

# Molecular Epidemiology of ‘Norwalk-Like Viruses’ Associated With Gastroenteritis Outbreaks in New Zealand

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Outbreaks of gastroenteritis are a major public health problem in New Zealand. The introduction of molecular detection methods has now shown that the ‘Norwalk-like viruses’ (NLVs) are the major cause of food and waterborne nonbacterial gastroenteritis. Reverse transcription and polymerase chain reaction (RT-PCR) were used to determine the presence of NLVs in faecal specimens from 83 nonbacterial gastroenteritis outbreaks occurring in New Zealand between August 1995 and July 1999. Further characterisation of the NLVs for epidemiological purposes was carried out by dot blot DNA hybridisation and DNA sequencing of representative outbreak strains. The majority of NLV strains occurring in New Zealand since August 1995 are similar to those occurring overseas. The predominant New Zealand strain is genetically similar to the Bristol/Lordsdale virus group. Several New Zealand outbreaks were attributed to Auckland virus, a Mexico-like NLV strain identified as the most likely cause of gastroenteritis after consumption of contaminated oysters in 1994. A new strain, designated Napier virus, has been identified in six outbreaks since 1996. A number of strains closely resembling internationally recognised strains, including Southampton virus, Saratoga virus; Desert Shield virus and Melksham virus have been associated with gastroenteritis outbreaks across New Zealand. Application of these typing methods has provided information on disease transmission for epidemiological investigations of public health significance. **J. Med. Virol.** 64:58–66, 2001. © 2001 Wiley-Liss, Inc.

**KEY WORDS:** human calicivirus (HuCV); Norwalk-like viruses (NLV); RT-PCR; outbreak investigation; nucleotide sequence; epidemiology

## INTRODUCTION

In New Zealand outbreaks of food-poisoning or gastroenteritis are a major public health problem. Until recently, the causal agents of many outbreaks were unidentified, and therefore often attributed to “viruses.” The small round structured viruses or ‘Norwalk-like viruses’ (NLVs) are widely recognised as the most common viral agents associated with foodborne and waterborne outbreaks of gastroenteritis [Caul, 1996a,b]. This group includes Norwalk virus, Southampton virus, Hawaii virus, Snow Mountain virus, Bristol virus, Lordsdale virus and other viruses generally named after the place of first identification. These viruses are now classified as members of one of four genera in the family *Caliciviridae* [Pringle, 1998].

The virus has an attack rate of about 50% and only a few virus particles are required to cause disease [Moe et al. 1999]. The only known host for Norwalk-like viruses is man. Transmission occurs generally by consumption of faecally contaminated food, indirectly by contact with contaminated waters used for growing, washing or irrigating foods, person to person by aerosol or direct contact, and by contact with contaminated surfaces and objects. Secondary aerosol and person-to-person transmission has been reported in institutions, resort camps and on cruise ships [Herwaldt et al. 1994; Khan et al., 1994]. Food-borne viral gastroenteritis has been associated with transmission via uncooked salads, bakery products (icing), fruit, water or ice, cold meats and especially raw or lightly cooked bivalve shellfish [Hedberg and Osterholm, 1993; Kohn et al., 1995; Jarman and Brown, 1995; Brieseman, 1996]. Enteric viruses accumulate in the tissues of filter-feeding shellfish grown in sewage-contaminated waters, and

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are not removed by depuration. NLVs have been implicated in many outbreaks of gastroenteritis associated with hotels, restaurants and institutions [McDonnell et al., 1995].

Until recently, lack of appropriate technology has hindered laboratory diagnosis of NLVs in clinical, environmental and food samples. The development of rapid, sensitive, specific molecular methods has now enabled NLVs to be identified from faecal specimens, but detection of NLVs in food and environmental samples is still difficult [Schwab et al., 2000; Rosenfield and Jaykus, 1999].

Investigation of the genetic structure of NLVs has now been studied extensively by many international groups [Wang et al., 1994; Norcott et al., 1994; Ando et al., 1995a, 2000; Berke et al., 1997; Green et al., 1997]. Molecular characterization of NLV strains has shown that they are genetically diverse and can be classified phylogenetically into 2 main genogroups. Genogroup I includes Norwalk virus, Desert Shield virus, and Southampton virus, and genogroup II includes Bristol virus, Lordsdale virus, Toronto virus, Mexico virus, Hawaii virus and Snow Mountain virus. Sets of generic primers that detect most known NLV types have been developed. Most sets are located in the RNA polymerase region that contains the YGDD motif common to all NLVs [Jiang et al., 1999]. Further discrimination of NLV strains for epidemiological investigations is obtained by typing and sequencing methods. Public health reference centres in the US and UK [Ando et al., 1995a, b; Green et al., 1995] have established typing systems based on hybridisation of PCR products with specific DNA probes, but confirmation and full identity of NLV types is best achieved by DNA sequencing of the amplified product. We describe the characteristics of NLVs identified in faecal specimens from 83 New Zealand nonbacterial gastroenteritis outbreaks occurring between August 1995 and July 1999. The typing information from hybridisation and sequencing has assisted in epidemiological analyses of gastroenteritis outbreaks of public health significance.

## MATERIALS AND METHODS

### Outbreaks and specimens

Eighty three outbreaks of nonbacterial gastroenteritis reported to public health services between August 1995 and July 1999 are included. An outbreak was defined as two or more cases with symptoms consistent with the criteria for NLV infection [Kaplan et al., 1982]. From 1997, outbreak data was recorded on the New Zealand outbreak surveillance system of the EpiSurv database at the Institute of Environmental Science and Research (ESR). Outbreak data was submitted weekly by public health services, then collated and analysed. In this report, only NLV outbreaks that were both laboratory and epidemiologically confirmed are included. Additional details of NLV outbreaks occurring between July 1997 and June 1999 are published elsewhere [Greening et al., 1999].

