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Isolation and Characterization of Two Hemolytic Phenotypes of *Vibrio damsela* Associated with a Fatal Wound Infection

JILL E. CLARRIDGE[1,2,3]* AND SONIA ZIGHELBOIM-DAUM[3]

Laboratory Service, Veterans Administration Medical Center,[1] and Departments of Pathology[2] and Microbiology and Immunology,[3] Baylor College of Medicine, Houston, Texas 77211

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Two hemolytic phenotypes of *Vibrio damsela*, isolated from the tissue of a patient with a fatal wound infection, were characterized. The patient had underlying disease, and the wound was associated with an injury inflicted during the handling of a catfish. The phenotypes were morphologically and biochemically similar except for their lecithinase, lipase, and hemolytic activities. When grown on rabbit blood agar, one phenotype (LZ) produced a large zone of hemolysis (10 mm) around the colony, whereas the other type (SZ) produced only a small zone (1 to 2 mm). On sheep blood agar, the differences in hemolytic activity were more subtle. By a modified CAMP test in which *V. damsela* was streaked perpendicularly to *Staphylococcus aureus*, it was determined that a factor elaborated by the LZ phenotype (but not the SZ phenotype) protected sheep erythrocytes from the hemolysis normally caused by *S. aureus* toxins. Cell-free filtrates of broth cultures of each phenotype had the same effects on erythrocytes as did the organisms themselves.

Vibrio damsela is a newly described species which has been associated with the marine environment and fish (16, 21). Most of the reported human wound infections due to *V. damsela* have occurred after handling fish or after exposure to seawater (7, 20). These infections, although sometimes serious enough to require debridement, have not had a fatal outcome. We report here the isolation and characterization of two hemolytic phenotypes of *V. damsela* from the tissue of a patient with underlying disease who sustained a superficial wound which led to the rapid onset of disease and death.

CASE REPORT

On the evening of 9 May 1984, an alcoholic 61-year-old male with a history of pancreatitis and insulin-dependent adult onset diabetes slightly injured his left hand while cleaning a catfish reportedly caught in a local freshwater lake. A few hours later he noted that his hand had become tender, swollen, and painful. At 2 p.m. the next day, when he sought medical attention at a community hospital, his leukocyte count was 18,400 cells per ml with a shift to the left, the platelet count was 115,000, and his hand was edematous with black discoloration. He was transferred to the Houston Veterans Administration Medical Center, where he appeared to be in acute distress with marked edema of the left forearm and bullae formation on the hand. The black discoloration had extended up to the elbow with contiguous erythema. The fingertips of the left hand appeared necrotic. A disarticulation of the left shoulder was performed. Tissue from the hand and fluid from the bullae were taken for culture. No other cultures were performed. Amikacin, clindamycin, and penicillin therapy was started. On the morning of 11 May, a few hours after surgery, it was noted that the shoulder area around the incision was erythematous and possibly necrotic. Extensive debridement of the left shoulder and chest with extension into the right anterior chest was performed; the scapula and clavicle were resected. The subsequent course of the patient was complicated by continued disseminated intravascular coagulation, acute renal failure, and hypercalcemia, and he subsequently died of a cardiac arrest 9 days after the initial injury. No autopsy was performed.

MATERIALS AND METHODS

Isolation and characterization of the organism. The tissue from the hand and fluid from the bullae were each cultured on the following media: 5% sheep blood Trypticase soy agar (SBA) (BBL Microbiology Systems), chocolate agar, and Columbia colistin-nalidixic acid agar, which were incubated in 6 to 8% CO_2; MacConkey agar, which was incubated in air; and Schaedler sheep blood agar, CDC anaerobe blood agar with phenylethyl alcohol, Schaedler kanamycin-vancomycin agar with 5% sheep blood, and bacteroides-bile-esculin agar, which were incubated anaerobically. The biochemical and morphological identification was accomplished by the API 20E system (Analytab Products, Plainview, N.Y.), the DMS–rapid NFT system (API System, S.A., La Balme les Grottes, France), and standard methods (14).

Hemolytic activity. The hemolytic activities of the phenotype colonies were determined after 24 h of growth on SBA or Casman rabbit blood agar (RBA). We tested the filtrates for hemolytic activity by dropping 5 to 50 μl of each filtrate on either SBA or RBA and measuring the size and intensity of the hemolytic zones at 24 h and 1 week. Both filtrates and colonies were examined by a modified CAMP test (14) in which the organisms or filtrates were streaked perpendicularly to *Staphylococcus aureus* ATCC 25923.

