Gold-standard diagnostics are tarnished by lytic bacteriophage

Running Header: Lytic vibriophage reduce *Vibrio cholerae* detection

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ABSTRACT

Background. A fundamental clinical and scientific concern is how lytic bacteriophage, as well as antibiotics, impact diagnostic positivity.

Methods. Cholera was chosen as a model disease to investigate this important question. Patients with diarrheal disease were enrolled at two remote hospitals in Bangladesh. Diagnostic performance was assessed as a function of lytic bacteriophage detection, as well as exposure to the first-line antibiotic azithromycin detected by mass spectrometry.

Results. Among diarrheal samples positive by nanoliter quantitative PCR for *Vibrio cholerae* (n=78/849), the odds that a rapid diagnostic test (RDT) or qPCR was positive was reduced by 89% (OR 0.108; 95%CI 0.002-0.872) and 87% (OR 0.130; 95%CI 0.022-0.649) when lytic bacteriophage were detected, respectively. The odds that a rapid diagnostic test (RDT) or qPCR was positive was reduced by more than 99% (OR 0.00; 95% CI: 0.00-0.28) and 89% (OR 0.11; 95% CI: 0.03-0.44) when azithromycin was detected, respectively.

Conclusions. Estimations of cholera burden may improve by accommodating for the negative effect of antimicrobial exposure on diagnostic positivity. Furthermore, the findings herein challenge our current approach to interpreting and developing bacterial diagnostics given variable rates of lytic bacteriophage and antibiotic exposure.

BACKGROUND

There are approximately 4.5 billion diarrheal diseases cases per year [1]. While the 2-4 million cases of cholera that occur annually represent a small fraction of the total cases [2], cholera inflicts high morbidity and mortality on populations with extreme poverty. Outbreaks begin when immunologically susceptible human hosts are exposed to the Gram-negative pathogen *V. cholerae* (O1 and O139 serogroups) from contaminated food or water [3]. Before modern rehydration regimens, mortality rates rose above 20% [4] from acute secretory diarrhea resulting from the action of cholera toxin [3]. Treated with oral or intravenous rehydration, mortality rates decrease to less than one percent [5, 6]. Antibiotics are recommended for cholera patients with moderate to severe dehydration [7, 8], but in practice most cholera patients are likely ordered antibiotics. Asymptomatic cases are detected by a rise in antibody titer but negative stool studies [9]. Recovered patients become susceptible over time as a function of the durability of their immune response [3]. *V. cholerae* are shed from the human host with increased infectivity [10, 11]. This 'hyper-infectivity' is suggested to drive the exponential phase of outbreaks [12].

Patients can shed *V. cholerae* specific lytic bacteriophage (ICP1, 2, 3); ICP1 is specific for O1 *V. cholerae* [13, 14]. These vibriophages are proposed to quench outbreaks based on data that a higher percentage of patients shed vibriophage during the collapse of an outbreak [15-17]. Diagnostically, culture and PCR approaches are the best available 'gold-standards' for the detection of *V. cholerae* [18, 19]. Alternative methods include direct immuno-fluorescence microscopy for the O-antigen polysaccharide (OPS) [20], RDTs that rely on OPS specific antibodies, and recently by nl-qPCR [21, 22].

The rationale for this study was based on the recognition that cholera RDTs have limited adoption because of variable performance for unknown reasons [19, 23-26]; immediately testing stool samples demonstrated broad sensitivities (58-100%) and specificity (71-100%). A modified

method enriches for *V. cholerae* in alkaline peptone water (APW) to increase specificity to 91-99% yet is associated with a decrease in sensitivity [23, 24, 27]. Both lytic phage and antibiotics have been postulated to impact diagnostics [26]. Using cholera as a model, we tested the hypothesis that lytic bacteriophage, and antibiotics, negatively impact diagnostics within the confines of a previously published clinical study [28]. In brief, the study was conducted from September to December 2015 at a district and sub-district hospital in the remote Northern district of Netrokona that is prone to seasonal cholera outbreaks. Inclusion criteria were patients at least two-months old and presented with acute (< 7 days) diarrhea (>3 loose stools in the 24 hours prior to admission) without complications.

METHODS

Subjects

This study was conducted with the confines of previously published studies in Bangladesh [28] and South Sudan [29]. Ethical approvals were obtained for the Bangladesh study at the Institutional Review Boards (IRBs) of Stanford University School of Medicine and the Institute of Epidemiology, Disease Control and Research, Bangladesh Ministry of Health and Family Welfare [28], and for South Sudan Study at the IRBs of Johns Hopkins Bloomberg School of Public Health and the South Sudan Ministry of Health, Directorate of Monitoring, Evaluation and Research [29]. Written informed consent was obtained from participants over 18 years, or guardians of participants.

Clinical Study

In Bangladesh, inclusion criteria were patients at least two-months of age presenting with acute (< 7 days) diarrhea (>3 loose stools in the prior 24 hours) without clinical complications. Sample collection occurred from September to December 2015 at a district and sub-district hospital in the remote Northern district of Netrokona that is prone to seasonal cholera outbreaks. In South Sudan, inclusion criteria were patients presenting at a cholera treatment center in Juba who

were at least 6 months-old, had diarrhea (>3 loose stools in the prior 24 hours) and no history of cholera vaccination. Samples were collected from August to September 2015.

