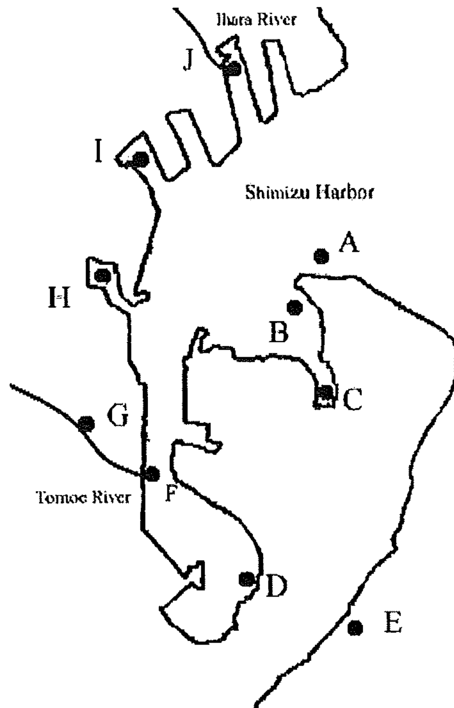

Fig. 1.　Sampling points in Shimizu Harbor area.
A: Masaki beach for sea bathing, B: Miho beach for sea bathing, C: The coast of 3797 Miho, D: The coast of Orido 4 chome, E: The coast of Orido 3 chome, F: The estuary of Tomoe river, G: Upper stream of Tomoe river, H: The coast of Minato-cho 1 chome, I: The coast of 149 Shimazaki-cho, J: The estuary of Ihara river.

ヵ所A：真崎海水浴場，B：三保海水浴場，C：三保3797番地海岸，D：折戸4丁目海岸，E：折戸3丁目海岸，F：巴川河口，G：巴川上流，H：港町1丁目海岸，I：島崎町149番地海岸，J：庵原川河口の表層海水，カキはDおよびHの岸壁，泥は海底2ヵ所および川底1ヵ所から採取し，海泥はDおよびHの海底，川泥はFの上流300M付近Gの川底からそれぞれ採取した(Fig. 1)．このうちC地点の採取場所には，浄水場からの浄水が流入していた．またGの巴川上流の検体採取場所は，巴川の河口から300M程度の上流であるが，清水港湾の潮の干満の影響を受け易く，海洋生物も生息していることから本調査報告では港湾の一部として扱った．

検体の採取時に海水はポリバケツを用いて3回ほど共洗いした後にくみ取り，カキは港湾の岸壁に付着しているものをはぎ取り，泥は柄杓ですくい取ったのち，それぞれ滅菌ポリ瓶および滅菌ポリ袋に入れ検体とした．検体はクーラーボックスで10℃前後に保冷して研究室に持ち帰り，直ちに供試した．採取時の海水は，採取直後の水温（積水化学，SS-31A），pH（新電元，KS701）および塩分濃度（積水化学，SS31-A）について測定した．

2.　海水，カキおよび泥のMPN値による汚染菌数の測定法

海水，泥およびカキの菌数は，10 m*l* のアルカリペプトン水を用いMPN法（3本法）により測定した．

海水は，試料の原液あるいはリン酸緩衝生理食塩水（PBS：日水製薬）で調整した希釈液10 m*l*，1 m*l*，0.1 m*l* および0.01 m*l*（0.1 m*l* 以下は各希釈段階の希釈液1 m*l* を用いた）を各々3本のアルカリペプトン水に接種した．カキと泥はそれぞれ10 gに滅菌PBS 90 m*l* を加えて10倍希釈したものを試料原液とした．10 m*l* の試験管系列に試料10 m*l* を接種する場合はアルカリペプトン水の濃度を常用の倍濃度としたものを用いた．

接種した各々のアルカリペプトン水は，37℃，24時間培養後，1白金耳をCHROMagar Vibrio培地（CV培地：関東化学）に画線塗抹し，発育した疑わしい集落について後述の方法によって生化学的性状を確認した．生化学的性状検査の結果，*V. vulnificus* の性状に一致した各集落を用いて方法4に示すPCR法に供試し，*V. vulnificus* が特異的に保有する溶血毒遺伝子（*V. vulnificus* cytotoxin-hemolysin: *vvh* 遺伝子）の有無を確認した．*vvh* 遺伝子の保有が確認されたMPN試験管は，*V. vulnificus* 陽性試験管とし，それらの試験管の本数をMPN表に照らし合わせてMPN値を算定し，試料の希釈倍数を乗じて菌数を求めた．

3.　分離菌株の生化学的性状試験

CV培地上に発育した鮮やかな青色を呈し，表面が盛り上がり，辺縁がスムースな *V. vulnificus* が疑われる集落を釣菌し，これを1%食塩加Triple Sugar Iron寒天培地（TSI培地：Oxoid）に移植して37℃，24時間培養した．

培養後，斜面部赤～黄変，高層部黄変，ガスおよび硫化水素陰性の性状を示した菌株を，食塩濃度を0%，1%，8%，10%に調整したNutrient Broth培地（NB培地：Becton Dickinson）に移植し，食塩耐性試験を実施した．その結果，*V. vulnificus* が疑われた株は，カジトン培地へ移植し，PCR用供試菌株として室温に保存した．

4.　PCRによる *vvh* 遺伝子の確認

PCR用保存菌株は，*vvh* 遺伝子（増幅産物：519 bp）を標的遺伝子としてHillら[8]の法に従ってPCRを行った．PCR用に供試したDNAの抽出は，保存菌株を標準寒天培地で再培養し，発育した集落から釣菌しPBSに浮遊させた．浮遊液は95℃で10分間加熱後，12,000 rpm，10分間遠心し，得られた上澄みを抽出DNA液としてPCRに供試した．

結　　果

1.　*V. vulnificus* のMPN値

V. vulnificus のMPN値および採取場所別平均MPN

値(AV)を Table 1 に示した．

V. vulnificus は，7 月から 12 月の海水の 51.6% (31/60)，カキの 75% (9/12) および泥の 94.4% (17/18) から検出された．

海水から検出された *V. vulnificus* の菌数は，検出下限値(＜3 MPN/100 ml)以下を除き，最も少ない検体は B（三保海水浴場）と D（折戸 4 丁目海岸）の 3 MPN/100 ml で，最も多い検体は G（巴川上流）の 280 MPN/100 ml であった (Table 1)．*V. vulnificus* は，8 月が 7 ヵ所，10 月が 8 ヵ所，11 月が 6 ヵ所から検出され，12 月は 4 ヵ所，7 月および 9 月が 3 ヵ所から検出された．採取場所別では，J（庵原川河口）5 回，B（三保海水浴場），F（巴川河口）および I（島崎町 149 番地海岸）から各 4 回検出された．また A（真崎海水浴場），C（三保 3797 番地海岸），D（折戸 4 丁目海岸）および H（港町 1 丁目海岸）から各 3 回，G（巴川上流）から 2 回検出された．一方，湾外の E（折戸 3 丁目海岸）の海水からは検出されなかった．

カキから検出された *V. vulnificus* の菌数は，最も多い検体では H（港町 1 丁目海岸）および D（折戸 4 丁目海岸）の 150 MPN/100 g であったが，検出下限値以下の検体もあった (Table 1)．

泥から検出された *V. vulnificus* の菌数は，最も多い検体は 8 月の G（巴川上流）600 MPN/100 g であったが，10 月の H（港町 1 丁目海岸）の検体においては，検出下限値以下 (＜30 MPN/100 g) であった (Table 1)．

2. 検体採取時の海水の水温，塩分濃度および pH

海水温，塩分濃度および pH は，変動を見るために調査月のデータ，標準偏差値および平均値を Table 1 に示した．7 月から 10 月までの 20℃以下の海水温は，7 月の A（真崎海水浴場）の 15.1℃で，ほかは 20℃を超えており，F（巴川河口）では 9 月に 30.8℃に達した．11 月以降はすべての場所において 20℃以下で推移した．

海水温が最も変動した場所は，F（巴川河口）で 21.7±6.8℃であり，次いで C（三保 3797 番地海岸）20.6±5.8℃，D（折戸 4 丁目海岸）の順であった．全採取場所では，月別に 3.6℃から 6.8℃の変動幅が見られた．

塩分濃度に大きな変動を示した検体採取場所は，J（庵原川河口）2.0±0.6%で，次いで C（三保 3797 番地海岸）2.3±0.5%，G（巴川上流）1.0±0.4%であった．その他の採取場所では，0.1%から 0.3%の変動幅で推移した．

検体採取場所の pH は，G（巴川上流）の pH 8.1±0.4 から J（庵原河口）の 9.1±0.1 の範囲に推移した．全採取場所の月別最低値は 12 月の G（巴川上流）の pH 7.5 で，それ以外の場所は，pH 8.1～9.2 の間で変動した．

考　察

V. vulnificus の全国的な分布については，限局的な環境中の調査報告があり，これらの報告では，本菌が汽水域などの塩分濃度が低い海水に多く存在すること[6]，水温が 20℃以上になると旺盛に増殖すること[1,6]，貝類や泥で多く検出されること[1,6,9]，および九州地方で汚染が高いことが報告されている[5,6]．

清水港湾には，巴川，庵原川，その他の小河川等が注ぎ込んでおり，これらの河川の存在により海水の塩分濃度が低下している．また清水港湾は深く切れ込んだ形状であるために外海からの海水が流入する度合いが少ないため外洋の海水温の影響による冷却効果が少ないことから外気温度の影響を受け海水温が上昇しやすい．したがって，清水港湾内には，*V. vulnificus* の生存に好適な環境が存在すると考えられる．

今回の調査では E（折戸 3 丁目海岸）を除き，夏期を中心に清水港湾内全体から *V. vulnificus* が検出されており，港湾の本菌による汚染が明らかにされた．また，汚染菌量が多い地域では塩分濃度は総じて低い傾向にあることが示され，D（折戸 4 丁目海岸：塩分濃度 2.4±0.3%）のカキと泥，F（巴川河口：塩分濃度 2.0±0.2%），G（巴川上流：1.0±0.3%）の海水と泥においては菌数が 100 MPN/100 ml (g) を超える月も見られた．しかし，塩分濃度と菌数の増減についての直接的な関連性を示すデータは得られなかった．一方，E（折戸 3 丁目海岸）では，いずれの検体からも菌が検出されなかった．その原因として E（折戸 3 丁目海岸）は，常に新鮮な外洋海流の影響を受けて有機物，プランクトンの死骸等が海底に蓄積されず，菌の増殖に適した場ではないこと，ならびに菌が増殖しても，その場に停滞することなく拡散されることなどが考えられる．

