Abstract

Viral gastroenteritis is a common disease with a high morbidity reported worldwide. Rotavirus, norovirus, and sapovirus are considered to be significant global enteropathogens. These viruses also are associated with sporadic outbreaks of gastroenteritis in such settings as kindergartens, schools, and nursing homes for the elderly. Surveys on rotavirus and norovirus were including in the routine diarrhea-associated outbreak diagnostic item in Taiwan. However, prevalence on sapovirus was unknown because of the absence of routine screening procedures and sensitive diagnostic tests. In this study, we describe diarrhea-associated outbreaks caused by difference kinds of viruses that occurred during 2011 in Taiwan.

Introduction

Viral gastroenteritis is a common disease with a high morbidity reported worldwide especially in infants and the elderly. The mortality among children due to gastroenteritis is greater in developing than in the developed countries. Acute gastroenteritis ranks consistently as one of the principal six causes of all deaths [Murray and Lopez, 1997; Parashar et al.,2003]. Rotavirus, norovirus, and sapovirus are considered to be significant global enteropathogens. These viruses also are associated with sporadic outbreaks of gastroenteritis in such settings as kindergartens, schools, and nursing homes for the elderly and among military recruits [Bon et al., 1999]. Currently there are vaccine available to prevent rotavirus infection but there is no for both norovirus and sapovirus which belong to the Family of *Caliciviridae.* The *Caliciviridae* Family is composed of small (27 to 40 nm) naked icosahedral viruses that possess a linear, single-stranded RNA (ssRNA) positive sense genome, about 7.3 to 8.5 kb in length and contains a number of important veterinary and medical pathogens [Knipe and Howley,2007]. The four genera of the family are *Norovirus*, *Sapovirus*, *Vesivirus*, and *Lagovirus*. Among these genera of *Caliciviridae* Family, noroviruses and sapoviruses cause acute gastroenteritis in human. Noroviruses are the leading cause of nonbacterial acute gastroenteritis outbreaks worldwide, a disease that usually occurs in family or community-wide epidemic. Characterized by diarrhea, vomiting, abdominal pain and fever, the illness resolves within 48 hours mostly, although virus shedding for a longer time for several weeks in some cases [Rockx et al., 2002]. The impact of the sapoviruses has not been fully established yet, but they do not pose a major role with the noroviruses in acute epidemic gastroenteritis. Although noroviruses and sapoviruses have been associated with gastroenteritis in infants and young children, the predominant etiologic agent of severe diarrheal illness in this young age group is the 70-nm rotavirus.

Norovirus was first identified as the etological agents of a gasteroenteritis in Norwalk, Ohio, USA in 1968 [Kapikian et al., 1972]. Subsequently, many *Calicivirus* strains identified in outbreaks of viral gastroenteritis have been named after the outbreak location. The epidemiology of Norovirus infection is not clear and poorly characterized due to the lack of a sensitive detection method. Even immunological assay developed in the late 70s and early 80s, they can not detect broad spectrum of norovirus infection and were not available widely [Richards et al., 2003]. Despite several efforts, the establishment of an *in vitro* cultivation of norovirus and sapovirus has failed up to this day. As a result, progress on the study of human norovirus pathogenesis and the molecular mechanisms used for viral genome translation and

replication has lagged behind that of many related positive stranded RNA viruses.

The RNA genome of norovirus is organized into three open reading frames, ORF1 to ORF3. ORF1 encodes a large nonstructure polyprotein processed by viral protease into 6 final non-structure protein products. ORF2 and ORF3 encode capsid structure proteins, VP1 (make up the S and P domains) and VP2 respectively [Jiang et al., 1992]. Norovirus is genetically diverse viruses can be segregated into five separate genogroups (GI, GII, GIII, GIV and GV) based on sequence comparison of regions of the genome (fig-1). Three genogroups, GI, GII, and GIV are known to infect humans. Owing to the significant strain diversity within these genogroups, *Norovirus* strains can be further divided into genetic clusters, or genotypes [Hall et al., 2011]. Despite the enormous genetic diversity of noroviruses, norovirus genogroup II/genotype 4 (GII/4, commonly known as Bristol/Lordsdale group) virus strains predominate across Europe and worldwide. Norovirus GII/4 strains are endemic in hospitals and long-term care facilities and recent reports suggest the epidemic spread of newly emerging GII/4 norovirus variants.

Sapoviruses are caliciviruses that together with the noroviruses, are the most common cause of acute viral gastroenteritis in adults. The type species is Sapporo virus and it is named after Sapporo, Japan where the virus was first discovered following an outbreak of gastroenteritis in an orphanage [Chiba et al., 1979]. Unlike norovirus, this virus generally only causes mild gastroenteritis in young children. Human Sapoviruses are primarily associated with 1.8 to 9% cases of pediatric acute gastroenteritis [Wang et al., 2005]. Genetically, Sapovirus is divided into five genogroups (genogroup I [GI], GII, GIII, GIV, and GV). GI, GII, GIV and GV infect humans while GIII infects porcine species (fig-2) [Hansman et al., 2007]. Despite extensive testing, human sapoviruses have been found only in sporadic cases and in one mixed outbreak in children between 1994 and 2007 in the Netherlands [Svraka et al., 2010].

