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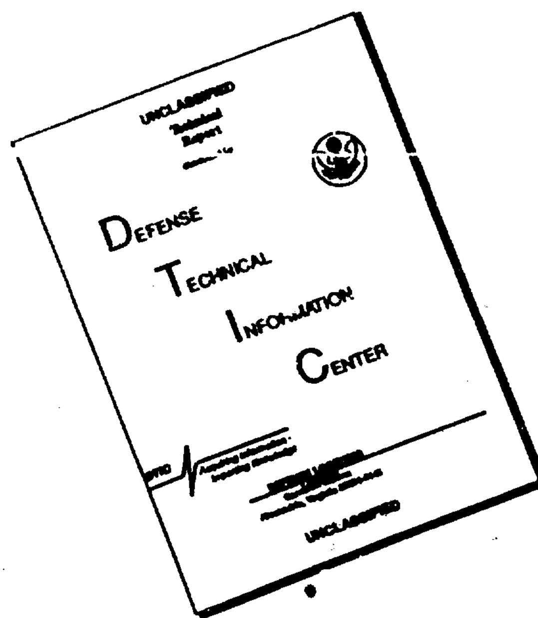

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Translated by Sp/6 Charles T. Ostertag Jr.

The Possibility of Using Specific Fluorescent Antibodies for Rapid Detection of the Cholera Vibrio in Water.

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In recent years, in native as well as foreign literature, a number of works have been published which suggest the use of fluorescent antibodies for the early laboratory diagnosis of infectious diseases and for indications of pathogenic microbes in the environment (Dashkevich and Mikhaylov, 1957; Mikhaylov and Li Li, 1958; Levina, 1958; Kabanova and Glubokina, 1958; Dashkevich, Dyakov and others, 1959; Thomason, Moody and Goldman, 1956; Thomason, Cherry and Moody, 1957; Halpern with coauthors, 1958; Winter and Moody, 1959).

The aim of our work was to obtain an anticholera fluorescent serum and investigate the possibility of using it for the rapid identification of cholera vibrios during laboratory investigation of material containing them. [1]

Diagnostic cholera O-serum was used for obtaining the fluorescent antibodies. It was produced at the Mikrob Institute and was obtained from horses. Rabbit immune sera were also used. The agglutinin titers of the sera ranged between 1:1000 and 1:7500. Globulin fractions of immune and normal sera were labeled with fluorescein-isocyanate. The globulin fractions were isolated by salting-out with ammonium sulfate at half-saturation and dry sodium sulfate up to a concentration of 1.23 M.

Conjugation of globulin with the fluorochrome was done by Coon's and Kaplan's method (1950). Fluorescein-isocyanate, prepared at the All-Union Scientific-Research Institute for Chemical Reagents [2], was used as the staining-fluorochrome. The serological activity of the prepared conjugates was determined by the agglutination reaction in test tubes and by the capability to cause luminescence of bacteria in smears treated with fluorescent serum for 20-30 minutes at room temperature.

Investigation of the processed preparations was carried out with the help of a MBI-1 microscope, equipped with an OI-17 luminescent opaque illuminator, and also an ML-1 luminescence microscope. In both cases they used an FK 90X objective, a 7X ocular, and FS-1 and ZhS-18 light filters. For eliminating the natural luminescence of commercial immersion oil, nitrobenzene was added in the proportion of (one part) of nitrobenzene to 3 parts of oil (Zubzhitskiy, 1957).

For control, the preparations were also studied in a phase contrast microscope to detect bacteria which didn't show up during luminescence microscopy.

The protein content in individual series of fluorescent serum prepared by us fluctuated from 0.6 to 0.9%, the titers in the agglutination reaction equaled 1:1600 - 1:3000. The cholera vibrios in smears treated with fluorescent serum, under dark field fluorescence examination, had the appearance of glistening yellow-green minute bacilli, slightly swollen and curved to various degrees. The peripheral part of the cell shone especially brightly.

