

## Rapid Identification of *Vibrio cholerae* by Darkfield Microscopy\*

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*This article describes a rapid, simple and reproducible method for detecting Vibrio cholerae in diarrhoeal patients. The method involves darkfield examination of a liquid stool specimen or a rectal swab immersed in broth and immobilization of V. cholerae by the addition of specific vibrio antisera. The authors state that in 80% of cases a definitive diagnosis is available within five minutes. There is no need for elaborate equipment or long training of technicians and the method is easily performed by one person in the field.*

The genus *Vibrio* is named for the unique, rapid to-and-fro motility which is characteristic of this group of organisms. In 1884, Robert Koch compared this vibrating, swarming movement to the exceedingly rapid progress of a host of gnats. In 1919, Sanarelli (quoted by Pollitzer, 1959) reported that vibrio movement was three to ten times more rapid than that of other common motile bacteria. However, diagnostic methods based on this characteristic have not been advocated since 1896 (Dunbar; quoted by Pollitzer, 1959); in fact, the value of direct microscopic examination of material from cholera patients is deprecated in the literature generally available (see Pollitzer).

Although proper treatment of the cholera patient does not depend on early recognition of the causative organism, early diagnosis is important for checking the spread of cholera and studying the disease. Ordinary bacteriological culture techniques usually require 18-24 hours before the specific diagnosis can be established. For the recognition of carriers, the added enrichment procedure prolongs the period to 36 or 48 hours. The fluorescent antibody technique (Finkelstein & LaBrec, 1959) makes a definitive diagnosis possible within one hour of taking a sample from the suspected patient, and the carrier can be recognized within seven or eight hours. This technique, however, requires costly equipment and a highly skilled observer. The present study was undertaken to determine whether early, reliable

diagnosis could be achieved by simpler techniques based on the vibrio's characteristic motility.

### MATERIALS AND METHODS

#### Equipment

The following equipment was used for these studies: a Baker research microscope equipped with a Trilux condenser, which provides both phase contrast and darkfield illumination; a Leitz Ortholux microscope with phase-contrast condenser; and a Cooke-McArthur microscope equipped with darkfield condenser. 10× and 40× objectives were used with 8× or 15× oculars. Microscope slides and cover-slips were chemically clean. The group- and type-specific cholera antisera contained no bactericidal preservative, as determined by lack of effect on heterologous vibrio strains. The titres of the sera used were such that a 64-fold dilution was the end-point in tube agglutination with living vibrios.

#### Media

Bile peptone broth with a pH of 9.2, and tellurite-taurocholate gelatin agar (TTGA) plates were prepared by the method of Monsur (1963). The non-suppressant broth was 1% trypticase (BBL) and 1% sodium chloride in distilled water; this had a pH of 7.2.

#### Examination technique

A small drop of the test material was placed on a clean slide by a bacteriological loop, a capillary pipette, or other means; a clean cover-slip was applied, and the slide examined under darkfield or phase illumination at 400×-600× magnification.

\* From the Pakistan-SEATO Cholera Research Laboratory, Dacca, East Pakistan. This work was supported in part by Research Agreement No. 196802 between the National Institutes of Health, Bethesda, Md., USA, and the Pakistan-SEATO Cholera Research Laboratory.

If organisms with the typical motility of vibrios were observed, two other preparations were set up: one was mixed with an equal volume of Inaba antiserum, and the other with anti-Ogawa serum; cover-slips were applied. If motility ceased in one of these preparations within three to five minutes and no change had occurred in the other, specific identification was completed. If the contrast between the two preparations was not definite, an additional mount was prepared with vibrio O group I antiserum. If this mixture had no effect on the vibrio motility, the organisms were presumed to be non-cholera vibrios.

With enrichment technique, the rectal swab tube containing bile peptone broth was held at 37°C, and the fluid was examined after various intervals with final examination after at least 18 hours of incubation.

The sensitivity of the darkfield method was measured by performing simultaneous darkfield readings and bacterial counts on 10-fold dilutions in enrichment broth of cholera stool or broth culture of vibrios. At least two observers made darkfield readings on coded samples; the readings were repeated after overnight incubation. Counts were performed by dropping a measured volume on the surface of TTGA plates and enumerating the colonies after overnight incubation.

#### *Clinical material*

Rectal swabs or faecal samples were obtained from 138 patients admitted to the ward of the Pakistan-SEATO Cholera Research Laboratory. By the laboratory's normal bacteriological routine, 107 of these patients were shown to be infected by *V. cholerae*. This routine consists of daily culturing directly on gelatin agar and TTGA plates, with subculturing to these media after overnight enrichment in tellurite bile broth (Monsur, 1963). The other 31 cases were bacteriologically negative for *V. cholerae* on all examinations.

The rectal swabs (plain cotton wool) were placed in test-tubes containing 0.3-0.5 ml of bile peptone broth and held at 37°C. Every time material was removed for examination, a drop was streaked on a TTGA plate, which was examined the following day independently by the diagnostic bacteriologists.

