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Incidence and Toxigenicity of *Aeromonas* Species in Retail Poultry, Beef and Pork

ANITA J. G. OKREND*, BONNIE E. ROSE and BARBARA BENNETT

Food Safety and Inspection Service, U.S. Department of Agriculture, Building 322, ARC-East, Beltsville, Maryland 20705

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ABSTRACT

Five enrichment broths and five selective and differential plating media were tested for efficiency of isolation of *Aeromonas* spp. from chicken, beef and pork. An overnight incubation of sample in Trypticase soy broth containing 10 μg of ampicillin/ml which was spread on starch ampicillin agar or on MacConkey mannitol ampicillin agar, gave the best results. A small survey was conducted on 10 samples each of chicken thigh-meat, ground beef, and pork sausage or ground unseasoned pork purchased from local food stores. Aeromonads were found in all of the samples in numbers ranging from 4.44×10^{-2}->4.44×10^{3}/g except for two of the pork products from which the organisms could not be isolated. Fifty-eight isolates from this survey were tested for hemolysin production and cytotoxin production; 36 isolates were tested for production of cholera-like toxin. Cytotoxin, as detected by mouse adrenyl Y1 cells and Chinese hamster ovary cells, was produced by 92.8% of the *Aeromonas hydrophila* isolates, by 84.6% of the *Aeromonas sobria* isolates and by 17.6% of the *Aeromonas caviae* isolates. Hemolysin production paralleled cytotoxin production in *A. hydrophila* and *A. caviae*. Of the *A. sobria* isolates, 69.2% were hemolysin producers. None of the isolates tested produced cholera-like toxin. It is not known whether the presence of cytotoxin- and hemolysin-producing *Aeromonas* species in retail meat and poultry has any public health significance, since to date there have been no reported outbreaks of *Aeromonas*-caused gastroenteritis traced to meat or poultry.

Approximately 35% of the known or suspected food poisoning outbreaks which occur in the United States each year are of unknown etiology *(3)*. It has been assumed that a large portion of these unclassified food poisoning cases are caused by species not customarily looked for during the routine investigation of an outbreak. In the last 10 years, there has been increased interest in identifying the causes of human gastroenteritis and this has expanded the list of bacterial species that are considered enteric pathogens. Two additional species, *Aeromonas hydrophila* and *Aeromonas sobria*, appear to verge on being considered major causes of human gastroenteritis, and thus could potentially be foodborne pathogens of significance. While most evidence to date strongly suggests that at least some strains of these species are enteric pathogens *(2,7,10,15,22)* and that water supplies are their source *(5,21)*, specific information on the possibility that foods also may be significant in the transmission of enterotoxigenic *Aeromonas* is unavailable. Such information would be critical for assessing the safety of food products such as meat and poultry.

In the present study, we evaluated methods for isolation of *Aeromonas* from meat and poultry products and conducted a small survey of retail beef, poultry and pork for the presence of the organism. These *Aeromonas* isolates were tested for ability to produce cytotoxin, hemolysin and cholera-like toxin.

MATERIALS AND METHODS

Evaluation of isolation and identification methods

Microorganisms. Five *Aeromonas* cultures (three clinical and two food isolates) were obtained from Dr. S. Palumbo at the Eastern Regional Research Center, Wyndmoor, PA. These isolates, three *A. hydrophila* and two *A. caviae* strains, were used in the development and evaluation of the media.

The cultures were grown in Trypticase soy broth (TSB; BBL Microbiology Systems) at 35°C overnight and centrifuged at 1075 × *g* for 30 min in a Sorvall RC-2 centrifuge, the supernatant liquid was decanted, and the pellicle resuspended in Butterfield's phosphate diluent (BPD; *23*). This suspension was further diluted in BPD to attain 50% transmittance (T) in a Bausch and Lomb Spectronic 20 at 400 nm. The 50% T suspension was diluted in BPD 9-ml dilution blanks and the 10^{-5}, 10^{-6} and 10^{-7} dilutions, which gave countable plates, were plated as described below.

