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Comparison of Vietnamese and US isolates of *Edwardsiella ictaluri*

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ABSTRACT: We compared *Edwardsiella ictaluri* from striped catfish in Vietnam with US channel catfish isolates. Biochemical analyses and sequencing of the 16S rRNA gene confirmed that the Vietnamese isolates were *E. ictaluri*. Comparison using rep-PCR fingerprinting demonstrated no significant differences between the isolates, but plasmid analysis indicated that the Vietnamese isolates grouped into 4 plasmid profiles, each different from the typical pEI1 and pEI2 plasmid profile found in the US isolates. Sequencing plasmids representative of the 4 profiles indicated that all contained derivatives of the *E. ictaluri* plasmid pEI1, whereas only 1 contained a plasmid derivative of the *E. ictaluri* plasmid pEI2. The pEI2 encoded type III secretion effector, EseI, and its chaperone, EscD, were found to be present on the chromosome in isolates lacking a pEI2 derivative. In addition, 1 isolate carried a 5023 bp plasmid that does not have homology to either pEI1 or pEI2. Furthermore, Vietnamese isolates were PCR positive for the type III and type VI secretion system genes *esrC* and *evpC*, respectively, and the urease enzyme, but were PCR-negative for the putative type IV secretion system gene *virD4*. A monoclonal antibody against the lipopolysaccharide of *E. ictaluri* ATCC 33202 did not react with the Asian isolates or with the more recent US isolates. Antibiotic resistance patterns were variable and did not correlate to the presence of any particular plasmid profile. Finally, the Vietnamese isolates were avirulent and had a significantly reduced capacity for intracellular replication within head-kidney-derived channel catfish macrophages.

KEY WORDS: *Edwardsiella ictaluri* · Catfish · Genotype · Plasmid · Virulence · Antibiotics

INTRODUCTION

Edwardsiella ictaluri causes enteric septicemia of catfish (ESC), a major disease of channel catfish *Ictalurus punctatus* (Hawke 1979, Hawke et al. 1981). In the USA, the channel catfish is the only commercially-relevant species affected by *E. ictaluri*, although *E. ictaluri* has been isolated from other siluriform fish, including walking catfish *Clarias batrachus* (Kasornchandra et al. 1987), tadpole madtom *Noturus gyrinus* (Klesius et al. 2003), brown bullhead *Ameieuris nebulosus* (Iwanowicz et al. 2006), and white catfish *I. catus* (Newton et al. 1988). Non-siluriform fish from which *E. ictaluri* has been isolated include the green knifefish *Eigenmannia virescens* (Kent & Lyons 1982), rosy barb *Puntius conchonius*

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(Humphrey et al. 1986), Bengal danio *Devario* (=*Danio*) *devario* (Waltman et al. 1985), zebrafish *Danio rerio* (Hawke et al. 2013), and Nile tilapia (Soto et al. 2012). Recent reports demonstrate that *E. ictaluri* infections are occurring in Asia, where *E. ictaluri* has been isolated from natural populations of ayu *Plecoglossus altivelis* in Japan (Sakai et al. 2008), cultured striped catfish *Pangasianodon hypophthalmus* in Vietnam (Crumlish et al. 2002, Bartie et al. 2012), striped catfish in Indonesia (Sakai et al. 2009), walking catfish in Thailand (Kasornchandra et al. 1987, Suanyuk et al. 2013), and yellow catfish *Pelteobagrus fulvidraco* in China (Ye et al. 2009, Liu et al. 2010).

Channel catfish isolates of *Edwardsiella ictaluri* carry 2 plasmids, pEI1 (4807 bp) and pEI2 (5643 bp) (Lobb & Rhoades 1987, Speyerer & Boyle 1987, Newton et al. 1988), both of which have been sequenced (Fernandez et al. 2001). The plasmid profiles of *E. ictaluri* previously isolated from non-catfish species, however, carried different sized plasmids (Newton et al. 1988, Reid & Boyle 1989) that hybridized to probes derived from pEI1 or pEI2, indicating that similar genetic information is carried on differently sized *E. ictaluri* plasmids. Isolates of *E. ictaluri* from channel catfish in the USA are also homogenous by biochemical (Waltman et al. 1986, Plumb & Vinitnantharat 1989, Liu et al. 2010) and serological (Plumb & Klesius 1988, Plumb & Vinitnantharat 1989, Bertolini et al. 1990) analysis. In the present study, *E. ictaluri* isolated from striped catfish *Pangasianodon hypophthalmus* in Vietnam were characterized using biochemical and molecular techniques to analyze their biochemical, genetic, and serological profile, and compared to US isolates. Furthermore, the Vietnamese isolates were used to infect channel catfish head-kidney-derived macrophages (HKDM) cultures to determine their capacity for intracellular replication, because the ability to replicate in channel catfish HKDM is essential for virulence (Thune et al. 2007). In addition, an immersion challenge was used to determine their ability to infect and kill channel catfish.

MATERIALS AND METHODS

Bacterial isolates and culture conditions

Edwardsiella isolates from Vietnam used in this study are listed in Table 1 and isolates from the USA are listed in Table 2. All *E. ictaluri* and the *E. tarda* isolate were cultured at 28°C in porcine brain heart infusion broth (Becton Dickinson) or on trypticase soy agar with 5% sheep blood (BA; Remel Products) started from stocks stored at −80°C and passed less than 5 times. *Escherichia coli* XL1-Blue MRF (Agilent Technologies) was used as the host strain for cloning and was grown at 37°C in Luria Bertani (LB) broth with aeration or on LB plates (Becton Dickinson).

Table 1. *Edwardsiella ictaluri*. Vietnamese isolates from striped catfish *Pangasianodon hypophthalmus*

Isolate	Year isolated	Region of isolation	Farm no.
CAF E1	2008	Sóc Trăng	6
CAF E2	2006	Châu Phú, An Giang	1
CAF E3	2007	Ômôn, Cần Thơ	4
CAF E4	2006	Châu Phú, An Giang	2
CAF E5	2008	Kế Sách, Sóc Trăng	7
CAF E6	2006	Châu Phú, An Giang	3
CAF E7	2008	Thốt Nốt, Cần Thơ	8
CAF E8	2007	Ômôn, Cần Thơ	5
CAF E9	2009	Cần Thơ	11
CAF E10	2008	Phụng hiệp, Hậu Giang	9
CAF 258	2008	Phụng hiệp, Hậu Giang	10
33	2011	Ômôn, Cần Thơ	12
36-1	2011	Ômôn, Cần Thơ	13
36-2	2011	Ômôn, Cần Thơ	13
19s	2011	Thốt Nốt, Cần Thơ	14
e02	2011	Thốt Nốt, Cần Thơ	15
180	2011	Thốt Nốt, Cần Thơ	16
2s	2011	Thốt Nốt, Cần Thơ	14
2s8	2011	Thốt Nốt, Cần Thơ	14

Specific pathogen-free (SPF) channel catfish

Egg masses were obtained from commercial channel catfish producers with no history of ESC outbreaks, disinfected with 100 mg l^{-1} free iodine, and hatched in closed recirculating systems in the Louisiana State University SPF aquatic laboratory.

