INFECTION AND IMMUNITY, Apr. 1970, p. 394-399
Vol. 1, No. 4
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# Comparative Hemolytic Activity of *Vibrio parahaemolyticus* and Related Vibrios

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Received for publication 17 October 1969

The hemolytic activities of 91 strains of *Vibrio parahaemolyticus* isolated from human diarrheal stools, sea fish, and sea water; 21 suspected *V. parahaemolyticus* cultures isolated from wound infections; 14 nonpathogenic marine vibrios; and 21 *V. parahaemolyticus* isolated from moribund blue crabs *Callinectes sapidus* were compared. Potentially pathogenic *V. parahaemolyticus* strains could be differentiated from the related nonpathogenic marine vibrios, because the former hemolyzed hamster, sheep, and human blood, whereas the latter were nonhemolytic. In addition, *V. parahaemolyticus* isolated from tissue infections could be differentiated from those of the first group isolated from sea fish or human stools, because the former exhibited primarily an $\alpha$-hemolytic reaction on chicken blood; the latter exhibited mostly $\beta$. It is suggested that *V. parahaemolyticus* isolated from blue crabs may be differentiated from the first group on the basis of their hemolysis of human blood. A useful schema of the differential hemolytic reactions, exhibited by *V. parahaemolyticus*, tissue infection vibrios, and nonpathogens on hamster, sheep, chicken, goose, and human blood is given. The patterns of hemolytic activity of these groups on special human blood-agar plates (Kanagawa hemolysis) resembled that seen on ordinary human blood-agar.

Since the first report of Fujino and co-workers (3) implicating *Vibrio parahaemolyticus* in a food poisoning epidemic, this halophile has been isolated from stools of patients, from sea fish and sea fish products, and from sea water in Japan (7). In addition, recent isolations of the vibrio in the continental United States, Germany, the Far East, and Hawaii suggest that the organism may enjoy ubiquitous distribution in the temperate seas of the world (1, 6, 7, 13, 14). Both Sakazaki and co-workers (9) and ourselves (11) have utilized morphological, cultural, biochemical, and serological characteristics to differentiate potentially enteropathogenic strains of *V. parahaemolyticus* from related nonpathogenic species. The hemolytic activity observed on ordinary 5% sheep blood-agar plates was a distinctive trait of *V. parahaemolyticus* and most strains of related halophilic vibrios. In contrast, Kato and co-workers (4) found that the vibrios shed in human diarrheal stools showed hemolytic activity, whereas those isolated from sea water or sea fish were nonhemolytic when plated on unautoclaved Brain Heart Infusion (BHI, Difco) agar containing 3% NaCl, 0.001% crystal violet, and 5% human group O blood. For the sake of clarity, Japanese workers have identified the hemolytic activity associated with strains of human origin and demonstrable on special blood agar as the "Kanagawa phenomenon." Sakazaki and co-workers have demonstrated that the "Kanagawa" type hemolysis is significantly associated with enteropathogenicity of *V. parahaemolyticus* but is not correlated with any other physiological, biochemical, or serological characteristic (10).

With the foregoing facts in mind, it seemed reasonable to us to make a comparative study of the hemolysis of blood from various species suspended in both ordinary and special blood-agar plates by *V. parahaemolyticus* and several groups of related vibrios.

## MATERIALS AND METHODS

**Cultures examined.** A total of 147 vibrio cultures were used for the present studies. They were classified in four groups on the basis of differential morphological, cultural, biochemical, and serological reactions.