V. vulnificus の清水港湾内における分布は，検体によって異なる傾向を示した．すなわち海水から検出された月別の検出頻度は，J（庵原川河口），B（三保海水浴場），F（巴川河口）および I（島崎町 149 番地海岸）の順で多かった．カキは，H（港町 1 丁目海岸）よりも D（折戸 4 丁目海岸）で菌数が多い傾向を示した．泥は，汽水域の G（巴川上流），D（折戸 4 丁目海岸）および H（港町 1 丁目海岸），の順に菌の検出検体数および平均検出菌量が多かった．

汽水域において *V. vulnificus* が多く検出される傾向は，宮坂らの報告[6]と一致した．

V. vulnificus の検体別の汚染菌数は，海水で 10 MPN/100 ml に満たないことが多かった．カキでは 30～150 MPN/100 g，泥は 100 MPN/100 g を超えるものが多かった．

清水港湾における *V. vulnificus* の検出率は，泥から 94.4%，カキから 75.0%および海水から 51.6%であった．このことから清水港湾内の海水における *V. vulnificus* は，Kneko ら[13,14]が報告した腸炎ビブリオの生態と同様に海水よりも泥・カキで多く検出された．海水，カキおよび泥の検出率に差が見られる原因としては，海水では増殖の場となるプランクトンが拡散される

Table 1. The number of *V. vulnificus* in seawater, oyster and sea mud, and environmental data in the sampling area

Sampling area	Parameter	Month in 2006						SD (AV)
		Jul.	Aug.	Sept.	Oct.	Nov.	Dec.	
A (Masaki beach for sea bathing)	Number of *V. vulnificus* in seawater (MPN/100 ml)	<3	16	<3	6.1	<3	9.2	(5.2)
	Temperature of seawater (℃)	15.1	20.5	27.7	23	18.8	15.4	20.1±4.8
	Salinity of seawater (%)	3	2.9	2.7	2.8	2.7	2.7	2.8±0.1
	pH of seawater	8.7	9.1	8.9	8.5	8.1	8.7	8.7±0.3
B (Miho beach for sea bathing)	Number of *V. vulnificus* in seawater (MPN/100 ml)	3	120	6.2	<3	6.1	<3	(25.6)
	Temperature of seawater (℃)	24	23.1	27	23.2	16.8	15.7	21.6±4.4
	Salinity of seawater (%)	2.8	2.5	2.9	2.8	2.6	2.6	2.7±0.2
	pH of sea water	8.8	9.1	8.8	8.7	8.4	8.9	8.8±0.2
C (The coast of 3797 Miho)	Number of *V. vulnificus* in seawater (MPN/100 ml)	<3	<3	<3	4	9.1	9.1	(5.1)
	Temperature of seawater (℃)	23.5	22.5	27.6	22.8	12.7	14.2	20.6±5.8
	Salinity of seawater (%)	2.7	1.6	2.7	2.7	1.8	2.5	2.3±0.5
	pH of seawater	8.5	8.8	8.6	8.3	8.1	8.6	8.5±0.3
D (The coast of Orido 4 chome)	Number of *V. vulnificus* in seawater (MPN/100 ml)	<3	14	<3	3	3	<3	(3.3)
	Number of *V. vulnificus* in oyster (MPN/100 g)	30	92	93	150	91	<30	(76.0)
	Number of *V. vulnificus* in sea mud (MPN/100 g)	270	91	150	420	40	30	(166.8)
	Temperature of seawater (℃)	25.5	23.5	28.2	22.7	16.3	14.1	21.7±5.4
	Salinity of seawater (%)	2	2.1	2.9	2.2	2.4	2.5	2.4±0.3
	pH of seawater	8.6	9.1	9.1	9.1	8.1	8.6	8.8±0.4
E (The coast in Orido 3 chome)	Number of *V. vulnificus* in seawater (MPN/100 ml)	<3	<3	<3	<3	<3	<3	(<3)
	Temperature of seawater (℃)	22.2	22.4	26.9	22.8	19.4	16.3	21.7±3.6
	Salinity of seawater (%)	2.8	2.8	3	2.9	2.9	3	2.9±0.1
	pH of seawater	8.7	9	8.7	8.8	8.6	8.9	8.8±0.2
F (The estuary of Tomoe river)	Number of *V. vulnificus* in seawater (MPN/100 ml)	6.1	4	<3	140	7	<3	(26.2)
	Temperature of seawater (℃)	24.2	23.4	30.8	24.2	14.1	13.2	21.7±6.8
	Salinity of seawater (%)	1.7	2.1	2.5	1.9	1.9	1.6	2.0±0.3
	pH of seawater	8.3	9.1	9	8.4	8.3	8.5	8.6±0.4
G (Upper stream of Tomoe river)	Number of *V. vulnificus* in seawater (MPN/100 ml)	<3	<3	<3	30	280	<3	(51.7)
	Number of *V. vulnificus* in sea mud (MPN/100 g)	91	600	300	160	110	60	(220.1)
	Temperature of seawater (℃)	24.7	23	28.9	22.5	17.2	13.5	21.6±5.4
	Salinity of seawater (%)	1	0.5	1	0.7	1.8	0.9	1.0±0.4
	pH of seawater	8.1	8.1	8.8	7.9	8.4	7.5	8.1±0.4
H (The coast of Minato-cho 1 chome)	Number of *V. vulnificus* in seawater (MPN/100 ml)	<3	7.3	7	60	<3	<3	(12.4)
	Number of *V. vulnificus* in oyster (MPN/100 g)	30	36	<30	150	<30	40	(42.7)
	Number of *V. vulnificus* in sea mud (MPN/100 g)	61	61	150	<30	40	30	(57.0)
	Temperature of seawater (℃)	25.2	24	29.6	24	19.5	13.8	22.7±5.4
	Salinity of seawater (%)	2.6	2.4	2.6	2.9	2.9	2.6	2.7±0.2
	pH of seawater	8.5	8.9	8.7	8.7	9	8.9	8.8±0.2
I (The coast of 149 Shimazaki-cho)	Number of *V. vulnificus* in seawater (MPN/100 ml)	<3	20	<3	2.1	4	6	(5.4)
	Temperature of seawater (℃)	24.7	23.7	28.3	23.7	20	14.8	22.5±4.6
	Salinity of seawater (%)	2.4	2.5	2.9	2.9	3	2.8	2.8±0.2
	pH of seawater	9.1	9	8.9	8.6	8.8	8.7	8.9±0.2
J (The estuary of Ihara river)	Number of *V. vulnificus* in seawater (MPN/100 ml)	9.2	9.1	3.6	7.3	<3	6	(5.9)
	Temperature of seawater (℃)	24.7	23.3	27.8	23.8	19	15.5	22.4±4.4
	Salinity of seawater (%)	1.2	2.5	2.2	1.2	2.3	2.8	2.0±0.6
	pH of seawater	9.1	9.1	8.9	9	9.2	9	9.1±0.1

SD: Standard deviation, AV: Average value of MPN

こと，カキではプランクトンが消化管内に濃縮されること，および泥では菌が付着したプランクトンなどが堆積している量が多いことが考えられる．また，これらの有機物を利用して腸炎ビブリオと同様に泥の中で菌が増殖[15]することも考えられる．したがって，今後は有機物が多い海泥，川泥，カキならびに底生動物などを採取し，それらが *V. vulnificus* の増殖の場になることを明らかにする必要があると考えられる．

V. vulnificus の分離培地に用いたCV培地では，本菌が強く疑われる鮮やかな青色集落として釣菌されたものが349株あった．このうちTSI培地による性状試験およびNB培地による食塩耐性試験で *V. vulnificus* の性状と一致した株は，181株(51.8%)みられ，そのうちPCRで *vvh* 遺伝子の保有を確認できたのが73株(40.3%)であった．*V. vulnificus* は，増菌培養の温度や使用する選択分離培地の違いによって，検出率に影響を及ぼすことが工藤らによって報告[5]されており，今後はこれらの方法を考慮した調査が必要であろう．

今回の調査において，海水温が26℃以上あった9月のA(真崎海水浴場)，F(巴川河口)，I(島崎町149番地海岸)の海水およびH(港町1丁目海岸)のカキでの菌量が少なかったが，その原因を明らかにすることはできなかった．しかし，静岡地方気象台は，検体採取日を含め，9月11日から4日間に渡り，清水地区に毎日降雨があったことを公表しており，それらが影響したのかもしれない．さらに分離培地によっては *V. vulnificus* を疑う集落の釣菌に個人差を生ずる恐れも考えられることから，今後はSDS-polymixin B sucrose agar[9]やCellobiose-polymixin B-collistin agar[8, 11]など有効性が認められている選択分離培地[4]を併用し，データのばらつきの原因を検証したいと考えている．

今回の調査で清水港湾内における *V. vulnificus* の汚染実態が明らかにされた．しかし，調査期間が短かったこと，および海水温が高いにもかかわらず *V. vulnificus* の検出率が低くデータにばらつきが見られたことなど，季節的な変動については解明できなかった．今後は年間を通して降雨と菌数の関係および増菌方法や分離培地を検討し，清水港湾内の *V. vulnificus* の消長を明らかにしたいと考えている．

文　献

1) 福島　博：島根県沿岸における *Vibrio vulnificus* の分布および市販魚介類の *V. vulnificus* 汚染状況．感染症学雑誌，80, 220-229 (2006).

2) Hill, W. E., Keasler, S. P., Trucksess, M. W., Feng, P., Kaysner, C. A. and Lampel, K. L.: Polymerase chain reaction identification of *Vibrio vulnificus* in artificially contamination oysters. Appl. Environ. Microbiol., **57**, 707-711 (1991).

3) 小野友道，井上雄二，横山真為子，栄　仁子，後藤和枝，河津俊彦，平野芳久，南　龍一，松井珠乃，小松崎　眞，大山卓昭，岡部信彦：熊本県で発生した *Vibrio vulnificus* 感染症の集積．病原体検出情報，22, 9 (2001).

4) Kitaura, T., Doke, S., Azuma, I., Imaida, M., Miyano, K., Harada, K. and Yabuuchi, E.: Halo production by sulfatase activity in *Vibrio vulnificus* and *Vibrio cholerae* O1 on a new selective sodium dodecyl sulfatecontining medium: a screening market in environmental surveillance. FEMS Microbiol. Lett., **17**, 205-209 (1983).

5) 工藤由起子：ビブリオ・バルニフィカスの検査法の検討と汚染実態，平成17年度厚生労働科学研究費補助金食品の安心・安全確保推進研究事業「細菌性食中毒の予防に関する研究」報告書，105-111 (2006).

6) 宮坂次郎，八尋俊輔，荒平雄二，濱洲大輔，甲木和子，徳永春樹：熊本県内の *Vibrio vulnificus* の環境分布と *Vibrio vulnificus* 感染症発生状況 (2001〜2004)，熊本県保健環境科学研究所報，34, 37-43 (2004).

7) 大澤　真，橘　洋正，有田真知子，橋本　徹，石田　直，本郷敏治，藤井寛之：*Vibrio vulnificus* による敗血症の1部検例．感染症学雑誌，76, 63-66 (2002).

