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# Norovirus Infection in A Taipei Regional Hospital and Its Laboratory Analysis

## Introduction

On 24 February 2004, the Center for Disease Control received specimens of several suspect diarrheal patients from a Taipei regional Hospital. Since 20 February, 17 patients and one nurse showed symptoms of watery diarrhea, fever and vomiting. By 3 March, a total of 37 cases had such symptoms. Upon investigation it was revealed that some of these patients were hospitalized and undergoing treatment after having been tracheotomized or intubated due to respiratory distress. According to the time of onset of the disease the incubation period was estimated to be 1-3 days. Subsequently, the Viral Laboratory received 36 rectal swabs and two fecal specimens of patients, 11 rectal swabs and one fecal specimen of the employees, and one rectal swab of a family member of the employee.

## The Background

Acute gastroenteritis is a health issue with global implications. Causes of gastroenteritis are many, including bacteria, parasites and viruses. Major causes of viral gastroenteritis are Norovirus (originally the Norwalk-like virus) of the Caliciviridae family, Sapovirus (originally the Sapo-like virus), Rotavirus A,

B and C, Astrovirus, and Adenovirus types 40 and 41. Other viruses that can be found in the intestinal tract include Enterovirus, Coronavirus, Hepatitis A virus, and Herpes virus. They can also induce diarrhea-like symptoms.

In industrialized countries in Europe, in the US and Japan, viral gastroenteritis is always the major cause of diarrhea. In Europe and the US for instance, viral gastroenteritis accounts for 60% and 80% respectively of all diarrhea cases each year(1,2). According to epidemiological investigations, Norovirus is the major pathogenic agent of non-bacterial gastroenteritis in adults; it is also the major pathogenic agent of food poisoning, accounting for 70-95% of all herd infections(3-5). Infection occurs more often in adults and older children, and herd infections are likely to occur in nursing homes, hospitals and schools. Cases are reported throughout the year, though more often in winter. Clinical symptoms are watery diarrhea, vomiting, abdominal pain and slight fever. In many patients, the major complaint is vomiting; the infection is also called the winter vomiting disease. The incubation period is 12-48 hours, and symptoms may last from 12-60 hours. Most patients recover in 12-60 hours. However the infection may be worse in infants, young children and debilitated patients as a result of dehydration. Infection is spread through fecal and oral transmission from water and food, particularly shellfish, contaminated by feces of the infected.

Norovirus is a virus of the Caliciviridae family, a single-ply RNA of 20 faces without outer shells(6). The gene is about 7.5-7.7 kb long, in three open reading frames (ORFs). Conventionally, the virus is examined with the electron microscope. This method requires a minimum amount of 106-107/mL in feces, sensitivity therefore is low, and low pathogenicity infections such as Norovirus infection are often undetected resulting in false-negatives. The rapid EIA method has been developed with a sensitivity of 104-106/mL in feces. As the gene and antigen of the virus have a large variability(7), the specificity of the

method is not high. Currently, RT-PCR testing is used more often (8), which is sensitive up to 101-3/g in feces. The gene variability of Norovirus is high, and to improve accuracy of testing, more pairs of nucleic acid primers should be developed and used for testing at the same time.

**Materials and Methods**

Handling of Specimens

Testing of diarrhea viruses often requires fresh feces to be transported from collection site to laboratory at a low temperature. In the present incident, the hospital transported only rectal swabs for testing, feces were just noticeable on the swabs, and no fecal matter could be taken for dissolution in PBS by standard operational procedures. Thus the quantity of viruses may have been affected. Specimens were handled in the following way: fecal specimens on the rectal swabs were mixed evenly with the transport media (about 1 mL) of the swab, pipetted under sterile conditions and placed in a distilled centrifugal tube, centrifuged at 4℃, 3000xg, for 15 minutes; the supernatant was collected and retained in two small tubes, labeled and dated, and maintained at -70℃.

ELISA Method

Norovirus analysis: The RIDACSCREEN® Norwalk-like Virus reagent of the γ-Biopharm was used for the testing of Norovirus. 100 μL of the supernatant fluid of the already processed fecal specimen was

collected and placed in the microwell of the RIDACSCREEN

Norwalk-like Virus at room temperature for one hour, rinsed five times. When the water in the microwell was dry, 100 μL of enzyme conjugate was added for reaction at room temperature for 30 minutes, rinsed again five times, dried, and 100 μL of substrate added, left in a light-free area for 15 minutes, then 50 μL of a stop solution was added. 450 nm of light absorption value was read by the

ELISA reader (μ Quant). When the light absorption value was larger than the cut-off value (negative control light absorption value plus 0.15), the result was read as positive.

