Starch-Ampicillin Agar for the Quantitative Detection of *Aeromonas hydrophila*

Interest in *Aeromonas hydrophila* as a food-borne and human pathogen is increasing. Isolation media from the clinical laboratory were evaluated for food use and either did not give quantitative recovery of *A. hydrophila* or did not permit ready differentiation of *A. hydrophila* from the background microflora. A new medium was developed which permitted quantitative recovery of *A. hydrophila* from foods. The medium consisted of phenol red agar base (Difco Laboratories), soluble starch (10 g/liter), and ampicillin (10 mg/liter). All foods surveyed contained *A. hydrophila*. Foods sampled included red meats, chicken, raw milk, and seafood (fish, shrimp, scallops, crab, and oysters). The count of *A. hydrophila* at the time of purchase ranged from 1×10^2/g (lower limit of detection) to 5×10^5/g. In most instances, the count of *A. hydrophila* increased during 1 week of storage at 5°C. The starch-ampicillin agar developed permitted rapid quantitative recovery of *A. hydrophila* from foods in the presence of very large numbers of competing microflora.

During the past several years there has been increasing interest concerning the possible role of species of the *Aeromonas hydrophila* group (*A. hydrophila*, *A. sobria*, and *A. caviae*) as a cause of human gastroenteritis; both clinical and laboratory investigations have suggested that the species is a significant enteric pathogen (5, 9, 10). To date, most studies into sources responsible for *A. hydrophila* gastroenteritis have concentrated on its transmission in water supplies (18). Recently, Buchanan (4) alternatively suggested that the species may represent a significant "new" food-borne pathogen and hypothesized that foods may be important in the dissemination of the microorganism. The basis for this hypothesis was the pathogenicity of the species in fish and reptiles (10), its frequent isolation from various foods of animal origin (1, 3, 7, 8, 11, 12, 14, 15, 20), and the psychrotrophic nature of the species (S. A. Palumbo, D. R. Morgan, and R. L. Buchanan, J. Food Sci., in press).

While *A. hydrophila* has been identified as part of the microflora of various foods, quantitative data on its incidence and extent in foods is generally lacking. As a preliminary step in obtaining this needed information, the initial purpose of the present investigation was to evaluate available media for the isolation of *A. hydrophila* in clinical samples to assess their suitability for the quantitative detection of the microorganism in food samples. However, early in this assessment it became apparent that currently available media did not permit the species to be quantitatively recovered, nor could they readily differentiate the species from other microorganisms associated with foods (particularly coliforms and pseudomonads). Therefore, our efforts were redirected towards engineering a medium that could fulfill these requirements. The present report describes the successful development of a simple, direct plating medium for the quantitative detection of *A. hydrophila* in foods and the application of the medium to assessing the incidence of the bacterium in foods of animal origin obtained at the retail market level.

MATERIALS AND METHODS

Microorganisms. A variety of clinical isolates of *A. hydrophila* previously described by Palumbo et al. (in press) were employed in the initial development and evaluation of the medium. Additionally, laboratory strains of *Pseudomonas fluorescens*, *Salmonella typhimurium*, and *Escherichia coli* were employed as representatives of important species that are commonly encountered as part of the background microbiota of foods.

Foods. Food samples were purchased at local retail markets in regular consumer packages. They were sampled on the day of purchase (zero time) and after 7 days of storage at 5°C. Sampling was achieved by aseptically transferring 20 g from the package to a sterile Stomacher 400 bag, adding 180 ml of sterile 0.1% peptone water, and blending in a Stomacher 400 for 2 min. Dilutions were then made with 0.1% peptone water and surface plated (0.1 ml) on the various test media. The raw milk samples were surface plated with a Spiral plater (model DU; Spiral Systems, Bethesda, Md.); the lower limit of detection was 2.2×10^1/ml.

Media. Total aerobic plate counts were made with APT agar (Difco Laboratories) in conjunction with a 48-h incubation at 20°C. Clinical media evaluated for the detection of *A. hydrophila* were those summarized by von Graevenitz and Bucher (22).

The starch-ampicillin agar (SA agar) developed consisted of phenol red agar base (Difco) (31 g), soluble starch (10 g) and distilled water (1,000 ml). After autoclaving at 121°C for 15 min and tempering to 50°C, ampicillin (Sigma Chemical Co.; A-9393) was added to achieve a concentration of 10 μg/ml. The medium was then poured into sterile petri dishes and allowed to harden. Dilutions of cultures or food samples were surface plated and subsequently incubated at 28°C for 24 h. After incubation, the plates were flooded (ca. 5 ml) with Lugol iodine solution (6), and amylase-positive colonies (those having a clear zone surrounding the colony) were scored as presumptive *A. hydrophila*. These colonies are typically 3 to 5 mm in diameter and yellow to honey colored.

