

Diarrhea and Intestinal Invasiveness of *Aeromonas* Strains in the Removable Intestinal Tie Rabbit Model

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Twelve *Aeromonas* strains were tested for virulence by using the removable intestinal tie adult rabbit diarrhea model. Mortality was 50% or greater for 7 of 12 strains; 23 of 37 rabbits that died developed diarrhea before death, and 11 of 27 surviving rabbits developed diarrhea. *Aeromonas* bacteremia was detected in 36 of 37 (97%) animals that died, but only in 2 of 27 (7%) survivors. Death, diarrhea, and bacteremia were all strongly strain dependent. Gastrointestinal lesions varied from moderate focal enteritis to severe multifocal necrosis and hemorrhage of the ileal mucosa, often accompanied by hepatic and splenic lesions. Intestinal colonization assays performed after infection indicated that the ileum was the most heavily colonized portion of the gut and the probable site of invasion. The application of the removable intestinal tie adult rabbit diarrhea model for intestinal challenge with *Aeromonas* strains has shown that some isolates are capable of invading the mucosa of rabbits, causing diarrhea and bacteremia. These data suggest that such strains may be important in causing human invasive diarrhea.

Bacteria belonging to the genus *Aeromonas* cause a variety of diseases in fish, amphibians, and mammals, ranging from diarrhea in piglets to fatal septicemias in fish and dogs and abortion in cattle (9, 13, 15, 38, 51). In humans, *Aeromonas* strains are common isolates from skin and wound infections in otherwise healthy persons (21, 29, 48, 50, 52). *Aeromonas* sepsis, which is frequently fatal in humans, is usually associated with malignancies or other chronic underlying illnesses (21, 23, 24). Bacteremias have also been reported in persons hospitalized for diarrhea (E. J. Kuijper, H. C. Zanen, and M. F. Peeters, Letter, Ann. Intern. Med. 106:640–641, 1987).

Aeromonas infection as a cause of diarrhea in humans is controversial. Although there are hundreds of reported cases of *Aeromonas* isolates from diarrheic stools, none satisfactorily eliminate the possibility of another cause of the illness. There are several distinct gastrointestinal syndromes commonly attributed to *Aeromonas* infection (22, 26). The most frequently reported is an acute, self-limiting watery diarrhea, sometimes accompanied by vomiting and/or fever. Perhaps the strongest evidence for a case of diarrhea caused by *Aeromonas* is the report of a choleralike illness with "rice-water" stools from a Thai woman who was admitted to a hospital in Paris and who showed a rise in neutralizing antibodies to *Aeromonas* enterotoxin (11). *Aeromonas sobria* was isolated from her stools. Less commonly, *Aeromonas* has been reported to produce a dysenterylike syndrome with bloody mucoid stools and abdominal pain, which is sometimes chronic or recurrent in nature.

Aeromonas organisms have been epidemiologically associated with acute diarrhea in some controlled studies (4, 18) but not in others (20, 39). In a recent hospital case control study of Peruvian children <2 years of age, *Aeromonas* strains were isolated from 52% of patients admitted with diarrhea and dehydration, compared with 9% of healthy controls (R. B. Sack, G. Pazzaglia, E. Salazar, A. Yi, and E.

Chea, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, C252, p. 435). Despite its high prevalence in some geographic areas and its strong epidemiological association with diarrhea in young children, *Aeromonas* infection has not been unequivocally linked to any outbreak of gastrointestinal disease.

If *Aeromonas* infection is not a common cause of diarrhea, it certainly has that potential. *Aeromonas* isolates have been reported to produce numerous virulence factors, including a cytotoxic enterotoxin (10); an enterotoxin which is cross-reactive with cholera toxin (8, 41, 47); and at least two hemolysins (34), one or both of which may be cytotoxic or enterotoxic or both (1, 12, 28). None of these virulence factors have been clearly associated with *Aeromonas* strains in human diarrheal disease. In human volunteer challenges, mild-to-moderate diarrhea could be induced in only 2 of 57 human volunteers with doses of up to 5×10^{10} organisms of five *Aeromonas* strains, each of which had demonstrated several of the known toxin activities (35).

If these toxins are virulence factors in *Aeromonas*-associated diarrhea, the *A. hydrophila* and *A. sobria* phenotypes are probably the only clinically important strains. Several investigators have reported a positive correlation between these species (or similar metabolic phenotypes) and the production of exotoxins (5, 6, 12, 25). This species-specific correlation with disease has at times led to confusion in the interpretation of survey results. Figura and co-workers (20), who were unable to find a significant association between *Aeromonas* infection and diarrhea, reported that only 38% of *Aeromonas* isolates from patients with enteritis produced enterotoxin, cytotoxin, or hemolysin. However, 69% of the strains isolated were *A. caviae*, a phenotype which normally does not produce these toxins. Among the 11 *A. hydrophila* or *A. sobria* strains isolated, 82% produced cytotoxin, enterotoxin, or both. The remaining 18% produced hemolysin only. In contrast, only one of the 26 *A. caviae* isolates (4%) produced cytotoxin.

