EPIDEMIOLOGY

Multiple outbreaks of Norwalk-like virus gastro-enteritis associated with a Mediterranean-style restaurant

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The role of diverse infectious agents, particularly Norwalk-like viruses (NLV), in three successive gastro-enteritis outbreaks in one setting (a restaurant) was evaluated. Methods included standard bacteriological tests, specific tests for Escherichia coli, tests for verocytotoxins, electron microscopy (EM) for viruses and reverse transcription-PCR (RT-PCR) methodology for NLV. No pathogenic bacteria were detected. Verocytotoxin genes, although detected by PCR in the first outbreak, could not be confirmed in the E. coli isolated, so they did not appear to be of significance. NLV was the main agent detected in each of the three outbreaks. DNA sequencing and phylogenetic analysis of the amplified products obtained from the RT-PCR positive specimens indicated that only one NLV strain was involved in each outbreak, but the NLV strains responsible for the three outbreaks were different from each other. PCR technology for detection of NLV proved highly sensitive, but failed to detect one specimen which was positive by EM. The restaurant associated with the outbreaks is a Mediterranean-style restaurant where food from a common platter is typically eaten with fingers. The findings indicate that NLV was introduced by guests or staff and was not due to a long-term reservoir within the setting.

Introduction

An important, but little studied, area of public health concern is the nature of infectious agents associated with multiple outbreaks of gastro-enteritis in a given setting (such as a restaurant). Two, not necessarily exclusive, hypotheses can account for such outbreaks. Firstly, a reservoir of the infectious agent remains within the setting and periodically infects cohorts of susceptible individuals. Secondly, new infectious agents are repeatedly introduced into the setting and rapidly spread among susceptible individuals.

Recent advances in molecular biology permit detailed analysis of viral infectious agents and enable a group of viral agents to be differentiated at the genetic level [1]. Thus, studies of the genetic relationship between viral agents associated with multiple gastro-enteritis outbreaks are now possible, whereas 10 years ago this would not have been the case.

In this investigation, detailed bacteriological and virological studies were performed on faecal samples from three separate gastro-enteritis outbreaks, which occurred over a period of 13 months, associated with one setting (a Mediterranean-style restaurant). The findings are reviewed in relation to the cause and origin of the three gastro-enteritis outbreaks.

Materials and methods

The setting

Restaurant X is a Mediterranean-style restaurant in suburban Melbourne, Victoria, Australia. The premises comprise two storeys with one function area downstairs and one upstairs. Food is usually put on platters, which are placed on tables. When one course is finished the
platter is removed and the next dish is served. Customers commonly eat with their fingers and move from table to table. Between May 1998 and June 1999 three gastro-enteritis outbreaks were associated with the restaurant.

**Outbreak 1**

On Sunday 10 May 1998, three functions were held at restaurant X; two groups (groups 1 and 2) were upstairs and a third group was downstairs. Individuals from both groups upstairs, but no one from the downstairs group, became ill. The food for all three functions was prepared in the same kitchen.

The upstairs functions began at 7 pm. The food for groups 1 and 2 was served on platters at five tables. Many guests ate with their fingers and broke bread with their teeth. Guests moved from table to table. Group 1 comprised 74 individuals of whom 52 reported illness; group 2 comprised 26 individuals of whom 24 reported illness. Four food handlers claimed to have no illness. Two band members acknowledged illness, one 4 days before the function, the other 35 h after the function.

The mean time interval between consumption of the meal and the onset of symptoms (‘incubation’ period) was roughly the same for groups 1 and 2: 32.8 SD 17.3 h (n = 52) for group 1 and 36.5 SD 19.2 h (n = 24) for group 2. However, in group 1, three guests reported an ‘incubation’ period of only 4 h, whereas the shortest ‘incubation’ period in group 2 was 10 h (one individual).

Symptoms reported by guests in groups 1 and 2 included diarrhoea, vomiting, muscle aches and abdominal pain. The illnesses commonly lasted 1–7 days.

Faecal specimens were collected from 39 individuals associated with this outbreak.

