Table 4. Mutations identified in novel INH-resistant associated genes, *iniB*, *iniA*, *iniC,* and *efpA* genes

katG, inhA, inhAr, oxyR-ahpC	DST result			*iniB*		*iniA*			*iniC*	*efpA*
				codon 222	codon 164-175	codon 19	codon 84	codon 634	codon 476	codon 47
				12-bp del	48-bp del	Lys→Asn	Ser→Arg	Arg→Pro	Arg→Cys	Pro→His
wild type	R*	57	mutation	**2**	**1**	**1**	**5**	**1**	**1**	**1**
			wild type	55	56	56	52	56	56	56
	S*	18	mutation	0	0	0	0	0	0	0
			wild type	18	18	18	18	18	18	18

*: R, resistant; S, susceptible

Molecular studies on virulence and drug resistance of leprosy: Molecular and sero-diagnosis of leprosy

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Summary:

Leprosy is a notifiable disease in Taiwan. During 2002 to 2011, we confirmed 81 new leprosy cases, with 5 to 12 cases reported annually. There were 56 (69.1%) multibacillary cases, 21 (25.9%) paucibacillary cases and 4 (4.9%) were not determined. Female individuals (50/81, 61.7%) are more venerable than male (P= 0.033). The majority (42, 51.9%) of cases was in the age group of 21-40 (P< 0.001), followed by 22 (27.2%) in the age group senior than 60 years old. Furthermore, 13 relapse indigenous cases were notified in 2002-2011. Since *M. leprae* can not be cultivated in vitro, smear microscopy is currently the only test used in clinical bacteriological laboratories for bacteriological diagnosis in Taiwan. The objectives of this project are to improve clinical diagnosis of leprosy, to develop diagnostic methods for leprosy and to participate in drug-resistance surveillance of leprosy. We conduct molecular diagnosis using *M. leprae* specific gene, drug-resistant gene sequencing of dapsone, rifampicin and ofloxacin, and serological diagnosis using MMP-II ELISA. Positive results were found in 4 cases using RLEP real-time PCR. Gene sequencing was performed to detect drug resistance of 4 cases. We found one case (D-10) was resistant to dapsone with mutation at codon 55 (CCC to CTC) of the *folP1* gene. The positive cut-off value was 0.233. The sensitivity and specificity of the MMP-II ELISA was 83.7% and 80%, respectively. We established a differential diagnosis algorithm for leprosy, and reveal drug-resistant patterns of *Mycobacterium leprae*. In addition, a sero-diagnostic test was also established for screening of leprosy. A strengthened laboratory detection system can improve clinical treatment and management of leprosy patients.

I. Purpose

The purposes of this collaborative project are to establish an algorithm for diagnosis of *Mycobacterium leprae*. We established molecular tests, a real-time and a nested PCR for detecting *M. leprae* and drug resistance to dapsone, rifampicin and ofloxacin. In addition, sero-diagnosis using MMP-II ELISA was evaluated in this study.

II. Methods

Study samples

In this study, 159 blade specimens and 110 blood samples were collected from suspected and/or enrolled leprosy cases from contract hospitals and/or from annual on-site health examination carried out by local health bureaus.

Molecular diagnosis of *Mycobacterium leprae*

DNA extraction

Paraffin-embedded or blade specimens was placed in microcentrifuge tubes containing 180 µl of buffer ATL, 20 µl proteinase K, mixed by vortexing, and incubated at 56°C until the tissue is completely lysed. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid before adding 200 µl Buffer AL to the sample. Mix again by vortexing for 15 sec., and incubate at 70°C for 10 min. Add 200 µl ethanol (96–100%) to the sample, and mix by vortexing for 15 sec. After mixing, carefully apply the mixture to the QIAamp Mini spin column. Close the cap, and centrifuge at 8,000 rpm for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube, and discard the tube containing the filtrate. Then add 500 µl Buffer AW1 and repeat the centrifuge program. And 500 µl Buffer AW2 added at full speed (14,000 rpm) for 3 min. Finally, 200 µl Buffer AE added at room temperature for 1 min, and then centrifuge at 8000 rpm for 1 min. The filtrate solution was collected for molecular assays.

Real-time PCR

For identification of *M. lepare,* we designed primers set based on *M. leprae*-specific repetitive element (RLEP). We developed a modified real-time TagMan PCR method[1] using probes designed at the Leprosy Research Center of National Institute of Infectious Disease (NIID), Japan for rapid screening of *M. leprae*. Primer-probe sets of RLEP were synthesized

by Applied Biosystems for the ABI 7500 real-time PCR system. Probes were labeled with TaqMan® MGB FAM™ markers. For each sample, the real-time PCR was performed by using 12.5μl of TaqMan® 2X universal master mix (Applied Biosystems, USA), 2μl of 10 μM RLEP-TM-F primer (5'-GCA GTA TCG TGT TAG TGA A-3'), 2μl of 10 μM RLEP-TM-R primer (5'-CGC TAG AAG GTT GCC GTA TG-3'), 2μl of 10 μM RLEP-TM probe (5'-FAM-TCG ATG ATC CGG CCG TCG GCG –TANRA-3'), 1.5μl RNase- and DNase-free water, and 5μl sample DNA, in a final total volume of 25μl per single well reaction. It was recommended to use at least 10 ng DNA from either purified DNA or inactivated bacterial lysate directly.