Table 2. *Edwardsiella* spp. US isolates from channel catfish *Ictalurus punctatus*

Species Region of isolation	Isolates
E. ictaluri	
Georgia	ATCC 33202
Louisiana	85-41, 88-108, 91-581, 93-146, 97-347
Mississippi	S03-830, S05-329, S05-518, S07-794, S07-885
E. tarda	
Louisiana	01-247

Fish were fed meal, 2 mm sinking, or 6.0 mm floating catfish feed, depending on size. Holding systems consisted of 350 l fiberglass tanks (n = 4) connected to 45 l biological bead filters (Aquaculture Systems Technologies). Water temperature was maintained at 28 ± 2°C, and water quality parameters (total ammonia nitrogen, total nitrate, pH, hardness, and alkalinity) were determined 3 times per week using a Hach aquaculture kit. Water quality was adjusted as necessary to maintain optimal conditions.

Bacterial identification

Presumptive identification was conducted by using Gram stain, cytochrome oxidase, catalase, and glucose fermentation in glucose motility deeps. The API-20E system (bioMérieux) was used for further biochemical analyses. Inoculation and interpretation of API-20E strips were done according to the manufacturer's instructions with the incubation of strips at 28°C for 48 h. To confirm the identification of the isolates as *Edwardsiella ictaluri*, the 16S rRNA gene was amplified from Vietnamese isolates CAF E3, CAF 258, 19s, and 2s, as well as the US isolate 93-146 and *E. tarda* 01-247, using primers SSU27 and SSU1492 as described by Pombert et al. (2009). Primers were used at a concentration of 0.5 mM. The cycling conditions were 1 cycle at 98°C for 30 s, 35 cycles of 98°C for 10 s, 57°C for 30 s, and 72°C for 45 s, followed by a final extension of 72°C for 5 min. Amplification of a single band in each reaction was verified by 0.6% agarose gel electrophoresis, and the amplified DNA was purified using the QIAquick PCR Purification Kit (Qiagen). Purified DNA was sequenced using the SSU27 and SSU1492 primers.

DNA techniques

Genomic DNA was purified from *Edwardsiella ictaluri* using the High Pure PCR Template Preparation Kit (Roche Applied Science). Plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen). All restriction enzymes were purchased from New England Biolabs. Polymerase chain reactions were done using Phusion High Fidelity DNA Polymerase (New England Biolabs). DNA sequencing was done using the BigDye terminator sequencing chemistry (Applied Biosystems) and a 3130 Genetic Analyzer from Applied Biosystems (Life Technologies). Sequence assemblies were constructed using Sequencher 5.0 (Gene Codes).

Plasmid profiles and sequencing

Plasmid DNA from each of the *Edwardsiella ictaluri* isolates was isolated using a Qiagen miniprep kit, and native plasmids were initially separated on 0.6% agarose gels. Subsequently, a mix of the plasmids from each isolate was cut with restriction enzymes that are known to cut pEI1 and pEI2 at single restriction enzyme sites, including *Bgl*II, *Dra*I, *Eco*RI, or *Nhe*I, and were separated on 0.6% gels. Based on the plasmid profiles, a representative plasmid of each size was cloned and sequenced. Briefly, plasmid DNA was digested with *Eco*RI, gel purified using a Qiagen gel extraction kit, cloned into the pBluescript vector (New England Biolabs), and transformed to *E. coli* XL1-Blue MRF by electroporation. Electroporants were selected on LB agar plates containing 200 µg ml^{-1} of ampicillin (Amp), 40 µg ml^{-1} X-gal, and 0.1 mM IPTG. Plasmid DNA from positive clones was purified as described above and the size was confirmed by *Eco*RI digestion and agarose gel electrophoreses. DNA sequencing was completed by primer walking into the cloned plasmids starting with the T3 and T7 primers of the pBluescript cloning vector. Open reading frames (ORFs) were predicted using ORF Finder (www.ncbi.nlm.nih.gov/gorf/gorf.html). Each predicted protein was analyzed using BlastP (http://blast.ncbi.nlm.nih.gov/Blast.cgi) with a minimum cut-off of 30% identity over 80% length coverage. Gene sequences were further compared and aligned with GenBank data using CLUSTAL W (www.ebi.ac.uk/clustalw).

Virulence gene amplification

Amplification of suspected and known virulence genes in *Edwardsiella ictaluri* was done for isolates CAF E3, CAF 258, 19s, 2s, and 93-146 using primers specific to the type III secretion system (T3SS) gene *esrC*, the type VI secretion system (T6SS) gene *evpC*, the type IV secretion system (T4SS) gene *virD4*, and genes *ureA–C* of the urease operon using primers listed in Table 3 at a concentration of 0.5 mM. Cycling conditions for each reaction were 1 cycle of 98°C for 30 s, 35 cycles of 98°C for 10 s, 56°C for 30 s, and 72°C for 2 min, followed by a final extension of 72°C for 5 min. Because the Vietnamese plasmid profiles lacked a pEI2 homologue in some isolates, and because pEI2 encodes an operon containing a putative T3SS effector, *eseI*, and its chaperone, *escD*, in US isolates (Fernandez et al. 2001, Thune et al. 2007), PCR was used to evaluate the possible absence of

Table 3. Oligonucleotides used as PCR primers to amplify representative genes of virulence-related pathogenicity islands of *Edwardsiella ictaluri*. T3SS: Type III secretion system; T4SS: Type IV secretion system, T6SS: Type VI secretion system

Primer	Pathogenicity island	Sequence
5′*esrC*	T3SS	5′-CGTTCATGGCTGCCACAG-3′
3′*esrC*		5′-AAACAGGAGGGTACAGGC-3′
5′*evpC*	T6SS	5′-ATGCCAAGTGGAATTCGCTG-3′
3′*evpC*		5′-CACCGCTTTGGCCATATTGA-3′
5′*virD4*	T4SS	5′-GTTGGCGGGTGTGTTTATCGTT-3′
3′*virD4*		5′-TCAGATTACGGGTCAGCTCGTT-3′
5′*ureA*	Urease enzyme	5′- CACCTGTAGATTTCAGCG-3′
3′ureC		5′-GACAGAGCATGATAAGCC-3′
5′eseI	T3SS effector	5′-ATGTTACCTATCAACCGCATCA-3′
3′*eseI*		5′-TGGGATGAAGACTCGCCGTACAGTGGAGGC-3′
5′escDeseI	T3SS effector and chaperone	5′-GTACCAACGCCGACTAATCCCTAACGCCTCCCAC-3′
3′*esdDeseI*		5′-TGGGATGAAGACTCGCCGTACAGTGGAGGC-3′

these virulence factors in Vietnamese isolates of *E. ictaluri.* Primers listed in Table 3 were used to amplify an internal fragment of *eseI*, and a second set to amplify from *eseI* into *escD* to confirm a similar operon structure. Total DNA of *E. ictaluri* 93-146 was used as a positive control for amplification. Amplification parameters were as follows for all primer pairs: 2 min denaturation at 94°C for 1 cycle, followed by 35 cycles of 94°C for 20 s, 56°C for 30 s, 72°C for 4 min, followed by a final extension for 5 min at 72°C. Amplified products were confirmed by size and DNA sequencing.