Sapovirus is now considered a common cause of viral gastroenteritis in infants and young children; however, its prevalence is largely unknown because of the absence of routine screening procedures and sensitive diagnostic tests. Recent studies using molecular detection methods, detecting the genetic material of the virus have shown that *Sapoviruses* are common causative agents of acute gastroenteritis in different geographic settings, accounting for up to 9% of cases of viral gastroenteritis in young children [Chiba et al., 1979]. Sapoviruses mainly affect babies and children under the age of five years in whom they may induce inapparent infection. Infections are not

associated with eating seafood. Mixed infections with sapoviruses, astroviruses, and noroviruses have been recorded in faeces of children with acute gastroenteritis in Argentina, England, Finland, Hungary, Japan, the Netherlands, Pakistan, Russia, and Spain [Vasickova et al., 2005]. In comparison with that of Noroviruses, the study of Sapoviruses is less advanced, which could be because Sapoviruses infect mainly young children and the illness is milder and, therefore, fewer laboratories study Sapoviruses. In addition, the methods for establishing the diagnosis of Sapoviruses remain limited. Thus, current understanding of Sapovirus epidemiology is preliminary. In contrast to the results of detection of Sapoviruses in stool specimens, the antibody prevalence studies showed that virtually all children are infected with Sapoviruses by the time they are 5 years of age, indicating that Sapovirus infection is widespread, although the illness most likely is sporadic with a high rate of asymptomatic infections [Moreno-Espinosa et al., 2004]. In Taiwan, the first sapovirus-associated outbreak was identified in college students with gastroenteritis that occurred during May 4–8, 2007. Symptoms of sapovirus infection are thought to be milder than symptoms of norovirus infections. However, in this outbreak approximately one third (17) of the 55 students reported symptoms of abdominal pain and 22 (40%) reported symptoms of vomiting. Viral genotype was performed by RT-PCR and sequence analysis. Phylogenetic analysis clustered this strain into genogroup I/genotype 2 (GI/2) [Wu et al., 2008].

Diagnostic Methods

The rapid definitive diagnosis of the causative agent(s) of viral gastroenteritis is important, particularly in hospital settings due to the possibility of ward closures, the disruption of planned procedures and the requirement for multidisciplinary teams to manage infection control systems, all placing an enormous burden on healthcare systems. Gastrointestinal virus detection was traditionally preformed by direct visualization of viruses by electron microscopy (EM), or alternatively using commercially available immunoassays. Molecular methods for the detection of genetic material of gastrointestinal viruses have been developed more recently, resulting in increased levels of detection of the causative agents of viral gastroenteritis.

The majority of clinical virology laboratories perform real-time reverse transcription-polymerase chain reaction (RT-PCR) assays for norovirus & sapovirus detection. These assays have not been cleared by FDA although commercial kits are available in the United States. Positive samples can be typed subsequently by DNA

sequencing of conventional RT-PCR products. FDA has recently cleared the RIDASCREEN Norovirus 3rd Generation (r-Biopharm, AG, Darmstadt, Germany) enzyme immunoassay (EIA) for preliminary identification of norovirus as the cause of an AGE outbreak. However, negative EIA results should be confirmed by RT-PCR reference methods [Hall et al., 2011].

Electron Microscopy

The application of EM to detect causative agents of viral gastroenteritis depends on direct observation of intact viral particles shedding in the stool of infected individuals. Electron microscopy requires highly specialized equipment, usually only available at large laboratory reference facilities. The method is also very tedious, labor intensive and requires highly trained and experienced staff to differentiate between virus types and requires up to 10^6 intact viral particles per ml of stool. Such high levels of viral particles are generally only detected for 12-48 h after the onset of symptoms, after which time the viral particles are often not present in sufficient quantity for detection. Thus detection rates using EM are estimated to be approximately 50%, with some reports of detection levels being as low as 24% [Duizer et al., 2007]. In addition, EM cannot distinguish viruses from within genogroups. So while visually a number of *Norovirus* strains, for example, may appear identical by EM, the viruses may well be genetically diverse and would not constitute the emergence of a *Norovirus* outbreak, but rather individual sporadic *Norovirus* cases.

Real-Time RT-PCR Assays

Molecular assays for the *in vitro* diagnostic testing of stool specimens for *Norovirus, Sapovirus* and *Astrovirus* are not currently commercially available. However, various molecular techniques have been developed in research laboratories and exploited for the development of highly sensitive and rapid assays for the detection of causative agents of viral gastroenteritis. The most conserved region of the norovirus genome is the ORF1-ORF2 junction region with a high degree of nucleotide sequence homology across strains. This character makes this region ideal for designing broadly reactive primers and probes for real-time RT-PCR assays [Katayama et al., 2002]. The majority of published assays are derived from the original publication, which has been modified into one-step and duplex assays for high throughput screening in clinical diagnostic laboratories. In addition to detecting norovirus RNA in clinical samples, relatively small optimizations of the primers and probes have resulted in sensitive assays to detect norovirus RNA in environmental samples (e.g., food and water). The extreme analytic sensitivity of RT-qPCR permits the detection of very

low titers of virus that might be present in samples from persons without disease caused by norovirus (i.e., asymptomatic infection). For this reason, low titer results (i.e., high cycle threshold [CT] values) should be interpreted with caution. While the detection limit of real-time RT-PCR assays can vary greatly depending on assay design, the detection of just one copy of the target viral RNA of interest is possible [Phillips et al., 2009].