Laboratory Procedures

For samples collected in Bangladesh, the methods have been previously described [21, 22]. In brief, the first stool sample voided was collected immediately after admission to avoid exposure to hospital administered antibiotics. The supernatants from *V. cholerae* positive stools were tested for antibiotic exposure using a LC/MS protocol for a 1100 series HPLC (Agilent Technologies) integrated with an LTQ XL ion trap mass spectrometer (Thermo Fisher Scientific) [21]. The stool samples were tested by RDT (Crystal VC, Span Diagnostics) after enrichment in APW for 6 hours or overnight [28]. The first and last samples collected per day were stored in Cary-Blair media (4°C) for culture at a central reference laboratory in Dhaka (icddr,b); samples were stored for up to 1 month. Aliquots (500 μl) from all patients were stored in 1.3ml RNA*later* (Invitrogen).

For Bangladesh samples, DNA was extracted using the MoBio Power Soil 96-well plate system (Qiagen; formerly PowerSoil). DNA extracts were screened in technical replicates for *V. cholerae* by qPCR in a 384-well Light Cycler (Roche) using *tcpA*^{set1} primers (Table S1) [21]. Samples that had CT values less than 25 were defined as positive. Samples with CT values from 25 to 31 were evaluated by PCR for *ompW* [8]. In parallel, nl-qPCR was performed in technical replicates with *tcpA*^{set1} and additional targets [21, 22]. Cyber Green master mix (Sigma Aldrich) was used for both qPCR and nl-qPCR however there was 1.8-fold more DNA in nl-qPCR reactions. Cycle thresholds for positivity for qPCR and nl-qPCR were 29 and 28, respectively. 16S rDNA analysis utilized previously published methods and data [21] on nl-qPCR *V. cholerae* positive samples for *tcpA* (Table S1). Lytic vibriophages ICP1, 2, and 3 were detected by PCR (Table S1). For samples collected in South Sudan, analyses for *V. cholerae*

have been previously described on DNA extracted from dried stool spots [29]. In addition, the extracts were analyzed by PCR for ICP1 and ICP3 (ICP2 PCR technically failed; Table S1).

Direct immune-fluorescence was performed as previously described on planktonic cells from RNA*later* preserved stool samples [30]. This fraction was obtained by a 15 seconds 100-G centrifugation to remove sediment from 500 μl of sample, one PBS wash, pelleting the supernatant fraction, and resuspension of the pellet in 500 μl of PBS with 3.7% formalin. Mock positive control stool samples were used for molecular and microscopy assays that consisted of *V. cholerae* set to concentrations relative to cholera stool (5e8 CFU/ml and 1e8CFU/ml) in 500 ml normal saline plus 1.3 ml RNA*later* (ratio used in stool storage).

Statistical analysis

Latent class modeling was used to estimate sensitivities and specificities of each diagnostic [31]. For prior information, the assumptions for sensitivities were the same for RDT, qPCR, nl-qPCR and culture (50-100%). Assumptions for specificities were 50-100% for RDT, 90-100% for qPCR and nl-qPCR, and 99-100% for culture [18]. Gibbs sampling with 100,000 iterations was used to generate posterior estimates with 95% credible intervals (CI). Fischer's exact test was used to evaluate associations between diagnostic type and detection of lytic bacteriophage / azithromycin. Both sample odds ratios and estimated sample odds ratios with a conditional Maximum Likelihood Estimate were computed. A two-sample Wilcoxin test was used to compare CT values between diagnostic positive and negative samples among samples positive for *V. cholerae* positive by nl-qPCR CT. Comparison of microbiota (16S rDNA analysis) by diagnostic result and exposure among nl-qPCR positive samples was conducted by PERMANOVA as previously described [21]. Missingness in the dataset is designated as 'NA' and is restricted to laboratory results. Statistical analyses were completed in Graphpad Prism 8.0.1 and R v3.4.1 / RStudio v1.1.0153 [32].

Data availability

Data analyzed in the manuscript have been made available in the online supplementary material.

RESULTS

Sensitivity and specificity estimates by latent class modeling

In Bangladesh, stool samples were collected from 881 of 961 enrolled patients. Among samples tested by RDT, qPCR, and nl-qPCR, the distribution of diagnostic positivity is provided (Fig 1A,B). The sensitivities and specificities of each diagnostic were estimated using a Bayesian latent class modeling framework, which enables estimation of diagnostic accuracy in the absence of a perfect reference standard by integrating data from multiple tests [31]. Estimates for sensitivity of RDT, qPCR, and nl-qPCR were 31.5% (95% CI:21.5–43.7), 64.1% (CI: 50.7-80.2) and 97.6% (95% CI: 89.0–100.0), respectively. The specificities were 99.6% (95% CI: 99.0–99.9), 99.9% (95% CI: 99.7–100.0) and 99.6% (95% CI: 98.3–100.0), respectively. Among the subset of samples randomly chose for culture (16 positive out of 251), sensitivity was 57.1% (40.4-73.2) and specificity 99.7 (99.3-99.9). Based on these results, nl-qPCR was selected as the best available reference standard for subsequent analysis and the receiver operator curve (ROC) is presented (Fig 1C).

Impact of lytic phage on diagnostic positivity

Among *V. cholerae* positive samples by nl-qPCR, 19.2% (15/78) and 1.3% (1/78) were positive for ICP1 and 2, respectively; ICP3 was not detected. Of 180 random samples negative by nl-qPCR, qPCR and RDT, two patients had ICP1 (one was culture positive) and one had ICP2. Among *V. cholerae* positive samples by nl-qPCR that lacked azithromycin, vibriophage were negatively associated with diagnostic positivity by RDT (OR 0.11; 95% CI: 0.002-0.87), qPCR (OR 0.13; 95% CI: 0.02-0.65) and direct immuno-fluorescent microscopy [30] (OR 0.18; 95% CI 0.02-1.031; Table 1). Frequencies of vibriophage detection were different between study sites

(Fischer's exact test; p = 0.033). Diarrheal samples from South Sudan were analyzed to increase generalizability [29]. ICP1 was detected in 10.2% (n=10/98) of all enriched samples, 24% of samples (n=7/29) that were PCR positive samples for *V. cholerae* and 5.7% (n=3/69) of samples that were RDT negative by PCR for *V. cholerae*. ICP1 was negatively associated with RDT positivity after enrichment (OR 0.00, 95%CI 0.00-0.64, p=0.010; Table S2); a statistically significant difference was not observed for unenriched samples. ICP3 was not identified. There were insufficient samples to assess phage impact on culture positivity.

