Monitoring Shellfish harvesting areas for Small Round Structured Viruses (SRSVs): Implications for combating the transmission of these viruses by molluscan shellfish

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Introduction

Increasing evidence suggests that SRSVs are a significant cause of gastroenteritis in the adult population. Although person to person transmission probably accounts for the bulk of cases, spread by the faecal-oral route through food or water vectors is likely to be important in the further dissemination of these viruses into vulnerable populations. Preventing such index cases may prove significant in combating this major cause of adult gastroenteritis. Waterborne transmission of SRSVs through faecal pollution of the aquatic environment is well documented through contamination of vectors such as molluscan shellfish. The shortage of pristine conditions dictates that in developed countries many marine water sources used for shellfisheries and recreational purposes are inevitably faecally contaminated. Legislative controls based on conventional bacterial indicators have failed to prevent shellfish transmitted outbreaks of viral-gastroenteritis caused by SRSVs. Monitoring shellfish harvesting areas for SRSV contamination may provide an alternative approach for more effective public health controls. We describe the application of a nested SRSV RT-PCR for monitoring viral contamination in a polluted oyster harvesting area over a one year period. The harvesting area was classified as Category B under EC legislation however it was known to be faecally polluted and to have been implicated in outbreaks of viral gastroenteritis. All oysters harvested from this area were purified (depurated) before sale for consumption. RT-PCR positive amplicons were characterised by cloning and sequence analysis. The public health significance of findings on the prevalence and diversity of SRSV strains contaminating this shellfishery is discussed in relation to the potential of this approach for more effective control of shellfish associated viral-gastroenteritis.

Materials and Methods

Harvesting area studies. Samples of 24 oysters were obtained from a commercial producer both before and after depuration on a weekly to fortnightly basis. All samples were received in the laboratory within 48 hours. On receipt 10 oysters were assayed for *E.coli* using standard methods (Dore and Lees, 1995) and the remaining oysters frozen at -20°C for subsequent analysis for SRSVs.

Shellfish Processing, virus extraction and purification and extraction of viral RNA. These procedure have been previously described in full (Lees *et al.*, 1994).

Nested SRSV RT-PCR. This method and its application to shellfish samples has been fully described previously (Lees *et al.*, 1995, Green *et al.*, 1998). Essentially the nested PCR uses 3 primers in the first round amplification, and two Genogroup specific primer sets in the nested PCR. They are known to detect approximately 90% of the SRSVs currently circulating in the UK.

Cloning and sequencing. All RT-PCR positive amplicons were separated from unincorporated oligonucleotide primers and nucleotides using Chromaspin 100 columns (Clontech Laboratories Inc) ligated into a pGem vector and transformed (pGem-T Vector System, Promega). Colonies from each sample were screened for inserts using colony PCR. A minimum of 5 positives clones from each sample were further purified using microsep 30k columns (Filtron Technology Corporation, MA), and both DNA strands sequenced using the ABI PRISM™ dye terminator cycle sequencing system (Perkin Elmer) and analysed on an ABI 310 genetic analyser.

Sequence analysis. Sequence data analysis was performed using the MegaAlign and EditSeq components of the Lasergene software (DNAStar, London U.K).

Results

Table 1. Summary of E. coli and SRSV results.

