Journal of Microbiological Methods 133 (2017) 23–31

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Review

Application of a paper based device containing a new culture medium to detect *Vibrio cholerae* in water samples collected in Haiti

Romain Briquaire [a,*], Rita R. Colwell [b,c], Jacques Boncy [d], Emmanuel Rossignol [d], Aline Dardy [e], Isabelle Pandini [f], François Villeval [g], Jean-Louis Machuron [a], Anwar Huq [c], Shah Rashed [c], Thierry Vandevelde [h], Christine Rozand [e,*]

[a] PAH – Les Pharmaciens Humanitaires, 84 rue de Charonne, 75011 Paris 11, France
[b] Institute for Advanced Computer Studies, University of Maryland College Park, College Park, MD 20742, USA
[c] Department of Cell Biology and Molecular Genetics, Maryland Pathogen Research Institute, University of Maryland College Park, College Park, MD 20742, USA
[d] Laboratoire National de Santé Publique, Rue Chardonnier #2 and Delmas 33, Port-au-Prince, Haiti
[e] bioMérieux, Novel Analytical Devices, Innovation Unit, 69280, Marcy L'Etoile, France
[f] 66A rue des Collonges, 69230 Saint Genis Laval, France
[g] bioMérieux, Microbiology R&D Clinical Unit, 69290, Craponne, France
[h] Veolia Foundation, 169 avenue Clemenceau, 92735 Nanterre cedex, France

ARTICLE INFO

Article history:
Received 18 October 2016
Received in revised form 15 December 2016
Accepted 16 December 2016
Available online 19 December 2016

Keywords:
Cholera
Vibrio cholerae
Water samples
Paper based analytical device

ABSTRACT

Cholera is now considered to be endemic in Haiti, often with increased incidence during rainy seasons. The challenge of cholera surveillance is exacerbated by the cost of sample collection and laboratory analysis. A diagnostic tool is needed that is low cost, easy-to-use, and able to detect and quantify *Vibrio cholerae* accurately in water samples within 18–24 h, and perform reliably in remote settings lacking laboratory infrastructure and skilled staff. The two main objectives of this study were to develop and evaluate a new culture medium embedded in a new diagnostic tool (PAD for paper based analytical device) for detecting *Vibrio cholerae* from water samples collected in Haiti. The intent is to provide guidance for corrective action, such as chlorination, for water positive for *V. cholerae* epidemic strains.

For detecting *Vibrio cholerae*, a new chromogenic medium was designed and evaluated as an alternative to thiosulfate citrate bile salts sucrose (TCBS) agar for testing raw water samples. Sensitivity and specificity of the medium were assessed using both raw and spiked water samples.

The *Vibrio cholerae* chromogenic medium was proved to be highly selective against most of the cultivable bacteria in the water samples, without loss of sensitivity in detection of *V. cholerae*. Thus, reliability of this new culture medium for detection of *V. cholerae* in the presence of other *Vibrio* species in water samples offers a significant advantage.

A new paper based device containing the new chromogenic medium previously evaluated was compared with reference methods for detecting *V. cholerae* from spiked water sample. The microbiological PAD specifications were evaluated in Haiti. More precisely, a total of 185 water samples were collected at five sites in Haiti, June 2014 and again in June 2015. With this new tool, three *V. cholerae* O1 and 17 *V. cholerae* non-O1/O139 strains were isolated.

The presence of virulence-associated and regulatory genes, including *ctx*A, *zot*, *ace*, and *toxR*, was confirmed using multiplex PCR. The three *V. cholerae* O1 isolates were positive for three of the four virulence-associated and regulatory genes. Twelve of the *V. cholerae* non-O1/O139 isolates were found to carry *toxR*, but none were *ctxA*+, *zot*+, or *ace*+. However, six of the *V. cholerae* non-O1/O139 isolates were resistant to penicillin, ampicillin, trimethoprim/sulfamethoxazole, nalidixic acid, and ciprofloxacin. The paper based analytical device (PAD) provides advantages in that standard culture methods employing agar plates are not required. Also, intermediary isolation steps were not required, including transfer to selective growth media, hence these steps being omitted reduced time to results. Furthermore, experienced technical skills also were not required. Thus, PAD is well suited for resource-limited settings.

© 2016 Elsevier B.V. All rights reserved.

* Corresponding authors.
E-mail addresses: romain.briquaire@ext.biomerieux.com (R. Briquaire), christine.rozand@biomerieux.com (C. Rozand).

http://dx.doi.org/10.1016/j.mimet.2016.12.014
0167-7012/© 2016 Elsevier B.V. All rights reserved.

Contents

1. Introduction . . . 24
2. Materials and methods . . . 24
   2.1. Design and evaluation of the new chromogenic medium . . . 24
      2.1.1. Bacterial strains . . . 24
      2.1.2. Media and reagents. . . . 24
      2.1.3. Evaluation of selective media . . . 25
   2.2. Design and field based evaluation of paper based solution . . . 26
      2.2.1. Sample collection . . . 26
      2.2.2. Paper based analytical device (PAD) . . . 26
      2.2.3. Conventional culture methods . . . 26
      2.2.4. Characterization of isolates . . . 27
3. Results and discussion . . . 28
   3.1. Designed and evaluation of the new chromogenic medium . . . 28
      3.1.1. Performance of the selective media using pure microbial strains . . . 28
      3.1.2. Performance using spiked water samples . . . 28
   3.2. Design and field based evaluation of the paper based solution. . . . 29
      3.2.1. Microbiological performance of PAD and standard reference method . . . 29
      3.2.2. Characterization of *Vibrio cholerae* strains isolated from Haitian water . . . 29
4. Conclusion . . . 30
Acknowledgements . . . 30
References. . . . 30

1. Introduction

Toxigenic *Vibrio cholerae* O1 and O139 (de Magny et al., 2011), historically are considered causative agents of cholera, a disease characterized by severe diarrhea, and have been implicated in major outbreaks and epidemics (Harris et al., 2012). *V. cholerae* O1 (El Tor and classical biotypes; Inaba and Ogawa serotypes) and O139 serogroups have been implicated in the seventh cholera pandemic (Mutreja et al., 2011). In 2014, a 47% increase in the number of cholera cases was reported, compared with 2013, with a total of 190,549 cholera cases, 2231 deaths, as reported to WHO (World Health Organization, 2014). In many developing countries, including Haiti, Bangladesh, and the Democratic Republic of Congo countries where cholera is endemic, *V. cholerae* O1 is prevalent in the aquatic environment, namely rivers, ponds, wells, sewage water, and estuaries (Chin et al., 2011; Huq et al., 1996; Islam et al., 2011; Ryan, 2013; Uchiyama, 2000). Transmission of cholera occurs via drinking water contaminated with *V. cholerae* (Jeandron et al., 2015), but transmission can also occur through improper food preparation (Chakraborty et al., 2013; Huq et al., 1990).

There are 200 serogroups, based on the O antigen, but only serogroups O1 and O139 have been linked to epidemic cholera (de Magny et al., 2011). Thus, both are considered a major public health threat, especially for developing countries with limited public health resources.

