Sensitive Microplate Assay for Detection of Bactericidal Antibodies to *Vibrio cholerae* O139

Stephen R. Attridge,[1]* Camilla Johansson,[2] Dang D. Trach,[3] Firdausi Qadri,[4] and Ann-Mari Svennerholm[2]

Department of Molecular Biosciences, University of Adelaide, Adelaide, South Australia 5005, Australia[1]; Department of Medical Microbiology and Immunology, Göteborg University, Göteborg, Sweden[2]; National Institute of Hygiene and Epidemiology, Hanoi, Vietnam[3]; and International Centre for Diarrhoeal Disease Research, Dhaka, Bangladesh[4]

A microplate assay for the detection of bactericidal antibodies to *Vibrio cholerae* O139 is described. The assay is sensitive, highly reproducible, specific, and convenient to perform. It has been used to demonstrate the induction of serum bactericidal antibodies in Vietnamese recipients of an oral, inactivated, bivalent O1/O139 vaccine, as well as in Bangladeshi patients with O139 disease. In both study groups there was a significant inverse correlation between the preexposure level of antibodies in serum and the magnitude of the subsequent bactericidal response. Although infection generated stronger responses than vaccination, the proportion of responders was similar among individuals with low background titers.

The potential to elicit serum antibodies with complement-dependent bactericidal activity has proven to be the most reliable indicator of the protective efficacy of orally administered cholera vaccines (7). The value of measuring such responses was initially suggested by seroepidemiological surveys of disease incidence among the inhabitants of areas where infections are endemic (10, 11). These surveys revealed a pattern of increasing levels of (naturally acquired) serum antibodies with age and an inverse correlation between the serum bactericidal titer and the cholera attack rate. This link was subsequently confirmed in a series of challenge trials in human volunteers, where it was possible to relate the protective efficacies of various oral vaccines to cohort and individual bactericidal responses (7, 14). Assessment of bactericidal responses in serum has therefore facilitated the development of vaccines against *Vibrio cholerae* O1.

The emergence and rapid spread of a second *V. cholerae* serogroup (O139) with epidemic potential (3, 12) provided the impetus for attempts to develop O139-specific vaccines by using approaches previously applied to O1 vaccine development. Early reports provided conflicting indications as to the likely value of measuring bactericidal responses in O139 vaccine recipients, however (4, 6, 9, 15). In a recent study (2), we used a tube-based bactericidal assay to study the comparative susceptibilities of *V. cholerae* O1 and O139 to lysis by antibody and complement. Whereas O1 strains were generally extremely sensitive to lysis, O139 bacteria displayed resistant or susceptible phenotypes, depending on assay conditions. Several features of the microplate assay systems used in laboratories that need to screen large numbers of serum samples appear to militate against the efficient detection of antibodies with lytic potential against *V. cholerae* O139 (2).

We describe here a modified microplate assay that is sensi-tive, highly reproducible, specific, and convenient to use. It has been successfully applied to detect the induction of bactericidal antibodies to *V. cholerae* O139 following vaccination or disease. In both instances there was a significant inverse correlation between preexisting antibody titers and the magnitude of the immune responses observed.

MATERIALS AND METHODS

Microplate bactericidal assay. A microplate assay for detection of bactericidal antibodies to *V. cholerae* O139 has been described previously (13). Briefly, colonies of O139 strain 4260B were cultured in brain heart infusion broth (BHI; Difco) for 3 h at 37°C, with shaking at 250 rpm. The bacteria were harvested by centrifugation and resuspended in saline; after measurement of the optical density (OD), the suspension was diluted into saline containing 20% guinea pig serum as the complement source to a final bacterial concentration of ca. 6×10^6 vibrios per ml. Aliquots (initially 25 µl but later 50 µl) of this reaction mix were added to equal volumes of serum samples which had been serially titrated (in saline) across the rows of a microtiter tray. After incubation for 60 min at 37°C, BHI (150 µl but later 100 µl) was added to each well, and the tray was returned to 37°C. After further incubation for ca. 3 h, the trays were inspected and the endpoints were determined visually as the highest serum dilutions causing complete inhibition of bacterial growth.

