Copyright ©

Kieler Meeresforsch., Sonderh. 8, 297-302. Kiel 1991

Viral and bacterial contamination of shellfish harvested in the natural environment

Ch. Jehl-Pietri*, J. Dupont and J. Munro***
*Unité 303, INSERM
1 avenue Jean Lorrain, F-06300 Nice, France
** IFREMER-DEL, Rue de L'Ile d'Yeu
BP 1049, F-44037 Nantes Cédex 01, France

Abstract

Fecal coliforms, Salmonella and antigens linked to hepatitis A virus were searched for in shellfish collected in farms or natural beds along the French coast. Statistical analysis was performed on 176 test samples harvested at 8 different stations. For fecal coliforms, there were significant statistical differences between stations ($F = 44.39$; $p < 0.001$). Salmonella was found more frequently in 2 of the stations and was isolated more often in stations where mean fecal coliform contamination was high. The presence of antigens linked to hepatitis A virus was low (detection in only 2 stations where mean fecal coliform contamination was also low). No relation between viral and bacterial markers was observed at any of the stations.

Introduction

There is ample epidemiological evidence that shellfish are key vectors in the transmission of viral and bacterial deseases to man (GOH et al. 1987, KONNO et al. 1983, RICHARDS 1985, KLONTZ et al. 1987, REEVE et al. 1989, SEKINE et al. 1989). Because of the great variety of contaminants, overall assessment of shellfish salubrity is difficult. Comparative studies of viral and bacterial contamination have been performed (ELLENDER et al. 1980a, 1980b, SOBSEY et al. 1980), but more data are required. The purpose of our yearlong study was to search for fecal coliforms (FC), Salmonella and the presence of antigens linked to hepatitis A virus (HAV) in a large number of shellfish samples and to consider possible relations between these microorganisms.

Material and methods

Sampling: Eight sampling stations were selected in different shellfish farming areas or natural beds along the French coast. In all of these sites, 3 types of shellfish were collected: oysters *(Crassostrea gigas)*, mussels *(Mytilus edulis* and *Mytilus galloprovincialis)* and cockles *(Cerastoderma edule)*. Shellfish samples

were obtained at each site every 2 weeks on average during a year's time for a total of 176 samples.

Preparation of samples: For each sample, 75 g of meat and shell liquor were ground in a mixer (Waring blender, Bioblock Scientific) for 45 sec. at 15,000 rpm.

For bacteriological analysis, the homogenate was diluted with 0,1 % (W/V) sterile tryptone water (two parts by weight). The initial suspension, obtained in this way, constituted the sample for analysis (3 ml of initial suspension corresponding to 1 g of meat and shell liquor).

For viral analyses, further steps were taken with the sample. Elution of viral particles was performed on 50 ml of the homogenate by 15 min. magnetic stirring in 150 ml of borate buffer 0.01 M containing 3 % beef extract (pH 9.0). After being clarified by centrifugation (10,000 g for 45 min. at +4 °C), the supernatant was collected and neutralized (pH 7.2) prior to viral particle precipitation by PEG 6000 (final concentration: 10 % W/V). After homogenization and incubation (one night at +4 °C), the mixture was clarified (10,000 g for 90 min. at +4 °C). The pellet was then resuspended (40 ml of elution buffer) and a second viral particle precipitation was performed. The final pellet, resuspended in 3 ml phosphate-buffered saline (PBS), pH 7.2, constituted the sample for analysis.

Bacteriological analysis: FC enumeration was performed in liquid medium according to the modified AFNOR NF V45110 method (circulaire interministérielle Agriculture/Mer, 1988). Results are expressed as the most probable number (MPN) of microorganisms per 100 g of shellfish (meat and shell liquor) calculated using the DE MAN table (1983). Detection of Salmonella was performed according to the modified ISO 6579 method (circulaire interministérielle Agriculture/Mer, 1988). The serovar of isolated Salmonella strains was determined by the National Reference Center for Salmonella at the Pasteur Institute (Paris, France).

Viral analyses: Screening for HAV-linked antigens was performed using a radioimmunoassay (RIA) (PURCELL et al. 1976). Antigen presence was revealed by iodine-125-labeled anti-HAV IgG. The P/N ratio between the number of cpm in the sample tested (P) and the mean cpm of negative samples (N) had to be equal to or greater than 2.1 for the sample to be considered positive. Radiocompetition specificity tests (FLEHMING et al. 1978) were carried out on all positive samples to confirm the presence or absence of HAV-linked antigens (ns/ps $\geq$ 2.1). These different methodologies used previously for detection of HAV in the environment have already been described in detail (PIETRI et al. 1987, 1988).

Statistical analyses: Comparison of stations according to mean FC contamination was performed using the F test of FISHER and SNEDECOR; detection percentages were compared using the X^2 test of PEARSON with YATES correction (SCHWARTZ 1986).

