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Hemagglutination: A Rapid Method for Differentiating *Vibrio cholerae* and El Tor Vibrios. (28043)

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At present, El Tor vibrios are differentiated from *Vibrio cholerae* in the laboratory primarily by their ability to hemolyze goat or sheep erythrocytes(1). However, the results of hemolysis tests have been extremely variable depending on the modification of the test used and the nature of the strains being tested. For example, strains from the 1961 epidemic in the Philippines and Hong Kong were non-hemolytic at 72 hours of incubation in "the original Greig test"(2) used by Goodner and his co-workers (personal communication) although other investigators have found these strains to be hemolytic in modifications of the test using 24 hour cultures (3,4). Some reason for this discrepancy was suggested by the work of de Moor(5) who reported the disappearance of hemolysin from broth cultures of the Celebes El Tor vibrios between the second and fourth days of incubation. This observation was repeated recently by Feeley and Pittman(6) who found the Philippine and Hong Kong cultures to be hemolytic or non-hemolytic depending on the time the broth cultures were tested. This characteristic varied somewhat with the different media used. de Moor(5) also had noted that the hemolytic activity of the Celebes vibrios increased during laboratory maintenance. Recently, some strains from the 1962 resurgence of cholera El Tor in the Philippines were found to be non-hemolytic when first isolated, but the strains revealed their hemolytic capacity in subsequent tests (C. Z. Gomez, personal communication).

The interpretation placed on the variable results of this single biological test and the implications thereof have had far reaching consequences since, in 1957, the Committee on International Quarantine stated its opinion that El Tor infection should not be included in the term "cholera"(7). As a result of the widespread epidemic of cholera caused by El Tor vibrios in the Far East in 1961, it is now acknowledged that "cholera, under the definition of quarantinable diseases in Article 1 of the International Sanitary Regulations, should include cholera due to the El Tor vibrio"(7).

Although it is now recognized that epidemic cholera may be caused by either *V. cholerae* or El Tor vibrios, for epidemiologic and other considerations it is still important to be able to differentiate between the 2 groups of organisms. Since other methods which have been devised for that purpose have proved unsatisfactory(8), a new method more reliable than the hemolysis test is still needed. Consistent results have been obtained by use of the group IV cholera bacteriophage(9) which is lytic for *V. cholerae* but does not lyse El Tor vibrios(8,10). However, the requisite phage and facilities for its propagation and maintenance are not universally available. Further, the results of this test are not available until overnight incubation following the primary isolation.

In preliminary studies, freshly isolated El Tor vibrios were observed to cause hemagglutination of chicken red blood cells in slide tests while the laboratory strains of *V. cholerae* tested were unable to do so. Subsequent

examination of larger series of El Tor and *V. cholerae* strains revealed the hemagglutination test to be a reliable means of differentiating these 2 groups of vibrios regardless of their source or history of laboratory maintenance.

Materials and methods. Hemagglutination test. The test was performed on microscope slides across which approximately 20 parallel lines were drawn with a Deco-Write ball point Craf-Tube (Craf-tint Mfg. Co., Cleveland, Ohio). A drop of physiological saline was applied with a 3 mm diameter bacteriological loop to the channels thus formed. Sufficient bacterial growth from agar cultures was emulsified in the drop to make a heavy milky suspension. A loopful of washed 2.5% chicken red blood cells in normal saline was then added and the slide was tilted back and forth to mix the cells and bacterial suspension. (Blood from unselected chickens in the U.S.A., India and the Philippines has performed satisfactorily when collected in Alsever's solution, heparinized or defibrinated. Preliminary tests indicate that erythrocytes from other species may also prove satisfactory.) If the test was positive, clumping of the erythrocytes was usually detectable almost immediately with the naked eye using transmitted illumination. A stereoscopic dissecting microscope was employed routinely, however, and was especially useful for confirmation of weaker reactions. Any clumping of erythrocytes within one minute was considered a positive reaction. The erythrocytes remained completely dispersed in negative reactions. Autoagglutination of degraded or rough strains in the saline did not interfere with the reaction. For early results, isolated suspicious colonies from the primary stool culture plates (nutrient agar, bile salts agar, Aronson's medium), with or without preliminary enrichment in alkaline peptone water, were tested for hemagglutination on the same slide in parallel with tests for agglutination in group and type specific cholera antisera. Tests with older laboratory cultures were performed using 18-24 hour growth on papain digest meat extract agar or nutrient agar.