Genomic fingerprinting

Repetitive sequence based PCR (rep-PCR) was conducted using the ERIC1R, ERIC2, and BOXA1R primers described by Versalovic et al. (1991, 1994). For each reaction, 200 ng of genomic DNA was used. The 25 µl reactions were run using Phusion Buffer HF supplemented with 3% (v/v) DMSO and 0.5 mM $MgCl_2$. Primers were added to a final concentration of 20 pmol for each ERIC primer or 40 pmol for the BOX primer. Cycling conditions were as follows: 1 cycle 98°C for 30 s, 35 cycles of 98°C for 10 s, 40°C for 30 s, and 72°C for 2 min, and a final extension at 72°C for 10 min. Equal aliquots of each reaction were separated on a 1.2% agarose gel containing Sybr Safe DNA Gel Stain (Invitrogen). Gels were imaged under UV light, and analyses were performed using Quantity One Software (Bio-Rad Laboratories). Lanes and bands were identified and compared in order to generate dendrograms by the unweighted pair-group arithmetic average method as described by Soto et al. (2012).

Western blotting

Ten µl of overnight cultures (about 2×10^6 cells) of *Edwardsiella ictaluri* of each of the US isolates identified in Table 2, as well as Vietnamese isolates CAF 3, CAF 258, 19s, and 2s were pelleted by centrifugation for 6 min at 6000 × *g*, washed twice with sterile phosphate-buffered saline, resuspended in NuPage LDS sample buffer (Invitrogen), and boiled for 5 min. The resulting lysate was centrifuged for 10 min at 16 100 × *g* to remove debris. Ten µl of each lysate were separated on NuPage 12% SDS-PAGE gels (Invitrogen). Separated samples were transferred to a polyvinylidene fluoride membrane using the iBlot Dry Transfer System (Invitrogen). Immunodetection was done using the Ed9 monoclonal antibody raised against the lipopolysaccharide (LPS) of the *E. ictaluri* ATCC type strain 33202 (Ainsworth et al. 1986) in conjunction with horseradish peroxidase-conjugated goat anti-mouse IgG polyclonal antibody (Thermo Fisher Scientific). The Super-Signal West Pico Kit was used as substrate for chemiluminescent detection.

Antibiotic susceptibility

Antibiotic sensitivity testing was performed for all of the Vietnamese isolates listed in Table 1, plus US isolates 93-146 and S05-329 using the disk diffusion method (CLSI 2006) for sulfamethoxazole/trimethoprim 23.75/1.25 µg, enrofloxacin 5 µg, florfenicol 30 µg, oxytetracycline 30 µg, chloramphenicol 5 µg, and nitrofuratonin 300 µg.

Macrophage infection assays

Channel catfish HKDM were collected from 400 to 800 g SPF catfish and used in a gentamicin exclusion assay as described by Booth et al. (2006). Briefly, triplicate cultures containing approximately 1×10^5 HKDM were infected with complement-opsonized *Edwardsiella ictaluri* isolates CAF E3, CAF 258, 19s, and 2s from Vietnam, as well as US isolate 93-146, at a multiplicity of infection of 1 bacterium to 10 HKDM. After 30 min, extracellular bacteria were killed by exposure to 100 µg ml^{-1} gentamicin for 1 h, after which the infected cells were incubated in media containing a 1 to 2 µg ml^{-1} static dose of gentamicin, depending on the sensitivity of the *E. ictaluri* isolate to gentamicin. At 0 (1.5 h post infection), 5, and 10 h, cells were lysed with 1% Triton X-100, and the lysates were serially diluted and plated on BA for bacterial enumeration. Fold replication was calculated by dividing the number of bacteria present by the number present at time 0. Experiments were repeated 3 times to verify the consistency of the results.

Immersion challenge

SPF fingerling channel catfish (n = 450, 18 g each) were stocked at a rate of 25 $tank^{-1}$ into 20 l tanks (n = 18), and 3 tanks were randomly assigned to challenge with each of US isolate 93-146, or Vietnamese isolates CAF E3, CAF 258, 19s or 2s, or broth-only treatments. Cultures were grown to an OD_{600} of 1.73 to 1.95, and 80 ml of the cultures were diluted in 4 l of water in each of the tanks, resulting in a challenge dose of 1.1×10^8 to 7×10^8 ml^{-1}. The fish remained immersed for 1 h before water flow was restored to the tanks. Deaths were recorded for each 24 h period after experimental infection until 3 consecutive days passed without a death. Liver samples from all dead fish were streaked on BA plates and incubated at 28°C for 48 h to confirm the presence of *Edwardsiella ictaluri.*

RESULTS

Isolation and identification

In 2008 and 2009, nursery ponds in the Cantho and An Giang provinces in Vietnam experienced mortality in striped catfish. There were few external signs, but upon necropsy, multifocal white spots were observed in internal organs, including the liver, head kidney, and spleen. A Gram-negative, rod-shaped bacterium was isolated from the internal organs of diseased fish, and biochemical analyses of the bacterium resulted in a presumptive identification of *Edwardsiella ictaluri*. Using API-20E strips, *E. ictaluri* isolates CAF E1, CAF E6, CAF 258, 33, and 36-2 from striped catfish were positive for citrate utilization, resulting in an API code of 4204000, while the other isolates were negative for citrate utilization and had the typical 4004000 API code. Interestingly, all of the isolates were non-motile in glucose motility deeps. Amplification of the 16S rRNA gene and DNA sequencing, however, confirmed that the isolates were *E. ictaluri*. A single 1381 bp product from the 16S rRNA gene was amplified from each isolate tested, and alignment of the resulting sequences demonstrated that the Vietnamese isolates were 100% identical to the US *E. ictaluri* isolate 93-146. The *E. tarda* 01-247 isolate had 99.6% homology to the *E. ictaluri* isolates, with 5 different base pairs.

Plasmid profiles and sequences

Plasmid analysis of the 19 Vietnamese *Edwardsiella ictaluri* isolates demonstrated 4 different plasmid profiles that each differed from the profile of the US isolates (Fig. 1), all of which carried plasmids the size of the typical pEI1 and pEI2 profile (GenBank accession nos. AF244083 and AF244084, respectively; Fernandez et al. 2001). All Vietnamese isolates carried at least 1 small (<10 kb) plasmid. Some plasmid preps from Vietnamese isolates also contained larger plasmids greater than 10 kb in size. In every occurrence of the larger plasmid, there were 2 bands, 1 just above the 10 kb marker of the supercoiled ladder and the second well above the 10 kb marker, possibly representing supercoiled and nicked stages of the same plasmid. Because the primary interest was comparing the smaller plasmids from the Vietnamese isolates to pEI1 and pEI2, the 10 kb plasmid was not further analyzed. Restriction digest analysis showed that 13 isolates had only 1 small plasmid of approximately 4 kb, 1 isolate had only 1 plasmid of approximately 5.6 kb, similar to the size of pEI2 in 93-146, 4 isolates had 2 plasmids of approximately 4 kb and 5.6 kb, and the final isolate had 2 plasmids of 4 kb and approximately 9 kb. Based on the grouping of the isolates by plasmid profile, representatives of each plasmid group, CAF E3 (4 kb only), CAF 258 (5.6 kb only), 19s (4 kb and 5.6 kb), and 2s (4 kb and 9 kb) were subjected to further testing as described below.