Various modifications were made to this assay procedure during the course of these studies. A spontaneous streptomycin-resistant variant of 4260B was selected for use as an indicator strain by growth on solid medium in the presence of antibiotic (200 µg/ml). Use of this variant allowed inclusion of antibiotic in both the killing and the growth phases of the assay to prevent growth of any chance contaminants (e.g., from serum samples). Initially, this strain was also grown in liquid (Luria-Bertani [LB]) medium, but subsequently the indicator bacteria were harvested from agar plates. LB plates (with streptomycin) were spread with ca. 10^4 bacteria taken from an early-log-phase culture in LB medium. After incubation for 17 to 18 h at 37°C, the bacteria were harvested into Mg^{2+}-saline (2 mM $MgCl_2$ in 0.85% [wt/vol] NaCl; pH 7.0), the OD of a suitable dilution was determined, and the suspension was diluted to a final concentration of ca. 2×10^5 per ml in the same diluent containing 20% guinea pig serum (and 200 µg of streptomycin per ml).

The assay diluent was changed to Mg^{2+}-saline, and test sera were titrated in twofold dilution series (in volumes of 50 µl) by using a multichannel pipettor. Paired sera were always tested together in adjoining rows of the same tray. In each tray control rows were allocated for titration of a standard rabbit anti-O139 serum, while additional wells received either no bacteria (−ve control for bacterial growth) or no serum sample (+ve control for growth). After serial dilution of the serum samples, each well received an equal volume (50 µl) of vibrio suspension, resulting in a final bacterial concentration of 10^5 per ml and a final

* Corresponding author. Mailing address: Department of Molecular Biosciences, University of Adelaide, Adelaide, South Australia 5005, Australia. Phone: 618-8303-4150. Fax: 618-8303-7532. E-mail: stephen.attridge@adelaide.edu.au.

complement concentration of 10%. Trays were incubated at 37°C for 60 min, at which time 70 µl of (prewarmed) 5× LB medium (containing streptomycin) was added to each well. Incubation was continued at 37°C until the OD_{600} of the +ve control wells—spectrophotometrically determined in a microplate reader (Labsystems Multiskan MS)—reached 0.35 to 0.38 (ca. 5.5 h).

The mean ODs of −ve and +ve control wells (*a* and *b*, respectively) were used to calculate an OD that represented a 70% inhibition of bacterial growth (OD = $a + 0.3[b − a]$). This value was then used to assign a lytic endpoint to each test sample, this being the highest dilution causing ≥70% killing. The assay was not accepted unless the titer of the standard antiserum fell within an acceptable range.

A fourfold increase in bactericidal titer was used as the criterion of a significant response. For purposes of calculating fold rises in titer or the geometric mean (GM) titer, samples with killing endpoints of <10 or <20 were assigned titers of 5 or 10, respectively. For some analyses Spearman's correlation coefficient (one tailed) was calculated by using Graphpad Prism software; a probability of 0.01 was regarded as significant.

Some assays were performed with the O1 serogroup strain T19479 to measure bactericidal antibodies in serum samples collected from cholera patients whose disease was caused by *V. cholerae* O1. These were conducted according to the modified microplate protocol described above, except that streptomycin was omitted.

Serum samples. Anti-O139 bactericidal responses were analysed in Vietnamese recipients of an oral, inactivated, bivalent O1/O139 cholera vaccine (16). Recipients (aged 19 to 24 years) were given two doses of vaccine, each comprising $1.25 × 10^{11}$ O1 and $0.5 × 10^{11}$ O139 bacteria, on days 0 and 14; serum samples were obtained on day 0 and day 28 and stored at −20°C until assay. We also determined the bactericidal titers of sera from Bangladeshi cholera patients (aged 5 to 58 years; median, 30 years) whose disease was confirmed bacteriologically to be caused by O139 strains. Sera were prepared from blood collected on the day of presentation (representing day 2 after onset of illness and referred to as acute phase) and during convalescence (days 21 to 22, *n* = 11; day 30, *n* = 4).