Although animal assays are currently used for virulence testing of *Aeromonas* isolates, none are practical for studying pathogenic mechanisms of diarrhea or the mucosal

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invasion which may occur in human intestinal infections. Mouse, rat, and rabbit intestinal assays have been used by various investigators to test for enterotoxin production by *Aeromonas* strains (7, 30, 33, 35, 39, 45). Extraintestinal assays, such as intraperitoneal inoculation of mice, have been reported as useful screening tests for virulence (14, 25, 27). *Aeromonas* isolates tested for invasiveness by the Sereny method (46) have been uniformly negative (28, 35, 39), although an *in vitro* tissue culture assay has shown some strains to be invasive to HEp-2 epithelial cells (19, 31).

We investigated the usefulness of the removable intestinal tie adult rabbit diarrhea (RITARD) model (49) as a possible screening procedure for detecting virulent *Aeromonas* isolates and as a tool for studying mechanisms of pathogenesis. Results indicate that a modified version of this animal model is useful for studies of pathogenesis because some isolates produce a bacteremia in <12 h followed by death in <24 h, often accompanied by moderate amounts of diarrhea, fluid accumulation in the small bowel, and lesions of gut mucosal epithelium. However, because the results correlate favorably with other, simpler methods, the modified RITARD procedure is not recommended for routine virulence screening of *Aeromonas* isolates.

(These data were presented in part at the 25th Annual U.S.-Japan Cooperative Medical Science Program, Tokyo, Japan, 14 to 16 November 1988 [G. Pazzaglia, A. L. Bourgeois, R. B. Sack, J. Froehlich, and J. Eckstein, *Aeromonas*: studies of invasiveness in the modified removable intestinal tie adult rabbit diarrhea (RITARD) model].)

MATERIALS AND METHODS

Bacterial isolates. *Aeromonas* strains L172, 51, and 55 were isolated from stools of diarrheic Sudanese adults. Strains 98, 99, 100, 102, and 105 were stool isolates from diarrheic Peruvian children. Strains 207 and 3647 were human diarrheic stool isolates from Bangladesh and Australia, respectively. Strain 6Y was from the stool of an apparently healthy Thai, and strain 59 was isolated from a water well in a rural village in Sudan. Six of the human diarrhea isolates (strains L172, 98, 105, 99, 102, 51, and 100) were reported to be from persons with dysentery for whom no other enteropathogen was detected. Strains 3647 and 6Y have been previously characterized and used in human volunteer challenge trials (35).

Isolates were subcultured from frozen stocks (-70°C) onto MacConkey agar and ampicillin sheep blood agar supplemented with $10\ \mu\text{g}$ of ampicillin per ml (ASBA) (42). Pure cultures were confirmed as containing *Aeromonas* strains by using conventional test procedures after overnight incubation at 35°C . All strains had reactions typical for the genus. For metabolic phenotyping, the strains were additionally characterized according to the taxonomic scheme of Popoff and Veron (40) at 28°C . Species identifications were based on Voges-Proskauer tests, H_2S from cysteine, gas from glucose, esculin hydrolysis, fermentation of salicin, utilization of arginine, and utilization of arabinose. Cytotoxin production was measured by using cell-free culture supernatants in the Y-1 adrenal cell assay (12).

Mouse pathogenicity. Mouse virulence testing of *Aeromonas* strains was by intraperitoneal challenges of 10- to 12-week-old mice, using methods described by Daily et al. (14). For each *Aeromonas* strain, 10 outbred ICR mice (21 to 25 g) (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) were each inoculated with 1×10^7 to 3×10^7 CFU. By using the criteria of Daily, strains having 50%

lethal dose (LD_{50}) values of $\leq 10^7$ CFU were considered highly virulent; strains having LD_{50} values of $>10^7$ CFU were considered to be of low virulence (48 h postinoculation).

Determination of autoagglutination phenotypes. *Aeromonas* strains were tested for their autoagglutination and precipitation properties by using the procedure described by Janda et al. (27). After individual strains were grown in filtered brain heart infusion broth (18-h static culture at 35°C), they were assigned a phenotype on the basis of their abilities to spontaneously pellet (SP^+) or to form a precipitate after being boiled for 1 h (PAB^+). Strain TF7 (kindly provided by T. J. Trust, University of Victoria, British Columbia, Canada) served as the positive control for both autoagglutination and precipitation testing.

Slide agglutination. *Aeromonas* strains were tested for agglutination in a 1:10 dilution of rabbit O antiserum prepared by the method of Leblanc et al. (32) against boiled cells (90 min at 100°C) of strain TF7. This *Aeromonas* strain has previously been identified as a member of serogroup O:11 (16).

Agglutination tests with TF7 antiserum resulted in strong 4^+ reactions against the homologous strain, as well as against *A. hydrophila* LL1, another well-characterized member of the O:11 serogroup (37).

Study strains were tested by mixing 1 drop each of boiled-cell suspension and TF7 antiserum on agglutination glass slides and rocking them manually for 2 min. Any visible agglutination was considered a positive reaction. Boiled cells of TF7 and LL1 were included in each test as positive controls. Suspensions of test strains were also evaluated in phosphate-buffered saline (PBS) (pH 7.0) and normal rabbit serum, which served as negative controls for the detection of autoagglutination. The possibility of nonspecific agglutination was minimized by testing study strains with O antiserum (1:8) prepared against strain L172. The L172 antiserum agglutinated 4 of 12 study strains but did not react with strains agglutinating in the TF7 or LL1 antisera.