Conventional RT-PCR Assays for Genotyping

Different regions of the genome have been used successfully for genotyping of noroviruses. However, typing based on the capsid gene (region C and region D) allows for better differentiation between strains (Fig-3). Although region C sequences have been used widely for genotyping strains by clinical diagnostic laboratories in the United States, Europe, and Japan, the resolution of this region is not sufficient to distinguish differences between certain GII.4 variants, which are the predominant strains associated with outbreaks. The gold standard for genotyping norovirus strains is full capsid sequencing [Vinje et al., 2004]. However, for clinical samples with high copy numbers, amplifying partial capsid sequences is more practical and is only slightly less discriminatory than full capsid sequencing. For this reason, laboratories participating in CaliciNet, CDC's electronic norovirus outbreak surveillance network, use region D genotyping.

While few national surveillance systems for acute gastroenteritis are in place, reference laboratories in many countries apply molecular diagnostic assays for the screening of stool specimens for causative agents of acute viral gastroenteritis, in particular for the epidemiological studies of large outbreaks of viral gastroenteritis including Taiwan CDC.

Enzyme Immunoassays

The low sensitivity of EM has resulted in the development of a number of commercially available immunoassays for the detection of gastrointestinal viruses, in particular for *Norovirus* detection. Enzyme immunoassays (EIAs) are, in general, quick to perform and are easily adaptable to a routine microbiology laboratory. EIA kits are, however, highly specific, and due to the levels of strain diversity among circulating gastrointestinal viruses, the estimated sensitivity for many antigen detection kits is low, albeit generally higher that the sensitivity obtained by EM detection. A large number of studies examining the use of commercially available

EIA for the detection of *Norovirus* antigen in stool specimens have recently been published, with huge variation in the reported sensitivity and specificity of the assays [Costantini et al., 2010; Morillo et al., 2011].

For detection of norovirus antigen in clinical samples, rapid assays (e.g., EIA) offer an attractive alternative to expensive and technically demanding molecular detection assays. However, the development of a broadly reactive EIA for noroviruses has been challenging because of the number of antigenically distinct norovirus strains and the high viral load required for a positive signal by these assays. Commercial kits, including IDEIA Norovirus EIA (Oxoid, Ely, United Kingdom), SRSV (II)-AD (Denka Seiken Co. Ltd., Tokyo, Japan), and RIDASCREEN include pools of cross-reactive monoclonal and polyclonal antibodies. In evaluations, the sensitivity of these kits when compared with RT-PCR has ranged from 36% to 80%, and specificity has ranged from 47% to 100% [Burton-MacLeod et al., 2004; Okitsu-Negishi et al., 2006]. Because of the modest performance of these commercial kits, particularly their poor sensitivity, they are not recommended for clinical diagnosis of norovirus infection in sporadic cases of gastroenteritis. The consensus regarding the use of EIA assays for the routine detection of *Norovirus* suggests that these assays are useful as rapid preliminary screening assays. However, a minimum of six specimens per outbreak should be examined and EIA-negative specimens should be further investigated with more sensitive molecular detection methods such as RT-PCR [Duizer et al., 2007]. EIAs for the routine detection of *Sapovirus* are not currently commercially available.

Materials and methods

Reporting of outbreaks of acute gastroenteritis in Taiwan.

All the stool samples used for this study were obtained from outbreak cases reported to the Taiwan CDC through the surveillance of viral gastroenteritis outbreaks in 2011. The occurrence of an outbreak is that in a community, region or specific setting of cases of illness with a frequency clearly excess of normal expectancy. The number of cases indicating presence of an out break will vary according to infectious pathogens, size and type of exposed population, previous exposure and lack of exposure to the disease, and time and place of occurrence. In our definition, the case of number is 2 with acute gastroenteritis. The stool samples were tested for the presence of norovirus, rotavirus and sapovirus.

RNA extraction and RT reaction.

Viral RNA was extracted from 200μl of the 10% stool suspension with the TANBead Viral Auto Plate (Taiwan Advanced Nanotech Inc.).

cDNA was prepared using the *Superscript III Reverse Transcriptase kit* (Invitrogen Corp., Carlsbad, CA). Briefly, 5 μl of extracted RNA was added to 15 μl of RT mixture containing 4 μl of 5× reverse transcription buffer, 2□μl of 0.1 M dithiothreitol ,2□μl of 20 mM deoxynucleoside triphosphates (dNTPs), 1 μl of 10 μM random hexamers, 200 U of *Reverse Transcriptase*, and 20 U of RNase inhibitor. The RT reaction mixture was incubated at 25°C for 10 min, 50°C for 60 min, and 85°C for 5 min to inactivate the enzyme.