One or more faecal specimens from reported outbreaks were referred for presence of NLVs via the Institute of Environmental Science and Research Public Health Laboratories, hospital laboratories and community laboratories. The specimens were held at 4°C before processing. A pea-sized portion of each faecal specimen was resuspended in 2 mL viral transport medium and 200 µL chloroform. After vortexing well, the suspension was centrifuged at 12,000 × *g* for 10 min at 4°C, then the supernatant transferred to a fresh sterile vial and stored at 4°C for the RNA extraction step.

### RNA extraction

Viral RNA was extracted using the High Pure Viral RNA kit (Roche Molecular Biochemicals Ltd.) or the silica-guanidinium isothiocyanate (GIT) extraction method of Boom et al. [1990] with the following modifications: 200 µL of faecal supernatant was added to 900 µL of GIT lysis buffer and 20 µL of size fractionated silica particles. The RNA was eluted from silica particles in sterile RNase-free distilled water. RNA extracts were stored at -70°C. Precautions were taken during all procedures to avoid contamination and degradation by RNases.

### Reverse-transcription

Viral cDNA was transcribed from RNA by reverse transcription (RT). Ten microlitres of RNA extract were denatured at 90°C for 4 min in a Perkin-Elmer 9600 or 2400 Thermal Cycler, then cooled rapidly on ice. Ten microlitres of a reverse transcriptase mastermix was added to give final concentrations of 10 U/µL reverse transcriptase enzyme (Superscript RT RNase H-, Life Technologies Inc., Bethesda, MD), 1 U/µL RNaseOut (Life Technologies Inc.), 0.01 M dithiothreitol (DTT), 7.5 ng/µL random primers (Life Technologies Inc.), 1 mM each of dATP, dCTP, dGTP and dTTP (Life Technologies Inc.) and 1 × First Strand RT buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl<sub>2</sub>; Life Technologies Inc.) Tube contents were mixed by microcentrifugation for 10 sec, then incubated at 42°C for 60 min. A denaturation step of 90°C for 4 min followed, then tubes were cooled and held at 4°C until the PCR mastermix was added.

### Primers and probes

Two forward primers, P3 (4673–4692 in the NV virus genome; Moe et al., 1994), and Mr4a (4554–4572, TV genome), and one reverse primer, Mr9b (4865–4882, TV genome) [Lew et al., 1994], from the RNA polymerase region were used. The Mr9b reverse primer is located in the conserved YGDD RNA polymerase motif common to all NLVs. The P3 and Mr9b primer pair amplified a 197 bp product primarily from genogroup I strains, whereas the Mr4a and Mr9b primers amplified a 328 bp product from genogroup II NLVs.

Specific oligonucleotide probes for typing of NLVs in the polymerase region were those published by Ando

et al. [1995a]. Three probes (63d, 65d, 69d) were combined to give the P1-A probe set. The remaining three probe sets were single oligonucleotides. All primers and probes were custom synthesised by Life Technologies Inc. Probes were 3'-end-labelled with biotin-14-ATP using terminal transferase enzyme (Life Technologies Inc.).

### Amplification by PCR

After the RT stage, 80  $\mu$ L of PCR mastermix was added to each tube to give final concentrations of 2.5 mM MgCl<sub>2</sub>, 50 mM KCl, 1.5 U/100  $\mu$ L Amplitaq polymerase (Perkin-Elmer, Norwalk, CT), and 20 pmol of each of the three primers. Amplification was carried out in a Perkin-Elmer 9600 or 2400 Thermal Cycler. After an initial denaturation step at 94°C for 2 min, 40 cycles of 95°C for 20 sec, 42°C for 20 sec and 72°C for 40 sec were applied, followed by a final extension step of 72°C for 5 min. Tubes were cooled and held at 4°C at the end of cycling.

Ten microlitres of each PCR product were electrophoresed on a 2% agarose gel (FMC Seakem) in 0.5  $\times$  TBE buffer, stained with ethidium bromide, then viewed and photographed on a UV transilluminator. A 100 bp DNA ladder (Life Technologies Inc.) was used as a size marker.

### Quality control

Negative and positive controls were included in each batch of specimens assayed. Both positive (known positive faecal sample) and negative (water) controls were taken through RNA extraction and RT-PCR procedures. A cloned fragment from the RNA polymerase region of 8FIIa strain of Norwalk virus (M87661) kindly donated by Dr. Suzanne Matsui, Stanford University, California, was used as a positive control. This fragment amplified with both forward primers to produce both genogroup I (197 bp) and genogroup II (328 bp) bands.

Anti-contamination procedures were followed for all RNA and DNA amplification procedures including use of dedicated laboratories and equipment for each stage of the process.

### NLV typing by dot blot hybridisation

Typing of PCR products was carried out by dot blot hybridisation. PCR products (2.5  $\mu$ L) were spotted on to four replicates of positively charged nylon membrane (Hybond N+, Amersham, Buckinghamshire, UK). Once dry, membranes were denatured for 2  $\times$  5 min on Whatman 3 MM chromatography paper saturated with 0.5 M NaOH, 1.5 M NaCl, and then neutralised in the same way with 1.5 M NaCl, 1 M Tris, pH 7.4. After drying, membranes were exposed DNA side down to UV light on a UV transilluminator for 4 min.

Membranes were pre-hybridised in 0.5% SDS, 0.1  $\times$  SSPE (3M NaCl, 200 mM NaH<sub>2</sub>PO<sub>4</sub> H<sub>2</sub>O, 20 mM EDTA) for 30 min at 56°C in a shaking waterbath. Each

oligonucleotide probe was diluted in pre-heated hybridisation buffer (0.1% SDS, 5  $\times$  SSPE, 56°C) to 0.5 pmol probe/mL hybridisation buffer, and added to a plastic bag containing the respective membrane. Membranes were hybridised at 48°C for 90 min. After hybridisation, membranes were washed twice in a shaking waterbath in 2  $\times$  SSPE, 0.1% SDS at 50°C for 10 min. A final wash in 0.5  $\times$  SSPE, 0.1% SDS at room temperature for 10 min on an orbital shaker was then carried out.

