose (LD_{50}) of 5.7×10^2 CFU in turbot following intramuscular injection.

Compared to the LEE in *Escherichia coli*, the EA181011 LEE is most similar to that of *E. coli* O157:H7 str. Sakai, which is 34 306 bp and contains 41 ORF. The EA181011 LEE contains the same 5 operons found in *E. coli* O157:H7 str. Sakai, with the genes arranged in the same order. The operons themselves, however, are not arranged in the same order. Previously, Nakamura et al. (2013) reported an *Edwardsiella* strain, FPC503, that carried an LEE that is highly similar to the LEE reported here in EA181011. Comparison of FPC503 to the subsequent description of the new species *E. piscicida* (Abayneh et al. 2013, Griffin et al. 2014) showed it belongs to the *E. piscicida*-like group, the same as the grouper isolates described here. Analysis of the recently reported *E. piscicida*-like genomes of strains RSB1309 from red sea bream (Oguro et al. 2014) and ET080813 from eels (Shao et al. 2015) revealed the presence of the same LEE PAI. Comparison of the sequences showed that the gene and operon arrangement of the LEE T3SS from FPC503, RSB1309, ET080813 and EA181011 are all highly conserved, and BLAST analysis indicates that all of the *Edwardsiella* strains that carry the enteropathogenic *E. coli* LEE T3SS are confined to the *E. piscicida*-like phylogroup.

Consideration of the various secretion systems indicated they are distributed along species lines. Unlike most other *Edwardsiella* spp., the *E. piscicida*-like phylogroup encodes all 3 T6SS and both of the T3SS. *E. ictaluri* and *E. piscicida* only encode T6SS1 and the *E. ictaluri* T3SS, and are lacking T6SS 2 and 3, as well as the LEE T3SS. The implications for the presence or absence of a given secretion system are unclear, and because of the differences in hosts for the 3 species, would be difficult to ascertain. The acid-activated urease that is essential for function of the *E. ictaluri* T3SS (Booth et al. 2009) is absent from the other 2 species, suggesting a differential pathogenesis for those species.

Shao et al. (2015) analyzed the *E. piscicida*-like isolates reported to date, as well as 5 new *E. piscicida*-like isolates from eels. Based on extensive phylogenetic analyses, genome level comparisons and DDH estimations, they proposed that the data comparing them to the other *Edwardsiella* species justified the characterization of a new species, *E. anguillarum*. An average nucleotide identity of 99.75% and a DDH value of 98.57% for the grouper isolate EA181011 genome (Reichley et al. 2015) compared to the genome of the proposed *E. anguillarum* sp. nov. type-strain ET080813 (Shao et al. 2015) confirm EA181011 fits into the proposed *E. anguillarum* sp. nov. classification.

It is noteworthy, however, that while 6 of the 9 isolates used to define the *E. anguillarum* group by Shao et al. (2015) came from eels, at least 5 of them are from the same location in China, whereas the other 3 came from sea bream in different locations in Japan. When combined with the data presented here for the grouper isolate in Israel and the isolation of strain from tilapia in salt water recirculating system in Louisiana, USA, 5 out of 11 currently known isolates of this phylogroup are from species other than eels (3 from Japan, 1 from the USA, and 1 from Israel). The peculiarities associated with this isolate and the association of other conspecific piscine isolates from multiple marine and brackish water species suggest a link of the entire *E. piscicida*-like phylogroup to the marine environment.

E. tarda is a known agent of gastroenteritis in humans (Janda & Abbott 1993). In Israel, several *E. tarda* serotypes were isolated from the aquatic environment (e.g. intestinal contents of water tortoises *Clemmys caspica* near the Jordan River, samples of water from Lake Kinnereth [Sea of Galilee], Jordan River, well water, sewage water; Sechter et al. 1983) as well as from human patients (Sechter et al. 1983, Hashavya et al. 2011, Dr. Yoram Keness, Emek Medical Center, Afula, pers. comm.). Only an Israeli *E. tarda* isolate from a 2008 case of human infection (vaginal flora of a mother and neonatal sepsis of her baby) could be compared both biochemically and in its 16S rDNA gene sequence with our grouper strain (data not shown). However, this clinical isolate (motile and able to grow at 42°C) did not appear to be an atypical *E. tarda* and was in no way similar to *E. piscicida*-like EA181011.

Ours would be the first description of pathology associated with *Edwardsiella* infections in groupers. It is also the first report of edwardsiellosis in marine fish in Israel. At present the source of contagion is unknown.

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