Drug-resistant gene sequencing

For drug-resistant detection, we sequenced the *folp1* gene for dapsone, the *rpoB* gene for rifampin and the *gyrA* gene for ofloxacin following published protocols[2-3]. Briefly, the first set of PCR amplification primer was used, and then a nested-PCR was performed with 10-fold diluted first PCR product. A resulting fragment was sequenced further with oligonucleotide primers. Thereafter, the PCR products were analyzed with an ABI 3730 automated sequencer (Applied Biosystems, USA), and the sequence data were assembled and edited using the Sequencing Analysis 5.2.0 software (Applied Biosystems, USA). In addition, site-directed mutagenesis technique was adopted to determine the effect of novel mutations on the dapsone susceptibility. Thereafter, the PCR products were analyzed with an ABI 3730 automated sequencer (Applied Biosystems, USA), and the sequence data were assembled and edited using the Sequencing Analysis 5.2.0 software (Applied Biosystems, USA).

Sero-diagnosis of loprosy

MMP-II ELISA

The ELISA for the detection of anti-MMP-II immunoglobulin G (IgG) antibodies was performed as leprosy center suggested[4-7]. Ninety-six well plates (Immunosorb, Nunc) were coated overnight, with MMP-II at a concentration of 4 μg/mL in coating buffer (pH 9.5) (Protein Detector ELISA kit, KPL). After blocking with blocking solution and incubated at 37 ℃ for 1 h., the plates were washed with phosphatebuffered saline containing 0.1% Tween 20 (PBST), and human sera diluted 100-fold were added and incubated at 37 ℃ for 2 h. After washing with PBST, biotinylated anti-human IgG (KPL) was added at a 1:1000 and incubated for 1 h. The plates were incubated with reagents ABC peroxidase staining kit (Thermo) for 30 min. After further washing with PBST, a substrate solution consisting of OPD/citric acid and

0.02% H2O2 in 0.1M citrate buffer was added until a yellow color developed and the OD was measured at 450nm using a spectrophotometer. Plate-to-plate variations in OD readings were controlled using a standard pool positive serum and if the positive control reading varied more than 0.05 O.D., then the test was repeated.

Statistical analyses

For statistical analysis, Receiver Operator Characteristics (ROC) curves were drawn to describe the relation between sensitivity and specificity at various cut-off levels using the MedCalc software.

III. Results

Epidemiology of Leprosy in Taiwan

During 2002 to 2011, we confirmed 81 new leprosy cases, with 5 to 12 cases reported annually[8]. There were 56 (69.1%) multibacillary (MB) cases, 21 (25.9%) paucibacillary (PB) cases and 4 (4.9%) were not determined. Female individuals (50/81, 61.7%) are more venerable than male (P= 0.033). Geographically, 42 (51.9%), 26 (32.1%), 11 (13.6%) and 2 (2.5%) cases were reported from northern, southern, central and eastern Taiwan, respectively. Of the 81 cases, 37 (45.7%) were indigenous cases and 44 (55.3%) were imported cases (Table 1). Majority (43/44, 97.7%) of imported cases were originated from South-East Asia, particularly from Indonesia (32/44, 72.7%) (P< 0.001), which reported 20,023 new cases in 2011[9]. In addition, of the total 425,660 foreign worker entered Taiwan as of December 2011, 175,409 (41.2%) were from Indonesia. Among 50 female cases, 37 (74%) (P< 0.001) were imported cases including 29 (58%) from Indonesia, 4 from Vietnam, 3 from Philippines and 1 from China. While among 31 male cases, 7 (22.6%) were imported cases including 3 from Indonesia, 3 from Thailand and 1 from Myanmar. The median age of male between indigenous and imported were 60.5 (range 31-83 years) and 33 (range 24-60 years) (P= 0.001) and that of female were 67 (range 45-81 years) and 27 (range 20-52 years) (P< 0.001), respectively. We did not observed any leprosy case younger than 19 years old. The majority (42, 51.9%) of cases was in the age group of 21-40 (P< 0.001), followed by 22 (27.2%) in the age group senior than 60 years old. Furthermore, 13 relapse indigenous cases were notified in 2002-2011. Of the 13 cases, 2 were reactivated after 5 years, one after 14 years, 6 after 35 years and 4 were unknown.