Rotavirus analysis: The RIDACSCREEN® Rotavirus reagent of the γ-Biopharm was used. 100 μL of the supernatant of the already processed fecal specimen was collected, placed in the microwell of the RIDACSCREEN Norwalk-like Virus, two drops of enzyme conjugate was added for reaction at room temperature for 60 minutes; the mixed solution was removed and rinsed five times with 300 μL of a rinsing solution. When water in the microwell was dry, two drops of substrate/chromogen was added, placed in a light-free area at room temperature for 15 minute; then one drop of stop solution was added again. 450 nm of light absorption value was read by the ELISA reader (μ Quant). When the light absorption value was larger than the cut-of value (negative control light absorption value plus 0.15), the result was read as positive.

RNA Extraction

The QIAmp Viral RNA kit of the QIAGEN was used for the extraction and purification of RNA. 140 μL of the supernatant of the already processed fecal specimen was collected, 560 μL of Buffer AVL was added for reaction at room temperature for 10 minutes, 560 μL pure alcohol was added for uniform vertexing. The composite fluid was sent

through a QIAmp spin column, and the filtered mixture was discarded.

The column was collected, washed with buffer AW twice, and RNA dissolved with buffer AVE.

Establishment of RT-PCR Standard Plates

As Norovirus cannot yet be isolated by cell culturing, standard virus strains cannot yet be obtained. To meet the experimental need of the RT-PCR positive

control group, the corresponding RT-PCR gene sections were synthesized into oligomers, and the two ends of the enzymes were made EcoR I and BamH I, to connect to pUC19 with T4 ligase to synthesize into 03Q401CDC1 plasmids, transferred to DH5αcells of E. coli for multiplication and frozen for keeping.

Designing of RT-PCR Primers

Norovirus analysis: As the gene variability of Norovirus is high, in the selection of primers, stability of gene sequences should be considered. The RNA dependent RNA polymerase sections and the N end of the nucleic protein of high stability were selected. Of the primers SR33, SR46, SR48, SR50 and SR52 are designed for the RNA dependent RNA polymerase sections (see Table 1), for the high variability of the virus, and to detect Norovirus of different genotypes, thus SR33 was used as a forward primer, SR46, SR48, SR50 as reverse primers of G1, and SR52 as a reverse primer of G2 for reaction separately with G1 or G2 to gain a product of 123 bp(2). Primer pairs Mon431, Mon432, Mon433 and Mon434 were designed for the sections between RNA dependent RNA polymerase and nucleic protein gene sequences of high stability (see Table 1) (CDC design).

Rotavirus analysis: The VP6 of the Rotavirus is the major glycoprotein, a group-specific protein used to divide Rotavirus into five groups of A-E. Of the 5 groups, groups A, B and C infect humans, and group A is the most common. Primers con1 and con2 (see Table 1) were therefore selected from the Group A VP6 section; the product was 201 bp(2).

RT-PCR

Norovirus analysis:

SR/RT-PCR: 5 μL of the virus RNA was extracted as plate for reactions with G1 (primers SR33, SR48, SR50, SR52) and G2 (primers SR33, SR46). The final concentration of the primers was 0.3 μM, placed at 95℃ for three minutes, and

the reaction tubes were put immediately on ice. A RT-PCR mixture containing 0.625 mM dNTP, 5U M-MuLV reverse transcriptase, 2.5U TaqDNA Polymerase, 40U RNase inhibitor, and buffers of 50mM Tris-HCl, 75 mM KCl, 3 mM MgCl2, and 10 mM dithiothreitol were added for a total volume of 50 μL, for reverse transcription at 42℃ for 60 minutes, and at 94℃ for three minutes, and then PCR for 40 cycles; at 94℃ for one minute, at 52℃ for 1.5 minutes, at 60℃ for two minutes, and at 72℃ for seven minutes.

Mon/RT-PCR: 5 μL of the virus RNA was extracted as plate and primers Mon 431, Mon 432, Mon 433 and Mon 434 were added for reaction. The final concentration of the primers was 0.6 μM; RT-PCR mixture containing 0.625 mM dNTP, 5U M-MuLV reserve transcriptase, 2.5U Taq DNA Polumerase, 40U RNase inhibitor, and buffer solution containing 50 mM Tris-HCl, 75 mM KCl, 3 mM MgCl2, 10 mM dithiothreitol were added for a total volume of 50 μL. Reverse transcription was conducted at 42℃ for 15 minutes, at 94℃ for three minutes for PCR for 40 cycles; at 94℃ for 30 seconds, at 52℃ for 1.5 minutes, at 60℃ for 30 seconds, and at 72℃ for seven minutes.

