Aeromonas hydrophila

Resistance of isolates to nine antibiotics was determined with the organisms recovered from broiler carcasses and chill water samples taken from a Georgia processing plant. Carcasses were randomly selected for recovery. Pre- and post-evisceration locations, immediately after immersion chilling, and immediately after evisceration. Cytotoxin activity was detected in 23.6% of 119 isolates. The majority of the multiple antibiotic-resistant isolates (76.4%) were resistant only to ampicillin and chloramphenicol. The remaining multiple antibiotic-resistant isolates (23.6%) were resistant to various combinations of 2, 3, or 4 antibiotics, most of which were recovered from carcasses immediately after evisceration. Cytotoxin activity was detected in 63.8% of all isolates using the Y-1 mouse adrenal tumor cell line. Cytotoxin positive isolates were recovered from all sampling locations including chill water. The highest cytotoxicity titers were shown among isolates recovered from carcasses immediately after evisceration. These data suggest bird fecal contamination as an important source of A. hydrophila in broilers and broiler processing plants rather than environmental contamination.

Aeromonas hydrophila has been detected in large numbers in virtually all types of environmental waters in the United States (17). The organism has been found in fresh water streams, lakes, polluted water and estuaries (1,6,29), in raw and chlorinated drinking water (7,8), and in bottled water (34).

Numerous studies abroad and in the U.S. have pointed to the involvement of Aeromonas spp. in cases of human gastroenteritis. Gracey et al. (16), in a year-long study of children in Australia, isolated Aeromonas spp. from 10.2% of 1,156 patients with diarrhea and from 0.6% of those without diarrhea. Moyer (26) reported a total of 248 strains of Aeromonas spp. isolated from 3,334 human fecal specimens submitted to a state public health laboratory over a two-year period. Numerous other studies have established an association of A. hydrophila with diarrheal disease in adults and especially in children (13,14,18,19,31).

The role which this organism plays in gastroenteritis is not clearly understood. Confusion lies in being unable to correlate cytotoxin, enterotoxin, hemolysin, and hemagglutinin production with clinical symptoms. Agger et al. (2) found 62% of A. hydrophila isolates in stool specimens from patients with diarrhea to be cytotoxigenic. Goodwin et al. (15) reported that A. hydrophila was isolated from the feces of 32 hospital patients with diarrhea, and that all 32 isolates produced enterotoxin. In a Canadian study, 66 out of 96 A. hydrophila isolates (69%) from patients' stools were cytotoxin and hemolysin positive (12). The incidence of enterotoxigenic or cytotoxigenic strains taken in other clinical studies ranged from 79-95% (21,22,36).

A number of foods have been evaluated at the retail level as potential vehicles of A. hydrophila, these include red meats, poultry, seafoods, milk, and produce. A survey of 10 samples each of chicken, ground beef, and pork sausage from local food stores found all but two pork products positive for aeromonads, and of the recovered A. hydrophila isolates tested for cytotoxicity, 92.8% were positive using mouse adrenal Y-1 cells and Chinese hamster ovary cells (30). Produce (10) and vacuum-packed pork (28) yielded cytotoxic Aeromonas spp.

Little information is available concerning food processing environments and the occurrence of A. hydrophila on product. Hood et al. (20) reported that conventional processing of oyster meats resulted in the overall reduction of the microbial load, but that A. hydrophila numbers increased after processing and storage. In our laboratory, A. hydrophila was recovered from 98% of fresh broiler carcasses, and 92% of all chill water samples taken from the same processing plant (4). The only measures taken other than general sanitation to control the microbial load on fresh poultry carcasses are chilling and chlorination. Little is known about the factors which promote the survival of this organism in the processing environment or on the final product. It has been reported in many studies that chlorination selects for multiple antibiotic-resistant bacteria in both water and wastewater treatment (3,27,35). Antibiotics have multiple uses in the production of food animals and may be...
perceived as a potential health problem if multiply resistant pathogens persist. This study examines the cytotoxic nature of *A. hydrophila* isolates obtained from various stages in a typical broiler processing operation, as well as their antibiotic resistance profile. These data may help us better understand the health implications of *A. hydrophila* on final products and factors which promote the survival and growth of this organism in the processing environment, and on the final product.

**MATERIALS AND METHODS**

*A. hydrophila* isolates

Isolates of *A. hydrophila* were recovered from fresh broiler carcasses taken from the processing line at a Georgia plant using the rinse method of Cox et al. (11). Carcasses were taken from selected steps in the processing sequence including pre-evisceration, post picking; post evvisceration, prechill rinse; chill tank exit; and finished product - boxed, iced, stored for 48 h post processing at 34°F (1.1°C). Grab samples of chill water were taken on random days using sterile polypropylene containers. All samples were iced and transported to the laboratory for analysis. Chill water and carcass rinse water samples were assayed for *A. hydrophila* using mAg agar, and the membrane filtration method of Rippey and Cabelli (32). All *A. hydrophila* isolates were confirmed on Ah medium (2) and also tested using API 20E strips (Analytab Products, Plainview, NY).