Impact of azithromycin on diagnostic positivity

Among Bangladesh samples positive by nl-qPCR but negative for bacteriophage, azithromycin was negatively associated with diagnostic positivity by RDT (OR 0.00; 95% CI: 0.00-0.28) and qPCR (OR 0.11; 95% CI: 0.03-0.44), but not by direct fluorescent microscopy (OR 0.54; 95% CI 0.14-1.97; Table 2). Azithromycin was negatively associated with culture positivity (OR 0.00, 95% 0.00-0.997; Table 2).

Absolute and relative *V. cholerae* concentration.

Absolute and relative *V. cholerae* concentration was assessed by nl-qPCR and 16S rDNA analysis, respectively. Among nl-qPCR positive samples, there was a significant inverse relationship between diagnostic positivity and *V. cholerae* concentration (Table S3). With no exclusions, fold-differences between positive and negative samples ranged from 21-fold (culture) to 79-fold (qPCR). The one exception was that phage exposure (azithromycin samples excluded) did not associate with a significant difference in the nl-qPCR Ct values between culture positive (n=13; Ct = 19.4, 95% CI 14.3-22.0) and negative samples (n=9; Ct =20.8, 95% CI 17.6-25.9; p=0.186). Statistically significant differences in microbiota (16S rDNA) were observed between RDT positive and negative stools with stratifications for bacteriophage (Fig S2A) and azithromycin (Fig S2B).

DISCUSSION

This study investigated the potential vulnerability diagnostics have when bacterial targets are exposed to lytic bacteriophage predation or antibiotics. Using cholera as a model system and nl-qPCR as a reference standard for *V. cholerae,* we found that the odds of a RDT, qPCR and microscopy diagnostic testing positive were reduced by more than 83% when lytic bacteriophage were present. Similarly, the odds of a RDT, qPCR and culture testing positive were reduced by more than 89% when the first-line antibiotic azithromycin was detected in stool by mass spectrometry. These results expose a vulnerability of gold-standard diagnostics that clinicians and microbiologists feared but lacked sufficient data to take evidence-based action.

We reason that the low inflection point in the ROC at approximately 0.7 sensitivity is multi-factorial (Fig 1C). We explored the effect on sensitivity and specificity of adding ICP1 detection as a proxy for *V. cholerae* detection (Fig 1C). Both qPCR, culture and the RDT moderately improved. The effects of lytic bacteriophage, antibiotics and host antimicrobial factors on diagnostic positivity are likely additive, especially given that these diagnostics target different biologic mechanisms. How duration of illness and severity of disease serve as determinants of diagnostic positivity also remain unknown. Time-series analyses of cholera patients with defined antimicrobial exposures are needed steps to further these lines of inquiry.

These findings should be viewed within the context of the limitations of the study. The procedures were chosen for feasibility at remote field sites. This delayed cultures up to one-month and precluded plaque assays. The higher detection rate of nl-qPCR compared to qPCR was multi-factorial, including the 1.8-fold difference in DNA. The positive nl-qPCR samples that were negative by qPCR and negative by *ompW* were unlikely to be false positives because *Vibrio* spp. were detected by 16S rDNA analysis in all 13 samples that did not have lytic vibriophage; those with vibriophage did not result (n=7/7). Among nl-qPCR positive and qPCR

189 negative samples, PCR detection for *tpcA* correlated with PCR detection of *ctxA* (cholera toxin*;*
190 n=5/5; Table S1). These toxin data, paired with serologic results that found only O1 *V. cholerae*,
191 makes the possibility of confounding from non-O1 *V. cholerae* unlikely. Despite these
192 limitations, the discovery that lytic bacteriophage negatively impacts diagnostics, even to the
193 point that samples will test positive for bacteriophage and negative for the pathogen, has broad
194 significance. One explanation is lytic bacteriophage and antibiotics inhibit bacterial growth below
195 the diagnostic limits of detection. Alternatively, bacteriophage nucleases, or host nucleases
196 responding to bacteriophage infection, may digest host chromosomal DNA to the point that PCR
197 fails [33, 34].

198 **Conclusion**

199 Within the cholera field, this study suggests that more nuanced analytical approaches are
200 needed to determine the true cholera burden during outbreaks, especially in the latter phases
201 when rates of concurrent lytic bacteriophage predation are likely higher [16, 17]. This may
202 require an approach that includes lytic bacteriophage detection as a proxy for pathogen
203 detection and a de-emphasis on diagnostic results with known antibiotic exposure. Outside the
204 cholera field, these data serve as a call-to-action to survey for lytic bacteriophage when
205 bacterial diagnostics have inconsistent performance, especially when there is discordance
206 between clinical presentation and diagnostic result. These efforts may justify a new line of
207 diagnostic development that targets both the prey (pathogen) and predator (bacteriophage).

