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Seasonal Distribution of *Vibrio parahaemolyticus* Serotypes Along the Oyster Beds in Hiroshima Coast

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Abstract The seasonal distribution of *Vibrio parahaemolyticus* serotypes was studied from April 1988 to March 1989 in the coast of Hiroshima Prefecture where oyster culture is carried out. The influence of various physico-chemical parameters and microbial indicators of pollution on the distribution of this halophile was investigated. The organism was frequently isolated in all the study area during the warmer months and its density declined towards the winter season remaining undetectable in months of January through March. The incidence and counts of *V. parahaemolyticus* were consistently higher in estuarine area (salinity 15.5‰) with the highest incidence of various serotypes. Totally 41 serotypes were identified whereas 46.1% of the total isolated strains (317) in this study were untypable. Some serotypes were constantly isolated from a particular area whereas some were found only in oysters or sediments but not in the water column of the same area. All the strains were Kanagawa phenomenon-negative. As some Kanagawa phenomenon-negative strains have been reported to be pathogenic, the presence of a diverse number of *V. parahaemolyticus* serotypes isolated in an area which is economically important in oyster culture should be of concern to health authorities.

INTRODUCTION

Since the famous Shirasu food poisoning in Japan during 1950, *Vibrio parahaemolyticus,* the causative agent has achieved a world wide attention in public health circles (JOSEPH *et al.* 1983). The isolation of this organism in diarrheal cases (HARVEY *et al.* 1987), sea foods (BINTA and NYAGA 1982, SCHANDEVYL *et al.* 1984) and the marine environs as reported by BAROSS and LISTON (1970), KANEKO and COLWELL (1973) and VENKATESWARAN *et al.* (1989b), has prompted more research on the organisms epidemiology, pathogenicity and ecology in many parts of the world. Recent studies suggest that *V. parahaemolyticus* also occurs in fresh water environments (BOCKEMÜHL *et al.* 1986, VENKATESWARAN *et al.* 1989a) and fish (SARKAR *et al.* 1983 and 1985). More than 50% of the reported enteritis cases in Japan are caused by this organism (JOSEPH *et al.* 1983). *V. parahaemolyticus* food poisoning is characterized by severe diarrhea with bloody stool which can sometimes be fatal (BLAKE *et al.* 1981). Although there are conflicting reports as to the role of this bacteria in fish diseases, it has been reported to cause death in shrimps at the Gulf of Mexico (VANDERZANT and NICKELSON

1973).

The pathogenicity of *V. parahaemolyticus* is associated with the Kanagawa phenomenon (Kp); demonstrated by human red blood cell hemolysis in Wagatsuma agar (Sakazaki *et al.* 1968). Epidemiology surveys have revealed that almost all clinical isolates are Kp-positive while their environmental counterparts are almost invariably Kp-negative (Sakazaki *et al.* 1968). Recently the Kp-negative strains have been incriminated in food poisoning cases and they have also been found capable of producing thermostable direct hemolysin (Honda *et al.* 1988). The potential danger posed by the presence of *V. parahaemolyticus* in the environment should not be ignored (Sarkar *et al.* 1987). Therefore, in considering the pollution of marine and estuarine environment, it is imperative to monitor the presence of this bacteria. Oyster culture is an important industry around Hiroshima coastal area and account for 85% of the Japan oyster culture output. Oyster seeding is done near the coastal line and is prone to pollution from nearby residential area and the in-flowing rivers. The current research was carried out to check the quality of water around the oyster seed-beds with special emphasis on the distribution of *V. parahaemolyticus.* In addition, environmental parameters were monitored to relate with the *V. parahaemolyticus* incidence.

MATERIALS AND METHODS

Area under study

Hiroshima Bay is located on the eastern side of Hiroshima city and is a part of the Seto Inland Sea (Fig. 1). Five stations were selected along the oyster seed-beds. Station 1 is nearest to the Hiroshima Port having the influence of Hiroshima Bay, stations 2 and 3 are near to residential areas, station 4 is away from the Port and residents while station 5 is at an estuarine area receiving domestic effluents.

