RESEARCH NOTE

DETECTION OF WU POLYOMAVIRUS AND NOROVIRUS GENOGROUP II IN STOOLS OF CHILDREN WITH ACUTE GASTROENTERITIS IN TAIWAN

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Abstract. To date, there has been no report of coinfection of human WU polyomavirus (WUPyV) with norovirus (NV). WUPyV has been detected in stool indicating pathogenicity of the gastrointestinal tract. Feces from 110 children (58 males, 52 females) with acute gastroenteritis admitted to Wei-Gong Memorial Hospital, Taiwan were screened for the presence of WUPyV and NV GII by PCR and RT-qPCR, respectively revealing three males with WUPyV only and one male with both viruses, the latter being first such report. There are no significant differences in clinical symptoms between patients with and without viral infection. Phylogenetic analysis based on WUPyV VP2 sequences indicated that the four samples are closely related to strains epidemic in China.

Keywords: norovirus, WU polyomavirus, coinfection, gastroenteritis, Taiwan

INTRODUCTION

Viral gastroenteritis is one of the most frequently encountered illnesses in children and adults worldwide (Eckardt and Baumgart, 2011). It is estimated that viral gastroenteritis is the cause of 30%-40% of infectious cases in developed countries (Hodges and Gill, 2010). An estimated 211-375 million episodes of acute gastroenteritis (AGE) occur annually in the United States, the majority of which are considered to have a viral etiology (Thielman and Guerrant, 2004; Ismaeel *et al*, 2007). There are more than 20 types of viruses known to cause AGE, among which norovirus (NV) frequently is associated with AGE (Hall *et al*, 2012).

Based on antigenic and genetic properties, NV, a member of Caliciviridae family, is classified into seven genogroups (GI-GVII) (Vega *et al*, 2014), but only GI, GII and GIV are associated with human infection (La Rosa *et al*, 2007), with GII being the most prevalent in AGE patients (Atmar and Estes, 2006). NV is a small (30-38 nm), round, non-enveloped single-

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stranded RNA virus of positive sense with a genome of 7.5 kb containing three open reading frames (ORFs), with ORF1 encoding six non-structural proteins, ORF2 major capsid protein VP1 and ORF3 minor capsid protein VP2 (Green *et al*, 2000; Thorne and Goodfellow, 2014). The highly conserved region in the NV genome is the ORF1/ORF2 junction, which has been used as a preferred target location for detection of NV by quantitative (q)PCR method (Stals *et al*, 2012). Although NV is associated with epidemics and sporadic AGE cases across all age groups, it causes more severe clinical manifestations in young children and the elderly than in other age groups (Bernard *et al*, 2014).

Polyomaviruses (PyVs) were isolated for the first time in 1971 in England and named BK and JC viruses (Padgett *et al*, 1971). Three further PyV types, namely, WUPyV, KIPyV and Merkel cell PyV, were detected subsequently (Gaynor *et al*, 2007; Jiang *et al*, 2009). WUPyV was discovered in Australia by shot gun sequencing of nasopharyngeal aspirate of a three-years-old child suffering from pneumonia (Gaynor *et al*, 2007). It belongs to the family Polyomaviridae and forms a small, non-enveloped, icosahedral particle, 40-45 nm in diameter, containing a 5,229 bp supercoiled double strand (ds)DNA genome with a GC-content of 39% and contains an early coding region encoding T-antigens (small Tag and large Tag), a late coding region encoding structural proteins (VP1, VP2 and VP3), and a non-coding control region (Gaynor *et al*, 2007; Johne *et al*, 2011). The role of WUPyV in disease pathogenesis remains unclear, but an ability of its detection in clinical specimens would help in the determination of the virus replication sites and routes of transmission and dissemination.

Previous studies of other PyVs, including BK and JC viruses and KIPyV, have demonstrated their presence in stool (Bofi *et al*, 2001; Allander *et al*, 2007), which suggests their potential for transmission through the gastrointestinal tract (Bofi *et al*, 2001). Because a number of children who had WUPyV display respiratory and gastrointestinal clinical signs, it was speculated that WUPyV might also be transmitted through the gastrointestinal tract (Han *et al*, 2007; Le *et al*, 2007; Johne *et al*, 2011). It has been suggested that the presence of WUPyV in stool samples might result from patients swallowing virus-containing nasal secretion or sputum (Ren *et al*, 2009).

Up till now there has not been any report of coinfections of NV GII and WUPyV in children with AGE. As both types of viruses are not able to be cultured (Kleines *et al*, 2009; Thorne and Goodfellow, 2014), coinfections cannot be demonstrated by such *in vitro* studies. Therefore, this study performed molecular biological methods to determine the prevalence of NV GII and WUPyV coinfection and to investigate phylogenetic characteristics of gastrointestinal viral strains among children with AGE in Taiwan.

MATERIALS AND METHODS

Case definition

AGE patients are defined as patients with clinical diarrhea (≥ 3 loose stools within a 24-hour period), which may be accompanied by abdominal pain, fever, nausea, or vomiting.