Infections and disease, as well as cholera can be caused by non-epidemic *V. cholerae* serogroups that are collectively referred to as non-O1/O139 *V. cholerae* and generally transmitted by consumption of contaminated water and raw or undercooked seafood (Mookerjee et al., 2015). Non-O1/O139 *V. cholerae* infections are continuously reported worldwide, emphasizing their clinical significance (Dutta et al., 2013). Non-O1/O139 *V. cholerae* strains generally do not carry the genes coding for cholera toxin, other virulence factors contribute to their pathogenicity, including the hemolysin gene *hlyA* and others, with outbreaks reported (Chatterjee et al., 2009; Hasan et al., 2012; Marin et al., 2013). Haley et al. recently published a paper on non-O1 *V. cholerae* isolated from 10 cholera cases in Florida (Haley et al., 2013).

Epidemic cholera was reported in Haiti in 2010, with poor information available on the previous occurrence or geographic distribution of toxigenic *Vibrio cholerae* in Haitian waters. However, a recent publication reported *V. cholerae* non-O1 in samples collected in a study of diarrhea in Haiti in 2008. The stored samples, when re-tested, confirmed the presence of *V. cholerae* in the samples (Liu et al., 2014). In a series of studies conducted in Haiti between 2011 and 2013, Kahler et al. (2015) suggested that ongoing environmental surveillance of *V. cholerae* could provide valuable data on the potential for cholera transmission via consumption of untreated water. Thus, knowledge of the ecology of *V. cholerae* in Haiti would provide useful data for water-related exposure risk management and informed decision making with respect to potential public health measures, such as vaccination campaigns and provision of safe drinking water, as well as informational materials. In general, environmental surveillance is important to understand the ecological dynamics of *V. cholerae* and to measure the contribution of waterborne transmission to cholera in Haiti.

To meet this need, a low cost, easy-to-use, and field-rugged solution is required to detect and enumerate *Vibrio cholerae* in water samples within 18–24 h, in remote settings without laboratory infrastructure and skilled staff.

To fulfill this need, the first objective of this study, was to develop and evaluate a *Vibrio cholerae* chromogenic medium for a single step detection, identification and differentiation of *V. cholerae* from other *Vibrio* species, with the intention to provide a more effective diagnostic medium than TCBS and ChromID™ *Vibrio* agar. Since agar plates are not usable outside laboratory, we have designed a new paper based device containing the new chromogenic medium previously evaluated with spiked water samples. To challenge this new microbiological tool, an on-field evaluation was made in Haiti.

2. Materials and methods

2.1. Design and evaluation of the new chromogenic medium

2.1.1. Bacterial strains

Strains of *V. cholerae* used in this study, with biotype, serogroup, and serotype, where known, are listed in Table 1. Bacterial and fungal strains comprising background flora in this study are listed in Table 2.

2.1.2. Media and reagents

ChromID™ *Vibrio*, Columbia agar (COS) and Tryptone Soya Agar (TSA) were obtained as pre-poured plates (bioMérieux; Marcy l'Etoile, France). TCBS agar plates were prepared from dehydrated powder according to manufacturers' instructions (Biokar; Beauvais, France). The plates were stored at 4 °C before use.

Table 1
Strains of *V. cholerae* used in this study.

V. cholerae strain	Biotype	Serogroup	Serotype	Source
93 02039	El Tor	O1	Ogawa	ATCC[a] 14035
09 06270	Unassigned	O1	Ogawa	bioMerieux strain collection
09 06271	Unassigned	O1	Ogawa	bioMerieux strain collection
09 06272	Unassigned	O1	Inaba	bioMerieux strain collection
12 09076	El Tor	O1	Inaba	ATCC 14033
12 09083	Unassigned	O1	Ogawa	NCTC[b] 10733
2SC5	Unassigned	O1		bioMerieux strain collection
09 06273	Unassigned	O139		bioMerieux strain collection
12 09074	Unassigned	O139		ATCC 51935
12 09078	Unassigned	O139		ATCC 51934

[a] ATCC, American Type Culture Collection, 10801 University Blvd. Manassas, VA 20110.
[b] NCTC, National Collection of Type Cultures, Public Health England, Wellington House, 133-155 Waterloo Road, London SE1 8UG.

The *Vibrio cholerae* chromogenic agar medium contains the following: growth-promoting mixture of peptone, yeast extract, and sucrose, 16 g l^{-1}; chemical as well as animal origin inhibitors, sodium cholate, sodium citrate, sodium thiosulfate, sodium chloride, ferric ammonium citrate, or bovine bile at 35 g l^{-1}, antibiotics (Vancomycin and Fosfomycin) at a concentration of 20 mg l^{-1}, glycosidase enzyme substrate, 0.05–1.15 g l^{-1}, and agar, 14 g l^{-1}. TRIS buffer is used to obtain pH 8.6. Plates are stored at 4 °C until use.

2.1.3. Evaluation of selective media

Media sensitivity and specificity were assessed as follows.

Table 2
Strains included as background flora for this study.

Bacterial and fungal strains	Collection	Origin	Source
Aeromonas caviae	92 11 064	Unknown	ATCC 15468
A. caviae	13 07039	Clinical isolate	bioMerieux strain collection
A. caviae	11 05005	Unknown	bioMerieux strain collection
A. caviae	11 05004	Unknown	bioMerieux strain collection
Aeromonas hydrophila	06 03112	Unknown	ATCC 7965
A. sobria		Environment isolate	bioMerieux strain collection
A. sobria	05 04205	Food isolate	bioMerieux strain collection
A. sobria	05 09001	Food isolate	bioMerieux strain collection
A. sobria	05 04112	Food isolate	bioMerieux strain collection
Aspergillus brasiliensis	08 01023	Food isolate	ATCC 16404
Bacillus subtilis	78 02084	Unknown	ATCC 6633
Candida albicans	130038640101	Clinical isolate	bioMerieux strain collection
C. albicans	130038742501	Clinical isolate	bioMerieux strain collection
C. albicans	90 08056	Clinical isolate	ATCC 10231
Enterobacter cloacae	130035675401	Clinical isolate	bioMerieux strain collection
E. cloacae	130036164201	Clinical isolate	bioMerieux strain collection
E. cloacae	81 12 012	Clinical isolate	ATCC 13047
Enterococcus faecalis	130038766801	Clinical isolate	bioMerieux strain collection
E. faecalis	130038078601	Clinical isolate	bioMerieux strain collection
E. faecalis	10 07027	Clinical isolate	bioMerieux strain collection
Escherichia coli	130038725901	Clinical isolate	bioMerieux strain collection
E. coli	130038729901	Clinical isolate	bioMerieux strain collection
E. coli	90 30 014	Unknown	bioMerieux strain collection
Proteus mirabilis	130037847201	Clinical isolate	bioMerieux strain collection
Pr. mirabilis	130037854701	Clinical isolate	bioMerieux strain collection
Pr. mirabilis	94 05058	Unknown	bioMerieux strain collection
Pseudomonas aeruginosa	130037863201	Clinical isolate	bioMerieux strain collection
Ps. aeruginosa	130038718101	Clinical isolate	bioMerieux strain collection
Ps. aeruginosa	75 09005	Clinical isolate	bioMerieux strain collection
Sphingomonas paucimobilis	SC9	Environment isolate	bioMerieux strain collection
Sp. paucimobilis	2SC14	Environment isolate	bioMerieux strain collection
Sp. paucimobilis	P34	Environment isolate	bioMerieux strain collection
Sp. paucimobilis	2P49	Environment isolate	bioMerieux strain collection
Sp. paucimobilis	2SCP6	Environment isolate	bioMerieux strain collection
Sp. paucimobilis	SC6	Environment isolate	bioMerieux strain collection
Vibrio alginolyticus	90 10 016	Food isolate	bioMerieux strain collection
V. alginolyticus	92 11 055	Food isolate	bioMerieux strain collection
V. alginolyticus	07 01002	Food isolate	bioMerieux strain collection
V. fluvialis	94 01053	Unknown	ATCC 33810
V. fluvialis	91 08055	Clinical isolate	CCUG[a] 20253
Vibrio mimicus	92 02028	Unknown	ATCC 700326
V. mimicus	92 02112	Unknown	ATCC 33655
V. mimicus	95 07021	Unknown	CCUG 13176
Vibrio parahaemolyticus	07 09108	Food isolate	bioMerieux strain collection
V. parahaemolyticus	07 07146	Food isolate	bioMerieux strain collection
V. vulnificus	83 08 05	Clinical isolate	ATCC 29306
V. vulnificus	07 07149	Food isolate	bioMerieux strain collection

[a] CCUG, Culture Collection University of Göteborg.

