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Aeromonas hydrophila in Shellfish Growing Waters: Incidence and Media Evaluation

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ABSTRACT

Levels of *Aeromonas hydrophila* determined for the shellfish growing area of Grays Harbor, Washington, ranged from 3 to 4600/100 g in oysters and from 3 to 2400/100 ml in water. Of isolates tested, 80% produced a hemolysin, a trait reported to correlate with enterotoxin production and pathogenicity. Two enrichment broths, Tryptic Soy Broth with ampicillin (TSBA) and Modified Rimler Shotts Broth (MRSB) were compared in combination with three solid agar media: Rimler Shotts (RS), Peptone Beef Extract Glycogen (PBG), and MacConkey's (MCA) agars. TSBA was far superior to MRSB in isolating this species from the environmental samples tested.

Aeromonads are considered indigenous inhabitants of aquatic environments *(5,6,7,12,15,24,25,35)*. They occur both in fresh and marine water environments; however, *Aeromonas* may not be truly indigenous to the marine environment, but may have a transient existence after entering salt water via river or sewage inputs *(17,24)*.

Aeromonas is a water-borne pathogen causing wound infections and possibly gastroenteritis. Wound infections have resulted after exposure to water contaminated with *Aeromonas* spp. *(10,11,13,27,33)*. Recently, *A. hydrophila* has been implicated in cases of gastroenteritis in humans after consumption of contaminated shellfish *(1,35)*. Therefore, consumption of raw or improperly cooked shellfish containing high levels of pathogenic *Aeromonas* may present a health hazard.

The distribution of *A. hydrophila* in shellfish growing waters has not been fully investigated. One of the factors in detecting *Aeromonas* spp. in shellfish growing waters successfully is the selection of a reliable enrichment medium. Numerous studies of selective media have been described for the isolation of *Aeromonas* spp. *(2,20,21,26,28,29,30,34)*. Also many of the media selective for enteric organisms also support the growth of *Aeromonas (16)*. The majority of the selective media for the isolation of *Aeromonas* has been developed for clinical studies, for which only qualitative data are provided. In order to determine levels of *Aeromonas* in foods and environmental samples, several investigators *(15,22,24)* have developed media for the quantitative detection of *Aeromonas* spp. The objectives of the investigation were to determine the prevalence of *A. hydrophila* in a shellfish growing area and to select the most reliable medium for the primary isolation of *Aeromonas* spp. from samples collected from this area.

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MATERIALS AND METHODS

Collection of samples. Samples of water (n=46) and oysters (n=23) were collected at 12 stations located in Grays Harbor Bay, Washington, over a 10-day period in December 1983 (Figure 1). Prior to this study, a preliminary study was conducted in May 1983, during which water (n=118) and oyster (n=29) from 48 stations were sampled (Figure 1). Surface water samples were collected by hand using sterile 250 ml glass bottles with a ground glass stopper (Wheaton Scientific, Millville, New Jersey). All oyster samples were collected using an oyster-tong grab sampler. Oysters were packed in sterile plastic bags. All samples were immediately placed on ice and analyzed within 3 h of collection. Analyses were conducted on site using a 32 ft. self-contained mobile microbiological laboratory. For each sampling site, temperature and salinity were measured using a hydrometer set (Kahlisco, Kahl Scientific Instrument Corporation, El Cajon, CA).

Media preparation

Trypticase Soy Broth. Trypticase Soy Broth with Ampicillin (TSBA) *(34)* was prepared according to the manufacturer's directions (DIFCO). After autoclaving at 121°C for 15 min, filter sterilized ampicillin (Sigma) was added to the broth to give a final concentration of 30 mg/liter.

*Modified Rimler Shotts Broth**. Modified Rimler Shotts Broth (MRSB) *(15)* contained in gm/L the following ingredients: maltose, 3.5; L-cysteine hydrochloride, 0.3; bile salts No. 3, 1.0; yeast extract, 3.0: sodium chloride, 5.0; and bromothymol blue, 0.03. The pH was adjusted to 7.0 and the broth autoclaved at 121°C for 15 min. After autoclaving, filter sterilized novobiocin (Sigma) was added to give a final concentration of 0.005 per liter.

