Epidemiol. Infect. (1992), **108**, 155–163
Printed in Great Britain

A 2-year survey of the prevalence of enteric viral infections in children compared with contamination in locally-harvested oysters

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(*Accepted 17 June 1991*)

SUMMARY

We studied, for two years, the prevalence of indigenous human enteric viruses in wild oysters gathered each month from the bottom of Mikawa Bay, Aichi Prefecture, Japan. Viruses were detected periodically in 9 out of 54 oyster pools prepared by the acid or polyethylene glycol precipitation method although all these 9 pools met current national bacteriological safety standards. Since most of the serotypes of the enteric viruses detected in the oysters were identical with those of viruses isolated from sick children living in the area, it is suggested that contamination of enteric viruses in the oysters would depend on the prevalence of enteric viral infections in the local inhabitants.

INTRODUCTION

Outbreaks of hepatitis and gastroenteritis have been associated with consumption of raw oysters [1–6]. In Japan, small round virus infections have been reported in several oyster-associated gastroenteritis outbreaks [7]. It has been suggested that bacteriological indicators such as standard plate counts and coliform indices may be poor indicators of the level of viral contamination [3, 8, 9]. However, these procedures are still being used to set national standards of microbial safety in oysters because other, more sensitive or simple methods for recovery of enteric virus have not been developed.

Enteric viruses such as enteroviruses, adenoviruses and reoviruses which can be propagated in cell culture systems will be more adequate indicators of the level of hepatitis A or small round virus contamination than bacteria. In recent years several methods have been suggested for recovering enteric viruses such as enteroviruses, adenoviruses and reoviruses which can be propagated from oysters in cell culture systems [10–12]. As an epidemiological application of a newly developed virological technique, we compared an active, prospective survey on the isolation of enteric viruses from commercially available oysters harvested in Mikawa Bay, Aichi Prefecture, Japan, with standard bacteriological tests. Seasonal distribution of the serotypes of the enteric viruses which were isolated were then compared with those found in sick children with enteric viral infections in six sentinel hospitals in the area.

https://doi.org/10.1017/S0950268800049608 Published online by Cambridge University Press

MATERIALS AND METHODS

Oysters

Between May 1987 and May 1989, oysters (*T. japonica*) were obtained on a monthly basis from the entrance of Mikawa Bay at a depth of 10 m in an area officially sanctioned for shellfish harvesting in Aichi Prefecture (Fig. 1) which has a population of 6220000. As part of a commercial operation, they were depurated in a flow-through system for 0, 24 or 48 h with u.v.-irradiated sea water drawn from a well at the seaside. Upon arrival in our laboratory, they were washed and scrubbed thoroughly in running tapwater, the outside of the shell treated with 70% alcohol and opened aseptically. The entire contents (100 g) of 10–15 oysters were collected and homogenized in a Waring blender at high speed with 1:1 (wt/vol) phosphate-buffered water (pH 7·2) for 1 min.

Bacteriological analysis

To correlate the virus data with a recognized biological pollution indicator, standard plate counts and faecal coliforms were determined for all the initial homogenates. Standard plate counts were made by a pour-plate method with plate count agar (Eiken Chemical, Tokyo). Most probable numbers (MPN) of faecal coliforms were determined by using four-dilution, five-tube replication of *Escherichia coli* broth (Eiken Chemical). Sample volumes of 2, 0·2, 0·02 and 0·002 ml of the oyster homogenates were inoculated into the appropriate tubes which were then incubated at 44·5 °C. Tubes showing production of acid and gas after 24 h were recorded as positive for the presence of faecal coliforms. The MPN index for faecal coliforms was computed by using published tables [13].