2.1.3.1. Performance evaluation using pure cultures. Bacterial colonies grown on COS at 37 °C for 18–24 h were resuspended in Maximum Recovery Diluent (MRD, bioMerieux) to prepare 0.5 McFarland suspensions. Serial decimal dilutions were made to obtain ~10^4 CFU/ml for the *V. cholerae* strains and ~10^7 CFU/ml for strains comprising background flora. A volume of 0.1 ml of *V. cholerae* and background flora suspensions were applied separately to the *Vibrio cholerae* chromogenic medium, TCBS agar, and ChromID™ *Vibrio* agar. All plates were incubated at 37 °C for 18–24 h.

2.1.3.2. Performance evaluation using V. cholerae-spiked water samples. A total of 50 randomly selected water samples (20 river, 20 hand pump, and 10 spring water) tested for presence of *V. cholerae* were used for spiking experiments. 9 ml aliquots of the water samples were inoculated with 1 ml of *V. cholerae* suspension at a final concentration of ~10^5 CFU/ml, prepared from each of the 10 *V. cholerae* strains, as described above. Spiked samples were vortexed and 0.1 ml volume of the mixtures was plated on *Vibrio cholerae* chromogenic medium, TCBS agar, and ChromID™ *Vibrio* agar, and incubated at 37 °C for 18–24 h. Water samples with addition of *V. cholerae* were also plated on the three media to serve as controls, i.e., to point out the presence of autochthonous *V. cholerae*. The number of *V. cholerae* cells added to water samples was confirmed by colony count on TSA.

2.1.3.3. Identification and enumeration of V. cholerae. Typical colonies of *V. cholerae* developed green color on *Vibrio cholerae* chromogenic medium, yellow on TCBS agar, and blue to blue-green or green on ChromID ™ *Vibrio* agar (Fig. 1). Colonies on each medium were enumerated (qualitative recovery) and colony color recorded. For all samples on the media being tested, each strain was classified as either presumptive-positive or negative (expressing or not, respectively, the colony characteristic) according to the following arbitrary color intensity classification. A strain was recorded as *V. cholerae* presumptive-positive with a color intensity value of >0.5 (Eddabra et al., 2011).

0 = no color

0.1 = trace color
0.5 = very pale color
1.0 = clear low intensity color
2.0 = medium intensity color
3.0 = intense color
4.0 = very intense color

Presumptive-positive *V. cholerae* colonies were picked and streaked on COS agar plates and the plates incubated at 37 °C for 18–24 h. Confirmation as *V. cholerae* was made by oxidase reaction and API 20E biochemical test results (bioMerieux) of colonies re-suspended in API NaCl 0.85% Medium (bioMerieux). Positive *V. cholerae* samples were also subjected to lateral flow immunoassay (Span Diagnostic) to determine whether serogroup O1 or O139.

2.2. Design and field based evaluation of paper based solution

2.2.1. Sample collection

Sampling sites in Haiti (Fig. 2) were selected where water was not treated with chlorine, bleach or disinfectant. A total of 185 water samples (spring, river, canal, sea, hand pump water) were collected. Sterile polypropylene bottles (500 ml; Nalgene, Rochester, NY) were used to collect water samples, providing a sufficient volume of water for the analyses, i.e., for the PAD test (3 ml per PAD) and to enumerate of *V. cholerae*. Water samples were stored in a cool box at a temperature of 10 to 15 °C until processing (not exceeding 8 h) (Rice et al., 2012).

A pre-sterilized plastic bottle was uncapped and submerged to fill to half volume, re-capped, shaken to rinse, and discarded. This was repeated 3 to 5 times. Rinsing was done downstream at every water sample collection site. The sample bottle was retrieved and capped, leaving, space for mixing.

2.2.2. Paper based analytical device (PAD)

The paper based analytical device (PAD) patented by bioMerieux is constructed from non-woven cellulose and dry impregnated with dehydrated chromogenic and culture media containing nutrients and inhibitors.

A dry impregnated PAD is placed in a PAD holder designed for the hydration of the PAD with a fixed quantity of the water. The PAD holder and PAD are packed in a thin aluminum foil to protect from humidity. Design of PAD holder allows homogeneous hydration of the PAD and elimination of excess water. Dry impregnation of culture medium inside a non-woven material was conducted by bioMerieux, Chemin de l'Orme, 69280 Marcy L'Etoile, France.

The PAD holder is designed to hold 3 ml of water, which eliminates the necessity for a syringe or vial to calibrate the volume of water added in the device. It is necessary only to add a water sample into the device designed for 3 ml water capacity. Via capillary action, 3 ml of water hydrates the dry impregnated PAD. Bacterial growth occurs inside the PAD, by utilizing dried *Vibrio cholerae* specific chromogenic culture media. Overnight incubation at a temperature of 35–37 °C for 18 to 24 h in a portable incubator is sufficient for growth of *V. cholerae*.

Green spots appearing on the PAD after incubation are counted, presumably representing colonies of *Vibrio cholerae*. The colonies are further characterized as described below.

2.2.3. Conventional culture methods

Conventional culture methods that are employed to isolate *V. cholerae* from environmental water samples rely on broth enrichment, followed by plating on selective media and confirmation using a series of biochemical tests (oxidase, API 20E, lateral flow). (Standard analytical protocol for *Vibrio cholerae* O1 and O139 in drinking water and surface water; EPA 600/R-10/139, October 2010.) A flow diagram is provided in Fig. 3. For each sample, 3 ml of water was analyzed. Overnight enrichment was performed using alkaline peptone water (APW) (20 g l^{-1} NaCl and 20 g l^{-1} peptone, adjusted at pH 8.6). Five APW replicates of each water sample were analyzed following the standard analytical

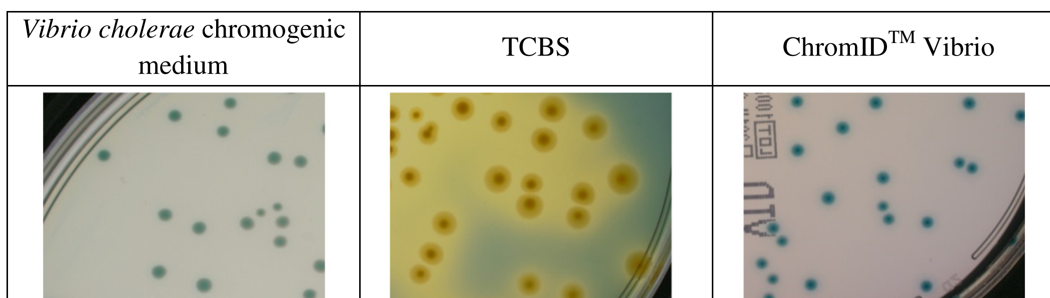

Fig. 1. Characteristic colony color of *V. cholerae* on *Vibrio cholerae* chromogenic medium, TCBS, and ChromID™ *Vibrio* medium.

