Hybridization of F+ RNA Coliphages detected in shellfish samples with oligonucleotide Probes to assess the origin of microbiological pollution of shellfish.

A.Vantarakis[1], D.Venieri[2], G.Komninou[2] and M.Papapetropoulou[2]

[1]Laboratory of Hygiene & Environmental Protection, Medical School, Democritus University of Thrace, Greece,[2] Laboratory of Hygiene, Medical School, University of Patras, Greece.
(E-mail address: *avanta@otenet.gr*)

ABSTRACT

Current measures for controlling the public health risks associated with bivalve molluscan shellfish consumption rely on the use of *Escherichia coli* to indicate the sanitary quality of shellfish harvesting areas. However, it has been demonstrated that *E. coli* is an inadequate indicator of the viral risk associated with shellfish. An alternative indicator organism, male-specific (F^+) coliphages have been investigated as viral indicators of faecal contamination that may provide source-specific information for impacted environmental waters. This study compared the distribution of *E. coli* and F^+ RNA bacteriophages in shellfish grown in harvesting areas of Greece and also examined the presence and proportions of the different subgroups of F^+ RNA coliphages in shellfish.

F^+ RNA bacteriophages were present in shellfish at higher concentrations than *E. coli*. Elevated numbers of F^+ RNA bacteriophages observed in the winter, concur with the known increased viral risk associated with shellfish harvested at that time of year in Greece. The majority of F^+ RNA coliphages detected in shellfish samples belonged to group IV which indicates the possible presence of animal faecal material in samples' harvesting area. The phages belonging to groups II & III (human waste & human faecal material respectively) ranged in low levels. Finally, 8% of the phages hybridized were found to belong to group I. The presence of group IV showed seasonal distribution (more in winter, less in summer) whereas the other groups did not show any difference.

Monitoring of F^+ coliphage subgroups may indicate the presence and major sources of microbial inputs to surface waters, but environmental effects on the relative occurrence of different groups need to be considered.

Keywords: F+ RNA coliphages; shellfish; hybridization; faecal pollution

INTRODUCTION

Faecal pollution can be a problem for estuaries that are associated with shellfish production and therefore with human health considering the consumption of these shellfish. For waters in which shellfish are grown, the ignorance of the precise source of faecal input makes it almost impossible to assess accurately the human health risk. Human faecal material is generally perceived as constituting a greater human health risk than animal faecal material, taking into account that it is more likely to contain human-specific enteric pathogens. Determining the source of faecal pollution enables us to apply appropriate management plans to remedy the problem and to prevent any further contamination of the shellfish.

Bacterial indicators have traditionally been used in waters and shellfish to define microbial health risk but their presence may not predict risk due to enteric viruses. Although some human enteric viruses can be enumerated in water or shellfish samples, currently there are no satisfactory enumeration methods for those that cause the majority of illnesses i.e. hepatitis viruses, Norwalk virus and the small round viruses (IAWPRC, 1991). Bacteriophages may have environmental survival characteristics similar to those of enteric viruses and therefore could be useful indicators of viral presence in natural waters. As bacteriophages are relatively easy to be enumerated, they are suitable for routine water or shellfish quality testing. Several bacteriophages have been suggested as being suitable viral indicators. These include somatic coliphages (Cornax *et al.* 1991) and F-specific coliphages (Havellaar *et al.* 1993) both of which infect *E. coli* cells. Somatic coliphages have been proposed by the U.S. Environmental Protection Agency as a viral indicator of faecal contamination of groundwater (USEPA, 2000) and male specific (F^+) coliphages may provide the additional benefit of distinguishing human and animal faecal sources of pollution.

F^+ RNA coliphages are composed of serogroups I through IV. Strains isolated from human faeces are usually groups II and III while groups I and IV are usually found in animal faeces (Furuse, 1987). The risk of viral gastroenteritis and hepatitis in humans resulting from ingestion of faecally contaminated shellfish is considered higher for human faeces than for the animal faeces, because enteric viruses from animal sources are not significant causes of human illnesses (Joklik, 1992). Hence determining if faecal contamination is of human or animal origin may aid in establishing its risk (Scott *et al*. 2002).

