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Vibrio vulnificus outbreaks in Dutch eel farms since 1996: strain diversity and impact

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ABSTRACT: *Vibrio vulnificus* is a potentially zoonotic bacterial pathogen of fish, which can infect humans (causing necrotic fasciitis). We analysed 24 *V. vulnificus* isolates (from 23 severe eel disease outbreaks in 8 Dutch eel farms during 1996 to 2009, and 1 clinical strain from an eel farmer) for genetic correlation and zoonotic potential. Strains were typed using biotyping and molecular typing by high-throughput multilocus sequence typing (hiMLST) and REP-PCR (Diversilab®). We identified 19 strains of biotype 1 and 5 of biotype 2 (4 from eels, 1 from the eel farmer), that were subdivided into 8 MLST types (ST) according to the international standard method. This is the first report of *V. vulnificus* biotype 1 outbreaks in Dutch eel farms. Seven of the 8 STs, of unknown zoonotic potential, were newly identified and were deposited in the MLST database. The REP-PCR and the MLST were highly concordant, indicating that the REP-PCR is a useful alternative for MLST. The strains isolated from the farmer and his eels were ST 112, a known potential zoonotic strain. Antimicrobial resistance to cefoxitin was found in most of the *V. vulnificus* strains, and an increasing resistance to quinolones, trimethoprim + sulphonamide and tetracycline was found over time in strain ST 140. Virulence testing of isolates from diseased eels is recommended, and medical practitioners should be informed about the potential risk of zoonotic infections by *V. vulnificus* from eels for the prevention of infection especially among high-risk individuals. Additional use of molecular typing methods such as hiMLST and Diversilab® is recommended for epidemiological purposes during *V. vulnificus* outbreaks.

KEY WORDS: Vibriosis · Biotyping · Molecular typing · *Anguilla anguilla* · Clinics · Zoonosis · Antibiotics · Necrotic fasciitis

INTRODUCTION

Vibrio vulnificus is a heterogeneous halophilic Gram-negative facultative aerobic bacterium from the family *Vibrionaceae,* which thrives in warm coastal waters and sewage (Cañigral et al. 2010) and sediment (DePaola et al. 1994) and has also been isolated from various invertebrates (Coleman et al. 1996, Hervio-Heath et al. 2002, Cañigral et al. 2010). Additionally, *V. vulnificus* has been isolated from diseased fish from brackish and marine waters, mainly from severely diseased eels (Kusuda et al. 1979, Biosca et al. 1991, Høi et al. 1998, Fouz et al. 2006, Esteve & Alcaide 2009), but also from diseased tilapia *Oreochromis niloticus* (Sakata & Hattori 1988, Bisharat et al. 1999, Fouz et al. 2002, Chen et al.

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2006), carp *Cyprinus carpio* (Bisharat et al. 1999, Mouzopoulos et al. 2008) and from various sea fish species (DePaola et al. 1994, Mouzopoulos et al. 2008, Rajapandiyan et al. 2009). In humans, *V. vulnificus* can be a zoonotic invasive pathogen (Austin 2010). It can cause severe disease, particularly when the immune system is compromised. Typically, disease in humans occurs after ingestion of contaminated shellfish (Baker-Austin et al. 2010) or after contact of injured skin with contaminated seawater or fish. The result may be primary wound infection, gastroenteritis and septicaemia, including necrotic fasciitis (Dalsgaard et al. 1996, Chiang & Chuang 2003, Kuo et al. 2007, Dijkstra et al. 2009), with a mean lethality of >50% when septicaemia occurs (Koenig et al. 1991, Chuang et al. 1992)

Strains of *Vibrio vulnificus* have been classified into 3 biotypes based on biochemical characteristics (Tison et al. 1982, Bisharat et al. 1999). Biotypes 1 and 2 include environmental (e.g. water, eels) and clinical isolates (Tison et al. 1982, Tsai et al. 1990, Roig & Amaro 2009). Biotype 1 has been isolated from seawater (Veenstra et al. 1993a,b, Hor et al. 1995, Hsueh et al. 2004), eels and oysters (Coleman et al. 1996) but has thus far been considered non-pathogenic for eels (Biosca et al. 1996, Coleman et al. 1996), although it can cause disease in tilapia and carp (Sakata & Hattori 1988). Biotype 2 has mainly been found in diseased eels (Tsai et al. 1990, Biosca et al. 1991). Biotype 3 was mainly isolated from tilapia in Israel and once in carp (Bisharat et al. 1999). In humans, biotype 1 was isolated mostly as a foodborne pathogen (Tison et al. 1982), while biotypes 2 (Amaro & Biosca 1996, Dijkstra et al. 2009) and 3 (Bisharat et al. 1999, 2007) were isolated from human wound infections, all associated with disease.