For detection, the membranes were immersed in a streptavidin-horse radish peroxidase conjugate (Streptavidin-HRP; Dako Corp., Buckinghamshire, UK) for 15 min on an orbital shaker at room temperature. Two further washes of 2  $\times$  SSPE, 0.1% SDS at room temperature on the orbital shaker followed. The conjugate was detected by the Amersham ECL chemiluminescent system. After exposure to the ECL reagents, membranes were exposed to autoradiographic film (Amersham Hyperfilm) overnight in a cassette. The film was developed and results recorded.

### DNA sequencing and analysis

For further identification, the RT-PCR amplified products were first purified using the QIAquick PCR purification kit (Qiagen, Chatsworth, CA) then sequenced with Mr4a or P3 forward primers or the Mr9b reverse primer on an ABI 377 or 310 DNA sequencer, using Big Dye-terminator cycle sequencing methodology (Perkin-Elmer Corp.; Applied Biosystems, Foster City, CA). Using the Entrez BLAST programme, nucleotide pairwise similarities were plotted for most New Zealand NLV strains against those deposited in the National Institutes of Health NCBI GenBank database. Clustal W v1.8 [Thompson et al., 1994] was used to create multiple alignment of the New Zealand isolates' (N = 34) nucleic acid (nt) and predicted amino acid (aa) sequences along with the corresponding region of reference NLVs: Norwalk [M87661], Southampton [L07418], Desert Shield [U04469], Saratoga [U07614], Napier [U91525], Melksham [X81879], Snow Mountain [L23831], Hawaii [U07611], Bristol [X76716], Lordsdale [X86557], Camberwell [AF145896], MX [U22498], and Auckland [U34381]. Figure 4 shows the aa multiple alignment of the sequences analysed. In case of identical nt sequences, only the longest of the two was included in the phylogenetic analysis. DNAML program in PHYLIP v3.52 [Felsenstein, 1993] utilising maximum likelihood algorithm was used to assess phylogenetic relationships among the nt sequences of the partial polymerase gene of the New Zealand isolates and with reference NLVs.

## RESULTS

### Incidence of outbreaks

Between August 1995 and July 1999, 83 outbreaks and infection clusters of viral gastroenteritis were reported to public health authorities. The majority of outbreaks were reported from the health districts in the

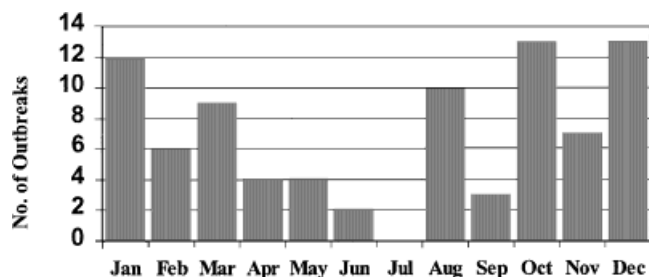


Fig. 1. Seasonality of NLV outbreaks: cumulative number of outbreaks occurring per month (August 1995–July 1999).

main population centres of Auckland, Wellington and Christchurch. Sixty (72.3%) outbreaks occurred over the warmer spring and summer months (October to March) (Fig. 1). Only two outbreaks were reported in the winter months of June and July. This is partly affected by bias because data was not included for June–July 1995.

The setting and probable mode of transmission for outbreaks is shown in Table I. Almost half (40, 48.8%) of the outbreaks were associated with food or waterborne transmission. This included one large outbreak caused by sewage-contaminated drinking water at a ski resort. No NLVs were recovered from the water supply or filtration units. The food type implicated most commonly was seafood, especially oysters, that were implicated in at least 6 outbreaks. Foods were not generally available for viral analysis and so there were no instances where the source was confirmed by detection of virus in a food or drink sample. The transmission factors implicated most commonly were contamination from an infected food handler (believed to be a factor in two-thirds of outbreaks where this data was recorded) and cross contamination from one food to another. Outbreaks in rest homes and hospitals accounted for a further 24 (29%) outbreaks. The actual mode of trans-

TABLE I. New Zealand NLV Gastroenteritis Outbreak Settings: August 1995–June 1999

Outbreak Setting	No.	%
Hospital/rest home	24	29
Catering/restaurant	40	48
Children's school/camp/creche	9	11
Person-to-person	3	4
Unknown	7	8
<b>Total</b>	<b>83</b>	<b>100</b>

mission in these settings is unclear, but secondary person to person spread is probably a significant factor.

### Distribution of NLV strains

Faecal specimens from cases were forwarded to ESR laboratories for analysis. Between August 1995 and July 1999, ESR identified NLVs in 229 (65%) of 355 faecal samples submitted for viral analysis from both sporadic and outbreak cases of gastroenteritis. Over the period of study, the largest number of outbreaks (55, 66.3%) has been attributed to genogroup II strains (Table II). In particular, 21 of 24 (87.5%) outbreaks occurring in hospitals and rest homes were caused by genogroup II strains. In contrast, members of both genogroups caused outbreaks associated with foodborne transmission. A single NLV strain was identified from each outbreak, and no mixed NLV infections were observed, although other microbial pathogens such as *Giardia*, *Cryptosporidium*, and *Campylobacter* sp. were also associated with a few of the NLV outbreaks (data not shown). Genogroup I strains were more common in 1997 and 1998; only a few members of this genogroup have been identified in 1999 outbreaks occurring up to July and they are unlike the strains identified previously in New Zealand (Table II, Fig. 2).

Seven NLV strains were responsible for the majority of New Zealand NLV outbreaks reported between August 1995 to July 1999 (Table III). Four of these belonged to genogroup II and the remainder to genogroup I.

### Molecular typing

All NLVs identified from each of the 83 New Zealand gastroenteritis outbreaks were typed first by hybridisation methods using the four specific oligonucleotide probes, P1-A, P1-B, P2-A, P2-B [Ando et al., 1995a]. Most New Zealand NLV genogroup II strains hybridised with the P2-B probe. Viruses from 7 outbreaks hybridised with the P2-A probe and viruses detected in 6 outbreaks hybridised with the P1-B probe. Only one strain hybridised with the P1-A probe, which is specific for Norwalk virus and closely-related genogroup I viruses. Several strains did not hybridise with any probe. Representative NLVs from each cluster of cases were sequenced.