8) Oliver, J. D., Guthric, K., Preyer, J., Wright, A., Simson, L. M., Siebeling, R. and Morris, Jr., J. G.: Use of colistin-polymyxin B-cellociose agar in the isolation of *Vibrio vulnificus* from the enviroment. Appl. Environ. Microbiol., **58**, 737-739 (1992).

9) 齋藤紀行，山田わか，渡邉　節，小林妙子，川野みち，田村広子，三品道子，菅原直子，佐藤由美，畠山　敬，谷津壽郎，秋山和夫，川向和雄：宮城県内の海水及び市販貝類からのビブリオ・バルニフィカスの検出．宮城県保健環境センター年報，第23号 (2005).

10) 篠田純夫：ビブリオ・バルニフィカスについて．日食微誌，17, 155-161 (2000).

11) Sun, Y. and Oliver, J. D.: The value of CPC agar for the isolation of *Vibrio vulnificus* from oyster. J. Food Prot., 58, 441-442 (1995).

12) 山本茂貴：ビブリオ・バルニフィカスによる重篤な感染症について．食品衛生研究，56, 25-28 (2006).

13) Kaneko, T. and Colwell, R. R.: Ecology of *Vibrio parahaemolyticus* in Chesapeake Bay. J. Bacteriol., **113**, 24-34 (1973).

14) Kaneko, T. and Colwell, R. R.: Incidence of *Vibrio parahaemolyticus* in Chesapeake Bay. Appl. Microbiol., **30**, 251-257 (1975).

15) Kumazawa, N. H. and Kato, E.: Survival of Kanagawa positive strains of *Vibrio parahaemolyticus* in a brackish-water area. J. Hyg. Camb., 299-307 (1985).

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Efficiency of real-time polymerase chain reaction assay to detect *Vibrio vulnificus* in seawater

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Abstract

The growth of *Vibrio vulnificus* in an enriched culture of seawater during the summer in Japan was monitored by a plating technique used as the culture method and a real-time polymerase chain reaction (PCR) assay as the molecular method. *V. vulnificus* was detected by the real-time PCR assay in the samples of August and September but not by the culture method. *Vibrio parahaemolyticus*, however, was detected among all of the samples with both the culture method and real-time PCR assay. In the analysis of the bacterial populations in enrichment culture, it was demonstrated that the growth of *V. vulnificus* on agar media was inhibited by the rapid growth of *V. parahaemolyticus* after 4 h of incubation and the 100 times larger initial populations of bacteria other than *V. vulnificus* and *V. parahaemolyticus*. These findings demonstrate that *V. vulnificus* detection by culture methods is a failure, and molecular methods are effective and detect *V. vulnificus* accurately.
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Keywords: *Vibrio vulnificus*; Real-time PCR; Detection

Introduction

Vibrio vulnificus is widely distributed in coastal and estuarine waters throughout the world and infections are reported in many countries (DePaola et al., 1994; Oliver et al., 1983; Hoi et al., 1998). Seafood was found to be related to approximately 40 fatal cases of *V. vulnificus* per year in the United States (Hlady and Klontz, 1996). In a recent surveillance of emergency medicine physicians in Japan, 425 cases of *V. vulnificus* sepsis were estimated per year (Osaka et al., 2004). Oishi et al. (2006) studied the epidemiological and clinical characteristics of *V. vulnificus* infections reported in Japan from 1975 to 2005. They identified 185 cases using medical article search engines. Approximately 40% of the cases were reported in four prefectures around the Ariake Sea in Kyushu, which is in the southern part of Japan. Therefore, we have been trying to detect *V. vulnificus* from the seawater by culture methods and real-time polymerase chain reaction (PCR) assay. However, we noticed that *V. vulnificus* was not detected in seawater using a technique of enrichment followed by

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culture methods, but was detected by real-time PCR assay. Subsequently we have monitored the growth during enrichment by culture methods, but by a real-time PCR assay.

In this study, we sorted the bacteria in seawater samples into three classifications, *V. vulnificus*, *Vibrio parahaemolyticus* and other bacteria, and enumerated the bacteria numbers by using a culture method and real-time PCR assay (Lyon, 2000).

To detect or identify *Vibrio* species, several genes that reflect the phylogenetic relationship, such as the ToxR gene (*toxR*), are commonly used. The *toxR*, which codes for a trans-membrane DNA-binding regulatory protein, is present on the ancestral chromosome (Provenzano et al., 2000). However, a partial sequence of *toxR* is different among *Vibrio* species. The different sequence of *toxR* for each *Vibrio* species has been used as an effective marker for the identification of *V. parahaemolyticus* (Kim et al., 1999) and *V. vulnificus* (Takahashi et al., 2005a, b). In this study, real-time PCR assays for *V. vulnificus* (Takahashi et al., 2005a, b) and *V. parahaemolyticus* (Takahashi et al., 2005a, b) were used.

Because 9–15% of the *V. vulnificus* strains can ferment sucrose and form yellow colonies (Tamplin et al., 1982; Cerdà-Cuéllar et al., 2001) and the rest do not ferment sucrose but rather form green colonies on thiosulfate citrate bile salt agar (TCBS) medium, it is impossible to distinguish *V. vulnificus*. Recently, chromogenic agar media such as CHROMagar Vibrio (CV; CHROMagar, Paris, France) (Hara-Kudo et al., 2001) medium has been utilized for isolating *V. parahaemolyticus*. This medium is also able to distinguish *V. vulnificus* from other bacteria by forming a different colony color. In an elementary study using CV, TCBS and modified cellobiose-polymyxin B-colistin agar media for isolation of *V. vulnificus* from seafood, CV medium was better than the other media. Thus we used it to isolate *V. vulnificus* in this study.

Materials and methods

Bacterial strains

V. vulnificus (VV16; from short-neck clam) and *V. parahaemolyticus* (VP15; from scallop) were used in this study. The strains were incubated in APW at 35 °C for 8 h to use for quantification in TaqMan PCR.

Seawater samples

A total of eight seawater samples were obtained from Kumamoto and Shizuoka prefectures from July to September 2006 (Table 1). They were packed in polyethylene bottles, kept in a styrene foam box, and transferred to our laboratory at a room temperature. Culture was started within 2 days.

Culture

A portion (10 ml) of seawater sample was added to 90 ml of APW pre-warmed at 35 °C, and incubated at 35 °C for 0, 2, 4, 6, 8, 10, 14, 18 and 24 h. To quantify the number of bacteria of each time, the culture fluid was 10-fold diluted with phosphate buffered saline (PBS; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 2% NaCl. Each dilution (0.1 ml) was plated onto CV agar. The plate was incubated at 35 °C for 22 h. Blue and purple colonies were suspected as *V. vulnificus* and *V. parahaemolyticus*, respectively. To identify each of the bacteria species, the PCR assay targeting for *toxR*, was carried out described below. Blue colonies and purple colonies on the CV agar plate were inoculated into triple sugar iron agar medium (OXOID Ltd., Basingstoke,Hampshire, UK), nutrient broth (Becton Dickinson, Sparks, MD), and nutrient broth supplemented with 3% and 8% NaCl.

Table 1. Seawater samples used in this study

Sample no.	Sampling date	Water temperature (°C)	Salt concentration (‰)	Detection of the *V. vulnificus* growth	
				Culture method	Real-time PCR assay
1	28 Jul.	30.0	13	+	+
2	4 Aug.	25.2	28	−	+
3	26 Aug.	26.4	12	−	+
4	30 Aug.	29.5	22	−	+
5	5 Sep.	28.0	25	−	+
6	12 Sep.	26.5	26	−	+
7	19 Sep.	25.5	25	−	+
8	26 Sep.	24.5	28	−	+

DNA extraction for TaqMan PCR assay

Enrichment culture of seawater and bacterial culture were centrifuged at 10,000*g* for 10 min. After the supernatant was removed, the pellet was re-suspended in 0.1 ml of 50 mM NaOH and heated at 100 °C for 10 min. The solution was neutralized by adding 16 μl of 1 M Tris–HCl pH 7.0 and then centrifuged at 10,000*g* for 10 min and the supernatant was transferred to a new tube and stored at −20 °C until the TaqMan PCR assay.

TaqMan PCR

To quantify the number of *V. vulnificus* and *V. parahaemolyticus* by TaqMan PCR assay, 8 h enrichment culture of *V. vulnificus* (strain no. VV16) and *V. parahaemolyticus* (strain no. VP15) were serially 10-fold diluted in APW. The DNA was extracted as described above. TaqMan PCR targeting of *toxR* of *V. vulnificus* and *V. parahaemolyticus* was performed by the methods of Takahashi et al. (2005a, b). To determine the viable cell counts of the overnight culture, 0.1 ml of the 10^{-4}–10^{-7} dilutions of the culture was plated onto tryptone soya agar (TSA; OXOID Ltd., Basingstoke, Hampshire, UK) containing 2% NaCl in duplicate. After incubation for overnight at 35 °C, the number of colonies was counted.

Results and discussion

A total of eight seawater samples obtained from July to September 2006 in Japan (Table 1) were monitored for the bacterial growth. The growth of *V. parahaemolyticus* detected by the culture method and the real-time PCR assay was similar in all of the samples (Fig. 1a). *V. parahaemolyticus* linearly grew to 14 h and stabilized thereafter. However, the growth of *V. vulnificus* was different in the different detection methods. In the sample of July 28, the population of *V. vulnificus* rapidly increased from 4 to 18 h. However, at 24 h, it was below the level detectable by the culture method (Fig. 1b), although *V. vulnificus* was detected by the real-time PCR assay. *V. vulnificus* might be dead by 24 h. In the samples of August and September, the growth of *V. vulnificus* was almost not detected in any of the samples by the culture method (Fig. 1c). However, by the real-time

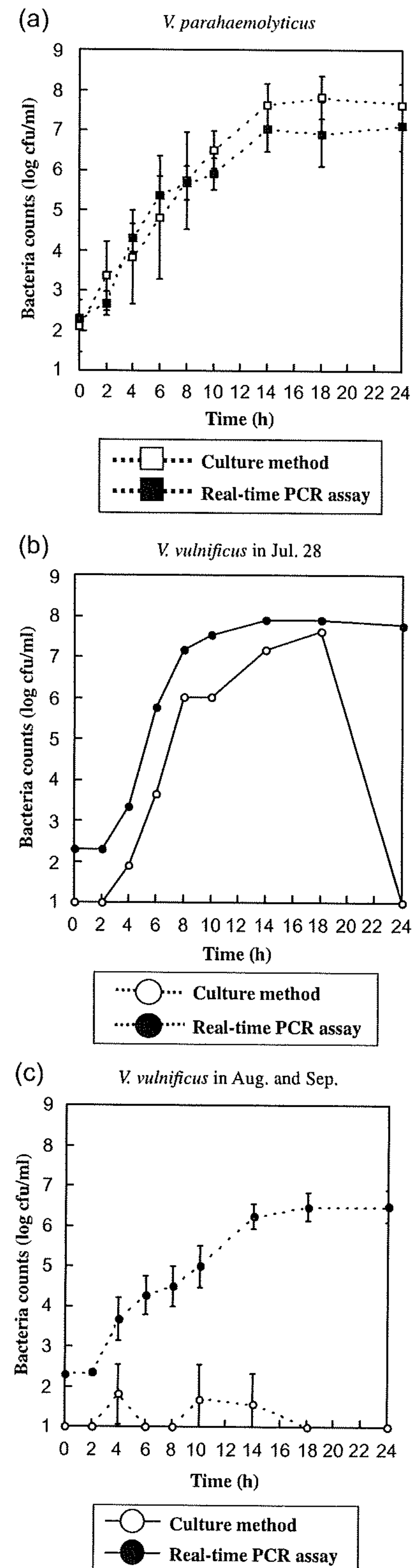

Fig. 1. Detection of *V. parahaemolyticus* and *V. vulnificus* by a culture method and real-time PCR assay. (a) *V. parahaemolyticus*: the mean of all of the samples in this study. (b) *V. vulnificus*: the sample of July 28. (c) *V. vulnificus*: the mean of the samples of August and September. Undetectable levels of the culture method and the real-time PCR assay were 1.0 and 2.3, respectively.