A final set of serum samples was prepared from Bangladeshi patients whose disease was caused by O1 serogroup *V. cholerae*. Again, acute-phase samples were obtained on day 2, with convalescent samples on day 22 or 30. In some cases an intermediate day 11 sample was also available; where two postinfection samples were tested, the sample with the higher titer was used to calculate the (peak) rise in bactericidal titer. All sera were heat inactivated (56°C, 30 min) before assay.

RESULTS

Development of improved microplate bactericidal assay. Previous studies using a tube vibriocidal assay showed that the assay diluent and the concentration of indicator bacteria are two critical determinants of the susceptibility of O139 strains to lysis by antibody and complement. The former influences the efficiency of complement fixation, while the latter can dramatically affect assay sensitivity (2). Accordingly, these were the first variables to be modified in the microplate assay protocol, with the diluent being changed from saline to Mg^{2+}-saline and the indicator concentration being reduced from $3 × 10^6$ to 10^5 per ml. Initial titrations suggested that each change increased the assay sensitivity by about one well (twofold dilution), giving a combined minimum fourfold enhancement of antibody titers (data not shown, but see below). In addition, to make the endpoint determination more objective, plates were read spectrophotometrically after the growth phase of the assay, and a formula was devised to define the lytic endpoints. Initially, this involved a calculation of 50% inhibition of growth, but this was subsequently altered to 70% in the interests of assay reproducibility. (Although plots of bacterial survival versus serum dilution were not drawn for the present titrations, previous experience has shown us that both 50 and 70% killing endpoints lie on the straight part of the survival curve.)

At this stage the combined impact of changing the assay

TABLE 1. Summarized data from a comparison of microplate assay protocols[a]

Fold increase in titer	No. of individuals with indicated titer increase	
	Original protocol	Modified protocol
<2	18	9
2–<4	3	7
≥4	3	8

[a] Paired serum samples from O139 vaccine recipients (*n* = 24) were tested for bactericidal antibodies by two microplate assay protocols, involving either saline diluent and indicator bacteria at $3 × 10^6$ per ml (original protocol) or Mg^{2+}-saline diluent and indicator bacteria at 10^5 per ml (modified protocol). For both methods, the streptomycin-resistant strain 4260B was used as the indicator strain; assays were read spectrophotometrically, and 70% killing endpoints were determined. The data indicate the numbers of individuals registering various titer increases according to each procedure.

diluent and the indicator concentration was assessed by performing parallel titrations of 24 pairs of serum samples collected from recipients of an inactivated whole-cell O139 vaccine. For both pre- and postvaccination sera, titers assigned by using the modified procedure were a median eightfold higher than those obtained by the original method. For nine sample pairs, the increase in titer was the same for both pre- and postvaccination sera, resulting in no change in the ratio of the two endpoints. In seven other cases, however, the modified assay allowed the detection of seroconversion in vaccinees regarded as nonresponders using the original method, reflecting proportionally greater titer increases in postvaccination sera (Table 1). When the same serum pairs were titrated in the tube assay previously shown (2) to detect bactericidal responses following O139 disease, seven responders were detected (data not shown). Among this subset were five of the responders identified by the modified microplate assay but none of the three identified by the original protocol.

Further modifications were introduced to make the assay more convenient to perform. When compared with the growth of the indicator strain in fresh liquid culture, overnight growth on agar allowed the harvest of bacteria several hours earlier in the day. In addition, rather than adding an equal volume (100 µl) of culture medium to all wells at the end of the killing phase of the assay, 70 µl of 5× LB medium was added. This inhibited further complement fixation by converting the diluent to a medium significantly less supportive of complement activation (2). Moreover, it provided a richer LB medium (2× versus 0.5×) to accelerate growth during the second phase of the assay. With these changes it became possible to perform an assay within 8 h.

Assay reproducibility and specificity. The reproducibility of the modified assay procedure was assessed by repeat titration of selected serum samples. Initially, eight samples were assayed on five separate occasions; the titers for six of these remained constant, while a twofold variation was observed with the remaining two samples (Fig. 1A). Subsequently, three serum pairs showing four- to eightfold increases in bactericidal titer were assayed three to four times. The estimated titer increases were constant for each serum pair, indicating that the assay is highly reproducible (Fig. 1B).

