J. Med. Microbiol. — Vol. 37 (1992), 262–267


# Production of haemolysis and its correlation with enterotoxicity in *Aeromonas* spp.

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**Summary.** A total of 147 clinical and environmental isolates of *Aeromonas* that included 14 *A. hydrophila*, 60 *A. sobria* and 73 *A. caviae* strains was tested for haemolysin production and its correlation with enterotoxicity; 108 isolates produced β-haemolysis. For *A. hydrophila* and *A. sobria*, titres of haemolysin were 16–128 HU/ml and for *A. caviae*, 16–64 HU/ml. In the ileal loop test, 82 (55·8%) strains of *Aeromonas* spp. produced enterotoxin. Of the β-haemolytic strains, 72·7% of *A. hydrophila*, 58·6% of *A. sobria* and 68·6% of *A. caviae* isolates caused fluid accumulation in rabbit ileal loops. One strain each of α-haemolytic *A. sobria* and *A. caviae*, one of non-haemolytic *A. sobria* and nine of non-haemolytic *A. caviae* also caused a secretory response. The β-haemolytic strains caused significantly more ($p < 0·05$) fluid accumulation than the α- and non-haemolytic isolates regardless of their species designation. The remaining 65 (44·2%) isolates belonging to the three species included α-, β- and non-haemolytic strains: they failed to cause fluid accumulation in the initial experiments but did so after one to three consecutive passages through rabbit ileal loops. Two α- and 13 non-haemolytic strains switched to production of β-haemolysis when they showed positive ileal loop reactions. However, on repeated subcultures or on storage in the laboratory, all of them reverted to their original haemolytic character and no longer produced enterotoxic activity.

## Introduction

*Aeromonas hydrophila* has been reported as an aetiological agent of diarrhoea in man.[1–16] Production of heat labile enterotoxin by strains of *Aeromonas* was first demonstrated in an adult rabbit ileal loop (RIL) model.[4,6,7,17] Subsequently, enterotoxin production by strains of *Aeromonas* was demonstrated in other animal and tissue-culture assays.[15–21] *A. hydrophila* and *A. sobria* have been reported to produce extracellular products such as haemolysin,[15,16,22,23] aerolysin,[24] cytotoxin[9,15] and various enzymes.[25–27] It has been reported that enterotoxic strains of *Aeromonas* spp. are β-haemolytic.[14–16,28–32] Most of these β-haemolytic strains were either *A. hydrophila* or *A. sobria* but rarely *A. caviae*.[14–16,28,30,32–34] Earlier studies indicated that enterotoxic and haemolytic properties of *Aeromonas* spp. were different entities[34] and were determined by separate genes located on different segments of the chromosome.[35] *Aeromonas* strains that caused little or no accumulation of fluid in the initial test were reported to switch to production of enterotoxin after consecutive passages through rabbit ileal loops.[4,6,7] However, no such effect of passage on the haemolytic character of this organism has yet been reported. The present study was undertaken to test for the production of haemolysis and enterotoxin by different species of *Aeromonas*, to look for any correlation between haemolytic and enterotoxic activities, and to test for changes in haemolytic character of strains showing enterotoxin production after passage through rabbit ileal loops.

Received 26 April 1991; revised version received 7 Dec. 1991; accepted 10 Jan. 1992.

## Materials and methods

### *Bacterial strains*

A total of 147 isolates of *Aeromonas* was included in the study. They were isolated from children and adults with diarrhoea (54), water from shallow tube wells (6), dug wells (6), the piped water supply (6), from sewage (6) and the river Ganga (20), and from superficial skin ulcers of fish (49). The organisms were identified by the method of Popoff[36] as *A. hydrophila* (14), *A. sobria* (60) and *A. caviae* (73). Strains were maintained in peptone agar stab cultures at room temperature and did not undergo more than three subcultures before testing for haemolysis and enterotoxin production.

