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Vibrio vulnificus in Denmark isolation, identification and characterization

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Forord

Denne rapport præsenterer et forskningsprojekt vedrørende forekomsten af *Vibrio vulnificus* i Danmark. Projektet er finansieret af Miljøstyrelsen og Ministeriet for Fødevarer, Landbrug og Fiskeri.

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Summary

Introduction

Vibrio vulnificus, a halophilic marine vibrio, is an opportunistic human pathogen that can cause severe wound infections and septicemias with mortalities as high as 60 % (Oliver, 1989; Hlady & Klontz, 1996).

V. vulnificus can be isolated from a wide variety of aquatic ecosystems, but the occurrence of the organism is favored by high temperatures (>20°C) and intermediate salinities (15-25 ‰) (Motes *et al.,* 1998). In temperate areas, *V. vulnificus* is less abundant than in subtropical waters, but *V. vulnificus* has been isolated from coastal waters or implicated in human infections during the summer months in Denmark, Sweden, Germany, Holland, and Belgium (Mertens *et al.,* 1979; Veenstra *et al.,* 1994; Hoyer *et al.,* 1995; Melhus *et al.,* 1995; Dalsgaard *et al.,* 1996b). *V. vulnificus* has also caused disease in eel farms in Japan, Spain, Norway, Sweden, and Denmark (Muroga *et al*., 1976, Biosca *et al.,* 1991; Høi *et al.,* 1998b; Dalsgaard *et al*., in press).

During the unusually warm summer in Denmark in 1994, coastal water temperatures exceeded 20°C for about four weeks (Dalsgaard *et al.,* 1996b). At the end of August 1994, 11 patients were admitted to hospitals with *V. vulnificus* infections; this was presented in the first report of a series of human *V. vulnificus* infections from a temperate zone (Dalsgaard *et al.,* 1996b). All patients contracted their disease after exposure to coastal water. In 1995 and 1997, a total of 3 and 4 *V. vulnificus* infections, respectively, were reported in Denmark. In 1996 and 1998, water temperatures were low and no cases were reported (Bruun, 1997). The clinical cases in 1994 prompted the Royal

Veterinary and Agricultural University, The Danish Institute for Fisheries Research, The Environmental Protection Agency and The Ministry of Food, Agriculture, and Fisheries to fund studies on the occurrence of *V. vulnificus* in Danish costal waters.

Overall conclusions

The overall conclusions of the studies were that *V. vulnificus* occur in Danish marine environments including coastal water, sediment, wild fish, and shellfish. Low concentrations of *V. vulnificus* were recorded and growth was favored when water temperatures exceeded 20°C for several weeks during warm summers. In this period health authorities should be aware of wound infections associated with *V. vulnificus* and other *Vibrio* spp. *V. vulnificus* was also associated with disease outbreaks in Danish eel farms (Høi *et al*., 1998b, Dalsgaard *et al*., in press). *V. vulnificus* was occasionally isolated from raw, frozen seafood imported from South East Asia and in Danish blue mussels but do not constitute a hazard to public health in Denmark providing correct handling and cooking.

Identification

The name *V. vulnificus* was given official taxonomic status in 1980 (Farmer, 1980). It is believed that *V. vulnificus* formerly often was misidentified as *V. parahaemolyticus* (Hollis *et al*., 1976). The species *V. vulnificus* comprises two biotypes which in the original definition differed phenotypically, serologically, and in host range (Tison *et al.,* 1982). *V. vulnificus* biotype 1 is ubiquitous to estuarine environments and is an opportunistic human pathogen (Oliver, 1989). Biotype 2 is typically recovered from diseased eels but is also reported to cause illness in humans after handling eels (e.g. Mertens *et al.,* 1979; Høi *et al.,* 1997).

Biochemical identification of *V. vulnificus* requires a number of biochemical assays which are costly and time-consuming (Colwell, 1984; Murray, 1995). The API 20E index, where information on *V. vulnificus* is based on 31 *V. vulnificus* strains only, differs from the reactions shown in several manuals for the lysine decarboxylase and Voges-Proskauer reactions (Barrow & Feltham, 1993;. Holt *et al.,* 1994; Murray, 1995; Sinding, 1998). In general, characters listed in reference manuals are based on a limited number of clinical strains and do not include the more heterogenous reaction patterns of environmental strains.

Molecular techniques

Molecular techniques, particularly specific oligonucleotide probes, constitute a very sensitive and specific tool for detecting *V. vulnificus.* An alkaline phosphatase-labeled oligonucleotide probe directed towards the cytolysin gene of *V. vulnificus* was constructed by Wright *et al.* (1985,1993). This probe, termed VVAP, demonstrated 100% specificity and sensitivity for clinical and environmental isolates of *V. vulnificus* and numerous investigators have shown that cytolysin is produced by all *V. vulnificus* strains, including both biotypes, and is species-specific (Kaysner *et al.,* 1987; Morris *et al.,* 1987a; Parker & Lewis, 1995; Biosca *et al.,* 1996c). The sequence of the cytolysin gene has also been used for constructing primers for PCR identification (Brauns *et al.,* 1991; Coleman *et al.,* 1996).

DNA probe detection

In the present project, suspected *V. vulnificus* isolates from environmental samples were more frequently identified as *V. vulnificus* by the VVAP probe than by the API 20E system (Dalsgaard *et al.,* 1996a). Sixty-six isolates identified as *V. vulnificus* with the specific DNA probe were tested with the API 20E strip according to the manufacturers instructions. A total of 29 API 20E profiles were obtained. Only four of these profiles, representing 20 isolates, reached the identification threshold of *V. vulnificus.* The reasons for these difficulties could be discrepancies between the species-specific reaction pattern in the API 20E database and the reactions

given in standard identification tables, the heterogeneity of environmental strains, and the limited number of *V. vulnificus* strains (31 strains) included in the API 20E database (Dalsgaard *et al.,* 1996a; Sinding, 1998).

Biotype 2 isolates are not included in the API 20E system and can not be identified by this system (Biosca *et al.,* 1993a). Furthermore, commonly used reference manuals do not recognize the existence of *V. vulnificus* biotype 2 strains since they do not include indole negative strains.

Conclusions

In conclusion, identification of *V. vulnificus* with the commercial biochemical testing kit API 20E is not reliable because *V. vulnificus* isolates are very heterogenous in their biochemical characters. Serological identification requires preparation of antibodies since no commercial *V. vulnificus*-specific antibodies are available. Colony hybridization with the *V. vulnificus*-specific oligonucleotide probe is specific, fast and cost-effective. Identification of *V. vulnificus* with oligonucleotide probes or PCR primers directed against DNA or rRNA sequences is recommended.

The choice of including a pre-enrichment step in isolation of *V. vulnificus* depends on four factors: (i) the expected concentration of *V. vulnificus* in the samples, (ii) if a quantitative or qualitative result is needed, (iii) the conditions of the cells, and (iv) the level and composition of background flora. The pre-enrichment step should improve the ratio of target to background flora before a selective plating step.

Pre-enrichment broths and selective agars

The isolation of pathogenic *Vibrio* spp. is usually accomplished by culture methods that start with pre-enrichment in alkaline peptone water (APW; 1% peptone, pH 8.6) with 1% NaCl to recover sublethal injured organisms, followed by plating onto thiosulfate-citrate-bile salts-sucrose (TCBS) agar (Colwell, 1984).

Alkaline peptone water with polymyxin B

Overnight pre-enrichment in APW with polymyxin B (20 U/ml; APWP) gave higher recovery rate than pre-enrichment in regular APW in combination with modified cellobiose polymyxin B colistin (mCPC) agar when analyzing samples of coastal water and sediment in Denmark (Dalsgaard *et al.,* 1996a). APWP and mCPC agar was subsequently used with success for the isolation of *V. vulnificus* from fresh and frozen seafood (Dalsgaard & Høi, 1997; Høi *et al.,* 1998c). However, when analyzing gills, mucus, and intestinal content from cultured diseased eels and wild fish from Danish coastal waters pre-enrichment in APW for 6-8 h proved more favorable than overnight pre-enrichment in APWP (Høi *et al.,* 1998c; unpublished results). Studies with heavily infected eels showed that direct plating of tissue samples homogenized in phosphate-buffered-saline (PBS) gave the same or sometimes even better recovery of *V. vulnificus* than by pre-enrichment (unpublished results). Other studies have also reported that different sample types requires different isolation strategies for *V. vulnificus* (Biosca *et al.,* 1997b; Kaysner *et al.,* 1989).

Studies of 50 *V. vulnificus* strains from various sources and countries showed that no strains had a minimal inhibitory concentration (MIC) lower than 779 U colistin/ml (Høi et al., 1998a); thus recovery should not be reduced by adding as high a concentration as 20

U colistin/ml to the enrichment broth (Høi *et al.,* 1998a). Colistin, which also is termed polymyxin E, shows a similar mode of action on bacteria as polymyxin B.

The use of a selective and indicative medium for isolation of *V. vulnificus* serves two purposes: (i) to allow growth of *V. vulnificus* while inhibiting growth of more abundant marine species, (ii) to allow differentiation of *V. vulnificus* from other bacterial species so suspect colonies can be further identified.

mCPC agar

Tamplin *et al*. (1991,1992) described a less selective modification of the CPC agar termed mCPC with a reduced concentration of colistin. This medium was reported to be effective in isolating *V. vulnificus* from environmental sources (Tamplin *et al.,* 1991; Tamplin & Capers, 1992; Dalsgaard & Høi, 1997; Høi *et al.,* 1998c). In Denmark, more than 95% of presumptive colonies on mCPC agar could be identified as *V. vulnificus* when taking into consideration the typical colony morphology of *V. vulnificus* on this medium (flat, yellow colonies of approximately 2 mm in diameter) (Høi *et al.,* 1998a; Høi *et al.,* 1998c). It was very important for the identification-success rate to include the criterion Aflat@ in the evaluation of suspect colonies (Høi *et al.,* 1998a; Høi *et al.,* 1998c). Høi *et al.* (1998a) recommended a new medium termed cellobiose colistin (CC) agar which gave a better recovery of *V. vulnificus* than TCBS, CPC and mCPC agar in laboratory studies using pure cultures and Danish water and sediment samples. TCBS agar gave a very low plating efficiency (1%) of both clinical and environmental *V. vulnificus* strains and should not be recommended for the isolation of *V. vulnificus* (Høi *et al.,* 1998a). This is in agreement with other reports of low recovery of *V. vulnificus* on TCBS (Brayton *et al.,* 1983; Beazley & Palmer, 1992).

Conclusions

In conclusion, the isolation strategy for recovery and enumeration of *V. vulnificus* from environmental samples depends on the sample type, the level of background flora, and the expected concentration of *V. vulnificus.* In Denmark, where *V. vulnificus* levels are generally low (less than 10 CFU per ml or gram), CC agar significantly increased the isolation rate of *V. vulnificus* from coastal water and sediment samples compared to mCPC agar when used in combination with pre-enrichment in APWP. More than 95% of the presumptive colonies on CC agar were identified as *V. vulnificus* with the VVAP probe indicating that identification and enumeration of *V. vulnificus* using CC agar only may be sufficient in laboratories where colony hybridization can not be done. TCBS gave very low plating efficiencies and can not be recommended for the isolation of *V. vulnificus.*

Disease in humans

V. vulnificus causes primary septicemias and wound infections in humans (Blake *et al.,* 1979). Most primary septicemias are associated with raw seafood consumption, especially raw oysters and in almost every case the patient has a chronic underlying disease. *V. vulnificus* differs from other food borne pathogens as it is seldom reported to cause diarrhea and vomiting (Hollis *et al.,* 1976; Blake *et al.,* 1979; Hlady & Klontz, 1996). *V. vulnificus* causes only sporadic disease and outbreaks have never been reported (Whitman, 1995).

The fatality rate is high with almost 60% of the patients with primary septicemia dying within a few days (Oliver, 1989). A high prevalence of liver disease and alcoholism among patients with septicaemia has supported a requirement of *V. vulnificus* for free iron via saturated transferrin or excess of iron (Hlady & Klontz, 1996). Other risk factors include the use of immunosuppressive agents, gastric diseases, and blood disorders (Oliver, 1989).

Wound infections

V. vulnificus causes wound infections by entering a pre-existing skin lesion during exposure to saline waters. Patients are often employed as fishermen or in other jobs with close contact to the marine environment (Dalsgaard *et al.,* 1996b; Hlady & Klontz, 1996). The fatality rate of reported cases is approximately 20% but amputation or surgical debridement is often necessary (Oliver, 1989).

V. vulnificus in Denmark

In Denmark, four of 11 patients in 1994 developed septicemia, of which one subsequently died. Nine patients exhibited skin manifestations and six underwent surgical debridement. Four patients contracted their disease during fishing and at least one patient had been handling eels (Dalsgaard *et al.,* 1996b). In 1995 and 1997 three and four patients were likely to have contracted their disease during fishing. None of the patients had consumed any seafood prior to infection.

Treatment

V. vulnificus is sensitive to most antibiotics and infections have been treated with antibiotics, e.g. ampicillin, tetracycline, chloramphenicol or third-generation cephalosporins (Klontz *et al.,* 1988; Chuang *et al.,* 1992; Fang, 1992; Dalsgaard *et al.,* 1996b). Antibiotic treatment is often ineffective unless initiated as soon as the first clinical symptoms appear (Oliver, 1989). However, in cases of serious wound infections, the primary treatment is a proper surgical debridement with antibiotics playing a secondary role (Dalsgaard *et al.,* 1996b).

Biotype 2 causes disease in eels

V. vulnificus biotype 2 causes serious economic losses in aquaculture in Denmark and other countries where eels are kept in brackish water around 20-24°C. The Danish production of eels in aquaculture is expanding with a production in 1999 of 3,000 metric tonnes. The majority of eel farms in Denmark use fresh water to culture eels. Disease outbreaks caused by *V. vulnificus* and other pathogenic *Vibrio* spp. have not been reported in Denmark with recirculating systems that use freshwater continuously. One exception is *V. anguillarium* that has been reported to casue infections in eels after salt-treatment (Mellergaard & Dalsgaard, 1987). It is preferable to culture eels in brackish water instead of fresh water since it leads to better growth rates, feed conversion and taste. However, brackish water can be a reservoir or vehicle of *V. vulnificus* biotype 2 and might facilitate the spread to cultured eels (Høi *et al.,* 1998c). Further, water temperatures in eel farms favor *V. vulnificus* growth (Høi *et al.,* 1998c). *V. vulnificus* biotype 2 was isolated from wound infections in humans and coastal water in Denmark in 1994 but was not isolated from diseased eels in Danish farms using brackish water until 1995 (Dalsgaard *et al.,* 1996b; Høi *et al.,* 1997; Høi *et al.,* 1998b; Høi *et al.,* 1998c; Dalsgaard *et al*, in press). Since 1995 recurrent outbreaks of *V. vulnificus* have occurred in two Danish eel farms both using brackish water. The disease has caused economic losses (Høi *et al.,* 1998b; Dalsgaard *et al.,* in press).

Although the disease is a septicemic infection and bacteria are easily isolated from blood samples from moribund eels (Amaro *et al.,* 1997), antibiotic treatment of *V. vulnificus* infections in eels has a limited effect and outbreaks are recurrent. No antibiotic resistance has been demonstrated so far (Dalsgaard *et al.,* in press). Changes to production in freshwater usually reduce the eel mortality, but in one Danish eel farm the infection with *V. vulnificus* is persisting at the present time. Research on survival and spread of *V. vulnificus* in eel farms and the efficiency of vaccination is needed to make culture of eels in brackish water profitable.

V. vulnificus has recently been isolated from the gills and intestinal contents of eels cultured in freshwater but these eels had been kept in brackish water in the past (unpublished results). These findings suggested that once *V.vulnificus* enter the eel farm and colonize the eels, then Na^+ or other cations present in the eel may be sufficient for growth of this halophilic bacteria. The concentration of Na^+ in blood an extracellular fluids in eels is approximately 150 mmol (8.8 g/L) which theoretically is sufficient for growth and persistence of *V. vulnificus* (Scholz & Zerbst-Boroffka, 1994).

Virulence factors

The high virulence of *V. vulnificus* can not be assigned to a single factor but is likely to be influenced by capsule production, ability to acquire iron in human serum, lipopolysaccharide (LPS) type, production of exoenzymes and exotoxins, and a susceptible host.

The two biotypes share many of the same virulence factors, including (i) the capsule, a protective surface antigen that allows cells to resist phagocytosis and lysis by human serum but not by eel complement (Biosca *et al.,* 1993b; Amaro *et al.,* 1994); (ii) various iron uptake systems, including siderophore production and the ability to utilize hemoglobin and hemin as iron sources (Amaro *et al.,* 1994; Biosca *et al.,* 1996b); and (iii) a cytolysin, with hemolytic activity together with potent proteases, which are active involved in the lesions produced in different organs (Amaro *et al.,* 1992).

Plasmids

A relationship between high molecular weight plasmids and eel virulence was first suggested by Biosca *et al*. (1997a), who found that a plasmid-free biotype 2 strain had a significantly higher LD_{50} in eels (the lethal dose of *V. vulnificus* that kills 50% of eels tested) than biotype 2 strains harboring high molecular weight plasmids. These findings are corroborated by Høi *et al*. (1998b) who found that 93 of 97 biotype 2 strains isolated from diseased eels contained one to three high molecular weight plasmids of varying sizes. Restriction digests of plasmids from a number of biotype 2 strains from Denmark revealed a high degree of homology (Lewin, 1998). The role of plasmids requires further studies.

Risk for eel farmers

Danish farmers when handling eels are exposed to *V. vulnificus* during outbreaks but at the present time no infections of eel farmers has been reported.

V. vulnificus in Danish coastal waters

In conclusion, human infection with *V. vulnificus* following exposure to coastal water in Denmark occur when water temperatures exceed 20°C and fishermen, especially eel fishermen, appear to be at the greatest risk. Consumption of raw shellfish has not been associated with *V. vulnificus* infections in Denmark, despite that *V. vulnificus* occasionally was isolated in low numbers. *V. vulnificus* presents a serious economic problem to the Danish eel farmers wanting to use brackish water and warrant for both therapeutic and prophylactic measures. Research is needed to understand the ecology of *V. vulnificus* in eel farms and to develop a vaccine against eel-pathogenic *V. vulnificus* strains.

A comprehensive environmental survey of *V. vulnificus* in Danish marine environments was done during 1996 (Høi *et al.,* 1998c). The aims of this survey were to investigate the occurrence of *V. vulnificus* in Danish coastal waters, shellfish, and wild fish (Høi *et al.,* 1998c).

From May to October 1996, water was sampled weekly at seven sites and sediment samples were collected weekly from two sites. Blue mussels (*Mytilus edulis*) and oysters (*Oestra edulis* and *Crassostrea gigas*) were sampled from July until December 1996 from a total of 13 sites. From July until October 1996, a total of 136 wild fish were analyzed, including 29 flounders (*Platichthys flesus*), 14 eel pouts (*Zoarches viviparus*), and 93 eels (*A. anguilla*) that were caught at various locations in Køge Bay (Høi *et al.,* 1998c).

In Denmark, biotype 2 strains were isolated from sediment and coastal water samples although the frequency of isolating such strains was very low (3 of 706 strains were indole negative and designated biotype 2). The low incidence of *V. vulnificus* biotype 2 strains in environmental samples may explain why its occurrence in coastal water has not been reported earlier. The isolation procedure may also influence which biotype is detected. Our study suggests differences in the ecology of the two biotypes: biotype 1 was isolated from various environmental sources whereas biotype 2 was rarely isolated. Thus, Danish marine environments are potential reservoirs of *V. vulnificus* biotype 2. The distribution of eel-pathogenic *V. vulnificus* strains in Danish coastal waters may have been underestimated because not all eel-pathogenic *V. vulnificus* strains are indole negative. This knowledge is particular important for fish farmers since the use of brackish water for culturing eels may introduce pathogenic *V. vulnificus* biotype 2 strains in the farms.

