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# Purification and Characterization of an *Aeromonas hydrophila* Hemolysin

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**A hemolysin produced by *Aeromonas hydrophila* CA-11, isolated from an environmental source, was purified by sulfopropyl–Sephadex C-25 chromatography at pH 5.0. This hemolysin caused fluid accumulation in infant mouse intestines and rabbit intestinal loops and killed Vero cells, as did the hemolysin produced by strain AH-1, isolated from a diarrheal case. In polyacrylamide gel electrophoreses at pHs 4.0 and 9.4 and in thin-layer isoelectric focusing, CA-11 hemolysin migrated as a single band to a position different from that of AH-1 hemolysin. Immunodiffusion tests indicated that CA-11 hemolysin was immunologically related to AH-1 hemolysin but possessed unique antigenic determinants. Neutralization tests with antihemolysin sera also demonstrated immunological cross-reactivity between AH-1 and CA-11 hemolysins. These results apparently indicate that the hemolysins produced by the two strains of *A. hydrophila* are immunologically and physicochemically different from each other.**

*Aeromonas hydrophila* produces a variety of biologically active extracellular substances, including enzymes, hemolysins, cytotoxin(s), and enterotoxin(s) (8, 16, 19). Such extracellular substances have been studied in relation to the pathogenicity of the organism. Many workers ascribed the enteropathogenicity of *A. hydrophila* to specific enterotoxigenic factor(s) (1, 5, 11). In a previous report, we demonstrated that a hemolysin produced by *A. hydrophila* isolated from a patient with diarrhea is a cytotoxic enterotoxin causing fluid accumulation in infant mouse intestines and rabbit ileal loops and eliciting cytotoxic effects to Vero cells (2). Some investigators reported that the hemolysin produced by *A. hydrophila* was closely related to its enterotoxigenicity (3, 10, 16), but only a few quantitative studies have been made on the hemolysin production of the organism. We tried to establish a simple immunological method for determining the hemolysin. During our efforts, we found that the hemolysin produced by another strain of *A. hydrophila* was antigenically similar to but distinct from the previously purified hemolysin.

In the present communication, we report the biological and immunological properties of the new hemolysin in comparison with those of the hemolysin reported previously (2).

## MATERIALS AND METHODS

**Bacterial strains.** *A. hydrophila* AH-1 and CA-11 were used. Strain AH-1 was isolated from a patient with watery diarrhea (2). Strain CA-11 was an environmental isolate and was given to us by G. N. Stelma, Center for Food Safety and Applied Nutrition, Food and Drug Administration, Cincinnati, Ohio. The strains were stored at room temperature in the maintenance medium, consisting of 0.3% yeast extract (Difco Laboratories, Detroit, Mich.), 1.0% Casitone (Difco), 0.5% NaCl, and 0.3% agar (pH 7.0).

**Hemolysin production.** Two media were used for hemolysin production; DY medium was composed of Davis minimal broth without glucose (Difco) and with 1% yeast extract (Difco) (2), and MG medium was the minimal-salts medium modified from that of Riddle et al. (15), consisting of (in grams per liter) NaCl, 6.0; $KH_2PO_4$, 3.0; $K_2HPO_4$, 6.96; $MgSO_4 \cdot 7H_2O$, 0.2; $(NH_4)_2SO_4$, 0.78; L-glutamic acid, 1.0; and $ZnSO_4 \cdot 7H_2O$, $8.6 \times 10^{-3}$. The stock culture was subcultured overnight in brain heart infusion broth (Difco) at 30°C.

One loopful of the culture was inoculated into 10 ml of fresh DY medium. After incubation overnight at 30°C, 2 ml of the culture was transferred to 200 ml of DY medium in an Erlenmeyer flask (1,000 ml), which was incubated at 30°C with shaking.