To evaluate the specificity of the modified O139 bactericidal assay, serum samples collected from patients with O1 disease
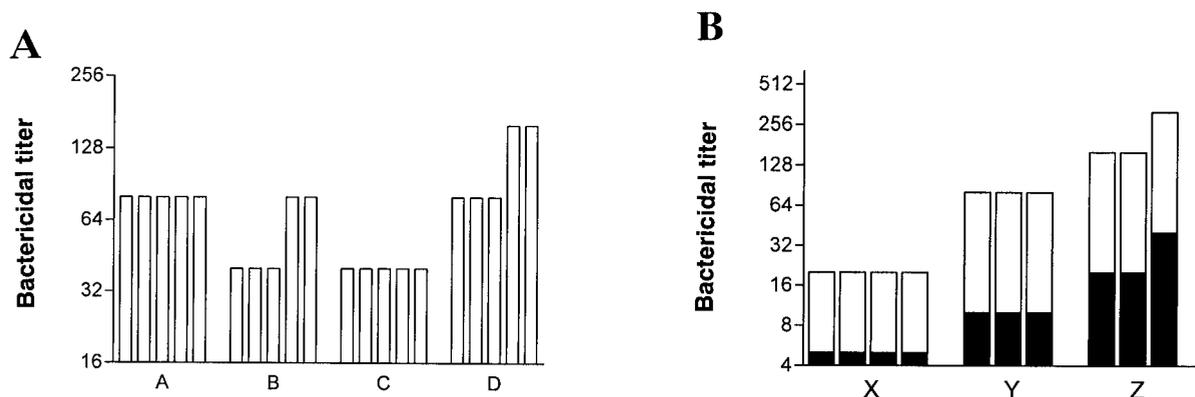
A

B

FIG. 1. Reproducibility of modified microplate assay protocol. (A) Eight sera were assayed on five separate occasions; the titers of six showed no variability (e.g., samples A and C), while the other two (samples B and D) showed a twofold range in titer. (B) Three serum pairs (X to Z) were each titrated three to four times, with the results shown. The titer preexposure is shown in black at the bottom of each histogram; the postexposure titer is represented by the open histogram. The ratio of postexposure to preexposure titers remained constant.

were initially screened for bactericidal responses against an indicator strain of homologous serogroup. These assays detected seroconversion to O1 in 14 patients, with a GM 27-fold rise in (peak) bactericidal titer. When these sera were reassayed against the O139 indicator strain, only one set showed a significant increase in anti-O139 bactericidal titer. In this patient there was evidence of seroconversion to O1 in a day 11 serum sample (32-fold titer increase, no increase against O139) and to both O1 and O139 in a day 22 serum sample (4- and 16-fold titer increases, respectively).

Bactericidal responses induced by O139 disease. Having detected seroconversion ($\geq$4-fold titer increase) in one-third of the O139 vaccine recipients by using the modified assay protocol (Table 1), it was of interest to determine the frequency and strength of bactericidal responses induced by the disease itself. Paired acute- and convalescent-phase serum samples from 15 O139 patients were titrated in the modified assay, and these results are shown in Table 2. Eleven patients showed $\geq$8-fold increases in lytic titer, while three others showed 2-fold increases. The median and GM responses of the group as a whole were 8- and 11-fold, respectively.

Examination of bactericidal responses after oral vaccination or infection with *V. cholerae* O1 has revealed an inverse correlation between the level of preexisting bactericidal antibody in serum and the magnitude of the responses observed (1, 5, 7). Similar trends were apparent when the magnitude of the immune responses detected in our two cohorts by the modified microplate assay were plotted against prevaccination or acute-phase bactericidal titers (Fig. 2). In both groups the correlation coefficients were highly significant ($r = -0.696$, $P = <0.0001$, and $n = 24$ for vaccine recipients; $r = -0.666$, $P = 0.0034$, and $n = 15$ for cholera patients).