Confirmation of isolates. Presumptive *A. hydrophila* iso-

TABLE 1. *A. hydrophila* and total aerobic count (per gram) of retail seafood at time of purchase (zero time) and after 7 days at 5°C

Food	Count at the following period			
	Zero time		Day 7	
	A. hydrophila	Total aerobic	*A. hydrophila*	Total aerobic
Butterfish	1.5×10^3	2.0×10^8	1.4×10^5	1.8×10^8
Buck shad	4.0×10^2	5.1×10^7	6.9×10^3	7.5×10^7
Crocker	1.6×10^5	3.7×10^8	2.0×10^6	2.0×10^9
Butterfish	2.4×10^4	1.2×10^7	3.8×10^5	5.1×10^8
Monk	1.0×10^2	1.4×10^6	5.0×10^5	1.1×10^9
Boston blue	1.5×10^2	1.0×10^4	3.3×10^5	5.0×10^8
Silver trout	1.5×10^2	4.0×10^4	5.0×10^5	3.3×10^8
Flounder	3.0×10^2	2.6×10^5	1.0×10^5	4.9×10^9
Cod fillets	1.9×10^5	1.2×10^7	1.7×10^6	2.0×10^9
Cod steaks	5.4×10^4	8.3×10^6	6.0×10^5	6.5×10^8
Blue	$<1.0 \times 10^2$	$<1.0 \times 10^4$	5.0×10^5	5.5×10^7
Raw shrimp sample 1	1.8×10^3	2.7×10^7	2.4×10^6	1.5×10^9
Raw shrimp sample 2	$<1.0 \times 10^2$	7.2×10^5	3.9×10^3	1.4×10^9
Scallop sample 1	6.3×10^4	5.0×10^6	1.5×10^7	1.9×10^9
Scallop sample 2	4.0×10^2	$<1.0 \times 10^4$	2.1×10^3	1.1×10^8
Raw shucked oysters sample 1	5.0×10^5	3.7×10^8	$<1.0 \times 10^5$	4.0×10^8
Raw shucked oysters sample 2	9.0×10^3	3.2×10^5	6.6×10^4	5.4×10^8
Raw shucked oysters sample 3	5.0×10^5	2.2×10^7	4.0×10^3	1.7×10^8
Crab sample 1	$<1.0 \times 10^2$	2.0×10^4	1.9×10^5	7.3×10^7

lated from food samples were confirmed by using the following protocols, tests, and media: gram strain, oxidase, catalase, DNase (16), *A. hydrophila* confirmation medium (13), resistance to vibriostatic agent 0/129, resistance to novobiocin, and API 20E strips (Analytab Products). The characteristics of the isolates were compared against those described by Popoff (16) and Popoff and Veron (17). Except where attempts were made to speciate isolates, all mesophilic aeromonads were designated as *A. hydrophila*.

RESULTS AND DISCUSSION

Evaluation of clinical media. With broth cultures of several clinical isolates of *A. hydrophila*, several clinical media previously evaluated by von Graevenitz and Bucher (22) for the recovery of *A. hydrophila* in clinical specimens were assessed for their ability to quantitatively recover *A. hydrophila* and to allow ready differentiation of the microorganism from a mixture of background flora organisms. Determination of percent recovery was based on differential counts on nutrient agar and the tested isolation medium. Recovery on xylose-sodium desoxycholate-citrate agar was typically 1 to 2 log cycles less than that on nutrient agar. This nonquantitative recovery appeared to be due to the desoxycholate, and other clinical media containing this component were not considered. Evaluation of inositol-brilliant green-bile salts agar indicated that while recovery rates were improved, it was extremely difficult to differentiate *A. hydrophila* from other species encountered as part of the microbiota of foods (*P. fluorescens*, *E. coli*, and *S. typhimurium*).

During the development of SA agar, a number of other observations were made that pertain to the quantitative performance of the clinical media described by von Graevenitz and Bucher (22). On medium containing toluidine blue, *A. hydrophila* colonies often took 48 h to appear and become characteristic. Incorporation of novobiocin (5 μg/ml) (19) decreased *A. hydrophila* by 1 to 2 log cycles. Further, this antibiotic made differentiation of *A. hydrophila* difficult (colonies are smaller and less characteristic).