Molecular diagnosis and drug-resistance detection of *M. leprae*

We analyzed 159 blade specimens from skin smears each from individual cases collected from 8 (A to H) sites in Taiwan in 2012 (Table 2). Positive results were found in 4 cases using RLEP real-time PCR. Gene sequencing was performed to detect drug resistance of 4 cases. We found one case (D-10) was resistant to dapsone with mutation at codon 55 (CCC to CTC) of the *folP1* gene (Table 3). Increased Ct value of the RLEP was observed from 23.86 in 2008 to 37.00 in 2012. Neither the *rpoB* gene conferring rifampicine resistance nor the *gyrA* gene conferring ofloxacin resistance was found in all 4 cases (Table 3).

Sero-diagnosis of leprosy

Of the 110 cases agreed to provide blood samples for MMP-II ELISA testing, 75 (68.2%) were male and the median age is 78.5 years old. There were 76 (69.1%) MB cases, 30 (27.3%) PB cases and 4 were unknown. The ROC curve indicated that the positive cut-off value is 0.233 (Figure 1A). The average O.D. values are 0.439, 0.318 for MB and PB, respectively (Figure 1B); while the average O.D. value for 10 health control is 0.149. The sensitivity and specificity of the MMP-II ELISA was 83.7% and 80%, respectively.

IV. Discussion

In this study, we established a diagnosis algorithm for leprosy, and reveal drug resistant patterns of *Mycobacterium leprae* using molecular methods. A real-time PCR was established with detection limit of 100 fg. We identified 4 leprosy cases in 2012. In addition, drug-resistant gene sequencing was performed to determine drug resistance of 4 *M. leprae* infected cases in 2012. After confirmation of one case for dapsone resistance, this patient can be treated with second-line medication to improve outcome. Nevertheless, we did not identify any mutation to rifampicin and ofloxacin in 4 confirmed cases. Taiwan has implemented national leprosy control program to eliminate leprosy sine the 1930s. Annual on-site health examination of cases, diagnosis and treatment with MDT remain key elements in our control strategies. In addition, foreign workers was requested by the Enforcement Rules of the Communicable Disease Control Act implemented in 1985 to provide health certificate at entry, and to have their health-check including leprosy at the sixth, the eighteenth and the thirtieth month during their stay in Taiwan. The prevalence rate has been less than 1 leprosy case per 10,000 populations in Taiwan since 2004. MDT treatment provides a simple yet highly effective cure for all types of leprosy. Results of

real-time PCR were consistent with histological classification of lepromatous leprosy. In addition, we proved that sero-diagnosis using MMP-II ELISA is useful for screening leprosy with a satisfactory sensitivity of 83.7%. For providing appropriate treatment, determination of drug-resistance for each confirmed case can ensure good treatment outcome become a leprosy diagnosis policy in Taiwan.

V. References

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Tables and Figures

Table 1. Demographic characteristics of 81 leprosy cases in Taiwan, 2002-2011

	Imported cases (N=44)	Indigenous cases (N=37)
Sex		
Male No. (%)	7 (15.9)	24 (64.9)
Female No. (%)	37 (84.1)	13 (35.1)
Male		
Median age (range), y	33 (24-60)	60.5 (31-83)
≦20	0	0
21-40	5	3
41-60	2	9
≧61	0	12
Female		
Median age (range), y	27 (20-52)	67 (45-81)
≦20	1	0
21-40	34	0
41-60	2	3
≧61	0	10
Nationality		
Taiwan	0	36
Indonesia	32	1
Vietnam	4	0
Others[a]	8	0
Type		
Multibacillary	31	25
Paucibacillary	10	11
Not determined	3	1

[a]Includes Thailand (3), Philippines (3), Myanmar (1), and China (1).

Table 2. Results of RLEP real-time PCR ,2012

Site	Cases no.	RLEP real-time PCR positive, Case no.
A	7	0
B	10	0
C	14	0
D	11	1
E	15	1
F	9	2
G	3	0
H	90	0
Total	159	4

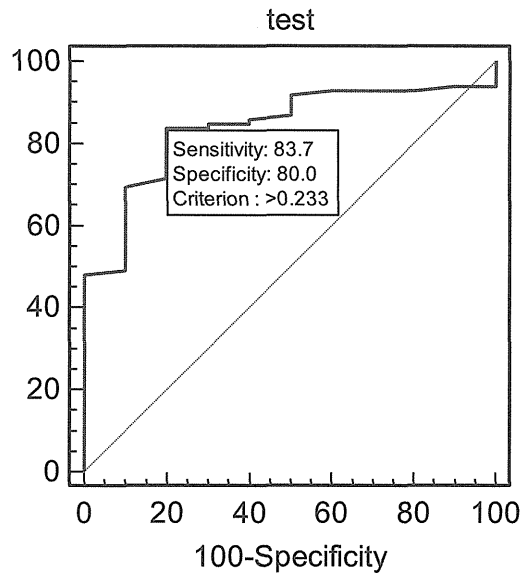
Table 3. Sequencing results of the drug-resistant genes of the RLEP positive cases, 2012