### *Preparation of culture filtrates for haemolysin and enterotoxin detection*

Culture filtrates of isolates that gave ileal loop reactions in initial tests with whole live cells were

**Table I.** Haemolytic activity of CFs of strains of *Aeromonas* before and after passage through rabbit ileal loops

| Species and haemolytic character (number of isolates) | Number (%) tested before passage | Haemolysin production (HU/ml) | Number tested after passage* | Haemolysin production (HU/ml) |
|---|---|---|---|---|
| *A. hydrophila* (14) | | | | |
| β-haemolytic | 11 (78·5) | 16–128 | 0 | ... |
| non-haemolytic | 3 (21·4) | ... | 3 | 16–128 |
| *A. sobria* (60) | | | | |
| α-haemolytic | 6 (10·0) | ... | 5 | 16–128 |
| β-haemolytic | 46 (76·7) | 16–128 | 0 | ... |
| non-haemolytic | 8 (13·3) | ... | 7 | 16–256 |
| *A. caviae* (73) | | | | |
| α-haemolytic | 2 (2·7) | ... | 1 | 16 |
| β-haemolytic | 51 (69·9) | 16–64 | 0 | ... |
| non-haemolytic | 20 (27·4) | ... | 11 | 16–64 |

* Non-β-haemolytic strains tested only after passage.

prepared by the method of Annapurna and Sanyal.[7] Briefly, 10 ml of Brain Heart Infusion Broth (BHIB; Difco) in a 50-ml conical flask was inoculated with five or six smooth colonies grown overnight on nutrient agar (NA). The flasks were incubated at 37°C in a water bath with shaking for 16–18 h with 80–120 oscillations/min. The cultures were centrifuged at 22000 *g* for 20 min at 4°C, and supernates were filtered through membrane filters (Millipore; 0·22 μm) and stored at 4°C. These culture filtrates (CFs) were used for haemolysin and enterotoxin assays. α-Haemolytic strains were grown at 25°C for 48 h for preparation of CFs because maximum production of this haemolysin takes place at this temperature.[22]

*Detection of haemolysis and titration of haemolysin*

Haemolysin production by *Aeromonas* strains was tested on sheep blood agar (5%) with a 4–5 h culture of each organism in BHIB. After incubation for 24 h at 37°C, the plates were examined for the presence of α- or β-haemolysis around the colonies. The production of haemolysin(s) by each strain was confirmed by the method of Smith[37] as modified by Rennie and Arbuthnott.[38] Briefly, sheep erythrocytes (SRBC) were washed three times in isotonic saline and a 2% suspension was prepared in 0·04 M phosphate buffered saline (PBS, pH 7·4). Haemolytic activity was determined by mixing 0·5 ml of two-fold serial dilutions of CF of each strain with an equal volume of SRBC 2% suspension, incubated at 37°C in a water bath for 2 h and left at 4°C for 12 h. The lysed portion was diluted four-fold with sterile normal saline and the optical density at 540 nm was measured in a colorimeter. Standardisation of the erythrocyte suspension was done by lysis of 0·5 ml of SRBC 2% with a few crystals of saponin. An optical density of 0·5 at 540 nm was considered to indicate a standardised SRBC 2%. The negative control was 0·5 ml of saline instead of CF. One haemolytic unit (HU) was defined as the amount of CF that caused 50% haemolysis under experimental conditions.

*Ileal loop test and passage through rabbit ileal loops*

Cultures and CFs of the 147 strains of *Aeromonas* spp. were tested in adult albino rabbits (Belgian strain) by the method of De and Chatterjee[39] as modified by Annapurna and Sanyal[6] for detection of enterotoxin production. Briefly, bacteria grown in BHIB for 3 h were diluted 10-fold in the same medium and inoculated into rabbit ileal loops in 1-ml doses containing $10^5$–$10^6$ cfu. A BHIB culture of toxigenic strain 569B of *V. cholerae* was used as a positive control and unseeded BHIB as a negative control. CF (1 ml) was tested in the same way. Each test was done in three rabbits. Rabbits were killed after 6 h.

Strains of *Aeromonas* that caused little or no accumulation of fluid in the initial tests were passaged through rabbit ileal loops according to the method of Sanyal *et al.*[40,41] Briefly, each strain was cultured aseptically from a rabbit ileal loop on NA and incubated overnight; five or six colonies were inoculated into BHIB and incubated for 3 h, and 1 ml of diluted culture was inoculated again into a rabbit ileal loop. The process was continued until a positive response was obtained.