PCR amplification of human Sapovirus genomes [Kitajima et al., 2010].

The first PCR was performed in 50 μl of reaction volume containing 5 μl of cDNA, 2.5 U of *Taq* DNA polymerase, and 20 pmol of SaV-124F, SaV-1F, SaV-5F, SV-R13, and SV-R14 primers. These primers generated approximately an 800-bp product. PCR amplification was performed under the following conditions: initial denaturation at 94°C for 2 min, followed by 35 cycles of amplification with denaturation at 94°C for 30 s, primer annealing at 48°C for 30 s, extension reaction at 72°C for 2 min, and then a final extension at 72°C for 10 min. The second PCR was performed in 50 μl of reaction volume containing 2 μl of the first PCR product, 2.5 U of Taq DNA polymerase, and 20 pmol of 1245Rfw and SV-R2 primers. PCR amplification was performed under the following conditions: initial denaturation at 94°C for 2 min,

followed by 35 cycles of amplification with denaturation at 94°C for 30 s, primer annealing at 48°C for 30 s, extension reaction at 72°C for 2 min, and then a final extension at 72°C for 10 min. These primers generated approximately a 420-bp product.

The PCR products were purified with a PCR purification kit (Qiagen), and both strands were sequenced with the BigDye cycle sequencing kit, version 3.1, and the 3130 genetic analyzer (Applied Biosystems). Nucleotide sequences were assembled using the program Sequencher version 4.1.4 (Gene Codes Corporation, Ann Arbor, MI) and aligned with MEGA version 4 (http://www.megasoftware.net/). The phylogenetic dendrograms from a bootstrap analysis with 1,000 replicates were generated by the neighbor-joining method.

Result and Discussion

Totally 714 fecal specimens from suspected cases with acute gastroenteritis reported to our system were collected from 101 reported diarrheal clusters in 2011. All the samples were screened for rotavirus and norovirus infection by RT-PCR. If no positive for these suspected cases, then RT-PCR specific for sapovirus would be applied. The detection rate of cases for viral pathogens including rotavirus, norovirus and sapovirus was 49.3% (352/714) among these cohorts. The most common pathogen was norovirus genogroup II about 65.3%, and other pathogens were rotavirus about 8.5%, and norovirus genogroup I about 7.8%. Some mix infections were indentified, about 5.1%. Surprisingly, 16 cases of sapovirus infection were detected and account for 4.5% of all suspected cases with acute gastroenteritis in this study period. Since we do not routinely screen and detect for sapovirus infection, we do not have the base line number for sapovirus infection. However 4.5 % sapovirus detection rate is higher than we expected.

The age of these patients reported as viral diarrhea clusters ranged from under 1 year old up to 97 years old. Most patients (44.8%) were under 15 years old (Fig 4). The highest pathogen-positive rate (80.6%) was found in the age group elder than 70 years old. However, the age of patients infected with sapovirus was spotted among two major age groups which were young children below 11 years old and adults above 50 years old (Table 1). What reasons for this phenomenon observed in our study need more study to resolve but may be difficult to find their risk behaviors which is highly suspected associate with occurrence of outbreak. Based on the month of the specimen collection, most cases were reported in January (Fig 5). The epidemic peak of norovirus infection occurred during January to April, from winter to early spring. The sapovirus was detected mainly in February. The most of viral diarrhea outbreaks occurred in elementary school and health care facilities. The major genotype of norovirus in diarrheal clusters was GII.2, followed by GII.4 (Fig 6). The association of facilities and genotype demonstrated that norovirus GII.2 were occurred particularly in elementary school and kindergarten and GII.4 were in health care facilities. Comparison the sequences of norovirus GII.2 with previous studies, there were no distinctly difference. And GII.4 genotype was in accordance with foreign study (Fig 7). Owing to the fact that norovirus GII.4 had rapid evolution ability, GII.4 variants changed every two or three years. We had found 2003, 2004 and 2006b variants in previous years. In this year, GII.4 strains were similar with 2010 variant. As to Sapovirus, each genotype seemed to have a restricted geographical distribution. Genotype GI/2 were found in north (Taipei and New Taipei City) and

northeast (Yilan City), and GI/3 were in south (Chiayi County).

Individual case of acute gastroenteritis caused by viral pathogens is not reportable disease in Taiwan. However, all outbreaks should be reported to the local or central health departments. We only detect Rotavirus and Norovirus infections routinely, not specific for sapovirus even we have identified sapovirus before. But because this co-operation project with Japan NIID, sapovirus detection was included in our surveillance system, once no formative results coming out from outbreak cases with acute gastroenteritis.

Genotyping of these viral pathogens have been demonstrated to be helpful in identifying links between clusters. Thus, we will also try to establish the genomic data base for future surveillance use.

