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Synthetic Oligodeoxyribonucleotide Probes to Detect Kanagawa Phenomenon-Positive *Vibrio parahaemolyticus*

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Synthetic oligodeoxyribonucleotide probes were used in the colony hybridization test to examine the association between the Kanagawa phenomenon (KP) and the thermostable direct hemolysin gene (*tdh*) of *Vibrio parahaemolyticus*. Representative *V. parahaemolyticus* strains with a variety of KP reactions and 17 other *Vibrio* species were examined for homology with four synthetic oligodeoxyribonucleotide probes (19 to 21 bases long) representing different regions of the *tdh* structural gene. Under stringent conditions, two of the probes were capable of distinguishing KP-positive *V. parahaemolyticus* from KP-negative or KP weak-positive *V. parahaemolyticus* which possesses mutated *tdh* genes. *Vibrio hollisae* strains hybridized with all four probes under reduced stringency, suggesting that they have *tdh*-related genes which are homologous but not identical to the *tdh* gene in all the regions examined. The results suggest that the colony hybridization test with the synthetic oligonucleotide probes is more suitable for the definitive determination of KP-positive strains than the hybridization with the larger gene probe or immunological assays.

Vibrio parahaemolyticus is an important etiologic agent of seafood-borne gastroenteritis (3). Although its pathogenic mechanism is not clearly understood, epidemiological surveys indicate a very strong association of the thermostable direct hemolysin (TDH) with disease, i.e., 88 to 96% of clinical isolates produce TDH, whereas only 1 to 2% of nonclinical isolates produce this hemolysin (10, 17). TDH production in these surveys was determined by using a special blood medium, Wagatsuma agar. Production of beta-hemolysis on this agar medium is called the Kanagawa phenomenon (KP); this reaction has been considered to be a hallmark of virulent strains (10). Because it is not easy to prepare Wagatsuma agar and because the interpretation of results is hampered by hemolytic factors other than TDH, better methods to detect TDH-producing isolates have been sought. False-negative results may be obtained by methods based on immunoprecipitation (4), owing to strain-to-strain variation in the level of TDH produced. The reversed passive hemagglutination test (16) and the enzyme-linked immunosorbent assays (5, 13) are very sensitive, and even some KP-negative (KP−) strains were shown to be positive in these assays. It is possible, however, that molecules which are immunologically related but not true TDH may be detected by these sensitive immunological assays.

In our previous study (13), a specific DNA probe for the gene encoding TDH (*tdh*) was constructed from an internal region of the sequenced *tdh* gene (14). However, detection of the *tdh* gene in *V. parahaemolyticus* with this probe did not completely correlate with the KP reaction. In addition to KP-positive (KP+) strains, some strains weakly positive for the KP (KP+^{w}) and some KP− strains were also gene positive. However, all probe-positive strains produced TDH detectable with anti-TDH serum by an enzyme-linked immunosorbent assay. These conflicting results raised the question of whether the gene probe detected strains producing TDH at low levels (possibly indicating a mutation in the control region) or detected mutated *tdh* structural genes, from which biologically less active or inactive TDH molecules are produced. A further question concerns the nature of the DNA sequences present in *Vibrio hollisae* which hybridized to the *tdh* gene probe (13).

In this study, we used synthetic oligodeoxyribonucleotides as hybridization probes in the DNA colony hybridization technique to further examine the association between KP reaction and *tdh* gene detection. Oligonucleotide probes allow the examination of very small regions of DNA sequences and the detection of even a single-base-pair mismatch under stringent conditions (20) and can therefore be highly specific. Probing with synthetic oligonucleotides for selected DNA sequences within the *tdh* structural gene enabled us to detect structural mutations in the *tdh* gene of KP+^{w} and KP− strains of *V. parahaemolyticus*. The hybridization test with the oligonucleotides also revealed substantial difference between the DNA sequence of the *tdh* gene of *V. parahaemolyticus* and the *tdh*-related genes of *V. hollisae*. The mutations in *V. parahaemolyticus* were localized in certain regions of the gene, and thus probes for these regions can be used to specifically detect KP+ *V. parahaemolyticus* in clinical and epidemiological studies.