**Antibiotic resistance profiles**

Antibiotic resistance profiles of *A. hydrophila* isolates were determined using the disc assay method of Bauer et al. (5). Broth cultures of isolates were swabbed onto plates of Mueller-Hinton agar and allowed to dry. Antibiotic-impregnated discs (Sensi discs, BBL Microbiology Systems, Cockeysville, MD) were placed on the agar, and plates were incubated 24 h at 32°C. Inhibition zones larger than 6 mm (disc diameter) indicated antibiotic sensitivity. Resistance to nine antibiotics was determined including: ampicillin (10 μg), cephalothin (30 μg), streptomycin (10 μg), kanamycin (30 μg), chloramphenicol (30 μg), naladixic acid (30 μg), tetracycline (30 μg), neomycin (30 μg), and gentamicin (10 μg).

**Assay of cytotoxic activity**

Cytotoxic activity of *A. hydrophila* isolates was determined using mouse Y-1 adrenal tumor cells (ATCC #CCL 79) according to modifications of the methods of Okrend et al. (34) and Sack and Sack (33). The Y-1 cells were grown in Eagle minimal essential medium in Earle balanced salt solution supplemented with 10% fetal bovine serum (FBS), 2.0 mM L-glutamine, 0.22% NaHCO3, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin, and 1.0 μg/ml fungizone. Cell culture media were obtained from Sigma Chemical Co. (St. Louis, MO), and FBS was from GIBCO (Grand Island, NY). Reagent grade 1 water of HPLC and cell culture quality produced by a Continental Ultrapure Water System was used throughout the project. Confluent Y-1 monolayers in 150-cm² flasks (Corning) were subcultured into 24-well microtiter plates (Costar) at the rate of 1.0 ml containing 2 x 10⁴ cells per well, and incubated at 37°C in a humidified, 5% CO₂, atmosphere for 1-2 d. *A. hydrophila* isolates were grown in brain-heart infusion broth (BHI, Difco Laboratories) overnight in a shaking water bath at 37°C. Cultures were centrifuged for 30 min in a microcentrifuge (Fisher Scientific, Pittsburgh, PA), and the supernatants were filtered through a 0.2-μm membrane filter (Ramin Instrument Co.). Culture filtrates were serially diluted with sterile BHI broth to test for cytotoxicity. Sterile BHI broth served as a negative control, while known cytotoxic strains of *Vibrio cholerae* and *A. hydrophila* served as positive controls. A 0.1-ml volume of sample filtrate or control was added to a Y-1 monolayer in a well without removal of the 1.0 ml of growth medium (2 wells per control or filtrate dilution). Inoculated microtiter plates were incubated at 37°C in a humidified, 5% CO₂ atmosphere for 48 h. At 24 and 48 h, each well monolayer was examined for cytotoxic activity (i.e., cell detachment, rounding, and shrinking). Each filtrate was diluted to the elimination of cytotoxin activity (cytotoxicity extinction). The cytotoxicity titer of each culture filtrate was expressed as the reciprocal of the highest filtrate dilution producing partial or complete destruction of Y-1 cell monolayers in 24 to 48 h.

**RESULTS AND DISCUSSION**

*Aeromonas hydrophila* was found in broiler carcasses and chill water in varying numbers at all sampling points throughout the processing operation, but isolates were not further characterized (4). In the present study, these *A. hydrophila* isolates were characterized with respect to antibiotic resistance and cytotoxic activities. The distribution of antibiotic resistance profiles of *A. hydrophila* isolates taken at various steps in the processing of the birds and from prechill and main chill tank waters is presented in Table 1. All isolates of *A. hydrophila* were resistant to ampicillin as expected since mAg agar relies on ampicillin as a selective agent. Greater than 99% of *A. hydrophila* are resistant to ampicillin (36). Isolates recovered in this study resistant to ampicillin alone comprised 53.8% of the total. Multiple antibiotic resistance (resistance to two or more antibiotics) occurred in 46.2% (55) of the isolates recovered. Of those which were multiply resistant, 42 (76.3%) were resistant only to ampicillin and cephalothin. The remaining 13 isolates (23.6%) were resistant to 2, 3, or 4 antibiotics of various combinations. Notably, most isolates exhibiting resistance to three or more antibiotics were recovered from rinse water of carcasses sampled immediately after evisceration. This observation parallels an increase in numbers of other bacteriological parameters measured at the post evisceration step (4). This emphasizes the impact that the carcass evisceration step has upon the level of bacterial contaminants on the carcasses. The highest percentages of multiple antibiotic-resistant isolates obtained from a given sampling location were recovered from carcasses sampled immediately after evisceration, and the chill water samples, 70.6% and 70.4% respectively (Table 1). This indicates that chill water may be an important route of cross-contamination of carcasses with *A. hydrophila* which are multiple antibiotic resistant.