Cell-free filtrates. Cell-free filtrates were prepared from *V. damsela* cultures grown in brain heart infusion broth with 0.5, 3, or 6% NaCl at 25 and 35°C. After 8 h, when a sample was removed to determine the number of CFU per milliliter, the broth was centrifuged and the supernatant was sterilized by passage through a filter (pore size, 0.22 μm). The effect of cell-free filtrate was also assessed by growing the cells on a membrane filter (0.45 μm; Millipore Corp.) placed on an SBA plate. After 24 h, the filter was removed and the extent of hemolysis was determined.

Temperature studies. Filtrates in 0.3-ml aliquots were heated in glass tubes at 45°C for 30 min, 60°C for 30 min, or 100°C for 10 min in static water baths. The small volume of

* Corresponding author.

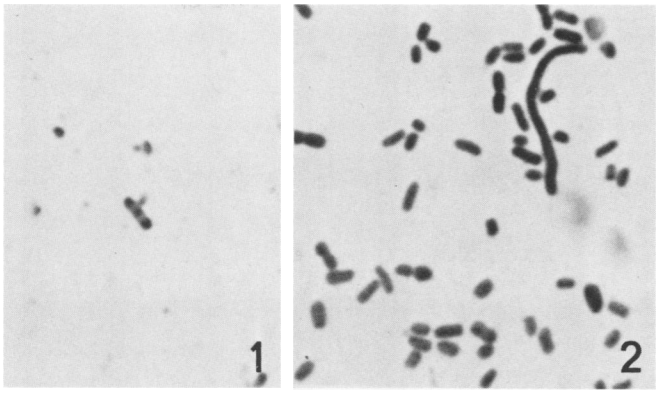

FIG. 1. Gram stain of ground tissue from hand, with bands of heavy and light staining on the single organism.

FIG. 2. Gram stain of organisms grown on an SBA plate. The elongate form was unusual.

filtrate and large volume of water at the given temperatures ensured rapid equilibration. Controls were incubated at 4 or 35°C for 30 min.

***V. damsela* reference organisms.** *V. damsela* ATCC 33538 (CDC 983-79) was obtained from the American Type Culture Collection. Strains CDC 1471-82, CDC 1421-81, and CDC 727-82 were obtained from the Centers for Disease Control.

RESULTS

No organisms were seen on Gram stain of the bullae fluid, and only two colonies grew on a single SBA plate. However, all the blood-containing plates showed hemolysis wherever the specimen had been streaked, even in the absence of growth in those areas. At several stabs there were no colonies, but a double zone of hemolysis was observed. The Gram stain of ground tissue showed large, vacuolated, noncurved gram-negative rods (Fig. 1). The tissue inoculum yielded heavy growth of a single organism on all plates except on the Columbia colistin-nalidixic acid, Shaedler sheep blood-phenethyl alcohol, and kanamycin-vancomycin agars. Two colony types with slightly different zones of hemolysis around the colony were observed on SBA and were subsequently isolated in pure culture. The large-zone colony type (LZ) and the small-zone colony type (SZ) were both identified as *V. damsela* by the criteria listed in Table 1 (24). All tests were conducted under standard conditions and in 2 to 3% NaCl. Of interest is that the urea hydrolase reaction was negative when assayed with 3% NaCl. These isolates differed from those in another study (16) in that, on primary isolation, they grew equally well at 25 and 35°C on SBA and grew poorly on MacConkey agar (no growth at 24 h and colonies of about 1 mm at 48 h). On casein-tyrosine-starch plates (usually used for *Nocardia* identification) and on DNase plates, the organisms grew only when NaCl was added and gave negative results. The triple sugar-iron-agar reaction was alkaline over acid with gas and no H_2S production. The API 20E system (Analytab) codes at both 25 and 35°C incubation were 2014004 and 2015004, which were interpreted with the API data base as *Pseudomonas* sp. fluorescence group. The DMS-rapid NFT system code 5300244 was not in this data base. The identification as *V. damsela* was confirmed by the Houston City Health Department laboratory.