**Outbreak 2**

A group of 27 people attended a 21st birthday party on the evening of Saturday 22 May 1999 at restaurant X. Of the 25 people interviewed, 16 reported illness.

The dinner began at 8.30 pm. The meal was a set menu including dips and platters. One individual developed gastrointestinal symptoms 2 h later while still at the restaurant. The remaining 15 individuals reported illness on the following 2 days with the majority (10) becoming ill on 24 May 1999. The mean ‘incubation’ period was 28.1 SD 13.2 h (n = 16). Symptoms included vomiting, lethargy, diarrhoea, nausea, abdominal pain, fever, anorexia and headache. The illnesses commonly lasted 1–4 days.

Faecal specimens were collected from six individuals associated with this outbreak.

**Outbreak 3**

A group of c. 100 individuals attended a christening function at restaurant X on the evening of Sunday 13 June 1999. Of these, 34 were interviewed, of whom 29 became ill and 5 did not. The meal began at 8.30 pm and comprised a buffet-style dinner. The earliest onset of symptoms was at 11 am on 14 June 1999, i.e., an ‘incubation’ period of c. 14.5 h. Fresh cases then occurred on 14 June (3 cases), 15 June (23), 16 June (1) and 17 June (1). Symptoms included fever, anorexia, nausea, vomiting, abdominal pain, diarrhoea, headache and lethargy. The illnesses lasted 1–7 days.

On 14 June some individuals ate together at another restaurant (restaurant Y). However, restaurant Y was excluded as the source of illness, as some individuals who were ill did not lunch at restaurant Y.

Faecal specimens were collected from 27 individuals associated with this outbreak. These included nine specimens from nine staff members of restaurant X (food handlers, band members, cleaners and a waiter).

**Bacteriology**

**Bacterial cultures.** All 72 faecal specimens from outbreaks 1–3 were examined for bacterial pathogens by standard microbiological procedures for species of Aeromonas, Campylobacter, Plesiomonas, Salmonella, Shigella, Yersinia and Vibrio, as well as Staphylococcus aureus and Bacillus cereus.

Specimens selected for detailed Escherichia coli characterisation included a subset of 22 faecal specimens from outbreak 1, all six faecal specimens from outbreak 2 and 18 of the 27 faecal specimens (i.e., excluding the 9 specimens collected from staff members) in outbreak 3.

Colonies from MacConkey agar, which resembled E. coli, were examined further by checking for agglutination with polyvalent antisera against commonly recognised enteropathogenic E. coli serovars as well as E. coli O157 antiserum. The faeces were also cultured on Sorbitol-MacConkey medium [2] and washed and unwashed sheep blood agars [3] especially to look for verocytotoxigenic E. coli. Colonies representative of all the different types found on these plates were picked out and these selected strains were further examined by full biochemical analysis and serotyping. The E. coli strains were fully serotyped for O and H antigens. All E. coli isolates were subjected to vero cell assay and enzyme-linked immunosorbent assay for their ability to produce verocytotoxins (VT).

**Verocytotoxin testing in faeces.** Two methods were used to test for the presence of VT in the 39 faecal specimens from outbreak 1: cell culture to detect VT and PCR to test for VT genes. No specimens were
tested from outbreaks 2 and 3. For cell culture studies, a filtrate of an overnight shake culture of faeces in Antibiotic Medium No. 3 (Oxoid) was placed on to VT-sensitive HeLa cells. They were then monitored for at least 48 h for the presence of a characteristic cytotoxic effect in the cell monolayer.

For PCR testing, a suspension of faeces was made up in Tryptone Soya Broth (Oxoid), then tested for VT by the method of Paton et al. [4]. The primers were designed to amplify conserved regions within the A subunit of Stx1 and Stx2 genes, so they should detect any bacterial strain carrying Stx1, Stx2 or both genes.

**Virological studies**

**Preparation of faecal specimens.** All 72 faecal specimens from outbreaks 1–3 were processed as described previously [5]. Both a clarified and an ultracentrifuged product were produced for each specimen.