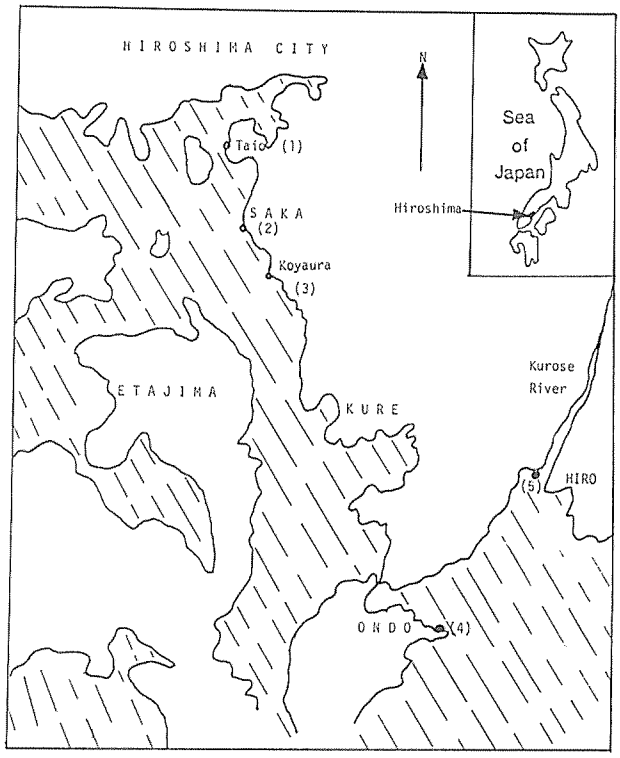

Fig. 1. Location of the sampling stations along the Hiroshima coastal area.

Sample collection

Surface water samples were collected once a month in sterile polyethylene bottles. Sediment samples were taken seasonally from station 1 and 4. Sediment (200 g) was scooped from near shore using the Shipex grab and aseptically tranferred to a presterilized container. Oyster samples were acquired from Hiroshima Prefecture Fisheries Research Station (HPFRS) during

winter months, November 1988 through March 1989. Opened oysters were purchased from a local supermarket which were already packed and certified for raw consumption. All the samples were transferred to the laboratory immediately and various bacteriological examinations were carried out within 6 hours.

Bacteriological analyses

Total viable counts (TVC) were determined using the 3-tube MPN method in nutrient broth for water and sediment samples while aerobic plate count (APC) was used for oyster samples. Appropriate samples (50 g) of oyster were aseptically weighed and transferred into 450 ml of 0.1% peptone water and homogenized by blender. The homogenate equivalent to 1 g (10 ml), was serially diluted for microbial analysis (SAKAZAKI *et al.* 1986). The sediment sample (20 g) was weighed into 180 ml of 0.1% peptone water and thoroughly stirred and after 20 min the clear supernatant was used for bacteriological analysis. Total coliforms (TC) and fecal coliforms (FC) were determined as already reported (NEUFELD, 1984). Enterococci was estimated using Azide Citrate broth as described elsewhere (HORIE *et al.* 1971).