In the Netherlands, *Vibrio vulnificus* biotype 1 has been isolated from marine coastal water samples (Veenstra et al. 1993b, 1994) and biotype 2 from humans with severe systemic infections (Veenstra et al. 1992, 1993a,b, Dijkstra et al. 2009). Previous reports on *V. vulnificus* contact zoonosis in the Netherlands (Veenstra et al. 1992, 1993b) described a patient with injured hands being infected with *V. vulnificus* after cleaning eels caught in a river. The isolated *V. vulnificus* strain biochemically resembled that of a *V. vulnificus* isolate from severely diseased eels in a Dutch seawater fish farm in 1987 (E. Liewes unpubl.), showing eels with pink-red fins, petechial haemorrhages, swollen spleen, red anus and mortality. Both isolates were considered to be biotype 2 (Veenstra et al. 1992).

The subtyping into 3 biotypes, based on differences in biochemistry, genetics, serology, and host range was critically reviewed by Sanjuán & Amaro (2007), who developed a PCR for detection of biotype 2 strains with simultaneous discrimination of zoonotic strains. Rapid, sensitive and accurate identification methods for zoonotic *V. vulnificus* strains were needed (Sanjuán & Amaro 2007, Sanjuán et al. 2009). Sanjuán et al. (2011) reported that sequence-based typing of 115 *Vibrio vulnificus* strains of biotypes 1, 2 and 3 subdivided them into 3 phylogenetic lineages which did not correspond to biotypes, with biotype 2 being polyphyletic. They proposed reclassifying the so-called biotype 2 to a pathovar, which groups the strains with pathogenic potential to infect and develop vibriosis in fish.

As various *Vibrio vulnificus* strains were isolated from Dutch eel culture, their possible zoonotic potential for aquaculture professionals and their genetic correlation between eel farms was questioned. Fast and reliable tracing methods to test for potential zoonotic strains were needed. Therefore, in this study, strain biochemical identification and molecular typing is given for 23 *V. vulnificus* isolates from disease outbreaks in Dutch indoor eel culture. These strains were compared with the isolated human strain (Dijkstra et al. 2009) from the eel farmer suffering from necrotic fasciitis. Strains were identified as *V. vulnificus* by 16S rRNA gene sequence analysis, and typed by biochemical tests (biotyping) (Tison et al. 1982, Bisharat et al. 1999), REP-PCR (Diversilab®) typing and high-throughput multilocus sequence typing (hiMLST; Sanjuán et al. 2011, Boers et al. 2012).

MATERIALS AND METHODS

Necropsy, parasitology and virology

Diseased eels from Dutch eel farms were brought to the laboratory for routine diagnosis. The anamnesis was recorded. The eels were checked for clinical symptoms and mechanical external damage and were then anaesthetized with 2-phenoxy-ethanol (0.2–0.5 ml l^{-1}). In 16 of 23 batches of eels, the presence of parasites was examined in blood smears, gill tissues and intestinal contents using a light microscope at 20 to 400× magnification. The eels were decapitated, the body cavity of each eel was opened, and abnormalities were recorded. Additionally, the swimbladder of each eel was examined for the presence of the nematode parasite *Anguillicoloides crassus*. In case of a pale spleen and petechial haemorrhages, eels were sampled for virus isolation, and processed as described by Davidse et al. (1999). If

cytopathic effect occurred, viruses were further typed by immunological testing using indirect immunofluorescence antibody testing (iIFAT) for eel virus European X (EVEX; van Beurden et al. 2011), immune peroxidase monolayer assay (IPMA; unpublished but similar to the method described by Davidse et al. 1999) for eel virus European (EVE) or by PCR for anguillid herpesvirus 1 (AngHV-1) (Rijsewijk et al. 2005).

