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Incidence of Motile Aeromonads From United States West Coast Shellfish Growing Estuaries

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ABSTRACT

The distribution of motile *Aeromonas* species in marine and tributary waters, sediment, and shellfish from 12 major estuarine areas in Washington, Oregon, and California with commercial or sport shellfish harvest was determined during the summer months. *Aeromonas* spp. were found in half of the total of 400 samples analyzed. Two enrichment broths, tryptic soy ampicillin broth (TSBA) and alkaline peptone water (APW), were compared for recovery of *Aeromonas* from Washington and Oregon samples. More *Aeromonas* were isolated using TSBA. For Washington and Oregon samples, recoveries using TSBA were 82 and 77% respectively compared to 31 and 50% using APW. For California samples, only APW was used with 28% samples positive. Of 767 isolates tested, 93.5% were positive for hemolysis, a trait reported to correlate with enterotoxin production and pathogenicity. Of the hemolysis positive strains, 59.5% were toxic to Y-1 adrenal cells.

The prevalence and distribution of motile *Aeromonas* spp. in fresh water habitats are well documented (*10,22,40,41*). This bacterium was once thought to be absent from the marine environment (*20*); however, numerous investigators (*11,22,26,28,50*) have shown species of *Aeromonas* to be widespread in marine systems along with Gulf and Atlantic Coast. Studies of motile *Aeromonas* spp. of the West Coast have been limited.

Evaluations of media to isolate *Aeromonas* have been conducted by food, environmental, and clinical microbiologists, resulting in an array of solid-agar and liquid-enrichment media recommended to isolate this organism from a variety of foods, soil, watersheds, and clinical specimens. Most recently, Abeyta et al. (*1*) reported that the use of tryptic soy broth with 30 mg/L ampicillin (TSBA) increased recoveries of *Aeromonas hydrophila* from oysters frozen for 1 1/2 years at -72°C. This medium gave significantly better recoveries of *A. hydrophila* from oysters and water compared to those using modified Rimler Shotts broth (*2*).

Most recently, the use of general purpose recovery broth alkaline phosphate water (APW) for enrichment of *Aeromonas* spp. has also been recommended (*49*). APW is useful for recovering *Aeromonas* from clinical specimens as well as those from livestock (*2*) and feces (*38*) and like TSBA will allow repair of injured cells. To date, the use of APW has not been evaluated for the recovery of *Aeromonas* spp. associated with marine systems. This study was conducted to determine the distribution of *Aeromonas* throughout the West Coast and to evaluate APW in comparison to TSBA in recovering motile *Aeromonas* spp. from shellfish growing estuaries.

MATERIALS AND METHODS

Sampling sites

Twelve major shellfish growing areas of the U.S. West Coast were sampled during the summer months and early fall of 1984 (Fig. 1). Samples consisting of 261 water, 100 sediment, and 39 shellfish were taken from 240 sites. All samples were placed on ice and analyzed within 6 h of collection using a self-contained mobile microbiological laboratory stationed near the estuarine system under study. Replicate samples were taken at many sites with some collected daily up to 4 d maximum at each site. Salinity and temperature were measured at each sample site (*31*). Site selections were in accordance with those of the National Shellfish Sanitation Program (NSSP) (*18*). Considerations were given to location of pollution (point and nonpoint) sources, location of shellfish and existing harvesting closure lines, and hydrographic factors such as water flow direction, time of travel and dispersion.

Collection of samples

Water. Surface water samples were collected using sterile 4-L screw-capped plastic bottles (Nalge Co., Rochester, NY). At some sampling sites, water was collected in a sterile 19-L polyethylene pail suspended from a rope and then transferred to a 4-L plastic bottle. Nalge 4-L plastic bottles were sterilized by autoclave for 15 min at 121°C. Polyethylene pails were sterilized by sodium hypochlorite (150 ppm), followed by an on-site rinse with sample water.

