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Comparison of Ribotyping and Randomly Amplified Polymorphic DNA PCR for Characterization of *Vibrio vulnificus*

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A total of 85 isolates of *Vibrio vulnificus* were characterized by ribotyping with a probe complementary to 16S and 23S rRNA of *Escherichia coli* and by randomly amplified polymorphic DNA-PCR (RAPD-PCR) with a 10-mer oligonucleotide primer. The RAPD-PCR results were scanned, and the images were analyzed with a computer program. Ribotype membranes were evaluated visually. Both the ribotyping and the RAPD-PCR results showed that the collection of strains was genetically very heterogeneous. Ribotyping enabled us to differentiate U.S. and Danish strains and *V. vulnificus* biotypes 1 and 2, while the RAPD-PCR technique was not able to correlate isolates with sources or to differentiate the two biotypes, suggesting that ribotyping is useful for typing *V. vulnificus* strains whereas RAPD-PCR profiles may subdivide ribotypes. Two Danish clinical biotype 2 strains isolated from fishermen who contracted the infection cleaning eels belonged to the same ribotype as three eel strains (biotype 2), providing further evidence that *V. vulnificus* biotype 2 is an opportunistic pathogen for humans. One isolate (biotype 2) from Danish coastal waters also showed the same ribotype as the eel strains. This is, to our knowledge, the first time the isolation of *V. vulnificus* biotype 2 from coastal waters has been described.

Vibrio vulnificus is an estuarine bacterium commonly found in coastal waters and in association with shellfish and the intestinal contents of fish (13, 18, 20, 34). This bacterium is known to cause septicemia and severe wound infections in patients with chronic underlying diseases or conditions which lead to immunosuppression (18, 25).

Isolation of *V. vulnificus* from coastal waters has been reported in Australia, Brazil, and the United States (18–20, 22, 33), but only a few studies have described the incidence of *V. vulnificus* in northern Europe (29, 30). However, during the unusually warm summer in Denmark in 1994, 11 clinical cases were reported (6, 9, 14), and all *V. vulnificus* isolates that were obtained from those patients are included in this study.

Several methods have been used previously to characterize *V. vulnificus*, including biotyping, serotyping, plasmid profiling, ribotyping, and randomly amplified polymorphic DNA-PCR (RAPD-PCR). Biotyping divides *V. vulnificus* into only two biogroups; biotype 1 is an opportunistic human pathogen, and biotype 2 is a primary eel pathogen. Therefore, biotyping provides only limited information of epidemiological value (5, 27). Five lipopolysaccharide serotypes and 10 capsular serotypes have been recognized among clinical and environmental isolates of *V. vulnificus* (16, 23), but many isolates remain untypeable (11). *V. vulnificus* biotype 1 strains rarely harbor plasmids (12), and plasmid profiling appears to be of limited value for epidemiological characterization of this biotype. However, the majority of *V. vulnificus* biotype 2 strains tested in a recent study harbored two plasmids, and a relationship between plasmid profile and geographical origin of the strains was found (5). Ribotyping and RAPD-PCR analysis of *V. vulnificus* were described by Aznar et al. (4), whose study showed that 16S and 23S rRNA targeted probes may be used to differentiate *V. vulnificus* isolates and that RAPD-PCR performed with an 18-mer universal primer can differentiate biotype 1 and 2 isolates.

This study compared the use of ribotyping with 16S and 23S rRNA probes and the use of RAPD-PCR with a 10-mer primer for characterizing clinical and environmental *V. vulnificus* isolates. Also investigated was the ability of ribotyping and RAPD-PCR to differentiate Danish and U.S. clinical strains.

MATERIALS AND METHODS

Bacterial strains. A total of 85 *V. vulnificus* strains were examined, including the type strain, ATCC 27562. Twenty-nine strains were isolated from humans in Denmark and the United States, three biotype 2 isolates originated from diseased eels in Norway and Sweden, and the remaining 52 isolates were isolated from samples of coastal waters and sediment collected in Denmark in late summer 1994 by methods described previously (8). Briefly, the samples were preenriched in alkaline peptone water (pH 8.6, 1% NaCl) with polymyxin B (20,000 U/liter) at 37°C for 24 h, and yellow colonies were selected after 24 h of incubation on colistin-polymyxin B-cellobiose agar (17) at 40°C. All *V. vulnificus* isolates used in the present study tested positive in colony hybridization with a *V. vulnificus*-specific, alkaline phosphatase-labeled DNA probe directed against a cytolysin-hemolysin gene performed under stringent conditions (35). Each strain except for the type strain, ATCC 27562, was assigned a number between 1 and 84 according to its location in the dendrogram shown in Fig. 1.