PCR assay, an increase of *V. vulnificus* population was detected from 4 to 24 h. The growth of *V. vulnificus* in the samples of August and September was weaker than that of July 28. To determine the reasons why the growth of *V. vulnificus* was not detected by the culture method in the samples of August and September, we more closely analyzed the monitored population of *V. vulnificus*, *V. parahaemolyticus* and the other bacteria by the real-time PCR assay.

The populations of *V. vulnificus* (Fig. 2a), *V. parahaemolyticus* (Fig. 2b) and the other bacteria (Fig. 2c) on July 28 were compared to those in August and September. In *V. vulnificus* (Fig. 2a), the values of the samples of August and September were lower after 4 h than those of the sample of July 28. In *V. parahaemolyticus* and the other bacteria, the populations had an increase in the samples of July 28, and also of August and September (Fig. 2b and c). The initial population of the other bacteria in the sample of July 28 was different from the mean of the samples of August and September (Fig. 2c), although the value was similar after 8 h of incubation. The populations of *V. vulnificus* in the sample of July 28 were quite different from the mean of the samples of August and September, although the populations were similar before 6 h.

Therefore, the details of *V. vulnificus*, *V. parahaemolyticus* and the other bacteria until 6 h of incubation were analyzed (Fig. 2d). The populations of *V. vulnificus*, *V. parahaemolyticus* and the other bacteria in the sample of July 28 similarly increased during the 6-h incubation. However, in the samples of August and September, there was a significant difference among the

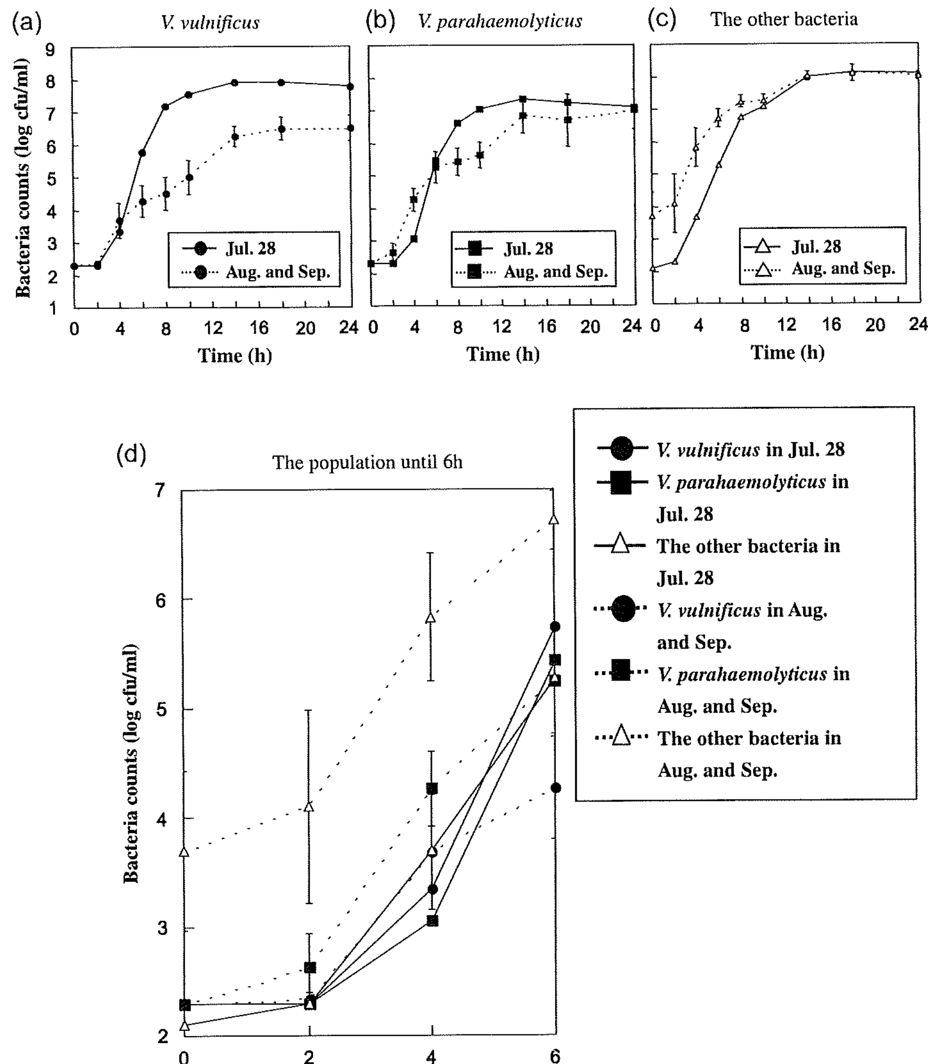

Fig. 2. Detection of *V. vulnificus*, *V. parahaemolyticus* and the other bacteria. (a) Quantification of the number of *V. vulnificus* using real-time PCR assay. (b) Quantification of the number of *V. parahaemolyticus* using real-time PCR assay. (c) Quantification of the number of the other bacteria using the culture method. (d) The numbers of *V. vulnificus*, *V. parahaemolyticus* and the other bacteria.

populations of *V. vulnificus*, *V. parahaemolyticus* and the other bacteria. We considered the reason that the growth of *V. vulnificus* in the samples of August and September was not properly monitored by the plating method and the growth was therefore found to be weaker than that of July 28. The mean of the population of *V. vulnificus* in the samples of August and September was similar compared with the samples of July 28 until 4 h, but the population on July 28 was more than 10 times larger than) the mean of the samples in August and September at 6 h. Until 4 h, *V. parahaemolyticus* in the samples of August and September grew more than 10-fold higher than in the sample of July 28. The initial population of the other bacteria in the samples of August and September was approximately 100 times higher than that of July 28.

In the sample of July 28, *V. vulnificus* was not detected at 24 h by the plating method. As a reason, it was considered whether the bacteria was decreased or had been eradicated. By the real-time PCR assay, the population of the bacteria did not change from 18 to 24 h. Therefore it is possible that the DNA of dead bacteria may also be extracted and quantified by the real-time PCR assay. In order to enumerate only live cells, RT-real-time PCR assay may be effective, although more extraction environments to prepare the assay are required.

APW has been successfully used as an enrichment medium for pathogenic vibrios including *V. vulnificus* (Donovan and Netten 1995), although the selectability is low. Therefore, during *V. vulnificus* growth in APW, the other bacteria also grow. The rapid growth of the other bacteria might be inhibitory towards *V. vulnificus* growth. It will be necessary to develop new selective enrichment media for *V. vulnificus* to inhibit the growth of the other bacteria.

In most of samples except for July 28, the water temperature was lower than 30 °C and salt concentration was higher than 22‰. Because *V. vulnificus* is very sensitive to temperature and salt concentration (Kelly, 1982, Tilton and Ryan, 1987), it is possible that the pathogen in most of samples except July 28 was dead. However, the growth of *V. vulnificus* in the all samples of this study was detected by the monitoring using a real-time PCR assay (Fig. 1). It indicates that the real-time PCR assay detected viable *V. vulnificus* but not DNA fragment from dead cells in all the samples.

In the present study, it is demonstrated that culture methods, including enrichment in APW and plating onto agar medium, are appropriate for detecting *V. parahaemolyticus* but not *V. vulnificus* in environmental samples. Molecular methods for detecting *V. vulnificus* are available to confirm the presence in a sample, although dead cells may also be detected. In addition, it is necessary to develop more effective selective culture methods utilizing enrichment and plating so as to better isolate *V. vulnificus*.

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References

Cerdà-Cuéllar, M., Permin, L., Larsen, J.L., Blanch, A.R., 2001. Comparison of selective media for the detection of *Vibrio vulnificus* in environmental samples. J. Appl. Microbiol. 91, 322–327.

DePaola, A., Capers, G.M., Alexander, D., 1994. Densities of *Vibrio vulnificus* in postharvest Gulf Coast shellstock oysters. Appl. Environ. Microbiol. 60, 984–988.

Donovan, T.J., Netten, P., 1995. Culture media for the isolation and enumeration of pathogenic *Vibrio* species in foods and environmental samples. Int. J. Food Microbiol. 26, 77–91.

Hara-Kudo, Y., Nishina, T., Nakagawa, H., Konuma, H., Hasegawa, J., Kumagai, S., 2001. Improved method for detection of *Vibrio parahaemolyticus* in seafood. Appl. Environ. Microbiol. 67, 5819–5823.

Hlady, W.G., Klontz, K.C., 1996. The epidemiology of Vibrio infections in Florida, 1981–1993. J. Infect. Dis. 173, 1176–1183.

Hoi, L., Larsen, J.L., Dalsgaard, I., Dalsgaard, A., 1998. Occurrence of *Vibrio vulnificus* biotypes in Danish marine environments. Appl. Environ. Microbiol. 64, 7–13.

Kelly, M.T., 1982. Effect of temperature and salinity on *Vibrio (Beneckea) vulnificus* occurrence in a Gulf coast environment. Appl. Environ. Microbiol. 44, 820–824.

Kim, Y.B., Okuda, J., Matsumoto, C., Takahashi, N., Hashimoto, S., Nishibuchi, M., 1999. Identification of *Vibrio parahaemolyticus* strains at the species level by PCR targeted to the *toxR* gene. J. Clin. Microbiol. 37, 1173–1177.