DISCUSSION

Our original microplate assay protocol (13) has been modified to derive a test with improved sensitivity and objectivity, which is convenient to perform, specific, and highly reproducible. Experience with a tube-based O139 bactericidal assay had shown the critical impact of assay diluent on the efficiency of

complement activation and the dramatic reduction in lytic titers which can result from high concentrations of indicator bacteria (2). Modifying these variables resulted in a median eightfold increase in titer when 24 paired vaccinee sera were assayed in parallel by both protocols. The response profile seen with the modified microplate protocol was similar to that obtained with the more cumbersome tube-based bactericidal assay. The latter has previously provided a guide to immune status, at least with respect to vaccinated individuals subsequently challenged with pathogenic O1 vibrios (14; S. R. Attridge, unpublished data).

In addition to this improvement in assay sensitivity, other protocol changes made the test more reproducible and convenient to perform. Spectrophotometric determination of the ODs made estimation of the lytic endpoints more objective and also provided a means for defining the duration of the growth phase of the assay. Harvesting bacteria from agar and using

TABLE 2. Bactericidal responses induced by *V. cholerae* O139 disease[a]

Patient	Bactericidal titer of:		Fold increase
	Acute-phase serum	Convalescent-phase serum	
1	<20	2,560	256
2	<20	640	64
3	<20	640	64
4	<20	320	32
5	<20	160	16
6	<20	80	8
7	20	320	16
8	20	160	8
9	20	160	8
10	20	<20	0.5
11	40	1,280	32
12	40	80	2
13	80	640	8
14	80	160	2
15	80	160	2

[a] The values show the bactericidal titers of acute- and convalescent-phase sera from *V. cholerae* O139-infected patients as determined by the modified microplate assay.
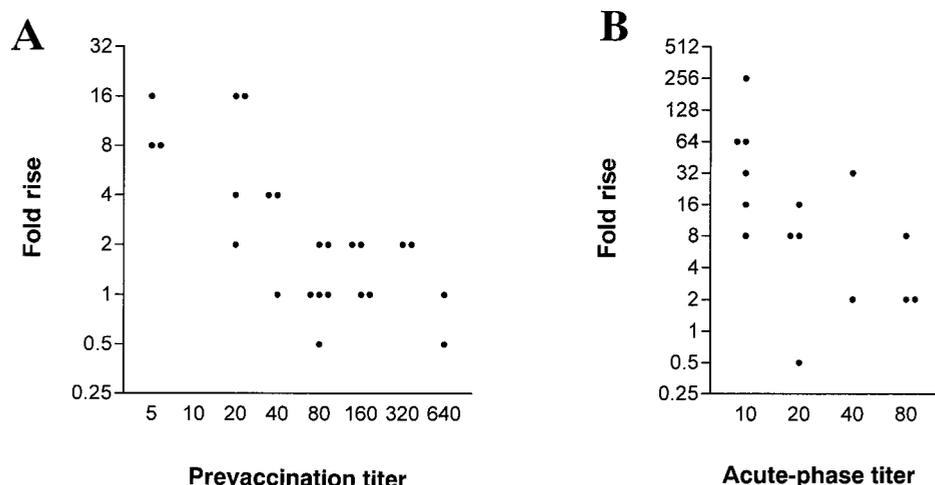
FIG. 2. Magnitude of anti-O139 serum bactericidal responses induced by vaccination or disease in relation to preexisting antibody titers. (A) Responses (fold rise in titer) of Vietnamese recipients of inactivated O139 vaccine, plotted in relation to prevaccination antibody titers. (B) Responses (titer of the convalescent serum divided by the titer of the acute-phase serum) of Bangladeshi cholera patients, plotted in relation to acute-phase antibody titers.

concentrated medium for the growth phase of the assay were both changes that reduced the time required to perform the test.

The modified protocol was demonstrated to be highly reproducible. Replicate assays of eight sera showed minimal interassay variation (Fig. 1A). When three serum pairs showing low but significant titer increases were subjected to repeated assay, the fold increase in lytic titer did not vary for any sample pair (Fig. 1B).