Chlorination is used routinely throughout the processing environment as a sanitizer to control microorganisms. Equipment surfaces are sprayed (i.e., automated eviscerating machine), a postevvisceration carcass spray is common, and chlorine is added to immersion chill tanks in the make-up water (20 ppm U.S.D.A. recommended in all cases), The occurrence of multiple antibiotic-resistant bacteria in other chlorine treatment processes has been thoroughly demonstrated, especially those of drinking water processes and wastewater treatment. Murray et al. (27) found resistance for up to nine antibiotics among *Escherichia coli* isolates from wastewater, sampled prior to and after chlorination,
with a high proportion of the bacteria isolated being multiply resistant. Antibiotic-resistant bacterial strains are apparent in agricultural environments. Mamber and Katz (25) found that antibiotic-resistant strains of aerobic and facultative anaerobic gram-negative enteric bacilli which colonize the intestinal tracts of chickens are not necessarily selected for by antimicrobial supplementation of animal feed, but may be dependent on their common presence in the environment of newly hatched chickens. This supports Linton's observation on the use of antimicrobials in the environment of newly hatched chickens. He claimed that it would be reasonable at this point but unable to persist as long in the lower temperature chill water and in iced storage. Likewise, isolates of higher cytotoxicity titer may simply be in greater numbers at this stage, and the probability of recovering them is greater.

These results are similar to those reported by Cumberbatch et al. (12) and by Agger et al. (2) who found 69 and 62% of the A. hydrophila isolates tested from human diarrheal cases, respectively, were cytotoxic. Others have reported a much higher incidence, i.e., 93-100% (15,22), among isolates from human sources. Clinical strains are usually more frequently cytotoxic or exhibit other virulence factors, such as enteropathogenicity and hemolysin production, as compared to environmental isolates. Turnbull et al. (36) reported only 53% of environmental isolates of A. hydrophila to be enterotoxigenic, while Burke et al. (9) demonstrated that 70.2% of environmental strains of this organism were enterotoxigenic. In a previous study conducted in our laboratory, we hypothesized that the A. hydrophila isolates recovered were not from environmental sources, but likely from bird fecal contamination within the processing operation (4). These data support that hypothesis. Cytotoxic isolates were recovered with the highest frequency early in the process stages and with highest cytotoxicity titer immediately following evisceration (post-evisceration step).

In conclusion, multiple antibiotic-resistant and cytotoxicogenic A. hydrophila were recovered from the broiler processing environment. Chlorination may be one factor which contributes to the persistence of these strains. In particular, cytotoxic strains are present at all stages in the process immediately following evisceration, and also on final product which is ready for shipment to retailers.

### TABLE 1. Distribution of antibiotic resistance among A. hydrophila isolates from a broiler processing operation.

<table>
<thead>
<tr>
<th>Antibiotic(s)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Pre-evisc. carcasses</th>
<th>Post-evisc. carcasses</th>
<th>Pre-chill water</th>
<th>Chill water</th>
<th>Post-chill carcasses</th>
<th>Iced (48 h) carcasses</th>
<th>Total isolates</th>
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<tr>
<td></td>
<td>(18)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>(17)</td>
<td>(6)</td>
<td>(27)</td>
<td>(10)</td>
<td>(41)</td>
<td>(119)</td>
</tr>
<tr>
<td>AM</td>
<td>10</td>
<td>5</td>
<td>4</td>
<td>8</td>
<td>7</td>
<td>30</td>
<td>64</td>
</tr>
<tr>
<td>AM, CF</td>
<td>7</td>
<td>4</td>
<td>2</td>
<td>19</td>
<td>2</td>
<td>8</td>
<td>42</td>
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<tr>
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<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>AM, NE</td>
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<td>1</td>
<td>0</td>
<td>1</td>
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<tr>
<td>AM, GM</td>
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<td>0</td>
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<td>0</td>
</tr>
<tr>
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<td>0</td>
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<tr>
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<td>0</td>
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<tr>
<td>AM, NE, GM</td>
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<td>0</td>
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<td>1</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
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<tr>
<td>Total MAR&lt;sup&gt;d&lt;/sup&gt;</td>
<td>8</td>
<td>12</td>
<td>2</td>
<td>19</td>
<td>3</td>
<td>11</td>
<td>55</td>
</tr>
</tbody>
</table>

<sup>a</sup> Total number of isolates tested was 119.
<sup>b</sup> AM - ampicillin, CF - cephalothin, S - streptomycin, K - kanamycin, C - chloramphenicol, NA - naladixic acid, TE - tetracycline, NE - neomycin, and GM - gentamicin.
<sup>c</sup> Total number of isolates from each processing location.
<sup>d</sup> Multiple-antibiotic resistant (MAR): isolates resistant to two or more antibiotics.
public health significance of their presence has yet to be determined in part because of the confusion associated with a failure, to date, to correlate the several virulence factors with clinical symptoms in humans.

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REFERENCES


