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METHODS FOR VIRUS DETECTION IN MOLLUSCS:

VALIDATION AND STANDARDISATION

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Abstract

Human pathogenic viruses accumulate within the edible tissues of shellfish grown in sewage contaminated waters. Current standards rely solely on bacteriological parameters and do not ensure the prevention of shellfishborne viral gastroenteritis and hepatitis. Among the causative agents of these diseases, Norwalk-like viruses and hepatitis A virus are the most relevant in terms of occurrence and severity, respectively. Currently available molecular procedures provide tools for the specific and sensitive detection of viral pathogens in shellfish, thus enabling a safer evaluation of its virological quality. However, quality control and quality assurance issues must be solved, as well as simplification and automatation, of molecular procedures before they could be adopted by routine monitoring laboratories., F-specific RNA bacteriophages are proposed as alternative indicator microorganisms. Yet, exhaustive work is required to ascertain their validity, since discordances between the occurrence of these surrogates and that of actual viruses in shellfish has been reported. In the meantime, the safest approach for the prevention of shellfishborne viral diseases, is to monitor for the presence of Norwalk-like viruses and hepatitis A virus in shellfish and shellfish growing areas.

Current water treatment practices are unable to provide virus-free wastewater effluents, consequently human pathogenic viruses are routinely introduced into marine and estuarine waters through the discharge of treated and untreated sewage (Rao and Melnick, 1986). During feeding and under favorable hydrographic conditions, molluscan bivalves filter contaminants from polluted water and accumulate them within their edible tissues (Abad et al., 1997).

A major public health concern posed by virus contaminated bivalves is that shellfish are often eaten raw, like oysters, or improperly cooked, like most of other molluscan shellfish, just steamed for a few minutes. Heat can render many viruses non infectious, however rotavirus and particularly hepatitis A virus may be found in cooked shellfish (Abad et al., 1997), and outbreaks of NLV diarrhea involving the consumption of stewed oysters have been documented (Kohn et al., 1997).

It has been shown that although shellfish depuration may be insufficient to completely remove viruses (Richards, 1988; Abad et al., 1997; Lees, 2000), it does contribute to reduce virus levels and hence the risk of infection due to shellfish consumption (Bosch et al., 1994).

According current European Union standards, conventional monitoring of shellfish and shellfish beds rely solely on bacteriological parameters (Anonymous, 1991). However, the control of bacterial parameters is an insensitive proxy for viral contamination, since hepatitis A and gastroenteritis outbreaks have been associated with the consumption of shellfish meeting legal standards (Mele et al., 1989; Le Guyader et al., 1996; Lees, 2000; Bosch *et* al., 2001), and enteric viruses have been detected in shellfish with bacterial counts meeting the current criteria for public consumption (Bosch et al., 1994; Le Guyader et al. 2000; Romalde et al., 2002).

It seems then reasonable to focus on the detection of actual virus pathogens in shellfish tissues and shellfish harvesting areas. Despite the variety of health-significant viruses found in shellfish, which include rotavirus, astrovirus and adenovirus, NLV and hepatitis A virus are the most relevant viral pathogens involved in shellfishborne diseases. NLV infections represent the vast majority of shellfish-related outbreaks, and hepatitis A is the most serious infectious disease caused by shellfish consumption. Although cell-adapted hepatitis A virus strains exist, primary isolation of wild-type strains through infectivity assays is not applicable to detection of the virus in shellfish. NLV assays are even more problematic since no cell system for this virus has ever been reported. Other obstacles arise from the small quantities of viruses present in shellfish samples, which nevertheless are sufficient to pose a health risk .

The advent of molecular techniques, and particularly procedures based on nucleic acid amplification through the polymerase chain reaction (PCR) provided tools for the specific and sensitive monitoring of health significant enteric viruses in shellfish, as those responsible for outbreaks of gastroenteritis and infectious hepatitis (Atmar et al., 1996; Green et al., 1998; Lees, 2000; Le Guyader et al, 2000; Bosch et al., 2001). However, virus extraction procedures are not always compatible with PCR detection of viruses: inhibitory substances are extracted and concentrated along with the viruses. PCR-based assay development has concentrated on refining virus extraction / or nucleic acid extraction and purification techniques to overcome this inhibitory problem. At the end, the degree of virus detection effectiveness achieved after RT-PCR is in fact the result of two related factors: the efficiency of recovery of the extraction procedure applied to the shellfish sample and the degree of final purity of the recovered virus. Table 1 lists different procedures for the processing of shellfish samples prior to the specific virus detection by molecular procedures. The first decision is to choose

between performing virus detection in dissected shellfish tissues or in whole shellfish meats. Studies on the localization of human enteric viruses in shellfish tissues, revealed that most of the virus could be found in the stomach and digestive diverticula (Romalde et al., 1994; Abad et al., 1997). Atmar and coworkers reasoned that removal of these organs for virus extraction might simplify and shorten the time needed to purify viral nucleic acid for RT-PCR (Atmar et al., 1996). Testing the stomach and digestive gland for virus detection presented several advantages in comparison with testing whole shellfish: less time-consuming procedure, increased test sensitivity, and decrease in the sample-associated interference with RT-PCR.