On Gram stain, organisms from a colony were observed as large, pleomorphic, and evenly stained gram-negative rods (Fig. 2). The colony size (about 3 mm at 24 h) and morphology of LZ and SZ were the same on both RBA and SBA, and the hemolytic zones were only slightly different on SBA (about 1 mm for LZ and 0.5 mm for SZ). However, on RBA and LZ had a hemolytic zone of about 10 mm, and the SZ hemolytic zone was about 1 to 2 mm. In addition, the LZ showed a line of increased hemolysis on SBA at the midpoint between two colonies, whereas the SZ did not (Fig. 3). Both the SZ and LZ strains grew to 1×10^8, to 3×10^8 CFU/ml in 8 h at 25 and 35°C and in 0.5 and 3% NaCl. The extent of organism growth in 6% NaCl was considerably less. The SZ filtrates from broth cultures grown at 25°C in 3% NaCl, at 35°C in 3% NaCl, at 25°C in 0.5% NaCl, and at 35°C in 0.5% NaCl all caused slightly less partial hemolysis on SBA than on RBA. Under the same four conditions, the LZ filtrates caused complete hemolysis on RBA and slightly more hemolysis on SBA than had the SZ filtrates. During prolonged incubation (3 days) at 25 or 35°C, the LZ filtrates produced concentric rings of hemolysis on RBA.

A modified CAMP test was used to examine the synergy between the hemolysins of *V. damsela* and those of *S.*

TABLE 1. Test results for the identification of *V. damsela* isolates

Test method(s)	Positive tests	Negative tests
API 20E system (35°C)	Urea hydrolysis,[a] arginine, dihydrolase, glucose utilization, nitrate reduction	Beta-galactosidase, lysine, and ornithine decarboxylase; indole, acetoin, and H_2S production; gelatin hydrolysis; tryptophan deaminase; utilization of mannitol, inositol sorbitol, rhamnose, sucrose melibine, amygdalin, and arabinose
DMA-Rapid NFT System (30°C)	Urea hydrolysis,[a] arginine dihydrolase, glucose fermentation, beta-galactosidase (weak), gelatin hydrolysis (at 3 days), malate and maltose assimilation	Esculin hydrolysis; assimilation of glucose, arabinose, mannose, mannitol, *N*-acetylglucosamine, D-gluconate, caprate, adipate, citrate, and phenylalanine
Standard methods	Urea hydrolysis; gelatin hydrolysis; growth on TCBS[b] agar; growth on salmonella-shigella agar; motility at 25 and 35°C; catalase, oxidase (slow), maltose, and glucose fermentation; growth in 6% NaCl-nutrient broth (variable and less than in 3%); acetoin production[c] methyl red test[c]; production of DNase[c], lecithinase, and lipase (SZ)	Growth at 4 and 42°C; growth in 0% NaCl-nutrient agar; indole production; phenylalanine, deaminase; hydrolysis of casein, tyrosine, and starch[c]

[a] In the presence of 3% NaCl the reaction was negative.
[b] TCBS, Thiosulfate-citrate-bile salts-sucrose.
[c] Tests were performed in the presence of 2 to 3% NaCl.

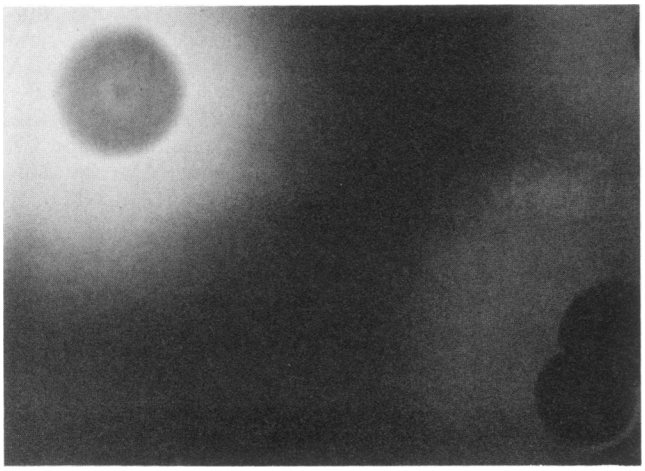

FIG. 3. Thin line of augmented hemolysis between two LZ colonies on SBA.