Phylogenetic analysis of the New Zealand isolates of the investigated period shows more detailed relationships among the strains analysed. Figure 3 shows the

TABLE II. Relationship of NLV Genogroup to Outbreak Setting

Setting	Genogroup I	Genogroup II	Unknown <sup>a</sup>	Total
Hospital/rest home	3	21	0	24
Catering/restaurant	14	25	1	40
Camp/school/creche	4	4	1	9
Person-to-person	2	1	0	3
Unknown	3	4	0	7
<b>Total</b>	<b>26 (31.3%)</b>	<b>55 (66.3%)</b>	<b>2 (2.4%)</b>	<b>83</b>

<sup>a</sup>not sequenced.

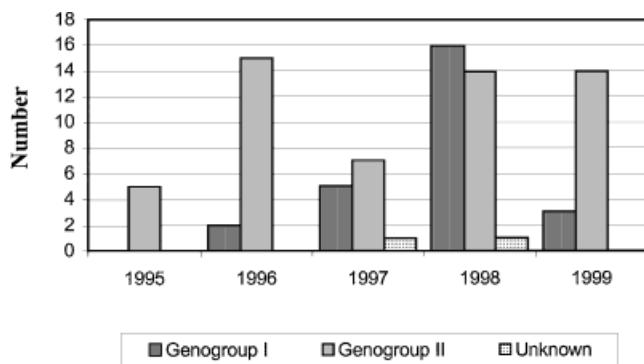


Fig. 2. Prevalence of genogroups I and II in New Zealand NLV outbreaks over time (August 1995–July 1999).

maximum likelihood phylogram for the partial polymerase gene nt sequences. Phylogenetic distances are expressed as expected number of substitutions per site and can be estimated using the scale. DNA sequencing of strains that hybridised with the P2-B probe showed that most had over 90% genetic similarity to the Bristol/Lordsdale virus subgroup, with many closely resembling the Australian Camberwell virus strain [Cauchi et al., 1996]. Strains resembling Melksham virus also hybridised with the P2-B probe. Strains hybridising with the P2-A probe were shown by sequencing to be identical to Auckland virus [Regli et al., 1995] that is similar to Mexico (MX) virus; Auckland virus was previously associated with outbreaks of gastroenteritis from contaminated local oysters [Jones and Graham, 1995; Jarman and Brown, 1995]. Strains hybridising with the P1-B probe were found to align with genogroup II strains in the phenogram (Fig. 3) and so were classed as genogroup II strains. Because of the lack of similarity to other NLV strains, a representative sequence was deposited in the GenBank International Database as Napier virus (accession number U91525), based on the site of first identification. This strain now seems to be similar genetically to 'Gwynedd virus' and other members of the newly designated NLV genogroup II/7

[Ando et al., 2000]. NLV strains that did not hybridise with any of the probes generally belonged to genogroup I and most closely resembled Southampton virus, Desert Shield virus or Saratoga virus.

Pairwise analysis from the consensus portion of the analyzed nucleotide (81nt) and predicted amino acid sequences (26aa, Fig. 4) resulted in the following ranges: from 58–100% nt identity, being 98\_229 and 99\_44 the most distant sequences. A number of sequences (18 pairs) shows 100% nt identity in this region. The lowest amino acid pairwise identity was 73% between MX and 98\_105. 258 identical (100%) pairs of amino acid sequences were shown in the pairwise matrix (not shown).

## DISCUSSION

This is the first report on RT-PCR detection and molecular epidemiology of NLVs in New Zealand. Previously there has been no published data on the incidence of NLV infection in New Zealand. Combined laboratory and epidemiological surveillance, however, shows that NLVs are an important cause of disease outbreaks in New Zealand, accounting for over 25 outbreaks and approximately 600 cases of gastroenteritis reported annually [Greening et al., 1999]. Viral gastroenteritis incurs high economic costs from lost productivity and impacts seriously on the capacity to staff healthcare institutions and the hospitality industry. Cases of acute gastroenteritis are a notifiable condition only if they are part of a suspected common source outbreak and so generally only large outbreaks are likely to be recognised, reported to public health agencies and investigated. Therefore the identified morbidity is likely to represent only a small proportion of the disease burden from NLV infection. The bulk of disease probably occurs as sporadic infection or small clusters rather than as defined common source outbreaks. A prospective population-based study in England found an annual incidence rate of NLV of 1,250/100,000 [Wheeler et al., 1999]. If this rate is applied in New Zealand, we could expect approximately 50,000 episodes of NLV gastroenteritis a year.

TABLE III. Major NLV Outbreak Strains Occurring in New Zealand Between August 1995–July 1999\*

NZ NLV strain	Genogroup	Probe type	1995	1996	1997	1998	1999	Total	%
BV/LV virus	II	P2-B	5	9	3	8	1	26	31
Melksham Virus	II	P2-B	0	1	0	1	9	11	13
Saratoga virus	I	UT	0	0	2	8	0	10	12
Auckland virus	II	P2-A	0	3	2	0	2	7	8
Napier virus	II	P1-B	0	2	2	2	0	6	7
Desert Shield	I	UT	0	0	0	6	0	6	7
Southampton	I	UT	0	1	2	1	0	4	5
SMV-like (Y13702)	II	P2-B	0	0	0	2	0	2	2
Other types	I and II	mixed	0	1	1	2	5	9	10
Not sequenced	II		0	0	1	1	0	2	2
Total strains			5	17	13	31	17	83	100%

\*NZ isolates that show greatest similarity to international NLV strains. BV/LV virus, Bristol virus/Lordsdale virus subgroup; UT, untypable with available probes.