The main objective of this study was the indication of the sanitary quality of shellfish harvesting areas: a) discrimination of faecal pollution (animal–human waste), b) determination of possible risk for public health and c) application of appropriate management plans for prevention of further contamination (sewage improvement schemes, appropriate water purification).

MATERIALS & METHODS

Shellfish sampling and preparation

63 mussel and 21 cockle samples were collected from 5 different common harvesting areas (A, B, C, D & E) throughout northern Greece. Samples were taken from each area at monthly intervals over a 12-month period (from Nov 2003 to Dec 2004). Samples were transported to the laboratory at ambient temperature and received within 24 h of sampling. Samples were analyzed for *E. coli* and F^+ RNA bacteriophage content on the day of receipt in the laboratory. On receipt, shellfish were thoroughly washed and scrubbed under running potable water. Dead and open shellfish not responding to percussion were discarded. A minimum of 10 mussels or 15 cockles were aseptically opened using a flame-sterilized shucking knife and meat and intravalvular fluid removed homogenized in Waring blender (Waring Products Division, New Hartford, CT, USA).

Escherichia coli analysis

The procedure applied for detection of *E. coli* was as described by Donovan *et al.* (1998), which consist on a two-stage, five tubes, three dilution most probable number (MPN) method. In brief, it requires firstly inoculation in minerals modified glutamate broth and further confirmation by subculturing positive tubes onto a chromogenic agar to detect β-glucuronidase activity.

F-specific RNA bacteriophage analysis

For the detection and isolation of F- specific RNA bacteriophages, the host strain, used, was a genetically strain of *Salmonella typhimurium*, WG49, containing the *E. coli* plasmid (F′ 42 *lac*::Tn5) that encodes pilus production and the reference phage: MS2. (Debartolomeis & Cabelli, 1991). The method is validated by ISO 10705-1.

Isolated phages were hybridized using the method described by Hsu et al. (1995). Fresh plates from shellfish were stored at 4^oC for at least 30 min. Hybond N^+ membranes (Amersham Pharmacia Biotech ltd, Buckinghamshire, U.K) were absorbed for at least 1 min. Four membranes were obtained for each plate by increasing the adsorption time by 1 min for each additional transfer. Membranes were agitated in a 0.05 $moll^{-1}$ NaOH solution for 1 min, followed by immersion in 0.1 $moll^{-1}$ NaAc (pH 6.0) for 5 min and fixed under ultraviolet light (312 nm) for 5 min. Prehybridization was carried out for 30 min at 37^oC in DIG Easy Hyb (Roche Diagnostics ltd, East Sussex, U.K). DIG labelled oligoprobes (Oswell DNA service, Southampton, U.K.) first described by Hsu et al. (1995) were added to fresh DIG Easy Hyb to a final concentration of 5 $pmolml^{-1}$. Hybridization was at 37 ^oC for at least 1h. After

hybridization membranes were washed for 10 min with two changes of 0.2 x SSC + 0.1% SDS, equilibrated in DIG wash buffer and immersed in blocking buffer (Roche) diluted 1 in 10 in 0.1 $moll^{-1}$ maleic acid. Alkaline phosphatase conjugated antiDIG Fab fragments diluted 1 in 10000 in blocking buffer were allowed to bind for 45 min at room temperature. Membranes were washed in two changes of 0.2 x SCC+0.1% SDS and stained with 50 μgml^{-1} nitro blue tetrazolium and 50 $mgml^{-1}$ bromo-4-chloro-3-indolyl-phospate. Bound probes were detected as dark spots on the filter.

The F+ RNA coliphages have been hybridized using oligonucleotide probes: MS2 (I) – GA (II) – Qβ (III) – SP (IV), which classify F^+ RNA coliphages into four groups. Members of groups II and III have been shown to be highly associated with human faecal contamination and/or domestic sewage, while group IV coliphages have a higher incidence in wastes associated with animal and livestock. Group I coliphages are present in faeces and sewage from both humans and animals.