The swabs of 53 patients were taken and examined within five minutes of each patient's admission; swabs from 33 were examined within five minutes and again after 4-30 hours' incubation at 37°C. Swabs from a further 52 patients were first examined microscopically after incubation for more than three

hours; these were re-examined at 18 hours or more if a specific diagnostic had not been established.

Rectal swabs were taken daily from 111 patients (89 of the *V. cholerae*-positive and 22 of the negative patients) to observe the persistence of vibrios. If a specific vibrio type had already been established, typing was not always repeated. The majority of these patients had been treated with tetracycline (Greenough et al., 1964).

#### RESULTS

With either darkfield or phase illumination, the motility of vibrios is easily visualized and clearly different from that of other organisms we have found in faecal samples. At 400×-600× magnification, the motility of the vibrio is so rapid that it cannot be held in the microscopic field. Some vibrios are observed to move in a gyrating, centrifuge-like manner. In the dark field, material positive for vibrios evokes a mental image of many shooting stars in a dark sky. At a magnification of 100×-150×, Koch's analogy of swarming gnats is seen to be apt, with busy milling in a scintillating field. The characteristic motility is perhaps clearer in darkfield than in phase-contrast microscopy. Because darkfield equipment is more generally available and less costly than that for phase contrast, our further studies were done with darkfield only.

In 16 separate dilution studies, the highest dilution in which vibrios were recognized had an average bacterial count of  $4.3 \times 10^5$  vibrios per ml, with a range in individual observations from  $2.3 \times 10^4$  to  $1.7 \times 10^6$ . After at least eight hours' incubation at 37°C, re-examination of these dilutions suggested that the sensitivity of the method with the enrichment step allows detection of an individual vibrio in the starting material. Similar results were obtained with the suppressant bile peptone broth at pH 9.2, and with the non-suppressant broth at pH 7.2.

Specific or group antisera immobilized cholera vibrios within three to five minutes; in many instances, motility had ceased before the mixture could be examined. The differences in the two preparations were unequivocal if the vibrio density was sufficient; in case of doubt, longer enrichment usually clarified the situation. Occasionally, though the difference between the two preparations was definite, some vibrio motility persisted. In several of these cases, both cholera and non-cholera vibrios were later found on the culture plates.

The swabs of 62 of the 107 *V. cholerae*-positive patients were examined within five minutes; 79%

## RESULTS OF DARKFIELD AND CULTURE STUDY ON SPECIMENS FROM HOSPITALIZED DIARRHOEA PATIENTS

Material tested	Darkfield (DF) and culture (Cult.) techniques							
	Observation and plating directly				Observation and plating after enrichment			
	DF+ Cult.+	DF+ Cult.-	DF- Cult.+	DF- Cult.-	DF+ Cult.+	DF+ Cult.-	DF- Cult.+	DF- Cult.-
<i>V. cholerae</i> -positive diarrhoea patients								
Specimens at admission from 107 patients	49	0	13	0	66	1 <sup>a</sup>	2 <sup>b</sup>	2 <sup>c</sup>
Specimens after admission from 89 patients	30	3	12	70	39	7	3	95 <sup>d</sup>
<i>V. cholerae</i> -negative diarrhoea patients								
Specimens at admission from 31 patients	0	0	0	24 <sup>e</sup>	0	0	0	14 <sup>e</sup>
Specimens after admission from 22 patients	0	0	0	24 <sup>f</sup>	0	0	0	27 <sup>f</sup>

<sup>a</sup> Reported as *V. cholerae* Ogawa by darkfield examination; reported as non-cholera vibrios by culture. Agglutination showed rise in titre from < 1:40 with both antigens to 1:320 and 1:40 against Ogawa and Inaba antigens, respectively.

<sup>b</sup> In one case, vibrios were identified but were reported as non-cholera vibrios.

<sup>c</sup> Both patients were positive in other specimens; one positive only on the second day.

<sup>d</sup> 26 tetracycline-treated patients were positive after routine enrichment technique in 15 ml broth.

<sup>e</sup> Two diagnosed as non-cholera vibrios.

One diagnosed as non-cholera vibrios.

were correctly detected by the direct technique. When the material was examined after enrichment, the darkfield findings agreed almost perfectly with the cultural results. Of 71 *V. cholerae* patients, 94% were recognized by darkfield examination, as compared with 96% recognized in the same material by a highly selective cultural method (see the table).

Subsequent daily swabs were taken from 89 of the positive patients; again, agreement with the cultures was excellent. More specimens were detected as positive after enrichment by darkfield than by culture (32% compared with 29%); the validity of these positive results is attested by two instances in which re-examination of the plates disclosed rare vibrio colonies obscured by overgrowing organisms. (These instances are tabulated as positive by both techniques.) One patient, reported as having *V. cholerae* Ogawa by darkfield, but as negative for cholera vibrios by culture, developed a significant rise in antibody titre against Ogawa (see footnote a to table).

All patients negative for *V. cholerae* were negative in all examinations (see the table). It is pertinent that non-cholera vibrios were recognized as such in three admission specimens from *V. cholerae*-negative patients, and in the subsequent specimens from two other patients. These were not confused with *V. cholerae*.