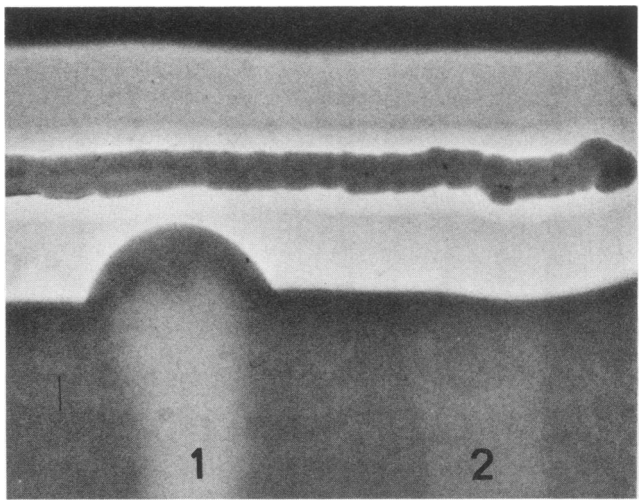

FIG. 5. Results of modified CAMP test with cell-free filtrate from LZ at site 1 and SZ at site 2 placed perpendicularly to the *S. aureus* streak.

aureus. The LZ and SZ phenotypes of *V. damsela* were each streaked perpendicularly to a beta-toxin-producing strain of *S. aureus* on an SBA plate. Although there was a slight increase in hemolysis with the SZ strain, the LZ strain prevented the hemolysis of the erythrocytes usually caused by *S. aureus* (Fig. 4). Similar results were achieved with the cell-free filtrates (Fig. 5) and with organisms grown on top of a Millipore filter placed on an SBA plate. The prevention of hemolysis depended upon the LZ filtrate reaching the sheep erythrocytes before the *S. aureus* toxin. If the LZ filtrate was placed on the SBA plate from 1 to 24 h before the addition of *S. aureus* filtrate, the whole area under the drop was protected from the *S. aureus* hemolysin. Since *V. damsela* grew faster and produced toxin sooner than *S. aureus*, when both organisms were inoculated at the same time on an SBA plate the usual pattern of protection was an X that crossed at the *S. aureus* inoculation line (Fig. 4a and 4b). If the *S. aureus* hemolysin was placed on the SBA plate first, the LZ filtrate could not protect that area. The filtrates showed no loss of activity after incubation at 4, 35, or 45°C for 30 min. However, SZ filtrates lost all activity after the 60 and 100°C treatments. After being heated to 100°C, but not

