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Identification of the Emerging Pathogen *Vibrio vulnificus* Biotype 3 by Commercially Available Phenotypic Methods

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Identification of the emerging pathogen *Vibrio vulnificus* biotype 3 has become a challenge for clinical laboratories in the last few years. In this study, the abilities of five commercial systems to identify this new species have been evaluated for the first time, using a unique collection of strains. Fifty-one well-documented wild strains of *V. vulnificus* biotype 3 were processed using API 20 NE, GNI+ Vitek 1 cards, ID-GNB Vitek 2 cards, Neg Combo 20 Microscan panels, and NMIC/ID-5 BD Phoenix panels. The numbers of strains identified as *V. vulnificus* by ID-GNB, NMIC/ID-5, and GNI+ were 50 (98.0%), 46 (90.2%), and 7 (13.7%), respectively. Neg Combo 20 Microscan panels and API 20 NE were unable to identify any of the strains of this emerging pathogen to the species level and mostly misidentifies them as other species of the *Vibrionaceae* family. Data on the phenotypic pattern of *V. vulnificus* biotype 3 when processed in all five systems as presented here could help clinical laboratories in identifying this new pathogen.

Since it was first described by Reichelt et al. in 1976 (9), for many years only two biotypes or serovars of the human pathogen *Vibrio vulnificus* have been recognized (1).

In the midsummer of 1996, we reported the first isolation of a new pathogen, *V. vulnificus* biotype 3, which causes septicemia and severe soft-tissue infections following contact with fish from artificial freshwater ponds (2). Since then, the identification of this emerging pathogen has become a challenge for clinical laboratories. With the phenotypic behavior of this new biotype being different from that of the *V. vulnificus* biotypes known so far, most of the commercial systems in use do not include the relevant data in their software, making correct identification of the new bacteria problematic.

In this study, the phenotypic pattern of this new biotype when tested with five commercial systems was defined, and the abilities of the current software of those systems to correctly identify *V. vulnificus* biotype 3 to the species level have been evaluated for the first time with a unique collection of strains.

MATERIALS AND METHODS

Identification systems. Fifty-one well-documented *V. vulnificus* biotype 3 strains isolated during the years 1996 to 1997 were processed by five commercial identification systems: API 20 NE, GNI+ Vitek 1 cards, ID-GNB Vitek 2 cards (BioMerieux, Marcy l-Etoile, France), Neg Combo 20 Microscan panels (Dade Behring Inc.), and NMIC/ID-5 BD Phoenix panels (BD Biosciences).

Given the fact that previous reports have already concluded that the use of API 20 E is inadequate for the identification of *V. vulnificus* (6), only the API 20 NE system was used in this study. API 20 NE strips were inoculated and processed following the manufacturer's recommendations, and the results were analyzed by using Apilab software, version 3.2.2.

Inoculated Vitek GNI+ cards were processed with a Vitek 1 system, version VTK-R 07.02.

Vitek ID-GNB cards were used with a Vitek 2 system, version VT2-R 02.03.

Microscan Neg Combo 20 panels were inoculated according to the manufacturer's recommendations for halophilic vibrios (0.125 ml of water plus Pluronic plus 0.1 ml of a 0.5 McFarland bacterial suspension in each well) and processed in a Microscan Walkaway 96 apparatus. Results were analyzed by using the Microscan DMS system, version 24.1.

Finally, inoculated NMIC/ID-5 panels were processed with the BD Phoenix system according to the manufacturer's instructions.

Bacterial strains. Fifty-one bacterial strains tested in the study were isolated from patients during the years 1996 and 1997 in laboratories serving the northern region of Israel, where all cases appear following contact with *Tilapia* fishes. The strains, which were previously identified at local laboratories, were eventually confirmed by the Central Laboratories of the Israel Ministry of Health in Jerusa-

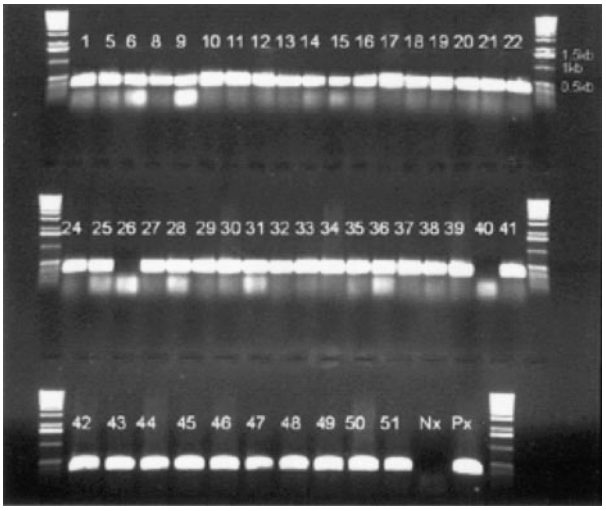

FIG. 1. PCR results for the CTH gene. Px, positive control strain; Nx, negative control strain.