Bacteriology: isolation

If present, eel skin lesions were flamed and cut open with a sterile scalpel after which specimens were taken for culture from the lesions with sterile disposable innoculation loops. These specimens were inoculated onto brain heart infusion (BHI) agar (Acumedia Neogen) with 5% (v/v) defibrinated sterile sheep blood (Bio Trading Benelux), and onto *Cytophaga* agar (based on lab-lemco powder L29, Oxoid, Thermo Fisher Scientific). Furthermore, specimens of liver, spleen and kidney of all eels were taken with disposable innoculation loops and inoculated onto BHI agar with 5% (v/v) sheep blood. The inoculated agar plates from skin and internal organs were incubated at 22°C for a maximum of 7 d. The agar plates were examined daily for bacterial growth. If no bacterial growth was observed, or if there was multi-bacterial growth, incubation was terminated after 7 d. If there was a predominant or pure culture of bacteria from organs and/or skin lesions, the bacteria were typed biochemically and molecularly identified. *Vibrio vulnificus* strains were stored at −80°C in peptone with 30% v/v glycerol until further confirmatory testing.

Bacteriology: typing and (molecular) identification

Identification. At the Central Veterinary Institute (Wageningen UR), *Vibrio vulnificus* strains were biochemically typed according to Barrow & Feltham (1993) and Austin et al. (1997), and molecularly identified by 16S rRNA gene sequence analysis with the MicroSEQ® 500 16S rDNA PCR Kit system according to the manufacturer's instructions (Applied Biosystems®). Identification of the isolated *V. vulnificus* strains was also performed at the Laboratory for Infectious Diseases, Groningen, with the Vitek 2® system with ID Gram negative (GN) card according to the manufacturer's instructions (bioMérieux). If identification of the isolate by Vitek 2® was ambiguous, 16S rRNA gene amplification and sequencing were performed for confirmation.

Biotyping. *Vibrio vulnificus* strains were biotyped by 2 laboratories according to biochemical characteristics (Tison et al. 1982, Bisharat et al. 1999): ornithine decarboxylase, indole production, D-mannitol fermentation, D-sorbitol fermentation, citrate (Simmons), salicin fermentation, cellobiose fermentation and *o*-nitrophenyl-galactopyranoside (Media Products).

REP-PCR typing. At the Laboratory for Infectious Diseases, Groningen, REP-PCR (Diversilab®) Microbial Typing was performed, in order to examine whether this could lead to more information about the relatedness of the investigated strains. For REP-PCR (Diversilab®) typing, the *Salmonella* REP PCR kit was used according to the manufacturer's instructions (bioMérieux).

HiMLST typing. For MLST typing, the hiMLST method of Boers et al. (2012) using a 454 Junior sequencer (Roche) was employed to sequence the 10 housekeeping genes *glp, pntA, pyrC, mdh*, *gyrB, metG, purM, dtdS, lysA* and *tnaA* as described by Broza et al. (2012). The oligonucleotides used for PCR amplification reported in the standardized MLST scheme by Broza et al. (2012) were equipped with universal tails for the hiMLST protocol. Allele variant numbers and corresponding MLST types (STs) were obtained by performing queries in the *Vibrio vulnificus* MLST database (available at http://pubmlst.org/vvulnificus). The ATCC®27562™ type strain of *V. vulnificus*, isolated from human blood, was included in the typing and tested for reference.

Antibiotic susceptibility testing. Antibiotic susceptibility testing of the isolated *Vibrio vulnificus* was performed with the Vitek 2 system®, using the AST-N048 or AST-N141 card according to the manufacturer's instructions (bioMérieux). Additionally, for antimicrobial susceptibility testing of the strains from eel farm no. E2 only, a disc diffusion test according to Clinical and Laboratory Standards Institute guidelines (CLSI 2006) was performed on Mueller Hinton non-supplemented agar plates. These were inoculated with a 0.5 MacFarland suspension of a fresh pure culture of the *V. vulnificus* isolate in 0.9% NaCl, after which Neo sensitabs (Rosco, via Beldico) of amoxycillin (30 µg), trimethoprim + sulphadiazine (5.2 + 240 µg), doxycycline (80 µg), erythromycin (78 µg), tetracycline (80 µg) and oxolinic acid (10 µg) were placed onto the plates. After 24 h at 22°C or 37°C, plates were measured for diameter (mm) of inhibited bacterial growth per disc, and data were compared with CLSI norms of resistance (sensitive, intermediate or resistant to the antibiotic).