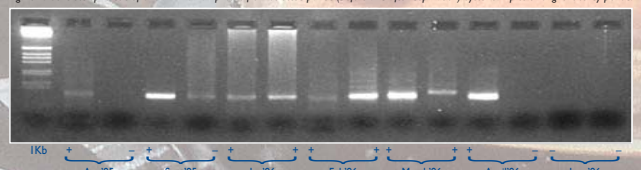
BEFORE DEPURATION				AFTER DEPURATION			
Date	E.Coli	RT-PCR	Confirmed (by sequence analysis)	Date	E.Coli	RT-PCR	Confirmed (by sequence analysis)
08-Aug 95	2400	+	Ongoing	08-Aug 95	<20	-	
15-Aug 95	>18000	+	Ongoing	17-Aug 95	<20	-	
22-Aug 95	>18000	-		24-Aug 95	<20	-	
30-Aug 95	2400	-		01-Sep 96	<20	-	
13-Sep 95	3100	-		15-Sep 95	<20	-	
26-Sep 95	220	+	Yes	28-Sep 95	<20	-	
10-Oct 95	2400	-		13-Oct 95	<20	-	
24-Oct 95	5400	-		26-Oct 95	<20	-	
07-Nov 95	5400	-		09-Nov 95	<20	-	
21-Nov 95	1100	-		28-Nov 95	<20	-	
09-Jan 96	1100	+	Yes	12-Jan 96	<20	+	tbd
16-Jan 96	110	+	Ongoing	18-Jan 96	70	+	Yes
23-Jan 96	200	+	Yes	25-Jan 96	20	+	Yes
31-Jan 96	10	+	tbd	02-Feb 96	<20	+	Yes
06-Feb 96	500	+	Yes	08-Feb 96	<20	+	Yes
14-Feb 96	700	-		16-Feb 96	<20	-	
20-Feb 96	500	+	Yes	22-Feb 96	<20	+	Yes
28-Feb 96	110	+	Yes	01-Mar 96	<20	+	Yes
06-Mar 96	5400	+	Yes	08-Mar 96	150	+	Ongoing
12-Mar 96	750	+	Yes	14-Mar 96	90	+	-
19-Mar 96	310	+	Yes	22-Mar 96	<20	+	Yes
27-Mar 96	<20	+	Yes	29-Mar 96	<20	+	Yes
16-Apr 96	1300	+	Yes	18-Apr 96	<20	-	
30-Apr 96	1300	-		02-May 96	<20	-	
05-Jun 96	<20	-		07-Jun 96	<20	-	
18-Jun 96	230	-		20-Jun 96	<20	-	
16-Jul 96	<20	-		19-Jul 96	<20	-	
13-Aug 96	22000	-		15-Aug 96	<20	-	
03-Sep 96	310	-		05-Sep 96	<20	-	
17-Sep 96	220	+	tbd	19-Sep 96	<20	-	
01-Oct 96	<20	+	tbd	04-Oct 96	<20	+	tbd
16-Oct 96	70	+	tbd	18-Oct 96	<20	+	tbd

tbd = to be done

Table 1 tabulates *E.coli* and SRSV results for all samples tested in this survey. The inadequacy of *E. coli* as an indicator of the viral risk associated with depurated shellfish is clearly demonstrated in table 1. Whilst high levels of *E. coli* were present in the majority of oysters sampled before depuration all samples were subsequently found to contain *E. coli* levels below the end-product standard of <230/100g following depuration. By contrast SRSVs were found to be present in oysters both before (56% positive) and after (38% positive) depuration.

Figure 1 shows SRSV PCR results for selected matched pairs (before and after purification) of samples taken throughout the study period. These results are presented chronologically in figure 2 together with the known outbreaks of gastro-enteric food poisoning associated with oysters from this harvesting area during the study period.

Figure 1. Gel electrophoresis of SRSV RT-PCR amplicons from selected paired (before and after depuration) oyster samples throughout study period.



A clear seasonal difference can be observed in numbers of SRSV positive oysters with the largest numbers of positive samples (73%) obtained during the winter period (October to March inclusive). This data concurs with the known winter association of gastro-enteric illness due to oyster consumption in the UK and also with the majority of the outbreaks associated with shellfish harvested from this area during the study period (figure 2). It is also noticeable that, for the most part, outbreaks from this harvesting area correlated with prior detection of SRSVs in monitored oysters. Of the SRSV positive samples during the winter period virus was successfully

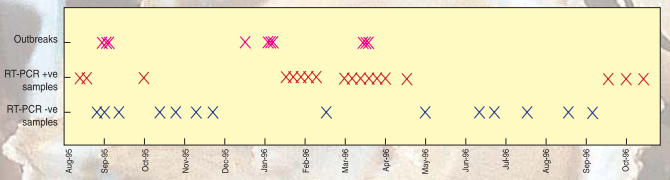


Figure 2. SRSV RT-PCR results throughout study period and occurrence of illness outbreaks associated with oysters from study area.