If the smears were treated with normal or heterologous (antiplague) luminescent sera, then the cholera vibrios didn't shine. The fluorescent anticholera serum which we prepared wasn't always strictly specific; when smears were treated with it there was observed a fluorescence of cells of paracholera vibrio and several representatives of the entero-typhoid group. However, this nonspecific reaction could be eliminated by conjugate dilution.

The level of dilution for each series of serum was established empirically, that is, in the experiments serum was used in dilutions at which there was no lowering in the intensity of luminescence of the cholera vibrios (1:6 - 1:12). While using such a diluted fluorescent serum, nonspecific luminescence was observed only in several strains of paracholera vibrios, however the fluorescence of the latter wasn't of such a brilliance and character as in the cells of the cholera vibrio (see picture). Taking into account all that has been written, we considered it possible to proceed in the concrete application of the fluorescent serum prepared by us and to simultaneously search for a method for the complete elimination of the nonspecific reactions which accompanied this. The present report is devoted to an explanation of the feasibility of using fluorescent anticholers serum for the indication of cholera vibrio in samples of water artificially contaminated under laboratory conditions.

In preliminary experiments, conducted with the aim of determining the sensitivity of the method, it was noted that during luminescence microscopy of smears, prepared from suspensions of cholera vibrio with a density of 1 million microbial cells in 1 ml, several fluorescing vibrios were detected in almost every field of vision. In smears from suspensions with a lesser density (500 000 - 100 000 microbes in 1 ml), single cells were encountered in occasional fields of vision. Therefore it was difficult to locate them.

In order to concentrate the suspensions of vibrios and make it easy to detect them in smears, we used membrane filters on which it was possible also to incubate cholera vibrios by placing the filters on a solid nutritive medium (Chibrikova with coauthors, 1958).

Being satisfied that in smears made from washings of filters immediately after filtration, one could detect cholera vibrios when the filter contained about 1 million organisms, and lesser numbers were detected after a 4-5 hour incubation of the filters on agar at 37°, we conducted a series of experiments, concluding in an investigation of various samples of water, both contaminated and not contaminated with cholera vibrios.

The water was obtained in various seasons, for the most part from wells serving for domestic needs. In each sample of water we determined the overall number of bacteria in 1 ml (seeding according to Koch). Then the water was divided into two parts. To one of these we added a suspension of a 24 hour culture of cholera vibrio in a definite amount.

The samples of contaminated and non contaminated water were investigated in like manner: First they were innoculated in peptone water (10 ml of basic peptone solution + 90 ml of the water under investigation), the innoculations were incubated at 37° and after 3, 4, 5 and 6 hours, smears, prepared from the surface of the medium, were treated with fluorescent serum; second, simultaneously those same samples of water were filtered through membrane filters (5 ml per filter, of the latter they took 5-6 specimens for one sample of water). From one of these they immediately washed off the absorbed bacteria and from the washing they made smears, and the remaining ones were placed on a Hottinger agar plate (pH = 7.4) and the washings were made after 3, 4, 5 and 6 hours of incubation at 37°. After treatment with fluorescent serum, smears from the washings were studied in blue-violet light.

For obtaining a washing, sterile tweezers were used to take the membrane filter from the filtering device or from the agar plate and to place it in an empty Petri dish, filtering surface up. A 0.1 ml physiological solution was applied to the filter and with the help of a small spatula made from a Pasteur pipette, a washing (scraping) was made from the entire surface of the membrane. The liquid obtained was subjected to study.

Besides the review of smears made out of the washings from membrane filters and out of peptone water, they conducted a simultaneous bacteriological investigation of the material, that is they made seedings on Hottinger agar (pH = 7.8) with the subsequent identification of a culture of cholera vibrio.

The cholera vibrios were detected in all the samples of water contaminated by various numbers of vibrios (see table), however positive results were obtained at different times.