Preparation of inocula. Three to five colonies were picked from overnight pure cultures and inoculated into 5 ml of brain heart infusion broth and placed on a roller drum (200 rpm at 35°C) for 12 h. Broth cultures were poured into 500-ml flasks containing 150 ml of Casamino Acids-yeast extract (CAYE) broth, plugged with cotton, and placed in a shaking water bath (200 rpm at 35°C) for 4 to 5 h. CAYE broth was prepared by adding 30 g of Casamino Acids, 6 g of yeast extract, 0.5 g of K_2HPO_4 , and 2 g of dextrose to 1 liter of distilled deionized H_2O and then was adjusted to pH 7.0 with concentrated NaOH. CAYE cultures were centrifuged, supernatants were removed, and the bacteria were suspended in PBS for a final concentration of approximately 10^9 viable organisms per ml. Syringes containing 10 ml of the whole-cell suspension were kept on ice until inoculation of the rabbit intestine (<1 h). Culture supernatants were not tested. Viable counts for inocula of bacteria were determined by quantitative plate counts by using ASBA and ranged from 1.0×10^9 to 7.0×10^9 CFU/ml.

Modified RITARD procedure. To ensure that rabbits were free of *Aeromonas* infection, rectal swabs taken from rabbits the day before surgery were swirled in 0.5 ml of PBS, and 0.1 ml of this suspension was directly plated on MacConkey agar and ASBA. Plates were visually inspected after 18 to 24 h of incubation for lactose-negative colonies on the MacConkey agar or suspect *Aeromonas* colonies on the ASBA. Suspect colonies were oxidase tested and then further characterized when indicated.

The modified RITARD procedure was performed as previously described (44). After food was withheld for 24 h, New Zealand White rabbits (1,250 to 1,650 g; 7 to 9 weeks of age; 3 to 4 weeks postweaning) were anesthetized with Innovar Vet intramuscularly. After injection of lidocaine along the abdominal midline, an incision was made along the midline and the cecum was permanently tied off. Sterile PBS was injected into the jejunum close to the ligament of Treitz and gently pushed through the small bowel past the mesoappendix. A temporary tie was placed around the ileum 2 to 4 cm proximal to the mesoappendix. The bacterial suspension was then injected at the same site as the PBS wash. The abdominal incision was partially sutured, allowing the ends of the temporary tie to protrude through a small opening at the distal end of the incision. After 1.5 h, the rabbit was given tincture of opium intraperitoneally. Two hours postchallenge, the temporary tie was removed and the small opening remaining in the abdominal incision was completely sutured. Challenged rabbits were returned to cages and fed and watered immediately. Rabbits were monitored hourly for the first 24 h postchallenge and every 3 to 4 h for the next 24 h. Surviving rabbits were monitored two to four times daily on days 3 to 7 and were usually sacrificed on day 7 postchallenge. Diarrheic stools were examined for frank or occult blood (Hemocult; Smith Kline Diagnostics, Inc., Sunnyvale, Calif.).

Blood cultures. Blood was aseptically drawn by syringe from the ear veins of surviving rabbits at 12 h postchallenge. Postmortem examinations of rabbits which died before 12 h were performed within 15 min, and heart blood was aseptically removed by syringe immediately after the chest cavity was opened. A portion of the blood specimen (0.1 ml) was directly plated onto ASBA and incubated for 24 h at 28°C; 0.1 to 0.5 ml was inoculated into brain heart infusion broth for 6 to 8 h of incubation at 35°C and then was subcultured onto ASBA for 24 h at 28°C.

Postmortem examination. Complete postmortem examinations, including preservation of organ tissues for histological examination, were performed on 24 *Aeromonas*-challenged rabbits within 15 min of death and on 6 surviving rabbits sacrificed at 18 to 30 h postchallenge. The six animals sacrificed at 18 to 30 h were typical of surviving rabbits undergoing uncomplicated recovery from challenge with nonlethal *Aeromonas* strains. (For analysis, the six sacrificed animals were considered survivors because they were challenged with nonlethal strains [55, 100, and 3647], showed no evidence of infection or illness, and were bacteremia-free at the time of sacrifice.) Two sham-infected rabbits (sacrificed 13 to 18 h postsurgery) and two unchallenged rabbits (all in the study weight range) were used as surgical controls and provided additional comparative tissues for histological studies.

At necropsy, the large and small intestines were tied off and removed; the fluid volume of the small intestine was measured and tested for the presence of occult blood. Portions of the jejunum, ileum, and proximal large intestine were removed and sectioned longitudinally. One half of each specimen was immediately placed in PBS (pH 7.0) on ice and reserved for colonization assays, which were performed within 4 h of death. The other half of each specimen was placed into 10% buffered Formalin and processed for histological examination. Tissue samples were also taken from the duodenum, cecum, colon, liver, spleen, kidneys, heart, and lung for histological study.

Fixed tissues were embedded in paraffin and cut into sections. Representative sections (6.0 μ m thick) were

stained with hematoxylin and eosin and then microscopically examined. Sections showing evidence of bacterial infection were additionally stained and examined by the methods of Brown and Brenn (2) and Brown and Hopps (3).

Colonization assay. Colonization assays of postmortem intestinal tissues were performed as previously described (44). A small portion (0.3 to 0.5 g) of tissue section was washed by swirling it 10 times in PBS. The section was weighed and placed in a homogenizing vessel with 2 ml of cold PBS. Each section was then homogenized for 1 to 3 min, and the homogenate was serially diluted 10^{-1} to 10^{-6} . Dilutions of homogenates were plated on MacConkey agar and ASBA and then incubated at 28°C for 18 to 24 h.