eliminated from only two samples during depuration with 63% of samples remaining positive (table 1 and figure 1). This concurs with evidence from many outbreaks showing that although bacterial indicators can be removed by depuration human enteric viruses are more problematical. By contrast the prevalence of SRSVs in oysters was considerably lower in the summer period (April to September inclusive) with only 31% of samples containing SRSVs. Interestingly depuration also appeared to be more efficient during the summer with SRSVs being successfully eliminated from all four SRSV positive samples following depuration (figure 1 and table 1). This is a novel finding which has potential significance for further studies on virus elimination during depuration.

PCR positive amplicons generated during this study were cloned and sequenced to investigate further the type and diversity of strains contaminating oysters. Of the samples completed to date those confirmed as SRSV sequence (the large majority) are shown in table 1. Some samples are still under analysis. Difficulties are mostly associated with obtaining sufficient template DNA for sequencing when virus levels are low. Of the 23 SRSV RT-PCR positive samples analysed between September 1995 and April 1996 about 80% have currently been confirmed by sequence analysis as SRSV (table 1). Sequence from these confirmed positives has been further analysed to investigate the type and diversity of SRSV strains. Sequence data from all isolates was compared with published sequences and clinical isolates using MegaAlign software. Alignment comparison with published sequences enabled determination of strain Genogroup (GI or GII) and, in some cases, provided an identification. However for other strains comparison with previous clinical isolates was necessary to determine strain identification.

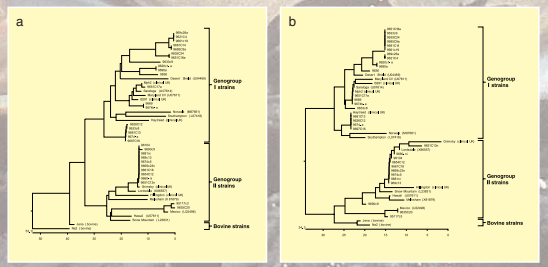


Figure 3. Phylogenetic trees showing relationships between SRSV isolates and published and clinical sequences. The phylogenetic tree and divergence/similarity plot was generated using the Clustal V algorithm within MegaAlign (DNA Star inc). Trees are based on the alignment of 78 nucleotides within the RNA polymerase excluding PCR primers. Dendrogram 3a shows sequence identity at the nucleotide level, dendogram 3b shows identity at the amino acid level.

Phylogenetic trees illustrating the genetic relationships of isolates with published sequences and with clinical isolates are shown at the nucleotide level in figure 3a and at the amino acid level in figure 3b. Isolate sequence identities at the nucleotide and amino acid levels are shown in table 2. Isolates could be clustered into groups sharing genetic relationship at the amino acid level and could be further subdivided into 10 'strains' sharing nucleotide identity of 95% or more. Stains were tentatively identified by comparison of both amino acid and nucleotide identities with published sequences and clinical isolates and are shown in table 2.

Table 2. Putative identification of SRSV strains detected in oyster samples.

Group	Strain	No of Isolates	Nucleotide identity within strains	Sequence identity with known strains Strain	Nucleotide identity	Amino Acid identity
Desert Shield Like (Genogroup I)	1	5	>97.4%	Desert Shield (U04469)	72.0%	92.3%
	2	2	100%		68.0%	92.3%
	3	1	–		68.0%	92.3%
	4	2	100%		66.0%	89.0%
	5	1	–		76.0%	92.0%
Maryland Like (Genogroup I)	6	1	–	M ph 2 (Clinical UK)	99.0%	100%
	7	2	100%	B291 (Clinical UK)	90.0%	96.0%
Haytread Like (Genogroup I)	8	5	>97.0%	Haytread (Clinical UK)	77.0% – 80.0%	96.0% – 100%
Lordsdale Like (Genogroup II)	9	10	>98.0%	Lordsdale (X86557)	>94.0%	96.0% – 100%
Mexico Like (Genogroup II)	10	2	97.4%	Mexico (U22498)	>94.0%	>96.0%

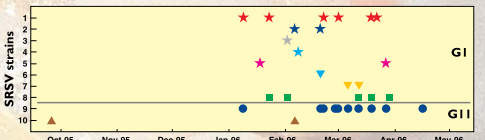
Table 3. Isolation and identification of SRSV strains before and after depuration.