Levels of V. vulnificus correlated with water temperatures

Low densities of *V. vulnificus* were detected at the seven costal sites in water (0.8 to 19 cell forming units (CFU)/liter) from June until mid-September and in sediment (0.04 to >11 CFU/g) (Høi *et al*., 1998c). The occurrence of *V. vulnificus* was strongly correlated to water temperatures, as reported by other researchers (Wright *et al.,* 1996; O'Neill *et al.,* 1992; Kelly, 1982). *V. vulnificus* was rarely isolated when water temperatures were below 15°C. However, *V. vulnificus* was detected in coastal waters at one mussel farm at 7°C which is lower than previously reported (Wright *et al.,* 1996).

The control of Danish coastal bathing water is based on presumptive *Escherichia coli* and coliforms as indicators of water quality. As reported elsewhere, analysis of the data collected in Denmark in 1996 did not reveal any correlation between presumptive *E. coli* and *V. vulnificus* (Høi *et al.,* 1998c).

V. vulnificus was mainly found in wild fish when water temperatures were high. The highest incidence of *V. vulnificus* was found in the gills from eels, but 4 of 75 samples from eelpouts and flounders did also contain *V. vulnificus.*

Low levels in mussels

Danish oysters and mussels and their surrounding waters were analyzed for *V. vulnificus. V. vulnificus* was isolated from both water and blue mussels from one cultivation site of a total of 13 areas examined. Concentrations of *V. vulnificus* in blue mussels were very low (# 10 CFU per gram of mussel tissue) and the low probability of detecting a viable *V. vulnificus* cell may explain why it was not isolated from other areas. Location of mussels in surface waters provides more favorable temperatures for growth of *V. vulnificus* during summer time, and the high level of nutrients may support growth and persis tence of *V. vulnificus,* even at low temperatures. *V. vulnificus* infections have not been associated with consumption of raw shellfish in Denmark or reported elsewhere in Europe. These findings suggest minimal risk associated with consumption of raw shellfish containing *V. vulnificus* in low numbers.

Conclusions

In conclusions, our findings of *V. vulnificus* in Danish wild fish during the summer suggest that fishermen, especially those with abrasions on their hands may be at risk for *V. vulnificus* wound infections. In 1994 and 1995, seven people contracted *V. vulnificus* wound infections while fishing or handling eels (Dalsgaard *et al.,*1996b; Bruun, 1997). *V. vulnificus* infections were not reported during the summer of 1996 when low concentrations (<2 CFU/100 ml) were observed in coastal waters. *V. vulnificus* levels were probably too low to cause infection, in susceptible individuals, and colder temperatures discouraged bathers from contact with coastal waters. Epidemiological data from 1994 and 1995 suggest that the risk of contracting a *V. vulnificus* infection following exposure to coastal water was correlated with high water temperature (>20°C). Thus, surveillance and monitoring efforts surveillance and monitoring efforts should be initiated when water temperatures exceed 20°C.

Low prevalence of V. vulnificus in frozen shrimp imported into Denmark

V. vulnificus is a naturally occurring bacterium in warm estuarine environments and should therefore be expected in shrimp produced in brackish-water aquaculture in tropical countries. The European Union imported approximately 75 metric tonnes of these warm-water shrimp through Denmark in 1995 (Dalsgaard & Høi, 1997). The prevalence of *V. vulnificus* in a total of 107 samples representing 37 consignments of frozen shrimp imported from South East Asia was determined. *V. vulnificus* was detected in three of 46 (7 %) frozen raw shrimp samples but was not recovered from any of the 61 frozen cooked products. Absence of *V. vulnificus* in frozen cooked shrimp products indicated proper processing such as adequate heat treatment and sanitation (Dalsgaard & Høi, 1997). The low prevalence of *V. vulnificus* in frozen raw shrimp products was likely due to its poor resistance to cold (Oliver, 1981; Boutin *et al.,* 1985; Parker *et al.,* 1994). Frozen shrimp products are usually kept at temperatures at -20EC before and after shipping, often for substantial periods, and a significant decrease in the number of *V. vulnificus* would be anticipated. In conclusion, the absence of *V. vulnificus* in frozen cooked shrimp products and the low prevalence of *V. vulnificus* in frozen raw shrimp suggested that *V. vulnificus* does not constitute a hazard to public health in Denmark (Dalsgaard & Høi, 1997).

Sammendrag

Introduktion

Den marine bakterie, *Vibrio vulnificus*, er en potentiel human patogen, som kan forårsage alvorlige sårinfektioner og blodforgiftning med dødeligheder på op til 60 % (Oliver, 1989; Hlady & Klontz, 1996).

V. vulnificus kan isoleres fra akvatiske økosystemer, hvor forekomsten af bakterien favoriseres af høje temperaturer (>20°C) og saltholdigheder på 15-25 ‰ (Motes *et al.,* 1998). Forekomsten af *V. vulnificus* er højere i subtropiske vandmiljøer end i tempererede områder, men *V. vulnificus* er også blevet isoleret fra kystområder eller har forårsaget humane infektioner i sommerperioden i Danmark, Sverige, Tyskland, Holland og Belgien (Mertens *et al.,* 1979; Veenstra *et al.,* 1994; Hoyer *et al.,* 1995; Melhus *et al.,* 1995; Dalsgaard *et al.,* 1996b). *V. vulnificus* er desuden årsag til infektioner hos ål opdrættet i ålebrug i Japan, Spanien, Norge, Sverige og Danmark (Muroga *et al*., 1976, Biosca *et al.,* 1991; Høi *et al.,* 1998b; Dalsgaard *et al*., in press).

Den usædvanlige varme sommer i Danmark i 1994 bevirkede, at vandtemperaturen i kystområderne var højere end 20°C i fire uger (Dalsgaard *et al.,* 1996b). I slutningen af august 1994, havde 11 patienter været indlagt på danske hospitaler med *V. vulnificus* infektioner; resulterende i den første publikation omhandlende en række af humane *V. vulnificus* infektioner fra et tempereret område (Dalsgaard *et al.,* 1996b). Alle patienter erhvervede sygdommen efter kontakt med vand fra kystområder. I 1995 og 1997, blev henholdsvis 3 og 4 *V. vulnificus* infektioner rapporteret fra Danmark (Bruun, 1997). I 1996 og 1998 var vandtemperaturerne lave, og i de år blev der ingen tilfælde rapporteret (Bruun, 1997). De kliniske tilfælde i 1994 foranledigede, at Den Kgl. Veterinær- og Landbohøjskole, Danmarks Fiskeriundersøgelser, Miljøstyrelsen og Ministeriet for Fødevarer, Landbrug og Fiskeri igangsatte undersøgelser, der skulle belyse forekomsten af *V. vulnificus* i danske kystnære vandområder.

Generel konklusion

Konklusionen af undersøgelserne var, at *V. vulnificus* var udbredt i danske marine miljøer omfattende kystnært vand, sediment, vildfisk og skaldyr. Der blev påvist relativt lave koncentrationer af *V. vulnificus*, og samtidigt at forekomsten blev favoriseret af, at vandtemperaturen var over 20°C i adskillige uger. I denne periode må sundhedsmyndighederne være opmærksomme på, at sårinfektioner kan være forårsaget af *V. vulnificus*. *V. vulnificus* blev i 1995 isoleret i forbindelse med sygdomsudbrud i danske ålebrug. *V. vulnificus* blev desuden isoleret fra rå, frosne skaldyr importeret fra Sydøst Asien og fra danske blåmuslinger, men i ringe antal. De formodes derfor ikke at kunne forårsage sundhedsproblemer.

Identifikation

Bakteriens navn *V. vulnificus* fik officiel taxonomisk status i 1980 (Farmer, 1980). Det antages at *V. vulnificus* tidligere blev fejlidentificeret som *V. parahaemolyticus* (Hollis *et al*., 1976). *V. vulnificus* omfatter to biotyper, som i følge den originale definition var forskellige fænotypisk, serologisk og med hensyn til værtspecificitet (Tison *et al.,* 1982). *V. vulnificus* biotype 1 forekommer udbredt i kystnære områder og er en potentiel human patogen (Oliver, 1989). Biotype 2 er typisk isoleret fra syge ål, men er også rapporteret at forårsage sygdom hos mennesker i forbindelse med håndtering af ål (Mertens *et al.,* 1979; Høi *et al.,* 1997). Biokemisk identifikation af *V. vulnificus* kræver et antal af biokemiske analyser, som er kostbare, tidskrævende og resultatet kan ikke forligge samme dag (Colwell, 1984; Murray, 1995). Det kommercielle forgæringssystem API 20E, hvor databasen vedrørende *V. vulnificus* alene er baseret på 31 stammer, afviger fra de karakterer, der findes i adskillige bakteriologiske identifikations

manualer (Barrow & Feltham, 1993;. Holt *et al.,* 1994; Murray, 1995; Sinding, 1998). Generelt er tabellerne i sådanne manualer baseret på et meget begrænset antal kliniske isolater, og omfatter derfor ikke de mere heterogene miljøisolater.

Molekylære teknikker

Anvendelsen af molekylære teknikker, især specifikke oligonukleotid-prober, udgør et sensitivt og specifikt værktøj til påvisning af *V. vulnificus.* En alkalisk fosfatase-mærket oligonukleotid- probe rettet mod cytolysin genet i *V. vulnificus* blev konstrueret af Wright *et al.* (1985,1993). Denne probe, benævnt VVAP, viste 100% specificitet og sensitivitet for kliniske og miljøisolater af *V. vulnificus,* og adskillige undersøgelser har vist, at cytolysin produceres af alle *V. vulnificus* stammer, inkluderende begge biotyper, og den er endvidere arts-specifik (Kaysner *et al.,* 1987; Morris *et al.,* 1987a; Parker & Lewis, 1995; Biosca *et al.,* 1996c). Sekvensen af cytolysin genet er også blevet anvendt til at udvikle primere til PCR identifikation (Brauns *et al.,* 1991; Coleman *et al.,* 1996).

DNA probe

I nærværende projekt, blev suspekte *V. vulnificus* isolater fra miljøprøver hyppigere identificeret som *V. vulnificus* ved VVAP proben end ved hjælp af API 20E systemet (Dalsgaard *et al.,* 1996a). 66 isolater identificeret som *V. vulnificus* med den specifikke DNA probe blev efterfølgende undersøgt med API 20E i henhold til producentens vejledning. Der blev påvist 29 forskellige API 20E profiler. Kun 4 af disse profiler, repræsenterende 20 isolater, havde scoringstal, så de kunne bestemmes som værende *V. vulnificus.* Årsagen til disse uoverensstemmelser kunne skyldes afvigelser mellem det artsspecifikke reaktionsmønster i API 20E databasen og reaktionerne, der findes i traditionelle manualer, den større grad af heterogenitet blandt miljøisolater, og det begrænsede antal af *V. vulnificus* stammer (31) der indgår i API 20E databasen (Dalsgaard *et al.,* 1996a; Sinding, 1998). Biotype 2 isolater er ikke inkluderet i API 20E databasen og kan derfor ikke identificeres ved hjælp af dette system (Biosca *et al.,* 1993a). Desuden findes *V. vulnificus* biotype 2 ikke i de fleste manualer, da disse ikke inkluderer indol-negative stammer.

Konklusion

Konklusionen er, at API 20E ikke er egnet til identifikation af *V. vulnificus,* da *V. vulnificus* isolater er meget heterogene i deres biokemiske reaktionsmønstre. Kolonihybridisering med den *V. vulnificus*-specifikke oligonukleotid-probe er en specifik, hurtig og økonomisk acceptabel metode til identifikation. Identifikation af *V. vulnificus* med oligonukleotid-prober eller PCR primere rettet mod DNA eller rRNA sekvenser kan anbefales.

Baggrunden for at inkludere et opformerings trin i forbindelse med isolering af *V. vulnificus* er baseret på følgende 4 forhold: (i) den forventede koncentration af *V. vulnificus* i prøverne, (ii) om et kvantitativt eller kvalitativt result er nødvendigt, (iii) bakteriecellernes tilstand, og (iv) mængde og sammensætning af baggrundsfloraen. Opformeringen forventes at forbedre påvisningen af den ønskede bakterie i forhold til baggrundsfloraen, før der sås ud på et selektivt agarmedium.

Opformering og selektivt agar medium

Isoleringen af patogene *Vibrio* spp. foretages sædvanligvis med metoder, hvori der indgår en opformering i alkalisk peptonvand (APW; 1% peptone, pH 8.6) tilsat 1% NaCl for også at påvise subletalt beskadigede organismer. Dette efterfølges af en udsæd på thiosulfate-citrate-bile salts-sucrose (TCBS) agar (Colwell, 1984).

Alkalisk peptonvand med polymyxin B

Opformering natten over i APW med polymyxin B (20 U/ml; APWP) gav bedre påvisningsresultater af *V. vulnificus* end opformering i almindelig APW kombineret med modificeret cellobiose polymyxin B colistin (mCPC) agar, når prøver af vand og sediment blev analyseret (Dalsgaard *et al.,* 1996a). APWP og mCPC agar blev efterfølgende anvendt med succes til isolering af *V. vulnificus* fra ferske og frosne skaldyr (Dalsgaard & Høi, 1997; Høi *et al.,* 1998c). Ved undersøgelse af gæller, slim og tarmindhold fra opdrættede syge ål og vildfisk fanget i kystnære områder viste opformering i APW i 6-8 timer sig mere velegnet end opformering natten over i APWP (Høi *et al.,* 1998c). Undersøgelser af kraftigt inficerede ål viste, at direkte udsæd af vævsprøver homogeniseret i fosfat-bufferet-saltvand (PBS) gav tilsvarende eller undertiden bedre påvisning af *V. vulnificus* end efter opformering (upubliserede resultater). Undersøgelser foretaget af andre forskere viser tilsvarende, at forskellige prøvetyper kræver forskellige isolationsstrategier til påvisning af *V. vulnificus* (Biosca *et al.,* 1997b; Kaysner *et al.,* 1989).

Undersøgelser af 50 *V. vulnificus* stammer isoleret fra forskellige miljøer og lande viste, at ingen af isolaterne havde en minimal inhibitory concentration (MIC) lavere end 779 U colistin/ml (Høi et al., 1998a). Det betyder, at fremvækst af bakterien ikke bliver reduceret ved tilsætning af en så høj koncentration som 20 U colistin/ml til opformeringsbouillonen (Høi *et al.,* 1998a). Colistin, der er lig med polymyxin E, påvirker bakterier på samme måde som polymyxin B.

Brugen af et selektivt og indikativt medium til isolering af *V. vulnificus* tjener 2 formål: (i) at tillade vækst af *V. vulnificus* samtidig med, at væksten af andre marine bakterier hæmmes, (ii) at differentiere *V. vulnificus* fra andre bakteriearter, så at suspekte kolonier kan identificeres.

mCPC agar

Tamplin *et al*. (1991,1992) beskrev en mindre selektiv modifikation af CPC agar benævnt mCPC med et reduceret indhold af colistin. Dette medium blev rapporteret at være effektivt til isolering af *V. vulnificus* fra miljøet (Tamplin *et al.,* 1991; Tamplin & Capers, 1992; Dalsgaard & Høi, 1997; Høi *et al.,* 1998c). I Danmark kunne over 95% af de suspekte kolonier på mCPC agar identificeres som værende *V. vulnificus,* når den karakteristiske kolonimorfologi af *V. vulnificus* på dette medium blev taget i betragtning (flade, gule kolonier, ca.2 mm i diameter) (Høi *et al.,* 1998a; Høi *et al.,* 1998c). Det var særdeles vigtigt for dette resultat at inkludere kriteriet Aflad@ i vurderingen af kolonimorfologien (Høi *et al.,* 1998a; Høi *et al.,* 1998c).

CC agar

Høi *et al.* (1998a) anbefalede et nyt medium kaldet cellobiose colistin (CC) agar, som gav en bedre påvisning af *V. vulnificus* end TCBS, CPC and mCPC agar ved laboratorie- undersøgelser med renkulturer og med vand og sediment prøver. TCBS agar gav en meget lav erkendelse (1%) af både kliniske og miljøstammer af *V. vulnificus* og kan derfor ikke anbefales til isolering af *V. vulnificus* (Høi *et al.,* 1998a). Dette stemmer overens med andre undersøgelser, som ligeledes rapporterer dårligere fremvækst af *V. vulnificus* på TCBS (Brayton *et al.,* 1983; Beazley & Palmer, 1992).

Konklusion

Konkluderende kan siges, at den isolationsstrategi, der skal anvendes til påvisning og kvantifisering af *V. vulnificus* fra miljøprøver er afhængig af prøvetype, baggrundsfloraen, og den forventede koncentration af *V. vulnificus.* I Danmark, hvor niveauet for *V. vulnificus* generelt er lavt (mindre end 10 kolonier pr. ml eller gram), øger CC agar signifikant påvisningen af *V. vulnificus* fra vand og sedimentprøver, sammenlignet med mCPC agar, brugt i kombination med opformering i APWP. Over 95% af de suspekte kolonier på CC agar blev identificeret som *V. vulnificus* med VVAP proben, hvilket viste, at identifikation og tælling af *V. vulnificus*

ved at anvende CC agar alene kan være tilstrækkelig i laboratorier, hvor kolonihybridisering ikke kan udføres. TCBS gav dårlige resultater og bør derfor ikke anvendes til isolation af *V. vulnificus.*

Sygdom hos mennesker

V. vulnificus forårsager primær blodforgiftning og sårinfektioner (Blake *et al.,* 1979). Hovedparten af de primære blodforgiftninger forekommer i forbindelse med indtagelse af råskaldyr, specielt råøsters, og i næsten hvert tilfælde har patienten i forvejen en kronisk sygdom. *V. vulnificus* er forskellig fra andre levnedsmiddelbårne patogener, da den sjældent rapporteres at forårsage diarré og opkast (Hollis *et al.,* 1976; Blake *et al.,* 1979; Hlady & Klontz, 1996). *V. vulnificus* forårsager kun sporadisk sygdom og udbrud er aldrig blevet rapporteret (Whitman, 1995). Dødeligheden er høj, ca. 60% af patienterne med primær blodforgiftning dør inden for få dage (Oliver, 1989). En høj prævalens af leversygdom og alkoholisme blandt patienterne med blodforgiftning hænger sammen med, at *V. vulnificus* har et behov for frit jern, som er til stede i disse patienter (Hlady & Klontz, 1996). Andre risiko faktorer inkluderer brugen af immunosupprimerende stoffer, ligesom sygdomme i ventriklen og blodsygdomme er disponerende (Oliver, 1989).

Sårinfektioner

V. vulnificus giver sårinfektioner ved at trænge gennem en eksisterende hudlæsion efter kontakt med havvand. Patienterne er ofte fiskere eller i andet arbejde med tæt kontakt til det marine miljø (Dalsgaard *et al.,* 1996b; Hlady & Klontz, 1996). Dødeligheden af rapporterede tilfælde er ca. 20%, men amputation eller andre kirurgiske indgreb er ofte nødvendige (Oliver, 1989).

V. vulnificus i Danmark

I Danmark udviklede 4 ud af 11 patienter i 1994 blodforgiftning, heraf døde én. Ni patienter fik sårinfektioner, og der blev foretaget kirurgiske indgreb på 6. Fire patienter erhvervede infektionen i forbindelse med fiskeri, heraf havde mindst én patient håndteret ål (Dalsgaard *et al.,* 1996b). I 1995 og 1997 fik henholdsvis 3 til 4 patienter sygdommen, alle i forbindelse med fiskeri. Ingen af patienterne havde spist skaldyr.

Risiko for åleopdrættere

Åleopdrættere kan i forbindelse med håndtering af ål komme i kontakt med *V. vulnificus,* men på nuværende tidspunkt er der ikke registreret smitte af *V. vulnificus* blandt danske åleopdrættere.