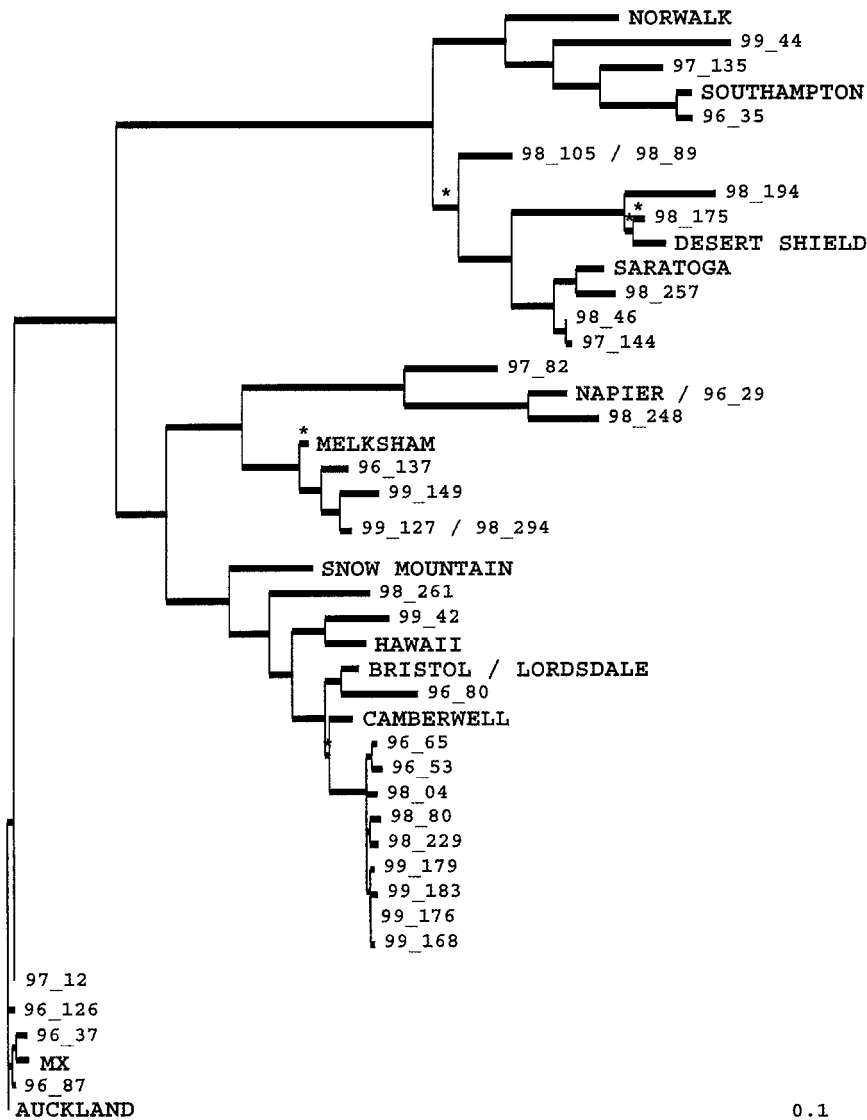


Fig. 3. Maximum likelihood phylogram for the partial polymerase gene nt sequences shows the genetic relationships among New Zealand NLVs. Phylogenetic distances are expressed as expected number of substitutions per site and can be estimated using the scale.

Non-significant branch lengths ( $P \geq 0.05$ ) are marked with asterisks or not shown. This is an unrooted tree. Identical sequences are presented as individual taxa.

The 83 laboratory-reported outbreaks occurred predominantly during the warmer months of the year (October to March). The seasonal disease pattern coincides with that observed in both Australia where the peak occurs between August and December [Wright et al., 1998] and in the UK. There the disease commonly occurs during the winter [Dedman et al., 1998] and has been termed 'winter vomiting disease' [Riordan, 1991]. Winter outbreaks are associated commonly with institutional settings such as rest homes and hospitals. In New Zealand, food-borne transmission was implicated in approximately half of the outbreaks and institutions in a third of outbreaks. Outbreaks were generally reported as food or waterborne or person to person transmission. For some outbreaks a combination of transmission modes was reported. The only foods

known to be at risk of contamination at source are the bivalve shellfish, mainly oysters and mussels. Oysters have been associated with New Zealand outbreaks in 1994–95 [Jarman and Brown, 1995], and in this current surveillance report, epidemiological analysis implicated bivalve shellfish in at least 6 outbreaks.

The majority of New Zealand NLVs identified belong to the genogroup II, members of which are genetically closer to Bristol virus, MX virus and Snow Mountain virus rather than the prototype Norwalk virus. The spectrum of NLV strains occurring in New Zealand has changed during the period of this surveillance report (Table III). The most common strains are related to genogroup II strains from the Bristol / Lordsdale virus subgroup (especially the geographically closer Camberwell (Australian) strain) and Melksham virus. In