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Disclaimer

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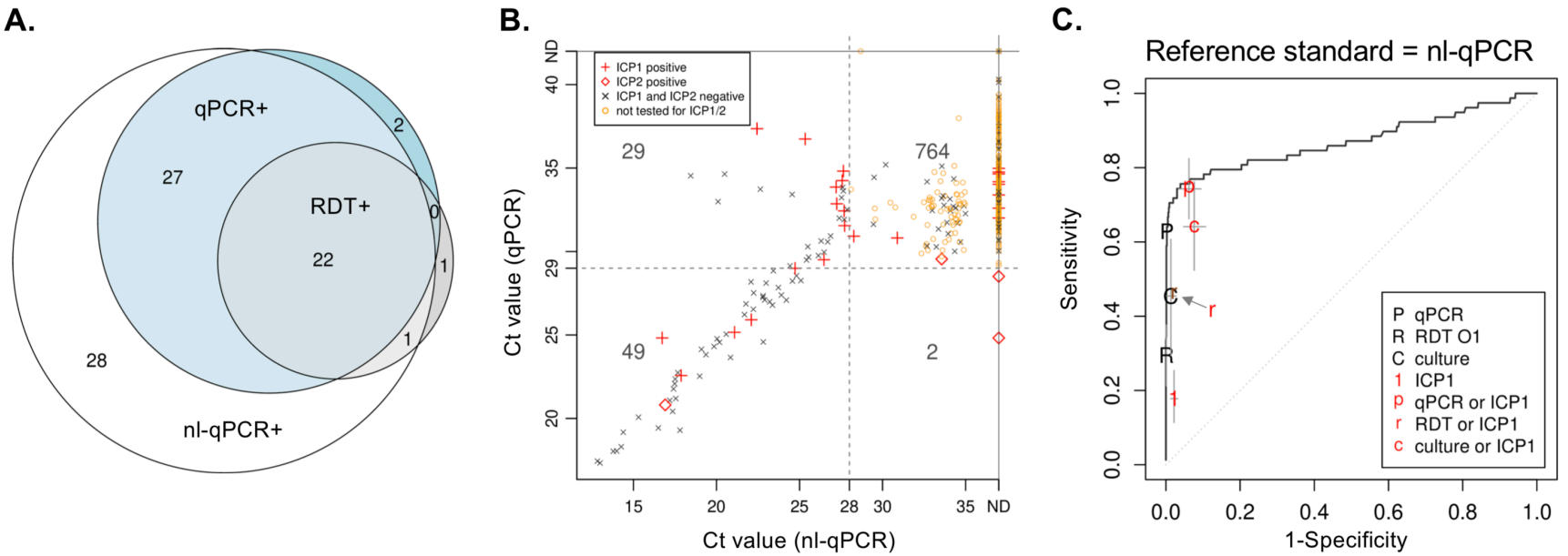
Potential conflicts of interest.

All authors: No reported conflicts.

FIGURE LEGENDS

Figure 1. Diagnostic evaluation. **A.** Euler diagram of diagnostic positivity for qPCR, nl-qPCR, and RDT; area within each circle is relative to the degree of positivity. **B.** Comparison Ct values between qPCR and nl-qPCR analysis with ICP1 and ICP2 metadata; horizontal and vertical dotted lines depict thresholds of positivity for each test; ND= not detected. **C.** Receiver operator characteristic (ROC) curve. Estimates of the sensitivity and 1-specificity of combining diagnostics are defined in the key and vertical bars from each symbol depict the 95% CI.

Insert 1 (Figure 1)



Insert 2 (Table 1)

Table 1.

Lytic phage negatively impact diagnostic positivity (azithromycin excluded)

Diagnostic (Dx)	N[a]	Dx positive among phage exposed	Dx positive among phage unexposed	OR[b]	OR_MLE[c]	CI[c]	p[d]
RDT	56	9% (1/11)	49% (22/45)	0.105	0.108	0.002-0.872	0.019
qPCR	56	36% (4/11)	83% (37/45)	0.124	0.130	0.022-0.649	0.005
Microscopy[e]	52	20% (2/10)	60% (25/42)	0.170	0.176	0.016-1.031	0.036
Culture[f]	22	--- (0/0)	59% (13/22)	---	---	---	---

[a] *V. cholerae* positive samples by nl-qPCR without azithromycin detected in the stool by mass spectrometry
[b] Sample odds ratio =OR
[c] Estimated odds ratio with conditional Maximum Likelihood Estimate (MLE); CI = 95th confidence interval
[d] Fischer's exact test
[e] Indeterminant samples were considered negative; limit of detection 100-1000 CFU/ ml
[f] Insufficient samples with phage for statistical analysis

Insert 3 (Table 2)

Table 2.

Azithromycin negatively impacts diagnostic positivity (phage excluded)

Diagnostic (Dx)	N[a]	Dx positive among azithro exposed	Dx positive among azithro unexposed	OR[b]	OR_MLE[c]	CI[c]	p[d]
RDT	63	0% (0/18)	49% (22/45)	0.000	0.000	0.000-0.282	<0.001
qPCR	63	33% (6/18)	82% (37/45)	0.108	0.113	0.026-0.437	<0.001
Microscopy[e]	58	44% (7/16)	60% (25/42)	0.529	0.535	0.139-1.973	0.378
Culture[f]	27	0% (0/5)	59% (13/22)	0.000	0.000	0.000-0.997	0.041

[a] *V. cholerae* positive samples by nl-qPCR without lytic bacteriophage (ICP1,2,3) detected in the stool by PCR
[b] Sample odds ratio =OR
[c] Estimated odds ratio with conditional Maximum Likelihood Estimate (MLE); CI = 95th confidence interval
[d] Fischer's exact test
[e] Indeterminant samples were considered negative; limit of detection 100-1000 CFU/ ml
[f] Insufficient samples with phage for statistical analysis