Following virus extraction, a variety of subsequent nucleic acid extraction and purification protocols may be employed (Table 1). Due to the small size of the PCR reaction volumes, a reconcentration step is incorporated prior to the molecular assay. Enhanced detection sensitivity may be achieved through nested PCR (Green et al., 1998). However, nested approaches may represent a frequent cause of carryover contamination in the PCR procedure.

Since non-specific amplicons may be generated, it is required to perform an ulterior confirmation of the PCR products by Southern blot hybridisation with an internal probe, restriction fragment length polymorfism (RFLP) or sequence analysis. The simplest procedure to ascertain the specificity of a PCR assay is to use probe hybridization with a labeled, virus-specific oligonucleotide homologous to a region of the virus genome located between the sites to which the PCR primers bind. The use of a probe hybridization assay may as well increase the sensitivity of the assay by allowing the detection of amplicons not visible on ethidium bromide-stained gels (Goswami et al., 1993). On the other hand, sequence analysis or RFLP of amplicons provide the tools to conduct molecular epidemiology studies, which are relevant in shellfishborne outbreak

situations (Le Guyader et al., 1996; Sugieda et al., 1996; Green et al., 1998; Sánchez et al., 2002). An additional improvement in this field is the development of hybridization on a DNA enzyme immunoassay (DEIA) will permit rapid, potentially automated PCR detection (Schwab et al., 2001)

Molecular techniques for the virological analysis of shellfish have unique or additional quality assurance and quality control requirements to ensure that the data generated are useful and reliable. Some laboratories have their own internal criteria for quality assurance but a consistent set of quality assurance procedures should be implemented in order to generate data that could be comparable and reliable. A major obstacle for acceptance of molecular techniques by regulatory agencies is a lack of confidence on data generated without adequate controls.

Since the potential presence of RT-PCR inhibitors is a perennial issue in shellfish monitoring, internal controls with small numbers of RNA transcripts containing the sequence to be amplified by the virus-specific primers have been developed to prevent false-negative results due to inhibitors persisting in the sample (Atmar et al., 1996; Schwab et al., 2001). The different length of the transcript amplicons allow their physical separation from the virus-specific amplicons after gel electrophoresis.

Internal standard may also be used in a quantitative RT-PCR format to quantify the number of copies of the virus genomes that could be detected in dilutions of extracted shellfish samples (Atmar et al., 1996). In these assays, the number of genome copies is calculated from the dilution at which the signal intensity of the virus-specific amplicon equals that of the transcript-derived amplicon.

Nucleic acid amplification-based techniques have been, and undoubtedly will continue to be, a major step forward in virus monitoring in shellfish. One major drawback of molecular techniques is that they fail to discern between infectious and non-infectious

particles which may be of critical relevance in the quality assessment of depurated or cooked shellfish. However, it remains unclear whether an altered virus capsid in an environment full of RNAses, such as shellfish tissues, may harbor an RNA molecule susceptible to be amplified by PCR.

Many users may find PCR cumbersome, since a single test entails many different manual steps, and will consider the technique as suitable only for academic or reference labs and inadequate for routine monitoring. However, over the last decade, PCR technology improved on several fronts. On the one hand, commercial PCR systems significantly improved convenience, and have been quickly adopted for diagnostic laboratories. Nevertheless, the most dramatic improvement comes from the emergence of rapid thermocycling combined with fluorescence monitoring of amplified product, collectively referred as “rapid-cycling real-time PCR”, which is now available in several commercially available instruments (Cockerill, III and Smith, 2002). Real-time PCR enables not only qualitative determination but also, and particularly, quantitative diagnostic assays. Although the generic determination of pathogens is the essence of diagnostic practices, the possibility to quantitatively detect virus agents represents a seminal refinement in routine monitoring virology.