10 μL of the Norovirus reaction product was extracted and placed in 1x Tris-acetate-EDTA (TAE) 2% SeaKem LE Agarose (BMA, BioWhittaker Molecular Application), with 100 volt electrophoresis for the analysis of the size of products.

Rotavirus analysis:

5 μL of the virus RNA was extracted as plate, primer mixtures were added for reaction at 97℃ for five minutes; the tubes were immediately placed on ice; RT-PCR mixtures containing 0.625 mM dNTP, 5U reverse teranscriptase, 2.5U Taq DNA Polymerase, 20U RNase inhibitor and buffers were again added for a total volume of 50 μL, placed at 42℃ for reverse transcription for 30 minutes.

The RT product was taken, PCR mixture containing 0.625 mM dNTP, primers con1 and con2 0.4 μM, 5U Taq DNA Polymerase, 20U RNase inhibitor and buffers were added, placed at 94℃ for three minutes for PCR for 40 cycles, at 94℃ for 30 seconds, at 42℃ for 30 seconds, at 72℃ for one minute, and at 72℃ for seven minutes.

10 μL of the reaction product was extracted and placed in 1x Tris-acetate-EDTA (TAE) 2% SeaKem LE Agarose (BMA, BioWhittaker Molecular Application) for with 100 volt electrophoresis for the analysis of the size of the product.

Sequence Analysis:

ABI PRISM (BigDye Terminator v 3.1 Cycle Sequencing Kit) was used for nucleic acid sequencing. A certain amount of the RT-PCR product, 1 μM of primer SR33, 1 μL of BigDye 3.1, and buffers were taken for a total final volume of 10 μL. The micro-centrifugal tube was placed at 96℃ for one minute, and then at 96℃ for 10 seconds, at 50℃ for five seconds, and at 60℃ for four minutes, for 25 cycles.

Purification of product: To avoid interference of floating markers in mixtures, the product of sequencing was first purified. To 10 μL for the sequenced product was added ddH2O of the same volume, 60 μL of 100% alcohol, 5 μL of 125 mM EDTA, and placed at room temperature for 15 minutes, centrifuged at 4000 rpm for 30 minutes; the supernatant was removed, and rinsed with 70% alcohol, centrifuged at 4000 rpm for five minutes, and 10 μL of Hi-diformamide was added to the dried sediment. Gene sequencing : the purified product was placed at 96℃ for two minutes, and put on ice immediately, ABI 3730 DNA Autosequencer was added again for nucleic acid sequence analysis.

Comparative Analysis of Virus Genes: Sequences were compared with the Norovirus sequences in the NCBI databank to verify their relationship.

**Results and Discussion**

On 24 February, the Virology Laboratory received from the DOH Taipei Hospital one rectal swab of a patient with a diarrhea syndrome listed on the surveillance and reporting system for emerging communicable diseases. Subsequently, the Laboratory received 36 more rectal swabs and two fecal specimens of patients, 11 rectal swabs and one fecal specimen of an employee, and one rectal swab of a family member of an employee. Epidemiological investigation revealed that several patients began to show symptoms of diarrhea on 20 February, and the number of cases increased thereafter. On 23 February, at the peak of the outbreak, employees and medical personnel of the Hospital also developed symptoms (see Figure 1). Clinically, the cases of this herd infection developed in a short period of time symptoms such as diarrhea, vomiting. With an incubation period of 1-3 days, and no common sources of food transmission detected, the incident was speculated to be either a Norovirus or Rotavirus infection.

The specimens, after centrifugation, were examined with ELISA method and sequence analysis by RT-PCR after extraction of the RNA. The Norovirus that infects humans has two genotypes, G1 and G2. The primers used, SR33, SR46, SR48, SR50 and SR52, could detect Norovirus and at the same time differentiate its genotypes. The RT-PCR products were of 123 bp length. Reactions of the specimens are shown in Figure 2a. Lane N is the negative control; Lane M is the 100 bp DNA Ladder Marker (Violet, Taiwan); Lanes 1-5 are the results of electrophoresis of the RT-PCR products; and Lane P is the positive control using synthesized 03Q401CDC1 standard strain as the plate. The RT-PCR showed Norovirus G1 positive; the RT-PCR product was thus confirmed to be Norovirus. After nucleic acid sequencing of the reaction product, the result was compared with the NCBI gene databank to confirm it as Norovirus (originally Norwalk-like

virus). Genes in the RNA dep polymerase sections were similar, and their sequences were 98% similar (Figure 3). The present study also used the primer pairs of the US CDC, Mon 431, Mon432, Mon433 and Mon434, for the parallel RT-PCR testing. Primers were selected from the area between the RNA dependent RNA polymerase and the N end of the nucleic protein. This area was the area of high stability of Norovirus sequences. Results of the reactions of the present study are shown in Figure 2b. The RT-PCR reaction product is 213 bp; Lane M is the 100 bp DNA Ladder Marker (Violet, Taiwan); and Lanes 1-6 are results of the electrophoresis of RT-PCR products.