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TABLE 1. Percentages of strains correctly identified as *V. vulnificus* by all systems

System	% (No.) of correct ID[a]	% (No.) of strains misidentified as:		% (no.) of strains not identified
		Other *Vibrio*	Not *Vibrio*	
API 20 NE	0 (0)	100 (51)	0 (0)	0 (0)
Neg Combo 20/Microscan	0 (0)	100 (51)	0 (0)	0 (0)
GNI+/Vitek 1	17.6 (9)	21.6 (11)	60.8 (31)	0 (0)
ID-GNB/Vitek 2	98.0 (50)	0 (0)	0 (0)	2.0 (1)
NMIC/ID-5/BD Phoenix	90.2 (46)	3.9 (2)	3.9 (2)	2.0 (1)

[a] ID, identifications.

lem as belonging to *V. vulnificus* biotype 3 by using an extensive phenotypic workflow proposed by J. J. Farmer from the Centers for Disease Control and Prevention (Atlanta, Ga.).

Eventually, all the strains were confirmed as *V. vulnificus* at the Nuffield Department of Clinical Laboratory Sciences of John Radcliffe Hospital, University of Oxford (Oxford, United Kingdom), using PCR for the cytotoxin-hemolysin (CTH) gene, as described by Brauns et al. (4). The DNeasy kit (QIAGEN GmbH, Hilden, Germany) was used to extract DNA, and the protocol for gram-negative bacteria was followed. Briefly, several colonies from a single clone of each bacterial culture were picked off into phosphate-buffered solution and centrifuged at 7,500 rpm (5,000 × *g*) for 10 min. The cell pellet was resuspended in 180 μl of tissue lysis buffer, and then 20 μl of proteinase K (10 mg/ml) was added and the sample was incubated at 55°C until the tissue was completely lysed. Next, 200 μl of lysis buffer was added, and incubation was at 70°C for 10 min. The DNA in the clear viscous lysates was precipitated with ethanol (95% [vol/vol]) and added to DNeasy mini-columns. Ethanol (70% [vol/vol])-based buffers (AW1 and AW2) were added sequentially to the columns and centrifuged at 8,000 rpm (6,000 × *g*). The supernatants were discarded, and the DNA was resuspended in sterile water and used for amplification.

PCR amplification was carried out with previously described oligonucleotide primers (4). Each 50-μl amplification reaction mixture comprised 10 ng of chromosomal DNA, 100 pmol of each PCR primer (MWG Biotech, Ebersberg, Germany), 10× PCR buffer with 1.5 mM $MgCl_2$ (QIAGEN GmbH), 0.5 U of *Taq* DNA polymerase (QIAGEN GmbH), and 1.6 mM deoxynucleoside triphosphates (ABgene, Epsom, United Kingdom). The reaction conditions were denaturation at 94°C for 1 min, primer annealing at 68°C for 1 min, and extension at 72°C for 1 min, for 30 cycles.

A volume (5 μl) of PCR-amplified DNA was separated on a 0.8% agarose gel containing 0.5 μg of ethidium bromide ml^{-1}. The gel was run in Tris-borate-EDTA buffer at 120 V for 30 min with a 100-bp DNA ladder (ABgene, Epsom, United Kingdom) as a size standard. DNA fragments in the gel were visualized under a UV transilluminator (Gel Doc 1000; Bio-Rad Laboratories Ltd., Bio-Rad House, Hemel Hempstead, United Kingdom).

In addition, two control strains were also tested with all systems but NMIC/ID-5 BD-Phoenix panels: *V. vulnificus* biotype 1 (CDC strain 9028A95) and *V. vulnificus* biotype 2 (L. Hoi, Denmark; strain 96-7-138), both from an Israel Ministry of Health strain collection.