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Publication list for this work

NIL

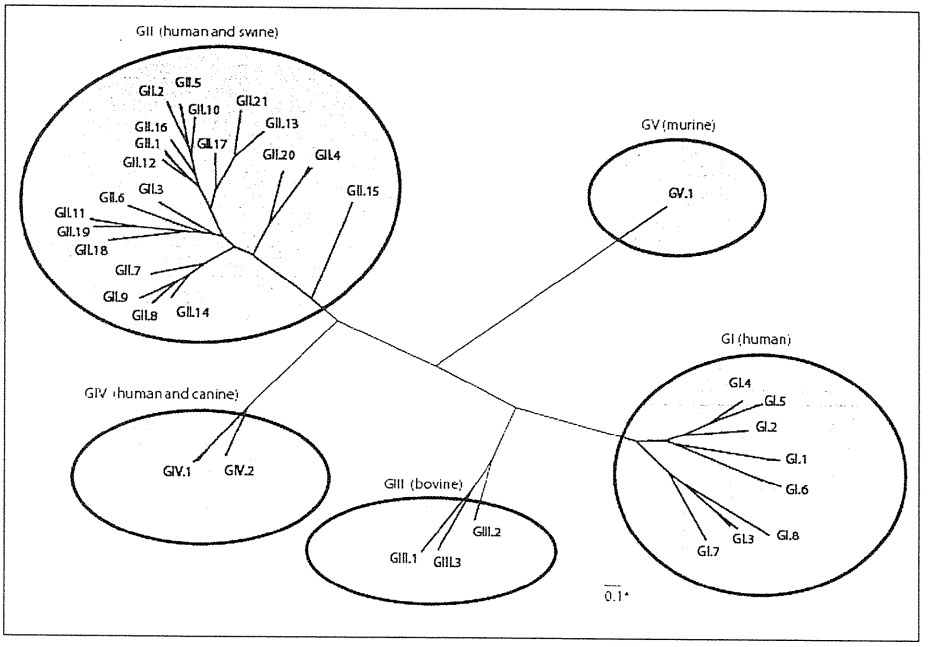


Fig-1. Classification of noroviruses into 5 genogroups (GI–V) and 35 genotypes based on sequence diversity in the complete capsid protein VP1 (CDC, MMWR 2011).

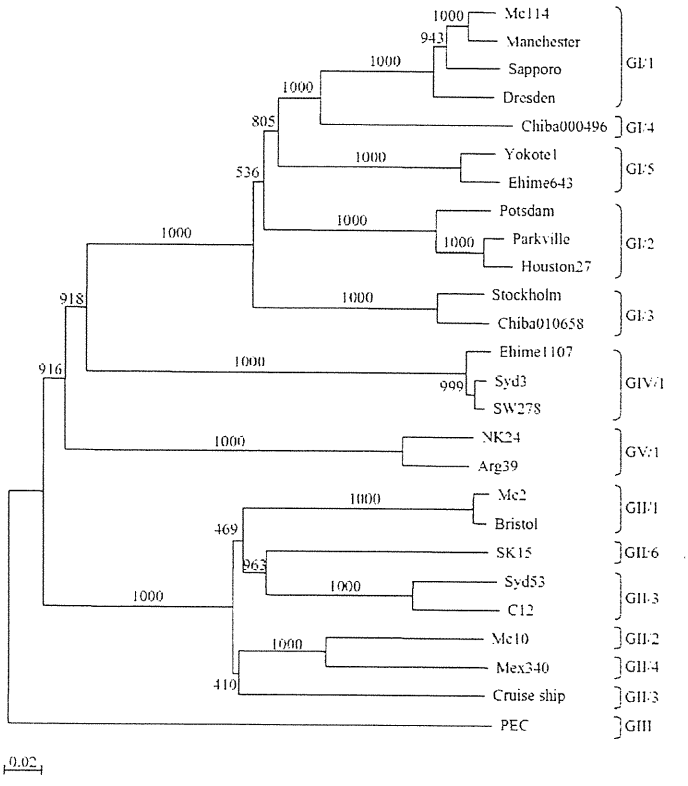
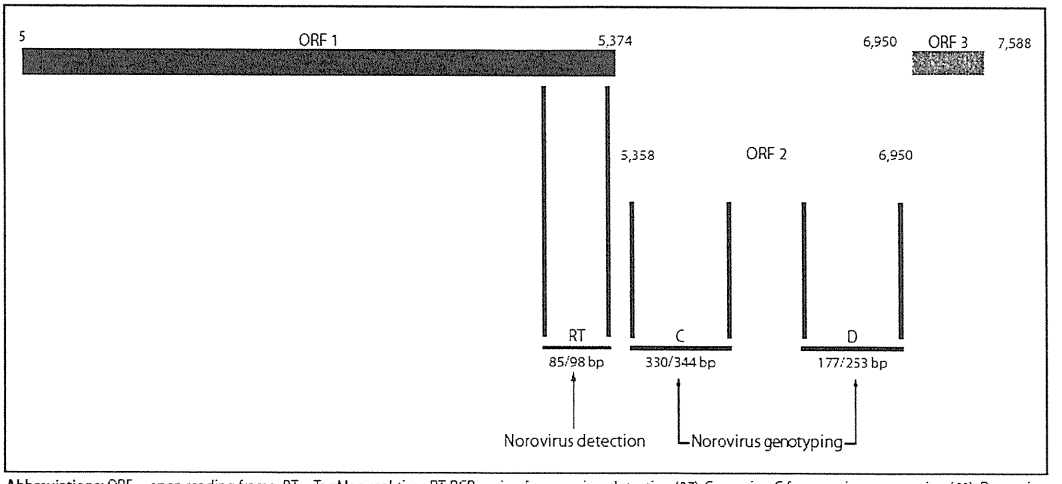


Fig-2. Phylogenetic tree of human sapovirus based on whole VP1 nucleotide sequences. (Rev. Med Virol 2007, 17:133-141).