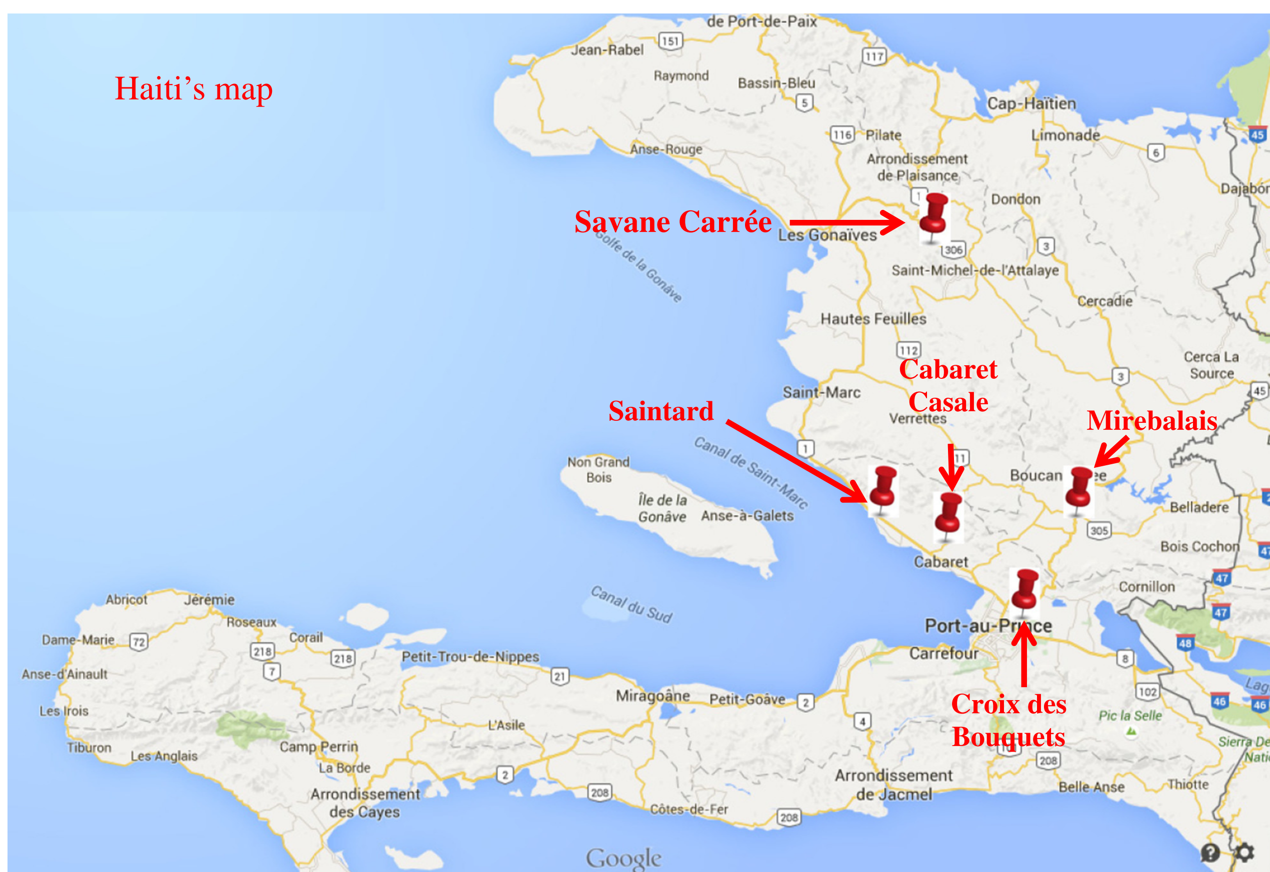

Fig. 2. Map showing sites in Haiti where water samples were collected in 2014 and 2015.

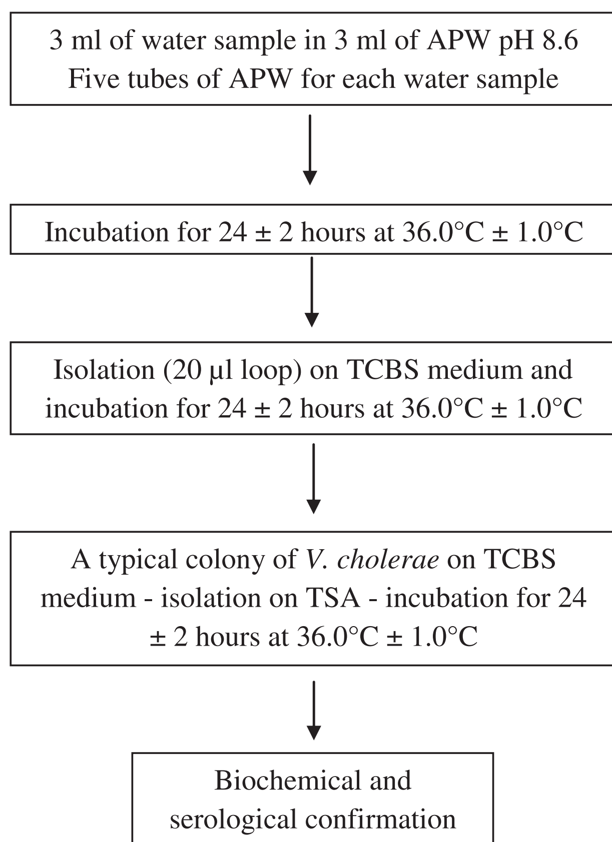

Fig. 3. Flow diagrams following EPA 600/R-10/139 recommended method.

protocol for *Vibrio cholerae* O1 and O139 in drinking water and surface water (Shah, 2010) whereby 10 ml of sample is diluted in 10 ml of 2X APW. However, in order to achieve equality with the PAD samples, 3 ml of sample was diluted in 3 ml of 2X APW.

A 20 µl loop-full of inoculum from the microaerophilic pellicle layer was streaked onto selective bacteriological media, namely thiosulfate citrate bile salts sucrose (TCBS) medium (Biokar; Beauvais, France). TCBS plates were prepared according to the manufacturers' instructions and stored at 4 °C until use. Yellow colonies on TCBS were recorded as *V. cholerae* and streaked on Tryptone Soya Agar (TSA) to obtain pure cultures. The TSA used was obtained as pre-poured plates (bioMérieux; Marcy l'Etoile, France) and stored at 4 °C until use. A simple typical *V. cholerae* colony on the TCBS medium was tested for oxidase and by the API20E gallery of tests.

2.2.4. Characterization of isolates

2.2.4.1. Identification confirmation. Identification of the individual strains prepared from the TCBS plates was achieved using the VITEK® MS (bioMerieux, Chemin de l'Orme, 69280 Marcy L'Etoile, France), an automated microbial identification system that incorporates mass spectrometry technology - Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) to provide rapid identification.