Isolation of *Vibrio parahaemolyticus* was performed as described by KANEKO and COLWELL (1973), and later modified by VENKATESWARAN *et al.* (1989b). The membrane filter (0.45 μm, Toyo Roshi Co., Japan), MPN procedure using alkaline peptone water as the enrichment medium was followed as described elsewhere (VENKATESWARAN *et al.* 1989b). Typical green colonies were picked and transferred to the VP medium (KAPER *et al.* 1980). The alkaline slant and acid butt producing organisms in VP medium were regarded as the presumptive *V. parahaemolyticus* and further biochemical tests were carried out to confirm the species (VENKATESWARAN *et al.* 1989c, WEST and COLWELL 1984). All the *V. parahaemolyticus* strains were screened for Kanagawa phenomenon reaction using the Biken agar (sodium chloride 4%, di-sodium hydrogen phosphate 3%, peptone 3%, glucose 0.5%, washed human red blood cells 5%, agar 1.5%, pH 7.3). Kanagawa phenomenon reaction was determined by beta hemolysis on the Biken agar after 24 h incubation at 37°C. Serotyping was carried out using the commercially available O and K serum (Denka Seiken, Japan) by slide agglutination. Cells grown overnight on brain heart infusion agar supplemented with 3% sodium chloride were harvested in 2 ml sodium chloride-glycerin buffer and autoclaved at 121°C, one hour for the O antigen. Typing for K antigen was done directly with life cells (SAKAZAKI and SHIMADA 1978).

Water temperature and pH were measured (Horiba pH meter D-12, Japan) while the salinity was estimated by salinometer NS-3P (Merbabu Trading Co., Japan). Chemical oxygen demand (COD) was also determined. Simple product moment correlation coefficients were performed on EPSON-PC 286LE computer, using Japan MICOM Institute program (Tokyo, Japan). The analysis were carried out as per the instructions of the manufacturer.

RESULTS

The range of variations in environmental parameters at various stations was given in Table 1. Surface water temperature at all stations varied between 9.9°C and 29.5°C, with the highest temperatures in August and lowest in February. Salinity was relatively high (>24.0‰) in four of the five stations with no major seasonal fluctuations. Station 5 which

is an estuarine region exhibited salinity changes according to seasons; high in winter (>26.0‰) and low (<16.0‰) in warmer months and this could be due to the flood water in warmer months. The pH, COD and TVC did not show any seasonal pattern.

Monthly distribution of bacterial indicators of pollution was as shown in Fig. 2. Enterococcal counts were low and most of the times remained undetectable during the present study. TVC, TC and FC were high in months of July, October and January in all the stations.

The monthly incidence of *V. parahaemolyticus* in various stations was as depicted in Fig. 3. The halophile was isolated in all the stations investigated. Seasonal distribution of *V. parahaemolyticus* exhibited a peak in summer irrespective of the stations examined, with a very high population during August and September (Fig. 3). *V.*

Table 1. Yearly mean and standard deviation (±) of abiotic factors in water samples.

Station	Temperature (°C)	pH	COD (ppm)	Salinity (‰)
1	18.30±5.40	7.87±0.67	0.77±0.53	24.3±0.39
2	18.29±5.40	8.03±0.74	0.95±1.00	26.3±0.25
3	18.11±5.60	7.86±0.76	0.75±0.59	24.3±0.37
4	18.51±5.40	8.06±0.62	0.55±0.50	28.6±0.08
5	19.49±6.50	8.01±0.68	1.11±0.46	16.8±0.84

