Performance of a Cholera Rapid Test in the Setting of High Lytic Phage and Antibiotic Burden: A Prospective Diagnostic Study

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Introduction

A *Vibrio cholerae* rapid test with high sensitivity and specificity would enable more effective evidence-based outbreak response[1], yet is currently not available[2]. Most *V. cholerae* rapid tests rely on antibodies that target the oligo-saccharide of *V. cholerae*[2]. The performance metrics of these dipstick tests use the gold standard of culture in reference laboratory[3-13] and field settings[8,9]. Limited studies have used PCR as a standard[3,14]. Immediately testing stool samples demonstrates relatively high sensitivity (58-100%) and generally lower specificity (71-100%). Modified methods use an incubation of 6-24 hours in selective media (alkaline peptone water (APW)) to increase specificity (91-99%) yet result in a modest reduction in sensitivity[5,6,12]. The broad performance range of these tests has hindered uptake and been postulated to be linked to antimicrobial agents[3]. The objectives of this study were to validate the POC test at a remote cholera outbreak using qPCR as the gold standard and rigorously test the impact of antimicrobial agents on performance. We chose to focus on two antimicrobial agents: lytic vibriophage because they can decrease viable bacterial counts in patient samples by 1000-fold[15], and clinically relevant antibiotics because they are ubiquitous and decrease the duration of illness, number of bacteria shed, and severity of disease[16]. This study is one of the largest studies to date to assess *V. cholerae* rapid-tests.

Collection Methods

This clinical study was conducted at a district and sub-district government hospital in the remote district of Netrokona (2.2. million people) in northern Bangladesh. **Inclusion criteria** were patients two-months of age and older who presented with acute (< 7 days) diarrhea (>3 loose stools in the 24 hours prior to admission). **Patients with comorbidities were excluded** (e.g. respiratory failure, severe malnutrition, sepsis). Patients were prospectively enrolled from September to December 2015 with brief disruptions (e.g. holidays, weekends, strikes). This study was approved by the Institute for Epidemiology, Disease Control and Research (IEDCR) at the Bangladesh Ministry of Health and Family Welfare and Stanford University IRB(s). The first stool and urine sample voided immediately after admission were collected. The intent was to collect samples prior to the admission of hospital antibiotics:

Urine: 1.5 ml was collected and stored at -20 deg C.

Stool: 2 ml was placed in 6 ml of RNAlater and stored at 4 deg C for up to 6 months at the field site. The remaining stool sample was tested with the POC test or stored in Cary Blair media.

Analytic Methods

Culture/ serotyping. A subset of stool samples (first and last patient per day) underwent targeted culture and sensitivity testing for *V. cholerae*, *Salmonella* spp. and *Shigella* spp. These results were used to guide clinical care, not as a gold standard for the POC test.

Modified dipstick assay. The CrystalVC™ test was selected for use in this study given its availability, stability, and establishment as the primary test deployed. An established protocol was further modified to accommodate for lack of an incubator and 8-hour work day. At the central laboratory, 5 ml of sterile APW was aliquoted into a ml sterile Falcon tube. A cotton swab was placed in a stool specimen and then transferred to the APW tube, the stick was broken leaving the cotton swab immersed in the APW tube, and the sample was incubated at room temperature (6 hours or overnight). After incubation, 2-4 drops from the meniscus of the APW media was transferred to the reagent bottle supplied by the kit using a disposable plastic pipette and the test was read by 15 minutes.

Molecular detection of *V. cholerae* and *V. cholerae* lytic phages. DNA from stool samples suspended in RNALater was extracted using the MoBio 96-well sample power soil kit. Positive controls were $5e^8$ and $1e^8$ CFU/ml *V. choleare,* vibriophage ICP1/2/3 isolates and no-template controls. DNA extracts were screened for *V. cholerae* in a 384-well qPCR format (Light Cycler, Roche) in technical replicates using established qPCR primers for *pilA*; standard curves were performed to assess limits of detection using the mock-stool sample controls. Samples that had CT values less than 25 were labeled positive for *V. cholerae*. Samples with CT values between 25 and less than 31 were independently screened using conventional PCR using established *ompW* primers.

Mass spectrometry detection of clinically relevant antibiotics. A qualitative method was developed to screen patients for 14 antibiotics and 3 common non-antibiotic medications used in the treatment of diarrheal disease. In brief, protein was precipitated with methanol, sonication, and centrifugation followed by further dilution in methanol/0.1% formic acid (1:1) and analysis (a.k.a. 'dilute and shoot'). Spectra were obtained by a unified LC/MS method using an 1100 series HPLC (Agilent Technologies) integrated with an LTQ XL ion trap mass spectrometer (Thermo Fisher Scientific).