In smears made from peptone water, cholera vibrios were detected in a small quantity, as a rule after a 5 hour incubation; after 6 hours so many vibrios had grown that they could be detected in the smears without difficulty. The exceptions constituted samples of water contaminated with a small number of vibrios (1000 and 5000 microbial cells in 1 ml of water). In these cases there were few vibrios in the smears even after 6 hours of incubation.

In smears made from washings of membrane filters, cholera vibrios were detected somewhat earlier; individual cells were found in almost all samples only 3 hours after growing on filters. After a 4 hour incubation, vibrios were detected without difficulty, again with the exception of water samples with a small number of vibrios (1000 in 1 ml of water). After a 5 hour incubation, vibrios, almost in a pure culture, were readily detected in all samples.

It was established that membrane filters, in comparison with a liquid enrichment medium, were more expedient for work. Besides this, when grown on filters, a positive result was obtained 1-2 hours earlier than during seeding of the same samples in peptone water.

In extending the time of incubation of cholera vibrios on membrane filters located in a nutritive medium up to 7-8 hours, it was possible to detect cholera vibrios in water containing individual cells in 1 ml, however, such a time doesn't meet the requirements of rapid diagnostics. In these cases, in our opinion, it is more expedient to use the accelerated method described in the work of Chibrikova, Kuznetsova and others (1958).

The presence, in investigated samples of water, of a rather abundant bacterial flora didn't hinder the appearance of cholera vibrios in our experiments.

In smears, treated with luminescent serum, made out of samples of water not contaminated with cholera vibrios, the main mass of aqueous bacteria didn't fluoresce and was visible only during phase-contrast microscopy; together with this, in the smears there was rather often encountered illuminated amorphous tiny lumps and occasionally brightly illuminated coarse thick bacilli (as part of the incubation of the investigated material, their number didn't increase) which were morphologically different from the cholera vibrio.

The possibility of non specific staining of bacteria is demonstrated also by Finkelstein and Labrec who used fluorescent serum with the aim of exposing cholera vibrios in the contents of the intestines of guinea pigs and human feces artificially seeded with cholera vibrio. They noted that when a large quantity of typical brightly fluorescent vibrios were present in smears, their exposure presented no difficulty, however in non specific staining the results could be questionable in samples that were minimally seeded.

It is necessary to note that young (3-4-6 hour growths) cells of cholera vibrio were detected especially easily during luminescence microscopy in that their illuminated cell walls were more broadly and brightly fluorescent than in vibrios from dry growth, and the cells themselves were coarser and of typical morphology. A brighter luminescence of young cells of cholera vibrio was noted during their cultivation both on solid as well as in liquid (peptone water and Hottinger broth) nutritive media. Apparently, cells from young cultures of cholera vibrio have surface antigens which react more actively with fluorescent antibodies as well as in agglutination reactions. This supposition agrees with the data of Chibrikova, Shchurkina, and Bazunova (1960), pointing out that live cells of cholera vibrio cultures of a 3-4 hour growth possess a higher agglutination ability than cells in dry cultures.

The stated material gives a basis to suppose that the method of identification of cholera vibrio with the help of fluorescent serum may be utilized for the rapid detection of cholera vibrio in water. The time required for the investigation depends on the number of vibrios in the water and may fluctuate from 1½-2 up to 5-7 hours. Since under natural conditions, the content of vibrios in the sample of water being investigated is unknown, it

is necessary to conduct an analysis both with preliminarily concentrated material and with non-concentrated material.

On the basis of the results obtained we consider it possible to recommend the following approximate scheme for investigating water for the presence of cholera vibrios.

1. From the sample of water being investigated, prepare two smears and treat them with luminescent serum; one with anticholera, the other with normal. Both smears are examined in a luminescence and a phase-contrast microscope. If there is a large number of vibrios in the water - around 1 million in 1 ml - a positive result can be obtained $1\frac{1}{2}$ hours after the onset of the investigation.