Sediment. Approximately 200 g were collected aseptically from the sediment-water interface with a grab sampler (Kahlisco, El Cajon, CA) or with sterile plastic spoons. Sediments were placed in sterile Whirl-Pak bags (Nasco, Fort Atkinson, WI).

Shellfish. Shellfish were collected using oyster tongs, a grab sampler, a shovel, or by hand. Shellfish consisted of the follow-

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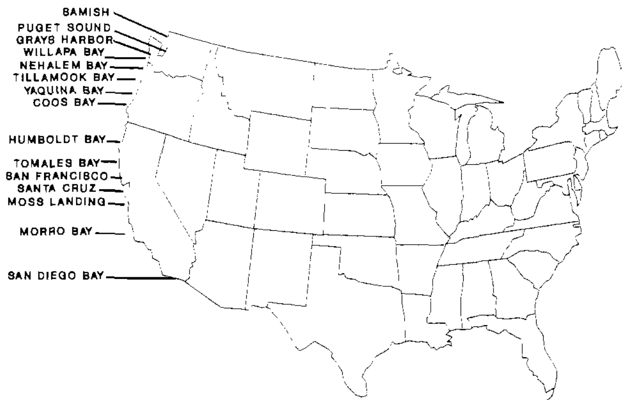

Figure 1. *U.S. West Coast estuaries sampled for motile* Aeromonas *spp.*

ing species: Pacific oyster (*Crassostrea gigas*); mussel (*Mytilus californicus*); *Mytilus edulis*; gaper clam (*Schizothaerus nuttali*); and cockle (*Clinocardium nuttali*).

Media preparation

Tryptic soy broth ampicillin (TSBA). TSBA (*48*) was prepared according to the manufacturer's directions (Difco, Detroit, MI). After autoclaving TSBA at 121°C for 15 min, filter sterilized ampicillin (Sigma, St. Louis, MO) was added to give a final concentration of 30 mg/L.

Alkaline peptone water (APW). APW was prepared by standard methods (*17*).

Peptone beef extract glycogen agar (PBG). PBG Agar (*37*) contained in g/L the following ingredients: peptone, 10; beef extract, 10; glycogen, 4; sodium chloride, 5; sodium lauryl sulfate, 0.1; bromothymol blue, 0.1; and agar, 15. The medium was heated to dissolve the solids, adjusted to pH 6.9-7.1, and autoclaved at 121°C for 15 min.

MacConkey's agar (MCA). MCA was prepared according to the manufacturer's directions (Difco).

Wagatsuma agar. Modified Wagatsuma agar contained in g/L the following: yeast extract, 3; bacto-peptone, 10; sodium chloride, 5; dipotassium phosphate, 5; mannitol, 10; crystal violet, 0.001; and Bacto agar, 15. The agar also contained 5% by volume of fresh human red blood cells.

Aeromonas hydrophila *medium* (AHM) - AHM (*27*) was prepared as follows in g/L: proteose peptone, 5; yeast extract, 3; tryptone, 10; L-ornithine hydrochloride, 5; mannitol, 1; inositol, 10; sodium thiosulfate, 0.4; ferric ammonium citrate, 0.5; bromocresol purple, 0.02; agar, 3. The medium was heated to dissolve solids, adjusted to pH 6.7, dispensed in 5-ml quantities in 13 x 100 mm tubes, and autoclaved at 121°C for 12 min.