Supplement Material:
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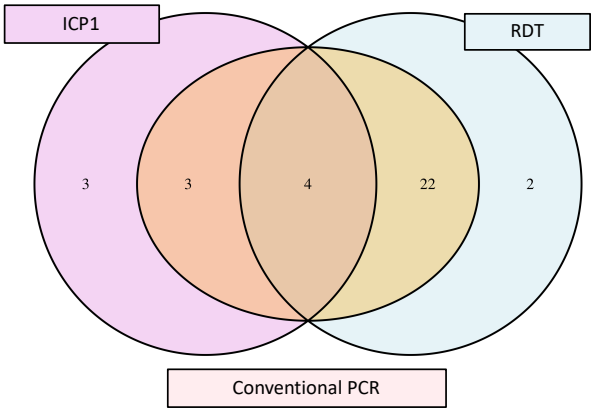
Figure S1

A. South Sudan Positivity



B. Bangladesh Positivity

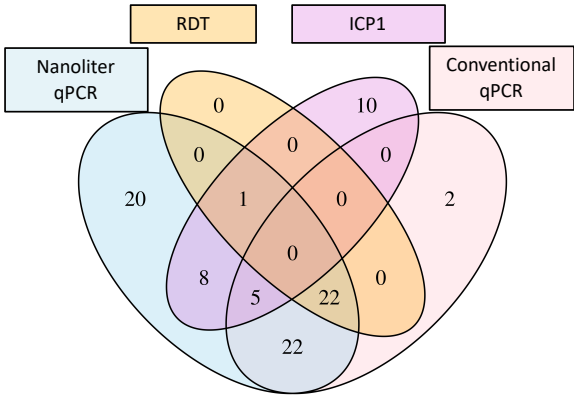
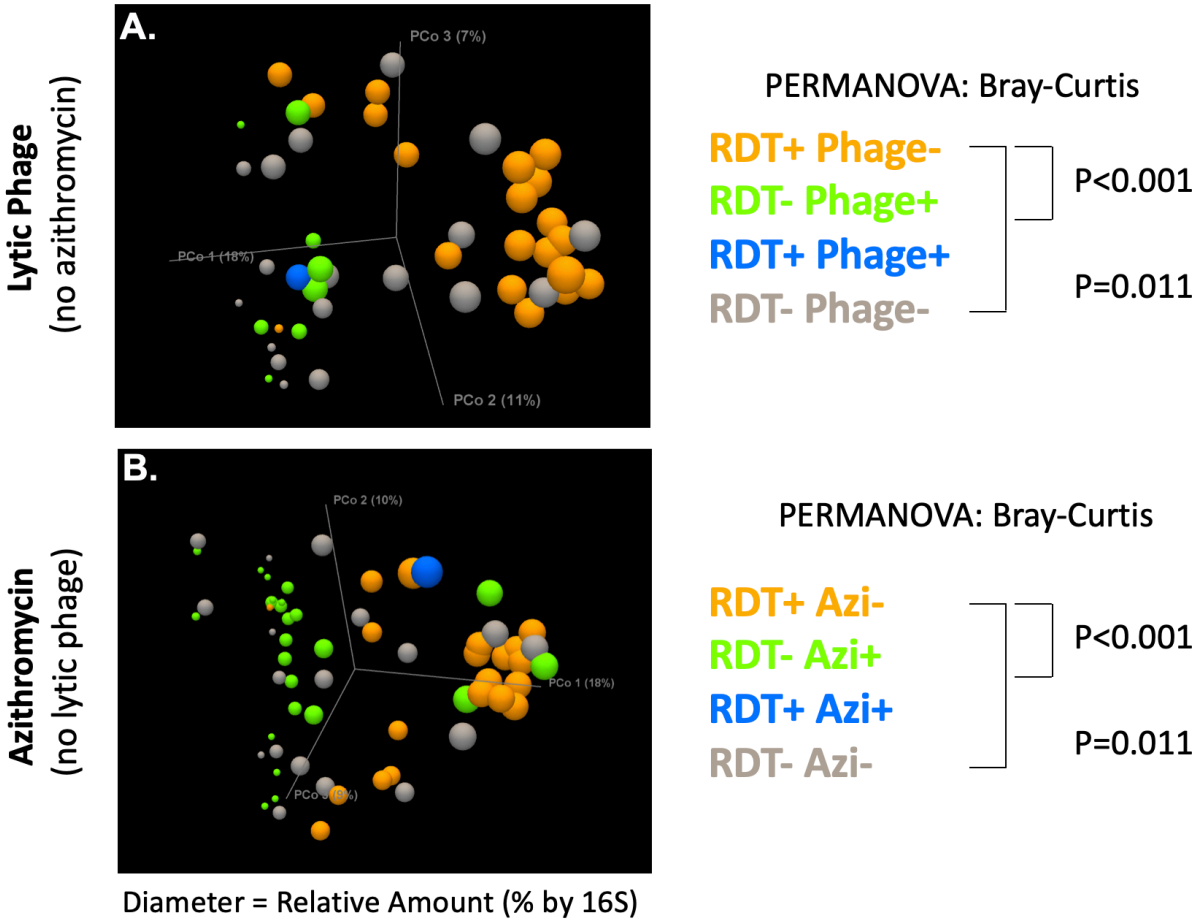


Figure S1. Comparison of diagnostic positivity and ICP1 detection in the libraries from South Sudan **(A)** and Bangladesh **(B).** RDT= rapid diagnostic test. Both settings used the Crystal VC test with enrichment. Data presented from S. Sudan is based on PCR performed at Institute Pasteur (Table S2).

