Potential of F-specific RNA bacteriophages to discriminate sources of faecal pollution in French shellfish

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In Europe, shellfish harvesting areas are classified according European Directive EC N°854/2004, using classic faecal indicator levels (*Escherichia coli)*. A better indicator is needed, however, because shellfish may contain other human pathogens. Other indicator organisms have therefore been evaluated and, among these, coliphages meet most of the requirements to serve as indicators and models for enteroviruses. In order to evaluate the capacity of coliphages as indicators, a total of 764 shellfish were sampled in 2004 from 70 shellfish harvesting areas monitored by the Ifremer microbiological surveillance network (REMI, http://www.ifremer.fr/envlit/), and tested for F-specific RNA bacteriophages (FRNAPH). Two species of bivalve mollusc were collected monthly: blue mussels (32%) and Pacific oysters (68%). Results showed the absence or weak presence of bacteriophages in most shellfish growing areas classified A (94% of shellfish were below 1000 PFU (Plaque Forming Units)/100 g). These data are important for evaluating the potential of FRNAPH to discriminate between sources of pollution. Genotyping of FRNAPH may differentiate between human and animal contamination. Our results show that genotyping should be possible in 43% of the studied sites when the total flesh is analysed. As digestive tissues are known to concentrate micro organisms, this percentage could be increased if analysis were performed on them. The FRNAPH method, used with other discrimination markers, could help both states and regions to make investment decisions on how to recover suitable sanitary quality in shellfish rearing areas. For example, contamination observed in clam samples collected in Daoulas estuary (Brittany) was shown to be of mainly animal origin. Similar results were obtained on oysters from Thau lagoon (Mediterranean coast), collected during dry weather.

Keywords: F-specific RNA bacteriophage, Shellfish, Faecal pollution, Discrimination

Introduction

Shellfish harvesting areas can be contaminated by faecal pollution inputs (Feldhusen et al. 2000; Le Guyader et al. 2006). They are presently classified into four categories according to classic faecal indicator (*Escherichia coli)* levels (CE 854/2004). Shellfish in class A, suitable for direct consumption, must have less than 230 *E. coli* per 100 g of total shellfish flesh (SF). Those with a higher level (B and C), have to be depurated or placed in class A

areas for at least 2 months before consumption. However, reports suggest that *E. coli* is not a sufficient indicator to ensure the absence of bacterial and, especially, viral pathogens (Chung et al. 1998; Havelaar et al. 1993). Moreover, *E. coli* cannot be used to identify the origin of faecal contamination (Sinton et al. 1998). A number of previous studies have proposed alternative indicators to assess the presence of viral contamination, including F-specific RNA bacteriophages (FRNAPH) (Doré et al. 2000; Noble et al. 2003; Grabow et al. 2001). The present study focussed on testing FRNAPH, even though they have some limitations (Miossec et al. 2001). Very few studies have been made on FRNAPH in shellfish (Legnani et al. 1998; Doré et al. 2003); It therefore appeared interesting to search for these viruses all along the coasts of France, in parallel with *E. coli* assessments. A total of 764 shellfish samples from 70 shellfish harvesting areas (classified A and B) monitored by the Ifremer microbiological surveillance network (REMI, http://www.ifremer.fr/envlit/) were sampled monthly for FRNAPH in addition to *E. coli* analyses. Temperature and rainfall data were also collected.

The value of FRNAPH for microbial source tracking (MST) has already been evaluated in faeces of different origins, in different effluents and in environmental waters (Gourmelon et al. 2007). These FRNAPH could be differentiated into four genogroups and our results showed that genogroups I and IV were generally specific to animal contamination while II and III were found in urban sewage, as shown by Furuse et al (1987) and Schaper et Jofre (2000). Here, we used FRNAPH genotyping with the aim of seeing whether this approach was possible in shellfish harvesting areas. Additional investigations were carried out on two selected sites: 9 batches of clams from North Finistere, and 19 batches of oysters sampled near an urban watershed in Thau lagoon (Remi Network, classified B).

The aims of this study were (i) to assess the occurrence of FRNAPH in shellfish growing areas classed A and B along the whole of the French littoral, (this represents around 90% of all shellfish production areas) (ii) to study the relation between *Escherichia coli* and FRNAPH concentrations, temperature and rainfall and (iii) to evaluate the interest of FRNAPH genotyping for identifying the origin of faecal contamination in shellfish.

1. Materials and methods

1.1. Studied Samples

Crassostrea gigas (48 areas), *Mytilus edulis* (22 areas) were sampled on a monthly basis between February 2004 and January 2005, from different shellfish growing areas (n = 70 stations) of the French monitoring network (REMI: http://www.ifremer.fr/envlit/) (figure 1). Ten Ifremer laboratories participated in collecting shellfish and analysing them for *E. coli*. Homogenates of shellfish were transported to our Ifremer microbiology laboratory for analysis of FRNAPH. Temperature and rainfall data were obtained from the Ifremer Quadridge network and the nearest Meteo France stations. Two other sites were also investigated for genotyping: Daoulas in North-Finistere, Brittany, with 9 batches of clams in a B classified area located near a rural watershed during 2007, and Thau lagoon, with 19 batches of oysters between February and April 2008 (Remi Network).

