Results (Continued)

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 NLV 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 NLV RT-PCR positive samples analysed between September 1995 and April 1996 about 80% have currently been confirmed by sequence analysis as NLV (table 1). Sequence from these confirmed positives has been further analysed to investigate the type and diversity of NLV 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.

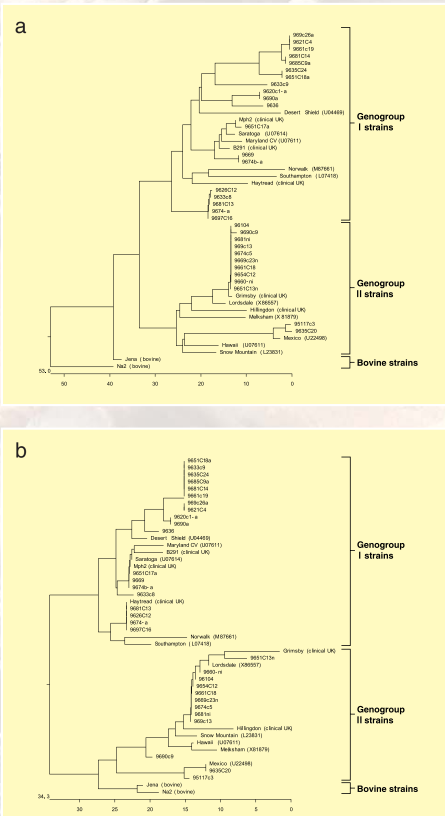


Figure 3. Phylogenetic trees showing relationships between NLV isolates and published and clinical sequences. The phylogenetic tree and divergence/similarity plot was generated using the Clustal V algorithm within MegaAlign (DNA Star inc). Trees are based on the alignment of 78 nucleotides within the RNA polymerase excluding PCR primers. Dendrogram 3a shows sequence identity at the nucleotide level, dendrogram 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 NLV 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	1	–		76.0%	92.0%
	5	2	100%		66.0%	89.0%
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%	Grimsby	>96.0%	>88.0%
Mexico Like (Genogroup II)	10	2	97.4%	Mexico (U22498)	>94.0%	>96.0%

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 NLV sequences or with clinical strains isolated in the UK. It was also noticeable that many samples contained more than one NLV 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.

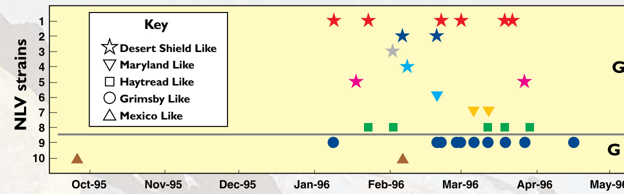
Table 3. Isolation and identification of NLV strains before and after depuration.

BEFORE DEPURATION: Date	Genogroup	Strain ID	AFTER DEPURATION: 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 GI	8 3
06-Feb 96	GII GI	10 2	8-Feb 96	GI	4
20-Feb 96	GII GI GI	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 7	08-Mar 96	GI	ongoing
12-Mar 96	GII GI GI	9 8 7	14-Mar 96	-ve	
19-Mar 96	GII GI GI	9 8 1	22-Mar 96	GI	1
27-Mar 96	GII GI	9 5	29-Mar 96	GI	8
16-Apr 96	GII GI	9 ongoing	18-Apr	-ve	

tbd = to be done

The chronological distribution of NLV strains isolated is shown in figure 4. It is clear that this harvesting area was contaminated with a variety of NLV 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 NLV 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 NLVs in shellfish harvested from a polluted area. We show that many samples were positive for NLVs 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 NLV strains. It is interesting however to note that following depuration less samples contained a mixture of strains suggesting at least partial removal of NLVs during depuration. This suggests that NLVs 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 NLVs 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 NLV episodes in the community with contamination of harvesting areas to investigate this further. It is also clear from our results that NLV 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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