297 **Figure S2**
298



299
300
301 **Figure S2.** Principal component analysis of 16S rDNA analysis of *V. cholerae* positive samples
302 by nl-qPCR analysis as previously described[21]. **A.** Among samples without azithromycin
303 detection, data are color coded based on RDT and phage positivity (right). **B.** Among samples
304 without bacteriophage detection, data are color coded based on RDT and azithromycin positivity
305 (right). PC1 is oriented horizontally and the icon size is set to the relative percentage of *V.*
306 *cholerae* detected in the microbiota (0-25%, 26-50%, 51-75%, 76-100%). For both the upper
307 and lower panels, statistically significant differences between groups were detected by
308 PERMANOVA (Bray-Curtis)[21]. *V. cholerae* positivity is defined by nl-qPCR positivity with
309 either tcpA primer sets to be consistent with prior analytic approach[21].
310
311

Table S1. Reagents

Reagent			
Bacterium	**Strain**	**Description**	**Reference**
V. cholerae	E7946	O1 serogroup, isolated from Bahrain*, SmR	Mekalanos [35]
V. cholerae	ENV2	Non-O1, from Haiti*, SmS	Rahman et al. [36]
V. cholerae	VC037; 280 NAG	Non-O1, from India*, ATCC25872, SmR	Felsenfeld et al. [37]
PCR target	**Primer name**	**Sequence 5' – 3'**	
ctxA	ctxA_F	CTCAGACGGGATTTGTTAGGCACG	Hoshino et al. [38]
	ctxA_F	TCTATCTCTGTAGCCCCTATTACG	
ompW	ompW_F	CACCAAGAAGGTGACTTTATTGTG	Nandi et al. [39]
	ompW_R	GAACTTATAACCACCCGCG	
tcpA	tcpA^{set1}_F	ACTAAGGCTGCGCAAAATCT	Grembi & Spormann[22]
	tcpA^{set1}_R	GCCTCATCAGCTGAAACCTT	
tcpA	tcpA^{set2}_F	ACACGATAAGAAAACCGGTCA	Grembi & Spormann[22]
	tcpA^{set2}_R	GCCTTGGTCATATTCTGCGA	
ICP1	ICP1gp58F	AACGCTGCTTTTCCTTTTGA	Seed et al. [14]
	ICP1gp58R	CCCAGCATTGAGGACACTT	
ICP2	ICP2_4F	CGCTAGTTCTGGCAGTGA GT	Alexandrova et al.[21]
	ICP2_4R	TCCGTTCCAGTTCCAACAGG	
ICP2	ICP2_24R	AGAAGTCGCAAACGGGGTAC	Alexandrova et al.[21]
	ICP2_24R	AACGTGGTTCTCGTGAGTGG	
ICP3	ICP3gp5F	ATTGTCGAGTGGGACAAAGG	Seed et al. [14]
	ICP3gp5F	ACCAACTCGACGCATAGCTT	
16S rDNA**	Maeda_1048_1067_F	GTGSTGCAYGGYTGTCGTCA	Maeda et al. [40]
	Maeda_1175_1194_R	ACGTCRTCCMCACCTTCCTC	
16S rDNA**	27F_Miseq	AATGATACGGCGACCACCGAGATCTACA*C**TATGGTAATT*ccAGMGTTYGATYMTGG CTCAG	Chung et al. [41]
	338rcbc1	CAAGCAGAAGACGGCATACGAGAT **ACGAGACTGATT***AGTCAGTCAG*aaGCTG CCTCCCGTAGGAGT	Chung et al. [41]

* Strain used as a control strain for testing specificity of *tcpA* primers set1 and set2.

** 16S rDNA primer pair used for nanoliter qPCR. Degenerate primers are coded per standard convention (http://arep.med.harvard.edu/labgc/adnan/projects/Utilities/revcomp.html). Example of 16S rDNA primer pair used for microbiome analysis. Degenerate primers are coded per standard convention. Structure of forward primer: (i) 5' Illumina adapter, (ii) *Forward primer pad*, (iii) Forward primer linker (lower case), (iv) Forward primer. Structure of reverse primer example: (i) Reverse complement of 3′ Illumina adapter (underlined), (ii) Golay barcode (bold text), (iii) Reverse primer pad (italics), (iv) Reverse primer linker (lower case), (v) Reverse primer.

Table S2. South Sudan: Impact of bacteriophage ICP1 on cholera RDT positivity

		Diagnostic Positive		Diagnostic Negative				
Diagnostic Test	Total	Phage Pos	Phage Neg	Phage Pos	Phage Neg	OR_{MLE}[a]	CI[a]	P[b]
Among VC PCR positive (Hopkins)[c]								
RDT (Direct)[d]	32	7	26	1	0	---	---	---
RDT (Enriched)	34	5	25	3	1	0.075	0.001-1.137	0.033
Among VC PCR positive (Pasteur)[c]								
RDT (Direct)[d]	29	6	22	1	0	---	---	---
RDT (Enriched)	29	4	22	3	0	0.000	0.000-0.641	0.010

[a] Estimated odds ratio with conditional Maximum Likelihood Estimate (MLE); CI = 95th confidence interval

[b] Fischer's exact test

[c] Conventional PCR was performed by Johns Hopkins University and Institute Pasteur for cross validation [29]. 'Direct' = tested directly from stool; 'Enriched' = grown in selective media (APW) prior to testing. Values reported in the main text and Figure S1 are derived from the Institute Pasteur data set.