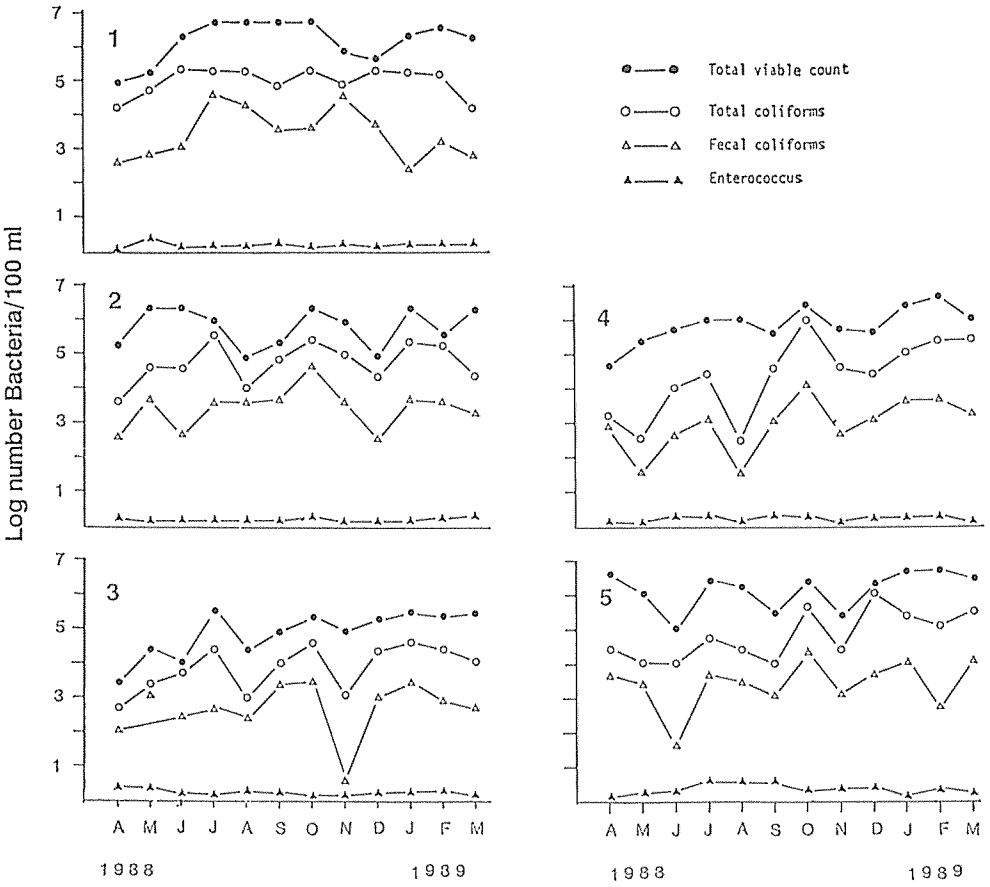

Fig. 2. Monthly distribution of bacterial indicators of pollution.

Table 2. Seasonal variation in the densities of *Vibrio parahaemolyticus* at various stations*.

Station / Season	1		2	3	4		5
	Water	Sediment	Water	Water	Water	Sediment	Water
Spring (Apr.-Jun.)	1.90	0.40	0.66	1.01	ND	ND	1.42
Summer (Jul.-Sep.)	4.93	2.40	3.57	3.45	2.78	1.10	4.29
Autumn (Oct.-Dec.)	2.94	0.21	1.15	1.94	1.06	0.20	2.44
Winter (Jan.-Mar.)	ND	ND	ND	ND	ND	ND	ND
Yearly mean	2.44	0.75	1.35	1.60	0.96	0.33	2.13

* Arithmatic mean. Bacterial counts are expressed as log number of MPN/100 ml for water and MPN/100 g for sediment. ND: not detected.

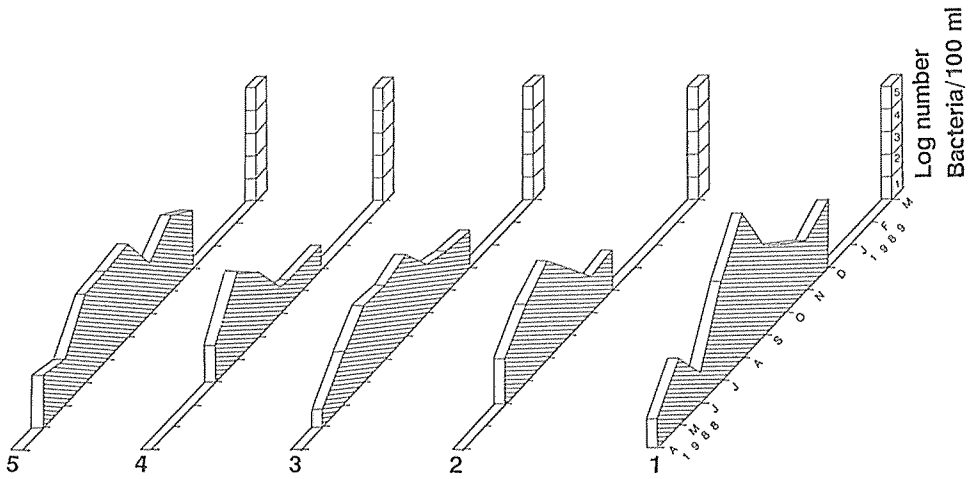

Fig. 3. Monthly distribution of *Vibrio parahaemolyticus* in various stations.