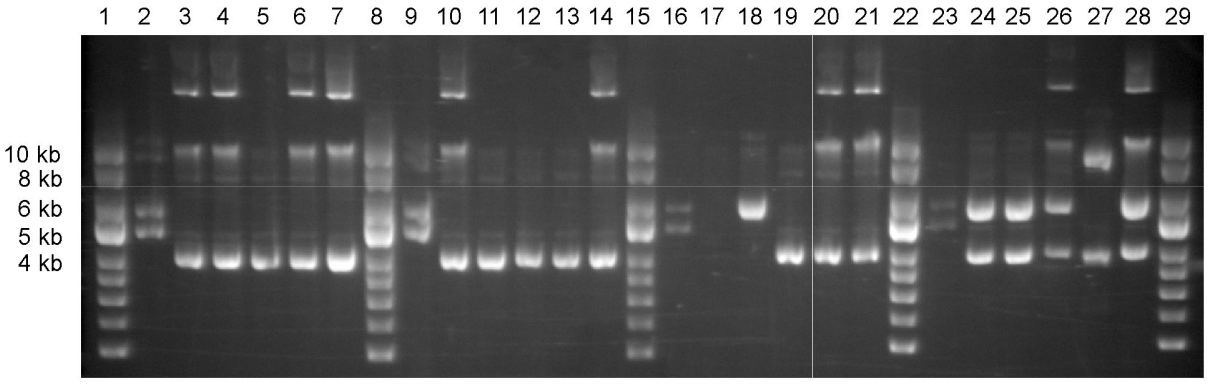

Fig. 1. *Edwardsiella ictaluri.* Uncut plasmid profiles from Vietnamese isolates. Lanes 1, 8, 15, 22, and 29, supercoiled DNA ladder; Lanes 2, 9, 16, and 23, plasmid preps from 93-146; Lane 17, *E. tarda* plasmid prep; Lanes 3–7, isolates CAF E1–CAF E5; Lanes 10–14, isolates CAF E6–CAF E10; Lane 18, isolate CAF 258; Lane 19, isolate 33; Lane 20, isolate 36-1; Lane 21, isolate 36-2; Lane 24, isolate 19s, Lane 25, isolate eO2; Lane 26, isolate 180; Lane 27, isolate 2s; Lane 28, isolate 2s8

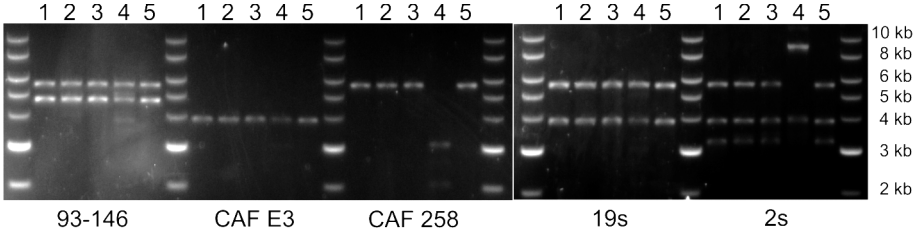

Fig. 2. *Edwardsiella ictaluri.* Restriction digestion of representative plasmids from *E. ictaluri* isolates. Plasmids from *E. ictaluri* isolates were digested with *Bgl*II (1), *Dra*I (2), *Eco*RI (3), *Nae*I (4), or *Nhe*I (5). Digests were separated on 0.6% agarose gels

Generally, the restriction enzymes that cut both pEI1 and pEI2 at a single site also cut each of the Vietnamese plasmids only once (Fig. 2). *Nae*I, however, cut the 5.6 kb plasmid of CAF 258 into at least 2 pieces, indicating at least 2 restriction sites in the plasmid, although the 5.6 kb plasmids of 93-146 and 19s were only cut once by *Nae*I. Digestion of the 9 kb plasmid of 2s with *Nae*I resulted in a single cut. Digestion of the 9 kb plasmid of 2s with the other 4 enzymes resulted in 2 bands, 1 of which was about 3 kb and the other of similar size to the 5.6 kb bands in the other isolates.

Sequencing results indicate that all isolates have a plasmid with some homology to pEI1 (Fig. 3) (Fernandez et al. 2001). Isolates CAF E3, 19s, and 2s contain plasmids with deletions of pEI1 orf2 and orf3, which encode a putative insertion element similar to IS402 and differ only by several nucleotides that are present or absent in the repeat sequence. The 2s-pEI1 sequence is available in GenBank (accession no. KC237288). Plasmid 2s-pEI1 and 19s-pEI1 also lost bp 2405 to 2428 and bp 2428 to 2439, respectively, that are present in CAF E3-pEI1 and contain sequences that are important for plasmid replication, incompatibility, and copy-number control in other plasmids (Lin & Meyer 1986). The pEI1-like plasmid in CAF 258 also has a deletion of orf2 and orf3, but a *tnpA IS26* transposase gene is inserted in their place. The CAF 258-pEI1 sequence is available in GenBank (accession no. KC237289). Importantly, all pEI1 based plasmids contain orf1, a putative effector of the *Edwardsiella ictaluri* T3SS that contains leucine-rich repeats (Fernandez et al. 2001) and is required for *E. ictaluri* virulence (Thune et al. 2007).

The only Vietnamese isolate that contains a pEI2 based plasmid (Fernandez et al. 2001) is 2s (Fig. 4A), which contains an extra 3461 bp compared with the typical pEI2. Isolates CAF E3, 19s, and CAF 258 do not contain a pEI2 derivative, which encodes the putative *Edwardsiella ictaluri* T3SS effector, EseI, and its chaperone, EscD. The genes *eseI* and *escD,* however, were amplified from genomic DNA from all isolates tested that did not carry a pEI2 based plasmid, and in the same operon structure as present on pEI2 (Fig. 4B). The 2s-pEI2 sequence is available in GenBank (accession no. KC286618). Database searches at the nucleotide level indicated that this region contains

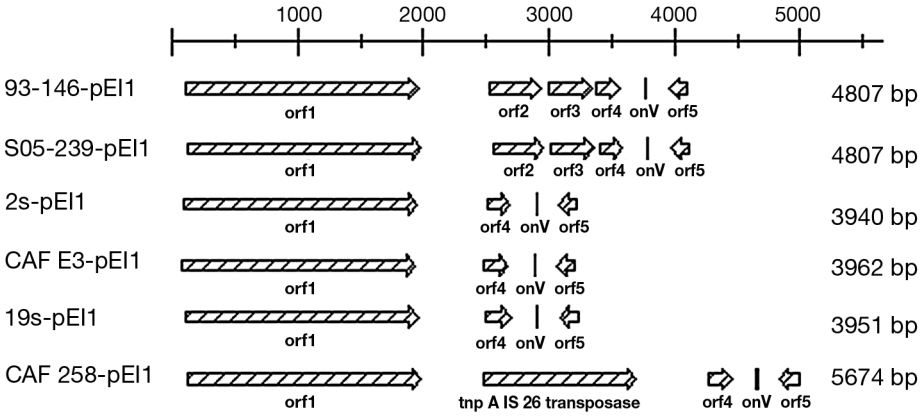

Fig. 3. *Edwardsiella ictaluri.* Plasmid maps of pEI1-based plasmids. Each open reading frame (orf) is drawn to scale. Base pair numbering begins from the *Eco*RI cut site that was used for cloning. Isolates 93-146 and S05-239 are US isolates from Louisiana and Mississippi, respectively. The Vietnamese isolate 2s is the isolate with 4 and 9 kb plasmids, CAF E3 represents the 13 isolates with a single 4 kb plasmid, 19s represents the isolates with 4 and 5.6 kb plasmids, and CAF 258 represents the single isolate with only a 5.6 kb plasmid