Statistical analysis. Statistical analysis was performed by using EPISTAT and MICROSTAT software. For tests of proportions, chi-square probabilities, when $df = 1$, included Yates's correction for continuity; Fisher's exact test was used for tests of proportions when expected cell values were <5 . Student's unpaired t test was used for testing differences between means. Tests of relationships between two variables were done by the least-squares method of linear regression.

RESULTS

Characterization of isolates. On the basis of the criteria of Popoff and Veron, four strains (6Y, 51, 59, and 102) were identifiable as *A. sobria*; four were of the *A. hydrophila* phenotype, except they did not utilize arabinose (strains L172, 98, 99, and 105); three strains were *A. caviae*, except they did not utilize arginine (strains 55, 100, and 207); and one strain could not be grouped with any degree of certainty (strain 3647).

Results from testing for hemolysis of rabbit, bovine, or sheep erythrocytes, pathogenicity in mice, O-antigen agglutination, SP/PAB phenotypes, and cytotoxicity are summarized in Table 1 for comparison with mortality outcomes in rabbits challenged by the RITARD method.

Weight limitation for challenged animals. With *Aeromonas* strain 59, a preliminary investigation (with 16 rabbits) of the RITARD procedure as a whole-animal model for evaluating *Aeromonas* strains indicated that only animals weighing 1,650 g or less provided useful and reproducible results. Use of low-weight rabbits (1,470 to 1,650 g) resulted in eight of eight (100%) deaths, compared with one of five (20%) deaths in intermediate-weight rabbits (1,700 to 1,760 g) and none of three (0%) deaths in larger rabbits (1,950 to 2,270 g). Death among low-weight (younger) rabbits (eight of eight) was significantly more frequent than death among heavier (older) rabbits (one of eight) ($P = 0.0007$). Among surviving rabbits, four of four intermediate-weight rabbits developed diarrhea, whereas none of three of the large rabbits developed diarrhea ($P = 0.029$). The likelihood of death was independent of variation in inoculum size, even after challenge doses were statistically adjusted to account for differences between weights of individual rabbits. These results were interpreted as an indication of a weight-age association for susceptibility to disease from *Aeromonas* challenge. Therefore, the results in the current report are limited to animals weighing 1,650 g or less, with death as the principal outcome variable.

RITARD challenges. The results of challenges in 64 rabbits (weight range, 1,270 to 1,650 g) using 12 strains of *Aeromonas* are summarized in Table 2. Death, diarrhea, and bacteremia were all strongly strain dependent ($P = 0.00002$, 0.005, and 0.00001, respectively). Overall, there was no significant difference in the prechallenge weights of rabbits dying ($\bar{x} =$

TABLE 1. Mortality, diarrhea, and phenotypic characteristics associated with 12 *Aeromonas* strains used to challenge 64 rabbits by the RITARD procedure

| Strain | Metabolic phenotype ^a | Source ^b | % Mortality by RITARD | % Causing diarrhea only by RITARD | Mouse LD ₅₀ of ≤10 ⁷ CFU | Y-1 cell cytotoxicity | SP/PAB, phenotype ^c | Serogroup O:11 | Beta-hemolysis on: | | |
|--------|----------------------------------|---------------------|-----------------------|-----------------------------------|--|-----------------------|--------------------------------|----------------|--------------------|--------------|--------------|
| | | | | | | | | | Sheep blood | Bovine blood | Rabbit blood |
| L172 | AH | DS | 100 | 0 | + | + | -/+ | - | + | + | + |
| 6Y | AS | HS | 100 | 0 | + | + | -/+ | + | + | + | + |
| 98 | AH | DS | 100 | 0 | + | + | -/+ | + | + | + | + |
| 105 | AH | DS | 100 | 0 | - | + | -/- | - | + | + | + |
| 59 | AS | WW | 80 | 13 | + | + | -/- | - | + | + | + |
| 99 | AH | DS | 75 | 25 | + | + | -/+ | + | + | + | + |
| 102 | AS | DS | 50 | 50 | - | + | +/+ | + | + | + | + |
| 51 | AS | DS | 0 | 67 | - | + | +/+ | + | + | + | + |
| 3647 | NT | DS | 0 | 25 | - | + | -/- | - | + | + | + |
| 207 | AC | DS | 14 | 0 | - | - | -/- | - | - | - | + |
| 100 | AC | DS | 0 | 50 | - | - | -/- | - | - | - | - |
| 55 | AC | DS | 0 | 0 | - | - | -/- | - | - | - | + |

^a AH, *A. hydrophila*; AS, *A. sobria*; AC, *A. caviae*; NT, not typeable.

^b DS, Diarrheic human stool; HS, healthy human stool; WW, well water.

^c SP, Spontaneous pelleting; PAB, precipitation after boiling (see text).

1,454 g) compared with the weights of those that survived (\bar{x} = 1,470 g) and no association between weight and the likelihood of death within the reported weight range. Likelihood of death was also independent of the size of the inoculum within the range used for challenges. None of the rabbits were colonized with *Aeromonas* before challenge.