Specimen collection

The study was conducted from August 2012 to July 2013 at Wei-Gong Memorial Hospital, Taiwan. Patients were given a follow-up questionnaire the week

after enrolment to obtain epidemiological information and clinical symptoms and to ascertain that AGE had occurred. Stools of 110 AGE patients were collected and stored at -20°C before being transferred on ice to the Department of Bioengineering, Tatung University, Taiwan where they were stored at -70°C until used.

This study was approved by the Human Subject Research Ethics Committee, Wei-Gong Memorial Hospital (approval no.101003). Written informed consent was obtained from adult participants and parents of minors.

Virus nucleic acid extraction

Nucleic acids were extracted from 200 µl of 10% fecal suspension in a balanced salt solution using a virus nucleic acid extraction kit (Geneaid, New Taipei City, Taiwan) according to the manufacturer's instructions. Extracted nucleic acids in 50 µl of RNase-free water were stored at -20°C until used.

PCR detection of WUPyV

Primers amplifying WUPyV 250 bp VP2 region were AG44 (5'-TGTTACAAATAGCTGCAGGTCAA-3') and AG45 (5'- GCTGCATAATGGGGAGTACC -3') (Zhao *et al*, 2010). For all PCR assays, standard precautions to avoid end product contamination were taken, including use of PCR hoods and maintaining separate areas for PCR set-up and analysis. PCR was performed in a 25-µl volume containing 10.5 µl of RNase-free water, 5 µl of extracted nucleic acids, 2.5 µl (10 µM stock) primers, 1 µl of dNTPs (10 mM stock), 5 U *Taq* DNA polymerase (IT'S Science, Taipei City, Taiwan), and 2.5 µl of 10X buffer (500 mM Tris-HCl pH 9.2, 160 mM ammonium sulfate, 25 mM $MgCl_2$ and 1% Tween 20). Thermocycling, performed in Thermo Electron Corp thermal cycler (Waltham, MA), was as follows: 94°C for 5 minutes; 40 cycles of 94°C for 30 seconds, 56°C for 45 seconds, and 72°C for 1 minute; with a final step of 72°C for 10 minutes. Clinical stool samples, positive for WUPyV and provided by the Wei-Gong Memorial Hospital, Taiwan were used as positive controls, and nuclease-free water (Qiagen, Taipei City, Taiwan) was used as a negative control. One positive and one negative control were included for each series of PCR assay. Amplicon was separated by electrophoresis in 2% agarose gels and visualized after staining with ethidium bromide.

One-step SYBR Green RT-qPCR detection of NV GII

One-step SYBR Green RT-qPCR was performed using a StepOne™ real-time PCR system (Applied Biosystems, Foster City, CA) and employing SYBR green fluorescent dye (PCR Biosystems, London, UK). NV GII ORF1 and ORF2 gene sequences were targeted using primers COG2F (5'-CARGARBCNATGTTYAGRTGGATGAG-3)' and COG2R (5'-TCGACGCCATCTTCATTCACA-3') (where B = C, G, or T; N = A, C, G, or T; R = A or G; Y = C or T) (Tajiri-Utagawa *et al*, 2009). An NV GII-positive stool sample provided by Wei-Gong Memorial Hospital was used as positive control and sterile deionized water as a negative control. A standard curve was created using 10-fold dilutions of NV GII RNA (Wei-Gong Memorial Hospital), the concentration of which was determined spectrophotometrically (UV-1700 spectrophotometer, Shimadzu, Kyoto, Japan) versus Ct values ($R^2 > 0.99$; slope of curve: -3.1 to -3.6; PCR efficiency: 90%-110%). Samples with a Ct value of ≤ 36 were considered to be NV GII-positive.

Statistical analysis

Samples were classified as WUPyV-positive or WUPyV-negative for viral

Table 1
Epidemiological and clinical features of AGE patients.

Parameter	Patients		*p*-value[a]	Patients
	WUPyV positive ($n = 4$)	WUPyV negative ($n = 106$)		WUPyV and NV GII coinfection ($n = 1$)
Gender				
Male	4	54		1
Female	0	52		0
Setting				
Outpatient	1	10	0.348	0
Emergency	0	62		0
Inpatient	3	34	0.110	1
Age (years)				
<2	0	37		0
2-10	4	49		4
>10-18	0	20		0
Season				
Spring	1	21	1.000	1
Summer	2	16	0.124	0
Fall	1	18	0.537	0
Winter	0	51		0
Fever >38°C				
Yes	1	53	0.618	1
No	3	53	0.618	0
Vomiting				
Yes	4	71		1
No	0	35		0
Stool type				
Watery	3	83	1.000	0
Bloody	0	2		0
Non-watery, non-bloody	1	21	1.000	1
Dehydration				
Moderate	0	1		0
Mild	2	30	0.579	0
No	2	75	0.579	1
Abdominal pain				
Yes	4	100		1
No	0	6		0

[a]Two-tailed chi-square, Fisher's exact test comparing WUPyV-positive and -negative cases.

pathogens. For categorical variables, chi-square test was used to examine differences in proportions between groups. Fisher's exact tests were used when the expected value for a cell was < 5. A *p*-value < 0.05 is considered statistically significant.