ID	Ct of RLEP	drug-resistant genes		
		*fol*P1	*rpo*B	*gyr*A
D-10 (2008)	23.86	R*	S	S
D-10 (2010)	29.00	R	NA	S
D-10 (2012)	37.00	NA	NA	NA
E-13	27.45	S	S	S
F-4	26.09	S	S	S
F-8	38.20	NA	NA	NA

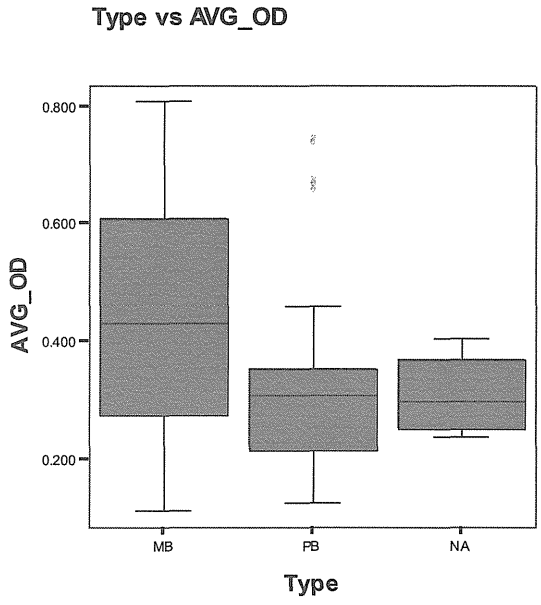
* indicate codon 55 of the *fol*P1 gene harbored CCC (Pro) to CTC (Leu)

Figure 1. (A)Receiver Operator Characteristics (ROC) curves describes sensitivity and specificity and the cut-off level; (B) OD values of MB and PB leprosy cases in Taiwan

(A)



(B)



VI. Publication list for this work

The poster has been accepted at the 23rd European Congress of Clinical Microbiology and Infectious Diseases.

Sapovirus epidemiological study & Quick diagnostic system for diarrheal viruses

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Summary: In this study, a total of 1454 stool specimens collected from 303 outbreaks of diarrhea in Taiwan were screened for viral agents in 2012. Forty-three percent (625/1454) was lab-confirmed with viral positive, including 175 Noroviruses (NoVs) and 9 Sapoviruses (SaVs) outbreaks. We also retrospective survey the outbreak specimens which were lab-confirmed with norovirus-negative during 2007 to 2011. There were at least isolated 13 different norovirus genotypes and 4 different sapovirus genotypes in the period. Norovirus strains belonging to the GII.4 were dominant. Furthermore, the GII.4 strains were isolated throughout Taiwan, demonstrating their widespread distribution. We also found that 24 sapovirus outbreaks belong to GI.1, GI.2, GI.3 and GV genotypes. Our results have shown for the first time that concluded noroviruses and sapoviruses in reporting diarrhea and foodborne outbreaks in Taiwan. It's an important evidence that viral causes of diarrhea outbreaks in Taiwan.

I. Purpose:

Diarrhea viruses are becoming important causes of acute gastroenteritis outbreaks. Up to 90% of nonbacterial gastroenteritis and related outbreaks were caused by NoVs, and rotaviruses worldwide. Transmissions of these viruses can be due to food-related, person-to-person close contact, and environmental contaminations. Noroviruses are the leading cause of nonbacterial acute gastroenteritis outbreaks worldwide, a disease that usually occurs in family or community-wide epidemic. Rotavirus was reported as major etiology which causes diarrhea of children under 5. Sapoviruses (SaVs) also belong to *Caliciviridae* family as Noroviruses (NoVs). However, the impact of the sapoviruses has not been fully established yet. Therefore, this study will focus on the establishment of surveillance of viral diarrhea including sapovirus in Taiwan, and collaborated together with NIID to evaluate the quick screening testing for setting-up the standard protocol to detect infectious agents. The results will be critical for displaying preventative measures and blocking further transmission of infectious pathogens.

II. Methods:

Sample collection and Reporting system in Taiwan

For Sapovirus surveillance study, the specimens were collected from reporting of diarrhea-associated outbreaks were by local health institute through food-borne outbreak reporting system and diarrhea syndrome outbreak reporting system.

For evaluation the detection methods for variations strains of Norovirus, freeze stool specimens were selected from sporadic cases with acute gastroenteritis during 2010-2011. All stool samples were examined for enteric bacteria and viruses.