MATERIALS AND METHODS

Bacterial strains. Forty-two *V. parahaemolyticus* strains isolated from diverse sources and 11 *V. hollisae* strains isolated from clinical cases were used in this study. Seventeen strains belonging to 16 other species of the genus *Vibrio* were also examined, including human-pathogenic vibrios (*V. cholerae* O1 569B, *V. cholerae* non-O1 N203OH, *V. fluvialis* DJVP 6957, *V. mimicus* 2031H, *V. vulnificus* E9315, *V. metschnikovii* 2167-78, and *V. alginolyticus* ATCC 17749) and marine- or fish-pathogenic vibrios (*V. aestuarianus* ATCC 35048, *V. campbellii* ATCC 25920, *V. gazogenes* ATCC 29988, *V. harveyi* ATCC 14126, *V. natriegens* ATCC

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TABLE 1. Nucleotide base sequence of the synthetic oligodeoxyribonucleotide probes

Probe	Nucleotide base sequence (5′ to 3′)	Location[a]	Calculated Td (°C)[b]
1	CCATCTGTCCCTTTTCCTGCC	330–350	66
2	GGTACTAAATGGTTGACATCC	504–524	60
3	CCAAGTAAAATGTATTTGG	685–702	50
4	GCATATGAGAGTGGTAGTGG	735–754	60

[a] Location corresponds to the position of the bases in the previously published nucleotide sequence of the *tdh* gene, in which mature protein is encoded between positions 321 and 815 (14).

[b] Tds, at which 50% of the probe is dissociated, were calculated by the method of Suggs et al. (18). The calculated Tds were used as guides to determine reasonable hybridization temperatures (see the text).

14048, *V. nigripulchritudo* ATCC 27043, *V. pelagius* ATCC 25916, *V. ordalii* SW-1669, *V. splendidus* ATCC 33125, and a psychrotrophic *Vibrio* 6CCC). These test strains represent the strains previously examined for the presence (gene+) or absence (gene−) of the *tdh* gene using the specific 415-base-pair (bp) gene probe (13). The sources of the strains were described previously (13, 15). The KP reactions of the *V. parahaemolyticus* strains were tested as previously described (13).

Colony blots. Two colony blots, one on a nitrocellulose filter and the other on Whatman no. 541 filter paper (Whatman, Inc., Clifton, N.J.), were prepared for each test strain. Colony blots on nitrocellulose filters (BA 85/20; Schleicher & Schuell, Inc., Keene, N.H.) were prepared as follows. Up to 30 test organisms were spot-inoculated onto an autoclaved nitrocellulose filter overlying an agar medium and incubated. Growth media and incubation conditions used were: L broth medium (9), 37°C, overnight for *V. parahaemolyticus* and other human-pathogenic vibrios; and brain heart infusion agar (Difco Laboratories, Detroit, Mich.) supplemented with 1% NaCl and 0.2% $MgCl_2$, 15°C, 2 days for marine- and fish-pathogenic vibrios. After incubation, the filter was removed from the plate and treated with NaOH, neutralized, and baked as described by Moseley et al. (12). Colony blots on Whatman no. 541 filter paper (7.0-cm diameter) were prepared essentially as described by Maas (7). Up to 30 test organisms were spot-inoculated onto an agar medium and incubated as described above. After incubation, a Whatman no. 541 filter was placed over the agar surface and incubated at room temperature for 1 to 2 h. The filter paper was removed and transferred to a glass petri dish containing Whatman no. 3 filter paper (11.0-cm diameter) dampened with a 3.5-ml solution of 0.5 M NaOH–1.5 M NaCl. The colony blot was steamed for 5 min, transferred onto a Whatman no. 3 filter dampened with a 3.5-ml solution of 1.0 M Tris (pH 7.0)–2.0 M NaCl, and incubated for 10 min at room temperature. The filter was air dried and stored at room temperature until used.

