Complexity of rice-water stool from patients with *Vibrio cholerae* plays a role in the transmission of infectious diarrhea

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At the International Centre for Diarrhoeal Disease Research, Bangladesh, one-half of the rice-water stool samples that were culture-positive for *Vibrio cholerae* did not contain motile *V. cholerae* by standard darkfield microscopy and were defined as darkfield-negative (DF⁻). We evaluated the host and microbial factors associated with DF status, as well as the impact of DF status on transmission. Viable counts of *V. cholerae* in DF⁻ stools were three logs lower than in DF⁺ stools, although DF⁻ and DF⁺ stools had similar direct counts of *V. cholerae* by microscopy. In DF⁻ samples, non-*V. cholerae* bacteria outnumbered *V. cholerae* 10:1. Lytic *V. cholerae* bacteriophage were present in 90% of DF⁻ samples compared with 35% of DF⁺ samples, suggesting that bacteriophage may limit culture-positive patients from producing DF⁺ stools. *V. cholerae* in DF⁻ and DF⁺ samples were found both planktonically and in distinct nonplanktonic populations; the distribution of organisms between these compartments did not differ appreciably between DF⁻ and DF⁺ stools. This biology may impact transmission because epidemiological data suggested that household contacts of a DF⁺ index case were at greater risk of infection with *V. cholerae*. We propose a model in which *V. cholerae* multiply in the small intestine to produce a fluid niche that is dominated by *V. cholerae*. If lytic phage are present, viable counts of *V. cholerae* drop, stools become DF⁻, other microorganisms bloom, and cholera transmission is reduced.

bacteriophage | biofilm | darkfield | mucin

Despite more than a century of investigation, much remains to be discovered about how pathogenic strains of *Vibrio cholerae* interact with the human host and how the biology of disseminating stool *V. cholerae* drives devastating cholera outbreaks. Cholera is an ancient secretory diarrheal disease caused by the O1 and O139 serogroups of *V. cholerae*. Today the burden of disease is estimated to reach as much as several million cases a year in both Asia and Africa, with fewer cases in Latin America (1). Despite the dramatic reduction of mortality rates due to the development of oral rehydration solution (2), the emergence of multiple drug-resistant *V. cholerae* (3, 4) may reduce the efficacy of antimicrobial treatment and alter the dynamics of outbreaks.

V. cholerae is a Gram-negative bacterial pathogen of the gastrointestinal tract. Cholera toxin is largely responsible for the massive fluid loss that may reach between 0.5 and 1.0 liter per hour (2). *V. cholerae* from human stool is hyperinfectious, compared with laboratory-grown bacteria, with a lower infectious dose in a murine model of infection (5–7). Mathematical models have quantified the importance of hyperinfectivity to the explosive nature of the biannual outbreaks of cholera seen so commonly in areas such as Bangladesh (8), but proof of direct person-to-person transmission of hyperinfectious *V. cholerae* is still lacking. One force driving the subsequent collapse of a cholera outbreak has been proposed to be the presence of

bacteriophage lytic for *V. cholerae*. Such lytic phage are shed by patients and are isolated in the environment at higher rates in the later stages of cholera outbreaks (9–11), suggesting, but not yet proving, a causal role of bacteriophage in the termination of epidemics.

Benenson *et al.* (12) introduced darkfield microscopy as a tool for the rapid identification of *V. cholerae* in rice-water stools in 1964. This technique involves examining fresh human stool under darkfield microscopy ($\times 400$ magnification) for vibrioid-shaped cells (0.75–2.0 μM) with distinct darting motility and designating these samples darkfield positive (DF⁺). The results are confirmed by using antibodies specific for *V. cholerae* LPS to neutralize motility of the observed organisms (12–14). Without enriching for *V. cholerae*, the sensitivity for diagnosis by darkfield microscopy ranges from 0.50 to 0.80, with a specificity >0.92 (12). The gold standard for diagnosis of cholera has been, and continues to be, overnight culture of stool on selective media for *V. cholerae*. We were interested in determining the differences in DF⁻ versus DF⁺ stools, where *V. cholerae* are harbored in DF⁻ samples that are culture-positive for *V. cholerae*, and what impact these findings may have upon transmission.