One drop of the overnight culture in brain heart infusion broth was spread over a heart infusion agar plate (diameter, 90 mm), which was incubated overnight at 30°C. The bacterial cells grown on the plate were suspended in a small amount of MG medium, which was poured into 1,000 ml of MG medium in a shallow stainless steel tray (27 by 39 by 9.5 cm). The tray and medium were incubated still at 30°C. Bacterial growth was determined photometrically at 640 nm.

**Assay for hemolysin.** Hemolytic activity was determined by the previous procedures with a slight modification (2). In brief, a sample (1 ml) diluted in 0.01 M Tris hydrochloride buffer (pH 7.2) containing 0.9% NaCl was mixed with an equal volume of a 1% rabbit erythrocyte suspension. After incubation for 90 min at 37°C, the mixture was centrifuged, and the $A_{540}$ of the supernatant was read. One hemolytic unit was defined as the minimum dose of the sample causing 50% hemolysis.

The hemolysin assay was performed also in a microtiter tray (Tomy Seiko, Tokyo). A 0.05-ml portion of cell-free culture filtrate diluted twofold serially in 0.01 M Tris hydrochloride buffer (pH 7.2) containing 0.9% NaCl was added to an equal volume of a 1% suspension of rabbit erythrocytes. The titer was expressed in $\log_2$ of the reciprocal of the highest dilution showing hemolysis.

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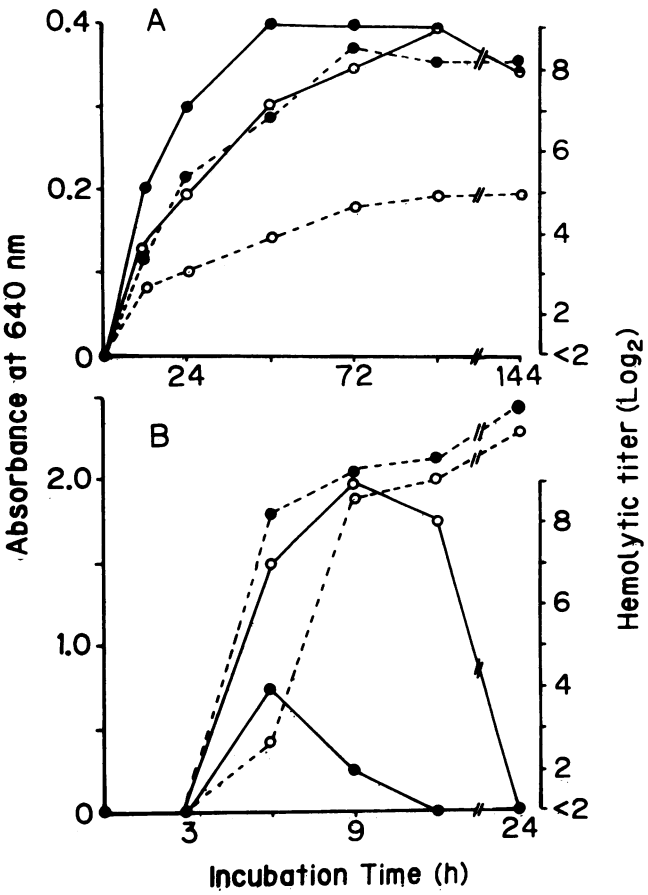


FIG. 1. Bacterial growth and hemolysin production by *A. hydrophila* AH-1 and CA-11 in MG medium without shaking (A) and in DY medium with shaking (B). Symbols: O- - - -O, growth of strain AH-1; O——O, hemolytic titer of strain AH-1; ●- - - -●, growth of strain CA-11; ●——●, hemolytic titer of strain CA-11.

