**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 outbreak response, yet is currently not available. Most V. cholerae rapid tests rely on antibodies that target the Oligo-saccharide of V. cholerae. The performance of these dipsticks tests use the gold standard of culture in reference laboratory¹ and field settings²-⁵. Many studies have used PCR as a standard⁶-⁹. Immediately following stool samples demonstrate relatively high sensitivity (98-100%) and generally lower specificity (71-100%). Modified methods used an incubation of 6-24 hours in selective media (alkaline protease water (APW)) to increase sensitivity (61%) yet result in a modest reduction in sensitivity⁷-⁹. The broad range of results of these tests has hindered uptake and been prototyped to be linked to antimicrobial agents. 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, and clinically relevant antibiotics because they are ubiquitous and decrease the duration of illness, number of bacteria shed, and severity of disease³. This study is one of the largest studies to date to assess V. cholerae rapid-tests.

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**Collection Methods**

This clinical study was conducted at a district and sub-district government hospital in the remote district of Netrakona (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 2015 to December 2015 with brief disruptions (e.g. hospital overloads, strikes). This study was approved by the Institute for Epidemiology, Disease Control and Research (EDCR) at the Bangladesh Ministry of Health and Family Welfare and the Ethics Review Committee of the Institute. Fresh stool and urine samples were collected 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 C.

Stool: 2 mL was placed in 6 mL of RNAlater and stored at 4 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.

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**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 CrystalV™ test was selected for use in this 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 milli 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 (8 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 5a and 14 PFU/ml V. cholerae, vibriophage ICPC2/3 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 pfh; 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 ompR primers.

**Mass spectrometry detection of clinically relevant antibiotics.** A modified method was developed to detect 14 antibiotics and 3 common non-antibiotic medications used in the treatment of diarrheal diseases. The protein was precipitated with methanol/sodium acetate, and centrifugation followed by further dilution in methanol/0.1% formic acid (1:1) and analysis (i.e. "Mile and shuttle"). Spectra were obtained using a UPLC Q-TOF LCMS method using an 1100 series LC/MS (Agilent Technologies) integrated with an LTQ XLQ ion trap mass spectrometer (Thermo Fisher Scientific). Data collection and statistical analysis. Data were collected by the field team using the Outbreak Responders software and team-based clinical and laboratory data collection/online in locations with limited connectivity.

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**Results**

**Diarrheal Case and Phase² Distribution**

The following distribution was used:

- Diarrheal cases (22), 7 with lytic phage ICPC1 (Netrakona Sadar sub-district, Northern Bangladesh). Five of six cases were within 17 days (1), diarrheal cases shown range from >7 days of the ICPC cases. Data visualized with Outbreak Responder heatmaps.

**Table:**

<table>
<thead>
<tr>
<th>Culture</th>
<th>V. cholerae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>22 (100%)</td>
</tr>
<tr>
<td>Negative</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

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**Method Development**

**Fig 1:** Diarrheal cases (green) and clustering of 6 diarrheal cases with lytic phase ICPC1 (Netrakona Sadar sub-district, Northern Bangladesh). Five of six cases were within 17 days (1), diarrheal cases shown range from >7 days of the ICPC cases. Data visualized with Outbreak Responder heatmaps.

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**Diarrheal Cases**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Culture</th>
<th>Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>Positive</td>
<td>ICPC2</td>
</tr>
<tr>
<td>Patient 2</td>
<td>Positive</td>
<td>ICPC1</td>
</tr>
<tr>
<td>Patient 3</td>
<td>Positive</td>
<td>ICPC2</td>
</tr>
<tr>
<td>Patient 4</td>
<td>Positive</td>
<td>ICPC1</td>
</tr>
<tr>
<td>Patient 5</td>
<td>Positive</td>
<td>ICPC2</td>
</tr>
<tr>
<td>Patient 6</td>
<td>Positive</td>
<td>ICPC1</td>
</tr>
</tbody>
</table>

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**Fig 1:** Diarrheal cases (green) and clustering of 6 diarrheal cases with lytic phase ICPC1 (Netrakona Sadar sub-district, Northern Bangladesh). Five of six cases were within 17 days (1), diarrheal cases shown range from >7 days of the ICPC cases. Data visualized with Outbreak Responder heatmaps.

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**Discussion**

- **Rapid test sensitivity was low (40%) but specificity was high (97%).**
- **Rapid test was likely negatively impacted by ICPC1 (p = 0.068).** ICPC1 cases clustered closely as determined by Outbreak Responder.
- **ICPC2 was present in almost all V. cholerae samples tested (>95%); the impact on the rapid test is unknown.** Lytic phase ICPC3 was not detected (data not shown).
- **Antibiotics were present in all V. cholerae samples tested; the impact on the rapid test is unknown.**

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**Limitations**

- **Small number of ICPC1 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 ICPC2 positive.** This makes assessing ICPC2 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 phase (ICPC1) may limit sensitivity. In addition, antibiotics are ubiquitous. These findings will guide future diagnostic development.

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**References**