Lyon, W.J., 2000. TaqMan PCR for detection of *Vibrio cholerae* O1, O139, non-O1, and non-O139 in pure cultures, raw oysters, and synthetic seawater. Appl. Environ. Microbiol. 67, 4685–4693.

Oishi, H., Ura, Y., Mitsumizo, S., Nakashima, M., 2006. A collective review of *Vibrio vulnificus* infection in Japan. J. Jpn. Assoc. Infect. Dis. 80, 680–689.

Oliver, J.D., Warner, R.A., Cleland, D.R., 1983. Distribution of *Vibrio vulnificus* and other lactose-fermenting vibrios in the marine environment. Appl. Environ. Microbiol. 45, 985–998.

Osaka, K., Komatsuzaki, M., Takahashi, H., Sakano, S., Okabe, N., 2004. *Vibrio vulnificus* septicaemia in Japan: an estimated number of infection and physicians' knowledge of the syndrome. Epidemiol. Infect. 132, 993–996.

Provenzano, D., Schuhmacher, D.A., Barker, J.L., Klose, K.E., 2000. The virulence regulatory protein ToxR mediates enhanced bile resistance in *Vibrio cholerae* and

other pathogenic *Vibrio* species. Infect. Immun. 68, 1491–1497.

Takahashi, H., Hara-Kudo, Y., Miyasaka, J., Kumagai, S., Konuma, H., 2005a. Developement of a quantitative real-time polymerase chain reaction targeted to the *toxR* for detection of *Vibrio vulnificus*. J. Microbiol. Methods 61, 77–85.

Takahashi, H., Iwade, Y., Konuma, H., Hara-Kudo, Y., 2005b. Development of a quantitative real-time PCR assay for estimation of the total number of *Vibrio parahaemolyticus* in contaminated shellfish and seawater. J. Food Prot. 68, 1083–1088.

Tamplin, M., Rodrick, G.E., Blake, N.J., Cuba, T., 1982. Isolation and characterization of *Vibrio vulnificus* from two Florida estuaries. Appl. Environ. Microbiol. 44, 1466–1470.

Tilton, R.C., Ryan, R.W., 1987. Clinical and ecological characteristics of *Vibrio vulnificus* in the Northeastern United States. Diagn. Microbiol. Infect. Dis. 6, 109–117.

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Rapid and Specific Detection of the Thermostable Direct Hemolysin Gene in *Vibrio parahaemolyticus* by Loop-Mediated Isothermal Amplification

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ABSTRACT

Several investigators have reported that thermostable direct hemolysin (TDH) and TDH-related hemolysin are important virulence factors of *Vibrio parahaemolyticus*, but it has been difficult to detect these factors rapidly in seafood and other environmental samples. A novel nucleic acid amplification method, termed the loop-mediated isothermal amplification (LAMP), which amplifies DNA with high specificity and rapidity under isothermal conditions, was applied. In this study, we designed *tdh* gene-specific LAMP primers for detection of TDH-producing *V. parahaemolyticus*. The specificity of this assay was evaluated with 32 strains of TDH-producing *V. parahaemolyticus*, one strain of TDH-producing *Grimontia hollisae*, 10 strains of TDH-nonproducing *V. parahaemolyticus*, and 94 strains of TDH-nonproducing bacteria, and the sensitivity was high enough to detect one cell per test. Moreover, to investigate the detection of TDH-producing *V. parahaemolyticus* in oysters, the LAMP assay was performed with enrichment culture in alkaline peptone water of oyster samples inoculated with TDH-producing *V. parahaemolyticus* and TDH-nonproducing *V. parahaemolyticus* and *V. alginolyticus* after enrichment in alkaline peptone water. These results suggest that the LAMP assay targeting *tdh* gene has high sensitivity and specificity and is useful to detect TDH-producing *V. parahaemolyticus* in oyster after enrichment.

Vibrio parahaemolyticus is a pathogen that causes foodborne infections associated with the consumption of fish or shellfish worldwide *(4, 9, 10, 22, 23)*. Most clinical strains of *V. parahaemolyticus* produce thermostable direct hemolysin (TDH) or TDH-related hemolysin. It is reported that these hemolysin variants are important virulence factors in pathogenic *V. parahaemolyticus (14, 16)*. Since TDH and/or TDH-related-hemolysin–producing *V. parahaemolyticus* organisms are present in seafood and the environment at low concentrations and in a small portion of microorganisms' flora, it is difficult to detect them in seafood or environmental samples *(11, 17)*. Testing for the Kanagawa phenomenon caused by TDH on Wagatsuma blood agar medium is cumbersome to carry out *(3, 20, 21)*. Although an enzyme-linked immunosorbent assay for detecting TDH has been reported *(6)*, the compositions of food or clinical samples inhibit the interaction between antigens and antibodies. Using a combination of enrichment and PCR assay, 8 h was required for the detection of pathogenic *V. parahaemolyticus* that had been artificially inoculated in UV-treated oysters *(1, 2)*. In 2000, Notomi et al. *(19)* reported a novel nucleic acid amplification method, termed the loop-mediated isothermal amplification of DNA (LAMP). Several researchers have reported that the LAMP assay is a more sensitive and rapid method than PCR assay *(5, 7)*. Furthermore, this assay has several advantages over PCR, nested PCR, and real-time PCR assays, as follows. The LAMP reaction is done under isothermal conditions between 60 and 65°C *(8, 13, 19)* using a simple incubator such as a water bath or block heater, which is sufficient for amplification. The reaction is highly specific for the target sequence because the primers recognize six distinct regions on the targeted DNA. The LAMP assay is suitable for detection of target genes both in laboratories and in field situations because LAMP produces a large amount of pyrophosphate ion as a by-product that is utilized to create a turbidity in the reaction mixture visible to the naked eye *(12)*. Finally, the reaction procedure is more economical and practical than both PCR and real-time PCR assays.

One of the notable characteristics of *V. parahaemolyticus* is rapid growth under optimum conditions. After a 3-h incubation, the cell numbers rise to approximately 10^6 times the original number. We noted that the number of *V. parahaemolyticus* rises over the detection limit with LAMP assays after a few hours of enrichment in alkaline peptone water. In this study, we designed a set of primers for detecting the *tdh* gene. This is the first report demonstrating the usefulness of the LAMP assay to detect TDH-producing *V. parahaemolyticus*. Detection of TDH-producing *V. parahaemolyticus* in oysters was investigated in combinations of enrichment procedures and LAMP assay in this study.

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MATERIALS AND METHODS

Bacterial strains and culture. The strains used in this study are listed in Table 1. In *V. parahaemolyticus,* 30 strains (1 strain each of serotypes OUT:K5, OUT:K29, OUT:K57, O1:KUT, O1:K1, O1:K25, O1:K56, O4:K12, O4:K13, O5:K68; 2 strains of serotypes O4:K9 and O4:K68; 3 strains of O4:K8; 13 strains of O3:K6) from clinical specimens; 10 strains (1 strain each of serotypes OUT:K22, OUT:K32, O4:K49, O4:K64, O10:K24, OUT:KUT; 4 strains of serotype O3:K6) from food samples; and 2 strains of serotype O3:K6 from the environment were used. One strain of *Grimontia hollisae* (ATCC 33564) was purchased from American Type Culture Collection (Rockville, MD). The strains of *Vibrio* spp. and *G. hollisae* were grown on heart infusion agar medium (Eiken Chemical Co. Ltd., Tokyo, Japan) with 2.0% (wt/vol) NaCl at 37°C for 18 h. The other bacteria were grown on 5% sheep blood agar medium at 37°C for 18 h. All strains were tested for the *tdh* gene by *tdh*-PCR assay *(21).* Forty-two strains of *V. parahaemolyticus* and *G. hollisae* were tested for TDH production by reverse passive latex agglutination (RPLA) assay with a kit (KAP-RPLA; Denka Seiken, Tokyo, Japan). To observe the Kanagawa phenomenon, 40 strains of *V. parahaemolyticus* and *G. hollisae* (Table 1) were tested for hemolytic activity by Wagatsuma blood agar medium *(3).*

Design of primer for LAMP. The LAMP primers were designed based on the published *tdh* sequences in *V. parahaemolyticus* (GenBank accession nos. AP005088, AY044107, AY044108, AY044109, AY044110, AY044111, AY044112, AY044113, AY044114, AY249144, D90100, D90101, D90238, M10069, S67841, S76724, X54340, X54341, X54342, and X54343), *G. hollisae* (GenBank accession no. M57900), *Vibrio mimicus* (GenBank accession no. M64120), and *Vibrio cholerae* non-O1 (GenBank accession no. M55316) (Fig. 1A). The forward inner primer (FIP) consisted of the complementary sequence of F1 (F1c) and F2 (Fig. 1B): 5′-CTTATAGCCAGACACCGCTGCGG-TTGACATCCTACATGACTGTG-3′. The backward inner primer (BIP) consisted of B1c, T linker and the B2: 5′-CGGTCA-TTCTGCTGTGTTCGTTCTTCACCAACAAAGTTAGCTAC-AG-3′. The forward outer primer (F3) and the backward outer primer (B3) were 5′-GTCTCTGACTTTTGGACAAACCG-3′ and 5′-CTACATTAACAAAATATTCTGGAGTTTCATCC-3′. The forward loop primer (FLP) and the backward loop primer (BLP) were 5′-CCGCTGCCATTGTATAGTCTTT-3′ and 5′-CAGATCAAGTACAACTTCAACATTCCT-3′.

LAMP assay. The LAMP assay was carried out in a total of 25 μl of reaction mixture containing 40 pmol of FIP, BIP, FLP, and BLP, 10 pmol of F3 and B3, 12.5 μl of 2× Universal LAMP reaction mixture (Eiken Chemical Co. Ltd.), 1 μl of *Bst* DNA polymerase, and 5 μl of template DNA. The mixture was mixed gently, and this was incubated at 65°C. The result of the reaction was evaluated at 60 min of incubation. The LAMP reaction synthesized an extremely large amount of DNA, and white precipitation appeared as a by-product *(12).* The increase of turbidity in the reaction mixture correlated with the amount of DNA amplification and was monitored with an LA-320C real-time turbidimeter for LAMP (Eiken Chemical Co. Ltd.).

PCR assay. The PCR assay for the detection of *tdh* gene was performed with primer 1 (5′-GGTACTAAATGGCTGACA-TC-3′) and primer 2 (5′-CCACTACCACTCTCATATGC-3′) as described by Tada et al. *(21).* The reaction was carried out in a total of 50 μl of reaction mixture containing 9.5 pmol each of the primers, 5 μl of 10× ExTaq-Buffer, 0.2 μM each of the four deoxynucleoside triphosphates, 2.5 U of Ex *Taq* polymerase (Takara Ex Taq, Takara, Ohtsu, Japan), and 5 μl of template solution. The amplification conditions were set at a first cycle of 96°C for 5 min, followed by 35 cycles of amplification consisting of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, followed by one cycle of 72°C for 7 min. All of the PCR products were confirmed by electrophoresis of 2% agarose gel under 1× Tris-borate-EDTA buffer to recognize the specific amplicon (251 bp).