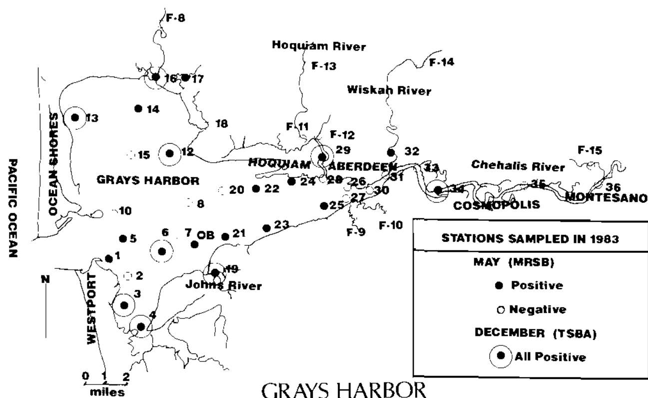

Figure 1. *Distribution of* Aeromonas hydrophila *in Grays Harbor Bay, Washington. Comparison of Modified Rimler Shotts Broth (MRSB) and Tryptic Soy Broth supplemented with Ampicillin (TBSA).*

*Note: The MRSB designation is not the original nomenclature. In Kaper, *et al. (15)* it is referred to as an enrichment broth. MRSB is basically a modification of Rimler Shotts agar *(30)*. For the sake of discussion in this report, it will be referred to as MRSB.

MacConkey's Agar (MCA). Prepared according to the manufacturer's directions (DIFCO).

Rimler Shotts Agar. Rimler Shotts Agar (RSA) *(30)* contained the following ingredients in gm/L: L-lysine-hydrochloride, 5.0; L-ornithine-hydrochloride, 6.5; maltose, 3.5; sodium thiosulfate, 6.8; L-cysteine-hydrochloride, 0.5; bromothymol blue, 0.03; ferric ammonium citrate, 0.8; sodium deoxycholate, 1.0; novobiocin, 0.005; yeast extract, 3.0; sodium chloride, 5.0 and agar, 13.5. The pH was adjusted to a pH of 7.0, the media boiled for 1 min, and cooled to 45°C before dispensing.

Peptone Beef Extract Glycogen Agar. Peptone Beef Extract Glycogen (PBG) Agar *(20)* contained in gm/L the following ingredients: peptone, 10.0; beef extract, 10.0; glycogen, 4.0; sodium chloride, 5.0; sodium lauryl sulfate, 0.1; bromothymol blue, 0.1 and agar 15.0. The media was heated to dissolve, the pH adjusted to 6.9-7.1 and autoclaved at 121°C for 15 min.

Wagatsuma agar. Modified Wagatsuma agar contained in gm/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 50 ml /L fresh human red blood cells.

Aeromonas hydrophila medium (AHM). AHM was prepared as described *(14)*.

Bacterial Analysis

Aeromonas hydrophila. Oysters were analyzed as described *(3)*. The 3-tube most probable number (MPN) method using TSBA and MRSB were used to measure the incidence of *A. hydrophila*. Appropriate dilutions of oysters and water were inoculated into enrichment broths and incubated at 35°C for 24 h. After incubation, the enrichment broth cultures from oysters were plated onto the agars RSA, PBG, and MCA. Enrichment broth cultures from water were plated onto PBG and MCA agars. Agars were incubated at 35°C for 24 h. Typical colonies were picked for multitest screening *(14)*. Strains which produced an alkaline surface, acid butt, and were motile, were considered presumptive positive and further tested for cytochrome oxidase. Identification of *A. hydrophila* strains was based on the following reactions: cytochrome oxidase (+), motility (+), mannitol fermentation (+), inositol fermentation (-), ornithine decarboxylase (-), hydrogen sulfite production in triple sugar iron (-), MacConkey growth (+), growth on 0% NaCl (+), arginine dihydrolase (+), methyl red at 35°C (+), methyl red at 26°C (±); indole production (+); and lysine decarboxylase (±). Biochemical tests were based on those included in the MICRO-IS, identification program IDDNEW [IBM 370 computer National Institute of Health] *(19)*.

Fecal coliforms and fecal streptococcus. Fecal coliforms and fecal streptococcus were estimated as described *(3)*.

Hemolysin assay. Test supernates (500 µl) of twofold dilutions in phosphate-buffered saline (PBS) were added to equal volumes of 1% washed (3x) rabbit erythrocytes (0.85% saline) in microliter trays. 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 positive. 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 and incubated at 35°C for 24 h.