[d] Insufficient sample size for statistical analysis

Table S3.
Fold-differences in target DNA detection among *V. cholerae* positive samples

		Diagnostic Positive	Diagnostic Negative		
Diagnostic Test	Total[1]	Median nl-qPCR Ct (n; IQR)	Median nl-qPCR Ct (n; IQR)	Fold-difference	p[2]
No exclusion					
RDT	78	19.0 (23; 16.5-21.7)	24.4 (55; 21.1-27.7)	42	<0.001
qPCR	78	20.2 (49; 17.4-22.9)	26.5 (29; 24.9-27.6)	79	<0.001
Microscopy[3]	71	20.3 (34; 17.3-23.0)	25.1 (37; 21.5-27.3)	28	<0.001
Culture	29	19.4 (13; 14.3-22.0)	23.8 (16; 17.7-25.5)	21	0.013
Includes samples with azithromycin (excludes phage samples)					
RDT	63	18.4 (22; 16.2-21.1)	23.9 (41; 21.3-26.2)	46	<0.001
qPCR	63	20.2 (43; 17.4-22.9)	26.2 (20; 24.5-27.5)	65	<0.001
Microscopy[3]	58	20.3 (32; 17.4-23.2)	24.5 (26; 20.7-26.2)	18	0.002
Culture	27	19.4 (13; 14.3-22.0)	23.8 (14; 17.8-25.5)	21	0.011
Includes samples with phage (excludes azithromycin samples)					
RDT	56	19.0 (23; 16.5-21.7)	24.6 (33; 21.1-27.0)	48	<0.001
qPCR	56	19.9 (41; 17.4-22.8)	27.2 (15; 25.4-27.6)	163	<0.001
Microscopy[3]	52	19.1 (27; 17.2-22.8)	24.8 (25; 21.5-27.2)	53	<0.001
Culture	22	19.4 (13; 14.3-22.0)	20.8 (09; 17.6-25.9)	2.8	0.186

[1] Nanoliter (nL) qPCR positive for *V. cholerae* (Ct < 28); a random subset were cultured or available for microscopy.
[2] Mann-Whitney U test (two-tailed)
[3] Indeterminant samples scored as negative; limit of detection 100-1000 CFU/ ml

References

[Main text and supplement references are combined]

1. Global Burden of Disease Collaborators. Estimates of the global, regional, and national morbidity, mortality, and aetiologies of diarrhoea in 195 countries: a systematic analysis for the Global Burden of Disease Study 2016. Lancet Infect Dis **2018**; 18:1211-28.
2. Ali M, Nelson AR, Lopez AL, Sack DA. Updated global burden of cholera in endemic countries. PLoS Negl Trop Dis **2015**; 9:e0003832.
3. Nelson EJ, Harris JB, Morris JG, Jr., Calderwood SB, Camilli A. Cholera transmission: the host, pathogen and bacteriophage dynamic. Nat Rev Microbiol **2009**; 7:693-702.
4. D'Herelle F, Malone R. A preliminary report of work carried out by the cholera bacteriophage enquiry. Indian Medical Gazette **1927**:614-7.
5. Kaper JB, Morris JG, Jr., Levine MM. Cholera. Clin Microbiol Rev **1995**; 8:48-86.
6. Musekiwa A, Volmink J. Oral rehydration salt solution for treating cholera: </= 270 mOsm/L solutions vs >/= 310 mOsm/L solutions. Cochrane Database Syst Rev **2011**:CD003754.
7. Leibovici-Weissman Y, Neuberger A, Bitterman R, Sinclair D, Salam MA, Paul M. Antimicrobial drugs for treating cholera. Cochrane Database Syst Rev **2014**:CD008625.
8. Nelson EJ, Nelson DS, Salam MA, Sack DA. Antibiotics for both moderate and severe cholera. N Engl J Med; 364:5-7.
9. Arifuzzaman M, Ahmed T, Rahman MA, et al. Individuals with Le(a+b-) blood group have increased susceptibility to symptomatic *Vibrio cholerae* O1 infection. PLoS Negl Trop Dis **2011**; 5:e1413.
10. Merrell DS, Butler SM, Qadri F, et al. Host-induced epidemic spread of the cholera bacterium. Nature **2002**; 417:642-5.
11. Nelson EJ, Chowdhury A, Flynn J, et al. Transmission of *Vibrio cholerae* is antagonized by lytic phage and entry into the aquatic environment. PLoS Pathog **2008**; 4:e1000187.
12. Hartley DM, Morris JG, Jr., Smith DL. Hyperinfectivity: a critical element in the ability of *Vibrio cholerae* to cause epidemics? PLoS Med **2006**; 3:e7.
13. Seed KD, Yen M, Shapiro BJ, et al. Evolutionary consequences of intra-patient phage predation on microbial populations. Elife **2014**; 3:e03497.
14. Seed KD, Bodi KL, Kropinski AM, et al. Evidence of a dominant lineage of *Vibrio cholerae*-specific lytic bacteriophages shed by cholera patients over a 10-year period in Dhaka, Bangladesh. MBio **2011**; 2:e00334-10.
15. Jensen MA, Faruque SM, Mekalanos JJ, Levin BR. Modeling the role of bacteriophage in the control of cholera outbreaks. Proc Natl Acad Sci U S A **2006**; 103:4652-7.
16. Faruque SM, Naser IB, Islam MJ, et al. Seasonal epidemics of cholera inversely correlate with the prevalence of environmental cholera phages. Proc Natl Acad Sci U S A **2005**; 102:1702-7.
17. Faruque SM, Islam MJ, Ahmad QS, et al. Self-limiting nature of seasonal cholera epidemics: Role of host-mediated amplification of phage. Proc Natl Acad Sci U S A **2005**; 102:6119-24.
18. Page AL, Alberti KP, Mondonge V, Rauzier J, Quilici ML, Guerin PJ. Evaluation of a rapid test for the diagnosis of cholera in the absence of a gold standard. PLoS One **2012**; 7:e37360.