**Purification of hemolysin.** The hemolysin of strain AH-1 was purified by the method previously described (2). For purification of the hemolysin of strain CA-11, a 48-h culture was separated by continuous centrifugation at 15,000 × *g* at 4°C. The supernatant was adjusted to pH 4.0 with 5 N HCl. To the supernatant (10 liters) was added sulfopropyl (SP)–Sephadex C-25 (6 g; Pharmacia, Uppsala, Sweden) preswollen in distilled water at pH 4.0. The mixture was stirred intermittently with a stirrer for 1 h at room temperature and allowed to stand to settle the SP-Sephadex gel to the bottom. After removal of the supernatant by siphoning, the hemolysin-adsorbed Sephadex gel was washed three times with 500 ml of 0.1 mM HCl each time and placed on top of a column of SP–Sephadex C-25 (1 g) equilibrated with 17 mM citric acid–33 mM $Na_2HPO_4$ buffer (pH 5.0). The column (1.5 by 24 cm) was further washed with 100 ml of the same buffer and eluted with a linear gradient of NaCl concentrations from 0 to 0.4 M in 1,000 ml of the buffer. Protein was determined by the method of Lowry et al. (12).

**Electrophoresis.** Polyacrylamide gel electrophoresis (PAGE) was carried out in a 6% gel at pHs 4.0 and 9.4 in the presence of 1 M urea by the methods of Reisfeld et al. (14) and Davis (6). Sodium dodecyl sulfate-PAGE was performed in a 6% gel by the method of Weber and Osborn (18). After electrophoresis, the gel column was stained in 0.2% Coomassie brilliant blue. Thin-layer isoelectric focusing was performed with a pH range of 3.5 to 10.0 by the method of Vesterberg (17).

**Preparation of antisera.** Antisera against hemolysins of strains AH-1 and CA-11 were obtained from rabbits weighing about 3 kg. A 1-ml portion of a purified hemolysin solution (20 μg/ml) was emulsified in an equal volume of Freund complete adjuvant (Difco) and injected subcutaneously into a rabbit. After 4 weeks, a 1-ml portion of the hemolysin solution without adjuvant was injected intravenously. The animal was bled 2 weeks after the last injection. Agar gel double-diffusion tests were performed by the method described elsewhere (2). The highest dilutions of the antisera forming a precipitin line to the homologous hemolysin (100 μg/ml) were 8- and 32-fold for anti-AH-1 and anti-CA-11 hemolysins, respectively.

**Other biological assays.** The rabbit ileal-loop and infant mouse tests were performed by the methods reported elsewhere (2). In the infant mouse test, a response was considered positive if the ratio of the intestinal weight to the remaining body weight (FA ratio) was higher than 0.09 based on the criteria for *Escherichia coli* heat-stable enterotoxin proposed by Dean et al. (7). Mouse lethal toxicity was assayed by the intravenous-injection method reported elsewhere (2). Cytotoxicity was determined with Vero cells labeled with $Na_2{}^{51}CrO_4$ (New England Nuclear Corp., Boston, Mass.). The amount of hemolysin required for 50% release of the radioactivity from $^{51}Cr$-labeled Vero cells was calculated (9).

**Neutralization tests.** Neutralization tests were performed by mixing the same quantities of the antiserum diluted twofold serially and a solution of the purified hemolysin, incubating the mixtures for 30 or 60 min at 37°C, and testing them for hemolytic activity and fluid accumulation in infant mouse intestines.

## RESULTS

**Production of hemolysin.** DY medium supported abundant growth of strains AH-1 and CA-11. The hemolysin production by both strains seemed to be rapid, reaching maximum levels very early in 6 and 9 h of incubation, respectively, and decreasing quickly thereafter. The amount of hemolysin produced by strain CA-11 in DY medium was about 1/30 that produced by strain AH-1 (Fig. 1B). The growth of both strains in MG medium gradually increased, but did not reach the levels attained in DY medium. The largest amounts of hemolysin produced by both strains in MG medium were attained shortly before maximum growth (Fig. 1A). Strain CA-11 produced hemolysin in a much larger quantity in MG medium than in DY medium.