Specificity of the LAMP and PCR assays. All of the strains listed in Table 1 were suspended in 2.5 % NaCl for the strains of *Vibrio* spp. and *G. hollisae* or 0.85 % NaCl for the other strains at a concentration of McFarland turbidity standard of 1, which is approximately 3.0×10^8 CFU/ml. These suspensions were diluted to approximately 1.2×10^4 CFU/ml in Tris-EDTA (TE) buffer. Then, each dilution was denatured at 95°C for 5 min and centrifuged at $2,000 \times g$ for 1 min. Five microliters of each supernatant was added in independent reaction tubes as a LAMP template and a PCR template. The results of the LAMP assay were compared with those of the PCR assay.

Sensitivity of LAMP and PCR assays. The five strains of TDH-producing *V. parahaemolyticus,* i.e., EKN5285 (serotype O3:K6 from sediment), EKN5301 (O4:K12 from a clinical specimen), EKN5302 (O4:K68 from a clinical specimen), EKN5731 (O3:K6 from a clinical specimen), and EKN5768 (O1:K1 from a clinical specimen), were tested for sensitivity in the LAMP and PCR assays. They were grown on heart infusion agar medium with 2.0% (wt/vol) NaCl at 37°C for 18 h. Colonies were suspended at a concentration of McFarland turbidity standard of 1 in 10 mM phosphate buffer (PB; pH 7.5) with 3% NaCl (3% NaCl-PB) and then serially 10-fold diluted to 10^{-4} to 10^{-7} in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and 3% NaCl-PB. Each of the TE buffer–diluted series was denatured at 95°C for 5 min and centrifuged at $2,000 \times g$ for 1 min. The supernatant was used as the template solution for LAMP and PCR assays in duplicate. In the 3% NaCl-PB diluted series, 100 μl of each dilution was plated onto heart infusion agar medium with 2% NaCl in duplicate. After incubation at 37°C for 18 h, the number of colonies was counted.

Detection of TDH-producing *V. parahaemolyticus* in artificially contaminated oyster samples. Fresh Japanese oysters (*Crassostrea gigas*) packed in polyethylene bags had been purchased from a retail market. These were transferred to our laboratories within 1 h and kept at 4°C for less than 6 h until they were used in the experiments. Approximately 100 g of Japanese oysters was homogenized by a blender in an ice bath for 1 min. The aerobic bacteria cells were enumerated by the spread plate technique on heart infusion agar medium with 2% NaCl. The homogenate was confirmed to be noncontaminated with TDH-producing *V. parahaemolyticus* by the LAMP and PCR assays described above. To each 25-g homogenized sample in stomach filter bags (Eiken Chemical Co. Ltd.) was added 225 ml of alkaline peptone water (pH 8.5). The colonies, grown on heart infusion agar with 2.0% (wt/vol) NaCl, of each TDH-producing *V. parahaemolyticus* (EKN5285) isolated from sediment, TDH-nonproducing *V. parahaemolyticus* (EKN5204) from a food sample, and *V. alginolyticus* (EKN5179) from a food sample were suspended in 2.5% NaCl at a concentration of McFarland turbidity standard of 1, which is approximately 3.0×10^8 CFU/ml. One of the homogenized oyster samples was inoculated with 0.1 ml of 10^{-6} dilutions of TDH-producing *V. parahaemolyticus,* 0.1 ml of 10^{-3} dilutions of TDH-nonproducing *V. parahaemolyticus,* and 0.1 ml of 10^{-2} dilutions of *V. alginolyticus.* The proportion of cell num-

TABLE 1. *Specificity of the LAMP assay for* tdh *gene*

			Detection of *tdh* gene or TDH[a]			
Bacterial species	Serotype	No. of strains	LAMP	PCR	RPLA	Kanagawa phenomenon
Vibrio parahaemolyticus	OUT:K5	1	–	–	–	NT
	OUT:K22	1	–	–	–	NT
	OUT:K29	1	–	–	–	–
	OUT:K32	1	–	–	–	–
	OUT:K57	1	–	–	–	–
	O1:KUT	1	–	–	–	–
	O1:K1	1	+	+	w	+
	O1:K25	1	+	+	+	+
	O1:K56	1	+	+	+	+
	O3:K6	19	+	+	+[b]	+
	O4:K9	2	+	+	+	+
	O4:K8	3	+	+	+	+
	O4:K12	1	+	+	+	+
	O4:K13	1	+	+	+	+
	O4:K49	1	–	–	–	–
	O4:K64	1	–	–	–	–
	O4:K68	2	+	+	+	+
	O10:K24	1	–	–	–	–
	O5:K68	1	+	+	+	+
	OUT:KUT	1	–	–	–	–
V. alginolyticus		3	–	–	NT	NT
V. anguillarum		1	–	–	NT	NT
V. cholerae		31	–	–	NT	NT
V. damsela		1	–	–	NT	NT
V. fluvialis		3	–	–	NT	NT
V. furnissii		1	–	–	NT	NT
V. metschnikovii		2	–	–	NT	NT
V. mimicus		5	–	–	NT	NT
V. pisoim var. *japonicus*		1	–	–	NT	NT
V. tryrogenes		1	–	–	NT	NT
V. vulnificus		2	–	–	NT	NT
Grimontia hollisae		1	+	+	–	+
Enterobacteriaceae						
Citrobacter freundii		2	–	–	NT	NT
C. koseri		1	–	–	NT	NT
Edwardsiella tarda		1	–	–	NT	NT
Enterobacter aerogenes		1	–	–	NT	NT
E. cloacae		1	–	–	NT	NT
Escherichia coli		1	–	–	NT	NT
E. fergusonii		1	–	–	NT	NT
E. hermannii		1	–	–	NT	NT
Hafnia alvei		1	–	–	NT	NT
Klebsiella oxytoca		1	–	–	NT	NT
K. pneumoniae		1	–	–	NT	NT
Morganella morganii		1	–	–	NT	NT
Proteus mirabilis		2	–	–	NT	NT
P. vulgaris		1	–	–	NT	NT
Providencia alcalifaciens		1	–	–	NT	NT
P. rettgeri		1	–	–	NT	NT
P. stuartii		1	–	–	NT	NT
Salmonella enterica		2	–	–	NT	NT
Serratia marcescens		3	–	–	NT	NT
Yersinia enterocolitica		1	–	–	NT	NT
Y. pseudotuberculosis		1	–	–	NT	NT
Pseudomonas aeruginosa		1	–	–	NT	NT
Gram-positive cocci						
Enterococcus avium		1	–	–	NT	NT
E. durans		1	–	–	NT	NT

TABLE 1. *Continued*

Bacterial species	Serotype	No. of strains	Detection of *tdh* gene or TDH[a] LAMP	PCR	RPLA	Kanagawa phenomenon
Enterococcus faecalis		2	–	–	NT	NT
Lactobacillus lactis		1	–	–	NT	NT
Micrococcus luteus		2	–	–	NT	NT
Staphylococcus aureus		1	–	–	NT	NT
S. capitis		1	–	–	NT	NT
S. epidermidis		1	–	–	NT	NT
S. haemolyticus		1	–	–	NT	NT
S. intermedius		1	–	–	NT	NT
S. warneri		1	–	–	NT	NT
S. xylosus		1	–	–	NT	NT
Streptococcus pyogenes		1	–	–	NT	NT
Listeria monocytogenes		1	–	–	NT	NT

[a] +, positive reaction; w, weak reaction; –, negative reaction; NT, not tested.
[b] Two strains exhibited weak reaction.

bers of these strains was 1:1,000:10,000, respectively. Another sample was not inoculated with *V. parahaemolyticus* or *V. alginolyticus,* to be used as a negative control. The inoculated bacterial cells were enumerated by the spread plate technique on heart infusion agar medium with 2% NaCl. The artificially contaminated and noncontaminated samples were incubated at 37°C. Following enrichment for 0, 2, 3, 4, 6, and 18 h, the culture was filtered with a stomach filter bag. One milliliter of filtered culture was transferred to a microcentrifuge tube and centrifuged at 2,000 × *g* at room temperature for 1 min. The supernatant was removed, and then the precipitate was stored at −20°C within 24 h until DNA extraction. The genomic DNA of bacteria and oyster were extracted using an EXTRAGEN II Kit (TOSOH Co., Tokyo, Japan) with a modified protocol as follows. The precipitate was resuspended in 250 μl of TE buffer, 20 μl of Reagent I of the EXTRAGEN II kit was added, and then, 1,000 μl of Reagent II was added, sequentially. The mixture was centrifuged at 12,000 × *g* at 4°C for 3 min, and the supernatant was discarded. The pellet was resuspended in 1,000 μl of Reagent II and centrifuged at 12,000 × *g* at 4°C for 3 min, and then the supernatant was discarded. The pellet was resuspended in 1,000 μl of Reagent II and centrifuged at 12,000 × *g* at 4°C for 3 min, and then the supernatant was discarded. The pellet was resuspended in 300 μl of Reagent III and centrifuged at 12,000 × *g* for at 4°C for 3 min, and the supernatant was discarded. Finally, the pellet was rinsed with 70% ethanol and dissolved in 100 μl of TE buffer. The solution including the DNA was denatured at 95°C for 5 min and centrifuged at 2,000 × *g* for 1 min. The supernatant was used as the template for the LAMP and PCR assays. These experiments were carried out in duplicate.