Specimen preparation

A total of 1454 stool specimens were collected from 303 outbreak patients suffering diarrhea in 2012 in Taiwan. Also, specimens of reporting outbreaks with NoV-negative during 2007 to 2010 were selected for sapovirus screening. A 10% (w/v) stool suspension was prepared by 1X PBS then centrifuged 3000 rpm for 15 min at 4℃. Clarified suspension would be prepared for RNA extraction and stored at -80℃ for next extraction.

RNA extraction and Reverse-transcript reaction

Viral RNA was extracted from 200 μL stool suspension using MagConpact (Roche) and dissolved in 100 μL of elution buffer with added 1μL (40U) RNase inhibitor. 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, 2μl of 0.1 M dithiothreitol ,2μl 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.

SaV PCR amplification

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.

NoV PCR amplification

For norovirus genogroup I (GI) and genogroup II (GII) PCR, G1SKF/G1SKR and G2SKF/ G2SKR primer sets were used as described(18). The PCR was performed in 25 μl of reaction volume containing 2.5 μl of cDNA, 12.5μl Mater Mix buffer (QIAGEN, Hot star), and 20 pmol of each primer. These primers generated approximately a 343-bp product. PCR amplification was performed under the following conditions: initial denaturation at 94°C for 15 min, followed by 40 cycles of amplification with denaturation at 94°C for 30 s, primer annealing at 50°C for 30 s, extension reaction at 72°C for 1 min, and then a final extension at 72°C for 10 min.

Real-time RT-PCR for Norovirus

Real-time quantitative RT-PCR(TaqMan). To prevent carryover contamination by NLV cDNA and to reduce nonspecific amplification, viral RNA extracted with a QIAamp viral RNA kit was treated with DNase I before RT. Viral RNA (12.5 μ l) was added to a reaction mixture (2.5 μ l) containing DNase I buffer (150 mM Tris-HCl [pH 8.3], 225 mM KCl, 9 mM MgCl2) and 1 U of RQ1 DNase (Promega Madison, Wis.). The reaction mixture was incubated at 37°C for 30 min to digest DNA and then at 75°C for 5 min to inactivate the enzyme. DNase I-treated RNA (15 μ l) was added to 15 μ l of another mixture containing 100 mM Tris-HCl (pH 8.3), 150 mM KCl, 6 mM MgCl2, a 1 mM concentration of each deoxynucleoside triphosphate, 10 mM dithiothreitol, 75 pmol of random hexamers (pdN6; Amersham Pharmacia Biotech, Piscataway, N.J.), 30 U of RNasin (Promega), and 200 U of SuperScript II RNase H (-) reverse transcriptase (Gibco BRL, Gaithersburg, Md.). RT was performed at 42°C for 2 h, and the enzyme was inactivated at 70°C for 15 min. cDNA solutions were stored at -20°C.

The real-time quantitative RT-PCR was carried out in 50 μ l of a reaction mixture containing 5 μ l of cDNA, 25 μ l of TaqMan Universal PCR Master Mix (Applied Biosystems) containing dUTP and uracyl *N*-glycosylase (UNG), a 400 nM concentration of each primer, and either 15 pmol of RING1(a)-TaqMan Probe (TP) and 5 pmol of RING1(b)-TP fluorogenic probes for NLV GI detection or 5 pmol of RING2-TP fluorogenic probe for NLV GII detection. PCR amplification was performed with an ABI Prism 7700 sequence detector (Applied Biosystems) under the following conditions: incubation at 50°C for 2 min to activate UNG, initial denaturation at 95°C for 10 min, and then 45 cycles of amplification with denaturation at 95°C for 15 s and annealing and extension at 56°C for 1 min. Amplification data were collected and analyzed with Sequence Detector software version 1.6 (Applied Biosystems).

In each operation, an NLV GI- or GII-specific standard curve was generated by a 10-fold serial dilution (107 to 101 copies) of purified NLV GI or GII cDNA plasmids. Plasmid standards containing PCR products of the ORF1-ORF2 junction were prepared with strains SzUG1 and U201 with primer sets G1FF-G1SKR and G2FB-G2SKR, respectively.

Direct RT-PCR Kit for Norovirus Detection

10% fecal sample suspended in saline and centrifuged for 5 min. 1μl centrifugal suspension mix thoroughly with 19μl Sample Treatment Reagent [G2]. Mixture was heated 90 °C for 1-2 min and cooled on ice. Reverse transcription(RT) containing 24.75μl RT Reagent [G2] and 0.25μl RT Enzyme [G2]. Add 25μl of the RT reaction mixture to 20μl preheated sample. Temperature conditions of RT reaction were 37°C for 30 min, 90°C for 5 min and cooling on ice. PCR reaction contained 4.75μl PCR Reagent [G2], 0.25μl PCR Enzyme, 1μl 125X SYBR Green I solution and 1μl 25μM ROX Reference Dye. Add 7μl of the PCR reaction mixture to 45μl RT reaction. PCR temperature conditions were denatured 95°C for 10 min and 45 cycles followed 95°C for 15 sec, 56°C for 30 sec and 72°C for 45 sec. Final extension was 72 C for 1 min.