SA agar. After our initial assessment that currently available clinical media would not meet the needs of food microbiologists, a medium was sought that would employ a cultural characteristic unique to *A. hydrophila*. Starch hydrolysis was selected since in gram-negative species associated with foods this enzyme activity is largely restricted to *Aeromonas* and *Vibrio* species. Soluble starch (10 g/liter) and ampicillin (10 mg/liter) were incorporated into either nutrient agar or phenol red agar base and compared against nutrient agar to determine quantitative recovery. Phenol red SA agar consistently gave recoveries comparable to nutrient agar, while nutrient agar with ampicillin or ampicillin and starch gave recoveries 1 to 3 log cycles less than nutrient agar. Thus, phenol red agar base was chosen as the basal medium, and starch and ampicillin were added as the differential and selective agents, respectively. The incorporation of ampicillin effectively suppressed coliforms and other members of the family *Enterobacteriaceae* but did not inhibit the growth of *P. fluorescens*. However, *A. hydrophila* was readily distinguishable by size and amylase reaction, and growth of pseudomonads was not considered detrimental to the usefulness of the medium.

In anticipation of difficulties differentiating *Aeromonas* species from amylase-positive *Vibrio* species, attempts were made to improve the selectivity of SA agar by incorporating novobiocin at a level of 5 mg/liter to inhibit the latter genus. However, its incorporation significantly lowered recovery rates for *A. hydrophila* and made differentiation of the species from the background flora more difficult. Shotts and Rimler (19) originally recommended the use of novobiocin (5 mg/liter) for the isolation of *A. hydrophila* and reported it to be satisfactory for use with a wide variety of samples. However, Rippey and Cabelli (18) found that *A. hydrophila* from a variety of sources were sensitive to the antibiotic.

It was alternatively considered that vibriostatic agent 0/129 could be incorporated into SA agar to eliminate potential false-positives due to amylase-positive *Vibrio* species. However, the compound cost would be likely to preclude its use in this manner.

The need for quantitative recovery of *A. hydrophila* precluded attempts to incorporate an agent that would prevent the growth of *Vibrio* species. However, actual experience with SA agar in conjunction with a wide variety of foods of marine and aquatic origin has indicated that initial concerns over differentiation of *Aeromonas* and *Vibrio* species were unfounded. To date, no presumptive *A. hydrophila* isolated from food samples have proven to be a *Vibrio* species upon taxonomic confirmation. Further, foods of primary interest in regard to *A. hydrophila* are those stored under refrigeration, and most vibrios will not grow at reduced temperatures (2). Those *Vibrio* species capable of growth at 4°C are amylase-negative and would not interfere with the detection of *A. hydrophila* on SA agar.

Food survey. Low-*Aeromonas*-count ground fish and beef was seeded with strains of *A. hydrophila* to give a final count

TABLE 2. *A. hydrophila* and total aerobic count (per milliliter or gram) of raw milk, sausage, chicken, and red meat at time of purchase (zero time) and after 7 days at 5°C

Food	Count at the following period			
	Zero time		Day 7	
	A. hydrophila	Total aerobic	*A. hydrophila*	Total aerobic
Raw milk sample 1	$<2.2 \times 10^2$	$<2.2 \times 10^5$	$<2.2 \times 10^2$	7.0×10^6
Raw milk sample 2	$<2.2 \times 10^1$	6.0×10^1	$<2.2 \times 10^3$	3.8×10^4
Raw milk sample 3	$<2.2 \times 10^1$	2.1×10^3	5.0×10^4	5.1×10^5
Sausage sample 1	1.0×10^2	4.8×10^7	$<1.0 \times 10^2$	2.4×10^8
Sausage sample 2	$<1.0 \times 10^2$	3.0×10^4	$<1.0 \times 10^2$	5.0×10^7
Sausage sample 3	$<1.0 \times 10^2$	3.5×10^2	2.5×10^2	1.2×10^8
Chicken sample 1	$<1.0 \times 10^2$	9.0×10^3	4.7×10^4	7.4×10^7
Chicken sample 2	1.7×10^3	2.8×10^5	5.0×10^5	1.0×10^8
Chicken drip sample 1	$<1.0 \times 10^2$	5.7×10^4	1.9×10^5	3.0×10^8
Chicken drip sample 2	3.3×10^3	1.0×10^6	1.0×10^6	4.5×10^8
Chicken liver sample 1	3.0×10^3	3.0×10^4	1.0×10^6	3.7×10^8
Chicken liver sample 2	1.2×10^3	1.5×10^6	1.0×10^6	3.5×10^8
Chicken liver drip sample 1	1.1×10^4	5.0×10^4	1.3×10^7	3.2×10^8
Chicken liver drip sample 2	2.8×10^4	2.0×10^7	1.0×10^7	6.5×10^9
Calf liver sample 1	6.5×10^2	4.0×10^7	5.0×10^2	4.5×10^8
Ground beef sample 1	2.8×10^3	1.4×10^7	8.3×10^4	1.6×10^9
Ground beef sample 2	1.7×10^3	1.9×10^6	6.8×10^4	3.4×10^9
Ground beef sample 3	4.5×10^4	1.0×10^7	5.0×10^5	2.6×10^9
Ground veal sample 1	8.2×10^4	2.8×10^8	3.4×10^5	1.6×10^9
Ground veal sample 2	3.1×10^4	1.2×10^6	2.1×10^5	7.0×10^8
Ground lamb sample 1	1.5×10^4	3.6×10^8	2.4×10^6	4.3×10^9
Ground lamb sample 2	6.0×10^2	1.3×10^6	1.0×10^6	2.6×10^9
Ground pork sample 1	1.1×10^4	1.5×10^8	1.5×10^6	6.7×10^9