	*	20	*	40	*	60	*	80	*	100					
norwalk	:	NHFADAYTAWDSTQNRQIMTSEFSIMSRLTASPELAEVVAQDLLAPSEMVDVGVIRVKEGLPSGFPCSTQVNSINHWIITLCLASEATGLSPDVVQSMYSFYFGDD										: 108			
napier	:	L.Y.	SR	Q.S.XSAA	MEV.V.FS.E	Q.E	QL	F.S.Q	V	W.A.L.S	MA.VS	: 102			
auckland	:	Y.Y.	SR	Q.AVLAA	ALE.V.FS.E	Q.QI.E	VV	FK.TIN	V	W.A.LL	V.G.II	AN.IY	: 108		
mx	:	Y.Y.	SR	Q.AVLAA	ALE.V.FS.E	Q.QI.E	VV	FK.TIN	V	H.G.A.LL	V.G.II	AN.MY	: 108		
sma	:	Y.Y.	SR	Q.AVLAA	ALE.VKFSPE	H.QI.E	V	FK.TIN	V	W.A.LL	V.N.A.II	AN.L	: 108		
bristol	:	Y.Y.	SR	Q.AVLAA	ALE.VKFSPE	H.QI.E	S.V	FK.SIN	V	W.A.LL	V.N.II	AN.L	: 108		
camberwell	:	Y.Y.	SR	Q.AVLAA	ALE.VKFSPE	H.QI.E	S.V	FK.SIN	V	W.A.LL	V.N.II	AN.L	: 108		
dsv	:	---	---	E.M.N	CK.N.S.A	S	S	D	L	V	L	Q	: 106		
hawaii	:	---	YR	CTAVL	AAALE.VKFSPE	H.QE	S.V	FK.SIN	V	W.T.LL	V.D	II	AN.L	: 101	
melksham	:	Y.Y.	SR	Q.AVLAA	ALE.VKFSPE	H.QI.E	S.VV	FK.SIN	V	W.A.LL	V.D	II	AN.L	: 108	
saratoga	:	F.Y.	---	S.E.M	P.N.CK.N	S.F.A	S	S	D	L	V	I	Q	: 108	
south	:	Y.	---	C	S	S	S	L	V	I	Q	I	: 108		
96_37	:	Y.Y.	SR	Q.AVLAA	ALE.V.FS.E	Q.QX.E	VV	FK.TIN	V	W.A.LL	V	---	: 89		
96_35	:	---	---	C	S	S	K	L	V	I	Q	I	: 91		
96_80	:	---	SR	Q.AVLAA	ALE.VKFSPE	H.QK.E	S.V	FK.SIN	V	W.A.LL	I.X	N	: 88		
96_87	:	---	SR	Q.AVLAA	ALE.V.FS.E	Q.QI.E	VV	FK.TIN	V	W.A.LL	V	---	: 84		
99_44	:	---	S	A	V	S	K	I	L	M	V	I	Q	LL	: 97
96_126	:	---	SR	Q.AVLAA	ALE.V.S.E	Q.QI.E	VV	FK.TIN	V	W.A.LL	V.G.II	---	: 90		
96_53	:	---	SR	Q.AVLAA	ALE.VKFSPE	H.QE	S.VV	FK.SIN	V	W.A.LL	V.N.II	---	: 90		
96_65	:	---	SR	Q.AVLAA	ALE.VKFSPE	H.QE	S.VV	FK.SIN	V	W.A.LL	V.N.II	---	: 90		
97_12	:	---	SR	Q.AVLAA	ALE.V.XS.E	Q.QI.E	VV	FK.TIN	V	W.A.LL	V.G.II	---	: 90		
98_04	:	---	SR	Q.AVLAA	ALE.VKFSPE	H.QE	S.VV	FK.SIN	V	W.A.LL	V.N.II	---	: 90		
98_229	:	---	SR	Q.AVLAA	ALE.VKFSPE	H.QE	S.VV	FK.SIN	V	W.A.LL	I	---	: 78		
98_248	:	---	SR	Q.S.LS	AAEMV.V.FS.E	Q.E	QL	F.S.Q	V	W.A.L.S	MA.VS	A	: 91		
98_261	:	---	SR	Q.AVLAA	ALE.VKFSPE	H.QE	S.V	FK.SIT	V	W.A.LL	I.N	II	: 90		
98_80	:	---	SR	Q.AVLAA	ALE.VKFSPE	H.QE	S.VV	FK.SIN	V	W.A.LL	V.N.II	---	: 90		
99_127	:	---	SR	Q.AVLAA	ALE.VKFSPE	H.QI.E	S.VV	FK.SIN	V	W.A.LL	V.D	I	: 90		
99_149	:	---	SR	Q.AVLAA	ALE.VKFSPE	H.QI.E	S.VV	FK.SIN	V	W.A.LL	V.D	I	: 90		
99_168	:	---	SR	Q.AVLAA	ALE.VKFSPE	H.QE	S.VV	FK.SIN	V	W.A.LL	V.N.II	---	: 90		
99_176	:	---	SR	Q.AVLAA	ALE.VKFSPE	H.QE	S.VV	FK.SIN	V	W.A.LL	V.N.XXII	---	: 90		
99_179	:	---	SR	Q.AVLAA	ALE.VKFSPE	H.QE	S.VV	FK.SIN	V	W.A.LL	V.N.II	---	: 90		
99_183	:	---	SR	Q.AVLAA	ALE.VKFSPE	H.QI.E	VV	FK.SIN	V	W.A.LL	V.N.II	---	: 90		
99_42	:	---	SR	Q.AVLAA	ALE.VKFSPE	H.QE	S.V	FK.SIN	V	W.A.LL	V.N.C	---	: 86		
98_105	:	---	---	S	D	M	V	I	---	---	---	---	: 54		
96_137	:	---	---	VV	FK.SIN	V	W.A.LL	V.D	XX	I	---	---	: 50		
97_135	:	---	---	L	V	---	---	---	---	---	---	---	: 48		
97_144	:	---	---	S	D	L	V	---	---	---	---	---	: 49		
97_82	:	---	---	RL	F.S.Q	V	W.A.L.S	M	VS	E	---	---	: 50		
98_194	:	---	---	S	D	L	V	L	RR	---	---	---	: 50		
98_257	:	---	---	S	D	L	V	I	---	---	---	---	: 50		
98_46	:	---	---	S	D	L	V	I	---	---	---	---	: 50		
98_175	:	---	---	D	L	V	L	Q	---	---	---	---	: 49		

Fig. 4. The multiple alignment for the amino acid sequences of the New Zealand isolates analysed is shown. The first rows include reference sequences followed by the study isolates. Norwalk sequence in the first row is shown in its entirety, in the rest of the sequences

only residues that differ at the given position are shown. Conserved residues are shown as dots. Dashes indicate unavailable portions of the sequences. The pairwise similarity values were calculated for the 26-aa consensus portion of the multiple alignment.