Data collection and statistical analysis. Data were collected by the field team using the Outbreak Responder software platform. This platform enables team-based clinical and laboratory data collection on/offline in locations with limited connectivity.

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Results

Diarrheal Case and Phage[ICP1] Distribution

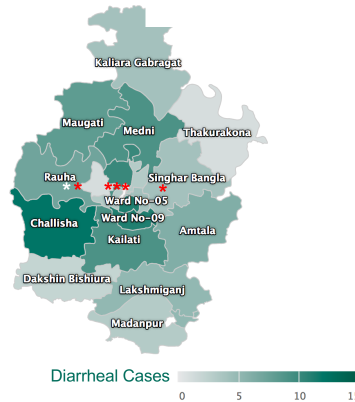


Fig 1. Diarrheal cases (green) and clustering of 6 cholera cases with lytic phage ICP1 (Netrokona Sadar sub-district, Northern Bangladesh). Five of six cases were within 17 days (*); diarrheal cases shown range from +/- 7 days of the ICP1 cases. Data visualized with Outbreak Responder/ highcharts.

Rapid Test Performance

	V. cholerae+	*V. cholerae-*	
Rapid Test +	22	2	24
Rapid Test -	33	808	841
	55	810	865

Sensitivity = 0.40; Specificity = 0.97; PPV=0.92; NPV=0.96
V. cholerae detected by qPCR for *tcpA*; CT values between 25 to less than 31 were further screened for *ompW*

Phage (ICP1) Impact on Rapid Test

	Rapid +	Rapid -	
ICP1+	0	5	5
ICP1-	22	28	50
	22	33	55

Fisher's Exact Test p = 0.068. Only VC+ samples shown.

Medication Distribution

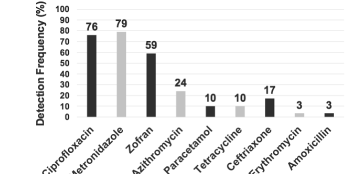
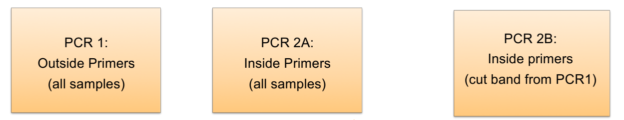


Fig 2. Medications in urine samples from 29 cholera patients at admission. All samples had >= 1 antibiotic.

Method Development



Standard Nested PCR for ICP2

Alternate: Ladder Positive

- PCR 1: External primers were used to amplify a small portion of gp6 of IPC2* (227 bp product).
- PCR 2A: Samples were amplified with internal primers (185 bp product); a subset were sequenced.
- PCR 2B: If PCR2A was negative and a ladder was present in PCR 1, the expected 227 bp band was excised (Fig 3 orange box) and amplified with internal primers; a subset were sequenced.
- Amongst cholera patients, 95% (52/55) were positive for ICP2; three were indeterminate.

*Control and external primers from A. Camilli et al. (Tufts U) and A. Ali (UF)

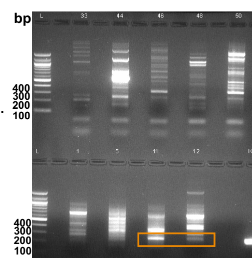


Fig 3. Representative patient samples (9) with ladder formation and ICP2 positive control band (bottom right, no ladder; 227 bp).

Discussion

- Rapid test sensitivity was low (40%) but specificity was high (97%).
- Rapid test was likely negatively impacted by ICP1 (p = 0.068). ICP1 cases clustered tightly as determined by Outbreak Responder.
- ICP2 was present in almost all *V. cholerae* samples tested (>=95%); the impact on the rapid test is unknown. Lytic phage ICP3 was not detected (data not shown).
- Antibiotics were present in all *V. cholerae* samples tested; the impact on the rapid test is unknown.

Limitations

- Small number of ICP1 cases (5) impacted statistical analysis; Case 6 was *V. cholerae* negative (data excluded). In addition biologic plaque assays were not done to assess efficiency of PCR methods.
- Almost all *V. cholerae* samples tested were ICP2 positive. This makes assessing ICP2 impact on diagnostics difficult because there is no negative control.
- Antibiotics are present in all *V. cholerae* samples tested. This makes assessing the impact on diagnostics difficult because there is no negative control.

Conclusion: Despite these limitations, these data reveal limitations of the cholera rapid test and expose that one phage (ICP1) may limit sensitivity. In addition, antibiotics are ubiquitous. These findings will guide future diagnostic development.

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