RESULTS

The results by PCR for 48 of the strains are shown in Fig. 1. Samples 2, 3, and 4 were tested separately and were also positive for the CTH gene. Samples 26 and 40, shown as negative for the CTH gene, gave a positive reaction when retested using an annealing temperature of 60°C.

The numbers of strains correctly identified by the different systems are shown in Table 1. The numbers of isolates misi-

TABLE 2. API 20 NE profile of the 51 strains of biotype 3[a]

Substrate	% (No.) of positive biotype 3 strains	Result with control strain		Expected result[c] (% positive)
		Biotype 1	Biotype 2	
Potassium nitrate	**100 (51)**	+	+	100
Tryptophan	**100 (51)**	+	+	95
Glucose	0 (0)	+	+	95
Arginine	0 (0)	−	−	0
Urea	0 (0)	−	−	1
Esculin	0 (0)	−	−	95
Gelatin	**100 (51)**	+	+	99
PNPG	0 (0)	+	+	99
Glucose	73 (37)	−	−	9
Arabinose	0 (0)	−	−	0
Mannose	61 (31)	−	−	10
Mannitol	0 (0)	−	+	9
N-Acetyl-glucosamine	6 (3)	−	−	1
Maltose	**100 (51)**	−	+	6
Gluconate	61 (31)	−	+	28
Caprate	0 (0)	−	−	0
Adipate	0 (0)	−	−	0
Malate	89 (45)	+	+	95
Citrate	33 (17)	−	+	91
Phenyl-acetate	0 (0)	−	−	0
Oxidase[b]	**100 (51)**	+	+	100

[a] Substrates that give a positive result with all 51 strains are boldfaced. PNPG, *p*-nitrophenyl-β-D-glucoside.
[b] External test.
[c] Expected result for *V. vulnificus* according to the insert chart provided by the manufacturer.

TABLE 3. Profiles of the 51 strains of biotype 3 in Vitek 1 using GNI+ cards[a]

Substrate	% (No.) of positive biotype 3 strains	Result for control strain	
		Biotype 1	Biotype 2
DP-300	0 (0)	−	−
Glucose (ox.)	**100 (51)**	+	+
Acetamide	0 (0)	−	−
Esculine	0 (0)	−	−
Plant indican	0 (0)	−	−
Urea	0 (0)	−	−
Citrate	0 (0)	−	−
Malonate	0 (0)	−	−
Tryptophan	0 (0)	−	−
Polymyxin B	0 (0)	−	−
Lactose	0 (0)	−	−
Maltose	**100 (51)**	+	+
Mannitol	0 (0)	+	+
Xylose	0 (0)	−	−
Raffinose	0 (0)	−	−
Sorbitol	0 (0)	−	−
Sucrose	0 (0)	−	−
Inositol	0 (0)	−	−
Adonitol	0 (0)	−	−
p-Coumaric	0 (0)	−	−
H_2S	0 (0)	−	−
ONPG	0 (0)	−	−
Rhamnose	0 (0)	−	−
L-arabinose	0 (0)	−	−
Glucose (ferm.)	**100 (51)**	+	+
Arginine	0 (0)	−	−
Lysine	78 (40)	−	+
Omithine	63 (32)	−	−

[a] Substrates that give a positive result with all 51 strains are boldfaced. ox., oxidation; ferm., fermentation; ONPG, *o*-nitrophenyl-β-D-galactopyranoside.

TABLE 4. Profile of the 51 strains of biotype 3 in Vitek 2, using ID-GNB cards[a]