parahaemolyticus was not detectable in water and sediment samples in all the five stations during the winter months of January through March (Fig. 3 and Table 2).

High densities of bacterial indicators of pollution as well as *V. parahaemolyticus* were seen in stations 1 and 5 and these microbial population were at minimum in station 4 among the various stations studied. The other two stations (2 and 3) were moderately polluted in terms of pollution indicator bacterial populations (Fig. 2).

Oyster samples procured from HPFRS and a nearby supermarket were examined during winter months starting from November 1988 through March 1989. Only 2 out of 8 samples (25%) were positive and none of the oyster samples purchased from the supermarket were observed to have *V. parahaemolyticus*.

Monthly variation in various serotypes were as shown in Table 3. No single serotype was continuously isolated throughout the period of the investigation. However, the serotype O10:K66 was recorded mainly during August through December irrespective of the stations examined and was the predominant serotype in this study. The distribution and frequency of various *V. parahaemolyticus* serotypes isolated from different sources were

Table 3. Distribution of *Vibrio parahaemolyticus* serotypes in various stations.

Station / Months	1		2	3	4		5	
	Water	Sedement	Water	Water	Water	Sedement	Water	Oyster
April	O4:K49 O4:K53	O4:K49 O4:K53	UT	UT	UT	UT	UT	ND
May	O4:K53	ND	O4:K42 O6:K18	O4:K42	UT	ND	O4:K34	ND
June	O1:K26 O1:K32 O1:K56 O4:K42	ND	O1:K26 O1:K32 O1:K41 O3:K6 O6:K18	O1:K32 O2:K28 O4:K34 O4:K42	O1:K32 O4:K42	ND	O6:K18	ND
July	O4:K34	O4:K42	UT	O3:K45	UT	O4:K42	O1:K25	ND
August	O4:K34 O4:K67	ND	O2:K3	O4:K55 O5:K50 O11:K51	O5:K50	ND	O1:K69 O4:K63 O10:K66	ND ND
September	O4:K11 O4:K13 O4:K34 O4:K42 O4:K63 O4:K67	ND	O1:K32 O3:K45 O7:K19 O11:K51	O3:K45 O5:30 O10:K66	O10:K66	ND	O1:K32 O4:K49 O4:K63 O10:K19 O10:K52 O10:K66 O11:K51	ND
October	O4:K63 O11:K40	O1:K69 O3:K45 O3:K48 O4:K55 O4:K63 O8:K39	O11:K51	O10:K66 O11:K51	UT	O1:K69 O3:K31 O3:K54 O4:K63 O5:K15 O5:K17 O10:K52 O10:K66	O3:K45 O3:K54 O4:K9 O4:K49 O5:K17 O5:K30	ND
November	O3:K48 O10:K66	ND	O10:K66	UT	O10:K66	ND	O2:K28 O5:K30 O5:K47 O8:K39 O10:K66	O2:K3 O2:K28 O3:K45 O5:K60 O9:K23 O4:K63
December	O2:K28	O3:K46	O2:K28	UT O5:K17	O3:K48	O3:K65	O9:K44 O10:K66	
January	Nil	ND	Nil	Nil	Nil	ND	Nil	Nil
February	Nil	ND	Nil	Nil	Nil	ND	Nil	Nil
March	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil

UT: Untypable with any of the known K antigens.
Nil.: Not detected
ND: Not done.

as shown in Table 4. Station 5 had the highest isolates, 82 strains (25.9% of the total) 32 of which were typable, followed by station 1 (66 strains with 39 typable strains) and station 3 (43 strains with 24 typable strains).