Diarrhea was considered to be any unformed stool and was usually moderate in volume, liquid but not watery, and transient (<24 h) for survivors. For all challenged rabbits, 23 of 37 (62%) rabbits which died developed diarrhea before death, and 11 of 27 (41%) surviving rabbits developed diarrhea ($P = 0.149$); the mean time to onset of diarrhea for rabbits which died was 16.8 h compared with 39.3 h for surviving rabbits ($P = 0.002$). Frank blood in feces was seen in 1 of 34 (3%) rabbits which developed diarrhea. Occult blood was detected in 9 of 20 (45%) rabbits with diarrhea which died (three rabbits were not tested) compared with 1 of 8 (13%) of surviving rabbits with diarrhea (three rabbits were not tested) ($P = 0.116$).

Moribund rabbits were sacrificed and recorded as deaths due to challenge. Of the 12 strains, 8 produced at least one death in challenged rabbits (Table 2), usually with accompa-

nying diarrhea if the rabbit survived for more than 12 h. Of the four nonlethal strains, only one failed to produce any sign of illness in challenged rabbits (strain 55). The time to death postchallenge ranged from 8 to 72 h, with 36 of 37 (97%) rabbits dying in 24 h or less (median = 12.0 h). *Aeromonas* bacteremia was detected in 36 of 37 (97%) animals which died, but in only 2 of 27 (7%) survivors ($P < 0.00001$).

Postmortem gross examination. Among those rabbits which died ($n = 37$), the fluid in the small bowel was moderate in volume (\bar{x} volume = 48 ml), of watery consistency, but slightly more viscous than that seen in a control rabbit challenged with enterotoxigenic *Escherichia coli* (volume = 125 ml). (Fluid volumes in the small bowels of two sham-infected rabbits challenged with PBS and sacrificed at 13 and 18 h measured 28 and 32 ml, respectively.) Visible or occult blood was detected in the small bowel contents of 24 of 28 (86%) animals that died and 2 of 6 (33%) surviving animals tested ($P = 0.018$).

The most common gross pathological findings at necropsy were necrosis and hemorrhage of the ileum, which were present in 22 of 24 (92%) animals that died and 0 of 6 of the surviving animals necropsied at 18 to 30 h postchallenge ($P = 0.00005$). Pinpoint foci of discoloration were grossly apparent throughout the liver in 10 of 24 (42%) animals which died and 0 of 6 survivors ($P = 0.065$). Similar lesions were seen in the spleens of 5 of 10 (50%) of the rabbits which had gross liver changes.

Histological examination. Abnormal histology was present in 22 of 24 (92%) rabbits which died after challenge. Gastrointestinal lesions varied from moderate focal enteritis in rabbits challenged with strains 59 (five of six) and 105 (two of three) to severe multifocal necrosis and hemorrhage of the ileal mucosa in rabbits infected with strains L172 (three of three), 6Y (three of three), 98 (two of two), 99 (three of three), 102 (three of three), and 207 (one of one). Representative examples of these lesions are shown in Fig. 1.

Bacterial invasion and necrosis of the underlying lymphoid tissues (Peyer's patches) were frequently observed (in 33 to 100% of rabbits challenged with each strain) in rabbits exhibiting severe ileitis after challenge with strains L172, 6Y, 98, and 102 (Fig. 1C and 2C). Disseminated foci of bacteria were often present (in 33 to 100% of rabbits chal-

TABLE 2. Death, diarrhea, and bacteremia in 64 rabbits challenged with 12 strains of *Aeromonas* spp. by the RITARD procedure

| Strain | No. of rabbits with outcome(s) indicated/no. tested (%) | | | \bar{x} No. of h to death | No. of rabbits that died/no. with bacteremia (%) |
|--------|---|--------------------|---------------|-----------------------------|--|
| | Death | Diarrhea and death | Diarrhea only | | |
| L172 | 5/5 (100) | 3/5 (60) | 0/5 (0) | 10.2 | 5/5 (100) |
| 6Y | 5/5 (100) | 5/5 (100) | 0/5 (0) | 12.4 | 5/5 (100) |
| 98 | 3/3 (100) | 3/3 (100) | 0/3 (0) | 34.0 | 3/3 (100) |
| 105 | 5/5 (100) | 2/5 (40) | 0/5 (0) | 11.5 | 5/5 (100) |
| 59 | 12/15 (80) | 5/15 (33) | 2/15 (13) | 12.9 | 11/12 (92) |
| 99 | 3/4 (75) | 2/4 (50) | 1/4 (25) | 11.7 | 2/3 (67) |
| 102 | 3/6 (50) | 3/6 (50) | 3/6 (50) | 14.3 | 3/3 (100) |
| 51 | 0/3 (0) | 0/3 (0) | 2/3 (67) | | |
| 3647 | 0/4 (0) | 0/4 (0) | 1/4 (25) | | |
| 207 | 1/7 (14) | 0/7 (0) | 0/7 (0) | 13.0 | 1/1 (100) |
| 100 | 0/4 (0) | 0/4 (0) | 2/4 (50) | | |
| 55 | 0/3 (0) | 0/3 (0) | 0/3 (0) | | |