RESULTS

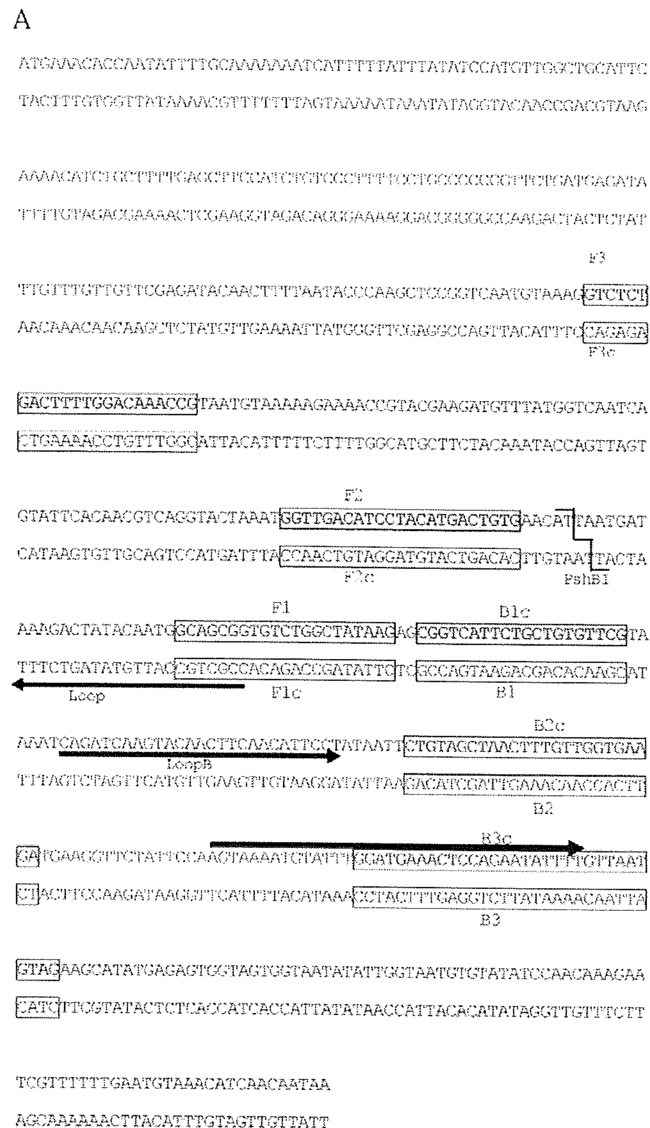
In the 32 strains of TDH-producing *V. parahaemolyticus* and one strain of *G. hollisae,* significant amplification was observed in the LAMP and PCR assays. In 10 strains of TDH-nonproducing *V. parahaemolyticus* and 94 strains of bacteria, amplifications were not observed in the LAMP and PCR assays (Table 1). In the RPLA assay, 32 strains of TDH-producing *V. parahaemolyticus* exhibited a positive reaction, and 10 strains of TDH-nonproducing *V. parahaemolyticus* and one strain of *G. hollisae* exhibited a negative reaction. There were no differences among the results for most strains in RPLA, LAMP, and PCR assays and the Kanagawa phenomenon. However, *G. hollisae* was positive for the Kanagawa phenomenon and in the LAMP and PCR assays but not in the RPLA assay.

The detection limits of the LAMP assay in five strains were determined. The sensitivity of the LAMP assay was compared with that of the PCR assay (Table 2). In both the LAMP and PCR assays, the 10^{-6} dilutions of all strains were positive and the lowest number of cells in the test tubes at this dilution was 0.9 CFU.

Detection of TDH-producing *V. parahaemolyticus* in oysters by the LAMP assay was investigated with oysters artificially contaminated with TDH-producing *V. parahaemolyticus* (1.2 CFU/g), TDH-nonproducing *V. parahaemolyticus* (8.0×10^2 CFU/g) and *V. alginolyticus* (2.4×10^4 CFU/g). The number of aerobic bacteria in oysters was 7.0×10^3 CFU/g. After incubation for 0, 2, 3, 4, 6, and 18 h at 37°C, the culture was subjected to DNA extraction. The *tdh* gene was amplified by LAMP and PCR assays in duplicate (Fig. 2). Figure 2A shows the results of turbidity measurement for the LAMP reaction for 60 min at 65°C in the artificially contaminated sample. One of the duplicated assays in the 3-h incubation sample and both of the duplicated assays in the samples of >4-h incubation resulted in significant amplification. The *tdh* gene was not amplified in the sample not artificially contaminated. The white precipitate of magnesium pyrophosphate in tubes after LAMP reaction was visible in the 4-, 6-, and 18-h incubation samples but invisible in the 0-h incubation sample (Fig. 2B). Figure 2C shows the electrophoresis of the PCR products. In the samples of 0, 2, 3, and 4 h of incubation, many nonspecific bands appeared. Specific amplicons (251 bp) were found in the samples of 6 and 18 h of incubation. Since the specific amplicons were obscure in the 6-h incubation sample, it was difficult to make a precise determination.

DISCUSSION

In this study, a set of primers was designed for detecting the *tdh* gene, and this is the first report demonstrat-



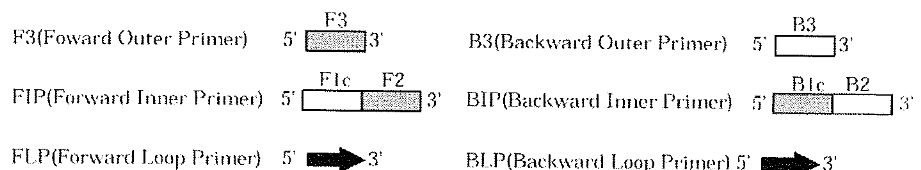


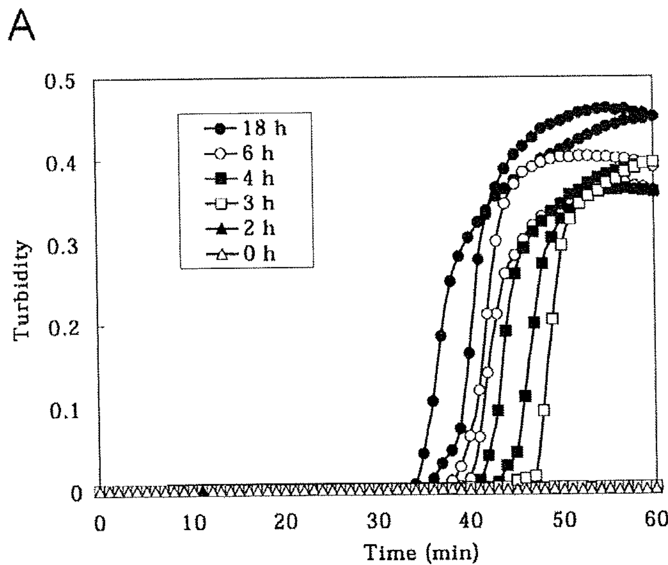
FIGURE 1. *(A) Nucleotide sequences of the* tdh *ORF (GenBank accession no. AY249144) used to design the six primers. The primer recognition regions of primers are shown by the boxes (inner and outer primers) and arrows (loop primers). F1c and B1c indicate the complementary sequences of F1 and B1, respectively. PshBI indicates the recognition site of the restriction enzyme* PshBI. *(B) Schematic diagram of the two outer (F3 and B3), two inner (FIP and BIP), and two loop (FLP and BLP) primers for LAMP.*

ing the usefulness of the LAMP assay to detect TDH-producing *V. parahaemolyticus*.

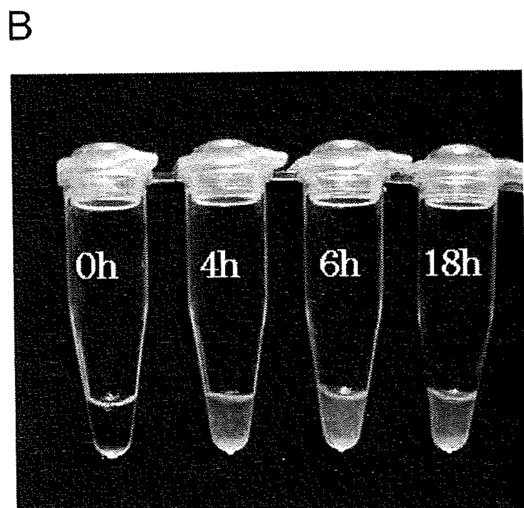
The specificity of the LAMP assay was the same as that of the PCR assay (Table 1), and the primer set designed in the LAMP assay has high specificity for detecting the *tdh* gene. Three strains of *V. parahaemolyticus* that exhibited a positive reaction in the LAMP and PCR assays had weak agglutination in the RPLA assay for TDH and were positive for Kanagawa phenomenon caused by TDH. On the other hand, one strain of *G. hollisae* that exhibited a positive reaction for the Kanagawa phenomenon was negative for the agglutination in the RPLA assay, although the results in the LAMP and PCR assays were positive. The LAMP and PCR assays might more clearly detect TDH-producing *V. parahaemolyticus* than does the RPLA assay. Tests using additional strains of TDH-producing *V. parahaemolyticus* should provide a more useful evaluation, although 32 strains were used in this study.

In the test for the sensitivity of LAMP and PCR assays, <1 CFU of TDH-producing *V. parahaemolyticus* in a test tube was detected (Table 2). The cultures of *V. parahaemolyticus* by incubation at 37°C for 18 h were used in the test. The cultures might be in stationary phase, including a high proportion of cells that do not produce CFU. However, the sensitivity was similar to the results of a test using log-phase culture by incubation for 5 h, which was performed in a preliminary study. Therefore, the test for the sensitivity of LAMP and PCR assays in this study was not affected

A



B



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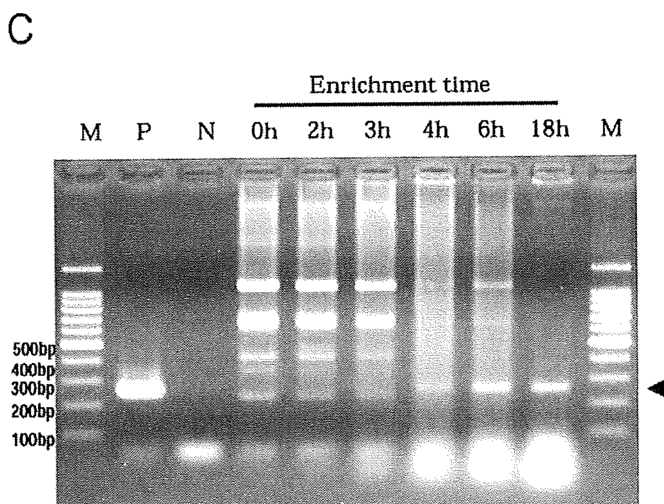

FIGURE 2. *Detection of the TDH-producing* V. parahaemolyticus *strain from artificially contaminated oyster samples. The Japanese oysters were inoculated with 1.2 CFU of TDH-producing* V. parahaemolyticus *(EKN5285) per g, 8.0 × 10^2 CFU of TDH-nonproducing* V. parahaemolyticus *(EKN5204) per g, and 2.4 × 10^4 CFU of* V. alginolyticus *(EKN5179) per g. (A) Turbidity measurements of the LAMP reaction with LA320C real-time turbidimeter for LAMP. The enrichment culture of oysters in APW (alkaline peptone water) for 2, 3, 4, 6, and 18 h were tested in the LAMP assay in duplicate. (B) Reaction tubes after LAMP assay for 60 min. (C) Agarose gel electrophoresis of the PCR products. The specific amplicons (251 bp) are indicated by arrowheads. M, 100-bp DNA ladder marker (Takara).*