Table 4. Distribution and frequency of occurrence of serotypes of *Vibrio parahaemolyticus* from various stations and oyster samples.

Station / Serotypes	1		2	3	4		5		
	Water	Sediment	Water	Water	Water	Sediment	Water	Oyster	Total
O1:K25							1		1
O1:K26	1		1						2
O1:K32	10		7	2	1		1		21
O1:K41			1						1
O1:K56	2								2
O1:K69		2				1	2		5
O2:K3			1					2	3
O2:K28	1			2			1	3	7
O3:K6			1						1
O3:K31						1			1
O3:K45		2	1	2			1	1	7
O3:K48	3	2				1			6
O3:K54						1	1		2
O3:K65							1		1
O4:K9							1		1
O4:K11	1								1
O4:K13	2								2
O4:K34	3			2			1		6
O4:K42	2	1	1	4	1	4			13
O4:K49	2	3					2		7
O4:K53	4	2							6
O4:K55		1		3					4
O4:K63	3	1				1	3	1	9
O4:K67	1								1
O5:K15						1			1
O5:K17				2		3	1		6
O5:K30				1			2		3
O5:K47							1		1
O5:K50				1	2				3
O5:K60								2	2
O6:K18			2			2			4
O6:K46			1						1
O7:K19			1						1
O8:K39		1			1				2
O9:K23								2	2
O9:K44								1	1
O10:K19							1		1
O10:K52						1	1		2
O10:K66	3		2	2	6	1	9		23
O11:K40	1								1
O11:K55			2	3			2		7
Typable	39	15	21	24	11	17	32	12	171
Untypable	27	9	13	19	10	10	50	8	146

Table 5. Correlation coefficient between *Vibrio parahaemolyticus* and biotic and abiotic factors*

Station	Temp.	pH	COD	Sal.	Rain.	TVC	TC	FC	ENT
1	0.878#	0.198	0.248	0.128	−0.304	0.171	0.293	0.743×	−0.309
2	0.912#	0.175	0.654×	−0.611	−0.286	−0.244	0.107	0.291	−0.323
3	0.932#	−0.080	0.544	0.030	−0.266	−0.069	−0.012	0.004	−0.167
4	0.677×	0.064	0.777×	−0.834#	−0.336	0.058	−0.240	−0.363	0.068
5	0.839#	−0.340	0.195	−0.477	−0.188	−0.482	−0.351	−0.103	−0.324

* Abbreviations: Temp., Temperature; COD, Chemical oxygen demand; Sal., Salinity; Rain., Rainfall; TVC, Total viable count; TC, Total coliforms; FC, Fecal coliforms; and ENT, Enterococci.

\# Significant at 99%.

× Significant at 95%.

Out of 317 strains, 171 (53.9%) were typable for K antigen while the rest were not typable but all the strains could be typed for O antigen. The number of individual serotypes recorded in this study were 41. The incidence of various serotypes varied from stations (Table 4). The freshwater influenced stations (1 and 5) which are eutrophic, experienced high numbers of serotypes (18 in station 5 and 15 in station 1 water samples). The moderately polluted stations (2 and 3) had 12 and 11 serotypes respectively whereas the water samples of the station which was less polluted (station 4) had only 5 different serotypes. The sediment samples of station 4 yielded 11 different serotypes as compared to 5 from water samples (Table 4). The sediment samples examined from station 1 and station 4 had 4 common serotypes and 11 different ones. Some serotypes found in water samples were not seen in the sediment samples of the same stations and vice-versa.