Meat samples. Raw meat products used for the initial evaluation of the isolation media were purchased at local retail markets in regular consumer packages. The foods tested included poultry, ground beef, ground pork, chicken livers and gizzards, and beef liver. The products were sampled either in the fresh or frozen state or both. Sampling for direct plating was done by aseptically transferring 25 g from the retail package to a sterile pint Mason jar, adding 225 ml of BPD and blending in an Osterizer blender for 2 min. Serial dilutions were made with BPD and plated on the various test media. When products were analyzed by enrichment methods, 25-g samples were asep-

tically transferred to 225 ml of enrichment broth and blended as described above.

Isolation media evaluation. Five selective and differential plating media were evaluated for isolation of *Aeromonas* using both pure cultures and meat samples. The media included: Columbia blood agar (GIBCO) containing 5% sheep blood and 10 μg of ampicillin/ml (Sigma) (CBA), peptone beef extract glycogen (PBG) agar *(14),* starch ampicillin (SA) agar *(17),* MacConkey xylose ampicillin (MXA) agar, and MacConkey mannitol ampicillin (MMA) agar. MXA and MMA agars, formulated in this laboratory, contained MacConkey agar base plus either 1% xylose or 1% mannitol and 30 μg of ampicillin/ml. All of the agar media except PBG agar were either streaked with a needle or surface-spread with a bent glass rod and incubated at 28°C for 18-24 h. PBG agar is a pour-plate medium which, after it has gelled and the agar surface dried, is overlaid with 10-15 ml of sterile nonnutrient agar. PBG agar plates were incubated at 37°C for 24 h. Determination of percent recovery of pure cultures of *Aeromonas* was based on differential counts on plate count agar (PCA; Difco) and the tested isolation medium. *Aeromonas* isolation rates from the meat samples were determined by comparison of the various isolation media.

Five enrichment broths were also evaluated for their efficiency in isolation of *Aeromonas:* alkaline peptone water, TSB, TSB with 10 μg of ampicillin/ml (TSBA), TSBA with 2% extra NaCl, and tryptone broth (8 g/L Difco tryptone, 5 g/L NaCl, pH 7.0). Incubation was at 28°C for 6 and/or 24 h. After incubation, the enrichment cultures were either streaked for isolation or serially diluted in BPD and the 10^{-5} -10^{-7} dilutions surface-spread on the plating media as described above.

Confirmation of isolates. Typical *Aeromonas* colonies from the various media were picked to triple sugar iron (TSI) agar and nutrient agar slants. After overnight incubation at 28°C, a few drops of a 1% solution of N,N-dimethyl-p-phenylenediamine monohydrochloride were added to the growth on the nutrient agar slant to determine the oxidase reaction. All oxidase-positive, glucose fermenters were tested in the following biochemical media: glucose and mannitol fermentation broths, Moeller's decarboxylase broths (with either lysine, arginine, or ornithine) and esculin agar. Aeromonads produce acid from mannitol, have variable reactions in lysine decarboxylase broth, negative reactions in ornithine decarboxylase broth and positive arginine dihydrolase reactions. The species were differentiated by their glucose fermentation and esculin hydrolysis reactions: *A. hydrophila:* gas from glucose, esculin hydrolysed; *A. sobria:* gas from glucose, esculin not hydrolysed; *A. caviae:* no gas from glucose, esculin hydrolysed.

Occurrence of aeromonads in retail meat and poultry products

Ten packages each of fresh chicken thighs, ground beef, and pork sausage or ground pork were purchased at local retail markets. The packages were held in the refrigerator (4°C) until time of analysis and were sampled either the day of, or the day after, purchase. Two packages each of chicken thighs, ground beef and pork were analyzed weekly for five weeks.