common with overseas observations [Fankhauser et al., 1998; Noel et al., 1999], a strain belonging to the genogroup II Bristol/Lordsdale virus subset, and now recognised as a 'global' strain common in hospital settings, has become the predominant strain in New Zealand over the study period. It has also been the most prevalent strain in New Zealand institutional outbreaks. In 1996, a large outbreak at a ski resort was traced to a drinking water (and ice) supply contaminated with sewage [Brieseman, 1996]. The NLV strain identified from faecal specimens amplified with both forward primers to produce two bands by gel electrophoresis, did not hybridise with any probes, and showed 97% homology with Southampton virus by DNA sequencing analysis. This strain has been identified sporadically since then. Two New Zealand NLV strains have been recognised. Auckland virus, a genogroup II strain closely resembling MX virus, was first identified in 1995 [Regli et al., 1995] after outbreaks associated with consumption of local oysters. Napier virus, that aligns in genogroup II but probes with P1-B probe was also first identified from an outbreak associated with contaminated oysters in 1996. This strain now seems to be genetically similar to the recently

identified genogroup II/7 'Gwynedd virus' cluster [Ando et al., 2000]. Strains of both Auckland and Napier virus have occurred sporadically across New Zealand since their first identification. In 1998, genogroup I strains have predominated. In particular, Desert Shield virus and Saratoga virus strains were responsible for 16 outbreaks, but have not been identified during 1999.

The development of sensitive molecular assays for detection and strain differentiation has been instrumental in clarifying the role of NLVs in many New Zealand outbreaks of gastroenteritis of public health significance. Traditional epidemiological and laboratory methods are unable to provide this information. Molecular typing methodology including RT-PCR, DNA hybridisation and DNA sequencing was used to determine whether a single NLV type or distinct types caused geographically separate clusters of infection. The diversity of strains occurring in New Zealand over 4 years is evident. As different NLV strains have appeared, the probe set has become of limited use for identifying strains.

Sequence analysis is now a tool accepted commonly for typing virus isolates (sequityping) and investigating outbreaks by tracing their source, and mapping relat-

ionships among them. Phylogenetic analysis can be used to further define relationships among molecular sequences obtained and their phylogenetic relationships with already known sequences. The power of these techniques depends on the genomic region analysed and the size of the sequences being compared. These are also the reasons for their limitations. Analysis of short, conserved sequences results in higher sensitivity, but low specificity, whereas longer sequences from a more variable genomic region result in higher specificity, but lower sensitivity. Sequences obtained during this investigation are from one of the most conserved regions of the calicivirus genome, the RNA-dependent RNA polymerase gene. The limitations of this less specific region are reflected in the sequence analysis results by 1) the high pairwise similarity values with number of identical sequences, 2) the short portion of the consensus alignment, and in the phylogenetic relationships by 3) the high number of branches with non-significant length. Sequencing of longer or more variable genomic region such as the capsid is necessary for further analysis of strains of particular interest.

The application of new molecular methods, sequence analysis tools and phylogeny provides information on disease transmission for epidemiological investigations. It is now possible to confirm the NLVs as causal agents and to examine the relatedness of NLV strains circulating in a community or a country. This can assist public health staff investigating outbreaks occurring in different locations that may have a common source. Such information will assist in the control of large outbreaks, be of significant benefit to public health authorities and may help in the development of improved food-handling practices, particularly in institutions and catering establishments.