Table 5 shows the correlation coefficient between *V. parahaemolyticus* and other biotic and abiotic factors. Statistical analyses did not show any relationship between *V. parahaemolyticus* and other bacterial flora in all the stations except station 1 where FC showed positive correlation. The pH range from 6 to 9 in all the stations with no apparent changes with seasons. No relationship between the pH and the distribution of *V. parahaemolyticus* was found. COD exhibited appreciable differences between the stations but no discernible seasonal patterns were seen. COD had positive correlation with the distribution of the halophile in stations 2 and 4 whereas salinity had negative correlation in the same stations. Rainfall, though higher in winter and spring, did not affect the distribution of *V. parahaemolyticus*.

DISCUSSION

The ecological studies on the distribution of *V. parahaemolyticus* narrated a clear temperature dependent seasonal distribution in estuarine and marine environment (Colwell 1977, Kaneko and Colwell 1973 and 1975, Molitoris *et al.* 1985, Thompson *et al.* 1976a). However the halophile was isolated without any interruption in nutrient enriched coastal area (Venkateswaran *et al.* 1989b). This organism was reported to be in high numbers during summer months and declined gradually towards winter in the majority of the field studies carried out in many parts of the world. The results of our study showed the same pattern (Fig. 3). There are reports on the attachment or adsorption behavior of *V. parahaemolyticus* with a variety of marine organisms from microscopic plankton communities

(KANEKO and COLWELL 1973, SARKAR *et al.* 1983, VENKATESWARAN *et al.* 1989b) to fish (SAKAR *et al.* 1985) when the surrounding conditions are unfavorable. The cycle is completed with an over-wintering process in sediments by these organisms (KANEKO and COLWELL 1978). This over-wintering process theory was contradicted by many researchers who observed the rich organic matter suspended in the water column might have helped in the over-wintering process of *V. parahaemolyticus* (WATKINS and CABELLI 1985).

By integrating the data obtained on the occurrence of *V. parahaemolyticus* serotypes in sea water, sediment and oysters during the present study (Table 4), a clear idea on the distribution of this organism emerges. This trend is in agreement with other researchers on the ecology of this organism (KARUNASAGAR *et al.* 1984, THOMPSON *et al.* 1976a). Sudden disappearance from the water column, sediments and oysters in months of January through March 1989 is primarily due to the low temperature. However, attachment of this bacteria to suspended and sinking particles (VENKATESWARAN *et al.* personal communication) should not be ruled out.

The temperature dependent seasonal distribution was discernible in all the samples analysed. The statistical analyses also revealed a significant positive relationship between *V. parahaemolyticus* densities and temperature. The simple correlation coefficient showed that COD exhibited positive relationship with these organisms in clean marine environment (station 4) and in the moderately polluted stations (2 and 3). The highly polluted environment (station 5) have no relationship with any of the environmental parameters analysed. However the relationship with the FC in station 1 suggests that the domestic effluents might have carried *V. parahaemolyticus.*

An interesting phenomenon noticed in this study was the negative statistical relationship between salinity and *V. parahaemolyticus.* These relationship was significant in stations 2 and 4. This may be attributed to the fact that the fresh water carried high nutrients which in turn enhanced the proliferation of *V. parahaemolyticus* in coastal area (VENKATESWARAN *et al.* 1989b). Such relationship was reported by WATKINS and CABELLI (1985). In addition, the highest *V. parahaemolyticus* incidence was reported in August during which the lowest salinity was recorded. This could be due to the flood freshwater which altered the sediment profile of the investigated environment. The turbulence caused by the flood might have released the high organic content from the sediment into the water column which subsequently proliferates the growth of the bacteria (VENKATESWARAN *et al.* 1989b).

Table 4 shows a clear distribution of serotypes within the sampled area and oysters. The organism was neither detected in sediment during winter nor in the water samples of the two stations examined. The lack of similarity in the incidences of serotypes between water and sediment samples contradicted the established over-wintering process of *V. parahaemolyticus* during winter conditions. The over-wintering process might be due to the attachment or adsorption phenomena with the microscopic zooplakton communities or the suspended and sinking organic matter which forms a suitable microbiota for the growth of *V. parahaemolyticus* during unfavorable conditions. MOLITORIS *et al.* (1985) concluded that the untypable environmental strains could not allow correlation of serotypes with geographical distribution or specific environmental parameters. A fair correlation can not be derived in this study also since 146 (46.1%) strains were untypable and the typable ones

were fairly distributed in all the stations.