An estimate of the numbers of aeromonads in the samples was done by the "indicated number" (IN) technique. Twenty-five gram samples of chicken thigh meat (half skin, half muscle), ground beef, pork sausage, or unseasoned ground pork were transferred to 225-ml quantities of TSBA in pint Mason jars and blended for 2 min in an Osterizer blender. The 250-ml samples were reapportioned in 225-ml, 22.5-ml and 2.25-ml quantities and (by manipulation of sample dilutions) in 0.225-, 0.0225- and 0.00225-ml quantities. The four smallest quantities were placed in tubes each containing 10 ml of TSBA. The IN broths were incubated at 28°C for 18 to 24 h. After incubation, we attempted to isolate *Aeromonas* species from each of these cultures using the spread-plate technique on SA and MMA agars. These plates were incubated at 28°C for 18 to 24 h, after which three typical colonies were picked from each medium and confirmed as above. One isolate of each *Aeromonas* species (*A. hydrophila, A. sobria, A. caviae*) from each meat and poultry sample was transferred to a nutrient agar slant and held for toxin production analysis as outlined below. The rationale of the IN method presumes that the isolation of the desired organism from the TSBA culture indicates at least one *Aeromonas* present in that meat portion. Using the smallest meat portion containing the organism, the IN/g can be calculated.

Preparation of samples for toxin assay. Each culture to be assayed was inoculated into 25 ml of brain heart infusion broth (BHI; Difco) in a 125-ml Erlenmeyer flask and was incubated overnight at 30°C on a reciprocal water bath shaker (Precision Scientific) at 80 strokes/min. The cultures were diluted in BHI to 50% T at 600 nm using a spectrophotometer (B & L Spectronic 20). These standardized suspensions were used to inoculate fresh flasks of BHI (0.1 ml/25 ml of broth) which were then incubated 24 h at 30°C on an environmental incubating shaker (New Brunswick Scientific) at 210 RPM. The cultures were centrifuged (Sorvall RC-2) at 11,950 × *g* for 30 min and the decanted supernatant liquid was filter-sterilized through 0.2-μm filters (Nalgene). The filtrates were tested immediately.

Cytotoxin test method. Mouse adrenyl Y1 cells (AY1) obtained from R. Grays, NMRI, Bethesda, Md., and Chinese hamster ovary cells (CHO; ATCC-CCL-61) were both used for toxin testing. The AY1 cells were grown in Eagle's minimal essential medium (MEM) with 10% fetal bovine serum (FBS), 1% glutamine and 0.1% gentamicin (Quality Biologicals, Gaithersburg, Md.). The CHO cells were grown in Ham's F12 with 10% FBS, 1% glutamine and 0.1% gentamicin (Quality Biologicals). Both the AY1 and CHO cells were inoculated at 2 × 10^5 cells/ml/well in 24-well plates (Falcon) and incubated overnight at 37°C under 5% CO_2. Each culture filtrate was tested both undiluted and diluted 1:8 in BHI, by adding 0.1 ml of filtrate to a well of each cell line. The cells were incubated overnight as above and observed for detachment, rounding and shriveling. The amount of toxic activity was graded on a scale of – to 4+. Wells containing uninoculated BHI broth instead of culture filtrate served as negative controls. A 4+ reaction had no normal cells in the well.

Hemolysin test. The method used was based on that of Burke et al. *(4,5).* A 1% suspension of washed rabbit erythrocytes was mixed with an equal volume of undiluted culture filtrate or filtrate diluted 1:4 in BHI, in a conical disposable centrifuge tube (0.3 ml of blood + 0.3 ml of filtrate). The mixture was incubated 1 h at 37°C and then 1 h at 5°C. The remaining cells were spun down in a clinical centrifuge at 2400 RPM for 5 min and 0.2-ml quantities of the supernatant liquid were transferred into a well of a 96-well plate. Hemoglobin concentrations were read by inspection and on a Titertek Multiskan MC photometer (Flow Laboratories, Inc.) at 540 nm. For visual readings, the results were recorded as (+) definite color, (–) no different than the control, and (±) a slight color change. A standard curve was prepared for each experiment as described in the Manual of Clinical Microbiology *(1)* using a 1% erythrocyte suspension and a 1% hemoglobin solution to make the var-

ious hemoglobin concentrations. The standard curve solutions were prepared in tubes and transferred to the 96-well plate containing the test samples.