In spite of these technical developments, it is arguably believed that guidelines restricting the levels of enteric viruses themselves could be of compliance restricted to laboratories with sophisticated facilities and well-trained personnel. Since concern is linked to viruses transmitted through the fecal-oral route, microorganisms present in the fecal flora were immediately proposed as indicators. Obviously the "ideal" indicator is the viral pathogen itself, however three bacteriophage groups: somatic coliphages, F-specific RNA (F+) bacteriophages and *Bacteroides fragilis* bacteriophages (IAWPRC, 1991; Havelaar, 1993; Jofre et al., 1995) have been proposed as surrogates, among which F+ phages appear as

the most promising candidates to evaluate the virological quality of shellfish (Lees, 2000). The International organization for standardization (ISO) has elaborated procedures for detecting all these bacteriophage types (ISO 10705-1, ISO 10705-2, ISO 10705-4). Shellfish associated with a large outbreak of hepatitis A reported in the East of Spain in 1999, with 184 serologically confirmed cases, was assayed for the presence of F+ phages, and the data compared with those of actual virus pathogens found in the same samples (Table 2). A matched case-control study signaled imported coquina clams, complying European Union shellfish standards, as the source of infection, which was confirmed by the detection by RT-PCR of HAV RNA in shellfish samples (Bosch et al., 2001). Other enteric viruses, such as rotaviruses and enteroviruses were also detected in the clams. The discrepancy observed between hepatitis A virus and F+ phages was 55%, while a 50% discordance was ascertained between generic enteric virus occurrence and F+. +. A study comparing the validity of E.coli, enterovirus and F-specific RNA bacteriophages as viral indicators demonstrated that these indicators did not fully prevent the viral risk (Miossec et al. 2001).

Additionally, when the comparative positivity for human enteric viruses and F-specific RNA phages was investigated in 101 randomly chosen shellfish samples from South and West coast of France, a good correlation between the occurrence of enteric viruses and F+ phages was observed in only 49% of the samples (Le Guyader et al., unpublished results). Forty one percent of these samples were positive for at least one type of enteric virus but negative for F+ phages, while 11% of the samples were found to be negative for enteric viruses but positive for F+ phages.

Exhaustive studies are required to ascertain the validity of a candidate indicator in a given situation, i.e., shellfish-growing waters, shellfish purification practices, etc… In the end we will probably give up our hopes of finding an all-purpose "universal" indicator for viruses

and resign ourselves to the use of different indicators, or actual cell-adapted laboratory strains of fastidious enteric viruses, for a given scenario.

The quest for a reliable virus indicator in shellfish is undoubtedly one of the objectives for the coming years. However, until this long-time pursued objective is attained, the safest approach to prevent shellfishborne virus outbreaks is to monitor for the presence of NLV and hepatitis A virus in shellfish and shellfish growing waters, specially in risk area where viruses are known to circulate or when the sewage was not adequately treated. For this purpose extensive work to develop simplified standardized methodologies for the detection of these health significant viruses is required.

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Table 1. Some procedures for the processing of shellfish samples prior to virus detection by RT-PCR.

Virus elution	Chloroform-butanol / cat-floc elution	Atmar et al., 1995
	Vertrel extraction	Mendez et al., 2000
Virus concentration	Organic flocculation	Sobsey et al., 1978
	Centrifugation	Sobsey et al., 1978
	Ultracentrifugation	Loisy et al., 1995
	PEG precipitation	Atmat et al., 1995
RNA extraction	Guanidium thiocyanate	Boom et al., 1990
	Qiagen	Shieh et al., 1999
	Trizol	Schwab et al., 2000
	Roche	Loisy et al., 2000

Table 2. Positivity of human enteric viruses and F+ bacteriophages in clams associated with a shellfishborne hepatitis A outbreak[a].

Sample	HAV	EV	AsV	NLV	RV	HEV	F+[b]
1	+	-	-	-	+	-	$9.7x10^4$
2	+	-	-	-	+	-	$1.1\ x10^5$
3	+	+	-	-	-	-	$1.5\ x10^2$
4	+	+	-	-	-		-
5	+	+	-	-	-	-	-
6	+	+	-	-	+	-	$>9.0x10^4$
7	+	+	-	-	-	-	-
8	+	-	-	-	+	-	-
9	-	+	-	-	-	-	$7.5\ x10^2$
10	+	-	-	-	-	-	-
11	-	-	-	-	+	-	$9.9\ x10^4$
12	-	-	-	-	-	-	-
13	+	+	-	-	-	-	-
14	+	+	-	-	+	-	-
15	+	-	-	-	+	-	$1.3\ x10^3$
16	-	-	-	-	+	-	-
17	+	-	-	-	+	-	$7.5\ x10^2$
18	+	-	-	-	+	-	$1.2\ x10^3$
19	+	-	-	-	+	-	-
20	-	-	-	-	+	-	$7.9\ x10^4$

[a]Details on the methodology employed for enteric virus detection in these samples are described elsewhere (Bosch et al., 2001). HAV: hepatitis A virus, EV: enterovirus, AsV: astrovirus, NLV: Norwalk-like virus, RV: rotavirus, HEV: hepatitis E virus.

[b]F+ data are expressed in plaque-forming units per 100 g of shellfish tissue.