Groups I, II, and III were described in our previous work (11) and included the original 110 cultures plus the following additions. Twelve strains of *V. parahaemolyticus*, K antigen types 1, 3, 4, 5, 18, 20, 22, 31, 32, and 33, were added to our collection of group I. Ten of these strains isolated from sea fish in Germany and one isolated in Japan were obtained from

TABLE 1. *Serological analysis of groups II, III, and IV vibrios with Vibrio parahaemolyticus anti-K antisera*

| Group | No. of strains | K antigens |
|---|---|---|
| II | 1 | 13 |
| | 4 | 17 |
| | 1 | 25 |
| | 1 | 33 |
| | 1 | 11, 13 |
| | 1 | 2, 6, 39, 40, 43, 45 |
| | 12 | None |
| III | 14 | None |
| IV | 3 | 17 |
| | 1 | 40, 42 |
| | 1 | 17, 27, 40 |
| | 1 | 5, 30, 40, 42 |
| | 15 | None |

L. Leistner, Budesanstalt für Fleishforschung, Kulmbach, Germany. The twelfth, a K-22, was obtained from Y. Miyamoto, Kanagawa Prefectural Public Health Laboratory, Yokohama, Japan. Four strains (B835, B308, B984, and B775) of suspected *V. parahaemolyticus* isolated from localized tissue infections apparently acquired by persons in recreational contact with the marine environment were included in group II. They were obtained from R. Weaver, National Communicable Disease Center, Atlanta, Ga.

Group IV consisted of 21 vibrios isolated from moribund Chesapeake Bay blue crabs *Callinectes sapidus* and identified by Krantz et al. (5) as *V. parahaemolyticus*. They were obtained from R. R. Colwell, Georgetown University, Washington, D.C.

The antigenic identity of these groups (Table 1) was established by slide agglutination using adsorbed, specific, high titer anti-K antisera prepared in our laboratory. Nine strains of group II vibrios and six group IV vibrios were agglutinated with antisera prepared against the K antigens of *V. parahaemolyticus*. The K antigen type appearing most frequently was K-17.

Colwell (2) analyzed the deoxyribonucleic acid base composition of *V. parahaemolyticus* and related vibrios. The mole per cent guanine plus cytosine for *V. parahaemolyticus* was 46 $\pm$ 1; for the 17 strains of group II used previously (11), 43.4 to 47.6; and for vibrios of group IV, 44 to 46. These data confirm the relationships that antigenic analysis has shown.

**Cultural methods.** The media and culture conditions used for maintenance of stock cultures as well as for routine growth were described previously (11).

Ordinary blood-agar plates were prepared by suspending 5% sterile defibrinated blood of 18 species of aquatic and terrestrial vertebrates in BHI plus 1.5% agar (Difco) by the usual methods. Blood from most species was obtained commerically (Colorado Serum Laboratory, Denver, Colo., and Gibco Laboratores, Inc., Madison, Wisc.). Turtle blood was provided by George Noland, University of Dayton. Blood of the green monkey, *Cercopithecus aethiops*, donated by Oscar Liu, Department of Health, Education, and Welfare, Marine Laboratory, Narragansett, R.I., was an ACD [acid-citrate-dextrose: one pint (480 ml) of whole blood was added to 120 ml of ACD solution (1.32 g of sodium citrate, 0.44 g of citric acid, and 1.47 g of dextrose per 100 ml)] preparation and was consequently used in 7.0% concentration. The blood of a nurse shark, *Ginglymostoma cirratum*, provided by W. J. Russell, Variety Children's Research Institute, University of Miami, was diluted 1:2 in modified Alsever's solution (2.05% dextrose, 0.8% sodium citrate, 1.42% NaCl, *p*H 6.1) and was therefore used in 10% concentration. For the suspension of shark blood, 2% sterile urea was added to the agar base to maintain cell tonicity.