**Purification of strain CA-11 hemolysin.** The hemolysin contained in culture supernatant was easily adsorbed onto SP–Sephadex C-25 preswollen at pH 4.0. Having adsorbed the hemolysin, the SP-Sephadex gel was packed into a column, and elution was performed at pH 5.0. The elution profile of the chromatography is illustrated in Fig. 2. The hemolytic activity was eluted as a single peak. The recovery and the specific activity of the hemolysin before and after chromatography are shown in Table 1.

**Characterization of strain CA-11 hemolysin.** In PAGE with a pH 4.0 gel, CA-11 hemolysin migrated as a single band at a higher velocity than AH-1 hemolysin (Fig. 3A). With a pH 9.4 gel, the mobility rate of the former was lower than that of the latter (Fig. 3B). In sodium dodecyl sulfate-PAGE, both hemolysins, not treated or treated with dithiothreitol, mi-

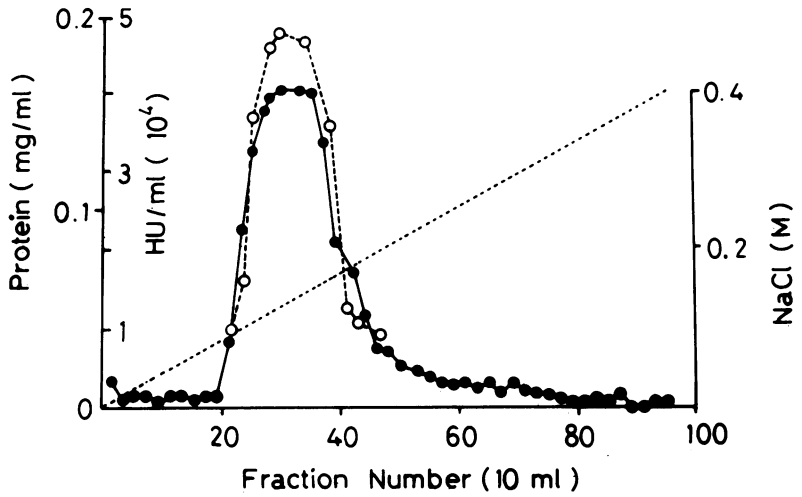


FIG. 2. Elution profile of hemolysin of strain CA-11 from SP-Sephadex. Elution was with a linear gradient of NaCl concentrations in 17 mM citric acid–33 mM $Na_2HPO_4$ buffer (pH 5.0). Symbols: ●, protein contents; ○, hemolytic activity; . . . . . , NaCl concentration.

grated in a single band to the same positions; their molecular weights were estimated at 50,000. In thin-layer isoelectric focusing, CA-11 hemolysin migrated as a single band, and the isoelectric point was found to be 7.60. The antiserum against the hemolysin of strain CA-11 was tested for reactivity with both the hemolysins. The agar gel double-diffusion tests demonstrated that anti-CA-11 hemolysin contained antibody recognizing AH-1 hemolysin and that CA-11 hemolysin possessed its unique antigenic determinant(s) as illustrated by spur formation (Fig. 4A). The converse was also the case; anti-AH-1 hemolysin recognized both hemolysins, and it gave a spur of AH-1 hemolysin over CA-11 hemolysin (Fig. 4B).

**Comparison of biological activities between hemolysins of strains CA-11 and AH-1.** In the rabbit ileal-loop and infant mouse tests, CA-11 hemolysin caused fluid accumulation, as did AH-1 hemolysin. The specific enterotoxigenic activities of CA-11 and AH-1 hemolysins were nearly the same. The minimum lethal doses for mice of the two hemolysins were also the same. The hemolytic activity and cytotoxicity of CA-11 hemolysin, however, were about two or three times higher than those of AH-1 hemolysin (Table 2). When both hemolysins (10 μg/ml) were treated with trypsin (twice crystallized; Sigma Chemical Co., St. Louis, Mo.) at 1 mg/ml or dithiothreitol (Sigma) at 5 mM for 30 min at pH 7.2 and 37°C, their hemolytic activities were as high as those before treatment. All these biological activities of CA-11 hemolysin were inactivated upon heating at 56°C for 5 min.