Cholera toxin. The assay for cholera-like toxin was done by the reversed passive latex agglutination method. We used reagents and instructions from a commercial kit (VET-RPLA SEIKEN, Denka Seiken Co. LTD., 12-1, Nihonbashikabutocho, Chuo-ku, Tokyo, Japan).

RESULTS AND DISCUSSION

Evaluation of the five plating media (CBA, PBG, SA, MXA, and MMA) with pure cultures showed no practical differences among the various media, but when meat was sampled some performed better than others. CBA proved unsatisfactory for routine isolation because not all strains of *Aeromonas* were hemolytic on sheep blood agar plates, which made the isolation of nonhemolytic aeromonads difficult. MXA agar was not suitable for isolation of *Aeromonas* from meat and poultry samples because of the many xylose-negative, non-aeromonads that grew on this medium. PBG agar also proved to be unsuitable because the nonnutrient agar overlay made picking colonies difficult. When samples were plated directly, recovery of *Aeromonas* was better on SA than on MMA agar. However, both media were equivalent when samples were first enriched in TSBA. *Aeromonas* colonies are honey yellow on SA agar and red on MMA agar.

Recovery of *Aeromonas* was facilitated by enrichment procedures, particularly when the samples contained freeze-injured or low numbers (10^0-10^2/g) of aeromonads. Retail raw meat and poultry samples usually have total bacterial counts of 10^4 to 10^8/g. Since many of these bacteria (i.e. pseudomonads) are not inhibited by the ampicillin in the direct plating media, plates with isolated colonies can only be achieved by serial dilution of the sample. This dilution process also reduces the number of aeromonads available for plating. Consequently, enrichment methods were necessary to increase the number of aeromonads. Using the percent recovery of typical colonies, confirmed colonies, and positive samples as our criteria, it was determined that TSBA performed slightly better than alkaline peptone water enrichment, but both enrichments were better than tryptone broth, TSB, and TSBA plus 2% extra NaCl. The extra NaCl did not significantly inhibit competing microflora in the enrichment broth. Overnight (18-24 h) enrichments at 28°C gave better recovery of aeromonads than did 6-h enrichments, thus we decided to use an 18-24 h enrichment in TSBA at 28°C for the retail meat survey samples. Surface plating rather than streaking the SA and MMA agar plates from the overnight enrichment broths also improved the recovery of low numbers of aeromonads.

The results of the analysis of the 30 retail meat and poultry samples for the presence of *Aeromonas* are summarized in Table 1. The 10 chicken thigh samples were all positive for *Aeromonas*, with indicated numbers ranging from 4.44×10^0 to $\geq 4.44 \times 10^3$ per gram. *A. hydrophila* was isolated from all 10 chicken samples; *A. sobria*, from six samples; and *A. caviae*, from six samples. *Aeromonas* was isolated from all the ground beef samples, with indicated numbers ranging from 4.44×10^1 to $\geq 4.44 \times 10^3$/gm. *A. hydrophila* was isolated from all 10 ground beef samples; *A. sobria*, from four samples; and *A. caviae*, from six samples. *Aeromonas* was not detected in two of the 10 pork samples. The indicated numbers of the remaining eight samples ranged from 4.44×10^{-2} to $\geq 4.44 \times 10^3$/gm. *A. hydrophila* was isolated from seven of the eight positive pork samples; *A. sobria*, from three samples; and *A. caviae*, from four samples. It is possible that the salt content or some other formulation parameter may prevent growth of aeromonads in some pork sausage/ground pork products during refrigerated storage. Since 30% of our samples did not reach extinction, we do not know how high some of the counts might have gone. Consequently, we can state only that the results of this survey establish that motile *Aeromonas* species are found in retail raw meat and poultry products, often in relatively high numbers.