BEFORE DEPURATION			AFTER DEPURATION		
Date	Genogroup	Strain ID	Date	Genogroup	ID
26-Sep 95	GII	10	28-Sep 95	– ve	
09-Jan 96	GII GI	9 1	12-Jan 96	tbd	
16-Jan 96	GI	ongoing	18-Jan 96	GI	5
23-Jan 96	GI	1	23-Jan 96	GI	8
31-Jan 96	GI	tbd	02-Feb 96	GII GII	8 3
06-Feb 96	GII GI	10 2	8-Feb 96	GI	4
20-Feb 96	GII GI GII	9 2 6	22-Feb 96	GII GI	9 1
28-Feb 96	GII GI	9 ongoing	01-Mar 96	GII GI	9 1
06-Mar 96	GII GI	9 1	08-Mar 96	GI	ongoing
12-Mar 96	GII GI GII	9 8 1	14-Mar 96	-ve	
19-Mar 96	GII GII GI	9 8 1	22-Mar 96	GI	1
27-Mar 96	GII GI	9 1	29-Mar 96	GI	8
16-Apr 96	GII GI	9 ongoing	18-Apr	-ve	

tbd = to be done

Although many of the isolates could be identified it was noticeable that the group of strains sharing nearest identity to Desert Shield could not be identified to a 90% or greater level of identity with any previously published SRSV sequences or with clinical strains isolated in the UK. It was also noticeable that many samples contained more than one SRSV strain often with a mix of Genogroups (table 3). Three samples contained both a Genogroup II strain and two different Genogroup I strains. Of the samples analysed Genogroup I strains were more prevalent than Genogroup II strains although 42% of samples contained a mixture of both Genogroups. It was also noticeable that depuration effected the distribution of strains with only 17% of samples post-depuration containing both Genogroups. The chronological distribution of SRSV strains isolated is shown in figure 4. It is clear that this harvesting area was contaminated with a variety of SRSV strains during the study period with, sometimes, many different strains occurring during a fairly short time period. Some strains were isolated periodically throughout the study period whereas others represented a single isolation.

Figure 4. Chronological occurrence, and type, of SRSV strains isolated from oysters during study period.



Discussion

This study investigates, for the first time, the feasibility of applying molecular techniques for the monitoring of SRSVs in shellfish harvested from a polluted area. We show that many samples were positive for SRSVs and furthermore that many samples contained a mixture of strains. A few samples each contained up to 3 different strains representing both Genogroups. This finding is surprising and may have implications for the importance of shellfish as a vector for the dissemination of SRSV strains. It is interesting however to note that following depuration less samples contained a mixture of strains suggesting at least partial removal of SRSVs during depuration. This suggests that SRSVs may be partially eliminated from shellfish during the depuration process and that this may be much more effective during the summer months. This finding has significance for future studies on virus removal during shellfish depuration. Further investigation of the removal of SRSVs during shellfish depuration will be facilitated by the application of PCR quantitation methods. These results also suggest that virus strains differ in their persistence in the harvesting area. Some strains were isolated on only one occasion whereas others were isolated on many occasions throughout the study period. Strain 9, Lordsdale-like, was repeatedly isolated over at least a 4 month period. It is not clear from this study whether such strains persist in shellfish following an initial contamination event or whether they are repeatedly seeded into the harvesting area. It would be interesting to correlate SRSV episodes in the community with contamination of harvesting areas to investigate this further. It is also clear from our results that SRSV contamination in this harvesting area followed a clear seasonal trend which correlates both with known outbreaks from this harvesting area and with the known winter association of illness from all outbreaks. In conclusion these results show that nested RT-PCR can identify virus contamination in shellfish harvesting areas and that this approach to virus monitoring could provide significantly enhanced levels of public health protection.

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