RESULTS & DISCUSSION

During this study a total of 84 shellfish (63 mussels and 21 cockles) samples were tested for *E. coli*, F^+ RNA bacteriophages as well as the serotypes of F^+ RNA bacteriophage. To assess the microbiological quality of shellfish *E. coli* results obtained from each site were used to classify the harvesting area into categories in accordance with the European Directive 91/492 (Anonymous, 1991). Table 1, shows the number of study sites classified into each category according to *E. coli* criteria. The majority of the samples were obtained from locations which were consistent with a category A classification.

47 of the shellfish samples (56%) tested in our laboratory were positive for F^+ RNA bacteriophages. The detection limit was 30 pfu/100g. The number of plaques in positive samples ranged from 150 up to 59760 pfu/100g. In 9 (43%) of the cockles and in 38 (60%) of the mussel samples analyzed, phages were detected. Interestingly, F^+ RNA bacteriophages but not *E. coli* counts in shellfish showed a strong seasonal effect with levels of F^+ RNA bacteriophages reduced during the summer months as also Dore *et al.* (2003) suggested (data not shown). It is clear that F^+ RNA bacteriophage levels in the winter months are significantly higher than in the summer months whereas *E. coli* values were more stable throughout the year. Also, the presence of *E. coli* did not correlate with the presence of bacteriophages ($P<0.005$) as also Dore *et al.* (2003) suggested.

F^+ coliphages can be isolated consistently from wastewater, swine and cattle waste animal waste lagoon liquids, waterfowl feces, and occasionally other domestic and fecal animal wastes. Furthermore, differences in the relative percentages of F^+ RNA coliphage

serogroups found in various waste and water samples may be useful for source tracking of specific inputs (Cole *et al*. 2003).

Phages from 34 (72%) of the positive samples were subjected to hybridization. The overall percentage of F^+ RNA coliphages classification with this method was 90%, with 746 out of 829 plaques having been hybridized successfully, a percentage as high as Hsu (1995) proposed.

The majority of F^+ RNA coliphages detected in shellfish samples belonged to group IV which indicates the possible presence of animal faecal material in samples' harvesting area. The phages belonging to groups II & III (human waste & human faecal material respectively) ranged in low levels. Finally, 8% of the phages hybridized were found to belong to group I (Figure 1).

The presence of group IV showed seasonal distribution (more in winter, less in summer) whereas the other groups did not show any difference (data not shown).

Among the sampling areas tested, the B area had the highest load of human waste & faecal material followed by A & E. The C & D had a low level of human faecal material. Animal waste was present in all harvesting areas (mostly in C, D & E) (Figure 2).

Screening the quality of shellfish samples throughout the sampling period and distinguishing the origin of the microbiological pollution according to the serotype enables us to assess the possible risk for human health from the consumption of human health

CONCLUSIONS

The serotyping of bacteriophages detected in shellfish samples indicates the sanitary quality of harvesting areas throughout the period of testing. Thus, it could be applied as a source tracking method, contributing to the improvement of the quality of seawater in which shellfish are grown. Generally, the speciation of F^+ RNA bacteriophages shows promise for tracking sources of faecal contamination, especially considering that the enumeration of F^+ RNA bacteriophages is well standardized and may be adopted in routine laboratories. Also, the accurate tracking of sources of faecal contamination will help to assess the exact risk for the human health of the consumption of shellfish.