Preparation of homogenates for virus assay

The homogenized oyster meat (8 ml) was suspended in 32 ml of distilled water and adjusted to pH 5·0 using 1 N-HCl. The samples (containing a concentration of 800–2000 mg of NaCl/l) were centrifuged at 1500 g for 20 min. The oyster meat pellets were treated by the method of Sobsey and Jensen (10) (see below). The supernatant fluid was adjusted to pH 7·2, and PEG 6000 (Katayama Chemical, Osaka) and NaCl were added to make a final concentration of 9% (wt/vol) and 0·5 M, respectively. The resulting suspensions were stirred for 4 h at 4 °C and centrifuged at 3000 g for 10 min. The pellets were suspended in Eagle's minimal essential medium (MEM) containing penicillin (200 U/ml) and streptomycin (200 μg/ml) to a final volume of 4 ml (PEG precipitation method). The oyster meat pellets (see above) were washed by resuspending in pH 7·5, 0·05 M glycine–0·15 M-NaCl (1:7 w/v). After centrifugation at 1500 g for 20 min, the supernatant was filtered through a Millipore AP25 fibre-glass filter. The filtered supernatant samples were adjusted to pH 4·5 using 1 N-HCl and centrifuged at 1500 g for 20 min. The sediments were suspended in 0·1 N-Na_2HPO_4 and treated with penicillin (200 U/ml) and streptomycin (200 μg/ml) to a final volume of 4 ml (acid precipitation method). The prepared samples were stored at 4 °C overnight and centrifuged at 1500 g for 10 min before virus assay.

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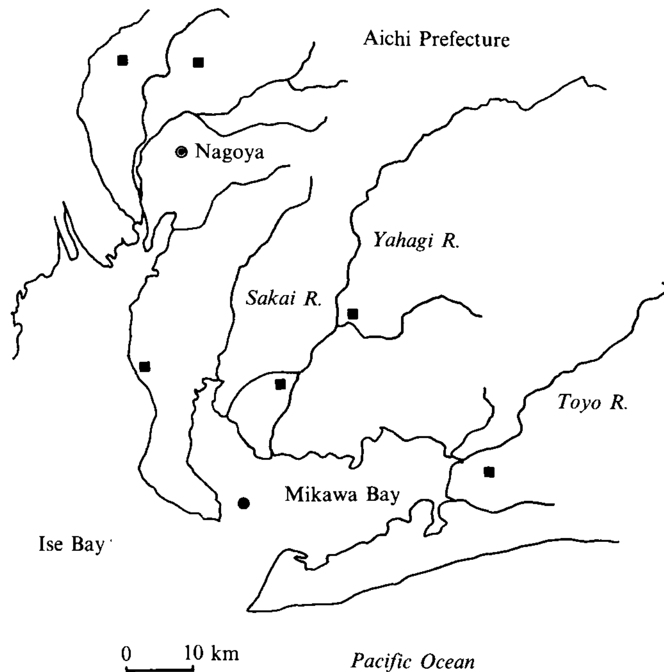

Fig. 1. Location of sample site. ●, Oyster sampling site; ■, paediatric clinic sites.

Samples from ill children

Children with aseptic meningitis, encephalitis, febrile illness, herpangina, exanthemata, respiratory infections or gastrointestinal symptoms who visited six paediatric clinics in Aichi prefecture (Fig. 1), from January 1987 to December 1988, were surveyed for enteric viruses. Specimens consisted of 1094 faecal samples and 1171 throat swabs from 1452 patients. Faecal extracts were prepared as 10% homogenates in veal-infusion broth with 0·5% bovine serum albumin, penicillin (200 U/ml) and streptomycin (200 μg/ml). Throat swabs were immersed in 3 ml of veal-infusion broth as described above. These samples were centrifuged at 10000 ***g*** for 20 min and the supernatants were collected for virus assay.