Negative staining electron microscopy (EM). All 72 ultracentrifuged faecal extracts were examined after negative staining essentially as described previously [5].

Reverse transcription and PCR for Norwalk-like viruses. Analysis by reverse transcription (RT)-PCR was performed on all the faecal specimens from the three outbreaks. In the case of outbreak 1, 5-µl samples of the clarified faecal extract were used, whereas in outbreaks 2 and 3, the faecal extract was made up to 100 µl with nuclelease-free water (NFW) before RNA extraction.

RNA was extracted from faecal specimens with guanidine isothiocyanate and acidified phenol/chloroform, followed by isopropanol precipitation, ethanol wash and resuspension of the pellet in 10 µl of NFW. This material was then used for RT-PCR.

Norwalk-like virus (NLV)-specific primers were designed in two ways. Initially, published sequences of NLV strains were aligned and examined for conserved regions. The GenBank accession numbers (strain identification/year/country of origin) of the published sequences used for primer design were as follows. Genogroup 1: L07418 (Southampton/91/UK), L23832 (Sa-1283/84/JPN), AF093797 (unknown/GMY), L23828 (KY-89/89/JPN) and U04469 (Desert Shield/90/SA). Genogroup 2: L23826 (925/92/UK), L23830 (OTH-25/89/JPN), U02030 (MV24/91/CAN), U22498 (Mexico 34/89/MEX), L23831 (Snow Mt Agent/76/US), X81879 (Melksham/89/UK), X86557 (Lordsdale/93/UK), U07661 (Hawaii/71/US) and U46500 (Camberwell/94/AUS). On the basis of this investigation separate primer sets were designed for NLV genogroups 1 and 2 [1,6].

However, primer NVZ, the second-round reverse primer used for outbreak 1, was based on the nucleotide sequence of a first-round amplified product from a faecal specimen collected in outbreak 1, because no product was obtained from these specimens with one of the initial second-round primers.

All the primers were designed to target a region within the putative RNA-dependent RNA polymerase gene found in open reading frame (ORF) 1 of the NVL genome. The nucleotide sequence of the primers used in the study, and their corresponding position on the published sequence of the prototype Norwalk strain (NV/88Fla/68/US), are shown in Table 1.

RT and the first round of the PCR were performed with the Superscript™ One-Step™ RT-PCR System (Gibco BRL, Gaithersburg, MD, USA). Reactions were performed with 20 µl of the Superscript 2× reaction buffer, 0.2 µM of each primer, Rnasin (Promega Corporation, Madison, WI, USA) 16 units, 0.7 µl of RT-Tag mix from the Superscript kit and 10 µl of the extracted RNA in a final volume of 40 µl made up with NFW. A GeneAmp 2400 thermocycler (Perkin-Elmer Corporation, Norwalk, CT, USA) was used to perform the RT step at 50°C for 30 min, followed by PCR at 94°C for 2 min and 35 cycles at 94°C for 30 s, 53°C for 30 s and 72°C for 30 s, and a final extension step at 72°C for 5 min. The primers (Table 1) used for genogroup 1 NVL were NV4547 and NV5283 (at 737 product), and for genogroup 2 NVL were NV4596 and NV5098 (at 503 product).