Behandling

V. vulnificus er følsom over for de fleste antibiotika, og infektionerne har været behandlet med antibiotika, f.eks. ampicillin, tetracyklin, kloramfenikol eller 3.-generation cephalosporiner (Klontz *et al.,* 1988; Chuang *et al.,* 1992; Fang, 1992; Dalsgaard *et al.,* 1996b). Antibiotika behandling er ofte ineffektiv, hvis den ikke påbegyndes, så snart de første kliniske symptomer optræder (Oliver, 1989). Imidlertid er den primære behandling ved alvorlige sårinfektioner ofte kirurgiske indgreb, hvor antibiotika spiller en sekundær rolle (Dalsgaard *et al.,* 1996b).

Sygdom hos ål

V. vulnificus biotype 2 forårsager økonomiske tab i akvakultur i Danmark og andre lande, hvor ål opdrættes i brakvand ved 20-24°C. Den danske produktion af ål i akvakultur har ekspanderet betydeligt de seneste år, og produktionen i 1999 forventes at blive ca. 3.000 tons. Hovedparten af ålebrug i Danmark anvender ferskvand ved opdræt af ål. I Danmark har sygdomme forårsaget af *V. vulnificus* og andre patogene *Vibrio* arter ikke tidligere forårsaget sygdomme i recirkulerede systemer, der anvender ferskvand, bortset fra infektioner med *V. anguillarum* i forbindelse med saltbehandling af glasål (Mellergaard & Dalsgaard, 1987). Ved at opdrætte ål i brakvand i stedet for ferskvand fås et bedre vækstpotetiale, en bedre foderudnyttelse og en bedre smag af produktet. Imidlertid kan brakvand være et reservoir for *V. vulnificus* biotype 2 og kan derfor være smittekilde for ål i akvakultur (Høi *et al.,* 1998c). Yderligere favoriserer vandtemperaturen i ålebrug vækst af *V. vulnificus* (Høi *et al.,* 1998c). *V. vulnificus* biotype 2 blev i 1994 isoleret fra sårinfektioner hos mennesker og fra danske kystnære områder, og i 1995 blev den isoleret fra syge ål i danske ålebrug, der brugte brakvand (Dalsgaard *et al.,* 1996b; Høi *et al.,* 1997; Høi *et al.,* 1998b; Høi *et al.,* 1998c; Dalsgaard *et al*, in press). Efter 1995 har der optrådt gentagne udbrud af *V. vulnificus* i to danske åleanlæg, der begge anvender brakvand. De gentagne sygdomsudbrud især på det ene anlæg har medført store økonomiske tab (Høi *et al.,* 1998b; Dalsgaard *et al.,* in press).

Skønt sygdommen manifesterer sig som en blodforgiftning, og bakterier let kan isoleres fra blodprøver fra døende ål (Amaro *et al.,* 1997), har antibiotikabehandling af *V. vulnificus* infektioner en begrænset effekt, og infektionen persisterer. Endnu er der ikke påvist antibiotikaresistens hos *V. vulnificus* (Dalsgaard *et al.,* in press). Ændring til produktion i ferskvand reducerer sædvanligvis dødeligheden, men i et dansk ålebrug har infektionen persisteret alligevel. Undersøgelse af overlevelse og spredning af *V. vulnificus* i ålebrug og effekten af vaccination er nødvendige tiltag for at gøre opdræt af ål i brakvand økonomisk rentabel. *V. vulnificus* er fornyligt blevet isoleret fra gællerne og tarmindhold af ål opdrættet i ferskvand, men disse ål havde tidligere været opdrættet i brakvand (upubliserede resultater). Dette tyder på, at hvis *V. vulnificus* én gang er kommet ind i et ålebrug og har koloniseret ålene, så er Na^+ og andre kationer i ålene tilstrækkelige for vækst og persistens af disse saltkrævende bakterier. Koncentrationen af Na^+ i blod og ekstracellulære vædsker i ålene er ca. 150 mmol (8.8 g/L), som teoretisk er tilstrækkeligt for vækst af *V. vulnificus* (Scholz & Zerbst-Boroffka, 1994).

Virulensfaktorer

Den høje virulens af *V. vulnificus* kan ikke tilskrives en enkelt faktor, men skyldes en kombination af kapselproduktion, evnen til at optage jern fra serumproteiner, lipopolysaccharide (LPS) type, produktion af exoenzymer og exotoxiner og en modtagelig vært. De to biotyper har adskillige fælles virulensfaktorer, inkluderende (i) kapsel, et beskyttende overfladeantigen, som gør cellerne resistente mod fagocytose og lysis af humanserum men ikke mod ålekomplement (Biosca *et al.,* 1993b; Amaro *et al.,* 1994); (ii) forskellige jernbindings-systemmer, inkluderende sideroforproduktion og evnen til at bruge hæmoglobin og hæmin som jernkilder (Amaro *et al.,* 1994; Biosca *et al.,* 1996b); og (iii) et cytolysin med hæmolytisk aktivitet samt proteaser, som er aktivt involveret i de læsioner, der produceres i forskellige organer (Amaro *et al.,* 1992).

Plasmider

En sammenhæng mellem højmolekylære plasmider og ålevirulens blev først antydet af Biosca *et al*. (1997a), som fandt, at et plasmid-fri biotype 2 isolat havde en signifikant højere LD_{50} i ål (den letale dosis af *V. vulnificus* som dræber 50% af de inficerede ål) end biotype 2 isolater, der indeholdt højmolekylære plasmider. Disse resultater blev bekræftet af Høi *et al*. (1998b), som fandt at 93 ud af 97 biotype 2 isolater fra syge ål indeholdt en til tre højmolekylære plasmider af varierende størrelser. Skæring med restriktionsenzymer af plasmider fra et antal af biotype 2 stammer fra Danmark viste en stor grad af homologi (Lewin, 1998). Plasmidernes betydning er endnu ikke afklaret.

Konklusion

Det kan konkluderes, at humane infektioner med *V. vulnificus* efter kontakt til kystnære vandområder i Danmark alene optræder, når vandtemperaturen er over 20°C, og fiskere, specielt ålefiskere, udsættes for den største risiko. Indtagelse af råskaldyr har ikke givet anledning til *V. vulnificus* infektioner i Danmark, skønt *V. vulnificus* lejlighedsvis blev påvist i ringe antal. *V. vulnificus* kan udgøre et økonomisk problem for åleopdrættere, der ønsker at anvende brakvand, og der er et stort behov for forebyggende og behandlingsmæssige tiltag over for sygdommen. Forskning er derfor nødvendig for at forstå økologien af *V. vulnificus* i ålebrug og at udvikle en vaccine mod ålepatogene *V. vulnificus* isolater.

V. vulnificus i danske områder

En omfattende miljøundersøgelse af *V. vulnificus* i danske marine miljøer blev foretaget i 1996 (Høi *et al.,* 1998c). Formålet med denne undersøgelse var at klarlægge forekomsten af *V. vulnificus* i kystnære vandområder, skaldyr, vildfisk (Høi *et al.,* 1998c). Fra maj til oktober 1996 blev der indsamlet vandprøver hver uge fra 7 lokaliter og sedimentprøver blev indsamlet hver uge fra 2 af lokaliteterne. Blåmuslinger (*Mytilus edulis*) og østers (*Oestra edulis* og *Crassostrea gigas*) blev indsamlet fra juli indtil december 1996 fra 13 lokaliteter. Fra juli indtil oktober 1996 blev der analyseret 136 vildfisk, bestående af 29 skrubber (*Platichthys flesus*), 14 ålekvabber (*Zoarches viviparus*) og 93 ål (*Anguilla anguilla*) som blev fanget på forskellige lokaliteter i Køge bugt og i Øresund i nærheden af Kastrup (Høi *et al.,* 1998c).

I undersøgelsen blev *V. vulnificus* biotype 2 stammer for første gang isoleret fra sediment og kystnære vandområder, men udbredelsen var meget beskeden (3 ud af 706 isolater var indol-negative og blev benævnt biotype 2). Den lave forekomst af *V. vulnificus* biotype 2 isolater i miljøprøver kan forklare, hvorfor denne organisme ikke er rapporteret tidligere. Isolationproceduren kan også påvirke, hvilken biotype der påvises. Ikke desto mindre er vandmiljøet et muligt reservoir for *V. vulnificus* biotype 2. Forekomsten af ålepatogene *V. vulnificus* i danske kystnære områder er sandsynligvis undervurderet, da ikke alle ålepatogene *V. vulnificus* er indol-negative. Denne viden er især vigtig for fiskeopdrættere, der anvender brakvand ved opdræt af ål, og herved kan få *V. vulnificus* ind i anlægget.

Forekomst af V. vulnificus er temperatur afhængig

Lav forekomst af *V. vulnificus* blev påvist i de 7 kystnære vandområder (0.8 til 19 kolonier/liter vand) fra juni indtil midten af september og i sediment (0.04 til >11 kolonier/g) (Høi *et al*., 1998c). Forekomsten af *V. vulnificus* var i høj grad korreleret til vandtemperaturen, hvilket også er rapporteret af andre (Wright *et al.,* 1996; O'Neill *et al.,* 1992; Kelly, 1982). *V. vulnificus* blev sjældent isoleret, når vandtemperaturen var under 15°C. Dog blev *V. vulnificus* påvist i et kystnært vandområde tæt ved et muslingeopdræt ved en temperatur på 7°C, som er lavere, end der tidligere er rapporteret i litteraturen (Wright *et al.,* 1996).

Kontrol af badevand i Danmark er baseret på undersøgelser af tilstedeværelsen af *Escherichia coli* og coliforme bakterier som indikatorer for vandkvaliteten. Som rapporteret andetsteds, viste analyser af data indsamlet i Danmark i 1996 ingen korrelation mellem *E. coli* og *V. vulnificus* (Høj *et al.,* 1998c).

V. vulnificus blev kun påvist i vildfisk, når vandtemperaturen var høj. *V. vulnificus* blev hyppigst fundet i gællerne fra ål, men 4 af 75 prøver fra ålekvabber og skrubber indeholdt også *V. vulnificus.*

Lav forekomst i skaldyr

Østers og muslinger produceret i Danmark og det omgivende vandmiljø blev undersøgt for forekomst af *V. vulnificus. V. vulnificus* blev isoleret fra både vand og blåmuslinger fra en lokalitet ud af ialt 13 lokaliteter, der blev undersøgt. Koncentrationen af *V. vulnificus* i blåmuslinger var meget lav (# 10 kolonier pr. gram muslingvæv), og den ringe sandsynlighed for at påvise én levende *V. vulnificus* celle kan forklare, hvorfor den ikke blev isoleret fra andre områder. Dyrkning af muslinger i de øverste vandlag giver mere gunstige temperaturer for vækst af *V. vulnificus* i sommerperioden, og det høje niveau af næringsstoffer giver gode betingelser for vækst og overlevelse af *V. vulnificus,* selv ved lave temperaturer.

V. vulnificus infektioner har endnu ikke været forårsaget af indtagelse af råskaldyr i Danmark eller rapporteret andetsteds i Europa. Hvilket viser, at der er en minimal risiko forbundet med at indtage råskaldyr, når de indeholder *V. vulnificus* i lavt antal.

Konklusion

Konkluderende viser vores undersøgelse med påvisning af *V. vulnificus* i vildfisk, at fiskere, specielt de med rifter på hænderne, har en risiko for at få *V. vulnificus* sårinfektioner. I 1994 og 1995, fik 7 personer *V. vulnificus* sårinfektioner i forbindelse med fiskeri eller håndtering af ål (Dalsgaard *et al.,* 1996b; Bruun, 1997). *V. vulnificus* tilfælde blev ikke rapporteret i sommeren 1996, da der var lave koncentrationer (<2 kolonier100 ml) i vandmiljøet. *V. vulnificus* niveauet var sandsynligvis for lavt til at forårsage infektion, selv i svækkede personer, og lave temperaturer opmuntrer ikke folk til at bade. Epidemiologiske data fra 1994 and 1995 antyder, at risikoen for at erhverve en *V. vulnificus* infektion i forbindelse med badevand var korreleret med vandtemperaturen. Derfor bør overvågning og monitering af forekomsten af *V. vulnificus* påbegyndes, når vandtemperaturen er over 20°C.

Lav forekomst af V. vulnificus i frosne importerede rejer

V. vulnificus er en naturligt forekommende bakterie i kystområder med høje vandtemperaturer, og kan derfor forventes at forkomme i rejer produceret i tropisk akvakultur.

EU importerede ca. 75 tons varmtvands rejer gennem Danmark i 1995 (Dalsgaard & Høi, 1997). Fore komsten af *V. vulnificus* i 107 prøver repræsenterende 37 sendinger af frosne rejer importeret fra Sydøst Asien blev undersøgt. *V. vulnificus* blev påvist i 3 ud af 46 (7 %) frosne rårejeprøver, men blev ikke påvist fra en eneste af 61 frosne kogte produkter. Fravær af *V. vulnificus* i frosne kogte rejeprodukter er tegn på en god produktions hygiejne samt tilstrækkelig varmebehandling (Dalsgaard & Høi, 1997). Den lave forekomst af *V. vulnificus* i frosne rårejeprodukter kan være forårsaget af bakteriens ringe resistens over for kulde (Oliver, 1981; Boutin *et al.,* 1985; Parker *et al.,* 1994). Frosne rejeprodukter opbevares sædvanligvis ved temperaturer på -20°C før og efter forsendelsen, ofte i lange perioder, og herved vil der forekomme en signifikant reduktion af mulige *V. vulnificus.*

Konklusion

Som konklusion kan siges, at fravær af *V. vulnificus* i frosne kogte rejeprodukter og den lave forekomst af *V. vulnificus* i frosne rå rejer tyder på, at *V. vulnificus* ikke udgør et sundhedsproblem i Danmark.

1 Introduction

Vibrio vulnificus, a halophilic marine vibrio, is an opportunistic human pathogen that can cause severe wound infections and septicemias with mortalities as high as 60 % (Oliver, 1989; Hlady & Klontz, 1996). The first fatal *V. vulnificus* infection was possibly reported in the 5th century B.C. by Hippocrates (Baethge & West, 1988). The king of the island Thasos in the Agean Sea got an acute infection which was characterized by a swollen foot with red and black skin lesions, rapidly progressive septicemia, and death on the second day. It is suggested that this infection was caused by *V. vulnificus* (Baethge & West, 1988).

In this century, most *V. vulnificus* infections have been reported from the United States, Japan, and Taiwan and since 1979 a number of cases in Belgium, Denmark, Germany, Holland, Israel, Korea, and Sweden, have been published (Mertens *et al.,* 1979; Yang et *al*., 1991; Veenstra *et al.,* 1992; Shin *et al*., 1993; Hoyer *et al.,* 1995; Melhus *et al.,* 1995; Dalsgaard *et al.,* 1996b; Bisharat & Raz, 1997).

V. vulnificus in Denmark

During the unusually warm summer in Denmark in 1994, coastal water temperatures exceeded 20°C for about four weeks (Dalsgaard *et al.,* 1996b). At the end of August 1994, 11 patients were admitted to hospitals with *V. vulnificus* infections; this was the first report of a series of human *V. vulnificus* infections from a temperate zone (Dalsgaard *et al.,* 1996b). All patients contracted their disease after exposure to coastal water; none had consumed any seafood. In 1995 and 1997, a total of 3 and 4 *V. vulnificus* infections, respectively, were reported in Denmark (Bruun, 1997). In 1996 and 1998, water temperatures were low and no cases were reported (Bruun, 1997). The clinical cases in 1994 prompted the Royal Veterinary and Agricultural Univer sity, The Danish Institute for Fisheries Research, The Environmental Protection Agency and the Ministry of Food, Agriculture, and Fisheries to fund studies on the occurrence of *V. vulnificus* in Danish costal waters.

V. vulnificus is furthermore also pathogenic to eels and the expanding Danish eel-farming industry recently experienced severe outbreaks of *V. vulnificus* infections causing significant economic losses (Dalsgaard *et al.,* in press). The use of brackish water in eel farms leads to higher growth potentials and thereby increased profits compared to the use of fresh water. However, water with low salinity of approximately 1% NaCl favors growth of *V. vulnificus* in eel farms where water temperatures usually are 23-24°C.

2 Identification of *Vibrio vulnificus*

2.1 Classification and taxonomy

V. vulnificus is a halophilic marine bacterium (Baumann & Schubert, 1984). As other members of the genus *Vibrio*, family *Vibrionaceae*, it is a Gram-negative rod, aerobic and facultatively anaerobic, motile by means of a polar sheated flagellum, and is oxidase and catalase positive (Baumann & Schubert, 1984).

The taxonomy of *V. vulnificus* was first investigated by Baumann *et al.* (1973). *V. vulnificus* was described as a group of Gram-negative, fermentative marine organisms which were assigned to group C-2. Hollis *et al*. (1976) designated the same organism as Alactose-positive *Vibrio*@ or AL+*Vibrio*@ since the ability to ferment lactose was one characteristic that could distinguish this species from *Vibrio parahaemolyticus* and *Vibrio alginolyticus.* Today it is known that the lactose fermentation reaction is negative in 25% of the *V. vulnificus* isolates (Baumann *et al.,* 1971). Strains within group C-2 were found to be genetically related, based on DNA/DNA hybridization and were assigned as a new species designated *Beneckea vulnifica* (A*vulnifica*@ means wound in Latin) (Reichelt *et al.,* 1976). Similar studies performed on the L+*Vibrio* concluded that this group was a species separate from *V. parahaemolyticus* and *V. alginolyticus* (Clark & Steigerwalt, 1977). In 1979, the transfer of *Beneckea vulnifica* (synonym = L+*Vibrio*) to the genus *Vibrio* was proposed and its name became *V. vulnificus* (Farmer, 1979). The name *V. vulnificus* was given official taxonomic status in 1980 (Farmer, 1980). It is believed that *V. vulnificus* formerly often was misidentified as *V. parahaemolyticus* (Hollis *et al*., 1976) The species *V. vulnificus* comprises two biotypes which in the original definition differed phenotypically, serologically, and in host range (Tison *et al.,* 1982) (Table 1).

V. vulnificus biotype 1 is ubiquitous to estuarine environments and is an opportunistic human pathogen (Oliver, 1989). Biotype 2 is typically recovered from diseased eels but is also reported to cause illness in humans after handling eels (Mertens *et al.,* 1979; Dalsgaard *et al.,* 1996b). The division into biotypes is no longer supported and a division into serovars has been suggested (Høi *et al.,* 1998b). The term Abiotype A is still used in the literature and will therefore also be used in this thesis. Chapter 5 includes the latest suggestions in this area and explains why Aserovar@ is a better term than Abiotype@.

Several studies have dealt with the phylogenetic relatedness of *V. vulnificus* and other *Vibrio* species based on sequencing of 5S, 16S and 23S rRNA as well as immunological relationship among superoxide dismutases (Baumann *et al.,* 1980; MacDonell & Colwell, 1985; Dorsch *et al.,* 1992; Aznar *et al.,* 1994). Three types of cluster analysis based on 5S rRNA sequences all generated dendrograms where *V. vulnificus* showed the highest similarity to *V. cholerae* (MacDonell & Colwell, 1985). In another study *V. vulnificus* was described to be more closely related to *V. parahaemolyticus* based on 16S rRNA sequences obtained by reverse transcription of cDNA (Fig. 1) (Dorsch *et al.,* 1992). A later more extensive study using alignment of 16S rRNA sequences reported that *V. vulnificus* did not belong to the core organisms of the genus *Vibrio* (Fig. 2) (Aznar *et al.,* 1994).