Statistical analysis. Statistical analysis included comparison of means ranks by the Kruskal-Wallis nonparametric procedure *(4)* and a multiple comparison test *(23)*. Geometric means were computed from transformed counts ($\log_{10}$ - count+1).

RESULTS AND DISCUSSION

Two separate studies during May and December of 1983 were conducted to determine the incidence of *A. hydrophila* in Grays Harbor Bay, Washington. For the first study, 118 water and 29 oyster samples were collected from 48 and 9 stations respectively (Figure 1). Samples were placed in modified Rimler Shotts Broth (MRSB) and transferred to MCA for the detection of *Aeromonas. Aeromonas hydrophila* were found in 17% and 38% of water and oyster samples respectively. All stations where oysters were collected were positive for the presence of *A. hydrophila*; however, only 33% of the stations where water was collected were positive for *A. hydrophila* during the 10-day sampling period.

Modified Rimler Shotts enrichment broth did not provide suitable enrichment for *A. hydrophila* especially when large numbers of other competing organisms were also present.

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This was initially observed by Kaper et al. *(15)*. To circumvent this problem, other enrichment broths in use were tested, and of those tested, TSBA had the highest recoveries of *A. hydrophila (1)*. Therefore, to improve recovery rates, a second study (December 1983) was conducted using TSBA enrichment broth. TSBA was compared with MRSB in conjunction with up to 3 agars for the detection of *Aeromonas* from the marine environment.

For the second study, samples from 12 stations were analyzed (Figure 1). Using TSBA, *A. hydrophila* ranged in oysters and water from 30 to 4600/100 g and 3.0 to 2400/100 ml respectively; whereas using MRSB, levels of *A. hydrophila* ranged from $\leq$ 3 to 73/100 g and $\leq$ or 210/100 ml in oysters and water respectively (Tables 1 and 2). All 12 stations were positive for *Aeromonas* from water and oyster samples. The increase in recovery observed in the second study was attributed to the TSBA enrichment broth.

A single observation was reported for each broth-agar combination. The MPN/100 g is shown in Table 1 for oysters and the MPN/100 ml for water in Table 2. Geometric means were calculated for each broth-agar combination. For oysters enriched in MRSB and then plated onto MCA, PBG, and RSA, the geometric mean was 2.1, 2.1, and 4.2 respectively. Whereas with TSBA, the geometric mean was 69, 97 and 51 respectively (Figure 2). The geometric means were lower for water samples. With MRSB, the geometric means for MCA and PBG were 3.8 and 5.0 respectively, and in TSBA, counts again were

TABLE 1. *Comparison of enrichment broths and plating agars on the recovery of* A. hydrophila *in oysters from Grays Harbor, Washington.*[a]

Sample		TSBA[b]			MRSB[b]		
number	Station	MCA[c]	PBG	RS	MCA	PBG	RS
1	3	62	0	36	30	0	0
2	3	110	93	0	0	0	30
3	3	61	750	210	0	0	0
4	3	73	150	110	0	36	30
5	4	36	36	36	0	0	0
6	4	210	280	30	0	0	0
7	4	210	430	91	0	0	0
8	4	0	30	30	0	0	0
9	4	36	91	36	0	0	0
10	6	94	160	62	0	0	0
11	60B	210	91	0	36	30	0
12	60B	200	110	110	0	0	0
13	60B	36	36	36	30	30	30
14	12	72	92	92	30	30	30
15	12	92	90	120	0	0	61
16	12	290	360	200	0	0	73
17	12	150	200	73	0	0	0
18	12	110	110	93	0	0	0
19	13	360	4600	4600	30	30	72
20	13	290	290	290	0	0	0
21	13	30	0	36	0	0	0
22	13	0	220	0	0	0	0
23	13	62	30	73	0	0	30

[a]Each value is a single observation per 100 g.
[b]Enrichments: TSBA (Trypticase Soy Broth Ampicillin)
MRSB (Modified Rimler Shotts Broth)
[c]Solid Agars: MCA (MacConkeys Agar)
PBG (Peptone Beef Extract Glycogen Agar)
RS (Rimler Shott Agar)

TABLE 2. *Comparison of enrichment broths and plating agars on the recovery of* A. hydrophila *in water from Grays Harbor, Washington.*[a]