<table>
<thead>
<tr>
<th>Primer name*</th>
<th>Primer sequence</th>
<th>Genogroup specified</th>
<th>Polarity</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>NV 4547 (22 mer)</td>
<td>5'-GATGCAAGTGAGATTACGAC/AAGCCCACT/TTGCGG-3'</td>
<td>1</td>
<td>Positive</td>
<td>4562–4583</td>
</tr>
<tr>
<td>NV 5283 (22 mer)</td>
<td>5'-ATCCACGCGGAAACTGCGCTGCGG-3'</td>
<td>1</td>
<td>Negative</td>
<td>5277–5298</td>
</tr>
<tr>
<td>NV5108 (19 mer)</td>
<td>5'-ATGGGGTTAAGTGAGATTACGAC/AGTTGCGG-3'</td>
<td>1</td>
<td>Negative</td>
<td>5105–5123</td>
</tr>
<tr>
<td>NV Z (21 mer)</td>
<td>5'-ATGGGGTTACGAC/GCTTGTTGCGG-3'</td>
<td>1</td>
<td>Negative</td>
<td>5040–5060</td>
</tr>
<tr>
<td>NV 4596 (21 mer)</td>
<td>5'-CA/TGGACGAC/AACCTGTA/GAGAAATCAGGG-3'</td>
<td>2</td>
<td>Positive</td>
<td>4611–4631</td>
</tr>
<tr>
<td>NV 4677 (23 mer)</td>
<td>5'-GTGTTCAAGTGAGATTACGAC/GTTGCGG-3'</td>
<td>2</td>
<td>Positive</td>
<td>4692–4714</td>
</tr>
<tr>
<td>NV 5098 (22 mer)</td>
<td>5'-ATGGAAAGTGAGATTACGAC/CTGAGCGG-3'</td>
<td>2</td>
<td>Negative</td>
<td>5092–5113</td>
</tr>
</tbody>
</table>

*For genogroup 1, NVL primers NV4547 and NV5283 were used in the first round and NV4547 and NVZ were used in the second round. For genogroup 2, NVL primers NV4596 and NV5098 were used in the first round and NV4579 and NV5098 in the second round. For sequencing, primers NV4547 and NVZ were used for genogroup 1 NVL and primers NV4677 and NV5098 for genogroup 2 NVL.

*Annexing site relative to Norwalk Reference Strain GenBank accession no. M87661.
The second round of the PCR was performed with 4 μl of 10× PCR buffer (Qiagen, Hilden, Germany), 0.2 μM of each primer, 0.2 mM of each deoxynucleotide (dATP, dCTP, dGTP, dTTP), Q buffer (Qiagen) 8 μl, Taq polymerase (Qiagen) 2 units and 2 μl of the first-round PCR product made up to a final volume of 40 μl with NFW. The Gene Amp 2400 thermocycler was used for amplification at 94°C for 5 min, then 25 cycles at 94°C for 30 s, 53°C for 30 s and 72°C for 30 s, followed by a final extension step at 72°C for 5 min. The primers used for genogroup 1 NLV were NV4547 and NV5108 (nt 562 product), except for outbreak 1 samples, for which NV5108 had to be replaced with NVZ (nt 499 product). For genogroup 2 NLV, primers NV4677 and NV5098 (nt 422 product) were used.

To visualise the RT-PCR products, 8 μl of each product were subjected to electrophoresis through an agarose 2% gel, stained with ethidium bromide and viewed on a UV transilluminator.

**NLV nucleotide sequence and phylogenetic analysis.**

The PCR products were purified with the JET-QUICK PCR Purification Spin kit (Genomed GmbH, Bad Oeynhausen, Germany) and sequenced with the Big Dye™ Terminator Cycle Sequencing kit (PE Applied Biosystems, Foster City, CA, USA) and the Applied Biosystems 373 automated DNA sequencer. Both plus and minus strands of the amplified products were sequenced with primers NV4547 (plus strand) and NVZ (minus strand) for genogroup 1 NLV, and NV4677 (plus strand) and NV5098 (minus strand) for genogroup 2 NLV. The software MacVector version 6.5.3 (Oxford Molecular Group, Campbell, USA) was used for the initial manipulation and analysis of nucleotide sequences.

Phylogenetic analyses of nucleotide sequence differences between the different NLV strains detected were performed with the software PHYLIP, version 3.5c [7]. All trees were built by the distance-based (maximum-likelihood matrix) neighbour-joining (NJ) method (functions DNADIST and NEIGHBOR), and drawn with the software TreeView version 1.5. The robustness of the NJ trees generated was assessed by bootstrap resampling of 1000 data sets that had been randomly resampled from the original alignment (functions SEQ-BOOT and CONSENSE). As different tree-building algorithms rely on different assumptions, the distance-based (Kimura matrix) NJ method and the maximum-likelihood (ML) method (function DNAML) were also used to increase the reliability of the derived tree topologies.