It has recently been shown that DF⁺, culture-positive stools harbor *V. cholerae* in both planktonic (individual bacterial cells freely suspended in a liquid medium) and nonplanktonic states (15). In addition to the planktonic fraction, the nonplanktonic aggregates contain infectious *V. cholerae* when tested in the infant mouse model (15). To expand on these findings, we examined patients with culture-confirmed *V. cholerae* O1 or O139 infection (VC⁺) and determined that more than half of their stools were DF⁻. We then investigated the location of *V. cholerae* O1 in DF⁻/VC⁺ stools, as well as whether the presence of bacteriophage lytic for *V. cholerae* O1 might affect the DF status of stool samples that were VC⁺. The results showed that in VC⁺ stool samples *V. cholerae* O1 were found in four distinct populations, and DF⁻ status correlated with a substantial reduction in viable numbers of *V. cholerae* and the presence of lytic phage. The DF status of the index case also correlated with disease transmission to household contacts.

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Fig. 1. *V. cholerae* culture results and darkfield status (DF) for rice-water stools collected from 2001 to 2005 at the Centre for Diarrhoeal Disease Research (Bangladesh). Data are separated by month; January is listed first. Open triangles, percentage of samples culture-positive for *V. cholerae* (VC⁺) and DF⁺; filled triangles, VC⁺/DF⁻; crosses, VC⁻/DF⁻; open squares, VC⁻/DF⁺. Within the VC⁺ stool samples, the distribution between DF⁺ and DF⁻ samples was significantly different for June (*, $P = 0.021$) and November (**, $P = 0.002$) by the Pearson χ^2 test.

Results

One-Half of the Culture-Positive Patients Shed DF⁻ Stool. A total of 658 patient stool samples were collected from 2001 to 2005. Of the 480 cases that were VC⁺ (73% of the total samples collected), 244 were DF⁻ (51%). As a predictive indicator for a sample to be culture-positive for *V. cholerae* O1 or O139, darkfield microscopy had a sensitivity of 0.49, specificity of 0.97, and predictive value positive and negative of 0.98 and 0.41, respectively.

The numbers of patient stools collected during each calendar month over the 4-year period of the study were as follows:

January, $n = 63$ and $VC^+ = 30$; February, $n = 56$ and $VC^+ = 24$; March, $n = 86$ and $VC^+ = 58$; April, $n = 88$ and $VC^+ = 68$; May, $n = 64$ and $VC^+ = 52$; June, $n = 57$ and $VC^+ = 48$; July, $n = 47$ and $VC^+ = 39$; August, $n = 48$ and $VC^+ = 40$; September, $n = 54$ and $VC^+ = 44$; October, $n = 46$ and $VC^+ = 39$; November, $n = 28$ and $VC^+ = 23$; December, $n = 21$ and $VC^+ = 15$. The distribution by month for the percentage of samples that were DF^+/VC^+ , DF^+/VC^- , DF^-/VC^+ , or DF^-/VC^- is depicted in Fig. 1. The percentage of samples that were DF^+/VC^+ and DF^-/VC^+ was significantly different only for the months of June (when the ratio of DF^-/DF^+ stools increased to 32/16; $P = 0.021$) and November (when the ratio of DF^-/DF^+ stools fell to 4/19; $P = 0.002$). Taken together, these data suggest that half of culture-positive patients produce DF^- stool over the year and that there is a divergence in the fraction of DF^+ and DF^- stools after the spring and fall cholera outbreaks (moving in the opposite direction). This suggests that the degree of DF positivity is not random, but relates to a biological variable(s).

Logistic regression analysis of the 480 cases that were VC⁺ from the 2001–2005 study revealed that increasing age was associated with a higher likelihood of a DF⁺ stool in VC⁺ patients; the odds ratio for DF positivity was 1.20 for each decade of life (95% confidence interval 1.04–1.38, $P = 0.009$). There was no association between the DF status and other variables examined, including consumption of antibiotics before obtaining the stool specimen, markers of severity of disease on presentation to the International Centre for Diarrhoeal Disease Research (Bangladesh), or baseline immunologic measurements of recent exposure to *V. cholerae* infection (data not shown).