## Results

The majority (108 of 147) of isolates—11 (78·5%) of *A. hydrophila*, 46 (76·7%) of *A. sobria* and 51 (69·9%) of *A. caviae*—produced β-haemolysis on sheep blood 5% agar (table I). α-Haemolysis was shown by only eight strains comprising six (10%) of *A. sobria* and two (2·7%) of *A. caviae*. Thirty-one strains were non-haemolytic including three (21·4%) of *A. hydrophila*, eight (13·3%) of *A. sobria* and 20 (27·4%) of *A. caviae*.

When CFs were tested for haemolytic activity, only those causing β-haemolysis on sheep blood agar plates showed lysis of 2% sheep erythrocytes (table I). The

**Table II.** Correlation between enterotoxic and haemolytic activities of *Aeromonas* strains

| Species and haemolytic character | Number of strains tested | Number showing fluid accumulation before passage | Fluid accumulated (ml/cm of gut) | Number showing fluid accumulation after passage* | Fluid accumulated (ml/cm of gut) |
|---|---|---|---|---|---|
| *A. hydrophila* | | | | | |
| β-haemolytic | 11 | 8 (72·7) | 0·5–1·7 | 3 (27·3) | 0·7–1·4 |
| non-haemolytic | 3 | 0 (0) | ... | 3 (100·0) | 1·0–1·5 |
| *A. sobria* | | | | | |
| α-haemolytic | 6 | 1 (16·7) | 0·5 | 5 (83·3) | 0·5–1·0 |
| β-haemolytic | 46 | 27 (58·6) | 0·6–1·3 | 19 (41·4) | 0·6–1·5 |
| non-haemolytic | 8 | 1 (12·5) | 0·4 | 7 (87·5) | 0·5–1·4 |
| *A. caviae* | | | | | |
| α-haemolytic | 2 | 1 (50·0) | 0·5 | 1 (50·0) | 0·5–1·0 |
| β-haemolytic | 51 | 35 (68·6) | 0·5–1·0 | 16 (31·4) | 0·6–1·2 |
| non-haemolytic | 20 | 9 (45·0) | 0·4–0·8 | 11 (55·0) | 0·5–1·0 |
| Total | 147 | 82 (55·8) | ... | 65 (44·2) | ... |

Figures in parentheses are percentages of total number of strains tested.
* One to three passages in rabbit ileal loops.

titres of haemolytic activity produced by *A. hydrophila* and *A. sobria* isolates were 16–128 HU/ml and by *A. caviae* isolates, 16–64 HU/ml (table I).

Cultures and CFs of 82 (55·8%) of the *Aeromonas* strains tested caused accumulation of fluid in the rabbit ileal loop in the initial set of experiments. Amongst the β-haemolytic strains, eight (72·7%) of *A. hydrophila*, 27 (58·6%) of *A. sobria* and 35 (68·6%) of *A. caviae* caused fluid accumulation (table II). A single strain each of α-haemolytic *A. sobria* and *A. caviae* isolates also showed this activity. Of the non-haemolytic strains, one *A. sobria* and nine *A. caviae* isolates caused a secretory response in rabbit ileal loops (table II).

The β-haemolytic strains of *Aeromonas* caused significantly more ($p < 0·05$, Student's $t$ test) fluid accumulation in rabbit ileal loops than the α- or non-

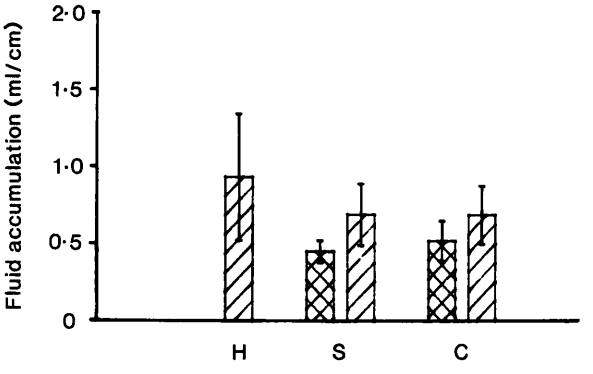


**Figure.** Enterotoxicity of β-haemolytic (▨) and α- or non-haemolytic (⊠) strains of *Aeromonas* without animal passage: **H**, *A. hydrophila* (eight strains); **S**, *A. sobria* (29 strains); C, *A. caviae* (45 strains).