2.2.4.2. Serological confirmation. For serological confirmation, a rapid dipstick test was used for *V. cholerae* O1 and *V. cholerae* O139 (Span Diagnostics (India) based on the Crystal VC® Rapid Dipstick (VC) test) (Harris et al., 2009).

The VC test was carried out following manufacturer's instructions, whereby a single colony from a TSA plate is picked and homogenized in a sample processing bottle included with the test kit. Four drops of processed sample from the sample processing vial are obtained by breaking

Table 3
Performance of three selective media for detection and presumptive identification of pure cultures of *V. cholerae.*

	TCBS	ChromID™ *Vibrio*	New *Vibrio cholerae* chromogenic medium
V. cholerae strains ($n = 10$)	10	10	10
Sensitivity[a]	100%	100%	100%
Background flora ($n = 47$)	10	1	0
Specificity[b]	79%	96%	100%

[a] Defined as the number of true positives detected on the medium as a function of the total number of true positives tested, expressed as percentage.
[b] Defined as the number of true negatives detected as a function of the total number of true negatives tested, expressed as percentage.

the tip and a VC test dipstick is placed vertically in the test tube. After 15–20 min, the dipstick is removed and test results judged positive, negative, or indeterminate (ambiguous) for *V. cholerae* O1 and for *V. cholerae* O139.

2.2.4.3. Serological confirmation. Antibiotic susceptibility was determined by disk diffusion on Muller-Hinton agar (BD, USA), according to Clinical and Laboratory Standards Institute guidelines for *V. cholerae* CLSI (Clinical and Laboratory Standards Institute) (2015a) and CLSI (Clinical and Laboratory Standards Institute) (2015b). *Escherichia coli* ATCC 25922 was used as a quality control strain. All strains were tested for resistance to ampicillin (10 μg), ciprofloxacin (5 μg), nalidixic acid (30 μg), penicillin (10 μg), sulfamethoxazole-trimethoprim (SXT) (23.75 and 1.25 μg, respectively), and tetracycline (30 μg).

2.2.4.4. DNA extraction and PCR amplification. Genomic DNA was extracted using the boiling protocol described previously by Ausubel et al. (2003). PCR was performed by using a 25 μl reaction mix containing 12.5 μl of GoTaq master mix polymerase (Promega) and 50 ng/μl DNA. For thermal cycling conditions, see Table 1. PCR amplicons were confirmed by sequencing performed by Eurofins Genomics and Invitrogen Vector NTI software was used to compare DNA sequences against the GenBank nucleotide database. *V. cholerae* O1 reference strains N16961, INDRE 91/1, and O395 and *V. cholerae* non-O1/O139 reference strains RC385, RC66, and AM-19226 and *V. cholerae* O139 reference strain MO10 were included as positive and negative controls where appropriate. DNA sequences were determined by Eurofins Genomics (Huntsville, AL, USA). Simpson's index of diversity was used to calculate sample diversity.

PCR experiments were carried out with published primers to detect the presence of genes related to *V. cholerae* identification/virulence/toxicity. There included *toxR* (toxin regulatory gene), *ompW* (encoding the outer membrane protein specific for *V. cholerae*), *ctxA* (encoding cholera toxin A subunit), *ctxB* (encoding cholera toxin B subunit), *zot* (encoding zonula occludes toxin), *ace* (encoding accessory cholera enterotoxin), *rfbO1* and *rfbO139* (lipopolysaccharide biosynthesis of O1 and O139 antigens) (Bhumiratana et al., 2014; Rajpara et al., 2013).

3. Results and discussion

3.1. Designed and evaluation of the new chromogenic medium

3.1.1. Performance of the selective media using pure microbial strains

Table 3 shows overall performance of the three media, when tested against *Vibrio* spp. and the background strains, i.e., strains from species likely also to be present in a non-contaminated water sample.

Sensitivity was 100% for all media. More precisely all *V. cholerae* strains produced presumptive colonies with respect to color indicator (>0.5). However, specificity of TCBS was 79%, since 10/47 non-*V. cholerae* strains were classified as positive (six *Sphingomonas paucimobilis*, two *V. alginolyticus* and two *V. fluvialis*). Specificity of ChromID™ *Vibrio* was 96%, with 1 strain (*E. cloacae*) recorded as false positive. In contrast, the *Vibrio cholerae* chromogenic medium provided 100% specificity. However, two *Vibrio vulnificus* strains revealed very pale coloration, recorded as 0.5 on the established scale, with a value that was, in fact lower than the limit of >0.5 for the two strains to be positive (Fig. 4).

3.1.2. Performance using spiked water samples

Table 4 shows overall performance of *V. cholerae* selective media against spiked water samples.

Ten *Vibrio cholerae* strains were used to spike environmental samples with an inoculum of ~10^5 CFU/ml. These samples were collected from a variety of water sources (spring, river, canal, sea, and hand pump water). All were detected using the *Vibrio cholerae* chromogenic medium without pre-enrichment. Medium coloration was easy to read. That is, it was easy to identify positive colonies, which appear green even in a mixture of background flora that appear as white colonies if able to grow on the medium.

TCBS and chromID™ *Vibrio* agar, however, did not inhibit bacteria naturally present in the water samples sufficiently without pre-enrichment in alkaline peptone medium.

On TCBS agar, *V. cholerae* colonies are bright yellow in a green-yellow background flora. Thus, it's difficult to differentiate the characteristic yellow color of *Vibrio cholerae* colonies. TCBS culture medium was originally developed for isolation of pathogenic vibrios (Kobayashi et al., 1963) and therefore, offers low sensitivity for environmental samples. Many species present in the natural environment or in clinic samples are able to ferment sucrose, as *V. cholerae*, hence differentiation is difficult.

ChromID™ *Vibrio* medium also has a green coloration so that the characteristic green colonies of *V. cholerae* are not easily differentiated from the colonies of the background flora.

Aeromonas hydrophila and *Enterobacter cloacae*, especially, cause false positives to be recorded and these species are natural inhabitant of the aquatic environment (Domingos et al., 2011).

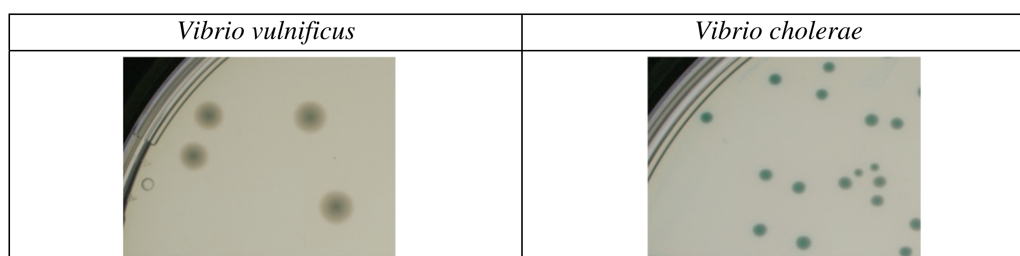

Fig. 4. Characteristic appearance of *V. vulnificus* and *V. cholerae* on *Vibrio cholerae* chromogenic medium.

Table 4
Performance of three selective media for detection and presumptive identification of *V. cholerae* in spiked water samples.