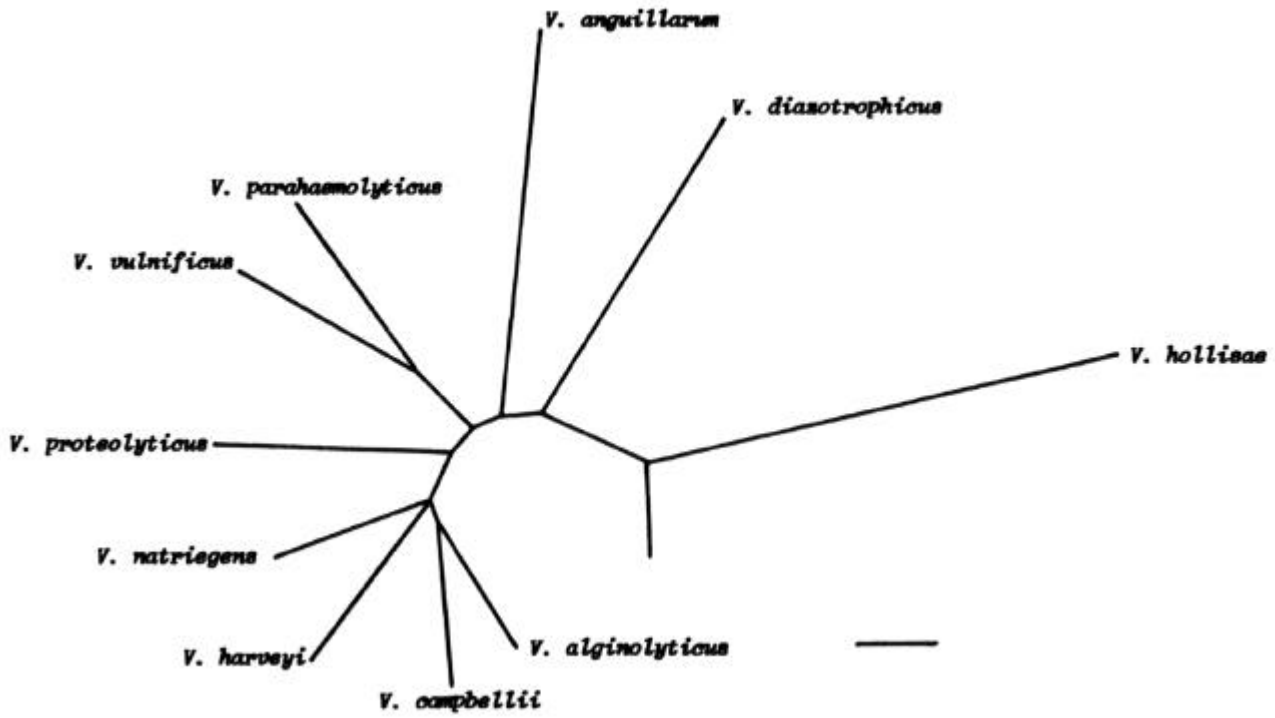


Figure 1: *Phylogenetic relationship among 10 Vibrio species based on 16S rRNA sequences (Dorsch et. al., 1992). Bar = 10 nucleotide exchanges.*

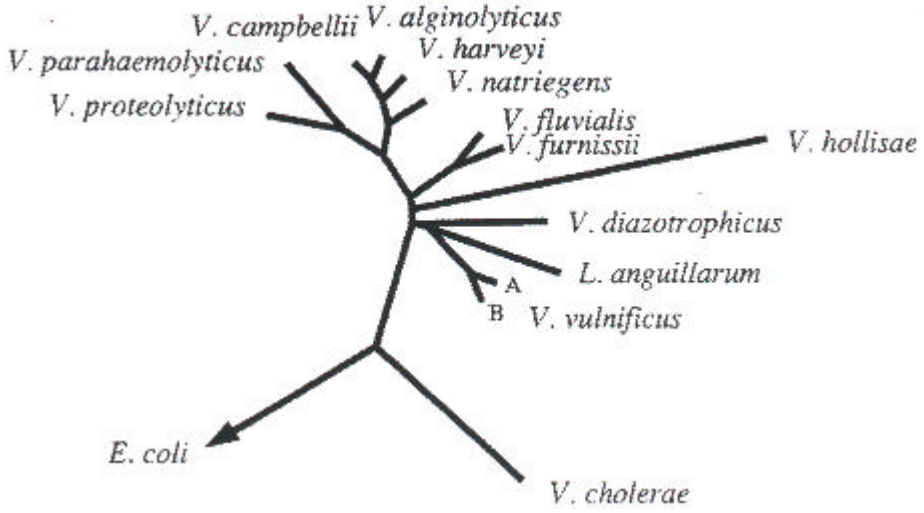


Figure 2: *Phylogenetic tree showing the phylogenetic relationship of V. vulnificus and other Vibrio species based on 16S rRNA sequences. A and B represent two subgroups of 16S rRNA detected in V. vulnificus (Aznar et. al., 1994)*

Data from comparative immunological studies of superoxide dismutases of vibrios supported that *V. vulnificus* is not closely related to the core organisms of the genus *Vibrio* (Baumann *et al.,* 1980). Fig. 2 shows that two subgroups (A and B) among *V. vulnificus* were identified based on 16S rRNA alignments (Aznar *et al.,* 1994). Strains of both biotypes belonged to group A and Aznar *et al.* (1994) concluded that the separation of *V. vulnificus* into two biotypes defined by biochemical and immunological properties does not reflect the genetical relationship of the strains. The phylogeny of the family *Vibrionaceae,* including *V. vulnificus* is at present not well established.

Table 1. *Characteristics of V. vulnificus biotype 1 and 2 strains as originally defined by Tison et al. (1982). Values are percentage positive strains.*

Test	*V. vulnificus* biotype 1 (9 strains)	*V. vulnificus* biotype 2 (3 strains)
Indole production (Kovács)	100	0
Ornithine decarboxylase (Møller)	89	0
D-Mannitol	44	0
D-Sorbitol	44	0
Growth at 42°C	44	0
Pathogenicity for eels	0	100
Pathogenicity for mice	100	100
Agglutination with rabbit antiserum raised against biotype 2 strains	0	100

2.2 Biochemical identification

Biochemical identification of *V. vulnificus* requires a number of biochemical assays which are costly and the result can not be obtained within the same day (Colwell, 1984; Murray, 1995). Table 2 shows selected biochemical characters of *V. vulnificus* and illustrates the difficulties in making a definitive identification based on biochemical reactions. Various references do not agree on reactions in certain biochemical tests. The API 20E index, which is only based on 31 *V. vulnificus* strains, differs from the reactions shown in three manuals in the lysine decarboxylase and Voges-Proskauer reactions (Table 2) (Barrow & Feltham, 1993;. Holt *et al.,* 1994; Murray, 1995; Sinding, 1998). In general, reactions listed in reference manuals are based on a limited number of clinical strains and do not include the more heterogenous reaction patterns of environmental strains, e.g. 3% of 66 environmental *V. vulnificus* isolates were positive in arginine decarboxylase even though this is not described in Manual of Clinical Microbiology or the API 20E index (Table 2) (Dalsgaard *et al.,* 1996a). Commercial kits for identification of *Enterobacteriaceae* can give false reactions when used to identify *V. vulnificus* since they may contain inadequate amounts of NaCl for growth of this halophilic *Vibrio* species (Horre *et al.,* 1996). To avoid this problem, a minimum of 0.5 % NaCl must be present or added to the medium (McLaughlin, 1995). MacDonell *et al.* (1982) recommended an addition of 2% marine salts to the API 20E system and incubation at 22°C. False positive reactions may be produced when the bacterial concentration in the diluent broth is too high (Davies *et al.,* 1995).

Suspected *V. vulnificus* isolates from environmental samples were more frequently identified as *V. vulnificus* by a DNA probe directed against the cytolysin gene than by the API 20E system (Dalsgaard *et al.,* 1996a). Sixty-six isolates identified as *V.*

vulnificus with the specific DNA probe were tested with the API 20E strip according to the manufacturers instructions (Table 2). A total of 29 API 20E profiles were obtained. Only four of these profiles, representing 20 isolates, reached the identification threshold of *V. vulnificus.* The reasons for these difficulties could be discrepancies between the species-specific reaction pattern in the API 20E database and the reactions given in standard identification tables, the heterogeneity of environmental strains, and the limited number of *V. vulnificus* strains (31 strains) included in the API 20E database (Dalsgaard *et al.,* 1996a; Sinding, 1998).

Table 2*. Biochemical characters of V. vulnificus (Barrow & Feltham, 1993; Holt et al., 1994; bioMérieux, 1995; Murray, 1995; Dalsgaard et al., 1996a)*

Reaction	Cowan and Steel's Manual for the Identification of Medical Bacteria	Manual of Clinical Microbiology (percentage positive)	Bergey's Manual of Determinative Bacteriology	API 20E version 3.1 (percentage positive)	Dalsgaard *et al.* (1996) (percentage positive)
Swarming	-[a]	-[b]	-[b]	NA[c]	NA
0% NaCl	-	0	-	NA	NA
1% NaCl	NA	99	+	NA	NA
6% NaCl	NA	65	d[d]	NA	NA
8% NaCl	NA	0	-	NA	NA
Growth at 4°C	NA	NA	-	NA	NA
Growth at 35°C	NA	NA	+	NA	NA
Growth at 40°C	NA	NA	+	NA	NA
Arginine decarboxylase	-	0	-	0	3
Lysine decarboxylase	+[e]	99	+[f]	68	90
Ornithine decarboxylase	+	55	d	90	33
Oxidase	+	100	+	99	100
Nitrate reduction	+	100	+	NA	NA
Gas from glucose	-	0	-	NA	NA
Indole	+	97	+	99	95
ONPG[g]	+	75	d	100	91
Voges-Proskauer	-	0	-	18	8
Urease	-	1	-	0	0
Citrate utilization	NA	75	d	81	0
H_2S production	NA	0	-	0	0

Gelatinase	NA	75	d	99	100
Resistance to O/129[h] 150ìg	-	2	-	NA	NA
Resistance to Polymyxin B	D[i]	97	+	NA	NA
Acid production from					
L-arabinose	NA	0	-	1	0
Cellobiose	NA	99	+	NA	NA
D-glucose	NA	100	+	100	91
myo-inositol	NA	0	-	0	3
Lactose	NA	85	d	NA	NA

Table 2*. Continued.*

Reaction	Cowan and Steel's Manual for the Identification of Medical Bacteria	Manual of Clinical Microbiology (percentage positive)	Bergey's Manual of Determinative Bacteriology	API 20E version 3.1 (percentage positive)	Dalsgaard *et al.* (1996) (percentage positive)
D-mannitol	NA	45	d	36	41
Melibiose	NA	40	d	0	3
L-rhamnose	NA	0	-	0	2
D-sorbitol	NA	0	-	0	2

85-100% of strains are negative; [b] 90% or more of strains are negative; [c] not avaliable; [d] 11-89% of strains are positive;
[e] 85-100% positive; [f] 90% or more of strains are positive; [g] ONPG, o-nitrophenyl-ß-D-galactopyranoside;
[h] O/129, vibriostaticum (2-4-diamino-6,7-diisoroylpteridine) ; [i] 16-84% of strains are positive.

Biosca *et al.* (1993a) reported that the API 20E system gave several false negative reactions when compared to conventional biochemically tests including the citrate test. Dalsgaard *et al.* (1996a) also reported that none of the 66 strains tested could utilize citrate in the API 20E test strip (Table 2). Changing the diluent in the API 20E from containing 0.9% NaCl (as recommended by the manufacturer) to 2% marine salts can promote citrate utilization (MacDonell *et al.,* 1982).

Biotype 2 isolates are not included in the API 20E system and can not be identified by this system (Biosca *et al.,* 1993a). The three manuals referred in Table 2 do not recognize the existence of *V. vulnificus* biotype 2 strains since they do not include indole negative strains.

Two studies have suggested that the API 20E can be used to identify the more common members of *Vibrionaceae* including *V. vulnificus* (Overman *et al.,* 1985; Overman & Overley, 1986). These studies are based on a limited number of *Vibrio* sp. including one *V. vulnificus* strain each of unknown origin.

2.3 Identification using serological and molecular methods

The US Food and Drug Administration (USFDA) is currently using an enzyme immunoassay (EIA) in an ELISA format to identify presumptive *V. vulnificus* subcultured from modified cellobiose polymyxin B colistin (mCPC) agar (US Food and Drug Administration, 1995). The assay uses a *V. vulnificus*-specific monoclonal antibody (MAb) directed against an intracellular epitope of *V. vulnificus* (Tamplin *et al.,* 1991; US Food and Drug Administration, 1995). No cross reactions to other *Vibrio* species and non-*Vibrio* species have been described and the ELISA format reduces assay time and facilitates handling of large numbers of test samples (Tamplin *et al.,* 1991). The cell line producing the *V. vulnificus*-specific monoclonal antibody (MAb) is available at the American Type Culture Collection (ATCC).

Detection by ELISA method

Parker & Lewis (1995) developed a sandwich ELISA assay for detection of *V. vulnificus* in environmental specimens using anti-hemolysin as capture and detector antibody reagents. The authors claimed that the sandwich ELISA offers time-saving and labor-saving advantages over the currently accepted EIA but so far no other researchers have chosen to use the sandwich ELISA (Parker & Lewis, 1995).

Agglunitiation with anti-flagellar antibody

V. vulnificus can be identified one step beyond primary isolation with anti-flagellar (anti-H) antibodies (Simonson & Siebeling, 1986). The anti-H antibodies are produced in rabbits immunized with the flagellar core protein prepared from *V. vulnificus.* The antibodies are coated onto *Staphylococcus aureus* Cowan 1 cells to permit visual coagglutination within the time frame of the slide test. The agglutination reaction is fast and reliable and has been optimized to include the use of MAbs. The anti-core H antibodies are not available commercially so the technique includes purification of the flagellar protein and immunization of rabbits which is time-consuming (Simonson & Siebeling, 1986).

Detection with DNA probes

Molecular techniques, particularly specific oligonucleotide probes, constitute a very sensitive and specific tool for detecting *V. vulnificus.* An alkaline phosphatase-labeled oligonucleotide probe directed towards the cytolysin gene of *V. vulnificus* was constructed by Wright *et al.* (1985,1993). This probe, termed VVAP, demonstrated 100% specificity and sensitivity for clinical and environmental isolates of *V. vulnificus* and numerous investigators have shown that cytolysin is produced by all *V. vulnificus* strains, including both biotypes, and is species-specific (Kaysner *et al.,* 1987; Morris *et al.,* 1987a; Parker & Lewis, 1995; Biosca *et al.,* 1996c). The sequence of the cytolysin gene has also been used for constructing primers for PCR identification (Brauns *et al.,* 1991; Coleman *et al.,* 1996). Two studies have argued that the use of the cytolysin gene as a target region in PCR amplification or as a target for an oligonucleotide probe is not suitable for the detection of *V. vulnificus* (Aznar *et al.,* 1994; Arias *et al.,* 1995). A non-essential gene, such as the cytolysin gene, could theoretically be lost or rearranged without affecting the viability of the bacteria. Instead, it has been suggested to use primers/probes directed against rRNA genes, since rRNA molecules are essential constituents

of all living organisms and are present in growing cells in very high numbers (Aznar *et al.,* 1994; Arias *et al.,* 1995). A recent comparative study of the VVAP probe to an oligonucleotide probe directed against a sequence in the 16S rRNA region showed that the rRNA probe was slightly more specific and sensitive than the VVAP probe for identification of *V. vulnificus* (Biosca *et al.,* submitted). The VVAP probe failed to detect two of 308 *V. vulnificus* strains tested and further hybridized with two isolates of 104 non-*V. vulnificus* strains tested (Biosca *et al.,* submitted).

Colony hybridization

Identification of *V. vulnificus* with colony hybridization offers at least three advantages compared to biochemical testing: (i) *V. vulnificus* usually produces strong signals with virtually no background with alkaline phosphatase-labeled oligonucleotide probes facilitating interpretations of hybridization membranes, whereas biochemical reaction patterns can be difficult to interpret, (ii) identification of suspect isolates with oligonucleotide probes is more reliable than conventional biochemical identification (Dalsgaard *et al.,* 1996a), (iii) colony hybridization is less time-consuming and cheaper than conventional biochemical identification, especially if the probes are used in a low concentration and reused as recommended by Høi *et. al.* (1998c).

VBNC state

During the colder months *V. vulnificus* apparently enters a viable-but-nonculturable (VBNC) state or at least a state where the bacterium cannot be cultured by ordinary bacteriological methods (Oliver, 1995). Detection by PCR or direct detection with flourescent tagged antibodies may solve the problem of nonculturability. However, the importance of VBNC cells in the ecology and virulence of *V. vulnificus* is not finally established and will be further discussed in section 3.1 (Brauns *et al.,* 1991).

2.4 Conclusions from Chapter 2

Identification of *V. vulnificus* with the commercial biochemical testing kit API 20E is not reliable because *V. vulnificus* strains produce heterogenous biochemical reaction patterns. Serological identification requires preparation of antibodies since no commercial *V. vulnificus*-specific antibodies are available. Colony hybridization with a *V. vulnificus*-specific oligonucleotide probe is specific, fast and cost-effective. Identification of *V. vulnificus* with oligonucleotide probes or PCR primers directed against rRNA sequences is recommended.

3 Isolation of *Vibrio vulnificus* from environmental samples

3.1 Use of pre-enrichment broths

The choice of including a pre-enrichment step in isolation of *V. vulnificus* depends on four factors: (i) the expected concentration of *V. vulnificus* in the samples, (ii) if a quantitative or qualitative result is needed, (iii) the conditions of the cells, and (iv) the level and composition of background flora. The pre-enrichment step should improve the ratio of target to background flora before a selective plating step.

Direct plating

The sensitivity of direct plating procedures are limited by the capacity of the agar surface of the plating medium to absorb inoculum. Therefore, the detection limit for direct plating procedures is higher than for procedures including a pre-enrichment step and samples with low concentrations (less than 10 CFU per gram) of *V. vulnificus* should be pre-enriched (DePaola *et al.,* 1997b). The sensitivity of direct plating procedures are also limited by the presence of selective substances in the agar, e.g. antibiotics, which may inhibit part of the target flora.

MPN methods

Results obtained by most-probable-number (MPN) methods including a pre-enrichment step are not as precise as results obtained by direct plating procedures. In one study, the measurement variance of a MPN procedure was 0.118 compared to 0.004 when using a direct plating procedure; and even a 10 tube MPN method would still have more than 8 times the measurement variance than the direct plating procedure (DePaola *et al.,* 1997b). Pre-enrichment procedures often give improved recovery of *V. vulnificus* compared to direct plating on selective agars but the choice of procedure should always depend on the sample type (Kaysner *et al.,* 1989; Biosca *et al.,* 1997b; DePaola *et al.,* 1997b; Arias *et al.,* 1998a).

Alkaline peptone water and TCBS agar

The isolation of pathogenic *Vibrio* spp. is usually accomplished by culture methods that start with pre-enrichment in alkaline peptone water (APW; 1% peptone, pH 8.6) with 1% NaCl to recover sublethal injured organisms, followed by plating onto thiosulfate-citrate-bile salts-sucrose (TCBS) agar (Colwell, 1984). Earlier studies of the environmental distribution of *V. vulnificus*, as well as clinical investigations, used this protocol, which was developed for other *Vibrio* spp. and not optimized for the isolation of *V. vulnificus* (Oliver *et al.,* 1982). Various enrichment broths have been tested for their capability to support the isolation of *V. vulnificus,* including APW with various salt concentrations, marine broth, salt-polymyxin B broth, Horie-s broth, Monsur-s broth and glucose-salt-teepol broth (Sloan *et al.,* 1992; Hagen *et al.,* 1994; Biosca *et al.,* 1997b; Arias *et al.,* 1998a). Overnight pre-enrichment in APW with 1% NaCl at 35-37°C generally gives the best recovery of *V. vulnificus* and this procedure is recommended in the Bacteriological Analytical Manual of the USFDA (US Food and Drug Administration, 1995).The use of APW in combination with

cellobiose-polymyxin B-colistin (CPC) agar and modified CPC (mCPC) agar has been reported to be effective in recovering *V. vulnificus* from oyster and water samples (Tamplin *et al.,* 1991; Tamplin & Capers, 1992).

