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Epidemiological Usefulness of Changes in Hemolytic Activity of *Vibrio cholerae* Biotype El Tor During the Seventh Pandemic

TIMOTHY J. BARRETT* AND PAUL A. BLAKE

Bacterial Diseases Division, Bureau of Epidemiology, Center for Disease Control, Atlanta, Georgia 30333

Hemolytic *Vibrio cholerae* biotype El Tor strains were isolated in the United States in 1973 and 1978 after they had supposedly disappeared worldwide during the 1960s and 1970s. We decided to examine the change in prevalence of hemolytic El Tor strains since the beginning of the seventh pandemic and evaluate the usefulness of hemolytic activity as an epidemiological marker. A total of 48 isolates of *V. cholerae* biotype El Tor isolated in the Eastern Hemisphere between 1960 and 1979, along with 1 Texas (1973) and 38 Louisiana (1978) isolates, were tested for hemolytic activity by each of four methods. One method (utilizing heart infusion broth with 1% glycerol) was slightly superior for detecting hemolytic activity. Titers obtained with this method ranged from <2 to 1,024. Of 13 (76.9%) strains from the earliest part of the current pandemic, 10 were hemolytic, compared with 1 of 26 (3.8%) strains isolated in the period from 1966 to 1979 in the Eastern Hemisphere, indicating that nonhemolytic El Tor strains have replaced the hemolytic variety there. In contrast, all 38 Louisiana isolates and the Texas isolate were strongly hemolytic. Hemolytic activity was concluded to be a useful epidemiological marker.

The seventh cholera pandemic, which began in 1960 to 1961 and continues today, was caused by the El Tor biotype of *Vibrio cholerae* O group 1. The classical biotype, the principal cause of cholera before the current pandemic, is now rarely found. Historically, the classical and El Tor biotypes were differentiated by the ability of the El Tor biotype to hemolyze erythrocytes. However, early in the seventh cholera pandemic, a number of authors reported nonhemolytic *V. cholerae* O1 isolates which otherwise resembled the El Tor biotype (3, 5, 7, 9, 12). The two biotypes are now generally distinguished by other characteristics. These include the Voges-Proskauer test, agglutination of chicken erythrocytes (10), and resistance to polymyxin B (13) and to Mukerjee phage IV (14). Gallut reported that by 1972 almost all El Tor isolates worldwide were nonhemolytic (11). Subsequently, interest in the hemolytic activity of El Tor strains apparently waned, and the prevalence of hemolytic activity for strains isolated since 1972 is unclear.

In 1973 and 1978, *V. cholerae* O1 infections caused by markedly hemolytic El Tor vibrios occurred in Texas (17) and Louisiana (4). When hemolytic *V. cholerae* strains were isolated after their supposed disappearance, we suspected that the 1973 and 1978 cases may have resulted from persistence of the organisms in the United States rather than from separate importations, and we decided to reexamine the phenomenon of hemolysis. We attempted to reconstruct the changes which have occurred during the past 20 years by using methods suggested by several authors to examine strains collected worldwide during the current pandemic.

MATERIALS AND METHODS

A total of 87 *V. cholerae* O1 strains were examined for hemolytic activity using four methods. These strains were collected between 1960 and 1979 and were resistant to polymyxin B and Mukerjee phage IV and toxigenic by the Y-1 adrenal cell assay (6). They included strains from the following areas (number of strains is shown in parentheses): Europe (2), Asia (28), Africa (8), Oceania (3), the Middle East (7), a case of cholera in Texas in 1973 (1), and strains of both human (11) and environmental (27) origins isolated in Louisiana in 1978 (2, 4), Since strains were obtained from several laboratories, storage methods varied, but most strains had been kept lyophilized since isolation.

Method 1. Feeley and Pittman (8) recommended growing cultures in heart infusion broth (HIB) (Difco; pH 7.4) at 35°C for 24 h, centrifuging, and titrating the supernatant. Serial twofold dilutions were made in phosphate-buffered saline (pH 6.8 to 7.0), and 0.5 ml of a 1% suspension of sheep erythrocytes was added to 0.5 ml of each dilution of supernatant. After they were incubated for 2 h in a 37°C water bath, the suspensions were kept overnight at 4°C and examined for hemolysis. Titers were recorded as the highest dilution at

which complete hemolysis occurred. Supernatants heated to 56°C for 30 min were also included to assure that any hemolysis reflected the presence of a heat-labile hemolysin.

Method 2. Barua and Mukherji (3) suggested that HIB with 1% glycerol was superior to plain HIB. We used the same methods and conditions described above, except we added 1% glycerol to the same lot of heart infusion broth (HIBG). This differs from the method described by Sakazaki et al. (15) in that they incubated cultures for 48 h.

Method 3. de Moor (5) used heart infusion agar with 5% washed sheep erythrocytes. Plates were inoculated with 1 drop of an overnight culture in HIB and incubated anaerobically at 37°C for 24 h.

Method 4. Plain Trypticase soy agar (BBL Microbiology Systems) with 5% sheep erythrocytes was inoculated with 1 drop of an overnight culture in HIB and incubated aerobically for 24 h at 37°C.

RESULTS

When results were read as the presence or absence of hemolytic activity, results obtained with all four methods agreed closely (Table 1). Three strains were nonhemolytic by HIB and aerobic blood plate but positive by HIBG (titers were 8, 16, and 32) and the anaerobic sheep erythrocyte plate.