**Neutralization tests.** The results of neutralization tests with the antisera against AH-1 and CA-11 hemolysins showed that the homologous hemolysin was more effectively neutralized than was the heterologous one. As for the hemolytic activity, either antiserum inhibited the homologous hemolysin about 15 times more efficiently than the heterologous one (Fig. 5). In the infant mouse test, however, the neutralizing activity of anti-CA-11 hemolysin appeared to be more effective than that of anti-AH-1 hemolysin over the homologous hemolysin (Fig. 6).

TABLE 1. Purification of *A. hydrophila* CA-11 hemolysin

| Material | Vol (ml) | Amt of protein (mg) | Amt of hemolysin (HU × $10^6$)[a] | HU/ μg | Recovery (%) |
|---|---|---|---|---|---|
| Culture supernatant | 10,000 | 470 | 14.0 | 30 | 100 |
| SP-Sephadex eluate | 260 | 31.5 | 9.6 | 305 | 68.6 |

[a] HU, Hemolytic units.

## DISCUSSION

In a previous study, we obtained a hemolysin from the supernatant of a young culture of *A. hydrophila* AH-1 in DY medium with vigorous aeration (2). Under the same conditions, strain CA-11 barely produced hemolysin. The minimal-salts medium of Riddle et al. (15) supplemented with L-glutamic acid as a carbon source (MG medium) and the prolonged still incubation, however, stimulated abundant production of hemolysin by both strains. CA-11 hemolysin produced in DY medium might have easily been inactivated during incubation, since the growth of strain CA-11 reached the same level as that of strain AH-1.

CA-11 hemolysin was purified by such simple procedures as mere batch-wise adsorption onto SP-Sephadex C-25 and

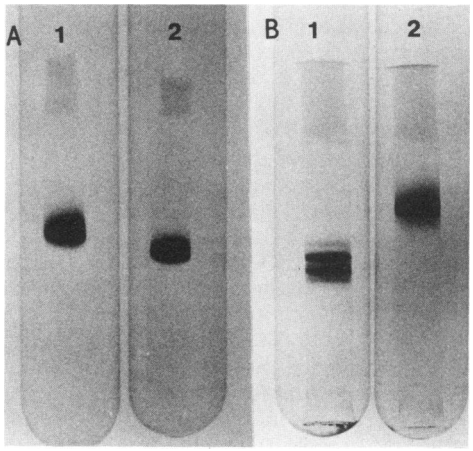


FIG. 3. PAGE of hemolysins at pHs 4.0 (A) and 9.4 (B). A 16-μg portion of purified hemolysin was applied to each column. Lanes 1, AH-1 hemolysin; lanes 2, CA-11 hemolysin.

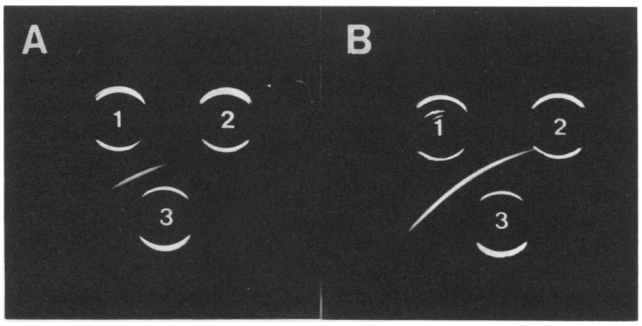


FIG. 4. Agar gel double-diffusion tests. 1, AH-1 hemolysin (216 μg/ml); 2, CA-11 hemolysin (107 μg/ml); 3, anti-CA-11 hemolysin in panel A and anti-AH-1 hemolysin in panel B.

subsequent elution. These procedures gave a satisfactorily high recovery of pure hemolysin.