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### REFERENCES

- Ando T, Noel JS, Fankhauser RL. 2000. Genetic classification of "Norwalk-like viruses." *J Infect Dis* 181:S336-S348.
- Ando T, Monroe SS, Gentsch JR, Jin Q, Lewis DC, Glass RI. 1995a. Detection and differentiation of antigenically distinct small round structured viruses (Norwalk-like viruses) by reverse transcription-PCR and Southern hybridisation. *J Clin Microbiol* 33:64-71.
- Ando T, Jin Q, Gentsch JR, Monroe SS, Noel JS, Dowell SF, Cicirello HG, Kohn MA, Glass RI. 1995b. Epidemiologic applications of novel molecular methods to detect and differentiate small round structured viruses (Norwalk-like viruses). *J Med Virol* 47:145-152.
- Berke T, Golding B, Jiang X, Cubitt DW, Wolfaardt M, Smith AN, Matson DO. 1997. Phylogenetic analysis of caliciviruses. *J Med Virol* 52:419-424.
- Boom R, Sol CJA, Salimans MMM, Jansen CL, Wertheim van Dillen PME, van der Noordaa J. 1990. Rapid and simple method for purification of nucleic acids. *J Clin Microbiol* 28:495-503.
- Brieseman M. 1996. Outbreaks of Norwalk-like virus infections linked to contaminated water at ski field. *NZ Public Health Report* 3:93.
- Caul EO. 1996a. Viral gastroenteritis: small round structured viruses, caliciviruses and astroviruses. Part I. The clinical and diagnostic perspectives. *J Clin Pathol* 49:874-880.
- Caul EO. 1996b. Viral gastroenteritis: small round structured viruses, caliciviruses and astroviruses. Part II. The epidemiological perspective. *J Clin Pathol* 49:959-964.
- Cauchi MR, Doultree JC, Marshall JA, Wright PJ. 1996. Molecular characterisation of Camberwell Virus and sequence variation in ORF3 of small round structured (Norwalk-like) viruses. *J Med Virol* 49:70-76.
- Dedman D, Laurichesse H, Caul EO, Wall PG. 1998. Surveillance of small round structured virus (SRSV) infection in England and Wales, 1990-5. *Epidem Infect* 121:139-149.
- Fankhauser RL, Noel JS, Monroe SS, Ando T, Glass RI. 1998. Molecular epidemiology of "Norwalk-like viruses" in outbreaks of gastroenteritis in the United States. *J Infect Dis* 178:1571-1578.
- Felsenstein J. 1993. PHYLIP (Phylogeny Inference Package). Distributed by the author, Dept. of Genetics, University of Washington, Seattle.
- Green SM, Lambden PR, Caul EO, Clarke IN. 1997. Capsid sequence diversity in small round structured viruses from recent UK outbreaks of gastroenteritis. *J Med Virol* 52:14-19.
- Green J, Gallimore CI, Norcott JP, Lewis D, Brown DWG. 1995. Broadly reactive reverse transcriptase polymerase chain reaction for the diagnosis of NLV-associated gastroenteritis. *J Med Virol* 47:392-398.
- Greening GE, Kieft C, Baker MG. 1999. Norwalk-like viruses (NLVs) a common cause of gastroenteritis outbreaks. *NZ Public Health Report* 6:73-77.
- Hedberg CW, Osterholm MT. 1993. Outbreaks of food-borne and waterborne viral gastroenteritis. *Clin Microbiol Rev* 6:199-210.
- Herwaldt BL, Lew JF, Moe CL, Lewis DC, Humphrey CD, Monroe SS, Pon EW, Glass RI. 1994. Characterisation of a variant strain of Norwalk virus from a food-borne outbreak of gastroenteritis on a cruise ship in Hawaii. *J Clin Microbiol* 32:861-866.
- Jiang X, Wang J, Estes MK. 1995. Characterization of NLVs using RT-PCR and a new antigen ELISA. *Arch Virol* 140:363-374.
- Jiang X, Huang PW, Zhong WM, Farkas T, Cubitt DW, Matson DO. 1999. Design and evaluation of a primer pair that detects both Norwalk- and Sapporo-like caliciviruses by RT-PCR. *J Virol Meth* 83:145-154.
- Jones N, Graham J. 1995. Outbreaks of gastroenteritis associated with oysters: a Norwalk-like virus most likely cause. *NZ Public Health Report* 2:25-26.
- Jarman J, Brown P. 1995. Outbreaks of gastroenteritis associated with consumption of oysters in Northland. *NZ Public Health Report* 2:26-27.
- Kaplan JE, Feldman R, Campbell DS, Lookabaugh C, Gary GW. 1982. The frequency of a Norwalk-like pattern of illness in outbreaks of acute gastroenteritis. *Am J Public Health* 72:1329-1332.
- Khan AS, Moe CL, Glass RI, Monroe SS, Estes MK, Chapman LE, Jiang XI, Humphrey C, Pon E, Iskander JK, Schonberger LB. 1994. Norwalk virus-associated gastroenteritis traced to ice-consumption aboard a cruise ship in Hawaii: comparison of and application of molecular method-based assays. *J Clin Microbiol* 32:318-322.
- Kohn MA, Farley TA, Ando T, Curtis M, Wilson SA, Jin Q, Monroe SS, Baron RC, McFarland LM, Glass RI. 1995. An outbreak of Norwalk virus gastroenteritis associated with eating raw oysters. Implications for maintaining safe oyster beds. *JAMA* 273: 466-471.
- Lew JF, Kapikian AZ, Valdesuso, Green KY. 1994. Molecular characterisation of Hawaii virus and other Norwalk-like viruses: evidence for genetic polymorphism among human caliciviruses. *J Infect Dis* 170:535-542.
- Moe CL, Gentsch J, Ando T, Grohmann G, Monroe SS, Jiang X, Wang J, Estes MK, Seto Y, Humphrey C, Stine S, Glass RI. 1994. Application of PCR to detect Norwalk virus in fecal specimens from outbreaks of gastroenteritis. *J Clin Microbiol* 32:642-648.
- Moe CL, Sobsey MD, Stewart PW, Crawford-Brown D. 1999. Estimating the risk of human calicivirus infection from drinking water. Presented in March at the International Workshop on Human Calicivirus, Atlanta, GA.

- McDonnell RJ, Wall PG, Adak GK, Evans HS, Cowden JM, Caul EO. 1995. Outbreaks of gastrointestinal disease associated with person to person spread in hotels and restaurants. *Commun Dis Rep* 5:R150–R152.
- Noel JS, Fankhauser RL, Ando T, Monroe SS, Glass RI. 1999. Identification of a distinct strain of “Norwalk-like viruses” having a global distribution. *J Infect Dis* 179:1334–1344.
- Norcott J, Green J, Lewis D, Estes MK, Barlow KL, Brown DWG. 1994. Genomic diversity of small round structured viruses in the United Kingdom. *J Med Virol* 44:280–286.
- Pringle CR. 1998. Virus taxonomy—San Diego. *Arch Virol* 143:1449–1459.
- Regli WJ, Green D, Jones N, Jarman J, Lewis G. 1995. First identification of Norwalk-like gastroenteritis in New Zealand by reverse transcription-PCR (RT-PCR). Presented at the NZ Microbiological Society Annual Conference, Dunedin.
- Riordan T. 1991. Norwalk-like viruses and winter vomiting disease. In: Morgan-Capnen P, editor. *Current topics in clinical virology*. London: Public Health Laboratory Service. p 61–94.
- Rosenfield SL, Jaykus L-A. 1999. A multiplex reverse transcription polymerase chain reaction method for the detection of food-borne viruses. *J Food Prot* 62:1210–1214.
- Schwab KJ, Neill FH, Fankhauser RL, Daniels NA, Monroe SS, Bergmire-Sweat DA, Estes MK, Atmar RL. 2000. Development of methods to detect “Norwalk-like viruses” and hepatitis A virus in delicatessen foods: application to a food-borne NLV outbreak. *Appl Environ Microbiol* 66:213–218.
- Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22:4673–4680.
- Viral gastroenteritis Sub-Committee of the PHLS Virology Committee. 1993. Outbreaks of gastroenteritis associated with SRSVs. *PHLS Microbiology Digest* 10:2–8.
- Wang J, Jiang X, Madore HP, Gray J, Desselberger U, Ando T, Seto Y, Oishi I, Lew JF, Green KY, Estes MK. 1994. Sequence diversity of small round structured viruses in the Norwalk virus group. *J Virol* 68:5982–5990.
- Wheeler JG, Sethi D, Cowden JM, Wall PG, Rodrigues LC, Tompkins DS, Hudson MJ, Roderick PJ, on behalf of the Infectious Intestinal Disease Study Executive. 1999. Study of infectious intestinal disease in England: rates in the community, presenting to general practice, and reported to national surveillance. *Br Med J* 318:1046–1050.
- Wright PJ, Gunesekere IC, Doultree, Marshall JA. 1998. Small round-structured (Norwalk-like) viruses and classical human caliciviruses in southeastern Australia, 1980–96. *J Med Virol* 55: 312–320.