TABLE 1. *Hemolytic activity of 87 V. cholerae O1 strains as determined by four methods*

Test	Hemolytic activity (no. of strains)[a]		
	Present	Absent	%
HIB	54	33	62.1
HIBG	57	30	65.5
Anaerobic sheep erythrocyte plate	57	30	65.5
Aerobic sheep blood agar plate	54	33	62.1

[a] Hemolytic activity is defined as a hemolysin titer of ≥2.

TABLE 2. *Distribution of hemolysin titers measured with HIB and HIBG methods for 87 V. cholerae O1 strains*

Titer	No. of strains by following method	
	HIB	HIBG
<2	33	30
2	1	1
4	1	0
8	2	4
16	12	10
32	21	21
64	13	18
128	3	3
256	1	0
512	0	0
1,024	0	1

TABLE 3. *Hemolytic activity of 48 Eastern Hemisphere, 1 Texas, and 38 Louisiana strains of V. cholerae O1 biotype El Tor by year of isolation and titer*

Source and yr of isolation	Hemolytic activity (no. of strains)		
	Nonhemolytic (titer of <2)[a]	Intermediate (titer of 2–8)[a]	Strongly hemolytic (titer of >16)[a]
Eastern Hemisphere			
1960–1962	2	1	10
1963–1965	5	2	2
1966–1968	2	0	0
1969–1971	7	1	0
1972–1974	4	1	1
1975–1977	7	0	0
1978–1979	3	0	0
Texas			
1973	0	0	1
Louisiana			
1978	0	0	38

[a] HIBG method.

Higher titers were generally measured with the HIBG method than with the HIB method; of the 57 strains shown to be hemolytic with any method, higher titers were measured for 35 (62.5%) with the HIBG method, whereas higher titers were measured for only 2 (3.6%) with the HIB method ($P < 0.001$, McNemar test). Hemolytic strains usually had hemolysin titers of 16 or higher in measurments with both the HIBG (87.7%) and HIB (92.9%) methods (Table 2).

To examine the changes that occurred in hemolytic activity of El Tor *V. cholerae* O1 isolates in the Eastern Hemisphere in the period from 1960 to 79 and to compare the levels of hemolytic activity of isolates from Louisiana and Texas with those of the other isolates, we wanted a method with which to measure titers. Since the HIBG method detected more positives and measured higher titers than the HIB method, we chose the former (Table 3). Of 13 (76.9%) strains isolated in the earliest years of the pandemic (1960 to 1962) in the Eastern Hemisphere, 10 were strongly hemolytic (titer of ≥16), compared with 1 of 26 (3.8%) strains isolated in the period from 1966 to 1979. All 38 Louisiana isolates and the Texas isolate were strongly hemolytic.

DISCUSSION

The reliability of various tests of the hemolytic activity of *V. cholerae* O1 has been dis-

cussed by many investigators (1, 8, 9, 12, 15, 16). In 1966, Finkelstein found hemolytic and nonhemolytic colonial variants in the same cultures (7, 9). In 1970, Sen and Shrivastava found hemolytic activity to be a stable property and argued that all nonhemolytic *V. cholerae* O1 were of the classical biotype (16). In 1971, Sakazaki et al. reported that 99% of the strains resistant to phage IV and polymyxin B that they tested had some hemolytic activity (15). In 1974, Adibfar and Preston suggested that nonhemolytic strains which otherwise resembled El Tor vibrios represent an "intermediate biotype" (1).

Our purpose was neither to determine an optimal method for hemolysin detection nor to debate whether nonhemolytic *V. cholerae* O1 strains should be included in the El Tor biotype. Regardless of whether all *V. cholerae* O1 biotype El Tor strains (strains resistant to phage IV and polymyxin B) can be manipulated into producing hemolysin, we have shown that the hemolysin titers of such El Tor strains can vary from <2 to 1,024 in a standard test (HIBG method). Our data also indicate that there has been a marked change in the prevalence of hemolytic activity of stored El Tor strains from the Eastern Hemisphere in the course of the seventh pandemic; nonhemolytic strains became predominant during the middle 1960s, and hemolytic strains have rarely been found since. Thus, the marked hemolytic activity of the Texas and Louisiana strains in 1973 and 1978 was distinctly unusual and provided the first indication that they might be related. Subsequently, the Texas and Louisiana isolates were found to be of the same phage type, a type unique to the United States, providing further evidence that they were related (4).

The fact that the Texas strain and all of the Louisiana strains (from patients, sewage, and the environment), which were of the same phage type (4) and presumably at one time came from a common source, had hemolysin titers of ≥16 suggests that, at least for these strains, the level of hemolysis measured may be a stable property under natural conditions. Although it is certainly possible that some of our older Eastern Hemisphere test strains lost their hemolytic ability during storage, the oldest (1960 to 1962) isolates were the most hemolytic, suggesting that storage probably had little effect on the results.

We conclude that when a *V. cholerae* O1 isolate is found to have hemolytic activity that is unusual for the area (currently, this would be hemolytic in the Eastern Hemisphere and nonhemolytic in the Western Hemisphere), this characteristic can be used in epidemiological studies to trace the spread of the strain. Although the HIBG method would be preferable because it can be used to measure titers, for practical purposes a standard blood plate can be used. Anaerobic incubation of the blood plate, as suggested by de Moor (5), is recommended to avoid hemodigestion by metabolic end products which may be confused with true hemolysis caused by the heat-labile hemolysin. Ideally, the identity of representative strains should later be confirmed biochemically, serologically, and by phage typing.

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