Vibrio strains. The majority of the strains were available at the Indian Institute for Biochemistry and Experimental Medicine. Additional stock cultures from the Walter Reed Army Institute of Research (WRAIR) were also examined. The identity of strains was evaluated by agglutination in cholera O group I antisera; by hemolysis test with sheep erythrocytes in the modification of the Greig method recommended by Burrows and Pollitzer(11) using 18-24 hour alkaline nutrient broth cultures; by fermentation of sucrose and mannose but not arabinose(11) and by the test of susceptibility to lysis by group IV choleraphage(8).

Results. Three hundred and twenty-seven laboratory strains of *V. cholerae* were tested. These included the 287 cited in Table I as well as 40 strains of diverse origin from the WRAIR stock culture collection. In addition to the laboratory strains, a number of fresh isolates of *V. cholerae* were tested at time of isolation during the 1962 cholera epidemic in Calcutta. None of the agar-grown *V. cholerae* cultures tested in this series were hemagglutinative in the slide test.

On the other hand, 349 laboratory cultures of El Tor vibrios (Table I), as well as a number of fresh isolates tested at time of isolation during the 1961 and 1962 epidemics of cholera El Tor in the Philippines were all hemagglutinative under the same conditions. The hemagglutination reactions varied somewhat in intensity among the El Tor strains. Usually, clumping of the erythrocytes was rapid and marked, but a few strains produced slower and weaker hemagglutination. Use of more concentrated bacterial suspensions gave a clearer result in these cases.

While the overwhelming majority of the strains tested fell into 2 categories, hemagglutinin + and hemolysin + or hemagglutinin – and hemolysin –, 2 El Tor strains deviated from this pattern.

One strain, Bandung 2/61, isolated in West Java in September, 1961, was forwarded to us as a "non-hemolytic El Tor strain." It also proved to be non-hemolytic and phage resistant when received by us, but after a period of laboratory maintenance it manifested hemolytic activity. At that time, 6 distinct colonial types were observed by the oblique light technic of Henry(12,13). Of 30 colonies representing the different colonial variants, all but one were weakly to moderately

TABLE I. Hemagglutination Reactions of Vibrio Strains.

Vibrio cholerae						El Tor vibrios				
Isolated				Hemagglutination		Isolated				Hemagglutination
Place	Year	Source	No. of strains	+	—	Place	Year	Source	No. of strains	+	—
Calcutta & Howrah	1955–62	Humans	197	0	197	Middle East	Unknown	Unknown	2	2	0
Bihar	1961	"	7	0	7	Indonesia	1938–62	Humans	42	42	0
Orissa	"	"	7	0	7			Water	14	14	0
Uttar Pradesh	"	"	21	0	21			Flies	3	3	0
Madras	"	"	7	0	7	Thailand	1959–60	Humans	2	2	0
Thailand	1959–60	"	15	0	15	Hong Kong	1961	"	64	64	0
Dacca	1960–62	"	33	0	33	Macao	"	"	1	1	0
						Philippines	"	"	193	193	0
							1962	"	20	20	0
						Calcutta	1958	Water	8	8	0
Total			287	0	287				349	349	0

hemolytic. The exception was not hemolytic when it was first tested, but manifested a feeble hemolytic activity after one sub-culture. All of the variants, including this one in both its hemolytic and non-hemolytic states, were hemagglutinative, lyso-resistant to group IV choleraphage and gave typical fermentation reactions. This strain could have been considered to be true *V. cholerae* at time of isolation because of its inactivity in the hemolysis test. However, the phage and hemagglutinin reaction suggested its real identity as an El Tor vibrio and its change in hemolytic activity confirmed that hypothesis.

Another strain, Mak 757, isolated in the Celebes during the 1937-38 El Tor epidemic, was designated an El Tor vibrio of the Hikojima serotype and lysotype I of Nicolle, *et al.* (14) when it was sent to us. However, in our hands, this strain was found to be non-hemolytic on repeated occasions over 7 months' propagation. The strain was hemagglutinative and lyso-resistant to group IV choleraphage. It was colonially homogeneous. Thirty colonies were picked and all reacted as did the parent strain. Based on the phage reaction, the hemagglutination test and its history of being isolated in an El Tor outbreak, we are led to the conclusion that this is an example of an El Tor strain which has lost its hemolytic property. However, in the absence of an earlier record of the hemolytic status of the strain (which might not have been tested at all) it is also possible that the strain was non-hemolytic since its isolation. Using only the criterion of the hemolytic test, it would likely be identified now incorrectly as true *V. cholerae.*

We have also tested 19 strains of so-called NAG (non-agglutinable, *i.e.,* vibrios which are not agglutinated by cholera O group I antisera) vibrios which were not further characterized. Fifteen of these strains were hemagglutinative in the slide test.