The number of cases tested was 36 patients, 11 medical personnel and one employee, and one family member of an employee. They were tested using the ELISA method (RIDACSCREEN Norwalk-like Virus reagent), and RT-PCR by SR and Mon. The results are shown in Table 2. Positive rates using the three methods were 29.17%, 89.5% and 18.75% respectively, with a large discrepancy. The reasons could be: 1) the ELISA method used the glycoprotein section for a single=ply antibody for the testing of specimens. Katayama et al.(9) reported that when ORF2 was used as gene section for glycoprotein, the variability was high, and it could reflect the degree of variability of the Norovirus antigens. 2) The SR primers were designed to meet all possible gene sequences of Noroviruses, and they were in the more stable regions of the RNA dep polymerase nucleic acid sequences. 3) The Mon primers were designed in the ORF1-ORF2 connecting area of high stability. 4) ELISA reagents, for difference in viruses of different regions, could produce positive findings different from those of the RT-PCR. 5) Differences in the positive rates of SR/RT-PCR and Mon/RT-PCR could be due to differences in gene specifics, or an insufficient amount of viruses, and the amount of PCR plates collected was too small, resulting in errors of manipulation. In addition, two specimens were Rotavirus

positive, the ELISA reagent used and the RT-PCR were so designed for the testing of Rotavirus group A single strain antibody and primers.

Most cases were patients with concomitant respiratory tract infection. They were 60 and above years old, with 70-80 year olds accounting for 35.4%. About 25% of the cases were employees of the Hospital. Age distribution of the patients and the employees is shown in Table 3. Results of the study show that the present source of infection of the herd outbreak was Norovirus. This speculation corresponded with the symptoms, clinical manifestations and incubation period. The pathogenicity of Norovirus is low (about 102 concentration of viruses); the infection is likely to spread in the environment and among humans. The present incident showed the importance of nosocomial infection, and the roles played by food handlers and caretakers of patients. In crowded public places such as nurseries and nursing homes, herd infection is likely to occur. Speculations are that after the diarrheal case on 20 February, the infection was spread by employees of the Hospital to other patients and medical personnel. From the viewpoint of public health, epidemiological studies and analyses of viruses causing acute gastroenteritis are incomplete, therefore the present study could be used as a point of reference for future investigations. The study could also serve as a model for the establishment of laboratory testing methods for future epidemiological investigations. In addition to strengthening health education of the public and of medical personnel, more should be done to develop indigenous studies.

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**Table 1. RT-PCR Primer Pairs**

| Nucleic acid primers | 5'→3' sequences | Localization |
|---|---|---|
| **Norovirus** | | |
| SR33 | TGTCACGATCTCATCATCACC | 4856-4876 |
| SR46 | TGGAATTCCATCGCCCACTGG | 4754-4773 |
| SR48 | GTGAACAGCATAAATCACTGG | 4754-4773 |
| SR50 | GTGAACAGTATAAACCACTGG | 4754-4773 |
| SR52 | GTGAACAGTATAAACCATTGG | 4754-4773 |
| | | |
| Mon431 | TGGACIAGRGGICCYAAYCA | 5093-5112 |
| Mon432 | TGGACICGYGGICCYAAYCA | 5093-5112 |
| Mon433 | GAAYCTCATCCAYCTGAACAT | 5305-5285 |
| Mon434 | GAASCGCATCCARCGGAACAT | 5305-5285 |
| **Rotavirus** | | |
| con 1 | TTGCCACCAATTCAAAATAC | 666-685 |
| con 2 | ATTTCGGACCATTTATAACC | 862-881 |

**Table 2. Sources of Specimens and Positive Rates of Norovirus**

| Sources | No. | Positive No.(%) | | |
|---|---|---|---|---|
| | | RT-PCR (SR) | RT-PCR (Mon) | RIDASCREEN |
| Patients | 36 | 34 (94.4) | 6 (16.67) | 13 (36.11) |
| Med personnel and employees | 11 | 8 (72.7) | 3 (27.27) | 1 (9.09) |
| Family | 1 | 1 (100) | 0 | 0 |
| Total | 48 | 43 (89.5) | 9 (18.75) | 14 (29.17) |