Ribotyping. We have previously observed that among seven restriction enzymes tested, *Hin*dIII provides the best band discrimination for *V. vulnificus* (9). Chromosomal DNA was extracted by the method of Pedersen and Larsen (21), and electrophoresis conditions, blotting procedures, hybridization with a digoxigenin-labeled cDNA probe complementary to 16S and 23S rRNA of *Escherichia coli* at 56°C, and detection procedures were as previously described (10). A 1-kb DNA ladder (Gibco BRL, Gaithersburg, Md.) was used as a molecular size marker. Ribotype patterns were considered to be different when there was a difference of one band between each isolate. Each ribotype was assigned an arbitrary number. The ribotyping results for 11 of the 12 Danish clinical strains included in this study have been described in a previous paper (9). The ribotype designations were changed in the present investigation, so ribotypes 1, 3, 4, 5, 6, and 7 in this study are equivalent to ribotypes 1, 2, 3, 4, 5, and 6, respectively, in the earlier study. All isolates were ribotyped twice.

RAPD-PCR. Ten 10-mer oligonucleotide single primers (Biosynthesis, Lewisville, Tex.), each with a G+C content of 50%, were screened for their ability to provide a suitable banding pattern (Table 1), and one primer (5′GGATCTGA AC3′) was found to give the best, with between 10 and 15 visible bands equally distributed in the molecular range 123 to 4,182 bp (31). All cell cultures were grown at 37°C to stationary phase in heart infusion broth (Difco, Detroit, Mich.). These cell cultures were used as the source of the template DNA. Each 25.0-μl RAPD reaction mixture contained the following reagents: 2.5 μl of 10× reaction buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl, 15 mM $MgCl_2$, and 0.01% gelatin; Promega, Madison, Wis.), 2.0 μl of diethyl pyrocarbonate-treated H_2O,

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TABLE 1. Sequences of 10 10-mer oligonucleotide single primers screened for RAPD-PCR analysis

Primer
5′GGA TCT GAA C3′[a]
5′TAG CAC AGT C3′
5′CCA AAC TGC T3′
5′CTT GAG TGG A3′
5′TCC TCA AGA C3′
5′AGC GTA ACC T3′
5′CCA CTT TGA G3′
5′GAG ATG ACG A3′
5′TCG CTA TCT C3′
5′TGA CTT GGC T3′

[a] Primer used in our study.

3.5 μl of $MgCl_2$ (25 mM), 8.0 μl of deoxynucleoside triphosphates (5 mM; Promega), 3.0 μl of primer (25 mM), 5.0 U of *Taq* polymerase (Promega), and 5.0 μl of cell culture. The RAPD reaction mixtures were overlaid with 15.0 μl of mineral oil to prevent evaporation during thermal cycling. Amplification was performed in a thermal cycler, model PHC-3 (Techne, Princeton, N.J.), programmed as follows: 1 cycle of 94°C for 5 min; 45 cycles of 94°C for 1 min, 36°C for 1 min, and 72°C for 2 min; and a final cycle of 72°C for 5 min. The RAPD products were electrophoresed on a 2.0% agarose gel containing ethidium bromide (6×10^{-6} mg/ml), and the gel was photographed under UV light. A 123-bp ladder (Sigma, St. Louis, Mo.) was used as a molecular size marker.