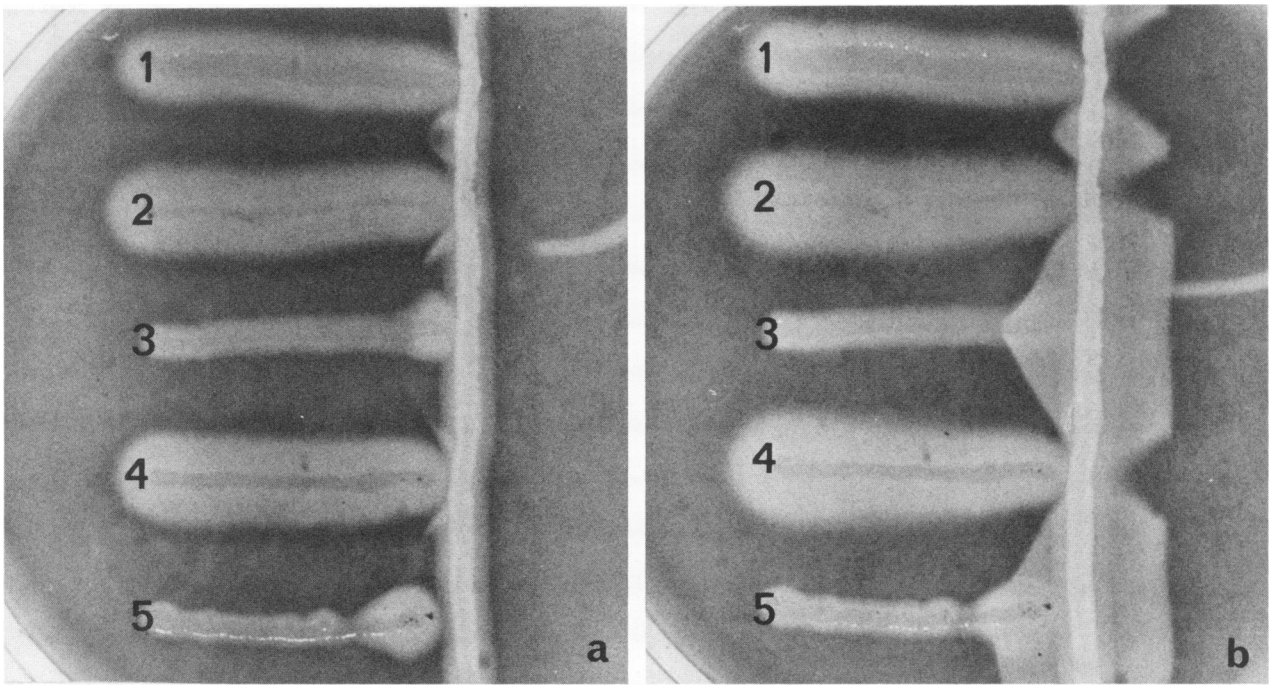

FIG. 4. Results of a modified CAMP test. *S. aureus* was streaked vertically on the SBA plate, and strains of *V. damsela* were streaked perpendicularly. (a) Plate after 24 h at 35°C, with augmented hemolysis at sites 3 and 5. (b) The same plate after overnight incubation at 4°C, with an absence of hemolysis at sites 1, 2, and 4. Numbers 1 through 5 refer to *V. damsela* strains LZ, CDC 737, SZ, CDC 1421, and ATCC 33538, respectively.

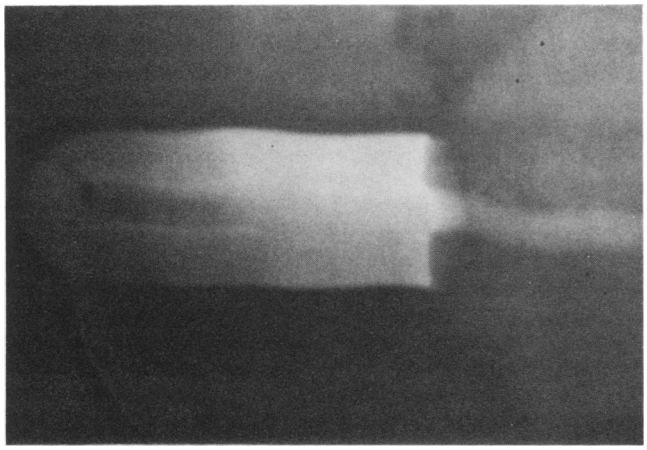

FIG. 6. Plate with lack of *S. aureus* inhibition by heated LZ filtrate. Samples (10 μl) of LZ filtrate heated to 100°C (left) and not heated (right) were placed on SBA. A millipore filter was placed on the samples and streaked with *S. aureus*. After 18 h of incubation at 35°C, the filter was removed.

after 60°C, the protective effect of the LZ filtrate was destroyed (Fig. 6).

In addition to their hemolytic differences, these *V. damsela* isolates were also dissimilar in enzyme activity. On a Nagler egg yolk agar plate, the LZ exhibited more lecithinase activity than did the SZ, and only the SZ showed detectable lipase activity.

The phenotypes were stable for over 3 months with weekly passages; each retained all of the characteristics described above. When stored at 4°C, the filtrates showed no detectable decrease in hemolytic activity for 2 months.

Antibiotic susceptibility testing by the standard Bauer-Kirby disk diffusion method (18) indicated that *V. damsela* was susceptible to ampicillin, carbenicillin, cephalothin, chloramphenicol, colistin, gentamicin, tetracycline, and tobramycin; results with amikacin were indeterminate. The broth in the MIC panel (Microscan) did not initially support growth. However, MICs (in milligrams per milliliter) were determined after the addition of NaCl to the wells (3% final concentration) as follows: ampicillin, ≤0.12; carbenicillin, ≤4; tetracycline, 0.25; cephalothin, 2; cefoxitin, 1; cefamandole, ≤0.5; chloramphenicol, 1; gentamicin, 2; tobramycin, 2; amikacin, 2; and trimethoprim-sulfamethoxazole, ≤0.25-4.8.

Examination of other strains of *V. damsela* indicated that strains CDC 1421-81 and CDC 727-82 produced the hemolytic and protective factors described for LZ and that strain CDC 983-79 resembled SZ (Fig. 4). Strain CDC 1471-82 was not beta-hemolytic.