DF⁻ Stools Have a 1,000-Fold Lower *V. cholerae* Viable Count, but a Similar Direct Count, to DF⁺ Stools. In stools collected in 2007, the median *V. cholerae* viable counts (cfu/ml) for DF⁺ and DF⁻ samples were 8×10^7 and 1×10^4 , respectively (Mann-Whitney *U* test, $P < 0.006$). The number of *V. cholerae* O1 in DF⁻ compared with DF⁺ stools was also determined by direct counts

Fig. 2. Populations of *V. cholerae* and non-*V. cholerae* organisms found in rice-water stool. (A and B) Planktonic *V. cholerae* from DF⁺/phage[−] (A) or DF[−]/phage⁺ (B) stool samples. (C and D) *V. cholerae* bound in mucus from a DF⁺/phage[−] (C) or DF[−]/phage⁺ (D) stool samples. (E) *V. cholerae* bound in an aggregate that does not counterstain for mucus; mucus in the same field is found in the upper left corner with no bacteria. (F) *V. cholerae* associated with ghost cells. For A–E, *V. cholerae* O1 antigen staining (grayscale images) is shown alongside merged images of *V. cholerae* (red), DAPI DNA labeling for total bacterial cell counts (green), and WGA staining for mucus (blue; in D, WGA blue color is reduced to allow DAPI to be visible). In F, phase contrast is alongside *V. cholerae* (green). (Scale bars: 10 μ m.)

Table 2. Vibriocidal antibody titer increase in household contacts grouped by the DF status of the household index case

Status	ΔVC_{Ab+}^*	$\Delta VC_{Ab-}^{\dagger}$
DF ⁺ index case	133	399
DF ⁻ index case	80	332

Data were collected as part of an ongoing study (27). Odds ratio = 1.38 and $P = 0.04$ for household contacts having a 4-fold or greater rise in vibriocidal antibody titers if the index case is DF⁺.

*Vibriocidal antibody titer increase of at least 4-fold from baseline.

[†]Vibriocidal antibody titer did not increase from baseline.

vibriocidal antibody titer and serial stool cultures that were negative for *V. cholerae* (Table 3). If the index case was DF⁻, the odds ratio for household contacts to develop DUE was 1.40 ($P = 0.04$) compared with household contacts of a DF⁺ index case.

Discussion

Unlike previous studies, which traditionally focused only on rice-water stool that was culture-positive for *V. cholerae* (VC⁺) and darkfield positive (DF⁺), our goals were to characterize the biology of DF⁻/VC⁺ samples and assess how the differences between DF⁺ and DF⁻ samples might impact the current concept of *V. cholerae* transmission. Our data suggest that half of VC⁺ rice-water stools from cholera cases are DF⁻. The DF status depends in part on the presence of lytic *V. cholerae* phage in the stool. In terms of transmission, if the DF status of a household index case is positive, household contacts are at an increased risk of infection with *V. cholerae*.

Of culture-positive cases, 51% (244 of 480) of the rice-water stool samples collected as part of our study from 2001 to 2005 were DF⁻. The distribution of DF⁻ and DF⁺ cases for each month was not significantly different except for the months of June and November; these 2 months usually follow the spring and fall cholera outbreaks (10). Although we did not assay stool for phage in the 2001–2005 period, the data in the spring 2006 study suggest that the DF status depends on the presence of lytic phage in the stool. The rise in the DF⁻ cases in June, just after the usual spring outbreak of cholera in Bangladesh (Fig. 1), supports the hypothesis that phage limit the production of DF⁺ stool, reducing transmission in households, and substantiate the model proposed by Faruque *et al.* (9) that host-mediated amplification of phage may provide self-limiting control over seasonal outbreaks of *V. cholerae* in Bangladesh. However, the rise in DF⁺ cases in November, at the end of the usual fall outbreak, suggests that the biology of the two yearly outbreaks is different; for example, the spring outbreak is at the end of the dry season, and the fall outbreak is at the end of the monsoons. Second, there is variability in the serotype and magnitude of outbreaks between years (21). Although we averaged data by month over 4 years, these annual and seasonal differences may contribute to divergent trends in the DF⁻ to DF⁺ ratio between outbreaks.