Computer analysis of RAPD-PCR results. RAPD-PCR gel photographs were scanned with an ImageMaster DTS scanner (Pharmacia, Uppsala, Sweden). The images were calibrated, and the presence or absence of bands was recorded in binary scores with RFLPScan software (Scanalytics, Billerica, Mass.). The match tolerance was set to 2.5% of the molecular weight of each band. The program TreeCon (28) was used on the output from the RFLPScan to produce a dissimilarity matrix based on $N(N-1)/2$ pairwise comparisons between N isolates. The genetic distances were calculated as $(N_x + N_y)/(N_x + N_y + N_{xy})$, where N_x is the number of bands present only in isolate x, N_y is the number of bands present only in isolate y, and N_{xy} is the number of bands common to isolates x and y. The dissimilarity matrix was used by TreeCon to perform unweighted pair group method using mathematic averages (UPGMA) cluster analysis and to infer a dendrogram. A bootstrap value of 100 was used to statistically evaluate the topology of the dendrogram.

RESULTS

Ribotyping. On the basis of *Hin*dIII ribotyping, the collection of *V. vulnificus* strains was separated into 36 different ribotypes (Table 2). Each ribotype contained between one and nine strains. Danish and U.S. strains belonged to different ribotypes. The 17 U.S. clinical strains were distributed among ribotypes 28 to 36. The 12 Danish clinical strains belonged to ribotypes 1, 3, 4, 5, and 6. Eight Danish environmental strains belonged to ribotypes containing 1 of the 12 Danish clinical strains. All three eel strains, two Danish clinical strains obtained from wound infections in fishermen cleaning eels, and one Danish environmental strain belonged to ribotype 6. Ribotypes 3 and 5 included only Danish clinical strains. The type strain, ATCC 27562, belonged to a unique ribotype, as did 25% (21 of 85) of the strains included in this study. When ribotyping was repeated with separate DNA extracts from each strain, no variation was found in the ribotype patterns. Most ribotypes were unique to strains isolated from samples from a particular coastal location. However, several ribotypes were found at more than one location. The clinical strain from the first fatal case of *V. vulnificus* infection in Denmark (6, 9) was ribotype 1, which also included five Danish environmental strains isolated from brackish water from the area where the patient had contracted the infection during fishing. Ribotypes 4 and 6, which included Danish clinical strains, were also found in Danish environmental strains.

RAPD-PCR. The dendrogram based on RAPD-PCR profiles is shown in Fig. 1, and examples of RAPD-PCR profiles are shown in Fig. 2. Forty-one of the *V. vulnificus* strains were separated into 12 clusters containing between two and seven strains each. Forty-four strains from both clinical and environmental sources were not included in any cluster. Eight of 12 clusters included clinical strains, and the percentage of clinical strains per cluster was between 33 and 100. Regardless of geographical origin, clinical strains were distributed throughout the dendrogram. RAPD-PCR was not able to differentiate Danish and U.S. strains, and the three eel strains were separated into two different clusters. One eel strain (no. 39) clustered with the type strain, a U.S. clinical strain (no. 40), and two Danish environmental strains (no. 41 and 42), while the two remaining eel strains (no. 63 and 64) clustered with one Danish environmental strain (no. 65). Therefore, based on RAPD-PCR patterns, *V. vulnificus* biotype 2 strains cannot be separated from biotype 1 strains (Fig. 2). The RAPD profiles were very heterogeneous, with similarities as low as 25%. RAPD-PCR was performed twice on each strain, and the banding patterns were stable.

TABLE 2. Ribotype and source of 85 *V. vulnificus* strains included in this study

Ribotype[a]	Source (isolate designation[s])
1	Danish, clinical (1, 4, 11, 28)
1	Danish, environmental (5, 12, 29, 30, 72)
2	Danish, environmental (31)
3	Danish, clinical (32)
4	Danish, clinical (45)
4	Danish, environmental (49, 52)
5	Danish, clinical (7, 15, 16, 53)
6	Danish, clinical (43, 47)
6	Danish, environmental (68)
6	Norwegian, eel (39, 63); Swedish, eel (64)
7	U.S., clinical (ATCC 27562)
8	Danish, environmental (54)
9	Danish, environmental (71)
10	Danish, environmental (8, 17, 44, 50, 83)
11	Danish, environmental (56, 80)
12	Danish, environmental (67)
13	Danish, environmental (2, 55, 57, 58)
14	Danish, environmental (33, 46, 73, 74, 77)
15	Danish, environmental (6)
16	Danish, environmental (41)
17	Danish, environmental (84)
18	Danish, environmental (3, 38, 59, 76)
19	Danish, environmental (42)
20	Danish, environmental (37)
21	Danish, environmental (18, 19, 51, 61, 65)
22	Danish, environmental (48)
23	Danish, environmental (35)
24	Danish, environmental (60, 81)
25	Danish, environmental (23, 36)
26	Danish, environmental (21, 66)
27	Danish, environmental (69, 75)
28	U.S., clinical (9, 14, 27, 40, 82)
29	U.S., clinical (70, 78)
30	U.S., clinical (79)
31	U.S., clinical (22, 62)
32	U.S., clinical (10, 13, 20)
33	U.S., clinical (26)
34	U.S., clinical (24)
35	U.S., clinical (25)
36	U.S., clinical (34)