Virus assay

BGM (Buffalo african green monkey kidney), HeLa, human diploid embryonic lung fibroblast and RD-18S cells cloned from RD cells [14] were used for virus isolation. Cells were grown in MEM supplemented with 10% fetal calf serum (FCS) and antibiotics (100 U of penicillin, 100 μg of streptomycin and 50 μg of kanamycin per ml), and were maintained in MEM with 2% FCS and antibiotics. The samples were seeded into these cells (0·1 ml/tube; 10 tubes of each cell type/oyster sample, 2 tubes of each cell type/human sample). After 24 h at 37 °C, the culture medium was changed and the incubation continued. The tubes were examined periodically for cytopathic effects, and the medium was changed at 3 or 4 day intervals. After 2 weeks, the cell cultures were frozen and thawed and inoculated into fresh tubes. The tubes were incubated and examined for a further

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Table 1. *Virus isolate identifications from 9 of 54 pools, total plate counts and the number of faecal coliforms from oysters*

Isolation period	Virus identifications	Total plate counts/g	Faecal coliforms/100 g	Depuration time (h)
March 1987	Reovirus 2	1200	230	0
June	Poliovirus 1	720	110	0
September	Coxsackievirus B3	120	<*	24
November	Adenovirus 3	5900	<	24
April 1988	Echovirus 3	310	<	48
	Echovirus 18	440	20	48
September	Echovirus 9	13000	1100	0
January 1989	Enterovirus†	160	130	48
March	Enterovirus†	850	230	0

* Less than 18. † Identity unknown.

2 weeks before being considered free of viruses. The final identification of any isolate was made by neutralization tests with intersecting-pool and type-specific antisera supplied from the National Institute of Health, Japan. Unclassifiable 'enterovirus-like agents' were those found to be stable in ether and after treatment at pH 3·5, not neutralized by these sera and confirmed as virus-like by electron microscopy using the negative contrast method.

RESULTS

Virus isolation from oysters

Fifty-four pools of oysters (1–4 pools per month) were examined. Virus isolations were made from nine pools during the months of March, June, September and November 1987, April and September 1988 and January and March 1989 (Table 1). The isolates included reovirus 2, poliovirus 1 (vaccine strain), coxsackievirus B3, adenovirus 3, echovirus 3, 9 and 18 plus two enterovirus-like agents, respectively. In Japan, oyster meat is required by law not to contain more than 230 faecal coliforms/100 g nor to exceed 50000/g in total plate counts. The MPN levels of faecal coliforms exceeded permissible levels in only one (1100/100 g) of the nine pools in which enteric viruses were detected.

Effect of viral depuration in oysters

Of the 54 pools, 10 were depurated for 24 h and 15 for 48 h. Figure 2 summarizes the results of standard plate counts, MPN of faecal coliforms and isolation of viruses from these 54 pools. Poliovirus 1, echovirus 9, reovirus 2, and an enterovirus-like agent were isolated from 4 of the 29 pools tested before depuration. The others were isolated from 5 of 25 pools depurated for 24 or 48 h. Oysters which were not depurated exceeded the limit for faecal coliforms four times with MPN values between 490 and 2400/100 g. The MPN limit of faecal coliforms was exceeded once (790/100 g) in oysters depurated for 24 h, where those samples after 48 h depuration were had counts lower than 130/100 g. The total plate counts of all samples were <27000/g and were within acceptable limits. The total plate counts and number of faecal coliforms in oysters with/without depuration did not parallel the virus isolations.