Phage may impact the DF status of stool by killing *V. cholerae* that are already present or simply blocking any growth of *V.*

cholerae. Of culture-positive samples, direct counts suggest that the number of *V. cholerae* cells is only slightly lower in DF⁻ samples than in DF⁺ samples. The physiologic state of DF⁻ *V. cholerae* is largely unknown, but many of the bacteria may be dead or possibly in the conditionally viable state recently described (15). The differential ratio for the number of *V. cholerae* to total number of bacteria in stool by direct counts was also significantly different between DF⁺ and DF⁻ samples. DF⁺/VC⁺ samples were dominated by *V. cholerae*, whereas in DF⁻/VC⁺ samples *V. cholerae* were outnumbered 10:1. Because the *V. cholerae* direct counts are approximately the same, non-*V. cholerae* bacteria have expanded in number in the DF⁻ samples. This expansion may be related to altered control of the microbial community by the host or the microbial milieu itself.

To understand more fully the nature of the DF status of stool, we explored the possibility that there may be a niche in stool that is protective against lytic phage. *V. cholerae* was found in four distinct populations in rice-water stool: planktonic, embedded in mucus, bound in nonmucinous microbial aggregates, and associated with ghost cells (Fig. 2). *V. cholerae* has long been documented to be associated with mucus (22, 23). The nature of the interaction between mucin and *V. cholerae* may be quite evolved. For example, *V. cholerae* harbors the enzyme neuraminidase (sialidase), which plays a role in pathogenesis by removing sialic acid from higher-order gangliosides (24), and cholera toxin can stimulate the disruption of and ejection of mucus by goblet cells (25). From our study of VC⁺ patients, the amount of mucus produced (by percent wet volume) in rice-water stools ranged widely from <0.1% to 46%, suggesting that a patient may pass substantial quantities of mucus during the course of infection. Therefore, *V. cholerae* in mucus may be important for transmission. Quantifying the counts for *V. cholerae* and non-*V. cholerae* in mucus was complicated by the uneven distribution of bacteria between fields and limited resolution at different focal planes. Qualitatively, the same trends that were observed in the planktonic counts were mirrored in the mucus fraction of the stool: a similar density of *V. cholerae* between DF⁺ and DF⁻ samples, but the ratio of non-*V. cholerae* to *V. cholerae* increased in DF⁻ samples (Fig. 2 C and D). These data suggest that phage may have access to *V. cholerae* embedded in the mucus; however, rigorous studies are required to determine whether mucus, aggregates, or ghost cells may act as sanctuaries from phage predation. Qualitative trends for the aggregates were not possible to assess because the aggregates were subject to extreme variability due to differences in size, number of *V. cholerae*, and diversity of non-*V. cholerae* in the aggregates.

At a population level, we were interested in how the DF dichotomy affects transmission. The results indicate that, if the household index case was DF⁺, the household contacts were at greater risk of *V. cholerae* infection. This finding is consistent with the observations that bacteria within DF⁻ samples are nonmotile and that viable counts are lower than in DF⁺ samples. How this finding will alter the modeling of outbreaks remains to be determined, but the results are important to incorporate because half of the VC culture-positive patients are DF⁻.

Unexpected in this study was the finding that non-*V. cholerae* bacteria expanded in number in DF⁻ samples. The identity of the non-*V. cholerae* is largely unknown, but our microscopic observations suggest that the non-*V. cholerae* bacteria consisted of a mixture of different, mainly Gram-negative bacilli and cocci (data not shown). These non-*V. cholerae* do not appear to be ETEC, because ETEC coinfecting samples were removed from the analysis and were equally distributed between DF⁺/VC⁺ and DF⁻/VC⁺ samples. We found that, if the index case was DF⁻/VC⁺, household contacts were at greater risk of diarrhea of unidentified etiology. There are at least two hypotheses for the cause of this diarrhea: the first is that the diarrhea is caused by a low inoculum of *V. cholerae* despite negative stool cultures and

Table 3. Incidence of DUE among household contacts grouped by the DF status of the household index case

Status	DUE	ND
DF ⁺ index case	177	117
DF ⁻ index case	245	116

Data were collected as part of an ongoing study (27). Odds ratio = 1.40 and $P = 0.04$ for household contacts experiencing DUE if the index case is DF⁻. DUE is defined as household contacts with clinical diarrhea but with no rise in vibriocidal antibody titer and serial negative rectal swabs for *V. cholerae*. ND, No diarrhea in household contacts.

no rise in vibriocidal antibody in the contact, as shown previously to occur in volunteer studies (26). Alternatively, the diarrhea might be caused by a member of the non-*V. cholerae* population that increases in number (up to 10^8 to 10^9 cells per milliliter) in the DF⁻/VC⁺ stools. Future studies on the identity of the non-*V. cholerae* in DF⁻ stools may resolve this debate.