REFERENCES

1. Anon, (1996). Water quality–Detection and enumeration of bacteriophage Part 1: Enumeration of F-Specific RNA bacteriophages. ISO 10705-1.
2. Anonymous, (1991). Council directive of 15 July 1991 laying down the health conditions for the production and placing on the market of live bivalve mollusks (91/492/EEC).Official journal of the European Communities **268,** 1-14.
3. Cole, D., Long, S.C., Sobsey M.D. (2003). Evaluation of F^{+}RNA and DNA coliphages as source specific indicators of fecal contamination in surface waters. *Appl. Environ. Microbiol.* **69(11)**, 6507-6514.
4. Cornax, R., Morinigo, M.A., Balebona, M.C., Castro, D., and Borrego, J. J. (1991). Significance of several bacteriophage groups as indicators of sewage pollution in marine waters *Wat. Res*. **25**, 673-678.
5. De Bartolomeis, J. and Cabelli, V.J. (1991). Evaluation of an Escherichia coli host strain for enumeration of F male-specific bacteriophages *Appl. Environ. Microbiol.* **57,** 1301-1305
6. Donovan, T.J., S. Gallacher, NJ. Andrews, M.H. Greenwod, J. Graham, J.E. Russel, D. Roberts, and R. Lee. (1998). Modification of the standard procedure used in the United Kingdom for counting *Escherichia coli* in live bivalve molluscs. Comm. Dis. Report. **1,** 188-196.
7. Dore, W.J., Mackie, M. and Lees, D.N. (2003). Levels of male-specific RNA bacteriophage and Escherichia coli in molluscan bivalve shellfish from commercial harvesting areas. *Let. Appl. Microbiol*.**36**, 92-98.
8. Furuse, K., (1987). Distribution of coliphages in the environment: general considerations, p. 87-123.In S.M.Goyal, C.P.Gerba, and G.Bitton (ed.) Phage ecology. Wiley-Interscience, New-York.
9. Havelaar, A. H., van Olphen, M., Drost, Y.C. (1993). F-specific RNA bacteriophages are adequate model organisms for enteric viruses in fresh water. *Appl.Environ.Microbiol*. **59**, 2956-2962.
10. Hsu, F.C., Shieh, Y.S., Duin, J., Beekwilder, M.J. and Sobsey, M. (1995). Genotyping male-specific RNA coliphages by hybridization with oligonucleotide probes. *Appl.Environ.Microbiol.* **61(11)**, 3960-3966.
11. IAWPRC, (1991). Review paper: Bacteriophages as model viruses in water quality control. *Wat.Res.* **25**, 529-545.

12. Joklik, W.K. (1992). Structure, components and classification of viruses, p. 749-782. In W.K.Joklik, H.P. Willett, D. B. Amos and C.M. Wiffert (ed.) Zinsser microbiology. Appleton & Lange, San Mateo, Calif.
13. Scott, T.M., Rose, J.B., Jenkins, T.M., Farrah, S.R. and Lukasik, J. (2002). Microbial source tracking: current methodology and future directions. *Appl. Environ. Microbiol.* **68(12)**: 5796-5803
14. U.S. Environmental Protection Agency, 2000. Proposed ground water rule EPA 815-F-00-03. Office of ground water, Environmental Protection Agency, Washington D.C.