APW with polymyxin B

Overnight pre-enrichment in APW with polymyxin B (20 U/ml; APWP) gave higher recovery rate than pre-enrichment in regular APW in combination with mCPC agar[1] when analyzing samples of coastal water and sediment in Denmark (Dalsgaard *et al.,* 1996a). APWP and mCPC agar was subsequently used with success for isolation of *V. vulnificus* from fresh and frozen seafood (Dalsgaard & Høi, 1997; Høi *et al.,* 1998c). However, when analyzing gills, mucus, and intestinal content from cultured diseased eels and wild fish from Danish coastal waters pre-enrichment in APW for 6-8 h proved more favorable than overnight pre-enrichment in APWP (Høi *et al.,* 1998c; unpublished results). This finding may be explained by a lower background flora in samples from healthy wild fish compared to samples of coastal water, sediment, and seafood and therefore there may be less need for adding antibiotics. Samples from diseased eels may be dominated by *V. vulnificus* and therefore a short pre-enrichment period may be sufficient. Recent studies with heavily infected eels showed that direct plating of tissue samples homogenized in phosphate-buffered-saline (PBS) gave the same or sometimes even better recovery of *V. vulnificus* than by pre-enrichment (unpublished results). Other studies have also reported that different sample types requires different isolation strategies for *V. vulnificus* (Biosca *et al.,* 1997b; Kaysner *et al.,* 1989). Arias *et al.* (1998a) reported that 3 h pre-enrichment in APW with 3% NaCl followed by streaking onto CPC agar was optimal for recovering *V. vulnificus* from seawater and shellfish samples from the Western Mediterranean coast and that this culture technique gave more positive results than detection by direct PCR. The high salt concentration in the pre-enrichment may favor isolation of *V. vulnificus* cells adapted to the high salinity in the Mediterranean (around 35 ‰).

Recently, the components of a possible enrichment broth were examined in laboratory testing using pure cultures. An enrichment broth containing 5% peptone, 1% NaCl, and 0.08 % cellobiose amended with 1 to 4 U colistin per ml (PNCC; pH 8.0) was suggested for future field studies (Hsu *et al.,* 1998). Studies with 50 *V. vulnificus* strains from various sources and countries showed that no strains had a minimal inhibitory concentration (MIC) lower than 779 U colistin/ml (Høi et al., 1998a); thus recovery should not be reduced by adding as high a concentration as 20 U colistin/ml to the enrichment broth (Høi *et al.,* 1998a).

3.2 Use of selective agars

The use of a selective and indicative medium for isolation of *V. vulnificus* serves two purposes: (i) to allow growth of *V. vulnificus* while inhibiting growth of more abundant marine species, (ii) to allow differentiation of *V. vulnificus* from other bacterial species present so suspect colonies can be further identified.

CPC and mCPC selective and indicative agars

New media have been developed and recommended for the isolation of *V. vulnificus* from the environment (Brayton *et al.,* 1983; Bryant *et al.,* 1987; Massad & Oliver, 1987; Miceli *et al.,* 1993). Cellobiose-polymyxin B-colistin (CPC) agar was first described in 1987 for isolation and differentiation of *V. vulnificus* (Massad & Oliver, 1987). The medium takes advantage of the colistin and polymyxin B resistance of *V. vulnificus* and the fermentation of cellobiose for differentiation. Further, high temperature incubation (40°C) eliminates many marine bacteria. CPC agar was clearly superior to TCBS, sodium dodecyl sulfate-polymyxin B-sucrose

[1]The agar used in Dalsgaard *et al.*, 1996a is mCPC agar and not CPC agar. A laboratory miscalculation was discovered after the manuscript was published.

(SPS) agar, and *V. vulnificus* enumeration (VVE) agar for the isolation of *V. vulnificus* from environmental samples (Sun & Oliver, 1995; Oliver *et al.,* 1992). Tamplin *et al*. (1991,1992) described a less selective modification of the CPC agar termed mCPC with a reduced concentration of colistin. This medium has been reported to be effective in isolating *V. vulnificus* from environmental sources (Tamplin *et al.,* 1991; Tamplin & Capers, 1992; Dalsgaard & Høi, 1997; Høi *et al.,* 1998c). In Denmark, more than 95% of presumptive colonies on mCPC agar could be identified as *V. vulnificus* when taking into consideration the typical colony morphology of *V. vulnificus* on this medium (flat, yellow colonies of approximately 2 mm in diameter) (Høi *et al.,* 1998a; Høi *et al.,* 1998c). However, in Spain only 8% of the presumptive colonies from CPC agar were identified as *V. vulnificus* by PCR (Arias *et al.,*1998a). Possible explanations for this disagreement could be: (i) lack of experience in recognizing presumptive colonies, (ii) the background flora in samples from the Mediterranean differs from the background flora in Danish environmental samples, (iii) the description of presumptive colonies as Ayellow colonies surrounded by a yellow halo@ is not sufficient, or (iv) the agar may have been made with different brands of reagents than used in Denmark or in a slightly different way, and this may have affected the colony appearance on the agar (Arias *et al.,* 1998a). In Denmark, we experienced that it was very important for the identification-success rate to include the criterion Aflat@ in the evaluation of suspect colonies (Høi *et al.,* 1998a; Høi *et al.,* 1998c).

Bactericidal activity of polymyxins

Arguments for using both polymyxin B and colistin in a *V. vulnificus*-selective agar have not been provided (Massad & Oliver, 1987; Tamplin *et al.,* 1991). Colistin and polymyxin B are both fatty acyl decapeptide antibiotics with bactericidal activity against most Gram-negative bacteria and are known by the name Apolymyxins@(Søgaard, 1982). The chemical composition of colistin and polymyxin B differs only in a single amino acid, and their mode of action and microbiological activity are identical (Søgaard, 1982). The basic polymyxin antibiotics act specifically on Gram- negative bacteria by electrostatic and hydrophobic interactions with anionic phospholipids and the lipid A group of lipopolysaccharide (LPS). Ultimately, a lethal effect is exerted by disruption of the cytoplasmic membrane resulting in leakage of periplasmic contents and small cytoplasmic molecules (Søgaard, 1982). The following mechanisms can be proposed to explain why *V. vulnificus* is relatively resistant to polymyxins: (i) polymyxins may not bind to the cell wall because of the fatty acid composition of the phospholipids in the outer membrane, (ii) the cytoplasmic membrane of *V. vulnificus* may contain low amounts of phosphatidylethanolamine, a substance that increases susceptibility to polymyxins, (iii) the access of polymyxins to the susceptible cytoplasmic membrane may be blocked by the polysaccharide capsule, or (iv) initial binding of polymyxins occur where divalent cations are present in the outer membrane, and the net ionic charge of the *V. vulnificus* membrane may differ from that of susceptible bacteria.

CC agar

Høi *et al.* (1998a) examined a collection of *V. vulnificus* strains for their sensitivity to colistin and recommended a new medium termed cellobiose colistin (CC) agar. CC agar gave a better *V. vulnificus* recovery than TCBS, CPC and mCPC agar in laboratory studies with pure cultures and with Danish water and sediment samples. *V. vulnificus* was isolated from 179 of 446 (40%) APWP pre-enrichments using CC agar and from 154 (35%) of the same 446 APWP pre-enrichments using mCPC agar in the investigation of 26 water samples and 14 sediment samples (Høi *et al.,* 1998a). The recovery rate on CC agar was significantly better than on mCPC agar (Høi *et al.,* 1998a). TCBS agar gave a very low plating efficiency (1%) of both clinical and environmental *V. vulnificus* strains and should not be recommended for the isolation of *V. vulnificus* (Høi *et al.,* 1998a). This is in agreement with other reports of low recovery of *V. vulnificus* on TCBS (Brayton *et al.,* 1983; Beazley & Palmer, 1992). Fig. 3 shows an overview of the methods used for isolation of *V. vulnificus* in Denmark.

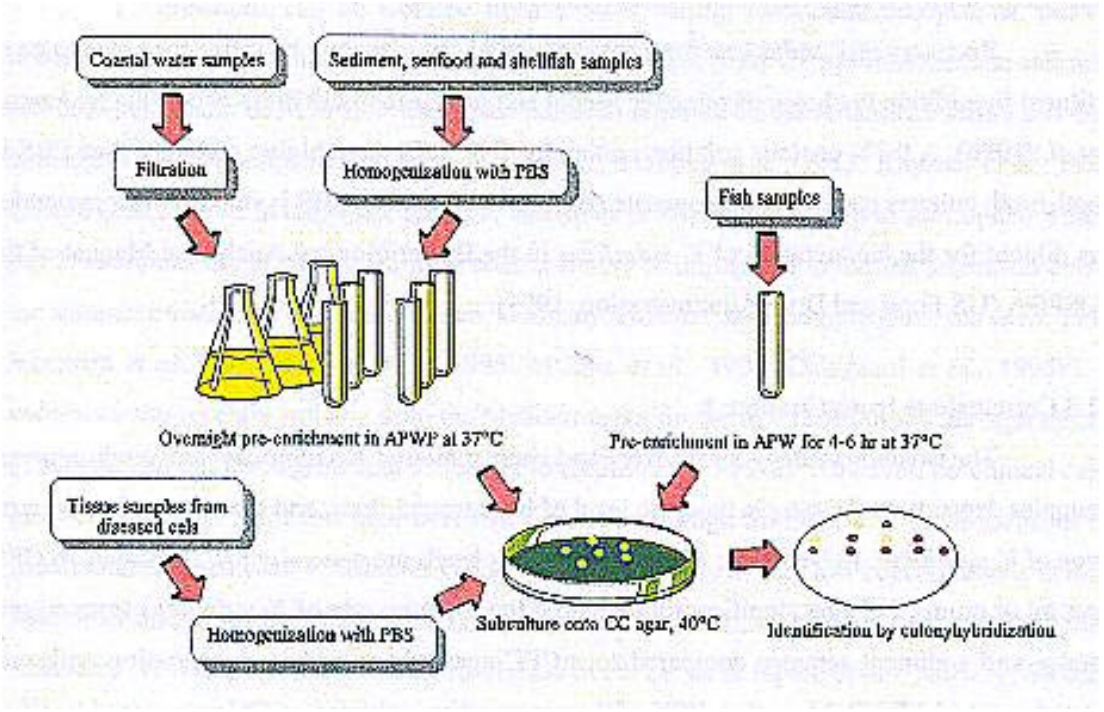


Figure 3: *Flow diagram for isolation of V. vulnificus in Denmark*

Although, *V. vulnificus* has been described to be resistant to colistin and polymyxin B (Massad & Oliver, 1987), plating efficiency[2] experiments showed that CC agar, which has the lowest concentration of colistin compared to the other media tested, gave the best recovery of *V. vulnificus.* The results showed that *V. vulnificus* was inhibited by increasing concentrations of colistin and polymyxin B and that a proportion of the *V. vulnificus* strains present in Danish marine environments were inhibited by the concentration of colistin and polymyxin B in CPC and mCPC agar, and that the use of CC agar increased the isolation rate of *V. vulnificus.* Even though MIC testing suggested that mCPC and CC agar both were suitable for the use in isolation of *V. vulnificus*, further investigations proved that CC agar was superior to mCPC in isolation of *V. vulnificus* from samples of coastal water and sediment (Høi *et al.,* 1998a). This underlines the importance of testing selective agars for "ruggedness" using a variety of naturally contaminated samples.

The confirmation rate of presumptive isolates from CC agar was as high as previously reported for mCPC (approximately 95%) (Høi *et al.,* 1998a).

Isolation strategy

Recovery of *V. vulnificus* from environmental samples may be influenced by the choice of diluent in addition to choice of selective media and pre-enrichment broth according to Azanza *et al.* (1996). A 0.1% peptone solution containing 3% NaCl gave higher recovery than PBS in both broth cultures and oyster homogenate (Azanza *et al.,* 1996). PBS is currently recommended as diluent for the enumeration of *V. vulnificus* in the Bacteriological Analytical Manual of the USFDA (US Food and Drug Administration, 1995).

[2]The term plating efficiency was defined as the percentage of CFU which can be recovered on a selective medium compared to the CFU encountered on a corresponding non-selective blood agar plate.

3.3 Conclusions from Chapter 3

The isolation strategy for recovery and enumeration of *V. vulnificus* from environmental samples depends on the sample type, the level of background flora, and the expected concentration of *V. vulnificus.* In Denmark, where *V. vulnificus* levels are generally low (less than 10 CFU per ml or gram), CC agar significantly increased the isolation rate of *V. vulnificus* from coastal water and sediment samples compared to mCPC agar when used in combination with pre-enrichment in APWP. More than 95% of the presumptive colonies on CC agar were identified as *V. vulnificus* with the VVAP probe. TCBS gave very low plating efficiencies and can not be recommended for the isolation of *V. vulnificus.*

4 Occurrence and virulence of *Vibrio vulnificus*

4.1 Ecology

V. vulnificus in aquatic ecosystems

V. vulnificus can be isolated from a wide variety of aquatic ecosystems, but the occurrence of the organism is favored by high temperatures (>20°C) and intermediate salinities (15-25 ‰) (Motes *et al.,* 1998). *V. vulnificus* has been reported on the Atlantic, Pacific, and Gulf coasts of the US (Kelly, 1982; Oliver *et al.,* 1982; Tamplin *et al.,* 1982; Kaysner *et al.,* 1987; O'Neill *et al.,* 1992). In temperate areas, *V. vulnificus* is less abundant than in subtropical waters, but *V. vulnificus* has been isolated from coastal waters or implicated in human infections during the summer months in Denmark, Sweden, Germany, Holland, and Belgium (Mertens *et al.,* 1979; Veenstra *et al.,* 1994; Hoyer *et al.,* 1995; Melhus *et al.,* 1995; Dalsgaard *et al.,* 1996b). *V. vulnificus* was recently isolated from the Mediterranean for the first time despite the high salinity (35 ‰) that do not favor growth of *V. vulnificus* (Arias *et al.,* 1998a). However, no clinical cases has been reported from this area in recent time even though millions of tourists swim in the Mediterranean each year. *V. vulnificus* occurs most likely only in very low concentrations in these waters because of the high salinity, and the concentration is apparently too low to cause human infections. *V. vulnificus* has also caused disease in eel farms in Japan, Spain, Norway, Sweden, and Denmark (Muroga *et al*., 1976, Biosca *et al.,* 1991; Høi *et al.,* 1998b; Dalsgaard *et al*., in press).

Reservoirs and vehicles of transmission

Oysters, clams, mussels, fish, plankton, as well as water and sediment have all been described as reservoirs and vehicles for *V. vulnificus* (Oliver *et al.,* 1983; Kaysner *et al.,* 1987; Oliver, 1989; DePaola *et al.,* 1994; Dalsgaard *et al.,* 1996a; Wright *et al.,* 1996; Biosca *et al.,* 1997b). *V. vulnificus* has been isolated from waters with temperatures from 7°C to 31°C and salinities between 1 to 35 ‰ (Kaysner *et al.,*1987; Wright *et al.,*1996; Arias *et al.,*1998a; Høi *et al.,*1998c). *V. vulnificus* tolerates wide ranges of salinities and temperatures and is abundant in water with temperatures above 20°C and salinities between 15 to 25 ‰ (Kelly, 1982; O'Neill *et al.,* 1992; Kaspar & Tamplin, 1993; Motes *et al.,* 1998). Additional factors (e.g., sunlight, pH, nutrient factors, presence of competing bacterial populations, and grazing) may also affect the distribution of *V. vulnificus* in the environment.

Grazing

Grazing by protozoa is one of the main biological processes that control bacterial density in marine environments but at the present time it is not known to what extent grazing effects the ecology of *V. vulnificus* (Barciana *et al.,* 1997). Bacteriophages lytic to *V.*

vulnificus have recently been reported in estuarine waters, sediments, plankton, shellfish, and the intestines of finfish from the Gulf of Mexico (DePaola *et al.,* 1997a; DePaola *et al.,* 1998). The number of plaque forming units (PFU) did not correlate with densities of *V. vulnificus* - the lowest number of PFU was found in intestinal contents from fish where the number of *V. vulnificus* was highest (DePaola *et al.,* 1997a). The greatest variety and abundance of phages lytic to *V. vulnificus* were isolated from Gulf of Mexico oysters. The abundance of phages ranged from 10^1 to 10^5 PFU per gram of oyster tissue (DePaola *et al.,* 1997a). The *V. vulnificus*-specific-phages from the Gulf of Mexico were able to lyse *V. vulnificus* isolated from diseased eels in Denmark which suggests that the unidentified attachment sites for these phages are present on *V. vulnificus* strains worldwide (Høi *et al.,* 1998b). Future research will probably reveal that phages play a significant role in the ecology and perhaps even in virulence of *V. vulnificus* as has been described for other bacteria (Hennes & Simon, 1995; Waldor & Mekalanos, 1996; Cochran & Paul, 1998).

No correlation with fecal indicators

Oliver *et al.* (1982, 1983) investigated the distribution and ecology of *V. vulnificus* along the East coast of the United States and found no correlation between the prevalence of *V. vulnificus* and faecal coliforms. Tamplin *et al*. (1982) described a negative correlation between *V. vulnificus* and faecal coliform; *V. vulnificus* was most frequently isolated from samples with less than three faecal coliform per 100 ml. An inverse correlation between faecal coliforms and counts of *V. cholerae* has also been described by Dalsgaard (1994).

Seasonal occurrence

The occurrence and prevalence of *V. vulnificus* is seasonal (Kelly, 1982; O'Neill *et al.,* 1992). The mechanism of the seasonal variation was speculated to be similar to that of *V. parahaemolyticus* which has been described to overwinter in bottom sediments and enter the water column again when warm temperatures return (Kelly, 1982). However, several studies have speculated that the disappearance of *V. vulnificus* in cold periods is not due to die-off but to entry into the Aviable but non-culturable@ (VBNC) state (Oliver, 1995). In this state, the cells have been shown to be viable with several direct viability assays but can no longer be cultured on routine media. VBNC cells can resuscitate and are virulent in an iron-overload adult mouse model according to Oliver & Bockian (1995). The majority of human infections occur in the warm summer months when densities of culturable *V. vulnificus* cells are high and the importance of VBNC cells in human infections remains to be definitively determined (Klontz *et al.,* 1988). The ability of VBNC cells to resuscitate when the stress (cold temperature) is eliminated or if reappearance is due to regrowth by a few non-detectable culturable cells is debatable (Nilsson *et al.,* 1991; Firth, *et al*., 1994; Oliver, 1995; Oliver *et al.,* 1996; Weichart & Kjelleberg, 1996). Recently Bloomfield *et al.* (1998) suggested a new plausible model to account at least partially for the VBNC phenomenon. They hypothesized that VBNC cells are not unculturable but that A*we are simply failing to provide appropriate conditions to support culture*@ (Bloomfield *et al.,* 1998). Further, they provided evidence that the transfer of Aso-called VBNC cells@ to a nutrient-rich agar cause cell death. Bloomfield *et al.* (1998) proposed A*that sudden transfer of cells to nutrient-rich agar at temperatures optimal for enzyme activity initiates an imbalance in metabolism, producing a near-instantaneous production of superoxide and free radicals. In the absence of phenotypic adaptation, the cells are not equipped to detoxify superoxide. As a result, a proportion or all of these cells die*@.

3.2 Clinical manifestations, epidemiology and treatment of *V. vulnificus* infections in humans

V. vulnificus causes primary septicemias and wound infections (Blake *et al.,* 1979). Most primary septicemias are associated with raw seafood consumption, especially raw oysters and in almost every case the patient has a chronic underlying disease. *V. vulnificus* differs from other food borne pathogens as it is seldom reported to cause diarrhea and vomiting (Hollis *et al.,* 1976; Blake *et al.,*

1979; Hlady & Klontz, 1996). *V. vulnificus* causes only sporadic disease and outbreaks (i.e., two or more culture- confirmed cases linked to a common meal or lot of oysters) have never been reported (Whitman, 1995). The fatality rate is high with almost 60% of the patients with primary septicemia dying within a few days (Oliver, 1989). A high prevalence of liver disease and alcoholism among patients with septicaemia may reflect a requirement of *V. vulnificus* for free iron via saturated transferrin or excess of iron (Hlady & Klontz, 1996). Other risk factors include the use of immunosuppressive agents, gastric diseases, and blood disorders (Oliver, 1989).