[a] *Hin*dIII was the restriction enzyme used for ribotyping.

Comparison of ribotyping and RAPD-PCR results. A comparison of the two fingerprinting techniques was performed by determining the location of all strains with the same ribotype in

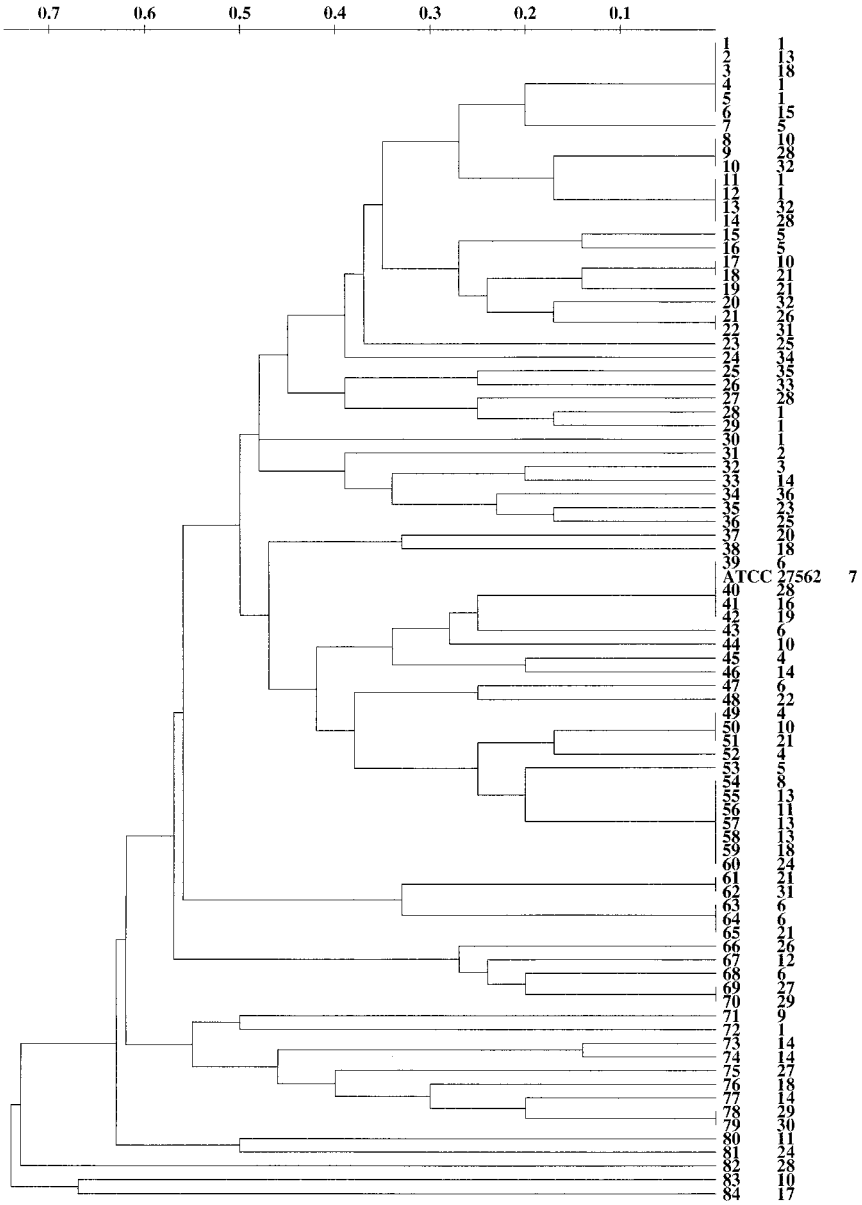

FIG. 1. Dendrogram based on UPGMA cluster analysis of RAPD-PCR profiles of 85 *V. vulnificus* strains. The scale measures dissimilarity (0.1 = 90% similarity). Numbers next to the dendrogram are isolate designations, and numbers to the far right are ribotype designations.