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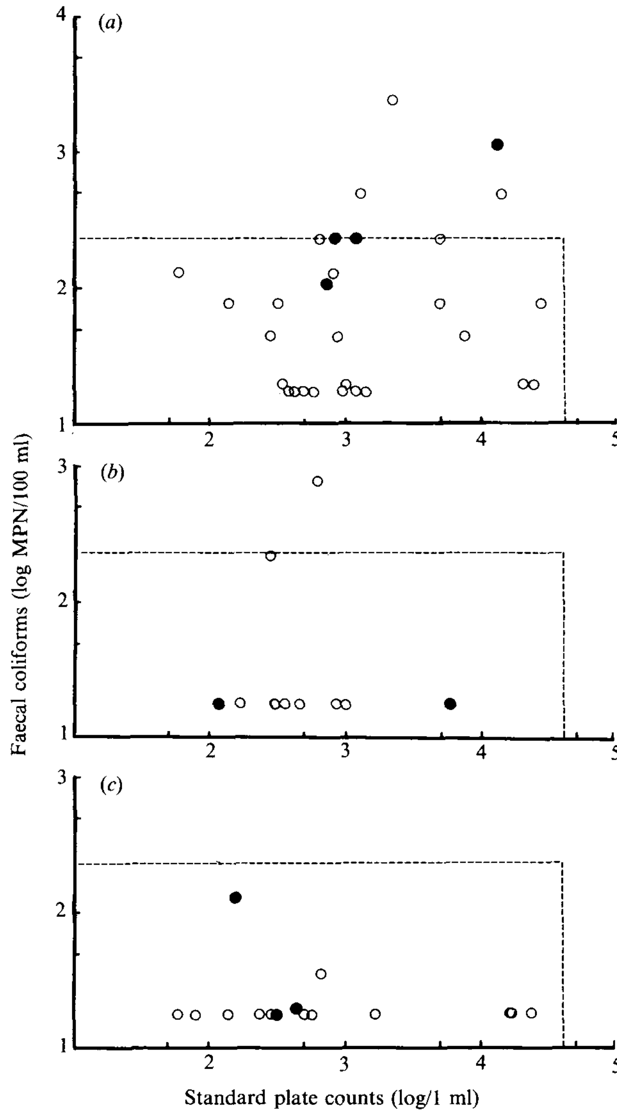

Fig. 2. Number of standard plate counts and faecal coliforms and presence of viruses in 54 pools of oysters depurated for 0 (*a*), 24 (*b*) and 48 (*c*) h. Specimen with (●) and without (○) virus; -----, the limits for bacterial standards of raw oysters recommended in Japan.

Table 2. *Comparison of the methods for detecting viruses in oysters*

Viruses isolated from the samples prepared by	
PEG precipitation	Acid precipitation
Coxsackievirus B3	Poliovirus 1
Echovirus 3	Reovirus 2
Echovirus 9	Two enteroviruses
Echovirus 18	
Adenovirus 3	

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Table 3. *Viruses isolated from 1452 ill children in 1987 and 1988*

Virus	Isolates (no.) in 1987	1988	Total
Poliovirus			
1	6	3	9
2	3	5	8
3	2	2	4
Coxsackievirus A			
2	0	10	10
3	1	0	1
4	10	1	11
9	0	1	1
10	2	1	3
15	0	1	1
16	8	11	19
Coxsackievirus B			
2	2	1	3
3	40	0	40
4	6	3	9
5	14	0	14
Echovirus			
3	0	19	19
6	0	1	1
9	15	5	20
16	0	1	1
18	0	87	87
21	0	5	5
22	5	3	8
25	4	0	4
Enterovirus			
71	11	8	19
Adenovirus			
1	5	0	5
2	10	7	17
3	22	6	28
5	1	4	5
11	2	0	2
Reovirus			
2	0	1	1
Total	169	186	355

Comparison of methods for recovering viruses from oysters

Of 9 virus isolates, 5 (coxsackievirus B3, echovirus 3, 9 and 18 and adenovirus 3) were isolated from the samples prepared by the PEG precipitation method and 4 (poliovirus 1, reovirus 2 and 2 enterovirus) by the acid precipitation method (Table 2). The former was prepared from the discarded supernatant of the latter. These results suggest that adequate assessment of virus pollution in oysters requires the use of both methods.

Virus isolation from ill children

In 1987 and 1988, 169 and 186 viruses were obtained from 732 and 720 ill children, respectively (Table 3). In 1987, the most prevalent isolates were coxsackievirus B3 (40 isolates) with adenovirus type 3 being the second most

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common (22 isolates). In 1988, the two most prevalent isolates were echovirus 18 (87 isolates) and echovirus 3 (19 isolates). Echovirus 9 was isolated from five ill children in 1988. These results suggested that the more prevalent viruses in this region had been acquired by the oysters harvested for this study.