For phylogenetic analysis, the same published sequences as for primer design were used except that M87661 (NV/88Fla/68/US) was used in place of AF093797 (unknown/GMY), and L23832 (Sa-1283/84/JP), L23826 (925/92/UK) and L23831 (Snow Mt Agent/76/US) were not used. Nucleotide sequences of genogroup 2 NLV detected from other gastro-enteritis cases in Victoria in 1998 and 1999 (Fig. 2) were also used in this study.

**Results**

**Bacteriology**

No Campylobacter, Plesiomonas, Salmonella, Shigella, Vibrio or Yersinia spp., Staphylococcus aureus or B. cereus were found in any of the faecal specimens tested from the three outbreaks.

In the subset of 22 faecal specimens tested for *E. coli* in outbreak 1, *E. coli* O157:H39 was found in four individuals and *E. coli* O7:H15 in six individuals; three individuals had both strains. Eight individuals had other *E. coli* types either alone or, in one case, in conjunction with the above two strains. Four of the faecal specimens tested by PCR for VT genes gave positive results. Only two of the four faecal specimens which tested positive by PCR for VT genes were available for isolation of *E. coli* and both yielded only non-vero-cytotoxicigenic strains of *E. coli* O7:H15; one of them also yielded non-vero-cytotoxicigenic *E. coli* O157:H39. One additional faecal specimen was positive for VT by cell culture, although it was negative by PCR. This specimen yielded only α-haemolytic strains of *E. coli* OR:H7, which were not vero-cytotoxicigenic. None of the isolated strains, including the O157 strains, were vero-cytotoxicigenic.

In outbreak 2 all six faecal specimens were tested for *E. coli*. No *E. coli* was isolated from one specimen; only non-vero-cytotoxicigenic commensal strains of *E. coli* were identified in the other five specimens.

In outbreak 3, tests for *E. coli* were performed on 18 faecal specimens; non-vero-cytotoxicigenic commensal strains of *E. coli* were isolated from 14 specimens.

**Virology**

**EM and RT-PCR analysis.** In outbreak 1, NLV was detected by EM in 4 of the 39 faecal specimens tested. The particles had the characteristic morphology of NLV [8], with a mean diameter of 33 nm (33.1 SD 1.7 nm, n = 23, based on a minimum of three particles from each of the four individuals).

Initial testing by RT-PCR produced positive results in only 4 of the 39 faecal specimens (not all EM positive) tested following first-round amplification with genogroup 1 primers, but all were negative in the second round. Based on the nucleotide sequence obtained from one of the first-round amplified products, a new second-round reverse primer, NVZ, was designed (see Materials and methods). With this primer, 26 of the 39 faecal specimens tested were positive. Faecal speci-
mens were provided for testing from two of the three individuals with a 4-h ‘incubation’ period. One of these was positive for NLV by RT-PCR.

The relationship between the detection of NLV and the time interval between onset of symptoms and specimen collection was examined for 21 cases in outbreak 1. The four specimens positive for NLV by EM (and RT-PCR) in this group were collected between 1 and 3 days following the onset of symptoms. The 13 specimens positive for NLV by RT-PCR (but negative by EM) in this group were collected between 1 and 3 days following the onset of symptoms. The four specimens in this group which were negative for NLV by both EM and RT-PCR were also collected between 1 and 3 days following the onset of symptoms. Taken together, these data suggest that virus quantity excreted, rather than the time interval between onset of symptoms and time of collection, is the crucial factor in determining the ability to detect NLV.

In outbreak 2, no virus was detected in any of the six faecal specimens by EM. However, four of the specimens were positive by RT-PCR with NLV genogroup 2 primers. No faecal specimen was received from the individual who had a 2-h ‘incubation’ period.

In outbreak 3, NLV was detected by EM in one (‘A’) of the 27 individuals tested. The particles had the characteristic morphology of NLV [8] with a mean diameter of c. 35 nm (35.4 SD 2.0 nm, n = 6).