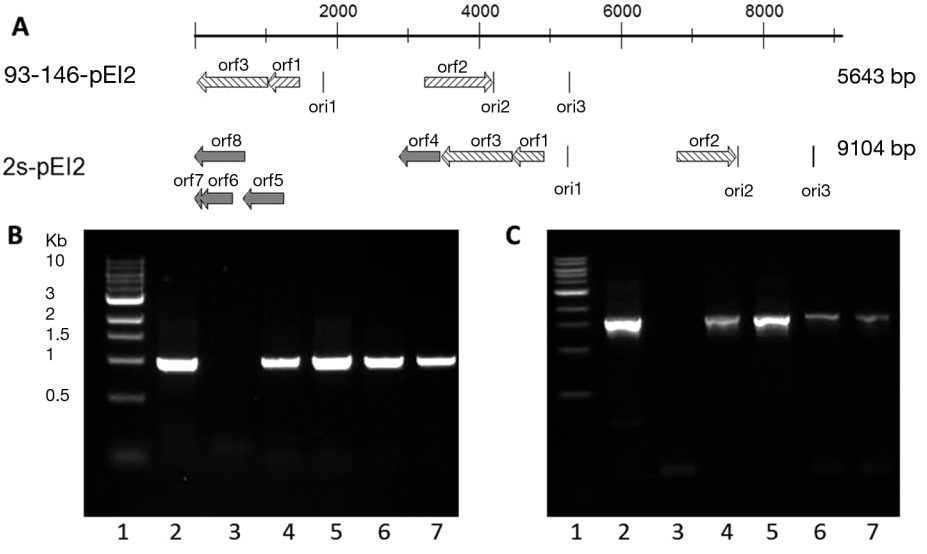

Fig. 4. *Edwardsiella ictaluri.* (A) Map of the pEI2-based plasmid. Base pair numbering begins from the *Eco*RI cut site that was used for cloning. (B,C) PCR detection of (B) *eseI* (expected 1019 bp) and (C) *eseDeseI* (expected 1748 bp). Lanes: 1, NEB 1 kb DNA ladder; 2, *E. ictaluri* 93-146; 3, no DNA control; 4, *E. ictaluri* 19s; 5, *E. ictaluri* CAF E3; 6, *E. ictaluri* CAF 258; 7, *E. ictaluri* 2s

5 ORFs with homology to proteins annotated in the protein database at the National Center for Biotechnology Information (NCBI) of the National Institutes for Health (www.ncbi.nlm.nih.gov) (Fig. 5A). Four of them are highly homologous to ORFs of *mobA*, *mobB, mobC*, and *mobD* from a variety of Gram-negative bacteria. The *mobA* gene encodes a relaxase/mobilization nuclease domain, while *mobB, -C*, and *-D* encode mobilization proteins. A putative origin of transfer (*oriT*) nick sequence was identified upstream of the *mob* genes (Fig. 5B). The fifth ORF identified encodes *repA*, a replication initiation protein.

Edwardsiella ictaluri 19s also contains a 5023 bp plasmid (designated pEI3) that does not have homology to either pEI1 or pEI2, and 9 ORFs were identified with homology to proteins in the NCBI database (Fig. 6). The 19s-pEI3 sequence is avail-

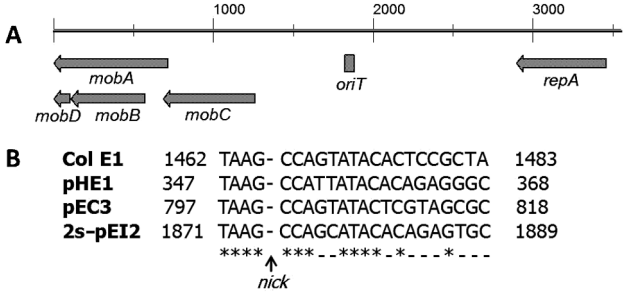

Fig. 5. *Edwardsiella ictaluri.* (A) Mobilization region of 2s-PEI2. The *mobA* gene encodes a relaxase/mobilization nuclease domain, while *mobB, -C*, and *-D* encode mobilization proteins. The fifth open reading frame identified encodes *repA*, a replication initiation protein. (B) Sequence alignments among the *nick* regions of plasmids Col E1, pHEl, pEC3, and 2s-pEI2. A putative origin of transfer (*oriT*) *nick* sequence proposed for 2s-pEI2 is located in the 15-bp TAA GCC AG/TC/T ATA CA/TC domain and is conserved in all plasmids

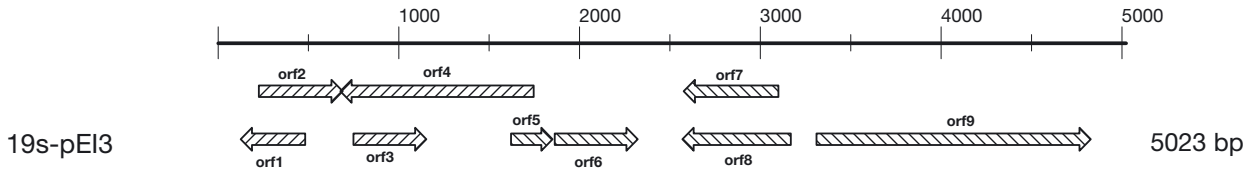

Fig. 6. *Edwardsiella ictaluri.* Plasmid maps of the trimethaprim resistance plasmid pEI3. Base pair numbering begins from the *Eco*RI cut site that was used for cloning. orf1: TnpM transposon modulator protein; orf2: cytosine-specific DNA methylase; orf3: hypothetical protein; orf4: integrase; orf5: aminoglycoside resistance; orf6: trimethoprim resistance; orf7: transposase; orf8: unknown protein; orf9: methyl-acceptin chemotaxis protein

able in GenBank (accession no. KC249996). orf1 has homology to a hypothetical protein with unknown function in *Escherichia coli* and *Corynebacterium* sp. The plasmid also encoded ORFs with homology to transposases, a transposon modulator protein, a DNA integrase, and 2 ORFs that encode antibiotic resistance genes. One of them has 100% identity over 92% coverage to a truncated AadA, the aminoglycoside resistance protein of *Salmonella enterica* and other Gram-negative bacteria. The second one has 99% identity over 97% coverage of dihydrofolate reductase (Dhfr1), which is important for thymidylate synthesis and DNA replication and confers a high level of resistance to the antibiotic trimethoprim.