Bacteriological analyses

Motile Aeromonas *species.* Water, sediment, and shellfish were analyzed as described (*3*). Shellfish and sediment samples were analyzed by adding 25 g portions each to 225 ml of both APW and TSBA broths and incubating at 35°C for 24 h. After incubation, the enrichment broth cultures were plated onto MCA and PBG agar plates. Agar plates were incubated at 35°C for 24 h. Typical colonies were picked for multitest screening (*27*). Strains which produced on AHM an alkaline surface, acid butt, and were motile were considered presumptive positive and tested further for cytochrome oxidase. Identification of motile *Aeromonas* strains was based on the following reactions: cytochrome oxidase (+); motility (+); mannitol fermentation (+); inositol fermentation (-); ornithine decarboxylase (-); hydrogen sulfite production in triple sugar iron (-); growth on MacConkey agar (+); growth without NaCl (+); arginine dihydrolase (+); methyl red at 35°C (±); methyl red at 26°C (±); indole production (+); and lysine dicarboxylase (±). Biochemical tests were based on those included in the MICRO-IS, identification program (*35*). Strains were further speciated using selected biochemical reactions (*39*), particularly salicin fermentation at room temperature, esculin hydrolysis, production of gas from glucose, and growth in potassium cyanide (KCN) broth.

Fecal coliforms. Fecal coliform estimations in shellfish and water were analyzed by the modified A-1 procedure (*4,24*).

Hemolysin assay. Cell-free preparations (500 µl) of twofold dilutions in phosphate-buffered saline (PBS) were added to equal volumes of 1% washed (3x) rabbit erythrocytes (Prepared Media Labs, Renton, WA) (0.85% saline) in microliter trays. Cell-free preparations were made by centrifuging the cultures at 10,000 x g for 30 min at 4°C, followed by filtration through a 0.45 µm membrane filter. Hemolysis was recorded after incubation for 1 h at 37°C and again after 24 h incubation at 4°C. Hemolysis of >50% of the erythrocytes was considered a positive reaction. Positive controls consisting of environmental isolates from earlier studies were run with each assay.

Hemolytic activity was also determined by the observation of alpha or beta hemolysis on modified Wagatsuma agar (spot agar plate method). Bacterial cultures were inoculated into 10 ml of TSB and incubated at 35°C for 24 h. Broth cultures were spotted onto Wagatsuma agar by using a sterile applicator stick and incubated at 35°C for 24 h.

Cytotoxicity tests. Aeromonas spp. (incubated for 24 h at 35°C in 16 x 125 mm test tubes containing Craig's medium) were inoculated into 125-ml flask containing 25 ml Craig's medium with 0.2 ml of culture and incubated with shaking at 200 rpm at 35°C for 24 h. Cultures were centrifuged at 9000 x g for 30 min, cells were discarded, and the supernatant liquid was filter-sterilized (0.45 - µm filter). Heat-treated filtrates were prepared by heating 2 ml supernatant liquid at 56°C for 30 min. Standard cell culture techniques were used to prepare the Y-1 cell culture (*33*).

Cytotoxin activity was observed by using a light microscope (40x), after 24 h of incubation and determination by the presence of completely rounded, granulated, and shriveled Y-1 cells. Cholera toxin (CT) controls (Schwarz/Mann, Orangeburg, NY) (1 ng CT/ml in PBS) were run with each assay.

RESULTS AND DISCUSSION

The study, conducted in conjunction with one of *Vibrio cholera* incidence (*31*), was initiated in Southern California in the San Diego Bay area (in July) with sampling proceeding from south to north (Fig. 1). For samples collected in California, APW only was used for enrichment. *Aeromonas* were recovered from 19% of the 219 samples and 28% of the 124 California sites with a distribution of 22, 10, and 17% in water, sediment, and shellfish, respectively (Table 1).

Recovery rates using APW were highest for the State of Oregon. *Aeromonas* were recovered from 50% of the 86 samples and 57% of the 60 sites. Distribution in water, sediment, and shellfish was 55, 40, and 33%, respectively (Fig. 2). For Washington state, recoveries were intermedi-

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TABLE 1. *Incidence of motile* Aeromonas *spp. and fecal coliforms from California estuaries*[a].