By RT-PCR, 12 of the 27 individuals were positive for NLV with NLV genogroup 2 primers. This included one staff member, a food handler. However, individual ‘A’, who was NLV positive by EM, was RT-PCR negative.

**NLV and VT**: In the five VT-positive faecal specimens from outbreak 1 tested for NLV, NLV was detected in two cases, both of which were positive by the VT gene PCR assay.

**NLV nucleotide sequence and phylogenetic analysis.** The nucleotide sequences of ORF1 segments (putative RNA polymerase region) amplified from 20 of the 42 faecal specimens positive for NLV by RT-PCR were determined. These included the four EM-positive faecal specimens from outbreak 1, four faecal specimens from outbreak 2 and 12 faecal specimens from outbreak 3. These nucleotide sequences were then aligned to find the longest common region, and a 342-bp segment was selected for subsequent analyses. All NLV identified from within the same outbreak showed no nucleotide variation within the 342-bp segment of ORF1. In contrast, marked differences were found in the same ORF1 region between the NLV strains from the three outbreaks.

Pairwise comparisons of identity between the nucleotide sequences of ORF1 segments from the NLV strains from the three outbreaks and other published sequences of NLV strains are listed in Table 2. Percentage nucleotide identities of the ORF1 segments between the NLV strains from outbreaks 1 and 2, outbreaks 1 and 3, and outbreaks 2 and 3 were 63%, 65% and 83% respectively. Published sequences that share the highest nucleotide identity with NLV strains from outbreaks 1, 2 and 3 were Desert Shield (79%), MV24 (75%) and Cambrewer (94%), respectively.

To assess the phylogenetic relationship between the NLV strains from the three outbreaks and other NLV strains, a number of tree-building algorithms were applied to the 342-bp ORF1 segments. The distance-based (maximum-likelihood distance matrix) NJ method was the principal algorithm used for analysis, and the unrooted tree generated is shown in Fig. 1. The radial presentation of the tree clearly allocated the NLV strains to the two genogroups, and trees derived by the NJ (Kimura matrix) and ML methods also

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**Table 2. NLV ORF1 segment nucleotide sequence identities**

|     | NV    | DSV   | KY89  | SHV   | OB1   | MV    | MXV   | OTH89  | MV24  | OB2   | H V   | L V   | C V   | OB3   |
|-----|-------|-------|-------|-------|-------|-------|-------|--------|-------|-------|-------|-------|-------|-------|-------|
| NV  | 100   | 73    | 74    | 75    | 73    | 62    | 58    | 59     | 59    | 59    | 62    | 59    | 60    | 59    |
| DSV | 100   | 71    | 70    | 79    | 63    | 60    | 61    | 61     | 59    | 61    | 61    | 61    | 60    | 60    |
| KY89| 100   | 73    | 73    | 62    | 61    | 61    | 62    | 59     | 63    | 63    | 62    | 61    | 61    | 60    |
| SHV | 100   | 74    | 64    | 65    | 65    | 66    | 61    | 65     | 61    | 65    | 61    | 60    | 61    |
| OB1 | 100   | 65    | 64    | 65    | 63    | 66    | 65    | 65     | 65    | 65    | 65    |
| MV  | 100   | 75    | 76    | 76    | 78    | 78    | 76    | 75     | 78    | 79    |
| MXV | 100   | 95    | 97    | 74    | 77    | 77    | 77    | 78     | 78    | 79    |
| OTH89| 100  | 96    | 97    | 74    | 77    | 77    | 77    | 78     | 78    | 79    |
| MV24 | 100 | 75    | 78    | 77    | 77    | 78    | 77    | 79     | 79    | 80    |
| OB2 |     | 100   | 80    | 83    | 82    | 83    | 82    | 82     | 83    | 83    |
| HIV | 100  | 85    | 85    | 85    | 85    | 85    | 85    | 85     | 85    |
| LV  | 100  | 91    | 91    | 91    | 91    | 91    | 91    | 91     | 91    |
| CV  | 100  | 94    | 94    | 94    | 94    | 94    | 94    | 94     | 94    |
| OB3 | 100  | 100   | 100   | 100   | 100   | 100   | 100   | 100    | 100   |

**Abbreviations and GenBank accession numbers**: NV (Norwalk virus, M876661), DSV (Desert Shield virus, U044699), KY89 (L23828), SHV (Southampton virus, L07418), OB (outbreak), MV (Melksham virus, X81859), MXV (Mexico virus, U22498), OTH89 (L23830), MV24 (U02030), HV (Hawaii virus, U07611), LV (Lordsdale virus, X86557) and CV (Cambrewer virus, U46500).