Sample		TSBA[b]		MRSB	
number	Station	MCA[c]	PBG	MCA	PBG
1	3	3	3	0	0
2	3	0	3.6	0	0
3	3	0	3.6	3.6	23
4	12	9.3	13	7.2	6.1
5	12	0	43	0	0
6	12	9.3	15	3.6	3.6
7	12	44	44	3	20
8	12	0	3.6	0	0
9	13	3	7.3	7.3	6.2
10	13	21	11	0	3
11	13	23	21	0	7.3

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12	13	0	43	15	93
13	13	7.3	21	3.6	15
14	13	9.1	3.6	3	3
15	16	240	240	3	3
16	16	240	240	6	6
17	16	15	43	3	0
18	16	15	15	3	3
19	16	15	11	6	9.2
20	16	1100	1100	11	36
21	16	44	24	9	0
22	16	15	15	0	0
23	19	9.1	43	9.3	210
24	19	16	16	6	6
25	19	3.6	7.3	0	0
26	19	3.6	15	0	0
27	19	6.2	11	0	3
28	19	24	290	6.1	15
29	19	7.2	15	11	6.1
30	19	460	44	6.1	6.1
31	19	28	1100	3	3
32	29	3.6	7.3	11	3.6
33	29	9	20	0	3.6
34	29	9.1	3.6	9.2	6.1
35	29	9.1	9.1	0	0
36	29	3	6.2	0	0
37	29	6.1	11	0	0
38	29	3	21	0	0
39	29	3.6	0	7.2	0
40	34	20	460	11	6.2
41	34	24	29	12	12
42	34	3	7.3	3	6
43	34	15	27	9.2	11
44	34	19	2400	3	6
45	34	19	29	16	9
46	34	24	44	6.1	9.3

[a]Each value is a single observation per 100 ml.
[b]Enrichments: TSBA (Trypticase Soy Broth Ampicillin)
MRSB (Modified Rimler Shotts Broth)
[c]Solid Agars: MCA (MacConkey's Agar)
PBG (Peptone Beef Extract Glycogen Agar)
RS (Rimler Shott Agar)

significantly higher, 12 and 25 respectively (Figure 3). Since duplicate observations were not recorded for each sample, an analysis of variance was not performed. Instead, the broth-agar means were compared by the Kruskal-Wallis *(4)* nonparametric procedure. The geometric mean as shown in Figures 2 and 3 differed significantly ($P = 0.05$) for both oyster and water samples. A multiple comparison test *(4)* demonstrated that the TSBA broth had significantly higher ($P = 0.05$) recovery than MRSB in both oysters and water.

No significant differences were noted among the three plating media, RSA, MCA, and PBGA, in combination with either TSBA or MRSB. The best broth-agar choice is TSBA-PBGA for both water and oyster samples. The PBGA media was also most productive for MRSB enrichment. Although PBGA did show better recoveries than RSA and MCA, RSA gave fewer false positives, and colony identification was easier on RSA.

The sensitivity of the three media for the isolation of *Aeromonas* spp. from oyster and water sources is shown in

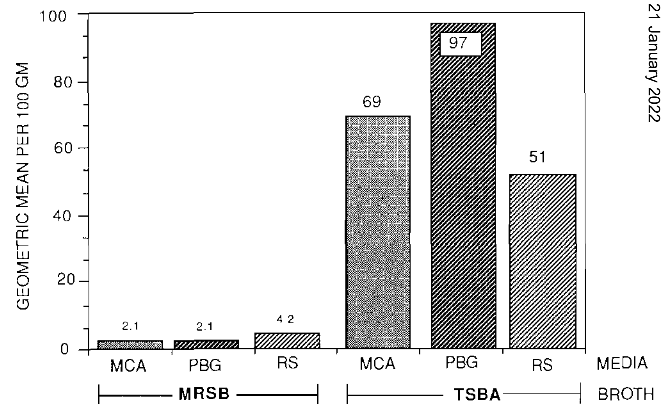

Figure 2. *Numbers of* A. hydrophila *in oysters as determined by two different broth-agar combinations. (a=MCA MacConkey's Agar; b=PBG, Peptone Beef Extract Glycogen Agar; c=RS, Rimler Shott Agar; d=MRSB, Modified Rimler Shotts Broth; e=TSBA, Trypticase Soy Broth Ampicillin).*