Synthetic probe. Sequences with GC-rich ends representing four different regions of the *tdh* structural gene were selected (Table 1). An automated phosphoramidite coupling method (2) was used to synthesize the oligodeoxyribonucleotides on an Applied Biosystems (Foster City, Calif.) model 380A DNA synthesizer as described previously (1). The 5′-dimethoxytrityl derivatives were purified by high-performance liquid chromatography as described by Broido et al. (1). The overall yield based on the amount of starting support-bound deoxyribonucleoside was about 18%. Purified oligodeoxyribonucleotides were labeled at the 5′ end (50 pmol of 5′ end per reaction) by the transfer of ^{32}P from [γ-^{32}P]ATP (ICN Biologicals Inc., Irvine, Calif.) with T4 polynucleotide kinase (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) as previously described (8). The labeled probe DNA was purified by chromatography on an NACS-52 PREPAC column (Bethesda Research Laboratories) according to the manufacturer's specifications, using 0.25 and 4.0 M ammonium acetate as the loading and eluting buffer, respectively. The specific activity ranged from 6×10^8 to 8×10^8 cpm/μg of probe.

Hybridization procedure. Hybridizations were done by a modification of the method of Wallace et al. (19). Because the lowest dissociation temperature (Td), the temperature at which 50% of the probe is dissociated, calculated for the probes was 50°C (Table 1), the blots were hybridized at 40°C overnight in a solution (5 ml per blot) consisting of 6 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0]), 5 × Denhardt solution (1 × Denhardt solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin), 1 mM EDTA (pH 8.0), 100 μg of boiled salmon sperm DNA (average size, 500 bases) per ml, and probe DNA (10^6 cpm per blot) at 40°C overnight. The blots were

TABLE 2. Results of the colony hybridization test with synthetic oligodeoxyribonucleotide probe 1[a]

Test organism	Gene[b]	KP[c]	Hybridization signal after washing at:					
			40°C	45°C	50°C	55°C	60°C	65°C
V. parahaemolyticus								
21 strains	+	+	+	+	+	+	+	−
3 strains	+	+[w,d]	+	+	+	+	+	−
6 strains	+	−	+	+	+	+	+	−
12 strains	−	−	+[w]	−	−	−	−	−
V. hollisae (11 strains)	+	NT[e]	+	+[w]	−	−	−	−
Other *Vibrio* species (n = 16)[f]								
V. aestuarianus	−	NT	+	+[w]	−	−	−	−
15 species	−	NT	+[w]	−	−	−	−	−

[a] Calculated Td of this probe was 66°C (Table 1).

[b] Presence (+) or absence (−) of the *tdh* gene as determined by the DNA colony hybridization test using a 415-bp DNA probe derived from an internal fragment of the *tdh* structural gene (13).

[c] KP reaction on Wagatsuma agar determined as described before (13).

[d] w, Weakly positive.

[e] NT, Not tested.

[f] See the text for details.

washed in 6 × SSC at a specified temperature as follows. The blots were rinsed briefly in 6 × SSC, incubated in 6 × SSC for 1 h, transferred to fresh 6 × SSC, and further incubated for 1 h. Then the blots were briefly rinsed in 2 × SSC at room temperature, air dried, and subjected to autoradiography at −80°C overnight with a single intensifying screen. The process of washing in 6 × SSC followed by rinsing in 2 × SSC and autoradiography was repeated with the same colony blots with increased washing temperature (starting at 40°C with 5°C increments) until hybridization could no longer be detected. The highest temperature at which hybridization was still clearly detected was considered the stringent hybridization condition.

RESULTS

The hybridization reactions of *V. parahaemolyticus*, *V. hollisae*, and 16 other *Vibrio* species with the four synthetic oligonucleotide probes are shown in Tables 2 to 5. The hybridization reaction with the larger 415-bp DNA probe and the KP reaction of the test organisms are also shown. Vibrios, except *V. vulnificus* (6), normally do not induce the KP reaction, and therefore the KP reactions of these organisms were not tested. They were included simply to examine the background level of the hybridization reactions. Under stringent washing conditions (60°C), probe 1 hybridized with all *tdh*+ *V. parahaemolyticus*, regardless of the KP (Table 2). Probe 3 showed a similar pattern and hybridized to all *tdh*+ *V. parahaemolyticus* at the stringent washing temperature (45°C, Table 3).

In contrast to probes 1 and 3, probes 2 and 4 clearly distinguished KP+ strains from KP+^{w} and KP− strains of *V. parahaemolyticus* (Tables 4 and 5). Under stringent conditions (55°C for probe 2, 55°C for probe 4), these probes hybridized to all KP+ strains and not, with one exception, to KP+^{w} or KP− strains. The lack of hybridization to KP− strains was seen even with strains which were positive with the 415-bp *tdh* gene probe. The one exceptional KP+^{w} strain, 1320, hybridized to both synthetic probes under stringent conditions and is discussed below.