Genomic fingerprinting

Rep-PCR amplification using the conserved primers ERIC1R and ERIC2 (Versalovic et al. 1991) and BOXA1R (Versalovic et al. 1994) showed homologous fingerprinting results from all 19 Asian and all 11 US isolates examined (data not shown). To demonstrate the pattern, the results of fingerprint analysis of the isolates chosen to represent the 4 plasmid profiles are presented in Fig. 7, with the fingerprint of the *Edwardsiella tarda* isolate being obviously different from the *E. ictaluri* isolates.

Virulence gene amplification

Amplification of genes representing *Edwardsiella ictaluri* virulence-related pathogenicity islands showed that the Vietnamese *E. ictaluri* isolates all contain the urease, T3SS, and T6SS genes. The T4SS gene, however, was not amplified from the Vietnamese *E. ictaluri* isolates (Fig. 8). A second set of primers for the type IV gene was used, but only confirmed that the Vietnamese isolates were negative (data not shown).

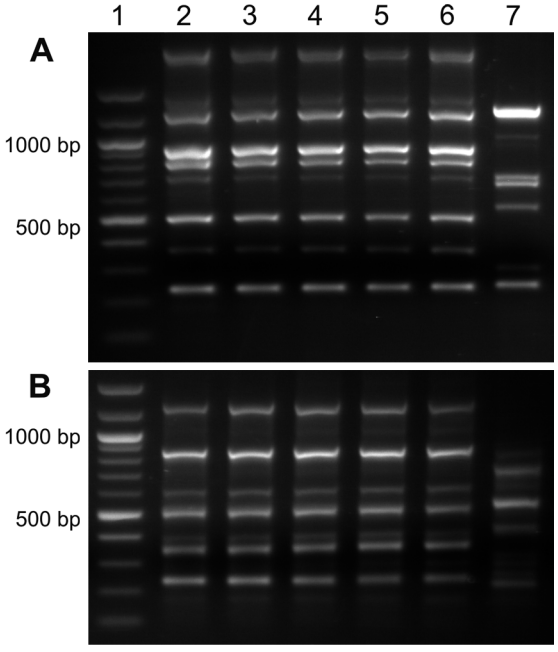

Fig. 7. *Edwardsiella* spp. Genomic fingerprinting pattern of selected isolates, with all *E. ictaluri* isolates having the same pattern, but *E. tarda* having a distinctly different pattern. Random PCR was conducted using (A) ERIC or (B) BOX primers. Lanes: 1, 100 bp ladder; 2, isolate CAF E3; 3, isolate CAF 258, 4, isolate 19s; 5, isolate 2s; 6, isolate 93-146; 7, *E. tarda* isolate 01-247

LPS serology

In addition to lacking *virD4*, the Vietnamese isolates were not recognized by Ed9, a monoclonal antibody generated against LPS from the *Edwardsiella ictaluri* ATCC type strain 33202 (Ainsworth et al. 1986) (Fig. 9). The antibody, however, was also not reactive against all of the US isolates from channel catfish. Only the US isolates ATCC33202, 85-41, 88-108, 91-581, and 93-146 were recognized by Ed9, while isolates 93-347, S03-830, and SO7-885 were not, indicating that there are differences in the LPS among US channel catfish *E. ictaluri* isolates. Based on this limited sample of isolates, the antibody was

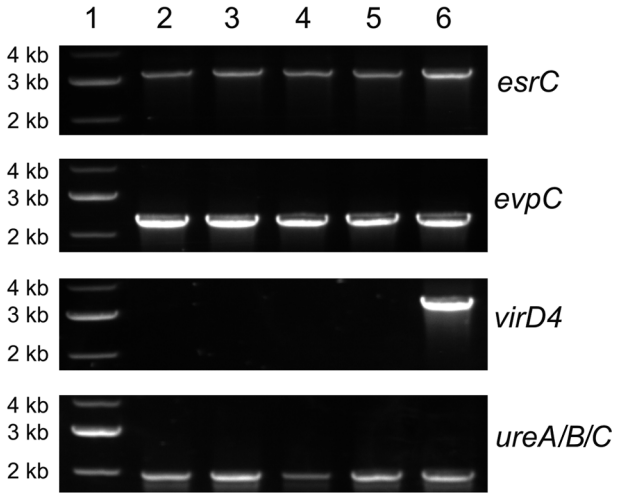

Fig. 8. *Edwardsiella ictaluri.* Presence of virulence factors. Genes representative of known *E. ictaluri* pathogenicity islands were amplified by PCR to determine their occurrence in Vietnamese isolates. Lanes: 1, 1 kb ladder; 2, isolate CAF E3; 3, isolate CAF 258; 4, isolate 19s; 5, isolate 2s; 6, isolate 93-146

only reactive against the 5 isolates from 1993 or before, whereas the 3 isolates from 1997 or later were non-reactive.

Antibiotic sensitivity

Table 4 shows antibiotic sensitivity for 2 US isolates and all of the Vietnamese isolates of *Edwardsiella ictaluri*. The US isolates were both sensitive to all 5 antibiotics. Eighty-nine percent of the 19 Vietnamese isolates were resistant to sulfamethoxazole/trimtoprim. All 19 isolates had reduced sensitivity to enrofloxacin, 47% were resistant to florfenicol, 31% to oxytetracycline, and 47% to chloramphenicol. All isolates were sensitive to nitrofuratonin.

Macrophage assay and immersion challenge

A channel catfish isolate of *Edwardsiella ictaluri,* 93-146, exhibited a 15-fold increase within 10 h following infection of channel catfish HKDM. The Vietnamese isolates, however, all replicated less than 4-fold in the same period (Table 5), indicating the ability to survive in HKDM, but a reduced

Table 4. *Edwardsiella ictaluri.* Antibiotic sensitivity of isolates based on the disk diffusion method (CLSI 2006). SXT: sulfamethoxazole/trimethoprim (23.75/1.25 µg); ENO5: enrofloxacin (5 µg); FFC30: florfenicol (30 µg); T30: oxytetracycline (30 µg); C5: chloramphenicol (5 µg); FM300: nitrofuratonin (300 µg)

Isolate	Antibiotic sensitivity (mm)					
	SXT	ENO5	FFC30	T30	C5	FM300
93-146	34	>40	40	30	26	30
S05-329	36	38	40	30	25	35
CAF E1	0	11	0	30	0	30
CAF E2	0	9	0	30	0	32
CAF E3	0	10	>40	32	28	34
CAF E4	0	0	0	0	0	30
CAF E5	0	15	0	34	0	36
CAF E6	0	12	0	36	0	38
CAF E7	>40	10	>40	34	32	40
CAF E8	0	10	>40	40	30	39
CAF E9	0	12	0	0	0	36
CAF E10	0	12	>40	40	28	36
CAF 258	0	20	0	40	0	40
33	0	20	>40	36	30	32
36-1	0	20	0	36	0	30
36-2	0	22	0	40	0	40
19s	0	12	>40	0	22	30
e02	0	22	>40	0	24	32
180	0	30	>40	0	30	38
2s	>40	30	>40	35	30	26
2s8	0	22	>40	0	24	36