Abbreviations: ORF = open reading frame; RT = TaqMan real-time RT-PCR region for norovirus detection (87); C = region C for norovirus genotyping (93); D = region D for norovirus genotyping (92); and bp = base pairs.

Fig-3. Norovirus genomic regions targeted by RT-PCR(CDC, MMWR 2011)

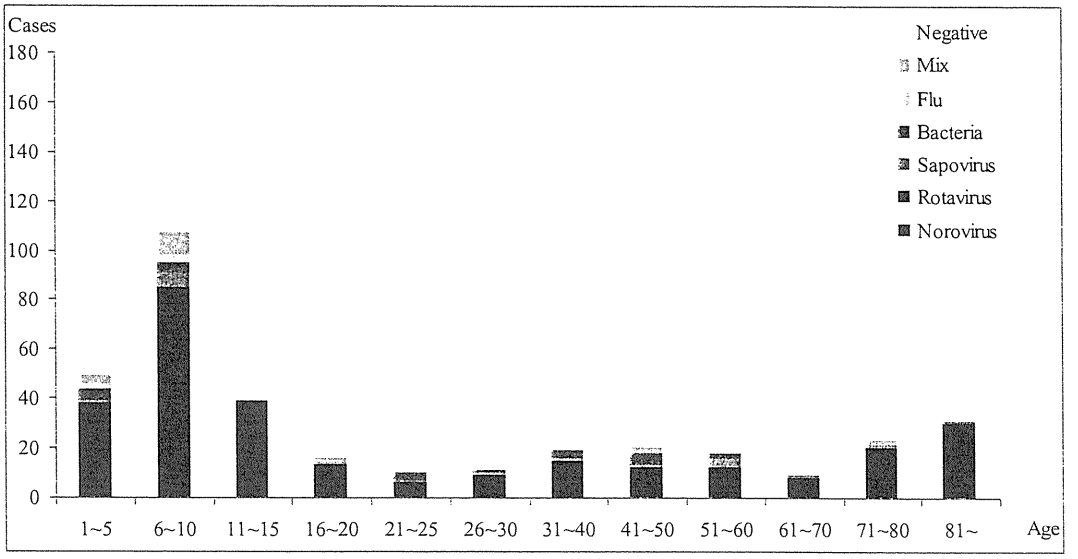


Fig-4. Age distribution of patients reported as diarrheal cluster in 2011.

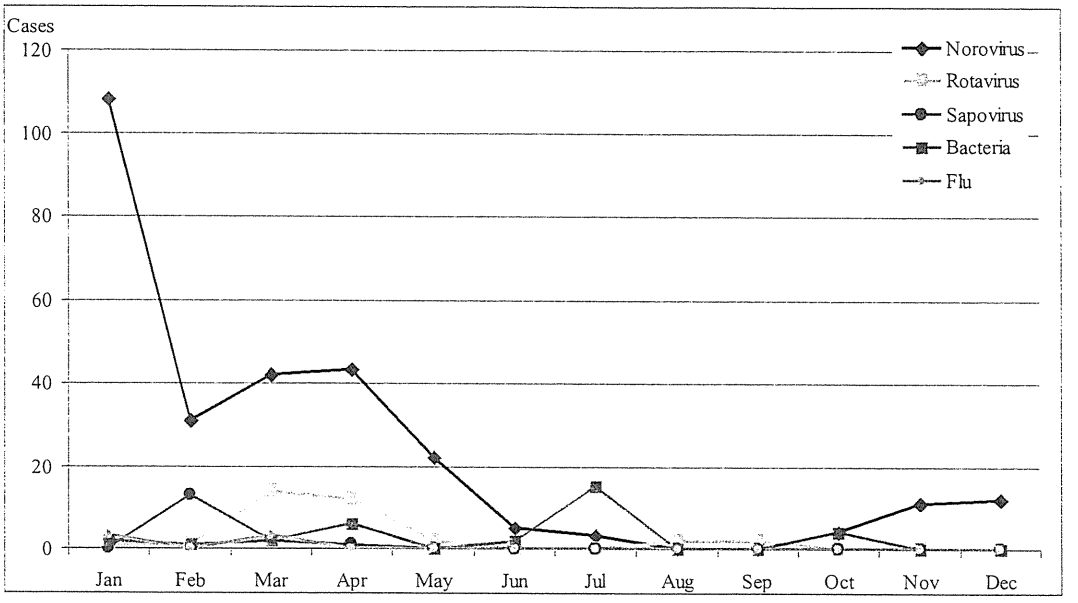


Fig-5. Time-line of the cases with diarrheal pathogens in 2011.