A recent report (8) described an O139 vibriocidal assay which determined 60% lytic endpoints against either wild-type or unencapsulated indicator bacteria (final bacterial concentration of 2.5×10^6 per ml; final complement concentrations of 10 and 2%, respectively). This was used to assess bactericidal responses after administration of wild-type or attenuated *V. cholerae* O139. Responses in both cohorts were very low and did not correlate with immune status upon rechallenge. Moreover, >50% of the control serum samples collected from volunteers exposed to bacteria other than *V. cholerae* O139 showed transient low-level activity in this assay (8). This finding prompted us to evaluate the specificity of our O139 bactericidal assay by testing serum samples collected from patients with O1 cholera. Such samples represent ideal specificity controls, given the extremely close relationship between the two pathogens. Only one of 14 serum sets showing seroconversion to O1 *V. cholerae* also registered as a responder against the O139 indicator strain, and even in this one instance subsequent field exposure to O139 could have occurred.

When the new assay procedure was applied to serum samples collected from O139 patients, the proportion (11 of 15 [73%]) and magnitude (GM = 23, median = 16) of significant responses were very similar to those obtained previously (2) by the tube assay (9 of 11 [82%]; GM = 29, median = 21). Clearly, seroconversion after O139 infection is readily detectable by either assay procedure, suggesting that our earlier goal (2) of combining the sensitivity of the tube assay with the screening power of a microplate procedure has been achieved.

Studies with *V. cholerae* O1 revealed significant inverse correlations between higher prevaccination or preinfection serum bactericidal titers and lower responses to subsequent (oral) vaccination or infection (1, 5, 7). Accordingly, the responses measured in the present study were plotted in relation to preexposure anti-O139 titers, and significant inverse correlations were noted in both cohorts (Fig. 2). Despite an initial concern that acute-phase sera collected from Bangladeshi patients on the day of presentation might already reflect the boosting of an immune system primed by environmental exposure, the titers seen with these sera were lower than those recorded for the sera prepared from Vietnamese recipients prior to vaccination. This suggests that the latter individuals had been environmentally primed, either by *V. cholerae* O139 or by other bacteria with cross-reacting O antigens. The higher preexposure antibody titers of the vaccination cohort compared to those of the cholera patients would appear to be a major factor limiting the seroconversion rate of 33% (8 of 24) observed with the inactivated O1/O139 vaccine. If only individuals with initial bactericidal titers of ≤40 are considered, the proportion of responders following vaccination (8 of 10 [80%]) is similar to that seen following infection (10 of 12 [83%]). This would argue that, during the initial period of vaccine evaluation, potential recipients should be prescreened to eliminate those with high levels of bactericidal antibodies; those who remain might give a clearer indication of vaccine immunogenicity.

REFERENCES

1. **Attridge, S. R.** 1991. Oral immunisation with *Salmonella typhi* Ty21a-based clones expressing *Vibrio cholerae* O antigen: serum bactericidal antibody responses in man in relation to pre-immunisation antibody levels. Vaccine **9:**877–882.

2. **Attridge, S. R., F. Qadri, M. J. Albert, and P. A. Manning.** 2000. Susceptibility of *Vibrio cholerae* O139 to antibody-dependent, complement-mediated bacteriolysis. Clin. Diagn. Lab. Immunol. **7:**444–450.

3. **Cholera Working Group.** 1993. Large epidemic of cholera-like disease in Bangladesh caused by *Vibrio cholerae* O139 synonym Bengal. Lancet **342:** 387–390.

4. **Coster, T. S., K. P. Killeen, M. K. Waldor, D. T. Beattie, D. R. Spriggs, J. R. Kenner, A. Trofa, J. C. Sadoff, J. J. Mekalanos, and D. N. Taylor.** 1995. Safety, immunogenicity, and efficacy of live attenuated *Vibrio cholerae* O139 vaccine prototype. Lancet **345:**949–952.

5. **Jertborn, M., A.-M. Svennerholm, and J. Holmgren.** 1986. Saliva, breast milk, and serum antibody responses as indirect measures of intestinal immunity after oral cholera vaccination or natural disease. J. Clin. Microbiol. **24:**203–209.