The purified hemolysin of strain CA-11 migrated as a single band in PAGE at pHs 4.0 and 9.4, and its mobility rates under these conditions were different from those of AH-1 hemolysin. The results were in accordance with the finding that the isoelectric point of CA-11 hemolysin was 7.60, while that of AH-1 hemolysin was from 5.28 to 5.58 in multiple forms (2). The molecular weight of CA-11 hemolysin was the same as that of AH-1 hemolysin. Immunodiffusion tests clearly showed that CA-11 hemolysin, although immunologically related to AH-1 hemolysin, possessed unique antigenic determinant(s). These results show that the hemolysins produced by *A. hydrophila* are immunologically and physicochemically different from each other. Wretlind et al. (20) reported that *A. hydrophila* produced two hemolysins with isoelectric points of 5.5 and 4.3. Their purified hemolysins were inactivated with trypsin or dithiothreitol; they were referred to as α-hemolysin (11). CA-11 hemolysin, however, had an isoelectric point different from those found by Wretlind et al. (20). AH-1 and CA-11 hemolysins were both resistant to treatment with trypsin or dithiothreitol. Besides, both hemolysins induced a clear zone around the wells in a rabbit blood agar plate. From these observations, AH-1 and CA-11 hemolysins might as well be referred to as β-hemolysin (11).

The purified hemolysin of strain CA-11 caused fluid accumulation in the infant mouse intestines and rabbit ileal loops and killed Vero cells, showing that it is also a cytotoxic enterotoxin. The specific activities of CA-11 hemolysin, however, were not the same as those of AH-1 hemolysin. CA-11 hemolysin possessed a hemolytic activity and cytotoxicity to Vero cells about two or three times higher than those of AH-1 hemolysin on the protein basis. Neutral-

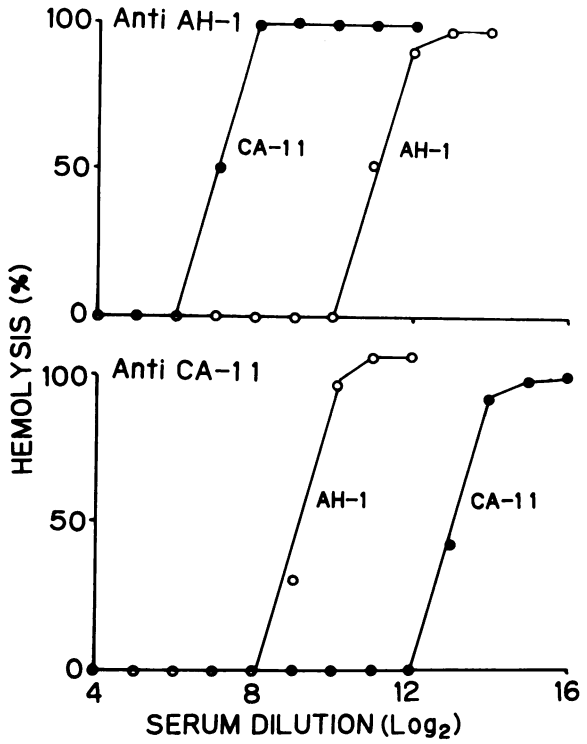


FIG. 5. Neutralization tests of hemolytic activities of AH-1 and CA-11 hemolysins with the two antihemolysin sera. Hemolysin (40 ng/ml) was mixed with an equal volume of serially diluted antiserum. After incubation for 30 min at 37°C, the hemolytic activities were determined as described in Materials and Methods.

ization tests with antihemolysin sera revealed immunological cross-reactivity between AH-1 and CA-11 hemolysins. The antiserum to each hemolysin more effectively neutralized the homologous hemolysin in terms of the hemolytic activity and the fluid accumulation in infant mouse intestines. The neutralizing activity of anti-CA-11 hemolysin was more effective than that of anti-AH-1 hemolysin, which may have been due to the different potencies of the antisera. Anti-CA-11 hemolysin formed precipitin lines with both hemolysins at a higher dilution than anti-AH-1 hemolysin.