Results and discussion

The mean number of FC found in the different samples (Table 1) ranged from 16 to 1,600 MPN/100 g according to station. The results were thus significantly different between stations ($F = 44,39$; $p < 0.001$). Stations 1, 4, 7 and 8 were on the whole more contaminated than stations 2, 3, 5 and 6, with respect to the usual threshold value of 300 FC (log = 2,48). Roughly 4.5 % of samples (most frequent-

Table 1. Mean fecal coliform contamination and percentages of detection of Salmonella and HAV-linked antigens in shellfish from each sampling station.

Sample collection sites	Number of collected samples	Mean logarithmic counts of fecal coliform (min.-max.)	Salmonella positive samples (%)	Ag. HAV positive samples (%)
1	24	2.78(1.73-4.48)	8.33	0
2	24	1.20(1.00-2.36)	0	0
3	24	1.41(1.00-3.08)	0	0
4	18	3.21(1.95-4.08)	5.56	0
5	24	1.62(1.00-3.38)	4.17	8.33
6	21	1.94(1.00-3.08)	4.76	4.76
7	19	3.12(1.95-4.82)	0	0
8	22	3.11(1.95-3.73)	13.64	0
		$F = 44.39$		
		$p < 0.001$		

ly those from stations 1 and 8) were contaminated by Salmonella. The serovars found were: diarizonae IIIb (twice), paratyphi B (twice), mbandaka, newport, regent, subspecies 1 4, 12: d:-.

HAV-linked antigens were detected in 1.7 % of the samples analyzed, and specificity tests confirmed the positive results found by RIA. For stations 5 and 6, respectively about 8.3 % and 4.8 % of samples were contaminated. Given the low frequency of detection of Salmonella and HAV-linked antigens, statistical analysis of these results was not feasible. However, when the sampling stations were divided into 2 groups determined above (Table 2) according to their rates of FC

Table 2. Percentages of detection of Salmonella and HAV-linked antigens in shellfish from the 2 groups of sampling stations.

Group of collection sites	Mean logarithmic counts of fecal coliforms	Number of results	Salmonella positive samples (%)	Ag-HAV positive samples (%)
A (2,3,5,6)	$\leq$ 2.48	93	2.15	3.61
B (1,4,7,8)	> 2.48	83	7.23	0

contamination, the percentage of Salmonella detection was different for the 2 groups. The frequency of Salmonella isolation was higher for the group of stations with a higher mean FC contamination. For HAV, the presence of virus-linked antigens was detected in the group of stations with the lower mean FC contamination.

Table 3. Percentages of detection of Salmonella and HAV-linked antigens in shellfish relative to the two categories of fecal coliform results.

Class of fecal coliform counts MPN/100 g	Number of results	Salmonella positive samples (%)	Ag-HAV positive samples (%)
≦ 300	105	0.95	1.90
> 300	71	9.86	1.41
		$X^2=9.93$ $p<0.01$ S	

A further analysis was made, all stations combined, in which the different FC enumeration results were grouped in two categories (Table 3) with respect to the threshold value of 300 FC/100 g. In this configuration as well, the percentages of Salmonella detection were significantly different for the two categories (X^2 = 9.93; $p<0.01$). However, the percentages of detection of HAV-linked antigens were very similar for both categories.

Previous studies have indicated an apparent correlation between FC contamination in water and the frequency of Salmonella isolation (BREZENSKI 1971, GELDREICH 1970, GRUNNET et al. 1970, MOORE et al. 1979). In general, the probability of finding Salmonella increases as CF rates rise, and this probability seems to be greater in saltwater than in freshwater (SMITH et al. 1973). However, Salmonella can also be found in water containing no or few FC (SELIGMAN and REITER 1965). Our observations in shellfish are in agreement with these findings. All stations combined, the percentage of Salmonella detection was higher for results above the threshold value of 300 FC. However, Salmonella were also isolated in a sample in which FC values were normal. Although systematic FC counts are of unquestionable practical value in assessing the bacteriological quality of water and shellfish, nevertheless, it should be recognized that this test is not infallible.

The results of our viral research show that the presence of HAV-linked antigens was low in all samples tested and that no statistical interpretation was thus feasible. It is difficult to detect relationships between bacterial and viral contamination of shellfish (SOBSEY et al. 1980, ELLENDER et al. 1980a, 1980b), so that most studies have been carried out on cell-culturable viruses. However, in a recent study (JOFFRE, pers. comm.) concerning the presence in mussels of HAV as well as enteroviruses, rotaviruses and FC (among other bacterial indicators), no correlations could be found for the coexistence of these microorganisms. Moreover, HAV was detected in an area of good bacteriological quality, which

was also the case in our study (HAV-linked antigens were detected at 2 of our stations with normal bacteriological levels).

It may be concluded that shellfish monitoring based both on bacterial and viral analysis would provide a better estimation of salubrity.

References

AFNOR-NF V45110, 1981 - Produits de la pêche - Dénombrement des coliformes fécaux dans les eaux conchylicoles et dans les coquillages marins vivants.

BREZENSKI, F.T., 1971. Estuary water quality and Salmonella. Proc. National Specialty Conference on Disinfection. Am. Soc. of Civil Engineers, New York.