DISCUSSION

Of the 26 *Vibrio* spp. recognized in *Bergey's Manual of Systematic Bacteriology* (1), 9 are presumably of clinical significance: *V. cholerae*, *V. parahaemolyticus*, *V. fluvialis*, *V. furnissii* (*V. fluvialis* II) (9), *V. mimicus* (5), and *V. hollisae* (16) cause or are associated with gastrointestinal disease (3). *V. vulnificus* is isolated from wounds and blood (2, 4, 19), *V. alginolyticus* is associated with wounds or otitis (3, 20), and *V. damsela* is associated with wounds (7, 16). Non-O:1 *V. cholerae* and *V. parahaemolyticus* have also been associated with wounds and septicemia (3, 4), and *V. damsela* and *V. anguillarum* have been associated with diseased (3, 16) and healthy (21) fish. *V. vulnificus* has been isolated from stool samples (19) but is rarely linked to diseases limited to the gastrointestinal system.

Of the six cases of *V. damsela* infection of human wounds presented by Morris et al. (17), five were associated with exposure to fish or salt water. Although no clinical isolations from other than blood or wounds have been reported for this species, the data may be biased by the difficulty in detecting *V. damsela* in a stool specimen. The isolates we studied grew well on thiosulfate-citrate-bile salts-sucrose and hektoen enteric media, but growth on Salmonella-shigella and MacConkey agars was slight at 24 h. When a stool sample is screened, the positive urea and alkaline-acid reactions on the triple sugar-iron-agar slant are not usually recognized as indicating a potential pathogen. However, this concept must be revised, as both *V. damsela* tested in normal saline and some strains of *V. parahaemolyticus* are urease positive. The strains of *V. damsela* we tested were urease negative in 3% saline; a similar observation was made for a vibrio isolate from a Senegalese fish (21). The Analytab Products identification code was misleading because it was not in the standard data base, and a reference to the larger data base available by telephone indicated that the number was a very good identification for a *Pseudomonas* sp. fluorescence group.

The two isolates (LZ and SZ) could be the result of a simultaneous infection by two unrelated strains of *V. damsela*. However, since the phenotypes resembled each other in biochemical characteristics which vary within the species, they are probably both variants of a single parent strain. Because of variation within a population, isolates which are selected for study may not reflect the infecting population. Kreger (13) obtained 19 isolates from a secondary source which showed excellent correlation between hemolysin (cytolysin) activity and experimentally produced disease in animals. However, there was no correlation between the severity of the disease in the original cases and the hemolytic activity of the isolates. Preliminary comparisons with other *V. damsela* isolates indicated that this LZ strain was similar to those reported to have a high hemolytic activity and virulence and that the SZ strain was similar to those with a low hemolytic activity (Fig. 4). It is possible that both types were present at the original isolate source in cases other than this one.

The existence of strains with different hemolytic capabilities has been documented for several species of vibrios as well as other organisms (8). There are thermostable and thermolabile hemolysins which are made by the Kanagawa-positive and -negative strains, respectively, of *V. parahaemolyticus* (23). A virulent strain of *V. vulnificus* produces a cytolysin active against rabbit erythrocytes, whereas an avirulent strain does not (13). There are two distinct hemolytic groups of *V. damsela* (12) which correspond to the LZ and SZ phenotypes discussed here. However, the dramatic protection of sheep erythrocytes against *S. aureus* hemolysins has not been documented before, although synergistic hemolysis is common.

S. aureus beta-toxin is phospholipase with specificity for sphingomyelin and lysophosphatidylcholine. It may be that the LZ toxin occupies or changes this target site on the erythrocyte, thus preventing *S. aureus* hemolysin activity.

Hemolytic activity is strongly associated with pathogenesis for *V. parahaemolyticus* (21) and *V. damsela* in an animal model (12) but not for *V. vulnificus* (10). *V. cholerae* produces a hemolysin which causes increased vascular permeability of rabbit skin, rapid death of mice, and hemolysis of rabbit erythrocytes (25). The vibrios elaborate a variety of

extracellular toxins and enzymes, many of which could cause cell damage (6, 11–13, 15, 22, 23, 25). The exact mechanism by which these factors cause the rapid and remarkable clinical manifestations documented here is not known.

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