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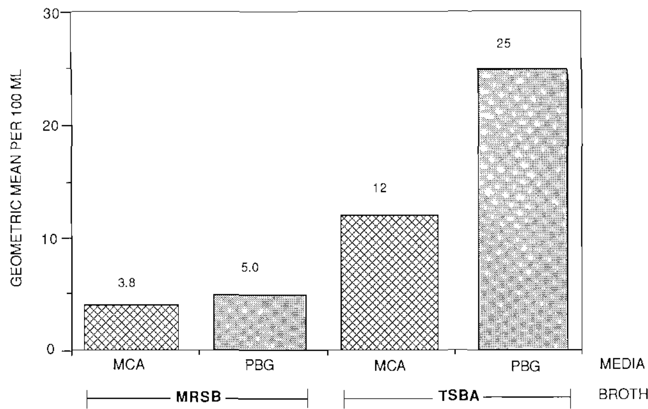

Figure 3. *Numbers of* A. hydrophila *in water as determined by two different broth-agar combinations. (a=MCA, MacConkey's Agar; b=PBG, Peptone Beef Extract Glycogen Agar; c=MRSB, Modified Rimler Shotts Broth; d=TSBA, Trypticase Soy Broth Ampicillin).*

Table 3. The rate of isolation in using 2 and 3 combination media were improved. The most effective combination of two media which gave an isolation rate of 100% was PBG combined with either MCA or RSA.

The efficacy of the multitest screening *A. hydrophila* medium (AHM) *(14)* was also determined. A total of 2,611 cultures were isolated from oysters and water. The isolates were first screened with cytochrome oxidase. Of the 2611 isolates, 54% (1,396) were positive for oxidase. Of the oxidase positive isolates, 76% (1,065) gave typical reactions in AHM. Ninety-five percent of the 1,065 isolates were confirmed as *A. hydrophila*; therefore, the AHM was an effective screening medium.

A seasonal cycle was partially observed for *A. hydrophila* in Grays Harbor (Table 4). Counts were lower in December, where in May, counts increased. Differences of levels are probably due to the influence of temperature. This observation has been reported by numerous investigators *(7,8,15,35)*.

Additional data (fecal coliforms, fecal streptococcus, salinity, temperature) were collected to identify possible correlations of these with *Aeromonas hydrophila* levels. Correlations among variables were computed with and without regard to stations. None of the linear correlations were significant at the P = 0.05 level. However, a trend was identified as to *Aeromonas* levels correlating with location. *A. hydrophila* counts were highest in rivers compared to bay waters in all cases. It appears that salinity and/or temperature would have an effect on levels of *Aeromonas* found; however, insufficient data were collected to establish significant correlation with these parameters.

Forty-nine isolates (31 oyster, 18 water) were randomly selected and tested for hemolytic activity. Eighty (80) percent of the strains tested produced a hemolysin, a trait reported to correlate with enterotoxin production and pathogenicity

TABLE 3. *Isolation rate of individual and combined solid agar media of* Aeromonas hydrophila *from oysters and water sources enriched in Trypticase Soy Broth Ampicillin.*[a]

Medium	Oysters	Water
MCA[c]	91.3	89.1
PBG[d]	91.3	98.0
RSA[e]	87.0	ND[b]
MCA/PBG	100	100
MCA/RSA	95.7	ND
PBG/RSA	100	ND
MCA/PBG/RSA	100	ND

[a]Percent positive data derived from that presented in Tables 1 and 2.
[b]Not done.
[c]MCA (MacConkey's Agar).
[d]PBG (Peptone Beef Extract Glycogen Agar).
[e]RSA (Rimler Shott Agar).

TABLE 4. *Seasonal distribution of* Aeromonas hydrophila *in oysters from Grays Harbor, Washington.*[a]

	May 1983	December 1983[b]
Number of samples	29	23
Positive (%)	38%	22%
MPN count range/100 g	0-930	0-36
Geometric mean	5.7	2.1
Water-Temperature mean (°C)	14.3	7.1

[a]Levels determined from MRSB/MCA.
[b]Data derived from Table 1.

(1,6,9,18,31,32). No further pathogenicity testing was conducted. No correlations were found between hemolysin strains and biotype.

In this study, *A. hydrophila* were distributed throughout an estuarine environment. In all cases, TSBA as a primary enrichment medium demonstrated higher recovery rates of *A. hydrophila* compared to those observed using MRSB.

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