All except one strain of *V. hollisae* did not react with any of the probes under stringent conditions but did react under reduced stringency (Tables 2 to 5). Strain 9041 reacted, under stringent conditions, only with probe 2 (55°C, Table 4).

Colony blots prepared on two different types of filters, nitrocellulose and Whatman no. 541, were compared because Whatman no. 541 is economical and easy to handle. The results presented in Tables 2 to 5 were obtained with nitrocellulose filters. No basic differences in results were seen between nitrocellulose and Whatman no. 541 filters in the colony hybridization procedure. In our hands, however, nitrocellulose paper gave more satisfactory results. Some nonspecific background noise in the autoradiograph was seen with the Whatman no. 541 filters, which made it difficult to judge the results after overnight exposure of the X-ray film. When exposure time was reduced to 3 to 4 h, the background was reduced, but signals were still not as clear as those obtained with nitrocellulose filters. In addition, some smudging of the colonies as reported by Maas (7) was also observed with Whatman no. 541 filters. A further problem with *Vibrio* species was that occasional strains swarmed on the agar surface. When Whatman no. 541 but not nitrocellulose filters were used, this swarming generated large, weak autoradiographic signals on the X-ray film.

TABLE 3. Results of the colony hybridization test with synthetic oligodeoxyribonucleotide probe 3[a]

Test organism	Gene[b]	KP[c]	Hybridization signal after washing at:		
			40°C	45°C	50°C
V. parahaemolyticus					
21 strains	+	+	+	+	−
3 strains	+	+^{wd}	+	+	−
6 strains	+	−	+	+	−
12 strains	−	−	−	−	−
V. hollisae (11 strains)	+	NT[e]	+^{w}	−	−
Other *Vibrio*[f] species (n = 16)	−	NT	−	−	−

[a] Calculated Td of this probe was 50°C (Table 1).
[b] through [f] See footnotes to Table 2.

TABLE 4. Results of the colony hybridization test with synthetic oligodeoxyribonucleotide probe 2[a]

Test organism	Gene[b]	KP[c]	Hybridization signal after washing at:				
			40°C	45°C	50°C	55°C	60°C
V. parahaemolyticus							
21 strains	+	+	+	+	+	+	−
1 strain (1320)	+	+^{wd}	+	+	+	+	−
2 strains	+	+^{w}	+	+	+	−	−
6 strains	+	−	+	+	+	−	−
12 strains	−	−	+^{w}	−	−	−	−
V. hollisae							
1 strain (9041)	+	NT[e]	+	+	+	+	−
10 strains	+	NT	+	+	+	−	−
Other *Vibrio* species (n = 16)[f]							
V. vulnificus	−	NT	+	+^{w}	−	−	−
15 species	−	NT	+^{w}	−	−	−	−

[a] Calculated Td of this probe was 60°C (Table 1).
[b] through [f] See footnotes to Table 2.

DISCUSSION

In our previous study (13), discrepancies between the KP and the presence of the *tdh* gene in *V. parahaemolyticus* were noted. Although the 415-bp DNA probe used was derived from a completely internal region of the *tdh* structural gene, some KP− and KP+^{w} strains of *V. parahaemolyticus* hybridized with the *tdh* gene probe. In addition, it was found that *V. hollisae* strains reacted with the gene probe, although the reaction was somewhat weaker than that of *V. parahaemolyticus*. One possible explanation for these findings is that the DNA probe hybridized with mutated *tdh* genes of KP− and KP+^{w} *V. parahaemolyticus* and the *tdh*-related genes possessed by *V. hollisae* because the hybridization conditions used, although of relatively high stringency, can still allow about 20%-base-pair mismatch (11). In the present study, the hybridization test with synthetic oligonucleotide probes, which have the potential to detect even a single-base-pair mismatch (18), demonstrated genetic differences among strains of *V. parahaemolyticus* and in *V. hollisae*. Among the four test probes, probe 4, specific for a region near the carboxy-terminal end of the *tdh* gene, clearly differentiated the KP+ *V. parahaemolyticus* strains from KP− or KP+^{w} strains. Under stringent conditions (55°C), this probe hybridized to all KP+ strains tested