RESULTS

Clinical observations, parasites and viruses

A total of 23 *Vibrio vulnificus* isolates were collected by diagnostic isolation from 23 batches of diseased eels from 8 Dutch eel farms since 1996. The coded farm of origin, strain number, year and month of isolation, mortality and subtyping are presented in Table 1. The eels showed predominantly (deep) ulcers, fin damage, haemorrhages and full sepsis. Clinical signs of *V. vulnificus* infection in diseased eels differed between MLST types 112 and 140, both from eel farm no. E2, and are presented in Fig. 1. In 11 eel batches, low to high numbers of ectoparasites (*Chilodonella* spp., *Trichodina* spp., *Ichthyobodo* spp., *Gyrodactylus* spp. and/or *Pseudodactylogyrus* spp.) were found. No endoparasites were found. The presence of virus, tested in 7 of the 23 eel batches that showed clinical signs of virus infection (pale spleen and petechial haemorrhages) showed that eels associated with 3 isolates (vv11, -12 and -15) were AngHV-1 positive, while eels associated with isolate vv18 were EVE positive. One AngHV-1 positive batch of eels was also found positive for *Edwardsiella tarda* (isolate vv11). One batch of eels (associated with isolate vv27) had vibriosis caused by both *V. vulnificus* and *V. anguillarum*.

Identification, bio- and molecular typing of *Vibrio vulnificus*

All isolates were biochemically identified as *V. vulnificus*, confirmed by 16S rRNA gene analysis at 2 laboratories. The biotype and molecular typing re-

Table 1. *Vibrio vulnificus*. Results from 23 outbreaks in Dutch eel farms, with year and month of isolation, mortality (low: <25%; medium: ~25–50%; high: >50%) and results of bacterial subtyping methods of the isolates (Biotyping, REP-PCR [Diversilab®] typing and hiMLST typing). Water temperature varied from 23 to 26°C. CVI: Central Veterinary Institute (Wageningen); na: not applicable; nd: not done; ST: sequence type; vv: *V. vulnificus* strain

Farm no.	Strain ID	CVI ref. no.	Year, month	Mortality	Biotype	Diversilab® Cluster	hiMLST typing (ST)
E1	vv30	09011798-1	2009, Jul	Low	1	G6	143
E1	vv1	09000993	2009, Jan	High	1	G4	143
E2[a]	vv21	05012456	2005, Jul	Low	2	G1	112
E2	vv17	05013697	2005, Aug	Chronic	1	G2	140
E2	vv13	06035079	2006, Dec	High	1	G2	140
E2	vv12	07019197	2007, Jul	High	2	G1	112
E2	vv10[b]	07020948	2007, Aug	High	2	G1	112
E2	vv9	07022913	2007, Aug	High	2	G1	112
E2	vv8	07025302	2007, Sep	Chronic	1	G2	140
H2	vv20	07025836	2007, Sep	–	2	G1	112
E2	vv6	08000838	2008, Jan	Medium	1	G2	140
E2	vv5[c]	08001704	2008, Jan	Low	1	G2	140
E2	vv3	08010193A	2008, May	Chronic	1	G2	140
E2	vv19	08010193B	2008, May	Chronic	1	G2	140
E3	vv11[d]	07020313	2007, Aug	High	1	G6	142
E4	vv16	05021752	2005, Dec	Medium	1	G2	140
E4	vv14	05023023	2005, Dec	Medium	1	G2	140
E5	vv18	617670	2002, Nov	High	1	G5	141
E5	vv15	05022408	2005, Dec	Medium	1	G5	141
E6	vv23	380581	1996, Jan	High	1	G3	nd
E7	vv24	392069	1996, May	High	1	G8	137
E8	vv28	532741	1999, Jun	Medium	1	G3	139
E8	vv27[e]	478867	1998, Jan	High	1	G7	138
E8	vv29	562669	2000, Jul	Chronic	1	G3	139
na		ATCC®27562™	Type strain from human		1[f]	nd	003

[a]Eel farm 2 (E2) suffered a severe eel disease, and a severe zoonosis in the eel farmer after contact (Dijkstra et al. 2009)
[b]See Fig. 1a
[c]See Fig. 1b
[d]Eel suffering an infection with *V. vulnificus* and *Edwardsiella tarda*, with AngHV-1
[e]Eel suffering an infection with *V. vulnificus* and *V. anguillarum*
[f]Biotype 1, according to www.atcc.org