*All values were calculated by using Clustal W from the software MacVector version 6.5.3.*
showed similar topologies. As expected, the NLV strain from outbreak 1 was placed amongst the genogroup 1 NLV strains, whereas the NLV strains from outbreaks 2 and 3 were placed amongst the genogroup 2 NLV strains. The NLV strain from outbreak 3 appeared to be most closely related to the Camberwell strain (94% nucleotide identity), and they in turn formed a clade with the Lordsdale strain. Although the NLV strain from outbreak 2 did not appear to be genetically related to any particular published sequence, it diverged from the same node as the clade that includes the outbreak 3, Camberwell and Lordsdale strains. The NLV strain from outbreak 1 formed a unique branch with the Desert Shield strain (79% nucleotide identity), and it in turn diverged from the same node as the clade that includes other genogroup 1 prototype strains. Bootstrap analysis of the tree provides good support for all the major clades on the tree. Although the bootstrap value for the node shared between the NLV strain from outbreak 2 and the clade that includes the outbreak 3, Camberwell and Lordsdale strains is
<30%, this entire clade shared the same ancestral node as the Hawaii strain with a bootstrap value >80%.

To further clarify the genetic differences between the NLV strains from outbreaks 2 and 3, phylogenetic analyses of the 342-bp ORF1 segments from these NLV strains, three published nucleotide sequences of genogroup 2 NLV strains, and other locally detected genogroup 2 NLV strains (i.e., NLV strains that have been associated with other gastro-enteritis cases in Victoria during 1998 and 1999) were performed. Using the published sequence of the Hawaii strain as the root, the distance-based (maximum-likelihood matrix) NJ method was the principal algorithm used to perform the analysis. The tree generated by this method is shown in Fig. 2, and the placement of the two NLV strains of this study in the tree clearly demonstrates their genetic differences. Similar topologies were obtained with other tree-building algorithms.

Discussion

This study examined in detail the infectious agents associated with three separate outbreaks of gastro-enteritis in one setting (a restaurant). No bacterial pathogens were found and the occasional detection of VT in outbreak 1, which could not be confirmed by the isolation of verocytotoxigenic E. coli, did not appear to be of clinical significance. If the diagnostic tests for outbreak 1 had been restricted to PCR or cell culture studies of the faeces for VT and to separate tests for the E. coli O157 antigen, then outbreak 1 might have been incorrectly designated as due to verocytotoxigenic strains of E. coli O157. It is noteworthy that in a previous study [9] VT-producing E. coli and Aeromonas were found in a NLV-related gastro-enteritis outbreak, but appeared to have no clinical significance.

NLV, a genus of viruses within the family Caliciviridae, are an important cause of gastro-enteritis in man [10,11] and are commonly associated with gastro-enteritis outbreaks [1,11]. Prolonged or recurring outbreaks associated with NLV have been reported in some settings [12,13]. Although neither of the latter studies determined the exact nature of the NLV, there was an inference in both studies that the same agent kept re-infecting new cohorts of individuals. NLV is a hardy virus, and can remain infective in the environment for long periods [14]. The results of the present study indicate that all three outbreaks in restaurant X were due to NLV, as this virus was found in a significant number of individuals in each outbreak. However, the three outbreaks were not due to a single agent re-infecting new groups of guests. Although no nucleotide variation was found between the 342-bp ORF1 segments of all NLV that belonged to the same outbreak, suggesting that all the gastro-enteritis cases from within the same outbreak were caused by a single NLV strain, there were substantial nucleotide differences found in the analysed ORF1 segments of NLV detected in different outbreaks (Table 2). Each outbreak was associated with its own strain of NLV.