TABLE 5. Results of the colony hybridization test with synthetic oligodeoxyribonucleotide probe 4[a]

Test organism	Gene[b]	KP[c]	Hybridization signal after washing at:					
			40°C	45°C	50°C	55°C	60°C	65°C
V. parahaemolyticus								
21 strains	+	+	+	+	+	+	+[w]	−
1 strain (1320)	+	+[wd]	+	+	+	+	+[w]	−
2 strains	+	+[w]	+	+	+[w]	−	−	−
3 strains	+	−	+	+	+[w]	−	−	−
3 strains	+	−	+	−	−	−	−	−
12 strains	−	−	+[w]	−	−	−	−	−
V. hollisae (11 strains)	+	NT[e]	+	+	+[w]	−	−	−
Other *Vibrio* species (n = 17)[f]								
V. vulnificus (n = 2)	−	NT	+	+	+[w]	−	−	−
6 species	−	NT	+	−	−	−	−	−
7 species	−	NT	+[w]	−	−	−	−	−
2 species	−	NT	−	−	−	−	−	−

[a] Calculated Td of this probe was 60°C (Table 1).
[b] through [f] See footnotes to Table 2.

but not to six of six KP− strains or two of three KP+[w] strains, all of which reacted with the 415-bp gene probe (Table 5). Probe 2 gave similar results, except that this probe also hybridized to 1 of 11 *V. hollisae* strains under stringent conditions (55°C, Table 4). These results indicate that significant differences exist between the KP+ and non-KP+ (KP− or KP+[w]) strains in at least two regions of the *tdh* structural gene. The one exceptional KP+[w] strain that hybridized to all four probes under stringent conditions was a seafood isolate, strain 1320. Unlike the other gene-positive KP− or KP+[w] strains examined, a highly concentrated culture supernatant of strain 1320 produced a line of identity with purified TDH in Ouchterlony immunodiffusion analysis (M. Nishibuchi and J. B. Kaper, unpublished observation). This low level of TDH production suggests a mutation in the control region of the *tdh* gene of strain 1320. Because the control region of the *tdh* gene is not clearly defined (14), this possible mutation in the control region was not examined in the present study. Therefore, we cannot rule out the possibility that gene-positive, KP−, or KP+[w] strains also carry mutations in the control region. This could cause the production of TDH-related molecules at low levels, which might still be detectable by sensitive immunological assays. Experiments are under way to investigate such possibilities.

Almost all *V. hollisae* strains hybridized to all four synthetic probes under reduced stringency but not under stringent conditions (Tables 2 to 5). These results indicate that *V. hollisae* contains nucleotide sequences which are similar but not identical to the *tdh* gene in all regions tested. This difference could explain why *V. hollisae* strains gave a weaker reaction with the 415-bp DNA probe than gene-positive *V. parahaemolyticus* strains (13) and why this species produces a molecule which is similar, but not identical, to TDH in Ouchterlony analysis (M. Nishibuchi and J. B. Kaper, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, B90, p. 33). One exceptional strain, 9041, reacted with probe 2 under stringent conditions, whereas the other strains did not (55°C, Table 4), demonstrating heterogeneity of the *tdh*-related gene in *V. hollisae* strains.

When other vibrio species were examined, *V. vulnificus* E9315 was found to hybridize to probes 2 and 4 under reduced stringency (Tables 4 and 5). *V. vulnificus* induces the KP on Wagatsuma agar (6), and the fact that probes 2 and 4 were specific for KP+ *V. parahaemolyticus* under stringent conditions suggests that these regions are important for the biological activity of TDH.

A wide variety of pathogenic bacteria can now be identified by using DNA probes. This technique is often simpler and more sensitive than complicated bioassays for the gene products (toxins or other virulence factors), and it also can be used for the direct examination of clinical, food, or environmental specimens for pathogenic species. Although there is no conclusive evidence that TDH is the major virulence factor of *V. parahaemolyticus*, the KP is a very important marker in clinical and epidemiological studies. The colony hybridization test using synthetic oligonucleotide probes (particularly probe 4) can distinguish potentially significant mutations in the *tdh* gene and is specific for KP+ strains. It is thus more suitable for definitive determinations than hybridization with the larger 415-bp gene probe or very sensitive immunological assays (5, 16), all of which may detect KP− or KP+[w] strains producing TDH-related molecules.

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