Circulaire relative à l'harmonisation du contrôle bactériologique des coquillages - Ministère de l'Agriculture, Direction Générale de l'Alimentation, Service Vétérinaire d'Hygiène Alimentaire / Secrétariat d'Etat à la Mer, Direction des Pêches Maritimes et des Cultures Marines (28 avril 1988).

DE MAN, J.C., 1983. MPN tables, corrected. Eur. J. Appl. Microbiol. Biotechnol. 17, 301-305.

ELLENDER, R.D., D.W. COOK, V.L. SHELADIA and R.A. JOHNSON, 1980(a). Enterovirus and bacterial evaluation of Mississippi oysters. Gulf Res. Rep. 6(4), 371-376.

ELLENDER, R.D., J.B. MAPP, B.L. MIDDLEBROOKS, D.W. COOK and E.W. CAKE, 1980(b). Natural enterovirus and fecal coliform contamination of gulf coast oysters. J. Food Protect. 43(2), 105-110.

FLEHMING, B., M. RANKE, H. FRANCK and H.J. GERTH, 1978. Application of a solid-phase radioimmunoassay and immune electron microscopy for hepatitis A in diagnosis and research. Med. Microbiol. Immunol. 166, 187-194.

GELDREICH, E.E., 1970. Applying bacteriological parameters to recreational water quality. J. Am. Water Works Ass. 62, 113-120.

GOH, K.I., S. DORAISINGHAM, E.H. MONTEIRO and A.E. LING, 1987. Acute hepatitis A in Singapore: importance of shellfish ingestion in a non-epidemic period. Ann. Acad. Med. Singapore 16(4), 591-594.

GRUNNET, K., A. GUNDSTRUP and G.J. BONDE, 1970. Isolation of Salmonella from a polluted marine environment: technique and enrichment media. Rev. Intern. Oceanogr. Med. 17, 165-177.

ISO 6579, 1981 - Microbiology - General guidance on methods for the detection of Salmonella.

KLONTZ, K.C., R.V. TAUXE, W.L. COOK, W.H. RILEY and I.K. WACHSMUTH, 1987. Cholera after the consumption of raw oysters: a case report. Ann. Intern. Med. 107(6), 846-848.

KONNO, T., T. CHIMOTO, K. TANEICHI, M. DENO, T. YOSHIZAWA, O. KIMURA, H. SIBAKI, M. KONNO and H. KOJIMA, 1983. Oyster associated hepatitis type A. Failure of certification of HAV in shellfish. Hokkaido J. Med. Science 58, 553-555.

MOORE, B., 1959. Sewage contamination of coastal bathing waters in England and Wales. A bacteriological and epidemiological study. J. Hyg. 57, 435-472.

PIETRI, Ch., J.M. CRANCE, J. PASSAGOT, E. BIZIAGOS, D. PUEL and R. DELOINCE, 1987. Application of a radioimmunological method for the detection of hepatitis A virus in shellfish. Hepatitis Scientific Memoranda, Memo-H-2332, 55-57.

PIETRI, Ch., B. HUGUES, J.M. CRANCE, D. PUEL, C. CINI and R. DELOINCE, 1988. Hepatitis A virus levels in shellfish exposed in a natural marine environment to the effluent from a treated sewage outfall. Wat. Sci. Tech. 20(11/12), 229-234.

PURCELL, R.H., D.C. WONG, Y. MORITSUGU, J.L. DIENTSTAG, J.A. ROUTENBERG and J.D. BOGGS, 1976. A microtiter solid-phase radioimmunoassay for hepatitis antigen and antibody. J. Immunol. 116, 349-356.

REEVE, G., D.L. MARTIN, J. PAPPAS, R.E. THOMPSON and K.D. GREENE, 1989. An outbreak of Shigellosis associated with the consumption of raw oysters. N. Engl. J. Med. 321(4), 224-227.

RICHARDS, G.P., 1985. Outbreaks of shellfish-associated enteric virus illness in the United States: requisite for development of viral guidelines. J. Food Prot. 48, 815-823.

SCHWARTZ, D., 1986. Methodes statistiques à l'usage des médecins et des biologistes, Flammarion Médecine Sciences Ed., Paris.

SEKINE, S., S. OKADA, Y. YAYASHI, T. ANDO, T. TERAYAMA, K. YABUUCHI, T. MIKI and M. OHASHI, 1989. Prevalence of small round structure virus infections in acute gastroenteritis outbreaks. Microbiol. Immunol. 33(3), 207-217.

SELIGMAN, R. and R. REITER, 1965. Enteropathogens in water with low *Escherichia coli* titer. J. Am. Water Works Ass. 57, 1572-1574.

SMITH, R.J., R.M. TWEDT and L.K. FLANIGAN, 1973. Relationships of indicator and pathogenic bacteria in stream waters. J. Water Pollut. Control Fed. 45, 1736-1741.

SOBSEY, M.D., C.R. HACKNEY, R.J. CARRICK, B. RAY and M.L. SPECK, 1980. Occurence of enteric bacteria and viruses in oysters. J. Food Prot. 43(2), 111-113.