Rapid Method of Determining Cholera Vibrio Biotype

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The characteristic motility of cholera vibrios, as viewed through a dark-field microscope, and the adhesiveness of chicken cell-positive vibrios provide a means for rapidly identifying and biotyping cholera vibrios. Dilute suspensions of vibrios, such as one might find in a fresh rectal swab specimen from a cholera patient, when mixed with a 0.25% suspension of chicken erythrocytes in saline, can be used to biotype the cholera vibrios without prior isolation in pure culture. This is accomplished by using a dark-field microscope through which the chicken cell-positive cholera vibrios are observed to attach to the scattered erythrocytes and to propel them with a characteristic flipping motion.

In 1964 Beneson et al. (2) described a method for rapidly identifying vibrios by using a dark-field microscope. Dark-field examination of rectal swab samples enabled them to identify cholera vibrios by their characteristic motility and, in this way, to confirm the clinical diagnosis of cholera in 80% of diarrheal cases within minutes. When sufficient concentrations of organisms were present, these investigators specifically characterized vibrios by immobilization with specific and group antisera. Rapid methods for biotyping cholera vibrios have not been described previously. To differentiate Classical from El Tor vibrios, it has been necessary to isolate these organisms in pure culture and test them for sheep cell hemolysis, chicken cell agglutination, resistance to polymyxin B, and/or sensitivity to phage IV (5).

The chicken cell agglutination test is a useful and simple test that is positive with many, but not all, El Tor and non-cholera vibrio biotypes (1, 4). This test requires highly concentrated vibrio suspensions usually obtained after plating and overnight incubation of the cholera patient's stool (obtained by rectal swab).

When low concentrations of chicken cell-agglutinating vibrios are mixed with dilute suspensions of chicken erythrocytes, the motile vibrios adhere to scattered erythrocytes and cause them to rotate like coins that have been flipped into the air. These "flipping" erythrocytes are easily perceived under a dark-field microscope as tiny flickering circles of light. Frequently, it is possible to visualize the tiny motile vibrios attached to the cells. The flipping movement of the vibrio-propelled cells is smooth and continuous as opposed to the abrupt, irregular jolting movement sometimes seen with high concentrations of nonagglutinating vibrios. These organisms collide with, but do not attach to, the chicken erythrocytes.

In this study, the dark-field test for chicken cell adhesiveness of vibrios is compared with the traditional chicken cell agglutination test, as a simple method for rapidly biotyping cholera vibrios.

MATERIALS AND METHODS

One hundred stock vibrio cultures and 373 rectal swabs were examined for chicken erythrocyte adhesiveness (vibrio's reaction as noted by the rapid biotyping method) by the technique described above, and the results were compared with those of the standard chicken cell agglutination test (4).

The stock cultures were obtained from the Bacteriology Branch of the Bangladesh Cholera Research Laboratory. Specimens from each stock culture were introduced into 1 ml of peptone broth and incubated for 30 min at 35 C. They were then tested under a dark-field microscope for chicken cell adhesiveness. Each organism had been characterized previously by its reaction with specific antiserum, standard chicken cell agglutination, phage IV typing, and sensitivity to polymyxin B. Of the 100 vibrios examined, 28 were Classical Inaba, 22 were Classical Ogawa, 9 were El Tor Inaba, 30 were El Tor Ogawa, and 11 were non-cholera vibrios. At the time of examination, the examiner was not aware of the identification of the test strains. After characterization of the 100 "unknown" stock specimens by the dark-field method, results of the chicken cell agglutination by these same organisms were compared with their chicken erythrocyte adhesiveness.

Three hundred and seventy-three rectal swabs from patients (routine admission) with suspected cholera were obtained at the Cholera Research Laboratory in
Dacca and examined. The swabs, taken at the time of admission, were placed immediately in 3 ml of peptone broth and incubated at 35 C; they were then examined after incubation periods varying from a few minutes to 20 h. Chicken erythrocyte adhesiveness, as demonstrated by dark-field examination, was compared with the results of the chicken cell agglutination test determined on the following day.

Identical routine techniques were utilized in examining the rectal swab and stock specimens. A drop of each specimen was placed on a clean glass slide without a cover slip. The slide was then placed on the stage of a dark-field microscope. Under microscope observation a second drop of 0.25% chicken erythrocytes was added, and the specimen was examined immediately for vibrio movement and or erythrocyte flipping. Erythrocyte movement, when present, was apparent within seconds after addition of red cells to the vibrio suspension. Rapid detection of this movement was essential since the cells either sank to the surface of the slide or lysed within 1 or 2 min.