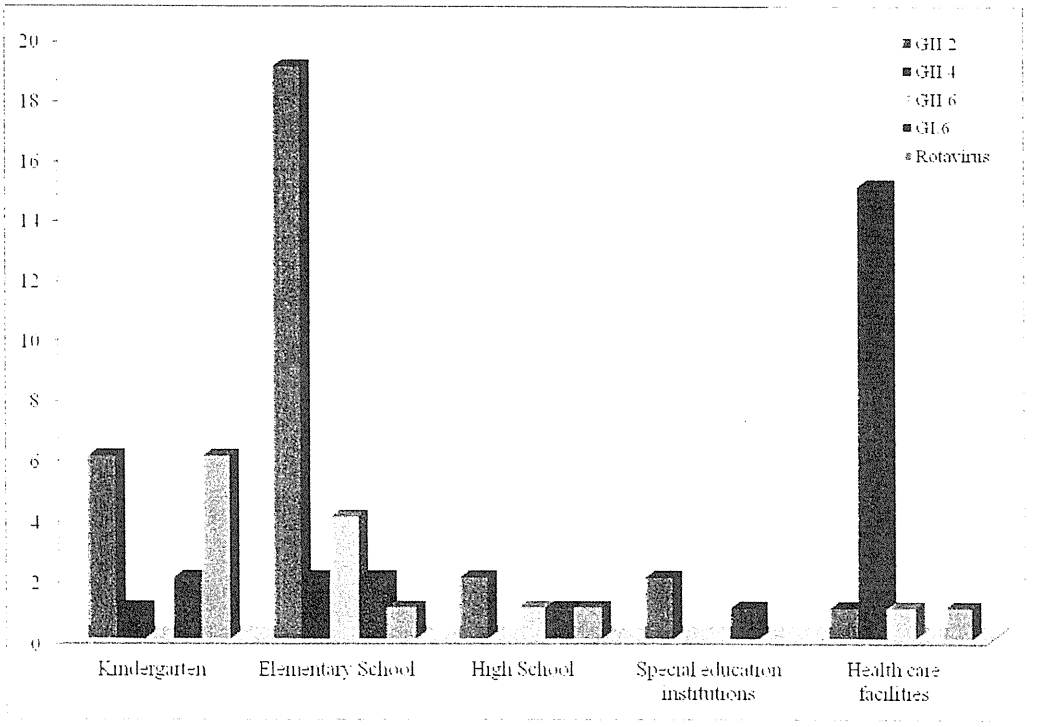


Fig-6. Numbers of norovirus outbreaks and corresponding facilities. Norovirus genotypes are indicated with different colors.