Estuary	No. of Sites Examined (%+)[b]	No. of Samples (%+)	Mean Water Temp (°C)	Mean Fecal Coliforms MPN/100 of Water	No. of Sample Type (%+) Water	Sediment	Shellfish
San Diego	26 (8)	63 (6)	26.1	71	32 (6)	26 (8)	5 (0)
Morro Bay	20 (25)	46 (15)	18.4	154	26 (19)	15 (7)	5 (20)
Moss Landing	8 (0)	11 (0)	23	NA	7 (0)	4 (0)	NA[c]
Santa Cruz	5 (100)	5 (100)	18.3	332	5 (100)	NA	NA
San Francisco Bay	21 (38)	25 (32)	22.3	192	20 (40)	2 (0)	3 (0)
Tomales-Bodega Bay	21 (19)	33 (18)	20.1	37	26 (15)	3 (67)	4 (0)
Humboldt-Arcata Bay	23 (48)	36 (31)	16.5	191	27 (30)	2 (0)	7 (43)
TOTAL	124 (28)	219 (19)			143 (22)	52 (10)	24 (17)

[a]Motile *Aeromonas* spp recovered using alkaline peptone water enrichment broth.
[b](%+), Percent positive.
[c]NA, Not analyzed.

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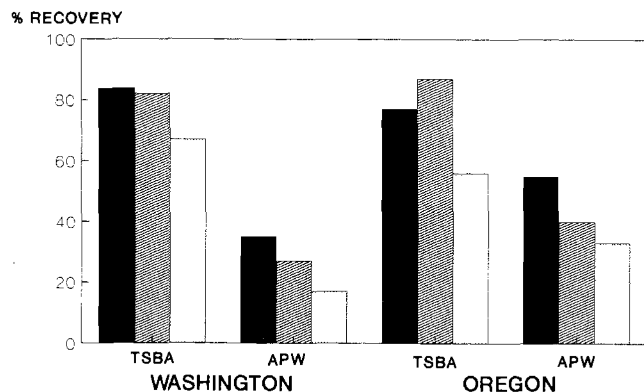

Figure 2. *Comparison of tryptic soy ampicillin broth (TSBA) and alkaline peptone water (APW) in the recovery of motile* Aeromonas *species from water, sediments and shellfish.* (▬ *water,* ▨ *sediment,* ▭ *shellfish).*

ate using APW with 31% of the 96 samples, and 43% of the 56 sites found to contain *Aeromonas* species with a distribution of 35, 27 and 17% in water, sediment, and shellfish, respectively (Fig. 2).

The lower recoveries for samples taken in California are most likely due to heavy confluent growth of other marine bacteria which occurred on the MCA and PBG agars and masked the presence of *Aeromonas*. Percent recoveries of *Aeromonas* species using APW in California, Oregon, and Washington were 20, 50, and 31, respectively (Table 2). Overgrowth by other marine organisms was not as pronounced for samples taken in Oregon and Washington, resulting in higher recoveries using APW. The presence of organisms capable of overgrowth in this enrichment scheme may be partially a function of temperature for surface water temperatures (Tables 1, 3) were highest in California (from 16.5 - 26.1°C) compared to Oregon (14.3 - 22.5°C) and Washington (10.2 - 16.3°C).

Von Gravenitz and Bucher (*40*) have recommended APW for recovery of motile *Aeromonas*, specifically at low temperatures (5°C). Our data suggest that APW is not effective for samples collected from areas where temperatures are high (Table 4). Because recoveries were low for samples from California using APW, a second enrichment medium, TSBA (in addition to APW), was used to recover *Aeromonas* from samples collected in Oregon and Washington.

Comparison of TSBS to APW for recovery of Aeromonas

Recoveries using two enrichment broths, TSBA and APW, were compared for combined samples from both States of Washington and Oregon. The incidence of *Aeromonas* using TSBA was much higher at 80% compared to 41% using APW (Fig. 2). While other investigators (*29,43*) have shown that APW significantly increases recovery of *Aeromonas* from clinical specimens, our data demonstrate that APW is not as effective for recovery of *Aeromonas* from environmental samples. Our previous studies (*1,2*) have shown TSBA to be an effective enrichment medium for isolating *Aeromonas* from environmental samples. In this study, use of TSBA consistently resulted in increased recoveries of this species from shellfish, sediment, and water compared to APW (Fig. 2).

