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Hemolytic Activity of and Lethal Toxin Production by Environmental Strains of *Vibrio parahaemolyticus*

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Repeated subculturing of Kanagawa-negative strains of *Vibrio parahaemolyticus* on Wagatsuma agar induced the production of a hemolysin which was not the thermostable direct hemolysin. Crude hemolysin exhibited a 30 to 40% lethal toxicity in mice after intraperitoneal injection. A 21-kilodalton protein band was observed with all the environmental isolates in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Results suggested that a certain percentage of environmental strains of *V. parahaemolyticus* is responsible for pathogenesis.

Despite extensive studies on the Kanagawa hemolysin of *Vibrio parahaemolyticus*, the exact pathogenic mechanism of this organism remains unclear. The virulence factor that has received the greatest attention is the thermostable direct hemolysin (TDH) or Kanagawa phenomenon (KP) hemolysin. A special blood agar medium, Wagatsuma agar (WA), is routinely used in clinical laboratories to test for hemolysin production. Epidemiological surveys have revealed that almost all clinical isolates of *V. parahaemolyticus* are KP positive (KP^+) and associated with the production of TDH, whereas nonclinical isolates are almost invariably KP negative (KP^-) (7, 8).

It is well established that TDH is responsible for the pathogenicity of the KP^+ strains of *V. parahaemolyticus*. Enigmatically, however, the association of KP^- strains with acute gastroenteritis has also been reported (9). The present investigation was carried out on the hemolysin(s) in KP^- strains of *V. parahaemolyticus* to find out whether it has a possible role in the pathogenesis of the disease.

The strains of *V. parahaemolyticus* used in this study and their sources of isolation are listed in Table 1. The strains were isolated from diverse categories of environmental specimens in the vicinity of Calcutta during a 1-year ecological study of *V. parahaemolyticus* (10). Initially, all the strains were nonhemolytic (KP^-) on WA (Eiken Chemical Co. Ltd., Japan) after 24 h of incubation at 37°C. However, after four to five subcultures, each strain produced a zone of hemolysis around the colony on WA. The method routinely used for the detection of KP on WA sometimes gives false-positive results, owing to the instability of erythrocytes or to the production of other hemolysins by these organisms (1). For this reason, a modified Elek test (2) was performed for the detection of TDH from KP^- strains of *V. parahaemolyticus*.

Crude hemolysin was isolated by culturing all of the induced strains in a previously described medium (3). The medium was composed of 3% sodium chloride, 1% peptone (Difco Laboratories, Detroit, Mich.), 0.5% dibasic sodium phosphate, and 0.5% glucose (pH 7.6 to 7.8). The medium supports the growth of *V. parahaemolyticus* at 37°C for 16 to 18 h with aeration. The culture filtrates were collected by centrifugation (8,000 rpm for 10 min). Crude hemolysin was isolated from the culture filtrates by the method of Honda et al. (3). Solid ammonium sulfate (35.1 g/100 ml) was added to the culture filtrates, which were then centrifuged (10,000 rpm for 15 min). The precipitate was dissolved in 0.01 M phosphate buffer (Na_2HPO_4-KH_2PO_4, pH 7.0), which was then dialyzed for 48 h against the same buffer and concentrated by lyophilization (Lyophilab model 80 MC; Scientific Instrument Co., Ltd., India). Particulate materials were removed by centrifugation (5,000 rpm for 5 min) from the concentrate and finally used as crude hemolysin.

Hemolytic activity was determined as described by Takeda et al. (12). The standard reaction mixture (2.5 ml) was incubated at 37°C for 30 min and then centrifuged (3,000 rpm for 5 min). Hemolytic activity was assayed by measuring the A_{540} of the resulting supernatant in a spectrophotometer. One hemolytic unit was defined as the amount of supernatant with an A_{540} of 0.5.

The sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles of crude hemolysins were compared by the technique of Laemmli (5) with a 3% (wt/vol) stacking gel and a 10% (vol/vol) separating gel. The amount of total protein was measured by the method of Lowry et al. (6). Lethal toxicity was observed by injecting crude hemolysins intraperitoneally into 4- to 6-week-old mice (ddo strain; average weight, 18 to 20 g), and the survival time of the animals was measured. The amount of protein injected was the same for each strain.

Initially, none of the strains were found to be KP^+. After four to five subcultures on WA, all of the strains acquired the ability to produce a weak hemolysin around the colony.

TABLE 1. *V. parahaemolyticus* strains used in this study

Strain[a]	Source	Serotype
E-2	Water	O4:K42
E-3	Water	O5:K17
E-38	Plankton	O2:K3
E-39	Plankton	O2:K28
E-44	Sediment	NT[b]
E-48	Fish	O4:K34
C-10	Clinical	O5:K15
C-34	Clinical	O2:K3

[a] E, Environmental; C, clinical. C-10 was from I. D. Hospital, Calcutta, India, and C-34 was from B. C. Roy Hospital, Calcutta, India.
[b] NT, Nontypable.