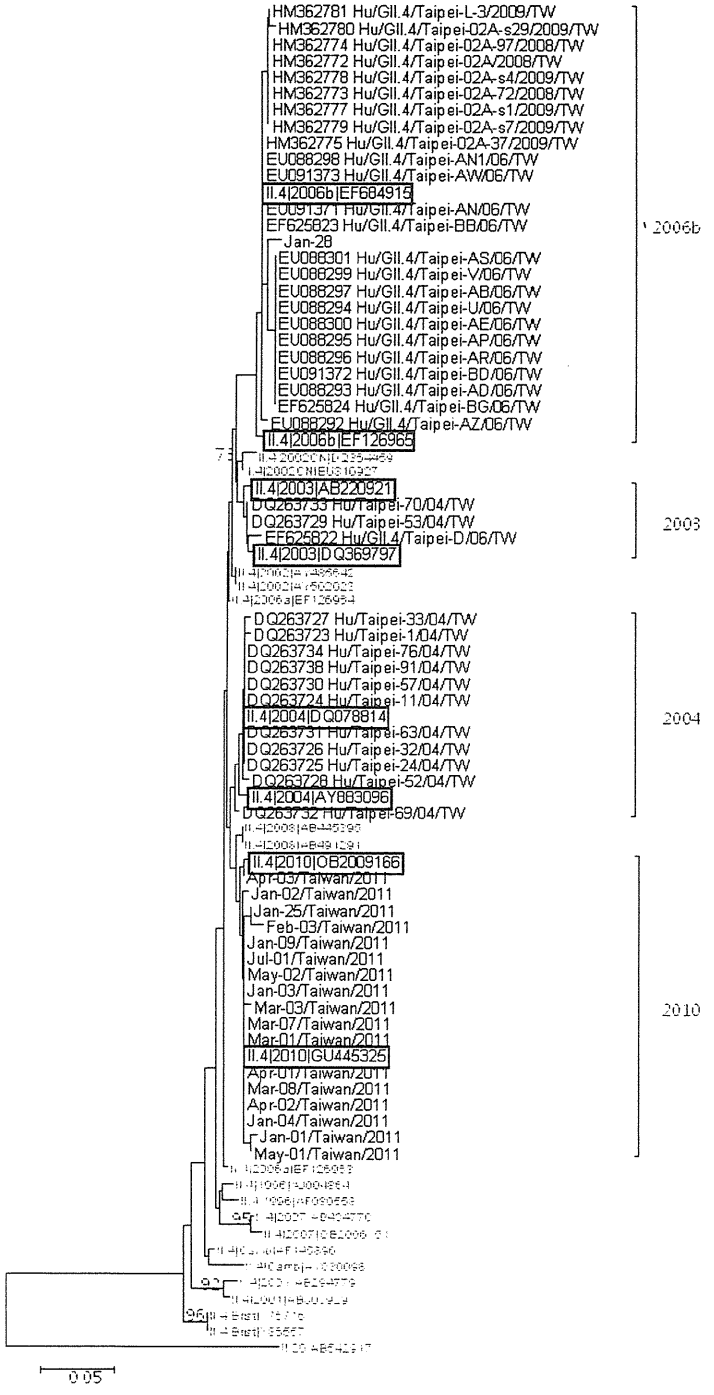


Fig-7. Comparison analysis of norovirus partial capsid nucleotide sequences using neighbor-joining tree analysis, showing different variants.

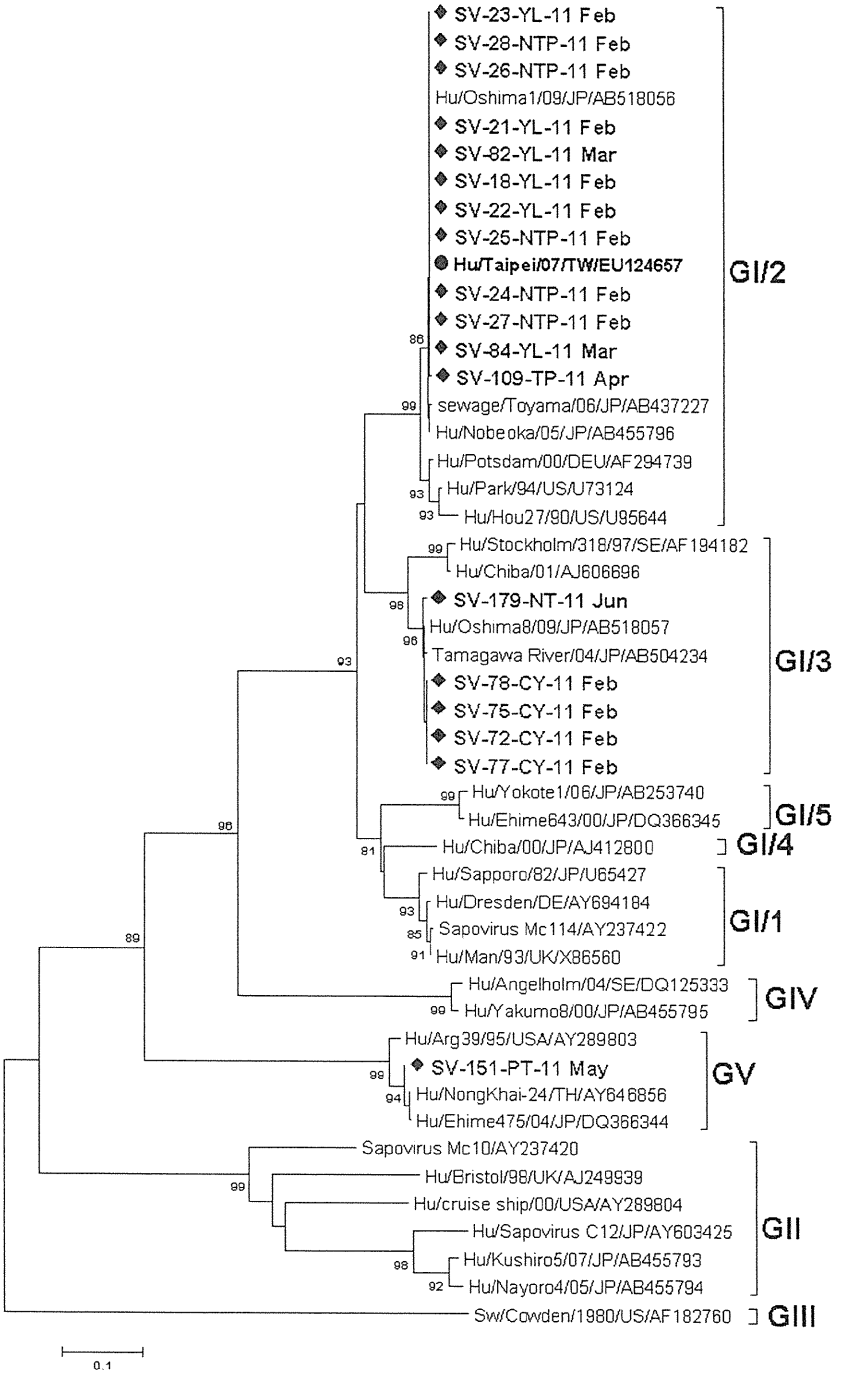


Fig-8. Analysis of Sapovirus partial capsid nucleotide sequences using neighbor-joining tree analysis, showing different genogroups and clusters.