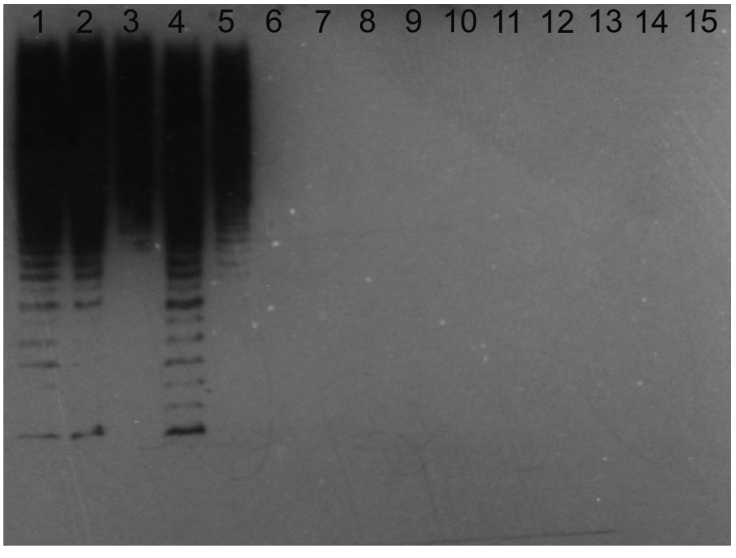

Fig. 9. *Edwardsiella ictaluri.* Western blot of lipopolysaccharide with Ed9 monoclonal antibody. Boiled whole cell lysates of isolates from the US and Vietnam were analyzed by Western blot using a monoclonal antibody derived against the *E. ictaluri* ATCC type strain 33202. Lanes 1–11, US isolates 93-146, ATCC 32202, 85-41, 88-108, 91-581, 97-347, S03-830, S05-329, S05-518, S07-794, and S07-885; Lanes 12–15, Vietnamese isolates CAF E3, CAF 258, 19s, and 2s

Table 5. *Edwardsiella ictaluri.* Intracellular replication of isolates. Values are the mean ± SE fold replication in head-kidney-derived macrophages for the combined data from 3 independent experiments, with 3 replicate wells per treatment per experiment. Values within a column with the same superscript are not significantly different ($p > 0.05$)

Isolate	5 h	10 h
93-146	5.0 ± 0.5 ^{A}	14.8 ± 1.8 ^{A}
CAF E3	3.0 ± 0.5 ^{B}	3.8 ± 0.4 ^{B}
CAF 258	2.0 ± 0.4 ^{B}	2.6 ± 0.6 ^{B}
19s	2.7 ± 0.4 ^{B}	3.1 ± 0.5 ^{B}
2s	2.0 ± 0.3 ^{B}	2.7 ± 0.5 ^{B}

competence for replication. Furthermore, immersion challenge of channel catfish with the US isolate 93-146 resulted in 35% mortality after 14 d (Fig. 10), while 3 of the Vietnamese isolates did not cause any mortality, and the fourth, 19s, caused only a single mortality.

DISCUSSION

Historically, *Edwardsiella ictaluri* has been primarily a pathogen of channel catfish cultured in the US. Recent isolation of *E. ictaluri* following disease outbreaks in Asia demonstrates an emerging distribution for this pathogen. It is not known, however, whether the recent outbreaks in Asia are caused by isolates similar to those that infect channel catfish in the US. Here we have demonstrated that the 19 isolates from striped catfish in Vietnam are biochemically and phenotypically very similar to US isolates, differing only in the utilization of citrate and in lacking motility. The citrate-positive phenotype, which results in an API code of 4204000, is unusual for channel catfish isolates of *E. ictaluri*, which typically do not utilize citrate and have an API code of 4004000. *E. ictaluri* isolates from zebrafish, however, also demonstrated citrate utilization (Hawke et al. 2012). Although *E. ictaluri* is typically motile, Bartie et al. (2012) described *E. ictaluri* isolates from Vietnam as non-motile, but did not provide API codes for comparison so that citrate utilization could not be determined. Identification as *E. ictaluri* in the work presented here was confirmed in selected isolates by 100% homology of the 16S rRNA sequences.

The plasmid profile of *Edwardsiella ictaluri* isolated from channel catfish in the US is homogeneous (Lobb & Rhoades 1987, Speyerer & Boyle 1987, Newton et al. 1988), including all of the isolates listed in Table1, and consists of the 4807 bp pEI1 and the 5643 bp pEI2 (Fernandez et al. 2001). Isolates from other fish species, however, show some diversity in their plasmid profiles. Reid & Boyle (1989) studied the plasmid profiles of non-channel catfish *E. ictaluri* isolates and found that probes derived from pEI2 hybridized to plasmids about 5.6 kb in size from non-channel catfish *E. ictaluri* isolates, whereas probes derived from a mix of pEI1 and pEI2 bound both the 5.6 and 4.8 or 4.0 kb plasmids in the non-channel catfish isolates. This suggests that *E. ictaluri* plasmids 4.0 or 4.8 kb in size can carry homologous DNA, and all plasmids about 5.6 kb in size are also homologous. Whereas Reid & Boyle (1989) found only 1 isolate without a 5.6 kb plasmid, 14 of the 19 isolates from Vietnam did not carry a 5.6 kb plasmid. None of the Vietnamese isolates carried a 4.8 kb plasmid, but 18 of 19 isolates carried a 4 kb plasmid. Sequencing representative plasmids of the 4 profiles identified all but one of them as derivatives of pEI1 and pEI2, although the single isolate with homology to pEI2 also carried an additional 3461 bp encoding a mobilization region that could mediate conjugative transfer. Bartie et al. (2012) described 3 apparent plasmid profiles for *E. ictaluri* isolates from Vietnam including a 35 kb plasmid. The use of restriction enzyme digestion to linearize

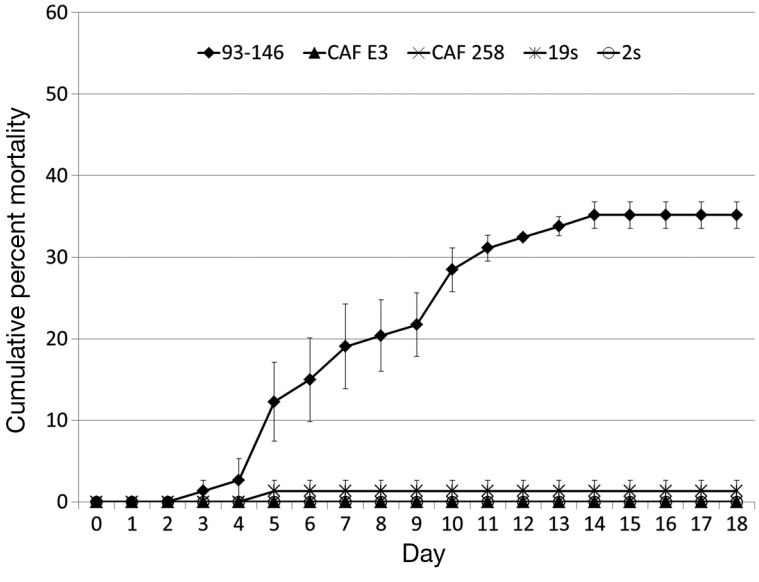

Fig. 10. *Ictalurus punctatus.* Channel catfish daily mean (±SE) cumulative percent mortality following immersion challenge with the *Edwardsiella ictaluri* US isolate 93-146 and the Vietnamese isolates CAF E3, CAF 258, 19s, and 2s to determine their ability to cause a lethal infection

both nicked and supercoiled forms of the plasmids, however, results in a more accurate determination of size and makes it difficult to compare to the previous results, which appear to include both nicked and supercoiled forms.