Fig. 1. *Anguilla anguilla*. Clinical pathology of diseased European eels infected with *Vibrio vulnificus* in Dutch indoor eel farms. (a) Eels infected with *V. vulnificus* biotype 2 (REP-PCR [Diversilab®] type 1, isolate vv10, MLST type 112; a zootonic strain) from farm E2 in 2007 (Table 1). The eels show abscesses developing from the interior, and full sepsis with haemorrhages. (b) Eels infected with *V. vulnificus* biotype 1 (Diversilab® type 2, isolate vv5, MLST type 140) from the same farm in 2008 (Table 1). The eels show deep ulcers developing from the exterior, haemorrhages and full sepsis

sults (REP-PCR and hiMLST) of the 24 *V. vulnificus* strains and the ATCC®27562™ type strain of *V. vulnificus* are presented in Table 1. Biotyping yielded groups of 19 biotype 1 strains and 5 biotype 2 strains (4 from eels, 1 from an eel farmer).

Analysis by REP-PCR (Diversilab®) typing resulted in 8 REP-PCR types, as presented in Fig. 2. REP-PCR type 1 contained the 5 biotype 2 strains (4 eel isolates and 1 human isolate of *Vibrio vulnificus* biotype 2 originating from eel farm no. E2, which suffered the zoonosis). HiMLST typing (Fig. 2), done on 24 strains, succeeded for 23 strains, but not for strain vv23. This method resulted in 8 MLST types, of which 7 (ST 137–143) were newly identified and after quality curation were accepted into the *Vibrio vulnificus* MLST database (http://pubmlst.org/vvulnificus). The human *V. vulnificus* strain was typed as MLST type 112, the same type as 4 of the *V. vulnificus* strains isolated from the eel farm with the zoonosis (see Table 1). Fig. 2 presents the minimal spanning tree of 23 of the 24 isolates based on hiMLST and Diversilab® results.

Antibiotic resistance testing

Table 2 presents the results of the antibiotic resistance testing with Vitek 2®, together with the results of the disc diffusion tests of strains from eel farm E2 only, with, as far as reported by the farmer and vet-