Substrate	% (No.) of positive biotype 3 strains	Result with control strain	
		B1	B2
Adonitol	0 (0)	−	−
L-arabinose	0 (0)	−	−
D-cellobiose	0 (0)	−	+
D-galacturonate	0 (0)	−	−
D-glucose	**100 (51)**	+	+
Glucose-1-phosp.	**100 (51)**	+	+
D-glucuronate	96 (49)	−	+
Myoinositol	0 (0)	−	−
5-Keto-D-gluconate	0 (0)	−	−
D-maltose	98 (50)	+	+
D-mannitol	0 (0)	−	+
D-melibiose	0 (0)	−	−
Palatinose	0 (0)	−	−
D-raffinose	0 (0)	−	−
L-rhamnose	0 (0)	−	−
Saccharose/sucrose	0 (0)	−	−
D-sorbitol	0 (0)	−	+
D-trehalose	**100 (51)**	+	+
Lysine	**100 (51)**	+	+
Decarboxylase			
Omithine decarbox.	71 (36)	−	−
Urease	0 (0)	−	−
Malonate	0 (0)	−	−
Tryptophane deaminase	0 (0)	−	−
Alpha-arabinosidase	0 (0)	−	+
Alpha-galactosidase	0 (0)	−	+
Alpha-glutamate	0 (0)	−	−
Beta-cellobiosidase	16 (8)	−	−
Beta-galactosidase	0 (0)	−	+
Beta-glucosidase	0 (0)	−	−
Beta-glucuronidase	0 (0)	−	−
Beta-mannosidase	**100 (51)**	+	+
Beta-*N*-acetyl-gluc.	**100 (51)**	+	+
Beta-N-acetyl-galac.	0 (0)	+	+
Beta-xylosidase	0 (0)	−	−
Glu-Gly-Arg-arylamidase	0 (0)	−	−
Gamma-glutamyl-trans.	0 (0)	−	−
L-lysine-arylamidase	**100 (51)**	+	+
Phosphatase	0 (0)	−	−
L-proline-arylamidase	**100 (51)**	+	+
L-pyrrolidonyl-arylam.	0 (0)	−	−
CBZ-Arg-arylamidase	0 (0)	−	−

[a] Substrates that give a positive result with all 51 strains are boldfaced. phosp., phosphatase; decarbox., decarboxylase; gluc., glucosidase; galac., galactosidase; trans., transferase; arylam., arylamidase; CBZ, carbobenzyloxy; B1, *V. vulnificus* biotype 1; B2, *V. vulnificus* biotype 2.

TABLE 5. Profile of the 51 strains of biotype 3 in BD-Phoenix, using NMIC/ID-5 cards[a]

Substrate	% (No.) of positive strains
Phenylalanine-AMC	**100 (51)**
4MU-NAG	**100 (51)**
Glutamic acid-AMC	0 (0)
Tryptophan-AMC	86 (44)
PYR-AMC	0 (0)
Proline-AMC	**100 (51)**
Arginine-AMC	**100 (51)**
Arginine-arginine-AMC	**100 (51)**
Glycine-AMC	**100 (51)**
Leucine-AMC	**100 (51)**
Lysine-alanine-AMC	**100 (51)**
Glutaryl-glycine-arginine-AMC	6 (3)
Glycine-proline-AMC	33 (17)
Colistin	0 (0)
Polymyxin B	0 (0)
Mannitol	0 (0)
Citrate	6 (3)
Acetate	6 (3)
Adonitol	0 (0)
Malonate	6 (3)
Ketoglutaric acid	0 (0)
Tiglic acid	4 (2)
Proline-NA	**100 (51)**
Gamma-L-glutamyl-NA	4 (2)
Bis(PNP) phosphate	**100 (51)**
PNP-β-D-glucoside	10 (5)
Allose	0 (0)
N-acetyl-galactosamine	4 (2)
N-acetyl-glucosamine	12 (6)
Sorbitol	4 (2)
Sucrose	2 (1)
Galacturonic acid	0 (0)
Maltulose	0 (0)
Rhamnose	0 (0)
Gentiobiose	6 (3)
Dextrose	10 (5)
Galactose	0 (0)
Fructose	8 (4)
Gluconic acid	4 (2)
Melibiose	0 (0)
Arabinose	0 (0)
Methyl-β-glucoside	2 (1)
Ornithine	0 (0)
Urea	8 (4)
Esculin	6 (3)

[a] Substrates that give a positive result with all 51 strains are boldfaced; AMC, fluorescent coumarin derivative; 4MU-NAG, 4-methylumbelliferone-*N*-acetyl-BD-glucosaminide; PYR, pyrrolidonyl-α-naphthylamide; NA, p-nitroaniline; PNP, *p*-nitrophenyl.

dentified as other *Vibrionaceae* or non-*Vibrio* species or not identified at all are also shown in the same table.

ID-GNB, NMIC/ID5, and GNI+ correctly identified 50 (98.0%), 46 (90.2%), and 9 (17.6%) strains, respectively. Microscan Neg Combo 20 and API 20 NE were unable to correctly identify any of the strains of this emerging pathogen.