	TCBS	ChromID™ *Vibrio*	New *Vibrio cholerae* chromogenic medium
Sensitivity	2/10	5/10	10/10
Specificity	51%	62%	95%
Readability	–	+	+++

3.2. Design and field based evaluation of the paper based solution

3.2.1. Microbiological performance of PAD and standard reference method

Water samples collected from the five sites included in this study were found to be positive for *Vibrio cholerae* by both PAD and the standard reference method. However, it is important to note the following findings.

Samples differed for each of the two methods because the water was tested by PAD right at the sampling site, whereas the water analyzed traditionally had to be transported to the laboratory for analysis. This is cost saving but, more importantly, it provided instant analyses on site.

The reference method requires TCBS, the premise of which is that isolation of sucrose fermenting colonies is sometimes not *Vibrio cholerae* and, more precisely, may be *Aeromonas hydrophila* or another species of *Vibrio*, i.e., *Vibrio fluvialis* or *Vibrio vulnificus*. Lack of specificity was not observed with PAD and its specific chromogenic substrate.

The chromogenic culture medium in the PAD remains dry during storage of the impregnated PAD, hence is stable, unlike media substrates or antibiotics. Thus, unlike agar plates, the PAD can be stored at room temperature for long periods. Furthermore, for the use PAD in the field a skilled technician is not required.

The total time to result (18 to 24 h) is significantly shorter with the PAD than the reference culture method, since transportation of samples to the laboratory and subsequent enrichment are not required. Incubation begins at the time of addition of water sample to the device.

3.2.2. Characterization of Vibrio cholerae strains isolated from Haitian water

Three strains of *V. cholerae* O1 and 17 *V. cholerae* non-O1/O139 were isolated from 185 surface and drinking water sources in Haiti during 2014 and 2015.

The presence of virulence-associated and regulatory genes, including *ctxA*, *zot*, *ace*, and *toxR* was confirmed using multiplex PCR. Results of genetic characterization of *Vibrio cholerae* isolates from the different sample sites are shown in Table 5.

The three *V. cholerae* O1 isolates were positive for either three or four virulence-associated and regulatory genes examined in this study. Twelve of the *V. cholerae* non-O1/O139 isolates were found to carry *toxR* but none were *ctxA*+, *zot*+, or *ace*+.

Table 5
Results of genetic characterization of *Vibrio cholerae* isolates from water samples collected at various sampling sites in Haiti.

Location of sample	Lateral flow O1/O139	FilmArray™ gastro intestinal panel	Organism	Serology	*ompW*	*rfb* O1	*ctxA*	*rfb* O139	*ctxAB*	*ctx*B type	*ace*	*zot*	Antibiotic resistance pattern
Savane Carrée (Artibonite)	O1	*toxR* +	*V. cholerae*	O1	+	+	+	−	+	*B7*	+	+	P^R, AM^R, NA^R, SXT^R
Savane Carrée (Artibonite)	O1	*toxR* +	*V. cholerae*	O1	+	+	+	−	+	*B7*	+	+	P^R, AM^R, NA^R, SXT^R
Savane Carrée (Artibonite)	O1	*toxR* +	*V. cholerae*	O1	+	+	+	−	+	*B7*	+	+	P^R, NA^R, SXT^R
Savane Carrée (Artibonite)	–	*toxR* −	*V. cholerae*	Non-O1/non-O139	+	−	−	−	−	−	−	−	P^R, AM^R
Savane Carrée (Artibonite)	–	*toxR* −	*V. cholerae*	Non-O1/non-O139	+	−	−	−	−	−	−	−	P^R, AM^R
Mirebalais (Centre)	–	*toxR* +	*V. cholerae*	Non-O1/non-O139	+	−	−	−	−	−	−	−	All sensitive
Mirebalais (Centre)	–	*toxR* +	*V. cholerae*	Non-O1/non-O139	+	−	−	−	−	−	−	−	All sensitive
Mirebalais (Centre)	–	*toxR* +	*V. cholerae*	Non-O1/non-O139	+	−	−	−	−	−	−	−	All sensitive
Mirebalais (Centre)	–	*toxR* −	*V. cholerae*	Non-O1/non-O139	+	−	−	−	−	−	−	−	P^R, AM^R
Mirebalais (Centre)	–	*toxR* +	*V. cholerae*	Non-O1/non-O139	+	−	−	−	−	−	−	−	NA^R, S^R, TE^R, SXT^R
Mirebalais (Centre)	–	*toxR* −	*V. cholerae*	Non-O1/non-O139	+	−	−	−	−	−	−	−	P^R, AM^R
Mirebalais (Centre)	–	*toxR* +	*V. cholerae*	Non-O1/non-O139	+	−	−	−	−	−	−	−	P^R, AM^R, NA^R, CIP^R, SXT^R
Mirebalais (Centre)	–	*toxR* +	*V. cholerae*	Non-O1/non-O139	+	−	−	−	−	−	−	−	All sensitive
Mirebalais (Centre)	–	*toxR* +	*V. cholerae*	Non-O1/non-O139	+	−	−	−	−	−	−	−	P^R, AM^R, NA^R, CIP^R, SXT^R
Pont Saintard (Ouest)	–	*toxR* −	*V. cholerae*	Non-O1/non-O139	+	−	−	−	−	−	−	−	P^R, AM^R
Cabaret (Ouest)	–	*toxR* +	*V. cholerae*	Non-O1/non-O139	+	−	−	−	−	−	−	−	All sensitive
Croix des Bouquets (Ouest)	–	*toxR* +	*V. cholerae*	Non-O1/non-O139	+	−	−	−	−	−	−	−	P^R, AM^R, NA^R, CIP^R, SXT^R
Croix des Bouquets (Ouest)	–	*toxR* +	*V. cholerae*	Non-O1/non-O139	+	−	−	−	−	−	−	−	P^R, AM^R, NA^R, CIP^R, SXT^R
Croix des Bouquets (Ouest)	–	*toxR* +	*V. cholerae*	Non-O1/non-O139	+	−	−	−	−	−	−	−	P^R, AM^R, NA^R, CIP^R, SXT^R
Croix des Bouquets (Ouest)	–	*toxR* +	*V. cholerae*	Non-O1/non-O139	+	−	−	−	−	−	−	−	P^R, AM^R, NA^R, CIP^R, SXT^R

P penicillin, *AM* ampicillin, *NA* nalidixic acid, *SXT* trimethoprim/sulfamethoxazole, *CIP* ciprofloxacin, *S* streptomycin, *TE* tetracycline.

Six of the *V. cholerae* non-O1/non-O139 isolates were resistant to penicillin, ampicillin, trimethoprim/sulfamethoxazole, nalidixic acid, and ciprofloxacin.

The multi-drug resistant *V. cholerae* non-O1/O139 strains isolated from Haitian water samples indicate that the natural environment may serve as a reservoir of multi-drug resistance genes (Chomvarin et al., 2014; Kruse and Sorum, 1994). A similar finding has been reported in Northeastern Thailand (Chomvarin et al., 2014) and in Ghana (Eibach et al., 2016). It is well known that conjugation and transfer of resistance plasmids (R plasmids) are a phenomenon occurring in the natural environment and also between bacterial strains in the human gut, and in animals, including fish, that are unrelated either via evolution or through ecological interaction, even in the absence of any antimicrobial agent. The presence in the environment of strains that are resistant to antimicrobial agents is an indication of a source of disease but also as a source from which R plasmids can spread to microorganisms of diverse origin (Kruse and Sorum, 1994). Consequently, *V. cholerae* non-O1/O139 should be included in monitoring *V. cholerae* with epidemic potential and antimicrobial-agent-resistance.