2. Simultaneously filter the water through 4-6 membrane filters, being guided in the choice of filterable volume by the "Instructions for the Investigation of Drinking Water for the Presence of Pathogenic Microbes", approved by the Chief State Sanitary Inspector of the USSR on 9/IV 1941. From one of the filters make a smear immediately and examine it with the help of fluorescence sera, the remaining filters are placed for growing in Hottinger agar (pH = 7.4-7.8) and incubated at 37°. Part of the filters should be placed in a differential medium with sucrose (Chibrikova with coauthors, 1958) in order to carry out their study by the accelerated method with an isolation of a pure culture out of individual colonies and their rapid identification in the same way as if the sample of water being investigated contained a small amount of cholera vibrios (5-10 specimens in 1 ml).

3. In a washing from a membrane filter without growing on a nutritive medium containing cholera vibrios in a concentration amounting to no less than 1 million, it is possible to detect them 2 hours after the onset of the investigation.

4. From filters, placed in Hottinger agar for growing, washings should be made after 3, 4, and 5 hours of incubation (when it is necessary to economize on filters, washings may be made repeatedly from one filter), and the smears investigated by the same method as was pointed out above. In this case, a positive result can be obtained in the time required for preparing the washing from the filter, treating the smear with serum (about 2 hours) and growing the culture (3,4,5 hours). The larger the number of vibrios absorbed in the filter, the less time is required for growing; a 4 hour incubation is sufficient for detecting tens of thousands of vibrios in 1 ml of water.

Conclusions

1. The rapid detection of cholera vibrio in water with the help of fluorescent antiserum makes only an approximate answer possible and should be accompanied by bacteriological investigation by the classical method as well as all other express methods.

2. Fluorescent antiserum can be used with success in the course of ordinary (classic) analysis for the rapid identification of vibrios in smears prepared either from peptone water used in the capacity of enrichment medium or from colonies suspected of belonging to cholera vibrio.

3. The method of the rapid identification of cholera vibrio with the help of fluorescent anticholera serum can be used with the aim of the rapid identification of cholera vibrios not just in water, but in other objects of the environment, particularly in the investigation of smears made from various surfaces and items.

Footnote 1. The work of Mikhaylov and Li Li, published in "Journal of Microbiology, Epidemiology and Immunobiology" in 1959 when we were conducting the main part of the present investigation.

Footnote 2. Under the leadership of G. I. Mikhaylov.

Legend to figure on page 10.

Illumination of microbes of a 6 hour growth, treated with fluorescent cholera serum (ML-1, 90X, 7X). a - culture of cholera vibrios; b - culture of paracholera vibrios.

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Detection of Cholera Vibrio in Artificially Contaminated Samples of Water with the Help of Fluorescent Serum

Sample of Water	Microbial Population in Water (Microbes in 1 ml)	Concentration of Cholera Vibrio in 1 ml (in microbial bodies)	Detection of Cholera Vibrio at Various Times after Seeding (in hours)							
			From Membrane Filters				From Peptone Water			
			3	4	5	6	3	4	5	6
Water supply	770	100 000	+	+	++	Not checked	–	–	+	++
From well # 1	26 000	200 000	+	++	++	" "	–	+	+	+
# 2										
a	80 000	200 000	+	++	++	" "	–	–	++	++
b	45 000	100 000	++	++	++	" "	–	+	+	++
v	63 000	50 000	+	++	++	" "	–	–	+	++
g	34 000	5 000	–	+	++	++	–	–	–	+
d	25 000	1 000	–	–	+	++	–	–	–	+
# 3										
a	72 500	100 000	+	++	++	Not checked	–	–	–	++
b	4 000	100 000	++	++	++	" "	–	–	++	++
v	2 700	50 000	+	++	++	" "	–	–	+	++
g	1 000	5 000	+	++	++	" "	–	–	–	+
d	1 500	1 000	–	+	+	" "	–	–	–	+

Designations: – vibrios not detected in smears; + individual cells of cholera vibrio in sharp fields of vision; ++ 10 and more cholera vibrios detected in each field of vision.