Special blood-agar plates were prepared according to two formulas. The first, described by Kato et al. (4), included 3.7% BHI, 2.5% NaCl, 0.001% crystal violet, and 5% human group O blood. The second, used by Wagatsuma (12), contained 0.3% yeast extract (Difco), 1.0% peptone (Difco), 7% NaCl, 0.5% $K_2HPO_4$, 1% mannitol, 0.001% crystal violet, 1.5% agar, and 5% sterile defibrinated rabbit or human group O blood. Ingredients of each medium, except the blood, were added to water and steamed until the agar was melted. The *p*H of Kato's medium remained at 7.4, whereas that of the Wagatsuma agar was adjusted to 8.0. Without autoclaving, the melted basal agar media were cooled to 50 C, blood was added, and plates were poured. Human blood was freshly drawn from laboratory staff donors of known ABO and Rh blood types.

**Determination of hemolytic activity.** Both ordinary and special blood-agar plates were point-inoculated at 5 to 10 radial positions with a small loop from 18-hr enrichment broth cultures. After incubation for 24 hr, at 37 C, for groups I, II, and IV and, at 30 C, for group III, colonies were washed off with buffered water and the hemolytic activity was recorded according to standard definition. On ordinary plates, green hemolysis was described as $\alpha$, clear hemolysis, including a combination of clear and greening, as $\beta$, and no hemolysis as $\gamma$. On special blood plates, where the inclusion of crystal violet prevented color differentiation, the presence or absence of visible hemolysis was recorded as positive (+) or negative (−).

**Preparation of antigens and antisera.** Antigens were prepared by growing strains of *V. parahaemolyticus* representing all K antigens on BHI agar slopes for 18 hr at 37 C. Growth was suspended, washed three times by centrifugation at 2,000 $\times$ *g*, and resuspended in formalinized saline (0.85% NaCl with 0.4% by volume of Formalin). The optical density of the suspension was adjusted to 0.25 at 620 nm in a Coleman Junior Spectrophotometer. Antigens were stored at 4 C in screw-cap tubes.

Antisera were produced by the intravenous injection of rabbits with eight successive, increasing doses of antigen starting with a volume of 0.5 ml until a final dose of 4.0 ml. The animal was rested for 1 week and bled by cardiac puncture. A booster dose of 2 ml of antigen was then given, and second and third bleedings were made 1 and 2 weeks later, respectively.

Antisera titers of 1:1,280 were obtained. After the antisera were heated to 56 C, interfering heterologous agglutinins were removed by specific adsorption. The sera were stored at −15 C in small volumes and diluted 1:2 in formalinized saline for use in slide agglutination.

## RESULTS

Preliminary experiments demonstrated that the patterns of hemolytic activity observed on ordinary rabbit, guinea pig, dog, horse, calf, swine, goat, turkey, and turtle blood-agar plates were duplicated by those seen on other species; consequently, blood from each of these species was not investigated further.

The hemolytic activity demonstrated by three groups of vibrios inoculated on ordinary blood-agar plates prepared from monkey, sheep, ox, hamster, chicken, goose, and shark blood is shown in Table 2. The blood of all seven species tested was hemolyzed by most ($\geq$ 71%) strains of group I *V. parahaemolyticus*. Hemolytic activity was predominantly $\beta$ upon monkey, hamster, chicken, and goose blood; primarily $\alpha$ on sheep and shark; and mixed $\alpha$, $\beta$, and $\gamma$ on ox blood. The tissue infection vibrios of group II exhibited hemolytic activity which resembled that of *V. parahaemolyticus* strains when tested on sheep, ox, hamster, and goose blood. They differed by exhibiting $\alpha$ hemolysis of chicken blood and mixed hemolysis of green monkey and shark blood. Most group III vibrios were not hemolytic on blood from the tested species. Exceptions were the $\alpha$ hemolysis of chicken blood-agar plates and the mixed hemolysis of monkey and goose blood plates.

The growth of all four groups of vibrios on ordinary blood-agar plates was excellent for all species of blood except shark. An average of more than 97% of all strains grew on plates prepared with human, monkey, sheep, ox, hamster, chicken, or goose blood. In contrast, 86% of all strains grew on nurse shark blood plates. This moderate inhibition of growth was probably caused by the high urea content of these plates and their resulting high osmolarity.