Some differences were noted between the two plating media, MCA and PBG, in combination with either TSBA or APW (Table 2). The overall best broth-agar choice was TSBA-MCA for shellfish, sediment, and water. Isolation rates expressed as percentages were calculated for each broth-agar combination. For shellfish, sediment, and water samples (Washington/Oregon estuaries) enriched in TSBA then plated onto MCA and PBG, the respective isolation rates were as follows: 53, 82, and 77% and 50, 51, and 66%. The isolation rates were distinctly lower for APW. With APW the respective isolation rates for MCA and PBG were: 7, 23, and 42% and 12, 27, and 14%. Overall, for all samples types enriched in TSBA or APW, the respective isolation rates for MCA, PBG, and combined MCA/PBG were 75, 61, and 80% and 35, 15, and 41%.

TABLE 2. *Isolation rate (%) of individual and combined solid agar media of motile* Aeromonas *spp. from water, sediment and shellfish sources enriched in trypticase soy broth ampicillin (TSBA) and alkaline peptone water (APW).*

Coastal State	Water		Sediment		Shellfish		All Sample Types	
	TSBA	APW	TSBA	APW	TSBA	APW	TSBA	APW
Washington	N = 57[a]		N = 33		N = 6		N = 96	
MCA[b]	79[c]	30	76	24	50	0	76	26
PBG[b]	72	88	55	9	67	17	66	9
MCA/PBG[e]	84	35	82	27	67	17	82	31
Oregon	N = 62		N = 15		N = 9		N = 86	
MCA	74	53	87	22	56	13	74	43
PBG	60	19	47	44	33	7	55	20
MCA/PBG	77	55	87	40	56	33	77	50
Total	N = 119		N = 48		N = 15		N = 182	
MCA	77	42	82	23	53	7	75	35
PBG	66	14	51	27	50	12	61	15
MCA/PBG	81	45	85	47	62	19	80	41
California	N = 150		N = 76		N = 24		N = 250	
MCA		19		17		13		18
PBG		5		5		4		5
MCA/PBG		21		18		17		20
Total	N = 269		N = 124		N = 39		N = 432	
MCA		34		21		9		29
PBG		11		19		9		5
MCA/PBG		37		37		18		19

[a]No. of samples examined.
[b]MacConkey's agar.
[c]Isolation rate expressed in percent.
[d]Peptone beef glycogen agar.
[e]Combined MacConkey's agar and peptone beef glycogen agar.

TABLE 3. *Incidence of motile* Aeromonas *spp. and fecal coliforms from Washington and Oregon estuaries*[a].

Estuary	No. of Sites Examined (%+)[b]	No. of Samples (%+)	Mean Water Temp (°C)	Mean Fecal Coliforms MPN/100 of Water	No. of Sample Type (%+) Water	Sediment	Shellfish
WASHINGTON							
Willapa Bay	24 (96)	39 (92)	16.3	107	27 (93)	8 (100)	4 (75)
Grays Harbor	4 (75)	4 (75)	15.0	23	2 (50)	NAc	2 (100)
Puget Sound (North)	13 (100)	25 (100)	10.2	41	13 (100)	12 (100)	NA
Puget Sound (South)	8 (88)	16 (69)	14.2	252	8 (63)	8 (75)	NA
Samish Bay	7 (100)	12 (92)	12.9	181	7 (100)	5 (80)	NA
TOTAL	56 (95)	96 (90)			57 (89)	33 (91)	6 (83)
OREGON							
Coos Bay	21 (67)	28 (68)	14.3	46	21 (71)	4 (75)	3 (33)
Yaquina Bay	19 (100)	25 (92)	17.9	33	12 (92)	10 (100)	3 (67)
Tillamook Bay	17 (94)	29 (90)	22.5	176	25 (92)	1 (100)	3 (67)
Nehalem Bay	3 (100)	3 (100)	20.6	NA	3 (100)	NA	NA
TOTAL	60 (87)	85 (84)			61 (85)	15 (93)	9 (56F)

[a]Motile *Aeromonas* spp recovered using both tryptic soy broth ampicillin and alkaline peptone water enrichment broths.
[b](%+), Percent positive.
[c]NA, Not analyzed.