19. Dick MH, Guillerm M, Moussy F, Chaignat CL. Review of two decades of cholera diagnostics--how far have we really come? PLoS Negl Trop Dis **2012**; 6:e1845.
20. Hasan JA, Bernstein D, Huq A, Loomis L, Tamplin ML, Colwell RR. Cholera DFA: an improved direct fluorescent monoclonal antibody staining kit for rapid detection and enumeration of *Vibrio cholerae* O1. FEMS Microbiol Lett **1994**; 120:143-8.
21. Alexandrova L, Haque F, Rodriguez P, et al. Identification of widespread antibiotic exposure in cholera patients correlates with clinically relevant microbiota changes. J Infect Dis **2019**.
22. Grembi J, Mayer-Blackwell K, Luby S, Spormann A. High-throughput multi-parallel enteropathogen quantification via nano-liter qPCR. Available at: bioRxiv **2019** 746446; doi: https://doi.org/10.1101/746446
23. Debes AK, Ateudjieu J, Guenou E, et al. Clinical and Environmental Surveillance for *Vibrio cholerae* in Resource Constrained Areas: Application During a 1-Year Surveillance in the Far North Region of Cameroon. Am J Trop Med Hyg **2016**; 94:537-43.
24. George CM, Rashid MU, Sack DA, et al. Evaluation of enrichment method for the detection of *Vibrio cholerae* O1 using a rapid dipstick test in Bangladesh. Trop Med Int Health **2014**.
25. Ley B, Khatib AM, Thriemer K, et al. Evaluation of a rapid dipstick (Crystal VC) for the diagnosis of cholera in Zanzibar and a comparison with previous studies. PLoS One **2012**; 7:e36930.
26. Alam M, Hasan NA, Sultana M, et al. Diagnostic limitations to accurate diagnosis of cholera. J Clin Microbiol **2010**; 48:3918-22.
27. Tuteja U, Kumar S, Shukla J, Kingston J, Batra HV. Simultaneous direct detection of toxigenic and non-toxigenic *Vibrio cholerae* from rectal swabs and environmental samples by sandwich ELISA. J Med Microbiol **2007**; 56:1340-5.
28. Haque F, Ball RL, Khatun S, et al. Evaluation of a Smartphone Decision-Support Tool for Diarrheal Disease Management in a Resource-Limited Setting. PLoS Negl Trop Dis **2017**; 11:e0005290.
29. Ontweka LN, Deng LO, Rauzier J, et al. Cholera Rapid Test with Enrichment Step Has Diagnostic Performance Equivalent to Culture. PLoS One **2016**; 11:e0168257.
30. Nelson EJ, Chowdhury A, Harris JB, et al. Complexity of rice-water stool from patients with *Vibrio cholerae* plays a role in the transmission of infectious diarrhea. Proc Natl Acad Sci U S A **2007**; 104:19091-6.
31. Islam K, Sayeed MA, Hossen E, et al. Comparison of the Performance of the TPTest, Tubex, Typhidot and Widal Immunodiagnostic Assays and Blood Cultures in Detecting Patients with Typhoid Fever in Bangladesh, Including Using a Bayesian Latent Class Modeling Approach. PLoS Negl Trop Dis **2016**; 10:e0004558.
32. R Core Team: A language and environment for statistical computing. R Foundation for Statistical Computing. Available at: http://www.R-project.org/. Accessed Dec 29, 2019.
33. McKitterick AC, Hays SG, Johura FT, Alam M, Seed KD. Viral Satellites Exploit Phage Proteins to Escape Degradation of the Bacterial Host Chromosome. Cell Host Microbe **2019**; 26:504-14 e4.
34. Warner HR, Snustad P, Jorgensen SE, Koerner JF. Isolation of bacteriophage T4 mutants defective in the ability to degrade host deoxyribonucleic acid. J Virol **1970**; 5:700-8.
35. Mekalanos JJ. Duplication and amplification of toxin genes in *Vibrio cholerae*. Cell **1983**; 35:253-63.

417 36. Rahman M, Jubair M, Alam MT, et al. High-frequency rugose exopolysaccharide production
418 by *Vibrio cholerae* strains isolated in Haiti. PLoS One **2014**; 9:e112853.
419 37. Felsenfeld O, Stegherr-Barrios A, Aldova E, Holmes J, Parrott MW. In vitro and in vivo studies
420 of streptomycin-dependent cholera vibrios. Appl Microbiol **1970**; 19:463-9.
421 38. Hoshino K, Yamasaki S, Mukhopadhyay AK, et al. Development and evaluation of a multiplex
422 PCR assay for rapid detection of toxigenic *Vibrio cholerae* O1 and O139. FEMS Immunol Med
423 Microbiol **1998**; 20:201-7.
424 39. Nandi B, Nandy RK, Mukhopadhyay S, Nair GB, Shimada T, Ghose AC. Rapid method for
425 species-specific identification of *Vibrio cholerae* using primers targeted to the gene of outer
426 membrane protein OmpW. J Clin Microbiol **2000**; 38:4145-51.
427 40. Maeda H, Fujimoto C, Haruki Y, et al. Quantitative real-time PCR using TaqMan and SYBR
428 Green for Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, Prevotella
429 intermedia, tetQ gene and total bacteria. FEMS Immunol Med Microbiol **2003**; 39:81-6.
430 41. Chung WS, Walker AW, Louis P, et al. Modulation of the human gut microbiota by dietary
431 fibres occurs at the species level. BMC Biol **2016**; 14:3.
432