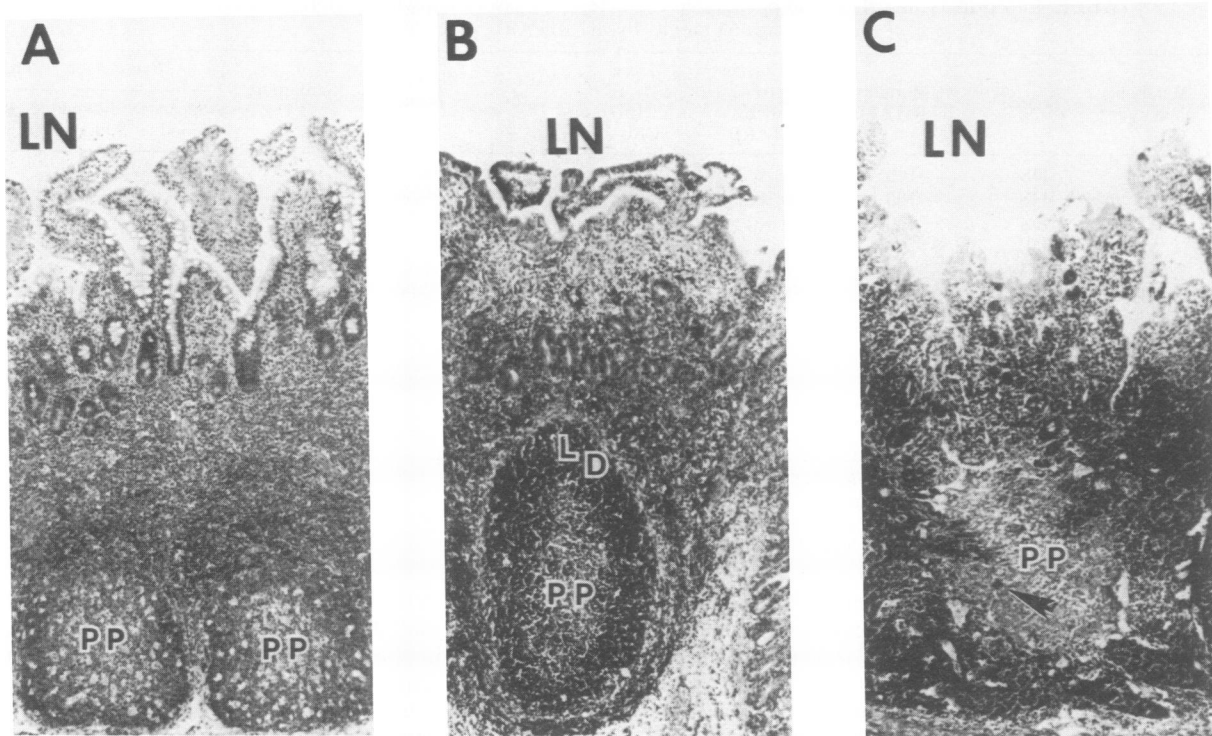


FIG. 1. Tissue cross-sections of distal ileum 14 to 18 h after sham infection (A) (normal tissue) or infection with *Aeromonas* strain 59 (B) (moderate enteritis) or 6Y (C) (extensive epithelial destruction and lymphocytic degeneration). Numerous bacteria are present in lymphoid tissues (arrow, panel C). LN, Lumen; PP, Peyer's patch; LD, lymphocytic degeneration. Magnification, $\times 12.5$.

lenged with each strain) in the livers of these animals at death. Splenic lesions were found only in animals challenged with strains 6Y (two of three) and 102 (three of three). Among those animals which died, only 2 of 22 (9%) showed signs of epithelial damage of the jejunum.

Liver and splenic lesions were characterized by intravascular bacterial colonies surrounded by a rim of necrotic hepatocytes or splenocytes, with minimal infiltration of heterophils (rabbit neutrophils) (Fig. 2A). Stained tissue sections demonstrated that the bacterial colonies in these lesions were gram-negative rods, similar in morphology to the challenge *Aeromonas* strains (Fig. 2B).

Tissues from three of six surviving rabbits sacrificed at 12 to 30 h postchallenge were histologically examined; there were no observable pathological changes in the livers, spleens, or Peyer's patches, although one rabbit demonstrated mild ileal changes. No lesions or other pathological changes were found in the sham or normal control rabbits.

Colonization. Although rectal swabs taken from challenged rabbits were usually negative for *Aeromonas* strains at 12 h postchallenge (6 of 21 tested positive), the majority (15 of 17) were positive by 24 h, and by 48 h, *Aeromonas* strains could be recovered from all rabbits (17 of 17) surviving to that time. Most surviving animals (16 of 17) remained stool culture positive during the 7 days of observation after challenge. Intestinal colonization after infection was not permanent; *Aeromonas* strains could not be detected in the stools of four of five surviving rabbits by day 10, even though *Aeromonas* isolates were recovered on day 6 or 7 from these same animals.

Results of colonization assays performed on 17 rabbits that died and 1 surviving rabbit (Table 3) indicated that for the challenged rabbits which died, the distal small bowel

(ileum) was most heavily colonized. The surviving rabbit (sacrificed on day 7 postchallenge) was heavily colonized but showed no measurable difference in the numbers of bacteria colonizing various tissues of the bowel. There were no significant differences between test strains in their abilities to colonize the intestines of challenged rabbits.

DISCUSSION

Aeromonas strains can be grouped into different virulence categories on the basis of their effects on rabbits challenged by the RITARD procedure. A survey of 12 *Aeromonas* strains has shown that some isolates can cause diarrhea with invasion of the mucosa followed by bacteremia and death. If the challenge strain is nonlethal or if the animal survives for longer than 12 h, colonization is often accompanied by transient diarrhea. For rabbits in the weight range of 1,200 to 1,650 g, the relative virulence of *Aeromonas* strains may be reproducibly determined by using death, death with diarrhea, and diarrhea only as outcome variables.