Sequencing for genotyping

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.

III.Results:

1) Surveillance of Sapovirus during 2007-2012

Though, the first sapovirus-associated outbreak was identified in college students with gastroenteritis that occurred during May 4–8, 2007 in Taiwan. Meanwhile, sapovirus is considered a common cause of viral gastroenteritis in infants and young children in the world. However, its prevalence is unknown because of the absence of routine screening procedures and sensitive diagnostic tests.

In this study, we screened the specimens from reporting diarrhea and foodborne outbreaks in 2012, and combined with the retrospective study of selected lab-confirmed NoV-negative specimens during 2007-2011. SaVs were isolated in a total of 24 outbreaks throughout Taiwan during 2007-2012. Most of the outbreaks separated in the west part of Taiwan (Fig1), demonstrating their widespread distribution. From the reporting system and outbreak investigation, we collected epidemiological informations. Review the reporting and investigation information of SaV outbreaks, half of outbreaks were reported in school (12/24), then restaurant (6/24), hospital (4/24), and populous institute. The most age distribution was age 0-10 then 11-20 years old. Half of the outbreaks showed age discrete which displayed the outbreak happened in the school, restaurant, populous institute and hospital. The transmission caused by person-to -person (workers to the patient) (Fig2). The monthly distribution of Sapovirus outbreaks was most in January to June, some in September and November. The monthly distribution curve wasn't so clear because of less outbreak numbers. Comparing to Norovirus-outbreaks happened in Taiwan, the Sapovirus-season was from winter to spring as Norovirus-season was since September to next March; most of the sapovirus infected patients were at younger age but norovirus could cause all ages (Fig 3).

By molecular analysis and sequencing, we isolated at least 4 different sapovirus genotypes GI.1, GI.2, GI.3 and GV. Sapovirus strains belonging to the GI.2 were dominant, were isolated from 18 of 24 (75%) outbreaks (Fig 4). Phylogenetic tree was generated by VP1 gene of sapovirus via neighbor-joining method using MEGA 4.0 software. Bootstrap values of 1000 replication were shown on the branches (Fig 5).

2) Evaluation of detection methods for variations strains of Norovirus

Freeze stool specimens were selected from sporadic cases with acute gastroenteritis during 2010-2011. All stool samples were examined for enteric bacteria and viruses. In this study, a

total of 113 specimens were selected, including 4 group which were genogroup I, genogroup II, mixed genogroup I and II, and control group. Those cases determined NoVs by RT-PCR and genotyping by sequencing. NoVs genogroup I included GI.2, GI.4, GI.6, GI.8, and GI untyped. NoVs genogroup II included GII.2, GII.3, GII.4, GII.7, GII.12, GII.16, and GII untyped. NoVs mixed genogroup included GI.1, GI.4, GI.10, GII.4, GII.12 mix as list in Table 1.

The evaluations were compared the tranditional RT-PCR with 2 kinds of real-time RT-PCR. The most dominant strains isolated in sporadic cases were GI.4 and GII.4 in Taiwan. Based on the traditional RT-PCR, the real-time RT-PCR(TaqMan) can recognize most of the genotypes but part of GI.4, GI.8, GII.2 and GII.4. However, the identified genotypes of Direct RT-PCR is lower than real-time RT-PCR(TaqMan) method. It cannot identify most of GI genotypes and GII.2. But the evaluation should be continued for testing sensitivity of all of the methods by quantitation of norovirus in each stool.

IV.Discussion:

Norovirus gastroenteritis remains a public health problem worldwide and is second ranked to rotavirus diarrhea in children visiting hospitals. Rotavirus is most caused diarrhea of children under 5, and mostly were published to be sporadic cases. However, Noroviruses were reported to cause outbreaks in restaurant, hospital, nursing center, school etc. in all ages. The prevalence of sapovirus infection was not clear in the world, but some manuscripts reported it caused sporadic or outbreaks in young children. In this study, we established the first overall surveillance data and viral strains in Taiwan during 2007-2012. Moreover, in order to improve the decision time schedule for policy control of a suspected diarrhea or foodborne outbreak. We evaluated and selected a proper diagnosis method for norovirus outbreaks, though it needs further testing for suggestion.