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Isolations were slightly improved by using two plating media (MCA/PBG). PBG was most effective in the isolation of *Aeromonas* specifically from samples collected where temperatures were lower.

TABLE 4. *Effects of temperature in the recovery of motile* Aeromonas *spp. from water in alkaline peptone water (APW) and tryptic soy broth with ampicillin (TSBA).*

State/Estuary	Mean Water Temp. °C	No. of Samples	% Recovery APW	% Recovery TSBA
Washington	13.7	57	35	84
Oregon	18.8	61	55	77
California				
Northern[b] Bays	19.2	78	24	NA
Southern[c] Bays	22.5	65	8	NA

[a]NA = Not analyzed.
[b]Northern Bays: Santa Cruz, San Francisco, Tomales-Bodega, Humboldt-Arcata.
[c]Southern Bays: San Diego, Morro, Moss Landing.

Effects of salinity

Aeromonas were isolated from waters with salinity ranging from 0 to 48 parts per thousand. There appeared to be a trend with densities of *Aeromonas* related inversely to salinity (Table 5). In waters with low salinity values, the probability of isolating *Aeromonas* increased. In our previous study (*2*) of a major shellfish growing area, it was demonstrated that salinity had an effect on levels of *Aeromonas*. *Aeromonas* counts were highest in river compared to bay waters. These findings are in agreement with Kaper et al. (*28*) showing the concentrations of *A. hydrophila* were inversely related to salinity. This suggests that *Aeromonas* are not truly indigenous to the marine environment but may have a transient existence after entering salt water via rivers or sewage inputs (*30,32,40,42*). In this present study, most of the tributaries drained through rural areas where farm animals and waterfowl were observed.

Aeromonas and fecal coliform levels

Aeromonas species were isolated from water samples collected at nearly every station of the Oregon and California waters. The recovery rate of *Aeromonas* for 112 water samples was 88%. The highest recovery rates were obtained at sites where the fecal coliform levels were also high (Table 6). Fecal coliform levels of water are the basis for classification of approved shellfish harvesting areas by Federal and State Agencies (*18*). The fecal coliform median or geometric mean MPN is not to exceed 14 per 100 ml of water and not more than 10% of the samples can exceed an MPN of 43 for a 5-tube dilution test (or an MPN of 49 per 100 ml for a 3-tube decimal dilution test).

The presence of fecal coliforms theoretically is correlated to the probable presence of pathogenic microorganisms in shellfish growing waters. All actual and potential growing waters are classified according to the fecal coliform levels determined by a sanitary survey to certify shellfish growing waters. Water samples collected in this present study were both from historically approved and nonapproved shellfish growing waters. A distinct pattern was evident in isolating *Aeromonas* from waters that exceeded the fecal coliform standard (Table 6). Thirty-six percent of water samples were within the acceptable standard fecal coliform range and 64% samples exceeded the standard. In water samples (N = 13) where fecal coliforms were not detected (<3 per 100 ml), 38% contained aeromonads; in samples exceeding the fecal coliform standard (>14 per 100 ml), 93% contained aeromonads. Association of levels of *Aeromonas* with fecal coliforms has been reported (*28*); however, in many cases *Aeromonas* spp. were isolated from samples collected in areas free of fecal coliforms. This suggests that *Aeromonas* are not necessarily of fecal origin but of other nonfecal origin. Several investigators have noted *Aeromonas* are naturally present in fish, reptiles, amphibians, and mammals (*6,19,23,36,44,45,47,51*). Thus, the presence of fecal coliforms is not always an adequate indicator of pathogenic microorganisms such as *Aeromonas* in shellfish waters.