Lack of correlation between bacterial indicators of pollution with *V. parahaemolyticus* shows that they are not useful indicators as to the presence of this pathogen in sea water and marine foods. This study agrees with the work of Hackney *et al.* (1980b) and Thompson *et al.* (1976a) who reported on the lack of significant positive correlation between the number of *V. parahaemolyticus* and bacteriological indices. Whereas oyster samples from the naturally growing oysters were positive for *V. parahaemolyticus,* opened and packed oyster samples from supermarket were all negative for the halophile. This could be due to the washing with fresh water or heat shock treatment done to oysters to facilitate easier opening of the shell (Thompson *et al.* 1976b).

All the strains (317) isolated in this study were Kp-negative. This is in agreement with many workers that almost all environmental strains are Kp-negative (Baross and Liston 1970, Binta and Nyaga 1982, Molitoris *et al.* 1985, Sarkar *et al.* 1985). A positive Kanagawa phenomenon is highly correlated with virulence in *V. parahaemolyticus* (Honda *et al.* 1980, Peters *et al.* 1982). However some Kp-negative strains have been incriminated in the diarrheal cases (Harvey *et al.* 1987, Honda *et al.* 1988). The Kanagawa phenomenon is not the only criteria for the pathogenicity of this bacterium. The Kp-positive phenomenon is not restricted to *V. parahaemolyticus* and it has been reported in other marine vibrios (Peters *et al.* 1982). Karunasagar *et al.* (1984) have reported the enhancement of *V. parahaemolyticus* virulence by lysed cell erythrocyte factor and iron regardless of the Kp phenomenon characteristic.

Cell adherence system as a method of differentiating virulent and avirulent strains of *V. parahaemolyticus* has been proposed by Hackney *et al.* (1980a), while Boutin *et al.* (1979), demonstrated that *V. parahaemolyticus* was capable of penetrating lamina propria of rabbit ileal villi causing varying tissue changes regardless of their Kanagawa phenomenon characteristic. Therefore until the genetic, ecology and epidemiological relationship of Kp-positive and Kp-negative *V. parahaemolyticus* strains is properly established, the potential danger posed by environmental isolates can not be ignored regardless of their Kanagawa phenomenon characteristic.

The ubiquity of this bacterium observed in the area under the study which is used for oyster farming as well as for recreation purposes indicates a public health problem. The present study also provides additional evidence that *V. parahaemolyticus* is indigenous to estuarine and coastal regions.

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広島県沿岸部のカキ養殖場における腸炎ビブリオ各種血清型の季節分布

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1988年4月から，1989年3月までの1年間にわたって，広島県沿岸部のカキ養殖海域5地点から表層水，セジメントおよびカキ（冬季間のみ）を採取し，腸炎ビブリオの季節分布を調べ，分離菌の血清型別を行った。さらに物理的化学的各種因子の影響と汚染指標菌との関係についても検討した。

腸炎ビブリオは夏季にはすべての地点で多数検出された。しかし，冬季になるに従って減少し，1～3月には検出されなくなった。腸炎ビブリオの出現と菌数は河口域地点で多く，出現する血清型も多かった。分離菌317株のうち，53.9%は型別可能で41の血清型に分類されたが，残りは型別できなかった。

分離株を同定の結果，地点によって常時出現する血清型とセジメントやカキからのみ分離されて，同地点の海水から出現しないような血清型もみられた。

分離した腸炎ビブリオ株はすべて神奈川現象陰性株であった。しかし，陰性株の中にも病原性を示すものがあるとの報告がみられることから，カキ養殖場における腸炎ビブリオの検査は重要である。