Taken together, our data suggest that cholera patients purge diverse populations of both *V. cholerae* and non-*V. cholerae* in rice-water stool. The sample complexity is in part related to the presence of lytic *V. cholerae* phage, but there are likely undiscovered factors that also influence whether a stool is DF⁺ or DF⁻. *V. cholerae* may multiply in the small intestine to produce a fluid niche that is dominated by *V. cholerae*. If lytic phage are present, the phage may drive *V. cholerae* and subsequently the patient into a DF⁻ state and allow for other microorganisms to grow.

We advocate that future studies include analysis of single patients to assess how the DF status changes over time, investigation of what role nonplanktonic populations play in transmission, and elucidation of the mechanisms that permit the expansion of the non-*V. cholerae* microbes. This study urges caution in treating rice-water stool as a “simple” homogenous source of *in vivo* pathogens because organisms in a single rice-water stool may be more diverse than previously appreciated. The household data presented here show that this complexity can affect transmission of *V. cholerae* and non-*V. cholerae* microbes in ways that were previously unknown and are likely to have additional consequences that remain to be uncovered.

Materials and Methods

Analyses of Darkfield Microscopy Data from Index Cases and Follow-Up of Household Contacts (2001–2005). The Dhaka Hospital of the International Centre for Diarrhoeal Disease Research, Bangladesh, carries out clinical research and services for patients with diarrheal illness in Dhaka, Bangladesh. In 2001 we began a study of the clinical features and immunological responses in patients with cholera cared for at the center and their household contacts (27, 28). Index patients were enrolled if they had a clinical syndrome consistent with cholera, were not participating in any interventional trials, and provided informed consent. Stool was examined by darkfield microscopy (described below) and cultured on selective TTGA media for *V. cholerae* O1 or O139 (29)—techniques most commonly used for detection of *V. cholerae*. During the study, the percentage of stool samples that were DF⁺/VC⁺, DF⁺/VC⁻, DF⁻/VC⁺, and DF⁻/VC⁻ were recorded. We tested for significant differences between these percentages for each month of the year using the Pearson χ^2 test. To evaluate the statistical significance of potential associations between patient characteristics and darkfield status of stools, logistic regression was used for continuous variables including age and baseline vibriocidal titer, and the Pearson χ^2 test was used for dichotomous variables.

Within 1 day of presentation of the index cases described above, a field team discussed enrollment with household contacts of an index patient—defined as individuals sharing the same cooking pot as the index patient for 3 or more days. Blood specimens for baseline vibriocidal antibody titer were collected from consenting household contacts immediately upon enrollment. Contacts were visited by the field team on days 2 through 7, 14, and 21 after presentation of the index case. Contacts were questioned at each visit about diarrheal symptoms, and rectal swabs were obtained for *V. cholerae* culture. Follow-up blood samples for vibriocidal antibody titers were obtained from contacts on study days 7 and 21. Vibriocidal antibody assays were performed with methodology previously described (28, 29) using guinea pig complement and the appropriate serotype of *V. cholerae* O1 or O139 as the target organism. A 4-fold or greater increase between the baseline and day 7 or 21 vibriocidal

antibody titer was considered a marker of recent infection with *V. cholerae* O1/ O139 (30–33). DUE was defined as more than three diarrheal stools per day and the absence of a 4-fold increase in vibriocidal antibody titer as well as negative rectal swab cultures on all days of follow-up for *V. cholerae*. Household contacts were excluded from the analysis if they did not complete 21 days of follow-up. The statistical significance of the difference in the proportion of contacts with 4-fold increases in vibriocidal titer and in the proportion of those with diarrhea of unidentified etiology, by DF status of the index patient in the household, was compared by using the Pearson χ^2 test.

Collection of Stools from Patients with Suspected Cholera at the Centre for Diarrhoeal Disease Research (Bangladesh) (2006 and 2007).