Wound infections

V. vulnificus causes wound infections by entering a pre-existing skin lesion during exposure to saline waters. Patients are often employed as fishermen or in other jobs with close contact to the marine environment (Dalsgaard *et al.,* 1996b; Hlady & Klontz, 1996). The fatality rate of reported cases is approximately 20% but amputation or surgical debridement is often necessary (Oliver, 1989).

In Denmark, four of 11 patients in 1994 developed septicemia, of which one subsequently died. Nine patients exhibited skin manifesta tions and six underwent surgical debridement. Four patients contracted their disease during fishing and at least one patient had been handling eels (Dalsgaard *et al.,* 1996b).

Treatment

V. vulnificus is sensitive to most antibiotics and infections have been treated with antibiotics, e.g. ampicillin, tetracycline, chloramphenicol or third-generation cephalosporins (Klontz *et al.,* 1988; Chuang *et al.,* 1992; Fang, 1992; Dalsgaard *et al.,* 1996b). Antibiotic treatment is often ineffective unless initiated as soon as the first clinical symptoms appear (Oliver, 1989). However, in cases of serious wound infections, the primary treatment is a proper surgical debridement with antibiotics playing a secondary role (Dalsgaard *et al.,* 1996b). A vaccine against *V. vulnificus* has been developed but has not been tested beyond the pre-clinical trials in mice (Devi *et al.,* 1995; Devi *et al.,* 1996). The vaccine antiserum is raised against the capsule of *V. vulnificus* and the achieved protection is capsule-type-specific. Clinical strains of *V. vulnificus* exhibit various capsule types and far from all types have yet been identified (Hayat *et al.,* 1993; Simonson & Siebeling, 1993). Because risk factors for *V. vulnificus* infection are unclear and illness are rare, vaccination may not be an appropiate effective control strategy (Blake *et al.,* 1979; Mertens *et al.,* 1979; Kelly & Avery, 1980; Oliver, 1989). However, a serosurvey in the Chesapeake Bay Region revealed that asymptomatic infection with *V. vulnificus* may be relatively common among persons like shellfish industry workers with high levels of exposure to shellfish (Lefkowitz *et al.,* 1992). Danish eel farmers are exposed to high concentrations of *V. vulnificus* during outbreaks but at the present time it is not known if asymptomatic infections of *V. vulnificus* occur in Denmark.

Warning labels

In California, Florida and Louisiana, where most cases of *V. vulnificus* occur, restaurants that serve raw oysters are required to post warnings. In the state of Louisiana all shucked shellfish products and shellstock must be labeled with the warning tag: *Raw oysters, raw clams, and raw mussels can cause serious illness in persons with liver, stomach, blood, or immune system disorders* (Dayal *et al.,* 1993).

Infectious dose unknown

The infectious dose of *V. vulnificus* is unknown and is due in part to the inability to match clinical isolates with isolates from implicated lots of oysters, the lack of opportunities to trace a human infection (especially wound infections) back to the implicated source and finally because human volunteer studies are too dangerous.

4.3 *V. vulnificus* infections in eels

V. vulnificus biotype 2 causes serious economic losses in aquaculture that keep eels in brackish water around 24°C (Amaro *et al.,* 1995). Under laboratory conditions, immersion challenge with 10^5 CFU/ml or between 10^2 and 10^5 CFU/fish by intraperitonal injection leads to the development of vibriosis in less than 24 h (Amaro *et al.,* 1995). The disease is a septicemic infection and bacteria are easily isolated from blood samples from moribund eels (Amaro *et al.,* 1997).

Pathological changes

The first isolates of *V.vulnificus* biotype 2 were recovered from Japanese eels (*Anguilla japonica*) between 1975 and 1977 (Muroga *et al.,* 1976; Nishibuchi *et al.,* 1980). The disease was characterized by reddening of the body, especially tails and fins, and hemorrhages of the dorsal area (Miyazaki *et al.,* 1977). In progressed cases, pathological changes could be observed in the gastrointestinal tract, gills, heart, liver and kidney (Miyazaki *et al.,* 1977).

V. vulnificus biotype 2 was first described in Europe in 1989 where recurrent outbreaks occurred in cultured European eel (*A. anguilla*) in Spain (Fig. 4) (Biosca *et al.,* 1991). Early clinical features were that the eels became lethargic, external lesions appeared first as petechiae on the abdomen, hemorrhages of the anal fin and a reddening in the opercular region. Large wounds (2-4 cm diameter) with central necrotic tissue occurred in some eels. Pathological changes and inflammation of internal tissues, liver, kidney, and in the abdominal cavity were observed (Biosca *et al.,* 1991). The eel farm in Spain experiencing these disease outbreaks used brackish well-water (17 ‰ NaCl and 22°C) in a recirculating system (Biosca *et al.* ,1991). The diseased eels had different origins and therefore it was impossible to ascertain the source of *V. vulnificus* (Biosca *et al.,* 1991). Earlier environmental studies in the area of the eel farm did not reveal the presence of *V. vulnificus* (Pujalte *et al.,* 1983; Ortigosa *et al.,* 1989; Garay *et al.,* 1985).

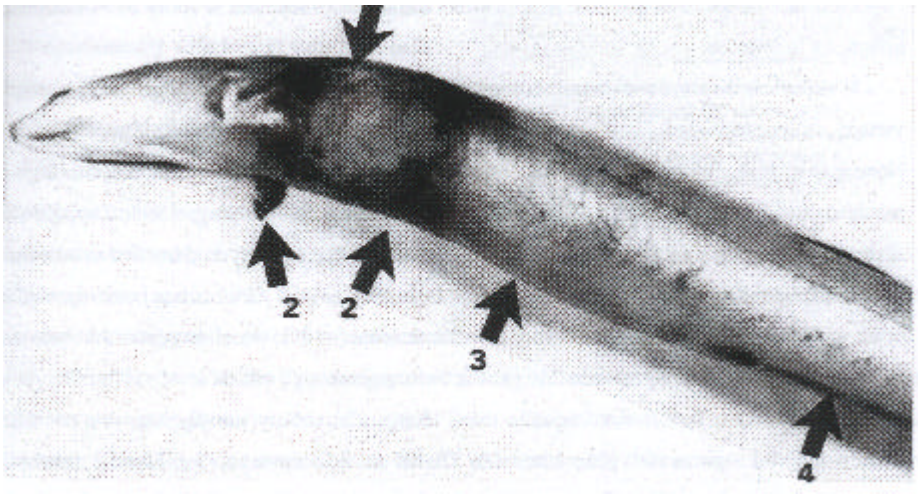


Figure 4: *Gross appearance of eel suffering from V. vulnificus biotype 2 infection: (1) Wound with central perforation (2) Reedening in the opercular region (3) distended abdomen (4) hemorrhegic anal fin (Biosca et. al., 1991).*

For information on *V. vulnificus* in Danish eel farms see section 4.5.

3.4 Virulence factors

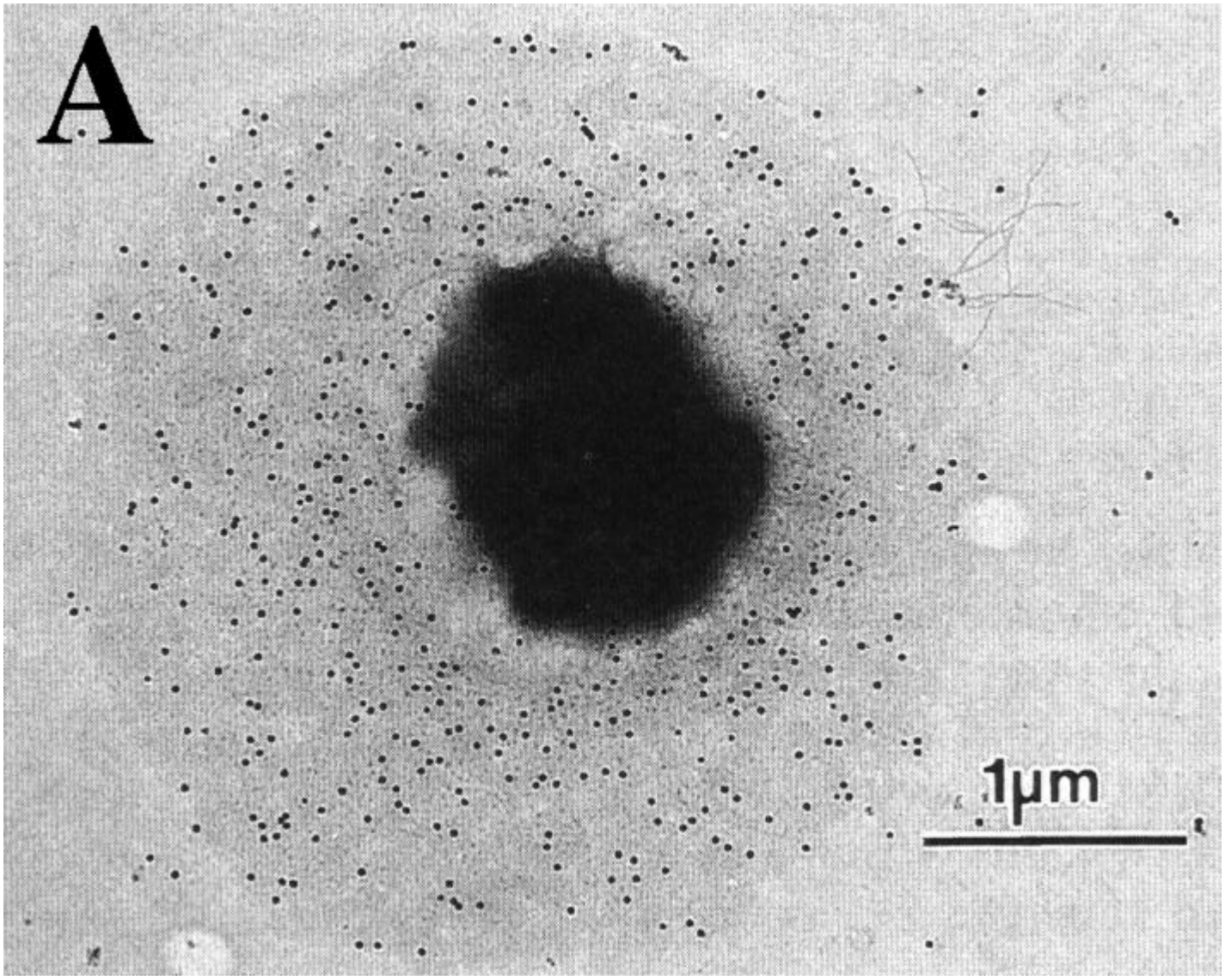
3.4.1 Virulence factors of *V. vulnificus* in human infections

Multifaceted bacterial and host factors affect the development and severity of *V. vulnificus* infections in humans.

Capsule production

V. vulnificus displays two distinct colony morphologies, opaque and translucent. The opaque variant is encapsulated, virulent to mice and capable of survival in human serum. Nonencapsulated, translucent strains arise spontaneous during routine bacteriological mani pulation of opaque strains (Fig. 5 and 6) (Simonson & Siebeling, 1993; Zuppardo & Siebeling, 1998). Opaque variants possess a ruthenium red-staining layer identified as an acidic polysaccharide (Kreger *et al.,* 1981). Reversion from both colony variants has been reported to occur under laboratory conditions at very low frequencies (~10^{-4}), which suggests that capsular production is controlled by a reversible genetic rearrangement (Yoshida *et al.,* 1985; Wright *et al.,* 1990). Genetic factors that regulate these changes in colony morphology are not fully understood. An epimerase[3] gene has been shown to be essential for capsule synthesis (Zuppardo& Siebeling, 1998). The loss of a functional epimerase, e.g. by transposition, can cause the disruption of capsule production by a depletion of precursors in the early stage of capsule production (Zuppardo & Siebeling, 1998).

[3]Sugars differing in configuration at a single asymmetric centre are *epimers*, e.g.glucose and mannose; *Epimerases* are enzymes that mediate the interconversion of epimers (Stryer, 1988).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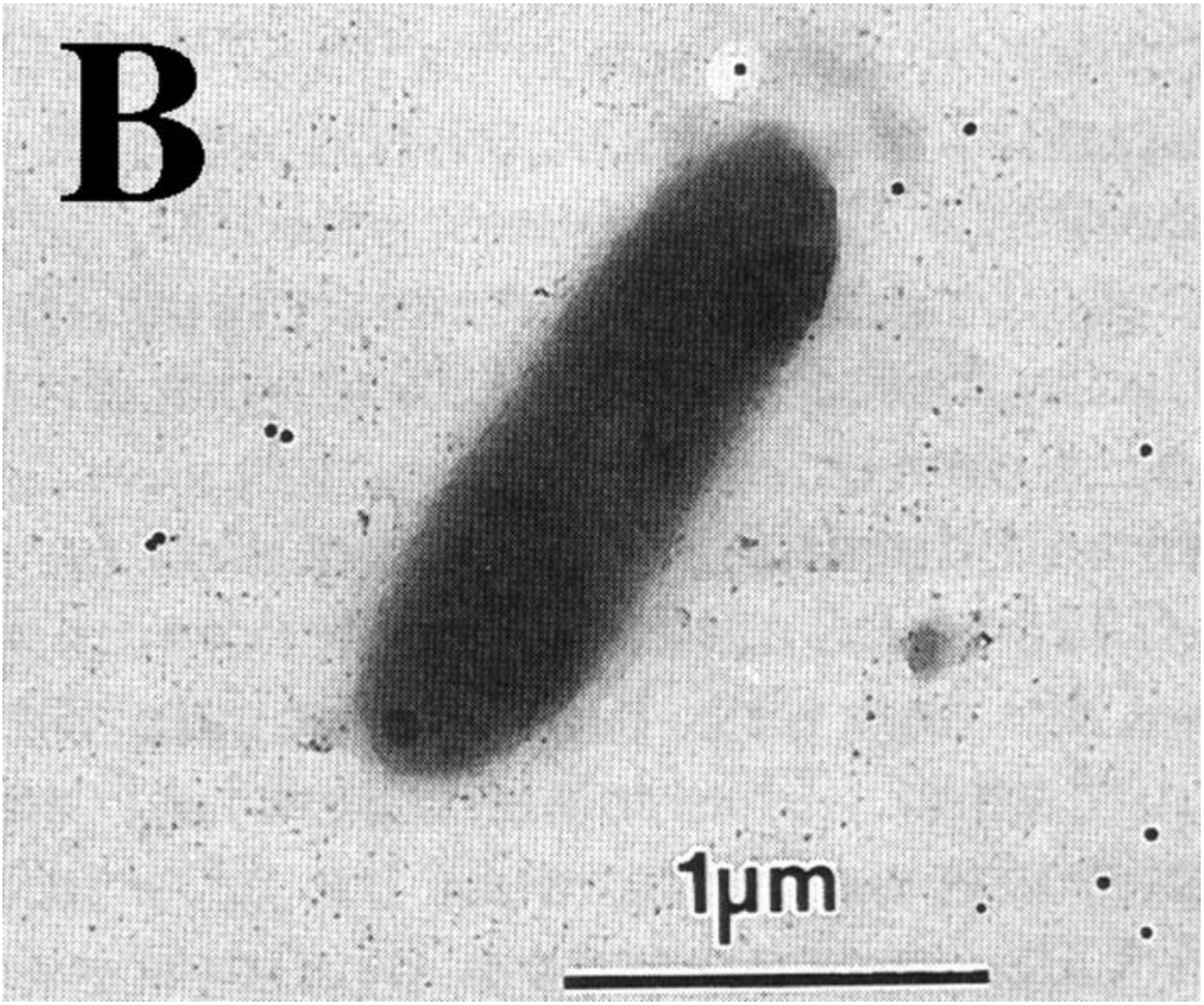

Figure 5: *Transmission electron micrographs of a opague (A) and translucent (B) variant of the same V. vulnificus strain reacted with anti-capsular rabbit sera and then stained with protein A-colloidal gold conjugates (Simmonson & Sibeling, 1993).*

The importance of capsule in *V. vulnificus* virulence has been demonstrated in a number of studies (Oliver, 1989; Simpson *et al.,*1987). The capsule permits *V. vulnificus* to evade nonspecific host defense mechanisms such as activation of the alternative pathway of the complement system and complement-mediated opsonophagocytosis (Tamplin *et al.,* 1985; Yoshida *et al.,* 1985; Shinoda *et al.,* 1987). Loss of capsule in transposon mutants is accompanied by a several log higher LD_{50} in mice and decreased serum resistance (Wright *et al.,* 1990; Zuppardo & Siebeling, 1998). Antibodies generated against capsule antigens protected vaccinated mice when challenged intraperitoneally with live *V. vulnificus* cells (Kreger *et al.,* 1984). However, the importance of capsule production for the virulence of *V. vulnificus* in humans is not fully elucidated as most environmental strains are encapsulated and yet vary in virulence to mice (Stelma *et al.,* 1992; Kaysner *et al.,* 1987). The presence of capsule may therefore be regarded as one among several virulence factors.

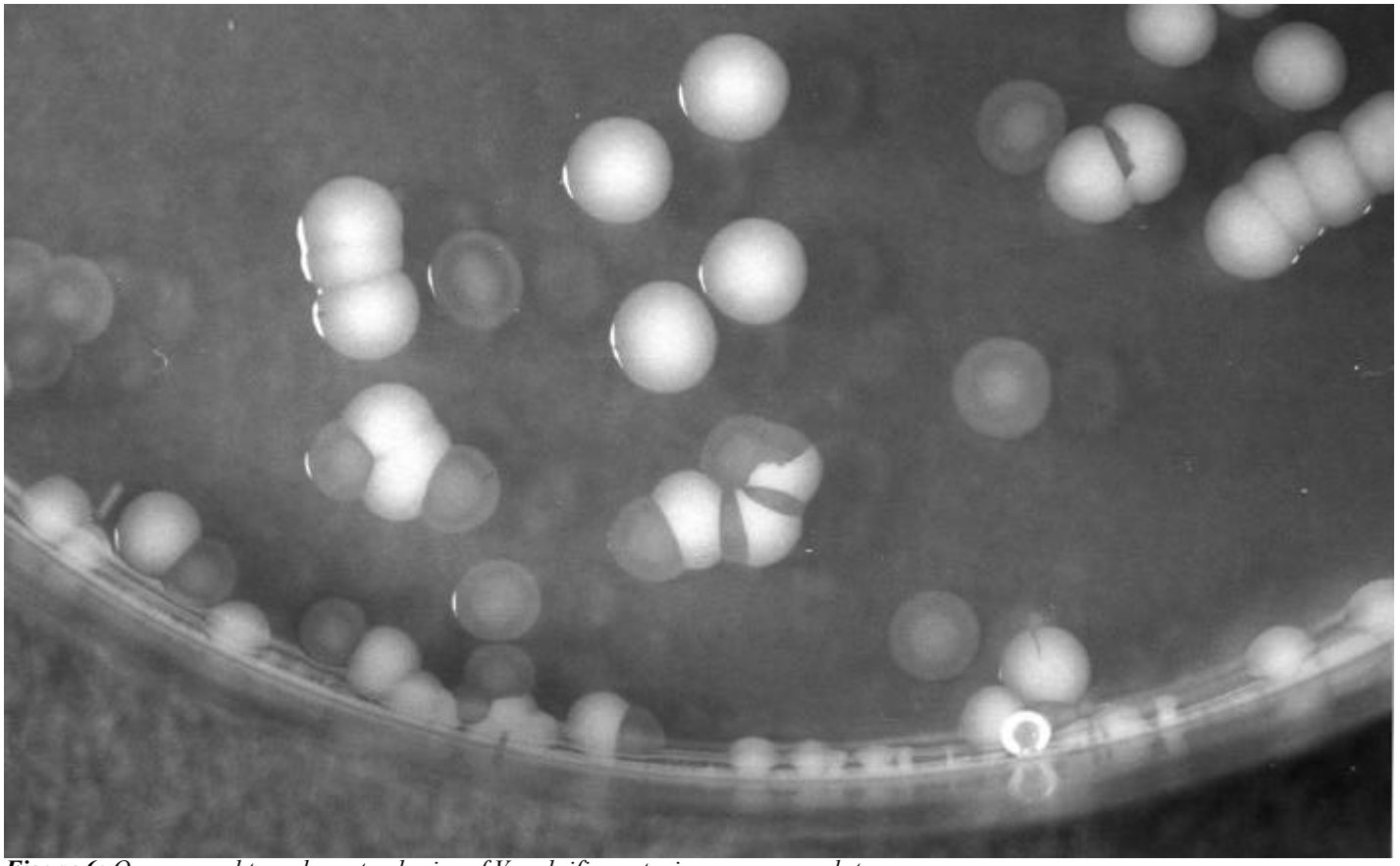

Figure 6: *Opaque and translucent colonies of V. vulnificus strain on an agar plate.*

Serum iron

Serum iron availability plays an important role in host susceptibility to *V. vulnificus.* Serum iron levels is strongly correlated to the size of *V. vulnificus* inoculum required to cause mortality in mice. The LD_{50} value dropped from 10^6-10^8 to less than 10^2 cells in mice injected with iron before bacterial challenge (Wright *et al.,* 1981). Virulent strains of *V. vulnificus* produce both phenolate and hydroxymate siderophores, which enable them to acquire iron from fully saturated transferrin, lactoferrin, ferritin, haemoglobin and haptoglobin complexes (Simpson *et al.,* 1987; Zakaria-Meehan *et al.,* 1988; Oliver, 1989; Stelma *et al.,* 1992). An exocellular protease has been described to cleave transferrin and lactoferrin thereby making bound iron more accessible to the siderophores (Okujo *et al*., 1996).