6. **Jertborn, M., A.-M. Svennerholm, and J. Holmgren.** 1996. Intestinal and systemic immune responses in humans after oral immunization with a bivalent B subunit-O1/O139 whole cell cholera vaccine. Vaccine **15:**1459–1465.

7. **Levine, M. M., and C. O. Tacket.** 1994. Recombinant live cholera vaccines, p. 395–413. *In* I. K. Wachsmuth, P. A. Blake, and O. Olsvik (ed.), *Vibrio cholerae* and cholera: molecular to global perspectives. ASM Press, Washington, D.C.

8. **Losonsky, G. A., Y. Lim, P. Motamedi, L. E. Comstock, J. A. Johnson, J. G. Morris, Jr., C. O. Tacket, J. B. Kaper, and M. M. Levine.** 1997. Vibriocidal antibody responses in North American volunteers exposed to wild-type or vaccine *Vibrio cholerae* O139: specificity and relevance to immunity. Clin. Diagn. Lab. Immunol. **4:**264–269.

9. **Morris, J. G., Jr., G. A. Losonsky, J. A. Johnson, C. O. Tacket, J. P. Nataro, P. Panigrahi, and M. M. Levine.** 1995. Clinical and immunologic characteristics of *Vibrio cholerae* O139 Bengal infection in North American volunteers. J. Infect. Dis. **171:**903–908.

10. **Mosley, W. H., A. S. Benenson, and R. Barui.** 1968. A serological survey for cholera antibodies in rural East Pakistan. 1. The distribution of antibody in the control population of a cholera-vaccine field-trial area and the relation of antibody titer to the pattern of epidemic cholera. Bull. W. H. O. **38:**327–334.

11. **Mosley, W. H., W. M. McCormack, A. Ahmed, A. K. M. A. Chowdhury, and R. K. Barui.** 1969. Report of the 1966–67 cholera vaccine field trial in rural East Pakistan. 2. Results of the serological surveys in the study population—the relationship of case rate to antibody titer and an estimate of the inapparent infection rate with *Vibrio cholerae*. Bull. W. H. O. **40:**187–197.

12. **Nair, G. B., T. Ramamurthy, S. K. Bhattacharya, A. K. Mukhopadhyay, S. Garg, M. K. Bhattacharya, T. Takeda, T. Shimada, Y. Takeda, and B. C. Deb.** 1994. Spread of *Vibrio cholerae* O139 Bengal in India. J. Infect. Dis. **169:**1029–1034.

13. **Qadri, F., G. Mohi, J. Hossain, T. Azim, A. M. Khan, M. A. Salam, R. B. Sack, M. J. Albert, and A.-M. Svennerholm.** 1995. Comparison of the vibriocidal antibody response in cholera due to *Vibrio cholerae* O139 Bengal with the response in cholera due to *Vibrio cholerae* O1. Clin. Diagn. Lab. Immunol. **2:**685–688.

14. **Tacket, C. O., B. Forrest, R. Morona, S. R. Attridge, J. LaBrooy, B. D. Tall, M. Reymann, D. Rowley, and M. M. Levine.** 1990. Safety, immunogenicity, and efficacy against cholera challenge in humans of a typhoid-cholera hybrid vaccine derived from *Salmonella typhi* Ty21a. Infect. Immun. **58:**1620–1627.

15. **Tacket, C. O., G. Losonsky, J. P. Nataro, L. Comstock, J. Michalski, R. Edelman, J. B. Kaper, and M. M. Levine.** 1995. Initial clinical studies of CVD112 *Vibrio cholerae* O139 live oral vaccine: safety and efficacy against experimental challenge. J. Infect. Dis. **172:**883–886.

16. **Trach, D. D., P. D. Cam, N. T. Ke, M. R. Rao, P. N. Dinh, P. V. Hang, N. V. Hung, D. G. Canh, V. D. Thiem, and J. D. Clemens.** Investigations of the safety and immunogenicity of a new killed oral cholera vaccine developed in Vietnam. Bull. W. H. O., in press.