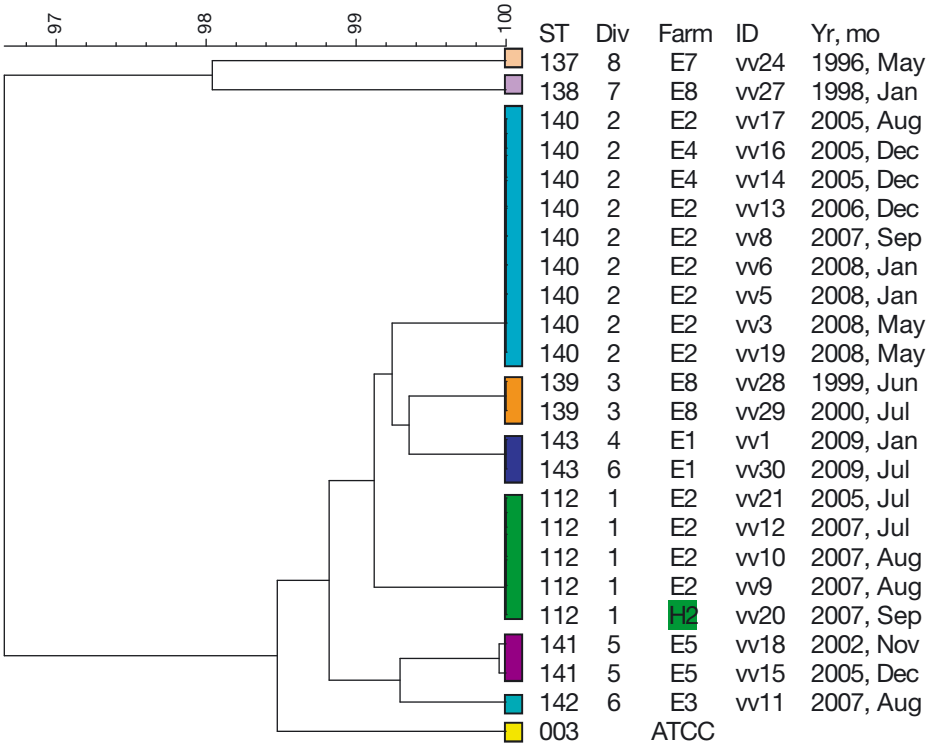

Fig. 2. *Vibrio vulnificus*. Minimal spanning tree based on molecular typing by hiMLST (Bisharat et al. 2007, Boers et al. 2012) and Diversilab® of 22 strains from eel outbreaks, and 1 (vv20) from a zoonotic case (necrotic fasciitis) in the involved eel farmer, and *V. vulnificus* type strain ATCC®-27562™. ST: MLST sequence type; Div: Diversilab® result; Farm column gives isolate source (E: eel; H: human) and farm no.; ID: *V. vulnificus* strain no.; Yr, Mo: year, month of isolation

erinarian, the related antibiotic treatments of the diseased eels at that farm.

DISCUSSION

In our study, both *Vibrio vulnificus* biotypes 1 and 2, but primarily biotype 1, were isolated from severely diseased eels from Dutch eel farms. The isolated biotype 1 strains showed a broad genetic diversity, Biotype 2 strains were all of 1 genotype (ST 112), and both biotypes were shown to be pathogenic to eels (Fig. 1) with different clinical pathologies. According to B. Fouz (pers. comm.), the eel disease clinical signs illustrated in Fig. 1a resemble those caused by *V. vulnificus* serovar E, a zoonotic strain from eels (Fouz & Amaro 2003), and those in Fig. 1b resemble those of serovar A, a strain from freshwater cultured eels only virulent for fish (Fouz et al. 2010). However, as we did no serotyping in this study, we cannot confirm

Table 2. *Vibrio vulnificus*. Vitek 2® and disc diffusion antibiotic resistance testing of 23 strains isolated from eels at various farms, and 1 from an eel farmer (vv20). Vitek 2® (left to right): cefazolin, cefoxitin, ciprofloxacin, norfloxacin, trimethoprim, trimethoprim/sulphamethoxazole. S: sensitive; I: intermediate sensitivity; R: resistant; X: not tested; Term: terminated. Disc diffusion assays (CLSI 2006) of strains isolated from eels of farm E2 were conducted at 22 and/or 37°C. 'Treatment' column shows antibiotic treatments confirmed to have been used (other antibiotics may also have been applied, but not reported). In the Vitek 2® assay, additionally, all strains were sensitive to ampicillin, amoxicillin/clavulanic acid, cefuroxim, axetil, ceftazidime, gentamicin, piperacillin (vv3 and vv18: X), piperacillin/tazobactam, ceftriaxone (vv3: X), cefepime (except vv14: R), imipenem, meropenem (vv3: X), amikacin (vv18: X), tobramycin (except vv15: I) and nitrofurantoin (except vv11 and vv18: I). In the disc diffusion assay, at 37°C, all strains were sensitive to amoxicillin, erythromycin and doxycycline (except vv4: R). TR+SU: trimethoprim + sulphadiazine; FLOR: florfenicol; FLU: flumequine; OXO: oxolinic acid; TET: tetracycline; i.v.: intravenous