of 10^4/g; recovery rates of *A. hydrophila* on SA agar ranged from 68 to 122%. After determining that SA agar quantitatively recovered clinical *A. hydrophila* from a variety of inoculated foods, the medium was used to assess the incidence of *A. hydrophila* in foods purchased at local retail markets. The *A. hydrophila* and total aerobic counts observed are summarized in Tables 1 and 2 for seafood and other products of animal origin, respectively.

A. hydrophila were detected in virtually all foods sampled. The only products which were negative (two of three samples) were fresh sausage products; in addition to being negative or very low, the *Aeromonas* count in fresh sausage essentially remained static during the 7-day storage period at 5°C. Whether the lack of *A. hydrophila* in these products is due to salt content (ca. 1.5%) or some other formulation parameter is unknown but is currently being investigated. The fresh sausage samples were also virtually the only product in which the level of *A. hydrophila* did not increase or showed a minimal increase after 7 days of refrigerated storage.

The sampling protocol employed in the current study has a lower limit of detection of 100 *A. hydrophila* per g for all foods except raw milk. In a few food samples the organism was not detected at the time of purchase but was subsequently observed after 7 days of refrigerated storage. This suggests that the organism was initially present at levels below the limits of detection. If the need arose, the sensitivity of the assay could be increased. For example, it should be feasible to employ an initial most probable number enrichment with alkaline peptone water (21), followed by detection on SA agar.

The level of *A. hydrophila* detected in the various food samples at the time of purchase ranged from less than 1×10^2/g to 5×10^5/g. Upon refrigerated storage for 7 days, the levels generally increased 10- to 1,000-fold, indicating that the *A. hydrophila* is capable of competitive psychrotrophic growth in a variety of foods. In a number of samples, *A. hydrophila* accounted for a significant portion of the total microbial population, with the greatest percentage (ca. 22%) being observed in chicken liver drip sample 1.

Representative amylase-positive colonies were picked from plates of the various food samples surveyed and were purified by restreaking on SA agar, followed by streaking on DNase agar. Amylase-positive, DNase-positive cultures were then subjected to biochemical characterization as described. Preliminary speciation based on a limited number of biochemical tests was performed, and 68% (44 cultures of 65) isolates keyed out as *A. hydrophila*, 12% (8 of 65) as *A. caviae*, and 20% (13 of 65) could not be unequivocally assigned to any of three mesophilic *Aeromonas* species. It is felt that this represents the actual occurrence of the various species of *Aeromonas* in food samples rather than a deficiency in the methodology. Since *A. caviae* and *A. sobria* along with *A. hydrophila* are considered human pathogens (21), food may represent the vehicle of transmission.

The results of the food survey quantitatively establish that *A. hydrophila* is ubiquitously associated with foods of animal origin, often in relatively high numbers. Further, the microorganism is competitive, even under refrigeration conditions currently considered adequate to prevent the growth of food-borne pathogens. The specific public health significance of these findings is unknown, since the determinants of virulence and minimum infectious dose for this organism have not been determined to date. However, the results strongly suggest that foods may play an important role in the etiology of human *A. hydrophila* gastroenteritis outbreaks, and future research should be directed to assess the significance of the species as a potential psychrotrophic food-borne pathogen.

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