**Table III.** Effects of passage on fluid accumulation and changes in haemolytic character of *Aeromonas* strains

| Species | Strain | Haemolytic type | Fluid accumulation (ml/cm) after passage no. | | | |
|---|---|---|---|---|---|---|
| | | | 0 | 1 | 2 | 3 |
| *A. hydrophila* | D-5485 | Non | ND | ND | ND | 1·4* |
| | F-10 | Non | ND | ND | 1·5* | 1·6 |
| | F-67 | Non | ND | ND | 1·0* | 1·2 |
| *A. sorbria* | D-5 | α | ND | ND | 0·44* | 0·6 |
| | D-72 | Non | ND | ND* | 0·44 | 0·6 |
| | W-38 | Non | ND | 0·5* | 0·6 | 0·8 |
| | W-16 | Non | ND | 0·7* | 0·8 | 0·9 |
| | F-36 | Non | ND | 0·6* | 0·75 | 0·9 |
| *A. caviae* | D-42 | Non | 0·4 | 0·8* | 0·95 | 1·1 |
| | D-421 | Non | ND | 0·65* | 0·78 | 0·94 |
| | D-425 | Non | ND | 0·55* | 0·75 | 1·0 |
| | W-3 | Non | ND | 0·4* | 0·85 | 1·0 |
| | W-9 | Non | ND | 0·6* | 0·7 | 0·85 |
| | F-51 | Non | 0·4 | 0·6* | 0·7 | 0·85 |
| | F-19 | α | ND | 0·65* | 0·78 | 0·94 |
| | F-61 | β | ND* | 0·55 | 0·75 | 0·95 |
| | F-75 | β | ND* | ND | ND | 0·65 |

ND, not detected (lower limit for detection 0·2 ml/cm).
Sources of isolates: D, diarrhoeal; W, water; F, fish.
* Change to haemolysis detected.

**Table IV.** Enhancement of titres of haemolysin of *Aeromonas* strains after passage through rabbit ileal loops

| Species | Strain | Haemolytic type | Hamolysin titres (HU/ml) after passage no. | | | |
|---|---|---|---|---|---|---|
| | | | 0 | 1 | 2 | 3 |
| *A. hydrophila* | D-5485 | Non | ... | ... | ... | 128 |
| | F-10 | Non | ... | ... | 32* | 64 |
| | F-67 | Non | ... | ... | 16* | 64 |
| *A. sorbria* | D-5 | α | ... | ... | 16* | 64 |
| | D-72 | Non | ... | 16 | 32* | 64 |
| | W-38 | Non | ... | 32* | 64 | 128 |
| | W-16 | Non | ... | 64* | 128 | 256 |
| | F-36 | Non | ... | 64* | 128 | 256 |
| *A. caviae* | D-42 | Non | ND* | 32 | 64 | 128 |
| | D-421 | Non | ... | 32* | 128 | 256 |
| | D-425 | Non | ... | 32* | 64 | 128 |
| | W-3 | Non | ... | 16* | 32 | 128 |
| | W-9 | Non | ... | 32* | 64 | 128 |
| | F-51 | Non | ND* | 32 | 64 | 128 |
| | F-19 | α | ... | 16* | 32 | 128 |
| | F-61 | β | 16 | 32* | 64 | 128 |
| | F-75 | β | 16 | 32 | 64 | 128* |

ND, not detected; ..., not tested.
Sources of isolates: D, diarrhoeal; W, water; F, fish.
* Enterotoxin produced.

haemolytic isolates, regardless of their species designation and source of isolation (figure).

After one to three consecutive passages through rabbit ileal loops, all 65 (44·2%) *Aeromonas* strains that caused little or no accumulation of fluid in the initial experiments did so (table II).