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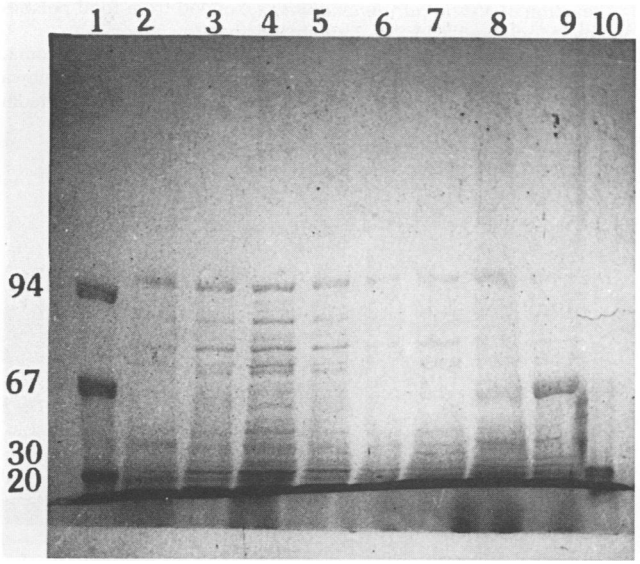

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles of crude hemolysin preparations from culture supernatants of *V. parahaemolyticus*. Concentrated (200-fold) dialyzed samples (40 to 50 μl) mixed with sodium dodecyl sulfate and 2-mercaptoethanol were boiled and electrophoresed. Lanes: 1, protein standards with molecular sizes shown in kilodaltons; 2, E-2; 3, E-3; 4, E-38; 5, E-39; 6, E-44; 7, E-48; 8, C-10; 9, C-34; and 10, purified TDH showing the 21-kilodalton protein band.

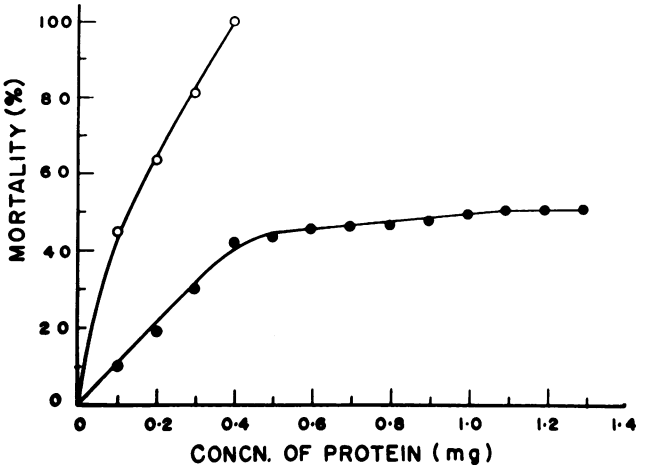

FIG. 2. Effect of different concentrations (CONCN.) of crude hemolysin from clinical (○) and environmental (●) *V. parahaemolyticus* on mouse lethality.

Subsequently, these strains were found to be Elek test negative, indicating that the hemolysin was not TDH. The hemolytic activity of all the environmental strains ranged between 20 and 30 hemolytic units. In contrast, the clinical strains (KP^+) used for comparison showed a high degree of hemolytic activity, at least threefold higher than that of the environmental strains. In sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the same protein profile was observed with all the environmental strains (Fig. 1). Each strain showed the presence of a protein band of ca. 21,000 daltons which correlated with the TDH band. The clinical strains (C-34 and C-10) included for comparison showed the presence of a strong protein band of 65,000 daltons (Can. J. Microbiol. in press), along with the existing TDH band (21,000 daltons). All of the environmental strains induced, at most, 40% mouse lethality within 14 to 24 h (Fig. 2), whereas a death rate of 100% (2 to 6 h) was observed with the clinical strains. In this experiment, 30% of the induced viable cultures of the KP^- strains showed a positive response in the rabbit ileal loop model, whereas uninduced viable cultures were not reactive. The crude hemolysins obtained from these induced cultures were weakly reactive in the ileal loops. It should be mentioned that all of the above-described findings were from the induced cultures, and significant results were not obtained from the uninduced cultures of *V. parahaemolyticus*.

Sakazaki (9) also observed that KP^- strains may produce an ileal loop response. Subsequently, Joseph et al. (4) reported that an ileal loop reaction along with inflammatory changes in the gut mucosa could be induced by *V. parahaemolyticus* strains, irrespective of their KP reaction. Moreover, diarrheal diseases caused by KP^- strains have also been documented (13, 14).

It is already established that KP^- strains do not produce TDH (11). In our study, crude hemolysins from KP^- strains were found to elicit 30 to 40% mouse lethality. In light of the above-described observations, it may be presumed that KP^- strains possess factors still unknown that may be related to pathogenicity.

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