DISCUSSION

Several studies on the occurrence of enteric viruses in oysters have been made worldwide [15]. Viruses have been isolated most commonly from heavily polluted areas closed to shellfish harvesting but have also been detected in some areas approved for shellfish harvesting [8, 9]. Our results indicate that uncultivated wild oysters grown on the sea bottom far from the estuary may still be contaminated by prevalent human enteric viruses. However, neither outbreaks of hepatitis nor viral gastroenteritis have been attributed to oysters in this area. In Japan, the number of patients with hepatitis A or viral gastroenteritis is less than those with enterovirus-like illness [16]. Hepatitis or gastroenteritis virus infection of man from oysters will depend on the prevalence of human enteric viruses found in the area. We believe further research is needed on the relationship between virus contamination in oysters and prevalence of human enteric viruses.

Reovirus has been found to be more frequently isolated in samples drawn from estuary water [17]. However, several studies have reported no isolation of reovirus from oysters when investigating the occurrence of enteric viruses [15]. In the present study, reovirus was isolated from only one oyster pool and one ill child. This result suggested that reovirus, prevalent in estuary water samples, may not be an adequate indicator of the actual viral disease hazard.

Recent studies have indicated that the faecal coliform index cannot be relied upon because coliform bacteria are more sensitive to treatment processes or natural inactivation factors than some of the more resistant enteroviruses [3, 8, 9, 18]. The standard plate count is very useful as a highly sensitive indicator of treatment deficiencies and secondary contamination, and is most valuable for monitoring disinfection processes because it covers a variety of bacterial spores which are more resistant than any known viruses [18]. In this study, viruses were isolated from oyster pools on occasions when the total plate counts and the MPN of faecal coliforms were as low as 120/g and 18/100 g, respectively. On the other hand, viruses were not isolated from oyster pools in which the total plate counts and the MPN of faecal coliforms were as high as 27000/g and 2400/100 g, respectively. Current monitoring capabilities have technical limitations, and more sensitive and rapid methods are needed for the detection of enteric viruses from oysters.

Liu and colleagues observed that lightly polluted shellfish were cleared of poliovirus after 24 h [19] and Metcalf and co-workers indicated that viruses usually were eliminated within a 24- to 48-h depuration period [20]. In this study, however, virus isolations were made in 4 of the 21 oyster pools which were depurated for 24–48 h, when few faecal coliforms were detected. Of the 9 oyster pools containing viruses, the MPN of faecal coliforms were more than 110/100 g in 4 non-depurated pools and lower than 20 in 4 of 5 depurated pools. These data indicate that uncultivated wild oysters from the bottom of the sea require more optimal environmental conditions for complete viral depuration.

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Our method, based on acid precipitation as described by Sobsey and Jensen [10], revealed four enteric viruses (poliovirus 1, reovirus 2 and two enteroviruses). As the authors reported, the method effectively recovered representatives of three different enteric viruses (poliovirus 1, reovirus 3, adenovirus SV-11), but the method's reliability for detecting all enteric viruses was open to question. In this study, five enteric viruses (coxsackievirus B3, echovirus 3, 9 and 18 and adenovirus 3) were isolated from the discard fractions of the method. Because the individual properties of each virus are not identical, the use of only one improved method to survey viral contamination in oysters may not be sufficient to provide a reliable measurement.

ACKNOWLEDGEMENTS

The authors are grateful to Dr M. Hara, National Institute of Health, Japan, for supplying the antisera to the viruses. We are indebted to the staffs of the following organizations: the Mihama Health Center; the Food and Veterinary Department of Aichi Prefecture; and the Department of Bacteriology of our laboratory.

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