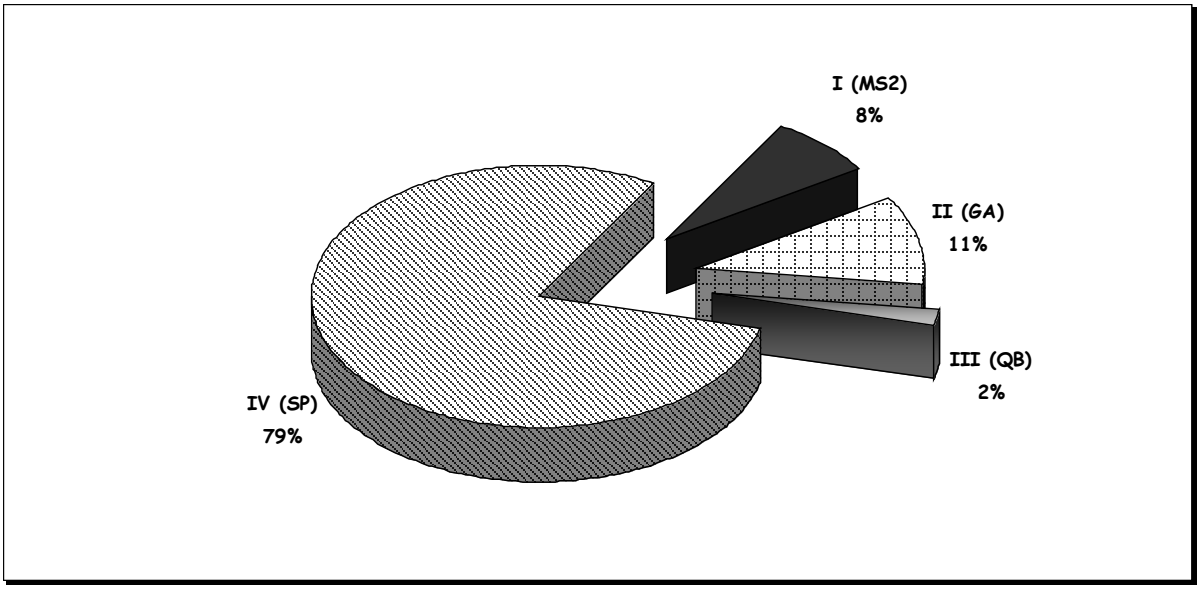

Figure 1: Percentage of serotypes of F-RNA coliphages isolated from shellfish Type I: MS2, Type II: GA, Type III:Qβ, Type IV: SP

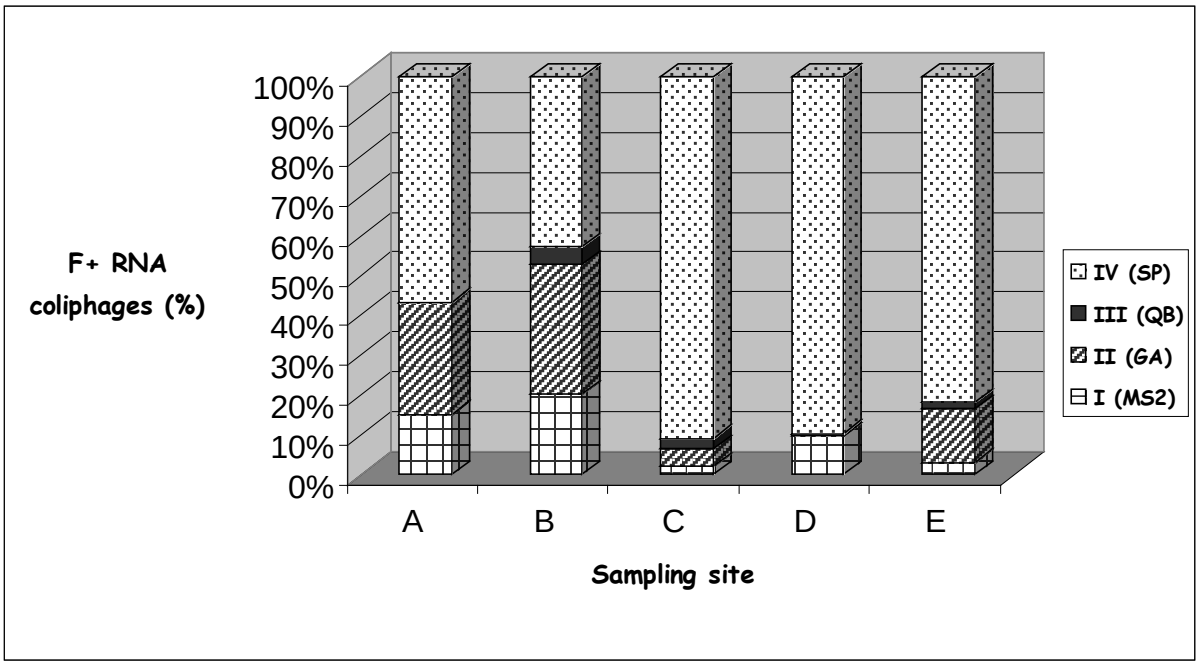

Figure 2: Percentages of F-RNA coliphage subgroups isolated from shellfish from each sampling site Type I: MS2, Type II: GA, Type III: Qβ, Type IV: SP

Classification	Number of samples analysed	Percentage of samples
A (<230cfu/100g)	67	79,7
B (230-4600cfu/100g)	13	15,5
C (>4600 cfu/100 g)	4	4,8

Table 1: Number of samples analysed in each classification category