The genetic differences between the three outbreak NLV strains were confirmed in the phylogenetic trees generated by the distance-based (maximum-likelihood) NJ method (Figs. 1 and 2). The NLV strains from outbreaks 2 and 3 are genetically related to the prototype NLV strains Camberwell, Lordsdale and Hawaii. This finding is not unexpected, as the majority of NLV detected in Victoria between 1980 and 1996 belonged to the Lordsdale/Camberwell-like group of viruses [1]. Nonetheless, when the ORF1 segments of these outbreak NLV strains were compared with other locally detected genogroup 2 NLV, these strains were clearly distinct (Fig. 2). On the other hand, the NLV strain from outbreak 1 was unusual in two aspects: (i) it belonged to genogroup 1 which is infrequently detected in Victoria [1], and (ii) the nucleotide sequence of the ORF1 region targeted for primer annealing was sufficiently different from those of the published sequences of genogroup 1 NLV strains used for primer design that a new primer was necessary. The most closely related well-known strain was Desert Shield (Fig. 1), and these two strains share a nucleotide identity of only 79%. When a BLAST (Basic Local Alignment Search Tool) search was performed on the 342-bp ORF1 segment of the NLV from outbreak 1, a nucleotide identity of 97% was obtained with a 198-bp ORF1 segment of the published sequence of Saratoga calicivirus 7 [15] that was initially detected in the USA (analysis not shown).

The difference in the NLV strains in separate outbreaks indicates that in each outbreak NLV was introduced into the restaurant rather than being continuously present. The incubation period for NLV infection has been stated to be c. 24–48 h [11], so that very short ‘incubation’ periods reported by one or a few guests in outbreaks 1 and 2 would strongly suggest that infection occurred before attendance at a suspect function. Thus the sources of infection in outbreak 1, where a NLV-positive guest reported an ‘incubation’ period of 4 h, and outbreak 2, where a guest reported an ‘incubation’ period of 2 h, could well have been these guests. The origin of infection in outbreak 3 is unclear. It is noteworthy that a food handler was NLV positive and may have been the source of the infection. The sharing of food from a common platter, often with finger handling, would facilitate the spread of the highly infectious NLV.

The application of RT-PCR technology to NLV detection has not only increased the sensitivity of detection, but has also made it easier to perform molecular characterisation. However, the results of this study emphasise that RT-PCR for NLV is subject to false negatives. Firstly, the NLV strain from outbreak 1
could easily have been missed if RT-PCR was the only method used for diagnosis. The annealing site for the second-round reverse primer (NV5108), normally used for genogroup 1 NLV detection in this laboratory, was sufficiently different to prevent the primer from annealing. A new second-round primer had to be designed. Secondly, in one instance (in outbreak 3), NLV was detected in a faecal specimen only by EM

Fig. 2. A phylogenetic tree showing the genetic relationship between different genogroup 2 NLV strains, based on sequence differences of the 342-bp ORF1 segments in the RNA genome. The tree was built with the distance-based (maximum-likelihood matrix) NJ method with the published sequence of the Hawaii strain as the root. The percentage of bootstrap support exceeding 50% (out of 1000) is indicated by numbers next to the corresponding node. The scale represents nucleotide substitutions per site.
and not by RT-PCR. As RT-PCR was able to detect NLV in other faecal specimens of the same outbreak, the most likely explanation for the false negative result in this case is the presence of inhibitors. The failure to detect NLV EM-positive samples by RT-PCR has been reported elsewhere [1]. It can be concluded that EM remains an important adjunct to viral gastro-enteritis investigations.

On the basis of the results obtained here, repeat outbreaks of viral gastro-enteritis do not necessarily indicate that a reservoir of a given agent is present in the setting. The possibility that the setting is particularly prone to the re-introduction of new infectious agents should also be taken into account. High standards of hygiene are especially important when guests take food from a common platter with their bare hands.

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References