Farm no.	Strain no.	Year, month	MLST type	Vitek 2®						Disc diffusion				Treatment
				Cefa	Cefox	Cipro	Norf	Trim	Trim/ sulf	TR +SU (22°C)	TR +SU (37°C)	TET (22°C)	OXO +FLU (22°C)	
E1	vv30	2009, Jul	143	S	R	S	S	S	S					
E1	vv1	2009, Jan	143	S	R	S	S	S	S					
E2	vv21	2005, Jul	112	S	R	S	S	S	S	S	S	S	S	TET (oral)
E2	vv17	2005, Aug	140	S	I	S	S	S	S	S	S	R	S	?
E2	vv13	2006, Dec	140	S	I	S	S	S	S	S	S	S	S	?
E2	vv12	2007, Jul	112	I	R	S	S	S	S	S	S	S	S	FLU (low dose), OXO (high) bath
E2	vv10	2007, Aug	112	I	R	S	S	S	S	S	S	S	R	FLU & TET bath
E2	vv9	2007, Aug	112	I	R	S	S	S	S	S	S	S	R	?
E2	vv8	2007, Sep	140	S	S	S	S	R	R	R	I	R	R	FLOR (S) bath
H2	vv20	2007, Sep	112	I	R	S	S	S	S	S	S	S	S	Doxycycline, ceftazidime, ciprofloxacin, clindamycin i.v. (Dijkstra et al. 2009)
E2	vv6	2008, Jan	140	S	S	S	S	R	R	R	R	R	R	?
E2	vv5	2008, Jan	140	S	S	S	Term	R	R	R	R	R	R	FLOR (S) bath
E2	vv3	2008, May	140	S	I	R	I	R	R	R	R	R	R	FLOR (S) bath
E2	vv19	2008, May	140	S	S	R	R	R	R	R	R	R	R	FLOR (S) bath
E3	vv11	2007, Aug	142	S	R	S	S	S	S					
E4	vv16	2005, Dec	140	I	R	S	S	S	S					
E4	vv14	2005, Dec	140	I	R	S	S	S	S					
E5	vv18	2002, Nov	141	I	R	S	S	S	S					
E5	vv15	2005, Dec	141	I	R	S	S	S	S					
E6	vv23	1996, Jan	nd	I	R	S	S	S	S					
E7	vv24	1996, May	137	I	R	S	S	S	S					
E8	vv28	1999, Jun	139	I	R	S	S	R	R					
E8	vv27	1998, Jan	138	I	R	S	S	S	S					
E8	vv29	2000, Jul	139	I	R	S	S	S	S					

assessment. In some cases, parasitic and viral infections were detected, but these were not related to the clinical symptoms seen in Fig. 1.

At eel farm E2 (Table 1), both biotypes of *Vibrio vulnificus* were found in the time period of the study, similar to Danish brackish-water eel farms (Høi et al. 1998, Dalsgaard et al. 1999). Until now, biotype 1 has been considered non-pathogenic to eels (Biosca et al. 1996). A great variety of biotype 1 strains was also found by Sanjuán et al. (2009) based on many isolates of *V. vulnificus*. They recommended using molecular typing to study the epidemiology of *V. vulnificus* infections (Sanjuán & Amaro 2007, Sanjuán et al. 2009). Although biotype 1 was considered to be primarily pathogenic to humans and mainly foodborne (Tison et al. 1982, Hsueh et al. 2004), based on our results of hiMLST, we found no identical published human strains in the database (http://pubmlst.org/vvulnificus). However, the isolated zoonotic biotype 2 strain ST 112 correlated perfectly to the ST 112 of the database.

The hiMLST typing revealed 8 different MLST types among the 24 isolates. The MLST and REP-PCR types showed a high concordance to each other. The 5 biotype 2 strains correlated exactly with the REP-PCR group 1 and MLST type 112. In the 19 biotype 1 strains, however, REP-PCR and hiMLST results mostly correlated well, but the 2 strains of REP-PCR group 6 belonged to 2 different MLST types (ST 142 and 143, respectively). Only 1 of the 8 MLST types was present in the online MLST database. The 7 other types carried 1 or more new allele variants, resulting in 7 new *V. vulnificus* MLST types. This suggests that many yet unknown *V. vulnificus* genotypes occur in eel farms. Most farms harboured a single genotype, and most genotypes were restricted to a single farm. At 2 eel farms, however, 2 different *V. vulnificus* strains were detected over time: at farm E8, ST types 138 (in 1998) and 139 (1999–2000), and at farm E2, ST types 112 (2005–2007) and 140 (2005–2008). The molecular similarity between the strains ST 140 at farm E2 (Fig. 2) suggests the persistence of this *V. vulnificus* strain at the farm over a 3 yr period. The MLST and REP-PCR types (Fig. 2) varied at different farms and over time. Possibly new strains were introduced into the farms over the years by transmission of strains which were acquired during stocking of new (wild) eels.