The hemolytic activity exhibited by four groups of vibrios tested on ordinary blood-agar plates prepared from several different human blood groups appears in Table 3. The blood from all groups, regardless of whether Rh-positive (Rh+) or Rh-negative (Rh−) was hemolyzed by most strains of group I *V. parahaemolyticus*. Hemolytic activity was predominantly $\beta$ when tested on blood groups A Rh+, B Rh+, O Rh+, and O Rh−, and mixed $\alpha$, $\beta$, and $\gamma$ on group B Rh−. The hemolytic activity exhibited by group II tissue infection vibrios was different from that of group I *V. parahaemolyticus*. The activity was primarily $\alpha$ on group B human blood, the activity was mixed $\alpha$, $\beta$, and $\gamma$ on A Rh+ and O Rh+ blood, and nonhemolytic on O Rh− blood. Again, most strains of the third group of nonpathogenic vibrios were nonhemolytic. The hemolytic pattern exhibited by group IV organisms differed from that of group I. Hemolysis was primarily $\alpha$ on group B blood and $\gamma$ on group A

TABLE 2. *Comparative hemolysis by vibrios*[a]

| Group | Reaction | Species of blood | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Green monkey | Sheep | Ox | Hamster | Chicken | Goose | Nurse shark |
| I | | (90)[b] | (90) | (90) | (90) | (90) | (90) | (30) |
| | $\alpha$ | 9 | 67 | 10 | 2 | 25 | 1 | 70 |
| | $\beta$ | 78 | 5 | 58 | 88 | 73 | 97 | 4 |
| | $\gamma$ | 13 | 28 | 32 | 10 | 2 | 2 | 26 |
| II | | (17) | (21) | (21) | (19) | (21) | (21) | (21) |
| | $\alpha$ | 41 | 71 | 19 | 5 | 81 | 0 | 55 |
| | $\beta$ | 30 | 19 | 29 | 95 | 19 | 100 | 5 |
| | $\gamma$ | 29 | 10 | 52 | 0 | 0 | 0 | 40 |
| III | | (14) | (14) | (14) | (14) | (14) | (14) | (14) |
| | $\alpha$ | 0 | 0 | 0 | 0 | 79 | 14 | 8 |
| | $\beta$ | 46 | 21 | 21 | 21 | 14 | 50 | 0 |
| | $\gamma$ | 54 | 79 | 79 | 79 | 7 | 26 | 92 |

[a] Hemolysis of ordinary blood agar plates prepared as described in the text. Hemolytic activity expressed as the per cent of strains grown on test agar.

[b] Numbers of strains tested expressed parenthetically.

TABLE 3. *Hemolysis of ordinary human blood-agar plates by vibrios*[a]

| Vibrio group | Reaction | Blood group | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | A Rh+ | | B Rh+ | B Rh− | O Rh+ | | O Rh− |
| | | 1 | 2 | | | 1 | 2 | |
| I | | (90)[b] | (90) | (88) | (88) | (46) | (91) | (88) |
| | α | 3 | 0 | 11 | 35 | 0 | 14 | 8 |
| | β | 81 | 87 | 66 | 49 | 74 | 73 | 64 |
| | γ | 16 | 13 | 23 | 16 | 26 | 13 | 28 |
| II | | (20) | (20) | (19) | (19) | (20) | (19) | (19) |
| | α | 5 | 25 | 84 | 84 | 20 | 32 | 0 |
| | β | 40 | 20 | 16 | 16 | 20 | 63 | 21 |
| | γ | 55 | 55 | 0 | 0 | 60 | 5 | 79 |
| III | | (14) | (14) | (12) | (12) | (14) | (13) | (12) |
| | α | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | β | 14 | 14 | 25 | 25 | 14 | 23 | 25 |
| | γ | 86 | 86 | 75 | 75 | 86 | 77 | 75 |
| IV | | (21) | (21) | (18) | (18) | (21) | (21) | (18) |
| | α | 10 | 29 | 72 | 67 | 10 | 57 | 22 |
| | β | 19 | 0 | 0 | 0 | 0 | 10 | 0 |
| | γ | 71 | 71 | 28 | 33 | 90 | 33 | 78 |

[a] Hemolysis of ordinary blood-agar plates prepared as described in the test. Hemolytic activity expressed as the per cent of strains grown on test agar.
[b] Number of strains tested expressed parenthetically.