TABLE 5. *Percent recovery of motile* Aeromonas *spp. from West coast water samples grouped into salinity ranges.*

Salinity Ranges (%)	STATE: Washington/Oregon No. of samples (%+)	STATE: California No. of samples (%+)
0-10	48 (96)	33 (64)
11-20	25 (88)	18 (22)
21-30	40 (85)	24 (8)
31-50	12 (50)	78 (12)

TABLE 6. *Recovery of motile* Aeromonas *spp. in marine waters (N = 112)[a] grouped into fecal coliform ranges (MPN/100 ml) used for certification of shellfish growing waters.*

MPN Range (per 100 ml)	Washington No. of samples (%+)[b]	Oregon No. of samples (%+)	Total No. of samples (%+)	No. of samples with fecal coliforms (%+)	No. of samples with aeromonas (%+)
<3.0	4 (50)	9 (33)	13 (38)	13 (12)	5 (5)
3.0 - 14	21 (90)	6 (83)	27 (92)	27 (24)	24 (21)
>14	31 (90)	41 (95)	72 (93)	72 (64)	67 (60)

[a]Total number of samples analyzed.
[b](%+), percent positive (Aeromonas detected).

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Virulence of testing isolates

A representative number of *Aeromonas* isolates from each state were tested for virulence by the mouse Y-1 adrenal cell test and the hemolysin assay. The production of cytotoxins and hemolysin by motile *Aeromonas* spp. is associated with virulence (*4,12,13,16,34,46*). Of 784 isolates (Washington N = 228, Oregon N = 375, California N = 120) tested, 91.4% were hemolytic. Heat-labile cytotoxin was found in 54% of the isolates tested (N = 224), and of these cytotoxic isolates 97% produced a hemolysin. Cells reacting in the Y-1 cell assay demonstrated a complete disruption and granulation (cell death). This activity was not altered when culture filtrates were held at 56°C for 30 min, as seen with cholera toxin giving a cytotonic response. Other investigators have tested environmental isolates of motile *Aeromonas* for toxin production (*1,8,9,28,47*). Direct correlations have also been reported between toxin production and biochemical properties such as lysine decarboxylation, acetoin from glucose, and arabinose fermentation (*8,13,28*). In our study, these relationships were not evident. For example, of 132 cytotoxin strains, 94.7% were lysine decarboxylase-negative; however, 74% were positive for the production of acetoin from glucose fermentation.

Speciation

Two hundred forty-four (31%) motile *Aeromonas* isolates were speciated. *Aeromonas caviae* (42%) predominated, followed by *A. hydrophila* (35%) and *A. sorbia* (24%). There have been few studies where environmental isolates of *Aeromonas* were speciated (*9,10,25*). Investigators (*7,14,25*) have suggested that it may be clinically important to distinguish *A. sobria* and *A. hydrophila* from *A. caviae*. Both *A. hydrophila* and *A. sobria* appear to be more inherently virulent than those organisms classified as *A. caviae*.

CONCLUSION

Motile *Aeromonas* spp. were found in estuaries of the West Coast. Results of this survey indicate that this bacterium is ubiquitious in the water column, sediment, and shellfish. The significance of aeromonads in the environment is not clearly known; however, these species do possess virulent properties. Motile *Aeromonas* spp. have received attention due to association with human disease (*15*). This was recently seen in contaminated shellfish samples implicated in outbreaks of gastroenteritis (*1*). It seems prudent to recommend to the food specialist that in cases of foodborne bacterial illness in which oysters are implicated, *Aeromonas* spp. should be included in the general screening for causative microorganisms. Selecting the most efficient isolation media is of great importance to maximize the recovery of motile *Aeromonas*. In this study, in all cases TSBA as a primary enrichment resulted in high recovery rates of this bacterium compared to those observed using APW.

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