Thirteen non-haemolytic and two each of α- and β-haemolytic strains showed a marked increase in fluid accumulation on each consecutive passage through rabbit ileal loops. The non-haemolytic and α-haemolytic strains switched over to the production of β-haemolysis when they showed a positive ileal loop reaction (table III) and the titres of haemolysin also increased on each consecutive passage (table IV). However, on storage for 2–3 weeks in peptone agar stab cultures at room temperature, or on repeated subculture, all the strains reverted to their original haemolytic characters (either α- or non-haemolytic), and no longer produced enterotoxic activity.

## Discussion

The majority of the strains of *Aeromonas* in this study produced β-haemolysis and this property was not limited to *A. hydrophila* and *A. sobria* but extended to *A. caviae* in almost equal proportion. This phenomenon may be explained by the genetic evidence that sequences homologous to the β-haemolysin gene are present in all species of *Aeromonas* including *A. caviae*.[42] Furthermore, β-haemolysin in *Aeromonas* spp. has been suggested to be a cytotoxin;[15,42,43] a recent report on the cytotoxicity of *A. caviae* strains indicates that most of them possess this property and the failure by earlier workers to detect this was probably due to the use of medium containing higher levels of glucose, which has deleterious effects on the organism.[44]

Several workers have shown that enterotoxin is produced by most β-haemolytic strains of *Aeromonas*.[14,15,28,30,45–47] However, the results of the present study indicate that a significant number of β-haemolytic strains (35%) did not show any enterotoxic activity when first tested ($p < 0{\cdot}05$). In addition, two of the eight α-haemolytic and 10 of the 31 non-haemolytic strains of *A. sobria* and *A. caviae* also produced enterotoxin in the initial tests. However, the β-haemolytic strains showed significantly more enterotoxic activity than the α- and non-haemolytic strains, independently of their species designation. Figura *et al.*[48] and Eko and Utsalo[33] also encountered a few enterotoxic non-haemolytic and non-toxic β-haemolytic strains in their studies. Thus, this study clearly demonstrates that the capacity for enterotoxin production in *Aeromonas* spp. is not confined only to the β-haemolytic trains but that the α- and non-haemolytic isolates also possess this property, although to a lesser extent.

All of the *Aeromonas* strains that failed to produce enterotoxin in the initial set of experiments (44·2%) produced the toxin after one to three consecutive passages through rabbit ileal loops, suggesting that all *Aeromonas* strains are potentially enterotoxic regardless of their species designation, source of isolation and type of haemolysin produced. Similar observations on switching to toxin production by a non-toxigenic strain on consecutive passage through a susceptible host were also made in our earlier studies with *Aeromonas* spp. and other enteropathogenic organisms.[6,7,40,41,49–53] Such a change may result from the existence of a repression—derepression phenomenon controlling expression of a toxin gene depending on a

particular micro-environment[54] such as occurs in *Vibrio cholerae*.

The observation that α- and non-haemolytic strains of *Aeromonas* switched to production of β-haemolysis after one to three consecutive passages through rabbit ileal loops, along with initiation of fluid secretion, indicates that this process may influence the control of β-haemolysin and toxin production. Moreover, the titres of haemolysin elaborated in the CFs increased with each passage. One non-haemolytic strain converted to β-haemolysin production after a single passage without any secretory effect. However, on the second passage the strain caused accumulation of fluid. These observations indicate that a repression and de-repression phenomenon may also be operative in the case of the β-haemolysin gene and that the rabbit ileal loop provides a micro-environment conducive to its expression; this is confirmed by reversion of these strains to their original α- and non-haemolytic characters. These data indicate that all *Aeromonas* strains, irrespective of their species designation and source of isolation, possess a β-haemolysin gene and may elicit a secretory response in the gut. Passage through the gut of a susceptible host probably controls the expression of β-haemolysin and enterotoxin production.

This study was supported by the Council of Scientific and Industrial Research, New Delhi in the form of a Fellowship to D.V.S. and by the Indian Council of Medical Research, New Delhi: grant no. 5/8-1/(49)/88 ECD/II. We gratefully acknowledge the secretarial assistance of Mr D. Venu Gopal.

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