TABLE 4. *Hemolysis of special human blood-agar plates by vibrios*

| Vibrio group | Blood group | Media[a] | No. of strains tested | Strains grown | | Hemolysis | | |
|---|---|---|---|---|---|---|---|---|
| | | | | Numbers | Per cent of strains tested | No. hemolytic | Per cent of strains tested | Per cent of strains grown |
| I | O Rh+ | W | 91 | 69 | 76 | 54 | 59 | 78 |
| | O Rh+ | K | | 86 | 95 | 66 | 73 | 77 |
| | O Rh− | W | | 62 | 68 | 40 | 44 | 65 |
| II | O Rh+ | W | 19 | 18 | 95 | 14 | 74 | 78 |
| | O Rh+ | K | | 19 | 100 | 17 | 90 | 90 |
| | O Rh− | W | | 17 | 90 | 14 | 74 | 82 |
| III | O Rh+ | W | 14 | 12 | 86 | 1 | 7 | 8 |
| | O Rh+ | K | | 7 | 50 | 1 | 7 | 14 |
| | O Rh− | W | | 12 | 86 | 1 | 7 | 8 |
| IV | O Rh+ | W | 20 | 17 | 85 | 11 | 55 | 65 |
| | O Rh+ | K | | 17 | 85 | 17 | 90 | 100 |
| | O Rh− | W | | 15 | 75 | 11 | 60 | 74 |

[a] W is Wagatsuma agar; K is Kato medium.

Rh+ and O Rh− blood. Activity was mixed α, β, and γ on one sample of O Rh+ blood, whereas most strains were nonhemolytic on the other. The hemolytic activity by the four groups of vibrios did not appear to be correlated with the Rh blood type.

The hemolytic activity of four groups of vibrios observed on special blood-agar plates prepared from human group O blood is shown in Table 4. An examination of the per cent hemolysis by the four groups on the basis of the number of strains grown demonstrates that most strains of groups

TABLE 5. *Differential hemolytic reactions of vibrios*[a]

| Vibrio group | Species of blood | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Human | | | | |
| | Hamster | Sheep | Chicken | Goose | A Rh+ | B Rh+ | B Rh− | O Rh+ | O Rh− |
| I | β | M[b] | β | β | β | M | M | β | M |
| II | β | α | α | β | M | α | α | M | γ |
| III | γ | γ | α | M | γ | γ | γ | γ | γ |
| IV | ND[c] | ND | ND | ND | γ | α | M | M | γ |

[a] Reaction is that shown by ≥71% of strains on ordinary blood-agar plates prepared as described in text.

[b] Mixed. The percentage of strains exhibiting any reaction is <71%.

[c] Not done.

I, II, and IV were hemolytic. On the other hand, most vibrios of group III were not hemolytic. Apparently the special agar media were more inhibitory than ordinary blood agar, the per cent of growth being as low as 50% in the case of group III strains on Kato medium. Group II tissue infection vibrios seem to be the group of strains most resistant to the higher osmolarity of special agar. In general, growth on Kato medium (3% NaCl) was less inhibited than that on Wagatsuma agar (7% NaCl).

A comparison of the data of Table 4 with those of Table 3 reveals that the hemolysis of human group O blood suspended in special agar plates by group I *V. parahaemolyticus* and group III vibrios closely approximates the sum of $\alpha$ and $\beta$ hemolysis on ordinary group O blood plates. However, a greater percentage of strains of group II tissue infection and group IV vibrios was hemolytic on group O blood suspended in special agar rather than in ordinary agar.