Further studies seem warranted to find the epidemiological significance of the two immunologically similar but distinct hemolysins. Hemolysin-producing *A. hydrophila* in fecal specimens has been correlated with diarrheal disease (3, 16). *A. hydrophila* is paid more attention as an environmental pathogen and is frequently found in water sources (4, 13, 16). The two hemolysins reported herein must constitute

TABLE 2. Biological activities of *A. hydrophila* hemolysins

| Source of hemolysin | Hemolytic activity (HU/μg)[a] | Minimum dose causing | | | |
|---|---|---|---|---|---|
| | | Fluid accumulation[b] (ng) | FA > 0.09[c] (ng) | Mouse lethality[d] (ng) | Cytotoxicity to Vero cells[e] (ng/ml) |
| CA-11 | 280 | 10 | 50 | 60 | 3.7 |
| AH-1 | 90 | 5 | 50 | 60 | 6.2 |

[a] HU, Hemolytic units.
[b] In 50% or more of intestinal loops ($n$ = 6) in rabbit loop test.
[c] In infant mouse test. FA, Ratio of intestinal weight/remaining body weight.
[d] Within 24 h.
[e] 50% release of $^{51}Cr$.

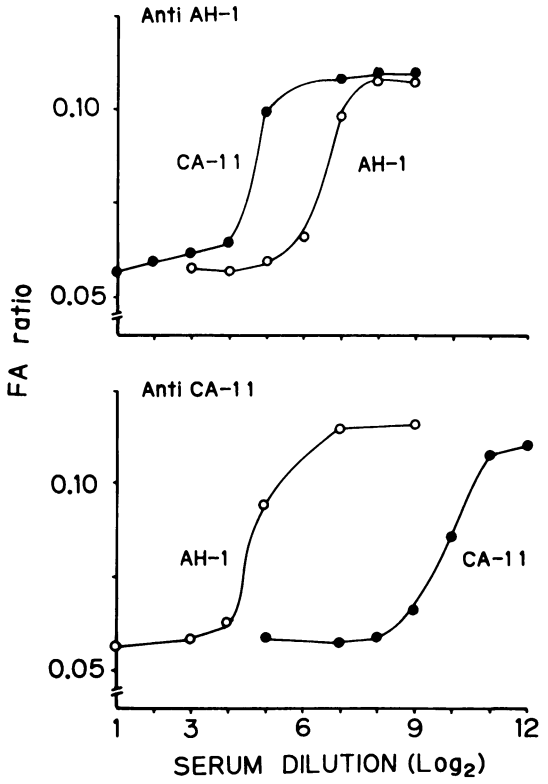


FIG. 6. Neutralization tests by infant mouse assay. Hemolysin (4 μg/ml) was mixed with an equal volume of serially diluted antiserum, and the mixture was incubated for 60 min at 37°C. After incubation, a 0.1-ml portion of the mixture was administered intragastrically. The animals were sacrificed 4 h after administration of the mixture. FA ratio, Ratio of intestinal weight/remaining body weight.

useful tools for epidemiological studies to clarify the enteropathogenic potential of *A. hydrophila*.