Non-encapsulated transposon mutants are not defective in iron acquisition but are less virulent in mice than encapsulated suggesting iron acquisition is less important than capsule production (Wright *et al.,* 1990). Biosca *et al.* (1996b) demonstrated that iron-uptake by *V. vulnificus* from human transferrin is independent of capsule production in both biotypes.

Other virulence factors

Published data on the role of other putative virulence factors include the production of a cytolysin (Gray & Kreger, 1985), a protease (Miyoshi *et al.,* 1987), a phospholipase (Testa *et al.,* 1984), piluslike structures (Gander & LaRocco, 1989) and LPS endotoxin (McPherson *et al.,* 1991). The cytolysin found in all *V. vulnificus* strains lyses mammalian erythrocytes and Chinese hamster ovary cells, produces a vascular permeability factor activity in guinea pig skin and is lethal to mice (Gray & Kreger, 1985). One study reported that mice and humans surviving *V. vulnificus* infections produced antibodies against the cytolysin, indicating that the cytolysin was produced *in vivo* (Gray & Kreger, 1986). However, Wright & Morris (1991) inactivated the structural gene for the cytolysin of a virulent clinical strain and found that it did not affect virulence in mouse models. Other authors have also been unable

to detect any correlation between production of cytolysin and virulence (Oliver *et al.,* 1986; Morris *et al.,* 1987; Massad *et al.,*1988). The protease exhibits both caseinolytic, elastinolytic, and collagenolytic activities (Kothary & Kreger, 1987; Miyoshi *et al.,* 1987), but is produced by both virulent and avirulent strains and differences in titre do not correlate with virulence in animal models (Morris *et al.,* 1987b). LPS from *V. vulnificus* is pyrogenic and can cause cardiovascular injuries leading to death in rats (McPherson *et al.,* 1991). *V. vulnificus* LPS, once in the blood compartment in humans, may trigger the sepsis cascade leading to septic shock and possible death. The septic shock may be mediated by several factors including (i) degranulation of neutrophils onto the endothelium surface which causes vessel wall damage, (ii) intravascular activation of the complement cascade by LPS through the alternative pathway, (iii) activated macrophages overproduction of
the cytokines IL-1 and TNF which cause endothelium injuries, and (iv) activate additional secondary inflammatory mediators (e.g. platelet activation factor and arachidonic acid) (Siebeling, 1997).

Plasmids

Plasmids play an important role in the virulence of *Enterobactericeae*. but their role in virulence of *V. vulnificus* biotype 1 has received little attention (see section 5.1).

Numerous studies have reported no differences in virulence characteristics of clinical and environmental isolates of *V. vulnificus* (see Chapter 5) (Oliver *et al.,* 1986; Tison & Kelly, 1986; Kaysner *et al.,* 1987; Stelma *et al.,* 1992). However, this is inconsistent with the low attack rate in susceptible populations consuming seafood contaminated with *V. vulnificus.* Less than one illness occur per 10,000 meals of raw Gulf oysters served to the highest risk population, people with liver diseases, suggesting that environmental strains are not equally virulent or not all people with liver disease are equally susceptible (Hlady, 1997).

The high virulence of *V. vulnificus* can not be assigned to a single factor but is influenced by capsule production, ability to acquire iron in human serum, LPS type, production of exoenzymes and exotoxins, and a susceptible host.

4.4.2 Virulence factors of *V. vulnificus* in eel infections

The two biotypes share many of the same virulence factors, including (i) the capsule, a protective surface antigen that allows cells to resist phagocytosis and lysis by human serum but not by eel complement (Biosca *et al.,* 1993b; Amaro *et al.,* 1994); (ii) various iron uptake systems, including siderophore production and the ability to use hemoglobin and hemin as iron sources (Amaro *et al.,* 1994; Biosca *et al.,* 1996b); and (iii) a cytolysin, with hemolytic activity together with potent proteases, which are active involved in the lesions produced in different organs (Amaro *et al.,* 1992). Exotoxins produced by both
biotypes are equally lethal for eels; they produce the main symptoms of vibriosis when injected intraperitoneally as crude extracts of extracellular products (Biosca & Amaro, 1996). The capsule of biotype 2 strains has been suggested to favor adherence to eel mucus and to be essential for virulence under natural conditions (Amaro *et al.,* 1995). The existence of different capsule types has been studied with 10 polyclonal capsular antisera in *V. vulnificus* strains from diseased eels in Denmark (Høi *et al.,* 1998b). The isolates were either non-typeable or possessed capsule type 9 which indicates that this type of capsule may enhance the capability of *V. vulnificus* to infect eels (Høi *et al.,* 1998b).

A relationship between high molecular weight plasmids and eel virulence was first suggested by Biosca *et al*. (1997a), who found that a plasmid-free biotype 2 strain had a significantly higher LD_{50} in eels than biotype 2 strains harboring high molecular weight plasmids. These findings are corroborated by Høi *et al*. (1998b) who found that 93 of 97 biotype 2 strains isolated from diseased eels contained one to three high molecular weight plasmids of varying sizes. Restriction digests of plasmids from a number of

biotype 2 strains from Denmark revealed a high degree of homology (Lewin, 1998). The role of plasmids in virulence requires further studies including DNA sequencing.

LPS

Biotype 2 strains constitute a relatively homogenous LPS based O serovar according to Biosca *et al.* (1997a). The O side chain of this LPS type may determine the selective virulence of biotype 2 for eels (Amaro *et al.,* 1997). The crude extract of this LPS type, termed serovar E, is not toxic to eels, which suggests that this surface antigen acts as a protective factor against nonspecific immune mechanisms such as nonopsonic phagocytosis and/or bactericidal action of serum complement (Amaro *et al.,* 1997). Biotype 1 strains are lysed by eel serum through activation of the alternative pathway of the complement system (Amaro *et al.,* 1997). Biotype 2 strains are resistant to non-immune eel serum whereas rough mutants of biotype 2 lacking
the O polysaccharide chain are sensitive to non-immune eel serum and avirulent for eels. The authors suggest that only strains possessing this particular type of LPS are able to colonize eels (Amaro *et al.,* 1997). To cause septicemic infections the strains must also be able to express other virulence factors (e.g. exotoxins and iron uptake systems) which may be plasmid encoded.

4.5 *V. vulnificus* in Denmark

4.5.1 Occurrence of *V. vulnificus* in coastal water, sediment, wild fish, and shellfish in Denmark

A comprehensive environmental survey of *V. vulnificus* in Danish marine environments was done during 1996 (Høi *et al.,* 1998c). The aims of this survey were to investigate the occurrence of *V. vulnificus* in Danish coastal waters, shellfish, and wild fish and especially to investigate the distribution of *V. vulnificus* biotype 2 strains (Høi *et al.,* 1998c).

From May to October 1996, water was sampled weekly at sites 1 to 7 (Fig. 8) and sediment samples were collected weekly from sites no. 1 and 2. Blue mussels (*Mytilus edulis*) and oysters (*Oestra edulis* and *Crassostrea gigas*) were sampled from July until December 1996 from a total of 13 sites (Fig. 7). From July until October 1996, a total of 136 wild fish were analyzed, including 29 flounders (*Platichthys flesus*), 14 eel pouts (*Zoarches viviparus*), and 93 eels (*A. anguilla*) that were caught at various locations in Køge Bay and in the waters close to location no. 2 (Fig. 7) (Høi *et al.,* 1998c).

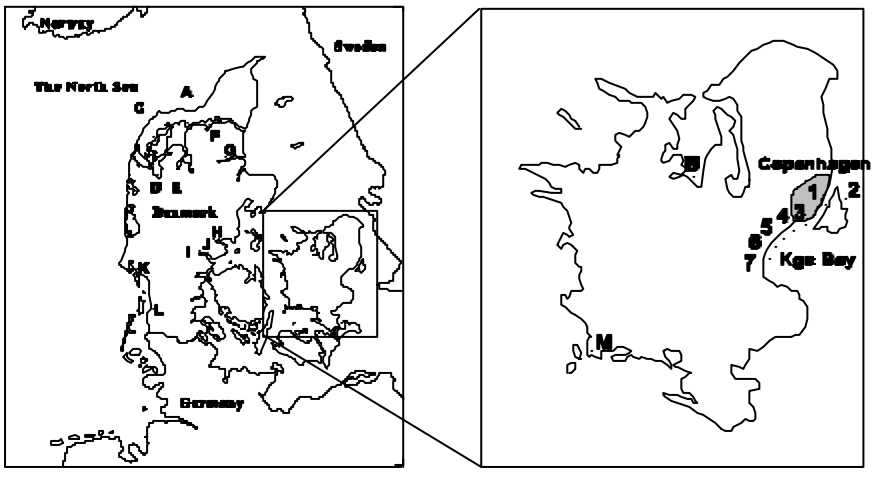

Figure 7: *Geographical location of sampling sites. Coastal water samples were collected at locations 1 to 7. Sediment samples were obtained from locations 1 and 2. Oysters were harvested in areas A and B and blue mussels in the areas C to M. Wild fish were caught in Køge Bay and at location 2.*

Biotype 2 in coastal water

Until recently, *V. vulnificus* biotype 2 had never been isolated from coastal water suggesting that disease was transmitted from one eel to another by direct contact, and that biotype 2 did not survive well in brackish water (Høi *et al.,* 1997).

In Denmark, biotype 2 strains were isolated from sediment and coastal water samples although the frequency of isolating such strains was very low (3 of 706 strains were indole negative and designated biotype 2). The low incidence of *V. vulnificus* biotype 2 strains in environmental samples may explain why its occurrence in coastal water has not been reported earlier. The isolation procedure may also influence which biotype is detected. A few biotype 2 colonies on a CC agar plate may not be picked for identification and characterization if numerous biotype 1 colonies are present on the plate. While virulence determinants, phenotypic properties, and physiology of the two bio types are similar (Amaro *et al.,* 1992; Biosca & Amaro, 1996; Biosca *et al.,* 1996a; Biosca *et al.,* 1996b; Biosca *et al.,* 1997a), our study suggests differences in their ecology: biotype 1 was isolated from various environmental sources whereas biotype 2 was rarely isolated from coastal water and sediment. Danish marine environments are potential reservoirs of *V. vulnificus* biotype 2. The distribution of eel-pathogenic *V. vulnificus* strains in Danish coastal waters may have been underestimated because not all eel-pathogenic *V. vulnificus* strains are indole negative (see Chapter 5). This knowledge is particular important to fish farmers since the use of brackish water for culturing eels may introduce serious *V. vulnificus* infections in the farms.

Occurrence correlated with to water temperature

The occurrence of *V. vulnificus* at the seven coastal sites was strongly correlated to water temperatures, as reported by other researchers (Fig. 8) (Wright *et al.,* 1996; O'Neill *et al.,* 1992; Kelly, 1982). *V. vulnificus* was rarely isolated when water temperatures were below 15°C. However, *V. vulnificus* was detected in coastal waters at a mussel farm at 7°C which is lower than previously reported (Wright *et al.,* 1996).

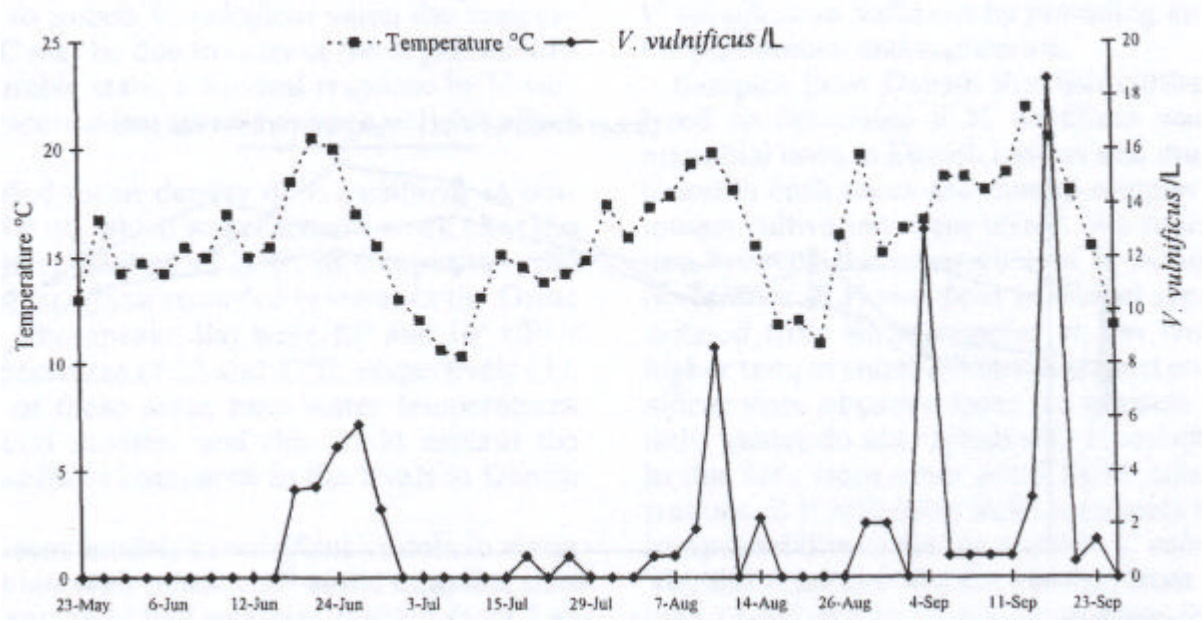

Figure 8: *Mean densities of V. vulnificus in samples of coastal water and water temperatures from the seven sites studied.*

Detection at low temperature

Detection of *V. vulnificus* at low temperatures as seen in the present study may be a function of the sensitivity of the isolation method and/or of adaptation of *V. vulnificus* to colder temperatures. Our inability to isolate *V. vulnificus* from water samples with temperatures below 7°C may be caused by inappropriate culture conditions for these cold-stressed cells or the absence of culturable cells (Oliver, 1995; Bloomfield *et al.,* 1998).

No correlation with fecal indicators

The control of Danish coastal bathing water is based on presumptive *Escherichia coli* and coliforms as indicators of water quality. As reported elsewhere, analysis of the data collected in Denmark in 1996 did not reveal any correlation between presumptive *E. coli* and *V. vulnificus* (Høi *et al.,* 1998c).

Low levels of V. vulnificus in Danish shellfish

Danish oysters and mussels and their surrounding waters were analyzed for *V. vulnificus. V. vulnificus* was isolated from both water and blue mussels from one cultivation area of a total of 13 areas tested (area G, Fig. 7). Temperature and salinity in area G was similar to others in the study. Concentrations of *V. vulnificus* in blue mussels were very low (# 10 CFU per gram of mussel tissue) and the low probability of detecting a viable *V. vulnificus* cell may explain why it was not isolated from other areas. However, cultivation of mussels in area G differed in several ways from the other areas investigated: (i) mussels are cultivated on suspended ropes. In this cultivation method mussels are raised above the sea-bed to give a more efficient exploitation of food at all depths as compared to cultivation on the sea-bed (ii) cormorants (*Phalacrocorax carbo*) are present in high numbers at the mussel farm (iii) the area is among the most nutrient rich waters in Denmark. Location of mussels in surface waters provides more favorable temperatures for growth of *V. vulnificus* during summer time, and the high level of nutrients may provide conditions favorable for growth of *V. vulnificus,* even at low temperatures.

Transmission through birds

The presence of high numbers of defecating cormorants above the mussel farm may be of importance for the prevalence of *V. vulnificus* in this particular mussel farm. One could hypothesize that when cormorants eat mussels containing *V. vulnificus,* the organisms may survive or even replicate in their intestines and be released back into the environment with feces. The body temperature of birds is 41-42°C which allows growth of *V. vulnificus* (Freeman, 1983). Further, *V. vulnificus* and *V. cholerae* have been isolated from cloacal swaps and bird droppings from a variety of seabirds from the Alabama coast of The Gulf of Mexico (DePaola, 1998). *V. vulnificus* was isolated from eight of 22 samples from seabirds, and concentrations as high as 10^6 CFU per gram of bird droppings were registered (DePaola, 1998). Birds have also been reported to be a potential vector for the fish pathogenic bacterium *Yersinia ruckeri* which has been isolated from the intestines of several fish-eating birds (Willumsen, 1989; Furones *et al.,*1993). The role of seabirds in the ecology of *V. vulnificus* in Denmark was investigated in the summer of 1997 (Hein, 1998). Thirty-seven cloacal swaps from seagulls and ducks (*Larus fuscus, L. Canus, L. Argentatus, Anas clypeata, A. Crecca, A. Platyrhynchos*) from two coastal locations on Zealand were investigated but all samples were negative for *V. vulnificus* (Hein, 1998). *V. vulnificus* was isolated from water samples relatively near one of the sampling areas in the same period as the birds were investigated (Hein, 1998). The negative result may be explained by one or more of the following factors: (i) the birds did not consume any *V. vulnificus,* (ii) some *V. vulnificus* cells may get killed during passage through the intestinal tract, and therefore intake of only a few *V. vulnificus* cells may not result in a positive cloacal sample, (iii) *V. vulnificus* may not survive passage through the bird species examined, or (iv) *V. vulnificus* occur with such low prevalence that more than 37 samples would have been necessary to isolate this bacterium. Further studies are necessary to determine if seabirds can be a reservoir or vehicle for *V. vulnificus* in Denmark.

US shellfish

Gulf of Mexico shellfish are implicated in nearly all *V. vulnificus* primary septicemia infections in the United States (Klontz *et al.,* 1988; Klontz *et al.,* 1994; Hlady & Klontz, 1996). High concentrations of *V. vulnificus* (10^5 -10^6 CFU per gram) are reported in raw oysters from April to October where more than 90% of primary septicemia cases occur (Klontz *et al.,* 1988; Tamplin, 1995; Hlady & Klontz, 1996). Wound infections due to occupational activities with contact to saline water have been reported to show a similar seasonal pattern (Hlady & Klontz, 1996). In Denmark, *V. vulnificus* primary septicemia has not been reported and all wound infections occurred in August during extremely warm summers (Dalsgaard *et al.,*1996b). *V. vulnificus* infections have not been associated with consumption of raw shellfish in Denmark or elsewhere in Europe. These findings suggest minimal risk associated with consumption of raw shellfish containing *V. vulnificus* in low numbers.