The data obtained with the RITARD challenges were similar to those obtained previously for mice (14, 25, 27), except that no diarrhea was produced in the murine model. In these studies, *Aeromonas* strains were grouped into high-virulence ($LD_{50}, \leq 10^7$ CFU) and low-virulence organisms on the basis of mortality obtained after intraperitoneal challenges. Of the seven most virulent strains tested in the present study, six had LD_{50} values of $\leq 10^7$ CFU in mice inoculated intraperitoneally, which is the suggested cutoff for virulence in this test. All of the five less virulent strains demonstrated LD_{50} values of $> 10^7$ CFU ($P = 0.008$). Five of the seven most virulent strains were PAB⁺, which has been reported as the phenotype frequently associated with inva-

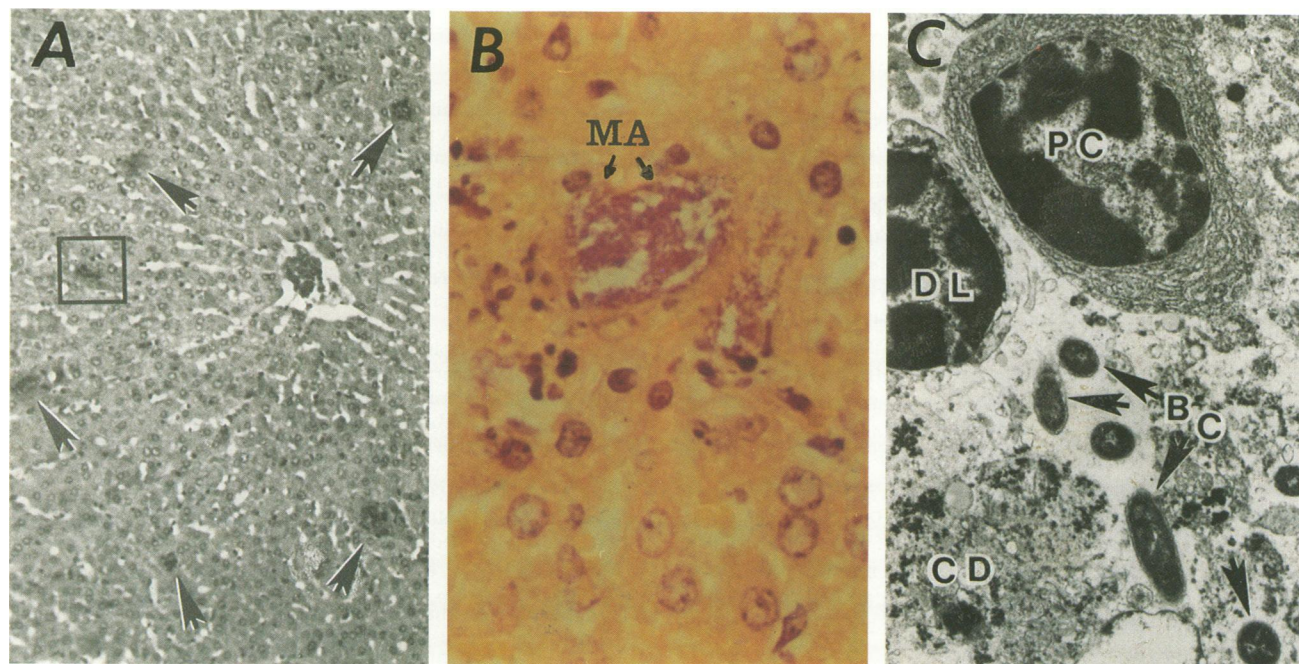


FIG. 2. (A and B) Tissue cross section of liver 14 h postchallenge with *Aeromonas* strain 6Y. Magnifications, $\times 10$ (A) and $\times 200$ (B). (C) Transmission electron micrograph of a necrotic Peyer's patch from the same animal as that used for panels A and B. Magnification, $\times 30,000$. Microabscesses (arrows, panel A) were present throughout the liver and were characterized by microcolonies of bacteria surrounded by necrotic hepatocytes and infiltrates of neutrophils (panel B and box, panel A). Clusters of bacteria were evident within necrotic Peyer's patches (C) and liver (not shown). In panel B (Brown and Hopps stain), gram-negative bacteria are red and tissue cells are yellow. MA, Microabscesses; BC, bacterial cells; DL, degenerated lymphocyte; CD, cellular debris; PC, plasma cell.

siveness in humans (27); only one of five of the less virulent strains was PAB⁺.

In the present study, there was a strong correlation between certain phenotypes and virulence in rabbits. On the basis of the results seen in Table 1, isolates which conform to the metabolic phenotypes of *A. hydrophila* or *A. sobria*, are hemolytic on sheep or bovine blood, and are cytotoxic to Y-1 adrenal cells have a high probability for virulence in rabbits (seven of eight strains). This is consistent with observations by other investigators who compared pre-

sumed virulent human isolates with isolates from healthy persons (6, 39). We are further investigating the applicability of these virulence markers as predictors of human disease.

The demonstrated ability of some *Aeromonas* strains to cause sepsis and death, when considered with previous reports of *Aeromonas* bacteremia in humans and other species, supports the notion that invasiveness may be an important virulence characteristic for *Aeromonas*-associated dysentery in humans. It is unknown at present whether the extensive damage to the rabbit intestinal mucosa seen after challenge with some strains is a prerequisite for invasion or whether invasion and tissue destruction are independent events.

