IMPROVED METHOD, USING STAPHYLOCOCCAL BETA-HEMOLYSIN, FOR DETECTION OF HEMOLYSIN(S) PRODUCED BY *VIBRIO CHOLERAE* BIOTYPE EL TOR

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INTRODUCTION

Greig (1914) described the first tube test to determine the hemolytic activity of non-cholera vibrios. He cultured the bacteria in an alkaline broth for 3 days and then added sheep red blood cells. Later, Feeley and Pittman (1963) used a 24 hour heart infusion broth (HIB) culture, titration of the centrifuged supernatant, and sheep red blood cells to detect hemolysin from *V. cholerae* biotype El Tor. Barua and Mukherjee (1964) found that the number of positive hemolysis tests could be increased compared to HIB alone when they added 1% glycerol to the medium (HIBG). However, Barrett and Blake (1980) found that the results obtained using HIB and HIBG were similar. Approximately 30% of their strains were non-hemolytic. Ganguly *et al.*, (1966) reported they further increased the number of positive tests by using brain heart infusion broth. Inconsistent hemolytic results have always been a problem and was discussed by Sakazaki *et al.*, (1971). They recommended the use of HIBG and brain heart-thioglycollate-cystine agar media jointly.

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We found that the sensitivity of *V. cholerae* biotype El Tor hemolysin detection could be increased using a broth culture containing staphylococcal B-lysin, HIB and sheep red blood cells. The results are reported herein.

MATERIALS AND METHODS

V. cholerae strains: The biotype El Tor *V. cholerae* tested, included serotypes Ogawa (28) and Inaba (28) cultured in thiosulfate citrate bile salts medium (Oxoid) from feces of cholera patients in the Infectious Diseases Hospital, Jakarta, Indonesia during 1979-1981. Seven classical strains (obtained from Dr. P. Echeverria, Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand, (1 Ogawa), Dr. Julius Surjawidjaja, Medical Faculty, Trisakti University, Jakarta, Indonesia (2 Ogawa), Dr. V. Basaca-Sevilla, Bureau of Research and Laboratories, Manila, Philippines (1 Ogawa, 1 Inaba) and Bio Farma, Bandung, Indonesia (1 Ogawa, 1 Inaba), were also tested. All isolates were held in nutrient agar butts as stock cultures.

Staphylococcal B-hemolysin: A *Staphylococcus aureus* strain that produced B-hemolysin on sheep blood agar and a positive CAMP (Christie *et al.*, 1944) test with a group B *Streptococcus* was cultured in HIB (Difco) at 37°C for 18-20 hours. The culture was centrifuged and the supernatant collected after filtration through a 0.22 μm pore size filter (Millipore). The crude filtrate (CF) containing B-hemolysin was stored at 4-8°C.

Titration of B-hemolysin: The CF (0.5 ml) was serially diluted two-fold with 0.5 ml 0.01M phosphate buffered saline (PBS : Na_2PO_4, 1.42 g; NaCl, 7.01 g; pH 7.4) containing 0.001 M magnesium chloride. Defibrinated sheep red blood cells (SRBC) not older than 1 week after collection, were washed 3 times with 0.15M NaCl and a 1.5% (v/v) suspension made in 0.15M NaCl. An equal volume (0.5 ml) of the CF and 1.5% SRBC were mixed in small tubes, incubated in a waterbath at 37°C for 80 minutes followed by 4°C for 60 minutes and examined for hemolysis. The lowest dilution that did not show hemolysis was used as the working dilution. This was usually 1 : 16.

Heart infusion broth lysin (HIBL) tube test: A portion of a *V. cholerae* colony from a subculture in brain heart infusion agar was added to 2 small tubes containing 0.5 ml HIB. The HIB cultures were incubated at 37°C for 2 hours, 1 tube heated at 56°C for 30 minutes to destroy any heat labile hemolysin, 0.5 ml of the B-hemolysin working dilution added to each tube and the tubes and contents incubated at ambient temperature, 15 minutes. One half ml of 1.5% SRBC was added and the tubes incubated in a 37°C waterbath for 80 minutes then 4-8°C for 30 minutes and the presence or absence of hemolysis recorded. Occasionally, the SRBC did not settle enough to read the test so the tubes were centrifuged for 5 minutes before recording the results.

HIB tube test: The method of Feeley and Pittman (1963) was used. The *V. cholerae* strains in HIB, pH 7.4, were incubated at 35°C for 24 hours and centrifuged. Five tenths ml of a 1% (v/v) SRBC suspension was added to 0.5 ml of the centrifuged supernatant, the mixture incubated in a 35°C waterbath 2 hours, placed in the refrigerator at 4-8°C and examined for hemolysis the next morning.

HIBG tube test: Glycerol (1%) in HIB was prepared (Barua and Mukherjee, 1964) and the HIB tube test procedure followed.

RESULTS

Partial to complete but readily recognizable hemolysis of the SRBC occurred in the HIBL tube test with 28/28 (100%) *V. cholerae* El Tor serotypes Ogawa and 28/28 (100%) Inaba by the end of the 30 minute, 4-8°C incubation period. Four (14%) Ogawa and 2 (7%) Inaba produced detectable hemolysis in HIB and 20 (71%) Ogawa and 18 (64%) Inaba in HIBG. The 1 strain of classical *V. cholerae* tested and the heated controls were negative by the 3 methods.