the RAPD-PCR dendrogram. We observed that, in all cases, strains with the same ribotype never occurred in a single cluster or even grouped close together in the RAPD-PCR dendrogram. Thus, there was no correlation between the two typing methods. Among strains showing the same ribotype, similarities between 100 and 25% were found based on RAPD-PCR. Further evaluation of the RAPD-PCR method was used to determine if any correlation existed between the sources of isolation and the RAPD profiles, but no such correlation was shown by the RAPD results.

DISCUSSION

Both ribotyping and RAPD-PCR showed that the 85 *V. vulnificus* strains examined were very heterogeneous. Buchrieser et al. (7) have previously described the high heterogeneity of a population of *V. vulnificus* from a single oyster, based on clamped homogeneous electric field gel electrophoresis, and our study supports their findings.

In our study, ribotyping was able to separate the Danish and U.S. strains, since no ribotype included strains from both countries. Two ribotypes included only Danish clinical strains, whereas the remaining Danish clinical strains had ribotypes that were identical to those of the Danish environmental strains. Our inability to detect any genotypic differences based on ribotyping between clinical and environmental strains is consistent with the observations of Tison and Kelly (26) and Stelma et al. (24), who found no significant differences between environmental and clinical strains based on biochemical characteristics, antimicrobial susceptibility patterns, or virulence characteristics. Our investigation reveals that no specific genotypic trait was present in clinical *V. vulnificus* strains.

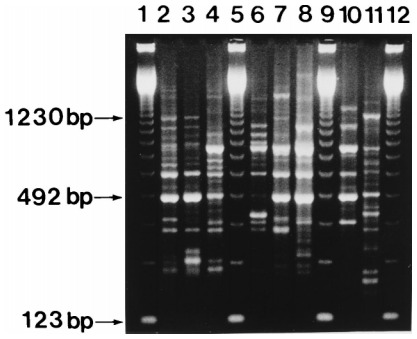

FIG. 2. RAPD patterns obtained from different *V. vulnificus* strains. Lanes 1, 5, 9, and 12, 123-bp molecular size standard. Lane 2, biotype 2 strain from a diseased Norwegian eel (no. 39); lane 3, biotype 2 strain from a diseased Swedish eel (no. 64); lane 4, biotype 1 reference type strain, ATCC 27562; lane 6, biotype 1 strain from sediment in Denmark (no. 41); lanes 7, 8, and 10, biotype 1 strains from coastal waters in Denmark (no. 54, 38, and 71); and lane 11, biotype 2 strain from coastal waters in Denmark (no. 68).

In an earlier study, researchers evaluated the usefulness of ribotyping and RAPD techniques as tools for epidemiological studies of *V. vulnificus* biotypes (4) and concluded that RAPD analysis is sufficient for differentiation of the two biotypes. This conclusion is not in agreement with our finding that RAPD-PCR was unable to differentiate biotype 2 eel strains from clinical and environmental biotype 1 strains. However, the protocol employed in our RAPD study differed significantly in two ways from the one in the previous study (4). First, we used a 10-mer instead of an 18-mer primer, and second, we used an annealing temperature of 36°C compared to the annealing temperature of 50°C used by Aznar et al. (4). Also, the ribotyping method used by Aznar et al. (4) differed in several ways from the one used in our study. Our choices of restriction enzyme, rRNA probes, and hybridization temperatures also differed from those of the previous study, which makes comparison of the results impossible. Aznar et al. (4), who used a probe directed against a sequence in 23S rRNA genes for hybridization following restriction with the enzyme *Kpn*I, were able to discriminate not only among *Vibrio* species and between the two *V. vulnificus* biotypes but also among individual strains. We found that our method was able to differentiate the two biotypes but not individual strains.