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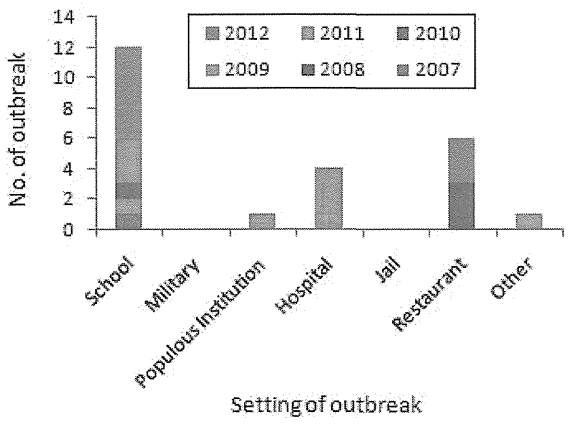
Fig1. Distribution of Taiwan Sapovirus during 2007-2012

Fig2. Epidemiology of Sapovirus-outbreak during 2007-2012, Taiwan



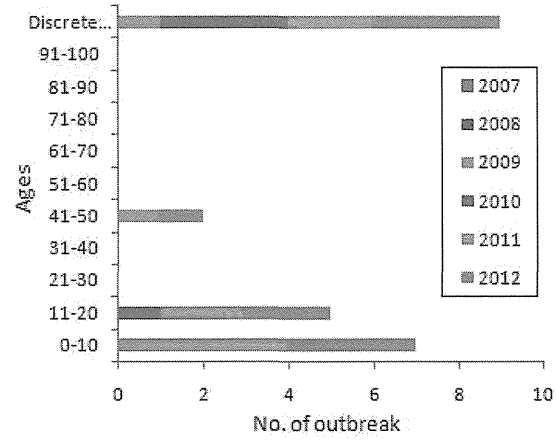


Fig 3. Monthly distribution of Norovirus and Sapovirus outbreaks in Taiwan, 2007-2012

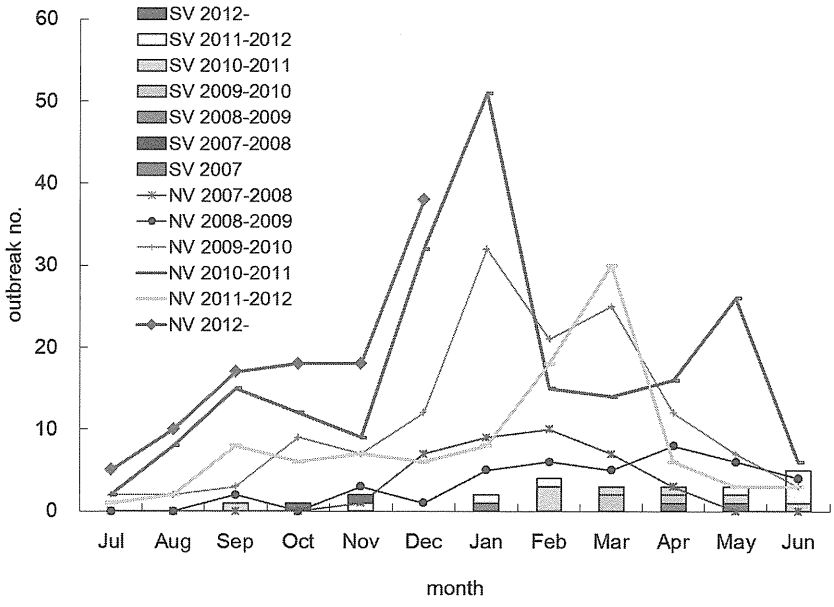


Fig 4. Genotypes isolated from Sapovirus-outbreaks in 2007~2012

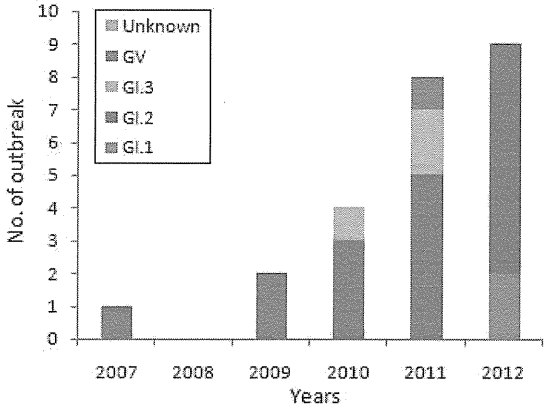


Fig 5. Phylogenetic analysis according to partial VP1 region of sapovirus

437 bp of all strains aligned and the tree was generated via neighbor-joining method using MEGA 4.0 software. Bootstrap values of 1000 replication were shown on the branches.