Correlation was excellent between the darkfield technique and matching cultures. However, discrepancies between darkfield results and those of routine bacterial methods were noted in significant number when the rectal swabs were tested after patients had had one or two days of tetracycline therapy. Ninety-five plain swabs incubated in 0.3-0.5 ml of bile peptone broth were negative by darkfield and matching culture; *V. cholerae* was demonstrated in 26 of the tellurite-impregnated swabs taken at the same time and incubated in a relatively large volume (1.5 ml) of bile peptone broth. It is likely that dilution of antibiotic by the excess medium permitted isolation of *V. cholerae* in these tetracycline-treated patients.

## DISCUSSION

A simple darkfield microscopic technique can make available to the clinician, investigator, or quarantine officer a specific diagnosis of cholera within minutes in approximately 80% of vibrio-caused diarrhoeal cases. When enrichment (in either selective or non-selective media) is added, nearly all cases recognized by culture are detected. Definitive information is available within 8-18 hours; in contrast, 48 hours may be required for a final negative report with cultural techniques.

The characteristic motility of the vibrio has long been appreciated; in 1960, Majid Khan (1962) used the hanging drop to diagnose cholera, but reported that this technique had real value only after six hours' incubation in alkaline peptone water. The use of darkfield illumination facilitates the visualization of typical motility; the addition of the specific antisera minimizes the risk of false positive results; indeed, in some instances the darkfield technique may detect vibrios when recognition by culture is prevented by overgrowth of other organisms.

The procedure can be simplified by testing with vibrio O Group I antiserum to establish whether the observed vibrios are *V. cholerae*; if desired, the vibrios can then be tested with the specific antisera. This sequence spares the more valuable antisera. For examination of antibiotic-treated patients, if the direct examination is negative, enrichment should be done with a relatively large volume of enrichment broth. The availability of compact portable microscopes (McArthur, 1945) makes the technique practicable even under the most primitive conditions.

The simplicity of the procedure places it within the grasp of any technician trained in basic microscopy, enabling him to detect vibrios literally within seconds. These attributes make the technique valuable in screening specimens for the presence of *V. cholerae*, minimizing delay in initiating epidemiological or special clinical observations.

This procedure has advantages over fluorescent microscopy in that less costly equipment is required, the processing of the specimen is minimal, and less technical skill is necessary for achieving accurate results. Neither direct microscopic method provides material for confirmation or further studies; however, the rectal swabs in enrichment fluid need only be transported to a central laboratory for strain isolation, if desired (Monsur, 1963).

In short, this diagnostic technique, based on equipment and media available in all general laboratories, makes diagnosis possible in minimal time, with an accuracy equal to that of the most selective vibrio culture system in cholera-suspect diarrhoea.

#### ACKNOWLEDGEMENTS

The authors are greatly indebted to Mr. M. Imdadul Huq for the invaluable bacteriological readings, and to Dr S. N. Zaman for performing the quantitative studies.

#### RÉSUMÉ

Bien que la mise en évidence du vibriion cholérique ne soit pas un préalable indispensable à l'application d'un traitement précoce contre la maladie, elle est nécessaire aux recherches sur la propagation du vibriion et l'étiologie du choléra. Il faut 18-24 heures pour qu'une culture donne des résultats utiles s'il s'agit d'un malade, et 36-48 heures avec la technique d'enrichissement pour déceler un porteur de germes. La technique des anticorps fluorescents permet le diagnostic en 1 heure et 7-8 heures respectivement, mais elle est coûteuse et compliquée.

Les auteurs ont entrepris des recherches en vue de la mise au point d'une méthode fondée sur l'immobilisation du vibriion par un immunsérum spécifique. En examinant au microscope sur fond noir des échantillons de selles liquides ou des prélèvements rectaux dilués dans le bouillon, on observe sans difficulté les vibriions animés de leur mouvement caractéristique. L'addition d'antisérum spécifique provoque l'immobilisation des vibriions de souches correspondantes, ce qui assure en 5 minutes un diagnostic spécifique dans 79 % des cas positifs à la culture.

94% de 71 cas de choléra, dont les prélèvements ont été examinés après enrichissement, ont été diagnostiqués par cette méthode, lors de leur admission à l'hôpital, alors que 96% l'étaient par culture sur milieu hautement sélectif. Dans une autre série d'expériences, 32% de 57 cas de choléra ont été décelés par la méthode d'immobilisation, contre 29% par culture. Cette méthode n'a donné aucune réponse faussement positive lors de l'examen de 31 cas négatifs à la culture, bien que 5 des malades aient été porteurs de vibriions non cholériques.

La méthode est assez sensible pour qu'une concentration de  $10^5$  vibriions par ml permette le diagnostic par immobilisation. Si l'on procède à un enrichissement, la présence de quelques vibriions seulement dans le matériel pathologique — d'un seul même peut-être — suffit au diagnostic. Lorsqu'il s'agit de cas traités par des antibiotiques, l'enrichissement doit être prévu de façon que l'antibiotique soit assez dilué pour ne pas inhiber la croissance.

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