During the cholera outbreaks of 2006 and 2007 we collected stool samples from an additional 104 and 39 adult patients (>15 years of age), respectively, with acute watery diarrhea and no prior treatment with antibiotics. These samples were examined by darkfield microscopy, cultured for *V. cholerae* by standard methods on 100 μ g/ml LB streptomycin or TTGA media (29), screened for coinfection with ETEC by standard methods (34), and examined for the presence of lytic bacteriophage and for direct microscopic counts of *V. cholerae* by immunofluorescence microscopy (see below). Only data from patients with *V. cholerae* O1 infection were analyzed. All human studies (2001–2007) described above were reviewed and approved by both the Research Review Committee and Ethical Review Committee at the International Centre for Diarrhoeal Disease Research (Bangladesh) and by the Human Research Committee at the Massachusetts General Hospital. All patients provided written informed consent.

Darkfield Microscopy and Bacteriophage Assays. Ten microliters of stool was immediately placed on a slide. Twenty fields were scored by DF microscopy (Olympus BH2, $\times 400$ magnification) for the presence of 0.5- to 2- μ m vibrioid-shaped cells that had darting motility characteristic of *V. cholerae* (12). Supernatants were prepared from stool samples by centrifugation at $26,892 \times g$ for 15 min at 4°C, filtered through a 0.22- μ m polystyrene filter (Millipore), and frozen at -80°C. To screen for vibriophages, lawns of the *V. cholerae* O1 isolates N16961 (serotype Inaba) and EN159 (serotype Ogawa) were prepared from log-phase broth cultures on Luria–Bertani broth agar 100 μ g/ml streptomycin plates. The plates were dried for 1 h at 37°C, and 10 μ l of stool supernatant diluted in PBS (neat, 1:10, and 1:100) was spotted onto the bacterial lawns. The plates were incubated at 37°C overnight, and the samples were scored as positive or negative for distinct plaques indicative of lytic *V. cholerae* O1 bacteriophage.

Fluorescence Microscopy. Agar slides were prepared by spotting 250 μ l of molten 1% agarose (LE Agarose; SeaKem) on a standard microscope slide and completely drying the agarose layer overnight. Ten microliters of each formalin-fixed stool sample (3.7% formalin) was spotted in triplicate on agar slides and dried for 15 min at 37°C. When necessary, dilutions of the stool were made in PBS to yield countable fields. To examine *V. cholerae* embedded in mucus or aggregates, the sediment fraction of formalin-fixed stool was washed three times in PBS using differential centrifugation at $100 \times g$ for 15 s to remove planktonic *V. cholerae* and loosely attached *V. cholerae*. Control micrographs for each sample were taken after sequential washes to verify that three washes were sufficient to remove planktonic *V. cholerae*.

Slides were fixed with 20 μ l of isopropanol for 5 min and gently washed with PBS. The following staining mixture was used: 1:4 dilution of a monoclonal antibody for the O1 antigen of *V. cholerae* LPS directly conjugated to FITC (New Horizons), 300

nM DAPI (Molecular Probes) for nucleic acid staining to count total bacterial cells, and 5 $\mu\text{g/ml}$ WGA lectin (Molecular Probes) for staining human mucus and Gram-positive bacteria. The mixture was applied (40 μl per sample) and incubated for 1 h at 37°C, washed in PBS twice for 5 min, rinsed in dH_2O , mounted overnight in Mowiol reagent [Calbiochem; 48 g/liter Mowiol, glycerol 12% (wt/vol), and 0.05 M Tris (pH 8.5)], and examined by immunofluorescence with a Nikon Eclipse 80i fluorescent microscope. *V. cholerae* and total bacterial cell counts were performed in an automated fashion by using IP-Open lab software and size criteria such that objects $<0.2 \mu\text{m}$ and $>10 \mu\text{m}$ were excluded. The average of two fields from the center of the drop zone was taken for each sample. The linearity and utility of using agarose slides for direct bacterial counts was confirmed by constructing a standard curve using *in vitro* cultured *V.*

cholerae, with viable counts (cfu/ml) plotted against direct counts per field (data not shown). The Pearson product moment correlation coefficient, r , for the standard curve between these counts was 0.99.

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