Fifty-eight *Aeromonas* isolates (28 *A. hydrophila*, 13 *A. sobria*, and 17 *A. caviae*) from the survey were tested for cytotoxin, hemolysin, and cholera-like toxin. Cytotoxin test results showed the AY1 cells to be slightly more sensitive than the CHO cells at filtrate dilutions of 1:8 (Table 2). Sixty-nine percent of the isolates were cytotoxic to the AY1 cells while 64% were cytotoxic to the CHO cells. The undiluted filtrate had the same

TABLE 1. *Distribution of* Aeromonas *in ten samples each of fresh beef, chicken, and pork.*

Range of Indicated numbers/g	Beef		Chicken		Pork	
	SA[a]	MMA[b]	SA	MMA	SA	MMA
Not isolated	0	0	0	0	2	3
4.44×10^{-2}-$<4.44 \times 10^{-1}$	0	0	0	0	1	0
4.44×10^{-1}-$<4.44 \times 10^{0}$	0	0	0	0	0	0
4.44×10^{0}-$<4.44 \times 10^{1}$	0	0	1	1	1	1
4.44×10^{1}-$<4.44 \times 10^{2}$	1	1	1	1	1	1
4.44×10^{2}-$<4.44 \times 10^{3}$	6[d]	6[d]	5[c]	6[c]	2[c]	2[c]
$\geq 4.44 \times 10^{3}$	3	3	3	2	3	3
Total	10	10	10	10	10	10

[a]SA - Starch ampicillin agar used for isolation.
[b]MMA - MacConkey mannitol ampicillin agar used for isolation.
[c]one of the ten samples not tested beyond this level.
[d]two of the ten samples not tested beyond this level.

TABLE 2. *Summary, by* Aeromonas *species, of isolates with positive reactions to hemolysin and cytotoxin tests.*

	Number of positive isolates						
	Hemolysin		CHO		AY1		No. of isolates tested
Dilution	1:1	1:4	1:1	1:8	1:1	1:8	
A. hydrophila	26 (92.8%)	19 (67.8%)	26 (92.8%)	24 (85.7%)	26 (92.8%)	26 (92.8%)	28
A. sobria	9 (69.2%)	6 (46.1%)	11 (84.6%)	10 (76.9%)	11 (84.6%)	11 (84.6%)	13
A. caviae	3 (17.6%)	3 (17.6%)	3 (17.6%)	3 (17.6%)	3 (17.6%)	3 (17.6%)	17
Totals	38 (66%)	28 (48%)	40 (69%)	37 (64%)	40 (69%)	40 (69%)	58

TABLE 3. *Summary, by product source, of isolates with positive reactions to hemolysin and cytotoxin tests.*

	Number of positive isolates						
	Hemolysin		CHO		AY1		No. of isolates tested
Dilution	1:1	1:4	1:1	1:8	1:1	1:8	
Chicken	13 (56.5%)	7 (30.4%)	13 (56.5%)	12 (52.1%)	13 (56.5%)	13 (56.5%)	23
Beef	15 (75%)	12 (60%)	16 (80%)	15 (75%)	16 (80%)	16 (80%)	20
Pork	10 (50%)	9 (45%)	11 (73%)	10 (50%)	11 (73%)	11 (73%)	15
Totals	38 (66%)	28 (48%)	40 (69%)	37 (64%)	40 (69%)	40 (69%)	58

cytotoxic effects on both tissue culture cell lines. As shown in Table 2, 92.8% of the *A. hydrophila* strains and 84.6% of the *A. sobria* strains were positive for cytotoxin when tested with AY1 cells. Few of the *A. caviae* strains produced cytotoxin; only 17.6% were positive. Cytotoxin-positive aeromonads were found in all three meat products (Table 3).