Postmortem histological examinations of intestinal tissues from some rabbits (100% of rabbits which were histologically examined after challenge with strain L172, 6Y, 98, 99, or 102) demonstrated gross signs of epithelial invasion, similar to those seen with *Shigella* species and other bacteria which are known to cause extensive destruction of the epithelial layer. Since other rabbits challenged with lethal strains (strains 59 and 105) demonstrated little or no epithelial damage to the ileum (Fig. 1B), it is probable that some invasive *Aeromonas* strains also have more subtle routes of entry which may result in little or no obvious tissue deformity. These less destructive routes may include entry via the Peyer's patches of the ileum and lymphatics or passage through the mucosa via other mechanisms.

The *in vitro* colonization assays performed on postmortem tissues support histological observations that the site of epithelial invasion is the ileum. Sections taken from individual rabbits show that preferential colonization by *Aeromonas* strains occurs a substantial distance distal to the site of inoculation. The colonization factors related to this phenom-

TABLE 3. Colonization results for 16 rabbits that died and 1 rabbit that survived after RITARD challenge with 9 *Aeromonas* isolates

| Strain | Vol (ml) of small bowel | CFU/g of tissue from: | | | % of rabbits tested with blood present in: | |
|--------------------|-------------------------|-----------------------|--------------------|----------------------|--|-------|
| | | Proximal small bowel | Distal small bowel | Proximal large bowel | Bowel fluid | Feces |
| L172 | 63 | 1.5×10^6 | 6.0×10^7 | 4.5×10^6 | 100 | 0 |
| 6Y | 75 | 4.5×10^5 | 6.0×10^6 | 4.5×10^5 | 80 | 0 |
| 98 | 55 | 7.0×10^5 | 1.6×10^7 | 5.4×10^6 | 100 | 0 |
| 105 | 58 | 1.5×10^7 | 5.0×10^8 | 6.5×10^6 | 100 | 100 |
| 59 | 28 | 1.8×10^8 | 3.5×10^8 | 8.0×10^7 | 20 | 20 |
| 99 ^a | 55 | 5.0×10^5 | 7.0×10^6 | 1.0×10^5 | 100 | 100 |
| 102 | 40 | 1.8×10^7 | 2.9×10^8 | 1.4×10^8 | 100 | 0 |
| 207 ^a | 28 | 1.0×10^7 | 9.0×10^7 | 2.0×10^7 | ND ^b | ND |
| 102 ^{a,c} | ND | 9.0×10^9 | 9.0×10^9 | 9.0×10^9 | 100 | 100 |

^a Single observation; other observations based on two or more challenged rabbits.

^b ND, Not determined.

^c Surviving rabbit sacrificed on day 7 postchallenge.

enon, such as differences in epithelial binding sites, have yet to be studied.

An age-related susceptibility of rabbits to mortality from infection by *Aeromonas* was observed. Adult rabbits are generally not susceptible to whole-animal challenge by invasive or enterotoxigenic bacteria. However, infant rabbits have been successfully used for oral, intragastric, and in-traintestinal challenge experiments with enterotoxigenic strains of *Vibrio cholerae*, *E. coli*, and *Bacteroides fragilis* (36, 43). Such models typically use rabbits <1 week old, whose susceptibility to diarrhea and invasion is probably much greater than that of the 7- to 9-week-old rabbits used in the current study. The effect of age on susceptibility to *V. cholerae* infection was investigated by Dutta and Habbu (17) by using a standard inoculum. They found that in-traintestinal challenge with vibrios produced fatal diarrhea in 100% of 10-day-old rabbits, 100% of 16-day-old rabbits, 50% of 21-day-old rabbits, and 0% of rabbits that were more than 30 days old. The age-related susceptibility of rabbits to challenge with *Aeromonas* strains may be due to the maturation of the rabbit mucosal and parenteral immune systems. Changes related to factors responsible for nonspecific systemic resistance to infection or increased colonization resistance in the intestine could also account for our findings.

Voluminous diarrhea was not the cause of death in challenged rabbits, but the diarrhea produced in some animals and postmortem gut fluid accumulation suggest that some *Aeromonas* strains are diarrheagenic. Many of the features associated with intestinal effects of *Aeromonas* strains in the RITARD model are consistent with those seen in human disease. It may be advantageous in the future to take steps to reduce the gram-negative shock aspects of the disease in our model so that a clearer picture of intestinal events may be obtained.

In the current study, strains L172, 6Y, 98, 105, 59, 99, and 102 appear to be the most virulent of the isolates tested; all produced mortality in 50% or more of rabbits challenged. Strain 6Y (isolated from a stool sample from a healthy human) was previously used in a human challenge experiment (36). Although 6Y was one of the most virulent strains we tested in rabbits, it produced mild diarrhea in only 1 of 20 humans challenged. However, strain 6Y was more frequently recovered from volunteers (11 of 20) compared with all other isolates used for human challenge. The high virulence of this isolate in animals, considered with its ability to survive passage and to colonize humans, suggests this strain is capable of causing human enteric disease under appropriate circumstances. Further human volunteer challenges, using various inoculum sizes and various challenge conditions, may demonstrate that strains shown to be diarrheagenic in rabbits can cause human diarrhea.

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