4. Conclusion

Public health authorities are made aware that cholera transmission can occur via aquatic reservoirs, notably during the rainy season. Also, the antibiotic resistance of environmental *V. cholerae* both O1 and non-O1 isolated should also be monitored.

Currently available diagnostics for *V. cholerae* do not work well for monitoring water in settings where low cost, easy-to-use, and field-rugged solutions are necessary. Reported here is the development of a field test that can be reliably performed in remote settings in the absence of laboratory infrastructure, and not requiring a skilled technician.

The new culture medium embedded in the diagnostic tool (PAD) was proved to be highly selective against most of the cultivable bacteria in the water samples, without loss of sensitivity in detection of *V. cholerae*. *Vibrio cholerae* chromogenic medium is superior in performance, compared to TCBS and chromID™ *Vibrio* agar and, very important to note is that it does not require a pre-enrichment step. Thus, to speed detection and identification of *V. cholerae*, the pre-enrichment step is eliminated and therefore, diagnosis is facilitated.

Thus, reliability of this new culture medium for detection of *V. cholerae* in the presence of other *Vibrio* species in water samples offers a significant advantage.

The paper based device (PAD) containing the new chromogenic medium was proved to be a useful test for *Vibrio cholerae* in water supplies in remote settings, especially resource poor countries. PAD provides a low cost, rapid, reliable test for *V. cholerae* detection.

This field test can be employed to guide corrective action, such as instituting chlorination when water samples prove positive for *Vibrio cholerae*. Moreover, an immunological rapid test can be applied directly on the presumptive *Vibrio cholerae* colonies to determine O1 and O139 serogroups.

Acknowledgements

The authors would like to thank, the Merieux Foundation for financial support. PAH (Pharmacie et Aide Humanitaire) and the Veolia Foundation for logistic in Haiti.

We also wish to acknowledge Pradip Patel, Kavi M. Ramjeet, Nathalie Picot and Sy Rotter for their assistance in reviewing the manuscript.

References

Ausubel, F., Brent, R., Kingston, R., Moore, D., Seidman, J., Smith, J., Struhl, K., 2003. Current Protocols in Molecular Biology. Green Publishing Associates and Wiley, New York.

Bhumiratana, A., Siriphap, A., Khamsuwan, N., Borthong, J., Chonsin, K., Sutheinkul, O., 2014. O serogroup-specific touchdown-multiplex polymerase chain reaction for detection and identification of *Vibrio cholerae* O1, O139, and non-O1/non-O139. Biotechnol. Res. Int. 2014:295421. http://dx.doi.org/10.1155/2014/295421.

Chakraborty, S., Alam, M., Scobie, H.M., Sack, D.A., 2013. Adaptation of a simple dipstick test for detection of *Vibrio cholerae* O1 and O139 in environmental water. Front. Microbiol. 4:320. http://dx.doi.org/10.3389/fmicb.2013.00320.

Chatterjee, S., Ghosh, K., Raychoudhuri, A., Chowdhury, G., Bhattacharya, M.K., Mukhopadhyay, A.K., Ramamurthy, T., Bhattacharya, S.K., Klose, K.E., Nandy, R.K., 2009. Incidence, virulence factors, and clonality among clinical strains of non-O1, non-O139 *Vibrio cholerae* isolates from hospitalized diarrheal patients in Kolkata, India. J. Clin. Microbiol. 47:1087–1095. http://dx.doi.org/10.1128/JCM.02026-08.

Chin, C.S., Sorenson, J., Harris, J.B., Robins, W.P., Charles, R.C., Jean-Charles, R.R., Bullard, J., Webster, D.R., Kasarskis, A., Peluso, P., Paxinos, E.E., Yamaichi, Y., Calderwood, S.B., Mekalanos, J.J., Schadt, E.E., Waldor, M.K., 2011. The origin of the Haitian cholera outbreak strain. N. Engl. J. Med. 364:33–42. http://dx.doi.org/10.1056/NEJMoa1012928.

Chomvarin, C., Jumroenjit, W., Tangkanakul, W., Hasan, N.A., Chaicumpar, K., Faksri, K., Huq, A., 2014. Genotype and drug resistance of clinical and environmental *Vibrio cholerae* non-O1/non-O139 in northeastern Thailand. Southeast Asian J. Trop. Med. Public Health 45, 1354–1364.

CLSI (Clinical and Laboratory Standards Institute), 2015a. Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria. Guideline M45, third ed. Clinical Laboratory Standards Institute, Wayne, PA.

CLSI (Clinical and Laboratory Standards Institute), 2015b. Performance Standards for Antimicrobiological Susceptibility Testing; Twenty-fifth Informational Supplement. M100-S25. Clinical Laboratory Standards Institute, Wayne, PA.

de Magny, G.C., Mozumder, P.K., Grim, C.J., Hasan, N.A., Naser, M.N., Alam, M., Sack, R.B., Huq, A., Colwell, R.R., 2011. Role of zooplankton diversity in *Vibrio cholerae* population dynamics and in the incidence of cholera in the Bangladesh Sundarbans. Appl. Environ. Microbiol. 77:6125–6132. http://dx.doi.org/10.1128/AEM.01472-10.

Domingos, M.O., Franzolin, M.R., dos Anjos, M.T., Franzolin, T.M., Barbosa Albes, R.C., de Andrade, G.R., Lopes, R.J., Barbaro, K.C., 2011. The influence of environmental bacteria in freshwater stingray wound-healing. Toxicon 58:147–153. http://dx.doi.org/10.1016/j.toxicon.2011.04.016.

Dutta, D., Chowdhury, G., Pazhani, G.P., Guin, S., Dutta, S., Ghosh, S., Rajendran, K., Nandy, R.K., Mukhopadhyay, A.K., Bhattacharya, M.K., Mitra, U., Takeda, Y., Nair, G.B., Ramamurthy, T., 2013. *Vibrio cholerae* non-O1, non-O139 serogroups and cholera-like diarrhea, Kolkata, India. Emerg. Infect. Dis. 19:464–467. http://dx.doi.org/10.3201/eid1903.121156.

Eddabra, R., Piemont, Y., Scheftel, J.M., 2011. Evaluation of a new chromogenic medium, chromID *Vibrio*, for the isolation and presumptive identification of *Vibrio cholerae* and *Vibrio parahaemolyticus* from human clinical specimens. Eur. J. Clin. Microbiol. Infect. Dis. 30:733–737. http://dx.doi.org/10.1007/s10096-010-1145-2.

Eibach, D., Herrera-Leon, S., Gil, H., Hogan, B., Ehlkes, L., Adjabeng, M., Kreuels, B., Nagel, M., Opare, D., Fobil, J.N., May, J., 2016. Molecular epidemiology and antibiotic susceptibility of *Vibrio cholerae* associated with a large cholera outbreak in Ghana in 2014. PLoS Negl. Trop. Dis. 10, e0004751. http://dx.doi.org/10.1371/journal.pntd.0004751.