RESULTS AND DISCUSSION

One hundred stock vibrio strains were tested for chicken erythrocyte adhesiveness by the dark-field method and compared with the chicken cell agglutination reactions of these same organisms (Table 1).

The results of the 373 rectal swab specimens biotyped by the dark-field methods were compared with the conventional chicken cell agglutination reactions of vibrios isolated from these swabs in pure culture (Table 2).

Results of the dark-field test correlate well with the standard chicken cell agglutination test. Of the 235 chicken cell-negative vibrios studied, none produced chicken erythrocyte flipping. Fifty-five of 88 chicken cell-positive vibrios examined produced a characteristic erythrocyte movement that was easily recognizable under a dark-field microscope, indicating a high degree of specificity and sensitivity of the rapid dark-field test for chicken cell-positive organisms.

Chicken cell-positive vibrios appear to be quite adhesive. In suspension with a 0.25% mixture of chicken erythrocytes in saline, the likelihood of collision of a vibrio with a chicken cell is high. Thus, it was not unusual to identify erythrocyte flipping in extremely low concentrations of chicken cell-positive organisms. In one instance (peptone broth suspension of a stock El Tor vibrio), a single vibrio was found in a drop under examination that was adhered to a flipping erythrocyte. Nonetheless, the position of vibrios attachment to erythrocytes is important, since the tiny flagella of the vibrios must be free to move in such a way as to give them the mechanical advantage necessary to flip the

| Table 1. Comparison of chicken erythrocyte adhesiveness and agglutination among 100 stock vibrio cultures. |
|--------------------------------------------------------|
| **Vibrio (no.)** | **No. adhered** | **No. agglutinated** | **False negatives (no.)** | **False positives (no.)** |
| NCV* (2) | 0 | 0 | 0 | 0 |
| Classical Inaba (28) | 0 | 0 | 0 | 0 |
| Classical Ogawa (22) | 0 | 0 | 0 | 0 |
| El Tor Inaba (9) | 8 | 9 | 1 | 0 |
| El Tor Ogawa (30) | 29 | 30 | 1 | 0 |
| NCV (9) | 9 | 9 | 0 | 0 |

* NCV, Non-cholera vibrio.

| Table 2. Comparison of chicken cell adhesiveness and agglutination among 373 rectal swabs obtained from patients with clinical cholera |
|--------------------------------------------------------|
| **Vibrio (no.)** | **No. adhered** | **No. agglutinated** | **False negatives** | **False positives** |
| None* (149) | 8 | 9 | 1 | 0 |
| El Tor Ogawa (9) | 1 | 1 | 0 | 0 |
| NCV (1) | 0 | 0 | 0 | 0 |
| Classical Inaba (184)* | 0 | 0 | 0 | 0 |
| Classical Ogawa (1)* | 0 | 0 | 0 | 0 |

* Each rectal swab specimen was examined initially for vibrio motility under a dark-field microscope. When motility was not observed, the specimen was read as negative for vibrios. In most cases, these were then not examined for erythrocyte adhesiveness.

* Twenty-eight rectal swabs also examined, but not included, that were positive for Classical Inaba vibrios by culture, as well as one rectal swab that was positive for Classical Ogawa vibrios by culture, were negative for vibrio movement upon initial dark-field examination. These were not tested for chicken cell adhesiveness since the initial dark-field examination incorrectly identified these as coming from non-cholera patients.

comparatively enormous erythrocytes. With extremely low concentrations of chicken cell-positive vibrios, the likelihood of attachment is high; however, the likelihood of erythrocyte flipping may be less because of improper attachment. This may have accounted for the false classification, by the dark-field technique, of the two stock El Tor vibrios since very low concentrations of vibrios were examined.

The rectal swabs examined upon the admission of the patients yielded the highest concen-
trations of vibrios after 4 to 6 h of incubation in peptone broth at 36 C. Although immediate identification and characterization of the vibrio were possible in many cases, the optimal time for successful dark-field examination of rectal swabs was after the incubation period.

The modified chicken cell agglutination test has several advantages over the traditional agglutination test which is routinely used to biotype cholera vibrios. It provides a more rapid means of characterizing the vibrio biotype. It is particularly suited to field work in remote areas where bacteriological facilities may be limited, if not totally lacking. In conjunction with dark-field immune immobilization with group-specific and type-specific antisera, it also may obviate the need for isolation of the cholera vibrio in pure culture before typing. Like the chicken cell agglutination test, however, it may not be used to differentiate non-cholera from cholera vibrios, since non-cholera vibrios may be either chicken cell positive or negative.

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