The hemolysin tests were read both by inspection and by optical density (OD) readings. The mean of the slope of the optical density standard curves was 1.87 with a standard deviation of 0.05. When this curve was compared with visual results, a definite positive by visual inspection corresponded at a 99% probability to 25% hemoglobin. The ± visual designations fell between 10 and 25% with a definite negative below 10% hemoglobin. There was good correlation between hemolysin and AY1 cytotoxin test results when the undiluted sterile culture filtrates were tested (Tables 2 and 3). Only one of the 58 cultures was hemolysin-positive/cytotoxin-negative, and only two were hemolysin-negative/cytotoxin-positive. The hemolysin/cytotoxin results of the other 55 cultures were the same, either both positive or both negative. This correlation agrees with the observations of Daily et al. *(7)* and Cumberbatch et al. *(6)*, both of whom reported good correlation between hemolysin and cytotoxin production. When the 1:4 sample dilution for the hemolysin test was compared to the 1:8 of the AY1 cytotoxin test, the correlation was not as good. Ten of the cytotoxin/hemolysin results disagreed [hemolysin (−) cytotoxin (+)].

We were unable to find any evidence of cholera-like toxin in the 36 cultures tested. This may be due to the small number of isolates tested. Shemada *(20)* found only 8 out of 179 (4.5%) *A. hydrophila* to have this toxin, so it is not surprising that it wasn't found among the 14 *A. hydrophila*, 8 *A. sobria*, and 12 *A. caviae* that were tested.

An attempted study of invasiveness using the method described for *Escherichia coli* in the Bacteriological Analytical Manual (BAM) *(9)* [with modification used by Lawson et al. *(13)* specific for *Aeromonas* growth] failed because the HeLa cells died in the presence of the bacteria.

The results of this study show that *Aeromonas* species are frequently found in raw meat and poultry products, and many of these organisms are hemolysin and cytotoxin producers. Burke et al. *(5)* reported a 97% correlation between hemolysin production and enterotoxin production as determined by the suckling mouse test, therefore we infer that many of these strains are enterotoxin producers.

The public health significance of these findings is unknown since an outbreak where cause and effect are clear has not yet been established. Wekell et al. *(24)* reported the isolation of *A. hydrophila* from oysters associated with 472 cases of gastroenteritis; the isolations were made a year and a half after the outbreak occurred. Un-

fortunately, the patients' stools had not been examined for *Aeromonas* species at the time of the outbreak, so a clear cause and effect relationship was not established. Furthermore, neither Pitarangsi et al. *(19)* who did *Aeromonas* feeding-challenge studies on rhesus monkeys nor Morgan et al. *(16)* who did *Aeromonas* feeding-challenge studies on human volunteers have been able to produce gastroenteritis. Studies have shown more cases of gastroenteritis, with *Aeromonas* occurring in the stools, occur in the summer months *(5,12)* than at other times of the year, which might indicate either water transmission, food abuse, or a combination of both.

Our work shows the ease and frequency of the isolation of these organisms from retail meat, and confirms the work of Palumbo et al. *(18)* indicating its presence in almost all fresh retail foods of animal origin. Since Drazek and Stern *(8)* have reported a very low incidence of aeromonads in the feces of beef, pigs, sheep, and turkeys, this may indicate that the organism is introduced during handling.

Gracey et al. *(11)* reported that significantly more enterotoxin-positive *Aeromonas* were isolated from cases of gastroenteritis than from well persons; therefore, the observation that 66% of our isolates were hemolysin positive and by inference enterotoxin-positive is a matter of concern. More research on virulence mechanisms of these organisms is needed before we can assess the significance of their presence in meats.

With an increase in awareness of aeromonads as possible causes of gastroenteritis, more laboratories are testing for their presence. This increase in surveillance may ultimately provide the answer to the public health significance of these organisms.

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