## DISCUSSION

In 1963, Sakazaki et al. (9) examined 1,522 suspected enteropathogenic vibrio cultures, which they classified as 1,146 *V. parahaemolyticus*, 336 *V. parahaemolyticus* biotype 2 (now referred to as *V. alginolyticus*; 8), and 40 *V. anguillarum*. They reported that all were hemolytic on ordinary sheep blood-agar plates. Our previous investigation (11) revealed that 71 of 79 strains of *V. parahaemolyticus* were similarly hemolytic and that the trait was shared by all of 17 tissue infection vibrios and 12 of 14 nonpathogenic marine vibrios.

Our present studies have extended these observations to include the hemolytic reactions of *V. parahaemolyticus* and three related groups of vibrios on ordinary blood-agar plates prepared from the blood of 18 species of aquatic and terrestrial vertebrates. Hemolysis of sheep blood suspended in ordinary blood plates was a characteristic trait possessed by 72% of group I *V. parahaemolyticus* strains, 90% of group II tissue infection vibrios, but by only 21% of the nonpathogenic marine vibrio cultures studied.

The comparative hemolytic activity exhibited by the four groups of vibrios grown on ordinary blood-agar plates prepared from the blood of eight species (Tables 2 and 3) facilitated the selection of the most significant differential reactions. The resulting differential hemolytic schema for vibrio identification is given in Table 5. Nonpathogenic marine vibrios of group III are easily identified by their $\gamma$ hemolysis on the blood of these five species. Group IV presents mixed $\alpha$, $\beta$, and $\gamma$ hemolysis on all human blood groups except group O Rh−, which showed $\gamma$ activity. Group I *V. parahaemolyticus* and group II tissue infection vibrios demonstrate similar hemolytic activity on hamster, sheep, and goose blood but can be differentiated on the basis of their hemolysis of chicken and human blood.

It is possible that the differences in hemolysis of human blood observed among these groups of vibrios may be related to some characteristic feature other than blood type. Therefore, further investigation of these differences may be warranted.

Kato et al. (4) reported that strains of *V. parahaemolyticus* isolated from human diarrheal stools showed "Kanagawa" type hemolysis, whereas those isolated from sea fish and sea water were nonhemolytic when they were plated on a special human blood-agar medium containing 3% NaCl and crystal violet. The association of "Kanagawa" hemolysis with enteropathogenicity was supported by Sakazaki et al. (10), who reported that 2,655 of 2,720 cultures (96.5%) isolated from human diarrheal stools, but only 7 of 650 cultures (1.0%) isolated from sea fish and sea water, were hemolytic on the special blood-agar. Neither biochemical nor serotypal relationships

were observed to correlate with the hemolytic activity.

The large percentage (Table 4) of group I *V. parahaemolyticus* which were "Kanagawa positive" approached the high figure Sakazaki reported for diarrheal stool isolates. This would be expected inasmuch as many of our group I strains were originally isolated from patients during epidemics of gastroenteritis. However, neither the group II tissue infection isolates nor the group IV blue crab isolates, both of which have been identified as *V. parahaemolyticus*, were associated with gastroenteritis. Nevertheless, both groups II and IV as well as the nonpathogenic marine vibrios of group III exhibited percentages of "Kanagawa positive" strains which were significantly higher than that reported by Sakazaki (10) for *V. parahaemolyticus* strains isolated from sea fish and sea water.

We have no explanation for the differences in hemolytic activity observed but suggest that further study of this characteristic trait of halophilic vibrios would be warranted.

ACKNOWLEDGMENTS

We thank R. Colwell, L. Leistner, Y. Miyamoto, and R. Weaver for providing cultures. We further thank O. Liu, G. Noland, and W. J. Russell for providing sterile blood.

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