1.2. Methods

1.2.1. Analysis of E. coli: Numeration of *E. coli* was estimated by the AFNOR standardized five-tube MPN method NF V 08-600 (Anon 2000) or impedance measurement (Anon 2002), on the homogenate of total shellfish flesh (SF).

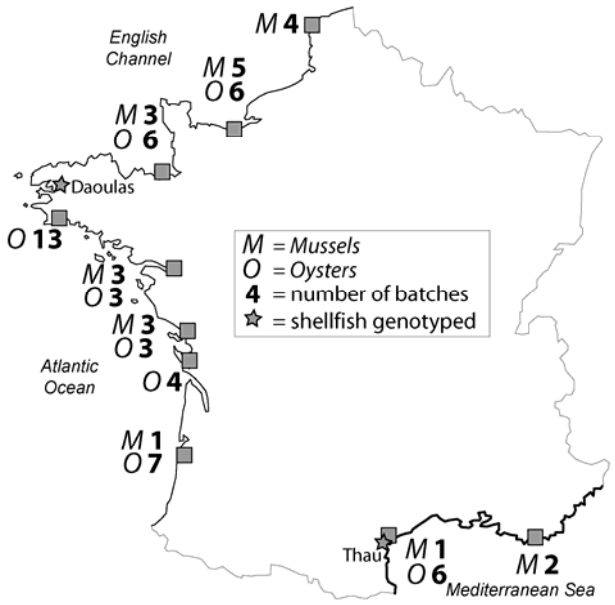

Figure 1. Location of shellfish sampling aeas.

***1.2.2. Detection of FRNAPH*:** Detection was realised on SF homogenate, and numeration done according to NF ISO 10705-1 with *Salmonella typhimurium* as bacterial host. Ten ml of supernatant were analysed; the results were expressed in Plaque Forming Units (PFU)/100 g and the detection limit was 30 PFU/100 g SF. Results of FRNAPH concentration were partitioned into 3 classes: [<100 PFU], [100 - 1000] and [> 1000 PFU $100g^{-1}$ SF].

***1.2.3. Genotyping**:* Twenty isolates were genotyped from each sample according to the laboratory protocol described in Gourmelon et al. (2007).

1.2.4. Data analysis: Data were analysed with Fisher tests using Statistica software version 6.1 (StatSoft; France).

2. Results

2.1. Concentration of *E. coli* along French coasts

A total of 764 data pairs (*E. coli* and FRNAPH) were obtained. For 72.1% of the total results (sites classified A and B), *E. coli* levels were ≤ 230 *E. coli*/100 SF. Data concerning shellfish sampled from A areas represented 37.8% of results (table 1). Out of these results, 90.3% of data were under 230 *E. coli*, 7.6% were between 230 and 1000 *E. coli*, and 2.0% were above 1000 *E. coli*/100 g SF. The proportion of shellfish in B areas was higher (62.17%). Of these, 61.1% of the samples were below 230 *E. coli*, 24.8% were

between 230 and 1000 *E. coli*, and less than 15% were above 4600 *E. coli* and concerned only oysters, mostly collected during summer (July to September 2004).

2.2. Occurrence of FRNAPH along French coasts

Results below the detection limit represented 49% of data (≤ 30 PFU). Results with less than 100 PFU/100 represented 57.2%. Among shellfish in A areas, 68.9% of samples were below 100 PFU (table 1), 25.3% were between 100 and 1000 PFU, and only 5.9% were above 1000 PFU/100 g. For shellfish in B areas, 50.1% of results were below 100 PFU, 31.6% were between 100 and 1000, and 18.3% were above 1000 PFU/100 g.

Table 1. Distribution of shellfish in areas classified A and B, number and percentage of E. coli and FRNAPH in each category.

Class of shellfish area	***E coli* CFU/100 g**			**FRNAPH PFU/100 g**		
A	< 230	230-1000	> 1000	< 100	100-1000	> 1000
n=289	261	22	6	199	73	17
37.83%	90.30%	7.60%	2.00%	68.90%	25.30%	5.90%
B						
n=475	290	118	67	238	150	87
62.17%	61.10%	24.80%	14.10%	50.10%	31.60%	18.30%
total						
n=764	72.12%	18.32%	9.55%	57.20%	29.19%	13.61%

2.3. Statistical results

Statistical analyses were performed to compare the occurrence of FRNAPH and *E. coli*. Links with other parameters, such as shellfish species, seasonal effect, location of sampling point, rainfall and water temperature were also sought. Results showed a significant correlation between logarithms of *E. coli* concentrations and the log of FRNAPH-specific concentrations (p-value<0.01 Fisher test). However, FRNAPH occurrence and concentration did not appear to be influenced by seaboard, shellfish species, shellfish rearing zone classification (A/B), rainfall or temperature.