For the single strain that ID-GNB was unable to identify, the Vitek 2 system proposed *Plesiomonas shigelloides* and *V. vulnificus* with the same probability rates and asked for further tests.

From the five strains not identified by NMIC/ID5 as *V. vulnificus*, only two were misidentified by the system as other *Vibrio* spp.

GNI+ misidentified 10 strains as other *Vibrio* spp. Thirty-one strains, all of them presenting the same phenotypic pattern, were misidentified as *P. shigelloides* (75% probability), with *V. vulnificus* as the second choice (19% probability).

Microscan Neg Combo 20 misidentified all the strains as other *Vibrionaceae*, most of them as *Vibrio mimicus* and *Vibrio parahaemolyticus*.

Finally, API 20 NE also misidentified all the strains as other *Vibrionaceae*, most of them as *Vibrio alginolyticus*.

Tables 2 to 6 show the phenotypic profiles presented by all 51 strains of biotype 3 tested with the API 20 NE, GNI+, ID-GNB, NMIC/ID5, and Microscan Neg Combo 20 systems, respectively. In addition, Tables 2, 3, 4, and 6 show the results for the control strains of biotypes 1 and 2 with the respective

TABLE 6. Profile of the 51 strains of biotype 3 in Microscan system, using Neg Combo 20 panels[a]

Substrate	% (No.) of positive strains	Result for control strain	
		Biotype 1	Biotype 2
Glucose	**100 (51)**	+	+
Raffinose	0 (0)	−	−
Inositol	0 (0)	−	−
Urea	0 (0)	−	−
Lysine	0 (0)	−	−
Tryptophan deaminase	0 (0)	−	−
Citrate	14 (7)	−	−
Colistin (4 mcg/ml) (gr.)	**100 (51)**	+	−
Sucrose	0 (0)	−	−
Rhamnose	0 (0)	−	−
Adonitol	0 (0)	−	−
H_2S	0 (0)	−	−
Arginine	0 (0)	−	−
Esculine	0 (0)	−	−
Malonate	0 (0)	−	−
Cephalotin (8 mcg/ml) (gr.)	14 (7)	−	+
Sornitol	0 (0)	−	−
Arabinose	0 (0)	−	−
Mellobiose	0 (0)	−	−
Indol	**100 (51)**	−	−
Omithine	0 (0)	−	−
VP	0 (0)	−	−
ONPG	0 (0)	−	+

[a] Substrates that give a positive result with all 51 strains are boldfaced. gr., growth; VP, Voges-Proskeuer; ONPG, *o*-nitrophenyl-β-D-galactopyranoside.

systems. Table 2 also shows the expected result for *V. vulnificus* with API 20 NE as it appears in the kit insert.

DISCUSSION

In the last few years, the routine use of molecular biology techniques such as nested PCR (7) has been playing a more important role in clinical microbiology laboratories in developed countries. However, this is not the situation in the majority of the clinical laboratories worldwide, especially in nondeveloped countries. Thus, the use of commercial phenotype-based identification systems will continue to be the mainstream in the next years.

In this study, the abilities of five widely used phenotype-based commercial systems to identify the emerging pathogen *V. vulnificus* biotype 3 were evaluated.

ID-GNB with Vitek 2 and NMIC/ID5 with BD Phoenix proved to be the best systems for correctly identifying this new biotype as *V. vulnificus* (98.0 and 90.2% of strains correctly identified, respectively). These two systems offer a wide range of phenotypic reactions, many of them enzymatic, and a considerable number of them were positive with the strains tested. Microscan Neg Combo 20, API 20 NE, and GNI+ with Vitek 1, however, offer a significantly smaller number of reactions, and the number of positive tests was minimal with the tested strains. However, the majority of the strains presented a consistent phenotypic pattern with each of the systems. Thus, clinical laboratories using these systems could tentatively identify strains showing the phenotype presented here as that of *V. vulnificus* or at least suspect the presence of this pathogen.

So far, this emerging pathogen has been reported only in Israel (2, 3, 5, 8).

However, since commercial systems may misidentify it as another member of the *Vibrionaceae* family, it is not unlikely that the presence of this pathogen in other countries could be underestimated. Thus, clinical microbiology laboratories should be aware of this possibility and must meticulously check the correct identification of any vibrio isolated from clinical sources.

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