The level of antibiotic resistance, in which 3 isolates were resistant to 3 of the 6 antibiotics, 7 were resistant to 4 of 6, and 2 were resistant to 5 of 6, potentially indicates extensive use of antibiotics in Vietnamese aquaculture or in aquaculture waters in the area. Of particular concern are the indications of chloramphenicol use, with resistance in 9 of the 19 isolates. Chloramphenicol use is banned in many countries because of the association of oral dosing in humans with irreversible bone marrow toxicity, and the development of aplasia and fatal anemia (Bywater 1991). In contrast to our results, Bartie et al. (2012) described multi-resistance to oxytetracycline, oxalinic acid, and sulfamethoxazole/trimtoprim in 69% of their isolates, with some remaining isolates having resistance to oxalinic acid alone, but none with resistance to amoxicillin, enrofloxacin, or florfenicol. Antibiotic resistance patterns in the current study, however, did not correlate to the presence or absence of any particular plasmids, including the >10 kb plasmid that was not further evaluated. For example, isolate 19s was resistant to the potentiated sulfonamide, presumably due to Dhfr1A encoded by pEI3. The plasmid in CAF E3, however, does not contain the plasmid-encoded antibiotic resistance gene, but is also highly resistant to sulfamethoxazole/trimtoprim.

Further analysis using rep-PCR with the ERIC and BOX primers indicated no differences in the genomic fingerprints, further substantiating that the isolates are homogeneous outside of their plasmid content. Griffin et al. (2011) surveyed diagnostic isolates from channel catfish in Mississippi using the same rep-PCR based ERIC (Versalovic et al. 1991) and BOX (Versalovic et al. 1994) primer sequences, finding few differences among the isolates. Bader et al. (1998) identified 4 genotypes of *Edwardsiella ictaluri* from channel catfish from several states in the southeastern USA using only ERIC primer II, whereas biochemical analyses showed no differences among the isolates. There was no correlation of genotype to geographical location. A similar study was conducted on *E. ictaluri* isolated from ayu in Japan using amplified-fragment length polymorphism analysis (Sakai et al. 2009), and of *E. ictaluri* isolates tested from Japan, Indonesia, and the USA, the Japanese and Indonesian isolates clustered differently from the US isolates, suggesting geographical divergence of the Asian and US isolates. A recent comparison of Vietnamese and US isolates of *E. ictaluri* (Bartie et al. 2012), using either rep-PCR genomic fingerprinting with their own $(GTG)_5$ primer design or macro-restriction analysis, demonstrated little discriminatory value within geographical regions, while macrorestriction analysis suggests that *E. ictaluri* originating in Vietnam constitutes a unique genetic group compared to US isolates.

Using primers designed against suspected and known *Edwardsiella ictaluri* virulence factors, a difference between the Vietnamese and US isolates was observed. Whereas primers for the pathogenicity islands that encode the T3SS, the T6SS, and the urease enzyme amplified representative genes, primers against the T4SS gene *virD4* did not, suggesting that the Asian isolates do not carry the *virD4* gene or possibly the entire T4SS locus. The T4SS is an important virulence factor for some bacterial pathogens (Juhas et al. 2008, Alvarez-Martinez & Christie 2009, Llosa et al. 2009), but mutation of *virD4* in *E. ictaluri* does not reduce intracellular replication or significantly reduce virulence in channel catfish (R. L. Thune et al. unpubl. data), although VirD4 may not be essential for T4SS function in *E. ictaluri*. Further testing is required to establish the presence of the full pathogenicity islands and their functionality.

The inability of the Ed9 anti-LPS monoclonal antibody to react with the Asian *Edwardsiella ictaluri* isolates indicates a difference in the LPS structure of these isolates. However, the finding that other US channel catfish *E. ictaluri* isolates are also non-reactive suggests that the difference in structure may not affect virulence. Because the monoclonal antibody recognizes a single epitope, the difference between the LPS structures may be minor. This finding does provide an indication that subtle heterogeneity may occur in the LPS of *E. ictaluri* isolates within the USA and elsewhere. The LPS results suggest that a change in LPS may have occurred between 1993 and 1997, as all 5 isolates between 1977 and 1993 reacted positively with the antibody, while the 3 isolated after 1997 were negative. A larger sample of isolates is required to determine whether this difference in reactivity with Ed9 is uniform, and further studies are required to determine whether the difference is significant relative to protection following vaccination, as LPS is a major Gram-negative bacterial antigen, and antigenic heterogeneity might preclude cross protection between different LPS strains.

The inability of the Vietnamese *Edwardsiella ictaluri* isolates from striped catfish to replicate as efficiently as the US isolate in HKDM or to cause mortality in channel catfish indicates that these isolates

have reduced or no virulence in channel catfish. In comparison, Dung et al. (2012) reported invasion of the FHM, CHSE-214, and R1 fish cell lines rather than macrophages, but did not evaluate replication. It is unlikely that the lack of the T4SS gene *virD4* is responsible for this virulence deficiency because a US isolate carrying a mutation in *virD4* retains its ability to replicate in HKDM (R. L. Thune et al. unpubl. data). It is unknown whether a channel catfish isolate of *E. ictaluri* would be pathogenic to catfish species cultured in Asia.

In summary, these data demonstrate that the *Edwardsiella ictaluri* outbreaks occurring in Asia are caused by *E. ictaluri* isolates with minor differences in genetic background than those from US channel catfish. The low levels of intracellular replication and lack of virulence *in vivo* suggest that the isolates may not be a threat to US channel catfish production, although the possible transfer of antibiotic resistant isolates would be problematic. The presence of the 2 secretory systems, however, could provide insight into differential virulence. In general, the apparatus of T3 and T6 secretion systems, the structural component that spans the bacterial envelope, is highly conserved in a number of bacterial pathogens. They function in conjunction with the translocon proteins to allow bacteria that are in contact with either a plasma or phagosomal membrane to inject specialized effector proteins across that membrane. Despite the conserved nature of the secretion machinery, the actual arsenal of proteins delivered to the host cell is unique to each pathogen. Thus, the effect of the translocated proteins on the host varies depending on the pathogen in question, and is defined by the particular set of effectors produced by that pathogen. Although the 2 effectors known to be encoded on the *E. ictaluri* plasmids (Fernandez et al. 2001) are present in the Vietnamese isolates, albeit 1 on the chromosome, not the plasmid, the presence or absence of other effectors is unknown.

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