**Table 3. Age Distribution of Cases**

| Age | Sex | No. (%) of Patients | No. (%) of Employees | Total (%) |
|---|---|---|---|---|
| Under 60 | M | 2 (4.17) | 0 | 2 (4.17) |
| | F | 1 (2.08) | 11 (22.92) | 12 (25.00) |
| 60~70 | M | 3 (6.25) | 0 | 3 (6.25) |
| | F | 3 (6.25) | 1 (2.08) | 4 (8.33) |
| 70~80 | M | 10 (20.83) | 0 | 10 (20.83) |
| | F | 7 (14.58) | 0 | 7 (14.58) |
| 80~90 | M | 4 (8.33) | 0 | 4 (8.33) |
| | F | 4 (8.33) | 0 | 4 (8.33) |
| 90 above | M | 0 | 0 | 0 |
| | F | 2 (4.17) | 0 | 2 (4.17) |

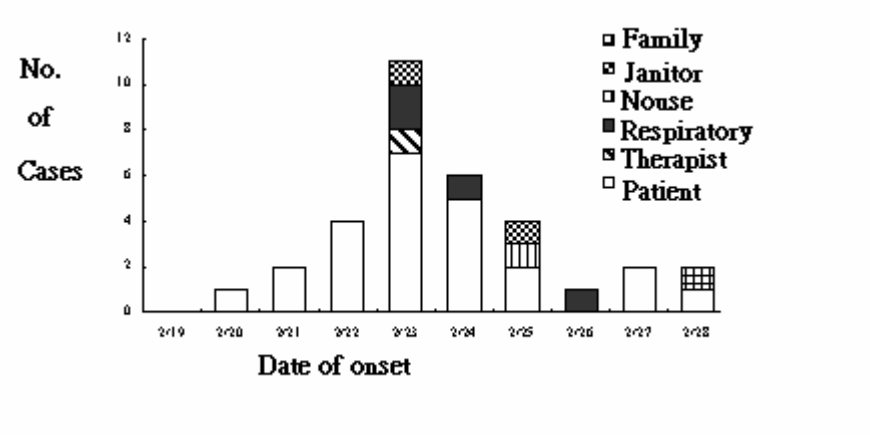


**Figure 1. Distribution of Dates of Onset of Cases**

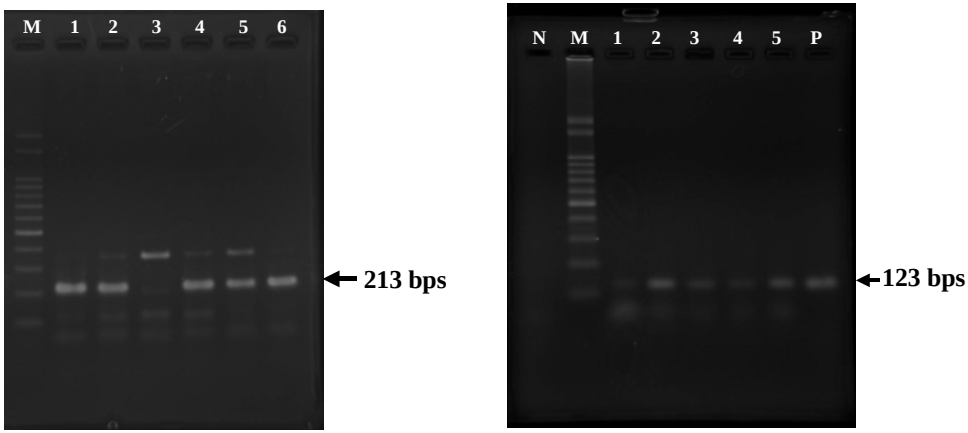


**Figure 2. RT-PCR Reactions**

A. SR/RT-PCR Norovirus G1 (primers: SR33, SR48, SR50, SR52); product is 123 bps. Bands 1-5 are specimens of patients.

B. Mon/RT-PCR Norovirus (primers: Mon431, Mon 432, Mon 433, Mon434); product is 213 bps. Bands 1-6 are specimens of patients.

gi|8388810|dbj|AB044360.1| Norwalk-like virus gene for RNA polymerase, partial cds,

Identities = 60/61 (98%)

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Query: 12  ataagccagtagcctcagacaatgcacaaagggtgattatccagtgatttatgctgttca 71
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||
Sbjct: 254 ataagccagtagcctcagacaatgcacaaagggtgattatccagtgatttatgctattca 195

Query: 72  c 72
           |
Sbjct: 194 c 194
```

**Figure 3. Sequences for Nucleotide-nucleotide BLAST in NCBI**