Amaro and Biosca (1) reported that *V. vulnificus* biotype 2, which previously has been considered an obligate eel pathogen, is also an opportunistic pathogen for humans. We found that two Danish clinical strains (no. 43 and 47) and one strain from Danish coastal waters (no. 68) belonged to ribotype 6, as did the three eel strains. Furthermore, all six strains belonging to ribotype 6 were biotype 2, as each showed a negative indole reaction in repeated tests, which is the main biochemical criterion for separating biotypes 1 and 2 (27). One of the Danish clinical strains has previously been reported to be indole positive (9), which may be explained by the use of a different broth in indole testing. These results indicate that fishermen handling eels are at risk for wound infections with *V. vulnificus* and that *V. vulnificus* biotype 2 should be considered a human pathogen. The two clinical strains (no. 43 and 47) were isolated from wound infections of fishermen who contracted the disease from handling eels. Danish strain 68 was isolated from coastal waters from a site commonly used for eel fishing, with a salinity of 8‰ and a temperature of 17°C. This is, to our knowledge, the first time *V. vulnificus* biotype 2 has been isolated from coastal water samples, although Amaro et al. (2) were able to show in a laboratory study the survival of this bacterium in brackish water. Two hypotheses as to why this bacterium has never previously been isolated from a marine environment are (i) that biotype 2 has been misidentified as biotype 1 and (ii) that it seldom occurs in the water because its natural reservoir is the eel. Our investigation of the occurrence of *V. vulnificus* in Danish coastal waters showed that biotype 2 strains can be isolated only at a very low frequency; however, our finding provides further evidence that *V. vulnificus* biotype 2 can survive in water and spread infection through water.

Our study shows that ribotyping can be used to differentiate biotypes 1 and 2, with ribotype 6 being unique to biotype 2 strains. Additional studies are planned to determine if all biotype 2 strains with ribotype 6 belong to the newly proposed serogroup E (5). Furthermore, we are investigating the ribotypes and biotypes of a collection of *V. vulnificus* strains isolated from healthy and diseased Danish eels.

RAPD-PCR was shown to be a very sensitive method for the characterization of *V. vulnificus*. The UPGMA cluster analysis based on RAPD profiles generated numerous clusters, each containing only a few strains. RAPD-PCR did not discriminate between clinical and environmental strains or between Danish and U.S. strains. Further, the clustering based on RAPD profiles was completely different from the grouping of strains based on ribotypes, and thus no correlation between the methods was detected. Biotyping of *V. vulnificus* cannot be supported by 16S rRNA sequencing data, suggesting that the separation of *V. vulnificus* strains into two biotypes does not reflect the natural relationship of the strains (3). However, sequences of genes encoding 16S ribosomal subunits may not reveal a genomic difference between biotypes. The RAPD-PCR assay differentiates strains based on differences in nucleotide sequences in the entire genome. We found that RAPD-PCR is unable to divide biotypes, which is in keeping with the observations of Aznar et al. (3) based on 16S rRNA sequencing. All biotype 2 strains showed the same ribotype, which is in agreement with other studies reporting that biotype 2 strains are genetically homogenous compared to biotype 1 strains (3, 4). Our results indicate that biotype 2 strains are not evolutionarily distant from biotype 1 strains, although biotype 2 strains are more genetically homogenous than biotype 1 strains.

RAPD-PCR has been shown in recent years to be useful for classifying a number of bacterial species (15, 32), but our results indicate that under the conditions employed in this study, RAPD-PCR is not appropriate for developing a typing scheme for *V. vulnificus*. However, this method can be applied to the identification of individual strains or to the subtyping of ribotypes because of its high sensitivity. Also, RAPD-PCR may be used as a diagnostic tool in tracing the source of infections associated with the consumption of seafood, because results can be obtained less than 24 h after sampling.

The clinical cases of *V. vulnificus* infections reported in Denmark have so far all been associated with exposure to seawater or with the handling of eels (9). Therefore, our current interest in Denmark is not to find a typing method able to identify subtle genetic dissimilarities between strains, as we lack the opportunity to trace clinical cases to their sources. Instead, a less sensitive typing technique, such as ribotyping, may be of value in grouping strains into clusters useful for investigating the epidemiology of *V. vulnificus* infections in Denmark.

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