Haley, B.J., Choi, S.Y., Hasan, N.A., Abdullah, A.S., Cebula, T.A., Huq, A., Colwell, R.R., 2013. Genome sequences of clinical *Vibrio cholerae* isolates from an oyster-borne cholera outbreak in Florida. Genome Announc. 1. http://dx.doi.org/10.1128/genomeA.00966-13.

Harris, J.R., Cavallaro, E.C., de Nobrega, A.A., Dos, S., Barrado, J.C., Bopp, C., Parsons, M.B., Djalo, D., Fonseca, F.G., Ba, U., Semedo, A., Sobel, J., Mintz, E.D., 2009. Field evaluation of crystal VC Rapid Dipstick test for cholera during a cholera outbreak in Guinea-Bissau. Tropical Med. Int. Health 14:1117–1121. http://dx.doi.org/10.1111/j.1365-3156.2009.02335.x.

Harris, J.B., LaRocque, R.C., Qadri, F., Ryan, E.T., Calderwood, S.B., 2012. Cholera. Lancet 379:2466–2476. http://dx.doi.org/10.1016/S0140-6736(12)60436-X.

Hasan, N.A., Choi, S.Y., Eppinger, M., Clark, P.W., Chen, A., Alam, M., Haley, B.J., Taviani, E., Hine, E., Su, Q., Tallon, L.J., Prosper, J.B., Furth, K., Hoq, M.M., Li, H., Fraser-Liggett, C.M., Cravioto, A., Huq, A., Ravel, J., Cebula, T.A., Colwell, R.R., 2012. Genomic diversity of 2010 Haitian cholera outbreak strains. Proc. Natl. Acad. Sci. U. S. A. 109: E2010–E2017. http://dx.doi.org/10.1073/pnas.1207359109.

Huq, A., Colwell, R.R., Rahman, R., Ali, A., Chowdhury, M.A., Parveen, S., Sack, D.A., Russek-Cohen, E., 1990. Detection of *Vibrio cholerae* O1 in the aquatic environment by fluorescent-monoclonal antibody and culture methods. Appl. Environ. Microbiol. 56, 2370–2373.

Huq, A., Xu, B., Chowdhury, M.A., Islam, M.S., Montilla, R., Colwell, R.R., 1996. A simple filtration method to remove plankton-associated *Vibrio cholerae* in raw water supplies in developing countries. Appl. Environ. Microbiol. 62, 2508–2512.

Islam, M.S., Mahmud, Z.H., Ansaruzzaman, M., Faruque, S.M., Talukder, K.A., Qadri, F., Alam, M., Islam, S., Bardhan, P.K., Mazumder, R.N., Khan, A.I., Ahmed, S., Iqbal, A., Chitsatso, O., Mudzori, J., Patel, S., Midzi, S.M., Charimari, L., Endtz, H.P., Cravioto, A., 2011. Phenotypic, genotypic, and antibiotic sensitivity patterns of strains isolated from the cholera epidemic in Zimbabwe. J. Clin. Microbiol. 49:2325–2327. http://dx.doi.org/10.1128/JCM.00432-11.

Jeandron, A., Saidi, J.M., Kapama, A., Burhole, M., Birembano, F., Vandevelde, T., Gasparrini, A., Armstrong, B., Cairncross, S., Ensink, J.H., 2015. Water supply interruptions and suspected cholera incidence: a time-series regression in the Democratic Republic of the Congo. PLoS Med. 12, e1001893. http://dx.doi.org/10.1371/journal.pmed.1001893.

Kahler, A.M., Haley, B.J., Chen, A., Mull, B.J., Tarr, C.L., Turnsek, M., Katz, L.S., Humphrys, M.S., Derado, G., Freeman, N., Boncy, J., Colwell, R.R., Huq, A., Hill, V.R., 2015. Environmental surveillance for toxigenic *Vibrio cholerae* in surface waters of Haiti. Am.J.Trop. Med. Hyg. 92:118–125. http://dx.doi.org/10.4269/ajtmh.13-0601.

Kobayashi, T., Enomoto, S., Sakazaki, R., Kuwahara, S., 1963. A new selective isolation medium for the vibrio group; on a modified Nakanishi's medium (TCBS agar medium). Nippon Saikingaku Zasshi 18, 387–392.

Kruse, H., Sorum, H., 1994. Transfer of multiple drug resistance plasmids between bacteria of diverse origins in natural microenvironments. Appl. Environ. Microbiol. 60, 4015–4021.

Liu, J., Winstead-Derlega, C., Houpt, E., Heidkamp, R., Pape, J., Dillingham, R., 2014. Pre-earthquake non-epidemic *Vibrio cholerae* in Haiti. J. Infect. Dev. Ctries. 8:120–122. http://dx.doi.org/10.3855/jidc.4524.

Marin, M.A., Thompson, C.C., Freitas, F.S., Fonseca, E.L., Aboderin, A.O., Zailani, S.B., Quartey, N.K., Okeke, I.N., Vicente, A.C., 2013. Cholera outbreaks in Nigeria are associated with multidrug resistant atypical El Tor and non-O1/non-O139 *Vibrio cholerae*. PLoS Negl. Trop. Dis. 7, e2049. http://dx.doi.org/10.1371/journal.pntd.0002049.

Mookerjee, S., Batabyal, P., Sarkar, M.H., Palit, A., 2015. Seasonal prevalence of enteropathogenic vibrio and their phages in the riverine estuarine ecosystem of south Bengal. PLoS One 10, e0137338. http://dx.doi.org/10.1371/journal.pone.0137338.

Mutreja, A., Kim, D.W., Thomson, N.R., Connor, T.R., Lee, J.H., Kariuki, S., Croucher, N.J., Choi, S.Y., Harris, S.R., Lebens, M., Niyogi, S.K., Kim, E.J., Ramamurthy, T., Chun, J., Wood, J.L., Clemens, J.D., Czerkinsky, C., Nair, G.B., Holmgren, J., Parkhill, J., Dougan, G., 2011. Evidence for several waves of global transmission in the seventh cholera pandemic. Nature 477:462–465. http://dx.doi.org/10.1038/nature10392.

Rajpara, N., Vinothkumar, K., Mohanty, P., Singh, A.K., Singh, R., Sinha, R., Nag, D., Koley, H., Kushwaha, B.A., 2013. Synergistic effect of various virulence factors leading to high toxicity of environmental *V. cholerae* non-O1/ non-O139 isolates lacking ctx gene: comparative study with clinical strains. PLoS One 8, e76200. http://dx.doi.org/10.1371/journal.pone.0076200.

Rice, E., Baird, R., Eaton, A., Clesceri, L., 2012. Standard Methods for the Examination of Water and Wastewater 22. American Public Health Association, Washington DC.

Ryan, E.T., 2013. Eyes on the prize: lessons from the cholera wars for modern scientists, physicians, and public health officials. Am.J.Trop. Med. Hyg. 89:610–614. http://dx.doi.org/10.4269/ajtmh.13-0173.

Shah, S.R., 2010. Standard analytical protocol for *Vibrio cholerae* O1 and O139 in drinking water and surface water. EPA 600/R-10/139. United States Environmental Protection Agency, Whashington, DC.

Uchiyama, H., 2000. Distribution of vibrio species isolated from aquatic environments with TCBS agar. Environ. Health Prev. Med. 4:199–204. http://dx.doi.org/10.1007/BF02931258.

World Health Organization, 2014. Cholera, 2013. Weekly Epidemiological Record 89 pp. 345–355.