DISCUSSION

The physiological, epidemiological and bacteriological importance of the presence of *V. cholerae* biotype El Tor hemolysin is still being debated. Honda and Finkelstein (1979) purified and characterized a heat labile protein-containing hemolysin that was cytotoxic in Y-1 adrenal cells, cardiotoxic in beating rat heart cells and rapidly lethal for mice after intravenous injection. Also, (Honda and Finkelstein, 1979; Gallut, 1974) *V. cholerae* El Tor hemolysin has been examined as an epidemiological marker and the presence of hemolysin is a criterion separating biotype El Tor from classical *V. cholerae*. Pollitzer (1959) recommended that only those hemolytic vibrios agglutinable with cholera O group 1 antiserum be classed as El Tor. However, the inconsistencies associated with hemolysin detection led to other means of separating El Tor from classical vibrios such as chicken cell agglutination, the Voges-Proskauer test, phage typing and polymyxin B resistance. None of these, except polymyxin B (Wachsmuth *et al.*, 1980) results, is entirely reliable (Feeley, 1965).

In 1944, Christie *et al.*, described a synergistic lytic phenomenon between staphylococcal B-hemolysin and a diffusable substance from group B streptococci in agar media containing sheep or ox erythrocytes. When the 2 different organisms were streaked perpendicular to each other but not touching and the cultures incubated, a lunar-shaped B-hemolysis pattern was formed within the juncture area of the inocula. This phenomenon later became known as the CAMP test. The diffusable substance from the streptococci is the CAMP factor. Besides identifying group B streptococci, this factor has been applied to separate pathogenic from nonpathogenic *Listeria monocytogenes* (Groves and Welshimer, 1977).

We found that a CAMP-like phenomenon occurred between staphylococcal B-hemolysin and *V. cholerae* biotype El Tor but not classical *V. cholerae* (unpublished results). Studies were then carried out to determine if hemolysis of sheep erythrocytes could be enhanced in a tube test by adding B-hemolysin to enhance the El Tor hemolysin or other factor (s). This might then provide another means to separate El Tor from classical vibrios.

It was found that all El Tor strains gave a positive tube hemolysis test and the 7 classical *V. cholerae* strains tested gave a negative reaction. The El Tor factor was shown to be heat labile at 56°C and the hemolysis magnesium dependent. Magnesium dependency of the B-hemolysin was shown before (Wiseman, 1965). The age of the sheep erythrocytes influenced the hemolysis reaction. Sheep erythrocytes could be used when they were prepared each day from defibrinated blood held at 4-8°C for no longer than 1 week after collection. However, those prepared and held longer than 1 day at 4-8°C resulted in equivocal hemolytic reactions. The timing of the HIBL test was not as critical. We found that the sensitivity of the HIBL reaction was the same when the initial HIB culture incubation time ranged between 2-24 hours. To reduce the time necessary for test completion, we choose 2 hours incubation in the initial step of the HIBL test. Fifteen minutes was chosen as the shortest time that could reliably be used, during the second step, for the incubation of the supernatant containing B-hemolysin and HIB culture. Extending this time did not influence the test results. Maximum hemolysis occurred when the SRBC were incubated with the HIB culture and B-hemolysin for 80 minutes. Likewise, a longer time period did not appear to influence the results. Refrigeration for 30 minutes was necessary to rapidly complete the partial hemolysis occurring at room temperature and allow any intact SRBC to settle. However, hemolysis occurred to the same degree if the test was incubated at ambient (23-25°C) temperature overnight without ever cooling to 4-8°C.

The working dilution of B-hemolysin was critical. Experiments showed that some false positive hemolysis occurred in those tubes containing any of the titered working dilutions showing hemolysis. Extending the dilution beyond the 1 dilution past the hemolysis end point produced some false negative and weaker positive reactions. However, we found that once prepared, the activity of the working dilution remained unchanged relative to test sensitivity for at least 1 month when stored at 4-8°C.

Whether the enhancement of hemolysis resulted from synergism between the staphylococcal B-hemolysin and *V. cholerae* biotype El Tor hemolysin remains to be determined. The active component(s) in the El Tor cultures was totally heat labile at 56°C when incubated 30 minutes. Preliminary experiments showed the activity was almost completely destroyed by heating at 56°C for 10 minutes. Heat lability of the purified El Tor hemolysin was shown before (Honda and Finkelstein, 1979). The sensitivity of the hemolysis reaction was also magnesium

dependent because equivocal results were obtained when the procedure was followed using components without added magnesium. Adding 0.001M magnesium to the PBS eliminated those equivocal results.

The HIBL tube hemolysis test appeared to be a better indicator than the HIB or HIBG tube tests to detect El Tor vibrio hemolysin. Since considerable attention has been given to qualitative changes in El Tor hemolysin production and the importance as an epidemiological marker, it may be worthwhile to reconsider the significance based on the results of the present study. This is especially true if the El Tor hemolysin identified by Honda and Finkelstein (1979) is shown to be the same as that detected in the HIBL, HIB and HIBG tube test.

SUMMARY

A tube test using brain heart infusion broth and staphylococcal B-lysin (HIBL) was devised to improve the detection of *Vibrio cholerae* El Tor hemolysin. Fifty six (100%) strains of *V. cholerae* serotypes Ogawa (28) and Inaba (28) were positive by the hemolysin test whereas 4 Inaba and 2 Ogawa were positive by a standard tube test using heart infusion broth (HIB) and 20 Ogawa and 18 Inaba were positive by another tube test using HIB containing glycerol (HIBG). Seven classical *V. cholerae* strains tested were negative by the 3 methods. The HIBL tube test was faster and more sensitive than the other 2 methods and showed that hemolysin was present that would otherwise have gone undetected by the other 2 methods using HIB or HIBG.

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