Antibiotic resistance profiling of the Dutch *Vibrio vulnificus* isolates (Table 2) showed that most *V. vulnificus* strains were resistant to cefoxitin, although this antibiotic had not been used in the eel farms. In addition, strains from the farm with the zoonotic case (E2) showed an increasing resistance to quinolones from August 2007 on, suggesting an acquired resistance after prolonged use of low-dose flumequine baths. Increased resistance of *V. vulnificus* can be explained by more or less frequent exposure to quinolone-related antibiotic treatment promoting the spread of more resistant bacteria (Ruiz 2003). The MLST type 140 strains from this farm suddenly acquired multi-resistance to several antibiotics including trimethoprim + sulphonamide, tetracycline and quinolones, from September 2007, the month in which the zoonosis occurred with the ST 112 strain. Improper antibiotic treatment of eel vibriosis or inadequate residue elimination at farms could favour the emergence of resistant strains (Roig et al. 2009). Fortunately, the *V. vulnificus* MLST type 112 strain was sensitive to most of the antibiotics tested, thereby providing a broad therapeutic option for treatment of the patient (Dijkstra et al. 2009).

In this study, the eel farmer developed a necrotic fasciitis from his *Vibrio vulnificus*-infected and diseased eels, as described by Dijkstra et al. (2009). From 23 *V. vulnificus* isolates from diseased eels, originating from 8 eel farms, 4 isolates from a single farm were MLST type 112 like the isolate from the related eel farmer (Table 1). Although *V. vulnificus*-related zoonotic cases are scarce, the impact can be high, especially in immuno-compromised patients (Dijkstra et al. 2009). The overall risk for warmwater eel culturists and related fish processors to acquire a contact zoonotic infection of *V. vulnificus* is dependent on human risk factors such as an underlying disease of the liver. Although the zoonotic strain (ST 112) was already present in diseased eels at eel farm E2 in July 2005 (Fig. 2), the eel farmer of that farm did not acquire the zoonosis until September 2007. This means that the eel farmer had already been at risk of acquiring a zoonosis for 2 yr, assuming he had intensive contact with infected eels and farm water. Possibly, skin injuries and a changed immunostatus of the farmer were reasons for acquiring the zoonosis. In a recent field study at 13 Dutch eel farms, no *V. vulnificus* was found on the skin of healthy eels (O. L. M. Haenen et al. unpubl.). This implies that the incidence of *V. vulnificus* infection in Dutch eel farms is low.

From a questionnaire in 2008 administered to various indoor Dutch fish farms (O. L. M. Haenen & I. Roozenburg unpubl. results), we know that employees of slaughter houses for indoor cultured fish sometimes work without gloves, resulting in frequent superficial hand injuries from tools. This fact, to-

gether with the lack of hygiene and awareness for zoonosis, creates a risk for contact zoonotic bacterial infections by bacteria such as *Vibrio vulnificus.* Often, patients do not mention or are not questioned about their contact with aquatic ecosystems or diseased fish in the anamnesis by their medical doctor (Aslam & Ng 2010). An additional risk is the potentially acquired resistance of *V. vulnificus,* as shown by MLST type 140 strains, probably resulting from chronic use of antibiotics as found in this study. More integrated work between the veterinary and medical branch on this subject is important to prevent human infections caused by *V. vulnificus.* Human infections with *V. vulnificus* are not yet notifiable in the Netherlands, although a proposal to make them notifiable has been made (Dijkstra et al. 2011). Human cases are notifiable in the USA (J. Evans pers. comm.).

In conclusion, our results show that since 1996, 23 *Vibrio vulnificus* variable strains were found in Dutch indoor eel farms associated with serious eel disease at 8 farms (approximately one-third of all Dutch eel farms), in relation to which 1 eel farmer suffered a severe *V. vulnificus* biotype 2 (ST 112) zoonosis. Biotyping, Diversilab® and hiMLST methods were all able to distinguish the zoonotic strain (biotype 2, REP-PCR type 1, ST 112) from the other strains. Seven new MLST types of *V. vulnificus* from eel were found and submitted to the database. The zoonotic potential of these new strains is not known. Both REP-PCR (Diversilab®) and hiMLST typing are recommended as epidemiological typing methods for the characterization of strains in veterinary and clinical laboratories. Resistance profiling of the isolates showed a diversity of antimicrobial resistance. Most *V. vulnificus* strains were resistant to cefoxitin, and increased resistance seemed to develop after prolonged treatment with quinolone-related antibiotics. Risk assessment and prevention are needed to protect farmers and processors of eels as well as brackish-water and marine fish. Virulence testing of *V. vulnificus* strains is recommended. Medics should be aware of the potential zoonotic risk of *V. vulnificus* infections in our temperate geographical zone associated with indoor eel farming.

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