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## LITERATURE CITED

1. **Annapurna, E., and S. C. Sanyal.** 1977. Enterotoxicity of *Aeromonas hydrophila*. J. Med. Microbiol. **10**:317–323.
2. **Asao, T., Y. Kinoshita, S. Kozaki, T. Uemura, and G. Sakaguchi.** 1984. Purification and some properties of *Aeromonas hydrophila* hemolysin. Infect. Immun. **46**:122–127.
3. **Burke, V., M. Gracey, J. Robinson, D. Peck, J. Beaman, and C. Bundell.** 1983. The microbiology of childhood gastroenteritis: *Aeromonas* species and other infective agents. J. Infect. Dis. **148**:68–74.
4. **Burke, V., J. Robinson, M. Gracey, D. Peterson, N. Meyer, and V. Haley.** 1984. Isolation of *Aeromonas* spp. from an unchlorinated domestic water supply. Appl. Environ. Microbiol. **48**:367–370.
5. **Cumberbatch, N., M. J. Gurwith, C. Langston, R. B. Sack, and J. L. Brunton.** 1979. Cytotoxic enterotoxin produced by *Aeromonas hydrophila*: relationship of toxigenic isolates to diarrheal disease. Infect. Immun. **23**:829–837.
6. **Davis, B. J.** 1964. Disc electrophoresis. II. Method and application to human serum proteins. Ann. N.Y. Acad. Sci. **121**: 404–427.
7. **Dean, A. G., Y. C. Ching, R. G. Williams, and L. B. Harden.** 1972. Test for *Escherichia coli* enterotoxin using infant mice: application in a study of diarrhea in children in Honolulu. J. Infect. Dis. **125**:407–411.
8. **Donta, S. T., and A. D. Haddow.** 1978. Cytotoxic activity of *Aeromonas hydrophila*. Infect. Immun. **21**:989–993.
9. **Horiguchi, Y., T. Uemura, S. Kozaki, and G. Sakaguchi.** 1985. The relationship between cytotoxic effect and binding to mammalian cultured cells of *Clostridium perfringens* enterotoxin. FEMS Microbiol. Lett. **28**:131–135.
10. **Hoštacká, A., I. Čižnár, B. Korych, and J. Karolček.** 1982. Toxic factors of *Aeromonas hydrophila* and *Plesiomonas shigelloides*. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A **252**:525–534.
11. **Ljungh, Å., and T. Wadström.** 1982. Aeromonas toxins. Pharmacol. Ther. **15**:339–354.
12. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
13. **Pitarangsi, C., P. Echeverria, R. Whitmire, C. Tirapat, S. Formal, G. J. Dammin, and M. Tingtalapong.** 1982. Enteropathogenicity of *Aeromonas hydrophila* and *Plesiomonas shigelloides*: prevalence among individuals with and without diarrhea in Thailand. Infect. Immun. **35**:666–673.
14. **Reisfeld, R. A., U. J. Lewis, and D. E. Williams.** 1962. Disk electrophoresis of basic proteins and peptides on polyacrylamide gels. Nature (London) **195**:281–283.
15. **Riddle, L. M., T. E. Graham, and R. L. Amborski.** 1981. Medium for the accumulation of extracellular hemolysin and protease by *Aeromonas hydrophila*. Infect. Immun. **33**:728–733.
16. **Turnbull, P. C. B., J. V. Lee, M. D. Miliotis, S. Van de Walle, H. J. Koornhof, L. Jeffery, and T. N. Bryant.** 1984. Enterotoxin production in relation to taxonomic grouping and source of isolation of *Aeromonas* species. J. Clin. Microbiol. **19**:175–180.
17. **Vesterberg, O.** 1973. Isoelectric focusing of proteins in thin layers of polyacrylamide gel. Sci. Tools **20**:22–29.
18. **Weber, K., and M. Osborn.** 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. **244**:4406–4412.
19. **Wretlind, B., L. Heden, and T. Wadström.** 1973. Formation of extracellular haemolysin by *Aeromonas hydrophila* in relation to protease and staphylolytic enzyme. J. Gen. Microbiol. **78**:57–65.
20. **Wretlind, B., R. Möllby, and T. Wadström.** 1971. Separation of two hemolysins from *Aeromonas hydrophila* by isoelectric focusing. Infect. Immun. **4**:503–505.