Role of fish in ecology

Bottom-feeding fish have been reported to play a role in the ecology of *V. vulnificus* in Alabama and Gulf of Mexico waters (DePaola *et al.,* 1994). High densities (10^8 CFU/g) of *V. vulnificus* were found in the intestinal contents of fish that consumed mollusks and crustaceans and it was suggested that fish may play an ecological role in the growth and transport of *V. vulnificus* (DePaola *et al.,* 1994). *V. vulnificus* was also isolated from the intestines from brackish-water fish caught on the west coast of India (Thampuran & Surendran, 1998). MPN counts in the Indian study ranged from 15 to 910 CFU per gram of intestinal content, but concentrations were probably underestimated since the selective agar TCBS, which offer poor recovery of *V. vulnificus,* was used (Thampuran & Surendran, 1998). In Denmark, *V. vulnificus* was isolated with higher a prevalence from gills (21%) ($P<0,001$, $\div^2$ test, SigmaStat**7** version 3.2) compared to intestines (5%) and mucus (10%) from the three fish species eel, eel pout, and flounder (Table 3).

Table 3. *Occurrence of V. vulnificus in samples of gills, mucus, and intestinal contents from different fish species collected in Denmark in 1996 (Høi et al., 1998c).*

Fish species	No. of positive samples / total no. of samples		
	gills	mucus	intestinal content
Eels	19/73	3/20	4/73
Eel pouts	2/27	0/6	0/27
Flounders	1/5	0/5	1/5
Total	22/105	3/31	5/105

Possible bactericidal effect of fish mucus

The low prevalence of *V. vulnificus* in fish mucus may be explained by a possible bactericidal effect since fish mucus has been reported to contain proteins (complement, antibodies) and glycoproteins that react with environmental antigens and serve as a defense barrier to bacterial colonization (Hjelmeland *et al.,* 1983; Alexander & Ingram, 1992). *V. vulnificus* biotype 2 strains were reported to be resistant to the antimicrobial activity of eel mucus in *in vitro* experiments (Amaro *et al*., 1995). The mucus solution used in these experiments were filter-sterilized, stored at -20°C, and dried onto disks which may decrease any existing antimicrobial effect. Further, no appropriate controls were included to test the mucus-assay. The low prevalence in the intestinal contents may be caused by competing bacterial populations and low pH.

The prevalence of *V. vulnificus* in samples from the 3 fish species were not statistically different ($\div^2$ test, SigmaStat**7** version 3.2). Detection of *V. vulnificus* in estuarine fish in Denmark supports their involvement in the ecology of *V. vulnificus* as suggested by DePaola *et al.* (1994). Migrating fish containing *V. vulnificus* may facilitate the spread to new areas where the bacterium can survive. Serotyping of the fish isolates revealed that healthy wild eels are asymptomatic carriers of eel-pathogenic *V. vulnificus* (biotype 2) strains in their gills (unpublished results).

Risk of infection correlated with water temperature

Findings of *V. vulnificus* in Danish wild fish during the summer suggest that fishermen, especially those with abrasions on their hands may be at risk for *V. vulnificus* wound infections. In 1994 and 1995, seven people contracted *V. vulnificus* wound infections while fishing or handling eels (Dalsgaard *et al.,*1996b; Bruun, 1997). *V. vulnificus* cases were not reported during the summer of 1996 when low concentrations (<2 CFU/100 ml) were observed in coastal waters. *V. vulnificus* levels were either too low to cause infection, even in susceptible individuals, and colder temperatures discouraged bathers from contact with coastal waters. Epidemiological data from 1994 and 1995 suggest that the risk of contracting a *V. vulnificus* infection following exposure to coastal water was correlated with water temperature. Thus, surveillance and monitoring efforts should be increased when water temperatures exceed 20°C.

4.5.2 Occurrence of *V. vulnificus* in frozen seafood imported into Denmark

Low and safe levels in tropical shrimp

V. vulnificus is a naturally occurring bacterium in warm estuarine environments and is therefore expected in shrimp produced in brackish-water aquaculture in South East Asia. The European Union imports approximately 75 metric tonnes [5] of these warm-water shrimp through Denmark each year (Dalsgaard & Høi, 1997). The prevalence of *V. vulnificus* in a total of 107 samples representing 37 consignments of frozen shrimp imported from South East Asia was determined. *V. vulnificus* was detected in three of 46 (7 %) frozen raw shrimp samples but was not recovered from any of the 61 frozen cooked products. Absence of *V. vulnificus* in frozen cooked shrimp products indicated proper processing such as adequate heat treatment and sanitation (Dalsgaard & Høi, 1997). The low prevalence of *V. vulnificus* in frozen raw shrimp products was likely due to its poor resistance to cold (Oliver, 1981; Boutin *et al.,* 1985; Parker *et al.,* 1994). Frozen shrimp products are usually kept at temperatures at -20EC before and after shipping, often for substantial periods, and a significant decrease in any number of *V. vulnificus* would be anticipated. The absence of *V. vulnificus* in frozen cooked shrimp products and the low prevalence of *V. vulnificus* in frozen raw shrimp suggested that *V. vulnificus* does not constitute a hazard to public health in Denmark if the shrimp products are correctly handled and cooked (Dalsgaard & Høi, 1997).

4.5.3 Occurrence of *V. vulnificus* in Danish eel farms

The Danish production of eels in aquaculture is expanding with a production in 1999 expected to double over 1998 production (3,000 metric tonnes). Danish eel production is dependent on import of elvers (*A. anguilla*) from France and the United Kingdom. The majority of eel farms in Denmark use fresh water to culture eels. Disease outbreaks caused by *V. vulnificus* and other pathogenic *Vibrio* spp. have not been reported in Denmark with recirculating systems that use freshwater continuously (Mellergaard & Dalsgaard, 1987). However, it is preferable to culture eels in brackish water instead of fresh water since it leads to higher growth rates, better feed conversion and taste. However, brackish water can be a reservoir or vehicle of *V. vulnificus* biotype 2 and might facilitate the spread to cultured eels (Høi *et al.,* 1998c). Further, water temperatures (approximately 24EC) in eel farms favor *V. vulnificus* growth (Høi *et al.,* 1998c). *V. vulnificus* biotype 2 was isolated from wound infections in humans and water in Denmark in 1994 but was not isolated from diseased eels in Danish farms using brackish water until 1995 (Dalsgaard *et al.,* 1996b; Høi *et al.,* 1997; Høi *et al.,* 1998b; Høi *et al.,* 1998c; Dalsgaard *et al*, in press). Since 1995 recurrent outbreaks of *V. vulnificus* have occurred in two Danish eel farms both using brackish water causing serious economic losses (Høi *et al.,* 1998b; Dalsgaard *et al.,* in press). Recent findings have shown that *V. vulnificus* also can cause disease in freshwater eel-farms if the farms have used intake of brackish water in the past (unpublished results).

Clinical findings in diseased eels

In the first outbreak in one of the farms, the diseased eels were lethargic and exhibited clinical signs typical of bacterial septicemia and also external hemorrhages in the ocular area and in some cases bilateral exophthalmia (Fig.10A). Erosive lesions developed on the operculum area and in the jaw region (Fig. 10B). The severe tissue necrosis seen in the jaw region of the eels has not been

[5] Due to the open market within the European Union (EU), no information is available about how many of the 75 tonnes are shipped from Denmark to other countries in the EU nor is it known how may tonnes of warm water shrimp Denmark imports from other members of the EU.

described in the literature and remains unexplained. When these *V. vulnificus* strains were injected intraperitoneally in challenge experiments, the eels died from septicemia within 1-2 days which was not enough time for a possible jaw necrosis to develop (Dalsgaard *et al*., in press).

Antibiotic treatment

Antibiotic treatment of *V. vulnificus* infections in eels has limited effect and outbreaks are recurrent. No antibiotic resistance has been demonstrated so far (Dalsgaard *et al.,* in press). Changes to production in freshwater usually reduce the eel mortality, but in one Danish eel farm the infection with *V. vulnificus* is persisting at the present time. Research investigating survival and spread of *V. vulnificus* in eel farms and the efficiency of vaccination is needed to make culturing eels in brackish water profitable. Future research should use routine culture methods as well as direct detection techniques to determine the role of non-culturable cells in the ecology of *V. vulnificus* in eel farms. *V. vulnificus* has recently been isolated from the gills and intestinal contents of eels cultured in freshwater but these eels had been kept in brackish water in the past (unpublished results). These findings suggest that once *V.vulnificus* enter the eel farm and colonize the eels, then the Na^+ ions present in the eel may be sufficient for growth and persistence of this halophilic bacterium. The concentration of Na^+ in blood and extracellular fluids in eels is approximately 150 mmol (8,8 g/L) which theoretically is sufficient for growth of *V. vulnificus* (Scholz & Zerbst-Boroffka, 1994).

4.6 Conclusions from Chapter 4

Human infection with *V. vulnificus* following exposure to coastal water in Denmark occur when water temperatures exceed 20°C and fishermen appear to be at the greatest risk. Consumption of raw shellfish has not been associated with *V. vulnificus* infections in Denmark, although *V. vulnificus* occasionally was isolated in low numbers. *V. vulnificus* was detected in a few imported frozen shrimp products and does not at present constitute a potential hazard to public health. *V. vulnificus* presents a serious economic problem to the Danish eel farmers wanting to use brackish water and warrant for both therapeutic and prophylactic measures. Research is needed to understand the ecology of *V. vulnificus* in eel farms and to develop a vaccine against eel-pathogenic *V. vulnificus* strains.

5 Characterization of *Vibrio vulnificus*

5. 1 Genotypic characterization

Genotypic techniques used for typing *V. vulnificus* include randomly amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) (Aznar *et al.,* 1993; Høi *et al.,* 1997), pulsed field gel electrophoresis (PFGE) (Buchrieser *et al.,* 1995; Tamplin *et al.,* 1996), ribotyping (Aznar *et al.,* 1993; Dalsgaard *et al.,* 1996b; Tamplin *et al.,* 1996; Høi *et al.,* 1997), and plasmid profiling (Davidson & Oliver, 1986). RAPD-PCR and PFGE results have shown that *V. vulnificus* biotype 1 is genetically very heterogeneous (Buchrieser *et al.,* 1995; Aznar *et al.,* 1993; Tamplin *et al.,* 1996; Høi *et al.,* 1997). PFGE and RAPD-PCR may be of use in retrospective epidemiological investigations and in ecological studies, but these techniques are too discriminatory for typing *V. vulnificus* biotype 1 strains (Høi *et al.,* 1997). Buchrieser *et al.* (1995) used PFGE to characterize *V. vulnificus* populations isolated from three single oysters. Ninety-five typeable *V. vulnificus* isolates were divided into 60 genotypes; isolates within a single oyster did not show higher similarity than isolates from different oysters. The study illustrates the high genotypic diversity among *V. vulnificus* isolates in the oysters (Buchrieser *et al.,* 1995).

A high degree of genotypic diversity was also observed when ribotyping and RAPD-PCR were used to characterize isolates from Danish coastal areas and sediment (Høi *et al.,* 1997). The Danish clinical and environmental strains could not be distinguished by ribotyping which indicates that no specific genomic characteristics are associated with clinical or environmental strains (Høi *et al.,* 1997). Ribotyping has been reported to be useful for differentiating biotype 1 and 2 isolates and for differentiating strains of different geographical origin (Aznar *et al.,* 1993; Høi *et al.,* 1996; Høi *et al.,* 1997; Arias *et al.,* 1997).

Plasmid profiling

The use of plasmid profiling for epidemiological purposes has only been investigated in a few studies. One study reported a low incidence of plasmids in 42 environmental and clinical *V. vulnificus* strains from the US whereas 8 of 11 strains isolated from wound infections in Denmark harbored plasmids of variable sizes (Davidson & Oliver, 1986; Dalsgaard *et al.,* 1996b). The plasmid content of *V. vulnificus* strains isolated from cases of primary septicaemia have so far not been studied. In addition, the methods for plasmid extraction and size measurements need improvements as several nucleases affects plasmid quality and indications of plasmid sizes vary considerably in repeated testing (unpublished results). As mentioned in Section 4.4.2, most *V. vulnificus* biotype 2 strains harbor at least two high molecular weight plasmids which may be associated with eel virulence (Biosca *et al.,* 1996c; Biosca *et al.,* 1997a; Høi *et al.,* 1998b; Dalsgaard *et al*., in press;). Curing and transfer experiments with these plasmids are necessary to determine their role in pathogenicity.

Prediction of virulence impossible

Jackson *et al.* (1997) reported that 50 isolates obtained from blood from each of two clinical cases showed 100% homology in Pulsed Field Gel Electrophoresis (PFGE) analysis with four different restriction enzymes. This suggest that a single *V. vulnificus* strain was responsible for each of the clinical cases. No methods is at the present time able to predict which strain is the Amost@ virulent or which persons within an at-risk population are the most susceptible to *V. vulnificus* infections. Host specificity has been hypothesized to be a key element of *V. vulnificus* diseases, since only 5 to 10 infections are reported in Florida each year among an at-risk liver-diseased oyster-eating population estimated to be over 70,000 (Jackson *et al.,* 1997).

Future research should include host specificity and not solely focus on

determining virulence markers in *V. vulnificus* by various typing techniques.

5.1.1 Origin of *V. vulnificus* biotype 2

Biotype 1 strains are genetically heterogenous compared to biotype 2 strains from a variety of countries, e.g. Denmark, Japan, Spain, Norway, Sweden, and Taiwan (Biosca *et al.,* 1997a; Aznar *et al.,* 1993a; Arias *et al.,* 1998a; Høi *et al.,* 1997). An eel-pathogenic clone of *V. vulnificus* may have acquired eel-virulence-genes[6] from other bacterial species or the genes may have evolved by positive selection (i.e. strains with the Abest@ virulence genes will outgrow strains with less efficient virulence genes) in Japan in 1975. The eel-virulence-genes might also have been acquired through transduction by a lysogenic bacteriophage which at the same time could have mediated a serotype conversion, a phenomenon that is described for *V. cholerae* (Manning *et al.,* 1994).

Role of eels

The annual spawning migrating routes of the Japanese eel (*A. japonica)* and the European eel (*A. anguilla)* do not intersect, therefore, the clone of eel-pathogenic *V. vulnificus* strains have most likely not been transferred directly from *A. japonica* to *A. anguilla* in the environment (Nielsen, 1997). A more likely explanation could be that this clone has spread to Europe with an import of live elvers and eels from Asia (Nagasawa *et al.,* 1994). The clone could have been transferred through Europe by shipments of live eels. This hypothesis does not explain how this clone has Aresisted@ genetic changes and retained a homogenous genetic profile since 1975. If strains belonging to the eel-pathogenic clone of *V. vulnificus* undergo naturally spontaneous mutations they might loose their capability to cause infections in eels and thereby Abe excluded@ of the clone. The genetic homogeneity of these strains might ensure that the eel-virulence-genes remain intact.

5.2 Serotyping, capsule typing and phage typing

Two serological typing schemes have been proposed for *V. vulnificus* (Martin & Siebeling, 1991; Shimada & Sakazaki, 1984). The typing scheme of Shimada *et al.* (1984) is based on direct agglutination of heat-killed *V. vulnificus* cells by polyclonal rabbit antisera that recognize 7 O serovars. Five different O serovars and 10 diffe rent capsule types are identified among *V. vulnificus* strains in the typing scheme proposed by Martin and Simonson which was applied to a collection of Danish clinical and environmental *V. vulnificus* isolates (Martin & Siebeling, 1991; Simonson & Siebeling, 1993). Anti-capsule reagents which detected 50% of clinical isolates recovered in the US did not recognize any capsule antigens of the Danish isolates (other than a few biotype 2) nor did anti-LPS MAbs react with the Danish isolates suggesting different *V. vulnificus* populations in the US and Denmark (Danieu *et al.,* 1996). Capsule types have also been studied by using high-performance anion exchange chromatography (HPAE) and nuclear magnetic resonance (Bush *et al.,* 1997; Hayat *et al.,* 1993) and these studies also indicate that numerous capsule types exist in *V. vulnificus.* Typing of *V. vulnificus* biotype 1 using reagents raised against different LPS types and capsule types are of limited value because of the high heterogeneity among this species which would require expensive production of many new antisera. Thirteen phages specific for *V. vulnificus* have been isolated from oysters collected in Louisiana, Alabama and Florida and used for phage typing of *V. vulnificus* from diseased eels (DePaola *et al.,* 1997a; DePaola *et al.,* 1998; Høi *et al.,* 1998b). The majority of *V. vulnificus* isolates from Danish eels were lysed by one or more of these phages which suggests a relatedness in *V. vulnificus* isolates from Gulf of Mexico and Denmark. One to six phage types were seen in each outbreak and none of the phages were specific for

[6]At the present time it is not known which genes are responsible for the selective virulence for eels of *V. vulnificus* biotype 2 strains

certain LPS or capsule types. No apparent correlation was observed between phage typing, ribotyping, and serotyping (Høi *et al.,* 1998b).

5.3 Substitution of the taxon "biotype" to "serovar"

Bisoca *et al*. (1997a) proposed to rename those strains previously classified as biotype 2 to that of serovar E based on three criteria: (i) serovar E strains express a homogeneous lipopolysaccharide (LPS)-based O serovar while biotype 1 strains are serologically heterogenous; (ii) the majority of serovar E strains are indole-negative and the majority of biotype 1 strains are indole-positive; and (iii) only serovar E strains are virulent for eels. *V. vulnificus* biotype 2 strains are genetically homogenous compared to biotype 1 strains and they usually harbor high molecular weight plasmids, which may be associated with virulence (Biosca *et al.,* 1996c).

Høi *et al*. (1998b) recently reported that *V. vulnificus* isolated from diseased Danish eels are more heterogeneous as shown by O-serovars, capsule types, ribotyping, phage typing, and plasmid profiling than those from Japan and Spain. At least three LPS-associated serovars were isolated from Danish diseased eels which indicate that grouping *V. vulnificus* biotype 2 into a single serovar is not adequate.

Indole reaction

The indole reaction has previously been used to distinguish between *V. vulnificus* biotype 1 and 2 (Biosca *et al.,* 1996c). One biotype 2 strain has previously been reported to be indole positive, and data from Denmark further demonstrate that indole production is not a reliable marker for biotype 2 since the majority of isolates from diseased eels in Denmark were indole positive (Høi *et al.,* 1998b). Eel-pathogenic *V. vulnificus* isolates, which are indole positive, seem to be unique to Danish eel farms and may have evolved from the "original" eel-pathogenic clone (Høi *et al.,* 1998b).

5.4 Conclusions from Chapter 5

Characterization of *V. vulnificus* has not identified particular traits that predict pathogenicity for humans but has illustrated the high genetic diversity among environmental *V. vulnificus* isolates and that high molecular weight plasmids may play a role in eel virulence.

V. vulnificus was originally divided into two biotypes differing phenotypically, serologically, and in host range but recent research has indicated that grouping into serovars is more appropriate.

6 Concluding remarks

V. vulnificus is widely distributed in Danish marine environments including coastal water, sediment, wild fish, and shellfish. Low concentrations of *V. vulnificus* were recorded in Denmark and growth was particularly favored when water temperatures exceed 20°C for several weeks during warm summers. In this period health authorities should be aware of wound infections associated with *V. vulnificus. V. vulnificus* was occasionally isolated from raw, frozen seafood imported from South East Asia and in Danish blue mussels but do not constitute a hazard to public health in Denmark providing correct handling and cooking. *V. vulnificus* presents a serious economic problem to the Danish eel farmers wanting to use brackish water and warrant for both therapeutic and prophylactic measures. Research is needed to understand the ecology of *V. vulnificus* in eel farms and to develop a vaccine against eel-pathogenic *V. vulnificus* strains.

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