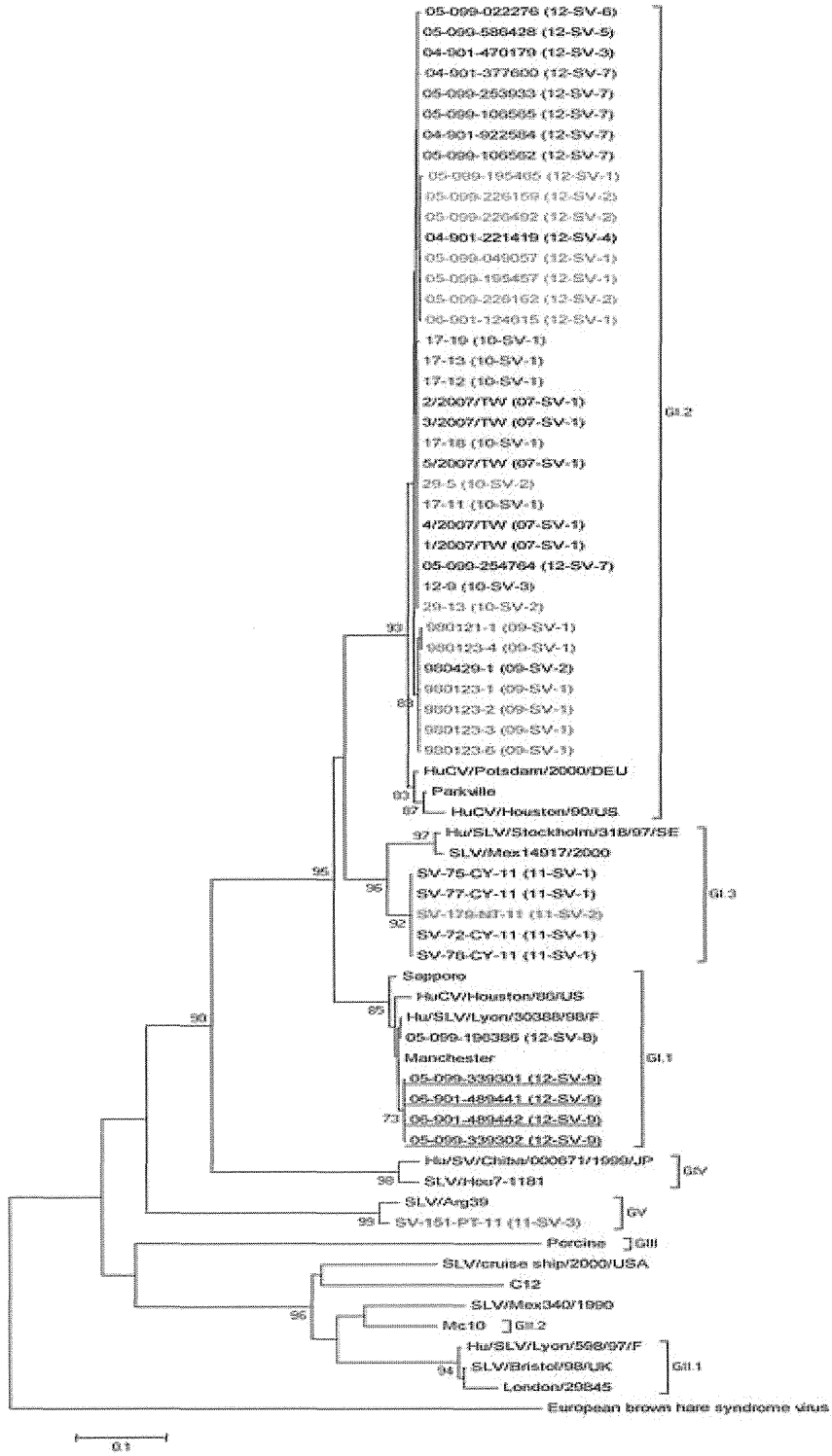


Table 1. Comparison of the traditional RT-PCR, Real-time quantitative RT-PCR(TaqMan) and Direct RT-PCR methods.

Genogroup	Genotype	RT-PCR (CDC)		Real-time PCR		Direct RT-PCR	
		%	pos/total	%	pos/total	%	pos/total
GI	unknow	100	1/1	100	1/1	0	0/1
	2	100	1/1	100	1/1	100	1/1
	4	100	11/11	27.3	3/11	9.1	1/11
	6	100	1/1	100	1/1	0	0/1
	8	100	8/8	50	4/8	0	0/8
GII	unknow	100	5/5	40.0	2/5	0	0/5
	2	100	4/4	75.0	3/4	50.0	2/4
	3	100	1/1	100	1/1	100	1/1
	4	100	60/60	93.3	56/60	85.0	51/60
	7	100	1/1	100	1/1	100	1/1
	12	100	4/4	100	4/4	100	4/4
	16	100	1/1	100	1/1	0	0/1
GI+GII	G1+G2.4	100	1/1	100	1/1	100	1/1
	G1+G2.12	100	1/1	100	1/1	100	1/1
	G1.1+G2.4	100	1/1	100	1/1	100	1/1
	G1.4+G2.4	100	1/1	100	1/1	100	1/1
	G1.10+G2.4	100	1/1	100	1/1	100	1/1