TABLE 2. *Sensitivity of the LAMP and PCR assays*

Serotype of *V. parahaemolyticus*/ strain	Dilution	No. of cells in tube	Reaction[a] (no. positive/total no.) LAMP	PCR
O3:K6/EKN5285	10^{-4}	420	NT	+ (2/2)
	10^{-5}	42	+ (2/2)	+ (2/2)
	10^{-6}	4.2	+ (2/2)	+ (2/2)
	10^{-7}	0.4	+ (1/2)	− (0/2)
O4:K12/EKN5301	10^{-4}	420	+ (2/2)	+ (2/2)
	10^{-5}	42	+ (2/2)	+ (2/2)
	10^{-6}	4.2	+ (2/2)	+ (2/2)
	10^{-7}	0.4	− (0/2)	− (0/2)
O4:K68/EKN5302	10^{-4}	260	+ (2/2)	+ (2/2)
	10^{-5}	26	+ (2/2)	+ (2/2)
	10^{-6}	2.6	+ (2/2)	+ (2/2)
	10^{-7}	0.3	+ (1/2)	+ (1/2)
O3:K6/EKN5731	10^{-4}	140	+ (2/2)	+ (2/2)
	10^{-5}	14	+ (2/2)	+ (2/2)
	10^{-6}	1.4	+ (2/2)	+ (2/2)
	10^{-7}	0.1	− (0/2)	+ (2/2)
O1:K1/EKN5768	10^{-4}	90	+ (2/2)	+ (2/2)
	10^{-5}	9	+ (2/2)	+ (2/2)
	10^{-6}	0.9	+ (2/2)	+ (2/2)
	10^{-7}	0.1	+ (1/2)	+ (1/2)

[a] +, positive reaction; −, negative reaction.

by cells that do not produce CFU. Nishibuchi and Kaper *(15)* reported that duplication and variation of the *tdh* gene are encoded in a chromosome of *V. parahaemolyticus*. The LAMP and PCR assays in this study were designed to detect both *tdh1* and *tdh2* genes. Thus, detecting <1 CFU of TDH-producing *V. parahaemolyticus* should be due to the duplication and variation of *tdh* gene.

It is difficult to isolate TDH-producing *V. parahaemolyticus* from seafood because a large number of TDH-nonproducing *Vibrio* spp. were contaminated with a small number of TDH-producing *V. parahaemolyticus* organisms *(11)*. It is expected that oysters are often contaminated with high populations of halophilic bacteria such as TDH-nonproducing *V. parahaemolyticus* and *V. alginolyticus*. Therefore, we investigated the sensitive detection by LAMP assay in oyster samples inoculated with these bacteria. In this study, a period of 6 h was required to detect TDH-producing *V. parahaemolyticus* in oysters, using a combination of enrichment for 4 h, DNA extraction for 1 h, and the LAMP assay for 1 h. It is noteworthy that TDH-producing *V. parahaemolyticus* in oysters were detected within a period of only 1 day by using this assay.

One of the unique characteristics of LAMP is the capacity it affords for visual judgment of nucleic acid amplification. Mori et al. *(12)* originally reported that amplification by this assay can be detected by confirming the presence of magnesium pyrophosphate, a white precipitate generated as a by-product during the reaction. Some researchers have reported that detecting a small amount of the white precipitate by observation with the naked eye is not always easy because the negative-reaction mixture has

white precipitate due to template-related obstacles such as proteins in the sample and the components of the culture medium. In this study, obstacles were removed from the solution of DNA template to make clear a negative reaction (Fig. 2B). The positive reactions in samples subjected to 4, 6, and 18 h of incubation were sharply contrasted to the negative reaction in 0-h incubation samples (Fig. 2B). In an analysis of PCR products by agarose gel electrophoresis, a specific amplicon (251 bp) was detectable in 6-h incubation but not 4-h incubation. The total time required from enrichment to detection was more than 10 h: enrichment required more than 6 h, DNA extraction required 1 h, PCR assay required 2 h, and electrophoresis required 1 h. Therefore, the PCR assay is not valuable to detect TDH-producing *V. parahaemolyticus* in oysters within a period limited to 1 day. Real-time PCR was much faster than PCR: it was possible to detect TDH-producing *V. parahaemolyticus* by using the sample preparation and real-time PCR within 1 day *(18)*; however, real-time PCR requires sophisticated equipment. LAMP amplification is performed under isothermal conditions, between 60 and 65°C *(8, 13, 19)*, using simple incubators such as a water bath or block heater, which is sufficient for DNA amplification. Visual judgment eliminates the need for special procedures postamplification such as electrophoresis.

This study demonstrates that the LAMP assay is effective in rapidly detecting TDH-producing *V. parahaemolyticus* with high sensitivity in seafood, and the assay has the capacity for use in the field or in the small-scale laboratory.

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REFERENCES

1. Bej, A. K., D. P. Patterson, C. W. Brasher, M. C. Vickery, D. D. Jones, and C. A. Kaysner. 1999. Detection of total and hemolysin-producing *Vibrio parahaemolyticus* in shellfish using multiplex PCR amplification of *tl*, *tdh* and *trh*. *J. Microbiol. Methods* 36:215–225.
2. Blackstone, G. M., J. L. Nordstrom, M. C. Vickery, M. D. Bowen, R. F. Meyer, and A. DePaola. 2003. Detection of pathogenic *Vibrio parahaemolyticus* in oyster enrichments by real time PCR. *J. Microbiol. Methods* 53:149–155.
3. Chun, D., J. K. Chung, R. Tak, and S. Y. Seol. 1975. Nature of the Kanagawa phenomenon of *Vibrio parahaemolyticus*. *Infect. Immun.*12:81–87.
4. DePaola, A., C. A. Kaysner, J. Bowers, and D. W. Cook. 2000. Environmental investigations of *Vibrio parahaemolyticus* in oysters following outbreaks in Washington, Texas, and New York (1997 and 1998). *Appl. Environ. Microbiol.* 66:4649–4654.
5. Hara-Kudo, Y., J. Nemoto, K. Ohtsuka, Y. Segawa, K. Takatori, T. Kojima, and M. Ikedo. 2007. Sensitive and rapid detection of Vero toxin-producing *Escherichia coli* using loop-mediated isothermal amplification. *J. Med. Microbiol.* 56:398–406.
6. Honda, T., M. Yoh, U. Kongmuang, and T. Miwatani. 1985. Enzyme-linked immunosorbent assays for detection of thermostable direct hemolysin of *Vibrio parahaemolyticus*. *J. Clin. Microbiol.* 22:383–386.
7. Imai, M., A. Ninomiya, H. Minekawa, T. Notomi, T. Ishizaki, P. Van Tu, N. T. Tien, M. Tashiro, and T. Odagiri. 2007. Rapid diagnosis of H5N1 avian influenza virus infection by newly developed influenza H5 hemagglutinin gene-specific loop-mediated isothermal amplification method. *J. Virol. Methods* 141:173–180.
8. Iwamoto, T., T. Sonobe, and K. Hayashi. 2003. Loop-mediated isothermal amplification for direct detection of *Mycobacterium tuberculosis* complex, *M. avium*, and *M. intracellulare* in sputum samples. *J. Clin. Microbiol.* 41:2616–2622.
9. Martinez-Urtaza, J., A. Lozano-Leon, A. DePaola, M. Ishibashi, K. Shimada, M. Nishibuchi, and E. Liebana. 2004. Characterization of pathogenic *Vibrio parahaemolyticus* isolates from clinical sources in Spain and comparison with Asian and North American pandemic isolates. *J. Clin. Microbiol.* 42:4672–4678.
10. Mead, P. S., L. Slutsker, V. Dietz, L. F. McGaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe. 1999. Food-related illness and death in the United States. *Emerg. Infect. Dis.* 5:607–625.
11. Miyamoto, Y., T. Kato, Y. Obara, S. Akiyama, K. Takizawa, and S. Yamai. 1969. In vitro hemolytic characteristic of *Vibrio parahaemolyticus*: its close correlation with human pathogenicity. *J. Bacteriol.* 100:1147–1149.
12. Mori, Y., K. Nagamine, N. Tomita, and T. Notomi. 2001. Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. *Biochem. Biophys. Res. Commun.* 289:150–154.
13. Nagamine, K., T. Hase, and T. Notomi. 2002. Accelerated reaction by loop-mediated isothermal amplification using loop primers. *Mol. Cell. Probes* 16:223–229.
14. Nishibuchi, M., A. Fasano, R. G. Russell, and J. B. Kaper. 1992. Enterotoxigenicity of *Vibrio parahaemolyticus* with and without genes encoding thermostable direct hemolysin. *Infect. Immun.* 60:3539–3545.
15. Nishibuchi, M., and J. B. Kaper. 1995. Duplication and variation of the thermostable direct haemolysin (*tdh*) gene in *Vibrio parahaemolyticus*. *Mol. Microbiol.* 4:87–99.
16. Nishibuchi, M., and J. B. Kaper. 1995. Thermostable direct hemolysin gene of *Vibrio parahaemolyticus*: a virulence gene acquired by a marine bacterium. *Infect. Immun.* 63:2093–2099.
17. Nordstrom, J. L., C. A. Kaysner, G. M. Blackstone, M. C. Vickery, J. C. Bowers, and A. DePaola. 2004. Effect of intertidal exposure on *Vibrio parahaemolyticus* levels in Pacific Northwest oysters. *J. Food Prot.* 67:2178–2182.
18. Nordstrom, J. L., M. C. L. Vickery, G. M. Blackstone, S. L. Murray, and A. DePaola. 2007. Development of a multiplex real-time PCR assay with an internal amplification control for the detection of total and pathogenic *Vibrio parahaemolyticus* bacteria in oysters. *Appl. Environ. Microbiol.* 73:5840–5847.
19. Notomi, T., H. Okayama, H. Masubuchi, T. Yonekawa, K. Watanabe, N. Amino, and T. Hase. 2000. Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res.* 15:E63.
20. Okuda, J., and M. Nishibuchi. 1998. Manifestation of the Kanagawa phenomenon, the virulence-associated phenotype, of *Vibrio parahaemolyticus* depends on a particular single base change in the promoter of the thermostable direct haemolysin gene. *Mol. Microbiol.* 30:499–511.
21. Tada, J., T. Ohashi, N. Nishimura, Y. Shirasaki, H. Ozaki, S. Fukushima, J. Takano, M. Nishibuchi, and Y. Takeda. 1992. Detection of the thermostable direct hemolysin gene (*tdh*) and the thermostable direct hemolysin-related hemolysin gene (*trh*) of *Vibrio parahaemolyticus* by polymerase chain reaction. *Mol. Cell. Probes* 6:477–487.
22. Wong, H. C., S. H. Liu, L. W. Ku, I. Y. Lee, T. K. Wang, Y. S. Lee, C. L. Lee, L. P. Kuo, and D. Y. C. Shih. 2000. Characterization of *Vibrio parahaemolyticus* isolates obtained from foodborne illness outbreaks during 1992 through 1995 in Taiwan. *J. Food Prot.* 63:900–906.
23. Yeung, P. S. M., and K. J. Boor. 2004. Epidemiology, pathogenesis, and prevention of foodborne *Vibrio parahaemolyticus*. *Foodborne Pathog. Dis.* 1:74–88.