2.4. Genotyping and *E. coli* levels in some shellfish

Concerning shellfish studied for genotyping, all samples of clams were positive for *E. coli* and FRNAPH, with a mean concentration of 2550 ± 4100 CFU and 880 ± 750 PFU/100 g SF respectively (table 2). A total of 213 isolates were recovered in 8/9 batches and genotyped as 75% genogroup I and less than 11% genogroup II. For Thau lagoon, mean *E. coli* and FRNAPH concentration were 1390 ± 1040 CFU, 2450 ± 4975 PFU/100 g SF respectively with 6 samples <LD. Nine samples could be genotyped with a total of 222 plaques isolated, and these harboured 97% genogroup I. No isolates of genogroup III or IV were detected.

Table 2. Numerations of E. coli and FRNAPH (CFU or PFU/100 g) and characterization of FRNAPH isolates; (percentages are given in brackets).

Sampling point	Date	*E coli*	FRNAPH	Number of isolates	GI	GII	NC*
Daoulas clams	12-19-06	20	2137	28	19 (68)	7 (25)	2 (7)
	01-22-07	9200	690	25	11 (44)	8 (32)	6 (24)
	02-22-07	170	990	30	26 (86)	2 (7)	2 (7)
	03-07-07	9200	1410	23	12 (52)	11 (48)	
	06-12-07	1300	450	20	20 (100)		
	07-17-07	130	150	25	19 (76)	6 (24)	
	09-11-07	93	150	6	6 (100)		
	10-25-07	ND	180	32	27 (85)	2 (6)	3 (9)
	12-12-07	330	1800	30	22 (72)	1 (2)	7 (26)
Thau oysters	02-11-08	130	30	0			
	02-11-08	210	30	0			
	02-18-08	480	30	0			
	02-18-08	2000	30	0			
	02-25-08	1900	250	20	20 (100)		
	02-25-08	3500	495	33	27 (82)	6 (18)	
	03-03-08	2000	40	1			
	03-10-08	650	390	12			
	03-15-08	1800	20700	30	30 (100)		
	03-17-08	1300	1620	21	21 (100)		
	03-17-08	1900	300	0			
	03-27-08	710	5460	24	24 (100)		
	03-27-08	470	1740	23	23 (100)		
	03-28-08	3400	5340	23	21 (91)		2 (9)
	03-28-08	2900	7920	25	25 (100		
	04-21-08	730	2010	23	23 (100)		
	04-21-08	830	150	0			
	04-28-08	980	30	0			
	04-28-08	570	30	0			

*NC: not characterized

3. Discussion and Conclusion

This study reflects the levels of FRNAPH in shellfish areas along French coasts. Overall, a good sanitary quality was observed, according to the *E. coli* indicator (90.3% and 61.1% from A and B areas, respectively, had <230 *E. coli*/100 g SF). Results showed the absence of FRNAPH or very little occurrence in most A or B shellfish growing areas (94.1% and 81.7% were below 1000 UFP, respectively). Genotyping could only be done on around 43% of the total samples. Twenty isolates of FRNAPH were needed to have a representative sample of the different FRNAPH genogroups present (Stapleton *et al.,* 2007). Moreover, the technique to detect FRNAPH in shellfish could be improved by examination of FRNAPH presence in digestive tissues. For example, Wolf et al, (2008)

obtained 62% recovery of FRNAPH in 295 shellfish samples. These data are important considering the potential use of FRNAPH to discriminate between sources of pollution in shellfish harvesting areas. This method, when used with other discrimination markers, could help states and regions to make investment decisions that would help to achieve sufficient sanitary quality in their shellfish production areas (Love et al., 2008). Contamination observed in clam samples collected in Daoulas estuary (Brittany) appears to be mainly of animal origin (5/8 samples harboured more than 70% of genogroup I genotypes, and the 3 others, a mix of genogroups I and II). These results are not surprising because the sampling was done near a rural zone downstream of a watershed with mainly agricultural activities. On the other hand, similar results were obtained on oyster samples in Thau lagoon during dry weather: animal contamination shown by presence of genogroup I alone in 7/9 samples. No isolates from genogroup III or genogroup IV were isolated. During our study, concentrations of *E. coli* were above 230/100 g in 17/19 samples and levels of FRNAPH were also high: 7 samples above 1000 PFU/100 g. In fact, the Thau site during this period was not under the influence of any urban areas or animal wastes, except for the presence of numerous seabirds. The animal (bird) contamination revealed by these results is presently being studied by other means of discrimination (Derolez et al. 2009, in press), and will be confirmed in the future. These first results are highly relevant to shellfish contamination studies and indicate the possibility of using FRNAPH markers to assess the origin of contamination in harvesting areas. Nevertheless, efforts still need to be made to improve the techniques: to raise the detection limit in shellfish, for example, and to better quantify the markers by developing quantitative real time PCR assays.

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