Discussion. Our attention was directed to the use of the hemagglutination test as a means of differentiating El Tor vibrios from *V. cholerae* as a result of a preliminary survey (Brinton and Finkelstein, 1960, unpublished observations) of cholera strains for the

presence of fimbriae(15) or pili(16) using hemagglutination as a presumptive test for these structures. Although all of the agar-grown *V. cholerae* strains included in that study were inactive in the direct slide hemagglutination test, following broth passage of 25 strains, it was possible to isolate 2 hemagglutinative variants from one strain, 20-A-11. Similar observations were also made independently by Bales and Lankford(17). They, however, found, in addition, that each of 17 cultures of *V. cholerae* was hemagglutinative in tube tests when cultivated in trypticase soy broth under forced aeration. They regarded this hemagglutinating activity as a manifestation of an adhesive antigen. It is important to note that the differentiation between *V. cholerae* and El Tor vibrios in the present study is dependent on the use of *agar-grown cultures in slide tests*. It may be mentioned here that electron microscopic examination revealed no evidence of pili in either the hemagglutinating *V. cholerae* variants or El Tor strains although flagellated vibrios could be seen adhering to, and seemingly forming bridges between, ghosts of red blood cells (Finkelstein and Hartman, 1961, unpublished observations).

Previously, other investigators(1) had observed hemagglutination by El Tor and cholera-like vibrios and Griffitts(18) reported observing microscopic hemagglutination in tube tests with 7 of 10 strains of *V. comma*. However, to our knowledge, no attempt to utilize hemagglutination in differentiating *V. cholerae* from El Tor vibrios has been recorded.

From the present study it is apparent that the chicken cell slide agglutination test with agar-grown vibrios is a rapid, simple and reliable method for distinguishing between El Tor vibrios and *V. cholerae*. Hemagglutination also appears to be a more stable and reproducible property of El Tor vibrios than their hemolytic activity. Although the observation that occasional strains of *V. cholerae* may yield variants with hemagglutinating activity in this test would seem to provide possible exceptions, in practice this did not occur in the large series of laboratory or freshly isolated cultures which were examined.

It is suggested that suspicious colonies from primary plating of specimens from cholera patients should be tested for agglutination in diagnostic antisera and, in parallel, for hemagglutination for rapid differentiation of *V. cholerae* and El Tor vibrios. Additional reliable tests such as susceptibility to lysis by group IV choleraphage may be applied subsequently for confirmation. The hemagglutination test may also provide a useful characteristic in classification of heterogeneous group of NAG vibrios which have sometimes been implicated in diarrheal disease.

Summary. Direct hemagglutination of chicken erythrocytes in slide tests using suspensions of agar-grown vibrios has been found to be a rapid and reliable means of differentiating the 2 "agglutinable" groups of vibrios which cause epidemic cholera, *V. cholerae* and El Tor vibrios. The test can be performed at the time of primary isolation to give immediate results. All El Tor strains which have been tested were hemagglutinative while no *V. cholerae* strains were hemagglutinative under the same conditions.

ADDENDUM. Since this manuscript was submitted, a recent note (Wahba, A. H., Takla, V., *Bull. Wld. Hlth. Org.*, 1962, v 26, 306) describing a new chemical flocculation test for cholera vibrio identification has come to our attention. The authors cite additional evidence of the unreliability of the hemolysis test as a means of differentiating *V. cholerae* and El Tor vibrios. In their examination of 36 strains, the test they propose differentiated between smooth *V. cholerae* and *"V. El Tor"* strains but was not applicable to rough strains.

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Measurement of Renal Red Cell and Plasma Transit Times in Acute Renal Failure. (28044)

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The disturbance of the renal circulation in acute renal failure is poorly understood. Recent investigations have shown that the persistence of renal failure in the oliguric stage of the disease is not due to renal ischemia as renal blood flow averaged 40% of normal when measured with the inert radioactive gas krypton^{85}(1,2) or with a constant infusion of indocyanine green into the renal artery(3,4). Both these methods are independent of tubular function or urine formation and contrast with the para-aminohippurate clearance and extraction technic which depends on tubular function and urine formation. The early results of renal blood flow in acute renal failure using PAH averaged only 3% of normal(5,6) and are probably invalid as the extremely low extraction of PAH by the oliguric kidney prevents accurate determination of renal blood flow.

If renal blood flow is adequate in acute renal failure then the possibility of intrarenal shunting of blood must be considered to explain the persistence of oliguria and loss of function. To investigate this problem further in man a method of measuring mean red cell and plasma transit times through the kidney has been developed and applied to the oliguric patient with acute renal failure.

Method. The method has been developed from a modification of the technic for measuring the splanchnic blood volume(7). If the effluent and affluent blood of an organ can be sampled rapidly over a period of time it is possible to estimate the quantity of an indicator trapped in the organ during the pe-

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