RESULTS

WUPyV was detected only in 4 (male)/110 (58 males, 52 females) stool samples of children with AGE, including 3 with only WUPyV infection and 1 with both WUPyV and NV GII infections (Table 1).

Table 2
Prevalence of WUPyV detected by PCR in AGE and other syndromes.

Country	Year[a]	Stool sample		Patient	Prevalence (%)	PCR assay		Reference
		Syndrome	*n*			Genome region	Primer name	
Taiwan	2014	AGE	110	C	4	VP2	AG44/AG45	Present study
China	2013	AGE	211	C	4	VP1	N/A	Li *et al*, 2013
Germany	2012	HSCT	37	C	3	VP1	N/A	Motamedi *et al*, 2012
Italy	2009	CS	84	N/A	7	Regulatory region	WU A	Bergallo *et al*, 2009
					7	Large T antigen	WU B	
					8	Regulatory region	WU C	
					25	VP2	WU D	
Italy	2009	HD	31	C/A	35	VP2	N/A	Babakir-Mina *et al*, 2009
Germany	2009	ARTD	14	C	14	Large T antigen	WU 2958s/ WU 2865a	Neske *et al*, 2009
Australia	2009	AGE	193	C	4	Regulatory region	WU C	Bialasiewicz *et al*, 2009
		Non-AGE	221	C/A	1	Large T antigen	WU B	
China	2009	AGE	377	C	0.5	VP2	AG48/AG49	Ren *et al*, 2009

AGE, acute gastroenteritis; HSCT, hematopoietic stem cell transplantation; HD, hematalogical disorder; ARTD, acute respiratory tract disease.

The clinical symptoms of the latter was not different from those of the other 3 patients. WUPyV prevalence among AGE patients is not statistically significant. Clinical symptoms of WUPyV-positive AGE patients included fever, vomiting, watery stool, dehydration and abdominal pain. WUPyV clinical symptoms among AGE patients also are not statistically significant.

DISCUSSION

To the best of our knowledge, this is the first description of WUPyV and NV GII coinfection in stool of children with AGE patients. Although the prevalence of NV GII and/or WUPyV was low (4%), this value is in keeping with other studies in AGE subjects worldwide (Table 2). Our observation that WUPyV infection in children with AGE showed no correlation between epidemiological features and disease is consistent with previous report (Ren *et al*, 2009). WUPyV also has been detected in stool of patients with non-AGE, such as acute respiratory tract diseases and hematological disorders, and patients with hematopoietic stem cell transplantation (Table 2). Although there is a paucity of information regarding the route of transmission of WUPyV, the presence of WUPyV DNA in feces indicates a fecal-oral route of transmission. Further-

more, fecal samples are as adequate as respiratory aspirates for detecting WUPyV infection and thus is a more convenient source for prevalent studies in the general population.

It still needs to be elucidated whether co-infection of WUPyV with other intestinal tract viruses plays a role in the pathogenesis of viral-associated AGE. Previous studies have suggested that WUPyV co-infection promotes the pathogenic effects of other infectious agents (Ren *et al*, 2009). Co-infections of NV GII and enteric viruses (sapo- and rotavirus) have been reported (Dai *et al*, 2011; Li *et al*, 2012), suggesting that NV GII is a frequent strain co-infecting with other types of enteric viruses. Further studies of more stool samples of AGE patients are needed to determine whether WUPyV and NV GII co-infections affects the clinical symptoms of the disease. During co-infection, the pathogenic potential of each virus type appears to be enhanced leading to a worsening of the patients' clinical manifestation (Liu *et al*, 2006; Bhavnani *et al*, 2012; Tang *et al*, 2014). Thus clinicians and public health researchers should consider the possible presence of viral co-infection in the etiology of AGE and monitor a broader range of enteric viruses.

The four WUPyV strains in this study are closely related to strains endemic in China (Yuan *et al*, 2008). It is crucial to emphasize that the four variants of WUPyV found in Taiwan have been implicated in sporadic gastroenteritis worldwide indicating a global spread.

There are at least three strengths in this study. First, all clinical departments of the hospital adopted a uniform case definition to enroll AGE patients and collect stool samples, and a formatted questionnaire was used to record the patients' biodata. Second, two enteric virus were analyzed by molecular methods, which allowed simultaneous identification of both viral agents. Third, genetic characterization of WUPyV isolates permitted phylogenetic analysis to be performed.

In summary, this study is the first to examine in Taiwan the presence of WUPyV and its co-infection with NV GII in stool of children with AGE. In addition phylogenetic analysis revealed that WUPyV in Taiwan were closely related to strains endemic in China. Further studies using a larger cohort of AGE patients are required to determine whether co-infection with these two enteric viruses will worsen further the clinical symptoms of AGE. Systematic surveillance and evidence-based studies also are required to determine the transmission routes and spread of WUPyV in Taiwan.

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Declaration of conflict of interests

The authors declare no conflict of interests.

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