CO-INFECTION OF ADENOVIRUS, NOROVIRUS AND TORQUE TENO VIRUS IN STOOLS OF PATIENTS WITH ACUTE GASTROENTERITIS

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Abstract. Up to now, there has been no report of co-infection of torque teno virus (TTV) with other enteric viruses playing a role in the pathogenesis of viral acute gastroenteritis (AGE). We investigated the proportion, epidemiological and clinical features of concurrent infections of adenovirus (ADV), norovirus (NV) and TTV in stools of 155 patients with AGE attending Wei-Gong Memorial Hospital, Miaoli City, Taiwan. The presence of the three viruses were determined using PCR-based assays. Some 55% of the patients were infected with at least 1 enteric virus, among whom 18% were co-infected, NV and TTV being the most common (62%). Rate of co-infectious in AGE patients is correlated statistically significantly ($p < 0.05$) with age, fever and drinking of spring water. Furthermore, AGE children with co-infection have a higher hospitalization rate (69%). To the best of our knowledge, this is the first report of ADV, NV and TTV triple co-infection in children (2) with AGE. This study also revealed that TTV co-infection promoted the pathogenicity of other infectious agents.

Keywords: acute gastroenteritis, adenovirus, co-infection, norovirus, torque teno virus, 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 AG, among which norovirus (NV) and adenovirus (ADV) frequently are associated with AGE (Hall *et al*, 2012; Chhabra *et al*, 2013), and torque teno virus (TTV) is highly prevalent in patients with AGE (Pinho-Nascimento *et al*, 2011).

Based on antigenic and genetic properties, NV, a member of the Caliciviridae family, is classified into 5 (I-V) genogroups

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(Patel *et al*, 2009), with genogroups I, II and IV being associated with human infections (La Rosa *et al*, 2007). 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).

ADV belongs to the Adenoviridae family, usually affecting children younger than 2 years and symptomatic adult infections are uncommon (Blacklow and Greenberg, 1991). Although ADVs are primarily recognized as pathogens of respiratory, ocular or genitourinary infections, serotypes 31, 40 and 41 can cause AGE (Matsushima *et al*, 2011). Previous studies have suggested that ADV-related cases might occur more commonly in the second half of the year (in Brazil), especially in children (Filho *et al*, 2007).

TTV is a member of the Anelloviridae family, and has been classified into at least five groups consisting of more than 27 genotypes as the result of an extremely wide range of sequence divergence observed among TTV isolates (Peng *et al*, 2002). TTV is common in asymptomatic and AGE patients.

Up till now there has not been any report of co-infections of NV, ADV and TTV in children and adults with AGE, especially in developing countries. In Taiwan, two studies on NV or ADV in AGE among children have been conducted, indicating viral enteric co-infections in 13.1% of hospitalized children with AGE (Chen *et al*, 2013; Tsai *et al*, 2014). As TTV and NV are not able to be cultured (Hino and Miyata, 2007; Thorne and Goodfellow, 2014), co-infections cannot be demonstrated by *in vitro* studies.

Therefore, this study was performed using molecular methods to determine viral co-infection prevalence in hospitalized patients with AGE and to investigate age distribution, seasonal trend and clinical characteristics of gastrointestinal viral co-infections among children and adults in Taiwan.

MATERIALS AND METHODS

Case definition

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

Specimen collection

Stools of 155 AGE patients attending Wei-Gong Memorial Hospital, Miaoli County were stored at -20°C and transferred to the Department of Bioengineering, Tatung University, Taipei City where they were stored as a 10% suspension in a balanced salt solution at -70°C until assayed (Tang *et al*, 2013). This study was conducted from August 2011 to July 2012 at Wei-Gong Memorial Hospital. Patients were given a follow-up questionnaire the week after enrolment to obtain the epidemiological features and clinical symptoms and to ascertain that AGE had occurred. This study was approved by the Human Subject Research Ethics Committee of the Wei-Gong Memorial Hospital, Miaoli County (approval no. 100003). Informed written consent was obtained from adult participants and parents of minors.

Viral DNA/RNA extraction

Nucleic acid was extracted from 200 μl of 10% fecal suspension using a viral nucleic acid extraction kit (Geneaid, New Taipei City, Taiwan) according to the manufacturer's instructions. Extracted DNA/RNA was stored in 50 μl of RNase-free H_2O at -20°C until assayed.

RT-PCR detection of NV

RT-PCR was performed separately using 10 µl nucleic acid, 10 µl of one-step RT-PCR master mic (Qiagen, Taipei City, Taiwan), 0.5 µl (10 µM) of JV12 (5'ATACCACTATGATGCAGATTA-3', nt. no. 4552-4572) and JV13 (5'-TCATCATCACCATAGAAAGAG-3', nt. no. 4878-4858) primers (Vinje and Koopmans, 1996), 4 µl buffer (Qiagen, Hilden, Germany), 0.4 µl (10 mM) of dNTPs, 3.8 µl of H_2O, and 0.8 µl (1.25 U/µl) of enzyme mix (RTase; Qiagen, Hilden, Germany). The thermocycling conditions (conducted in Thermo Electron Corp) were as follows: 50°C for 30 minutes; 95°C for 15 minutes; 40 cycles at 94°C for 30 seconds, 37°C for 1 minute, and 72°C for 1 minute; followed by a final step at 72°C for 10 minutes. The amplicons were analyzed by 2% agarose gel-electrophoresis at 100 V for 30 minutes and visualized under UV light after staining with ethidium bromide.

The amplicons were genotyped by DNA sequencing. NVs were identified based on the 327 nucleotide sequences of the RNA-dependent RNA polymerase (RdRp) region. All NV sequences were analyzed using the basic local alignment search tool (BLAST) and DNAMAN software. Phylogenetic trees with 1000 bootstrap replicates were generated using the neighbor-joining method by employing molecular evolutionary genetics analysis (MEGA), version 5.0. Reference strains were downloaded from the GenBank. Only bootstrap values >65 were considered significant.

Semi-nested PCR detection of ADV

Semi-nested PCR, was performed in a 25 µl reaction volume containing 17 µl of RNase-free water, 2 µl of template DNA, 1.5 µl of 10 µM Hex1 (5'-TTCCCCATGGCICACTAACAC-3') and Hex2 (5'-CCCTGGTA(GT)CC(AG) AT(AG)TTGTA-3') primers (Ko *et al*, 2003), 0.5 µl of 10 mM dNTPs, 0.25 µl of *Taq* DNA polymerase (15 U/µl, IT'S Science Corp, Taipei, Taiwan), and 2 µl of 10X buffer (500 mM Tris-HCl, pH 9.2, 160 mM ammonium sulfate, 25 mM $MgCl_2$, and 1% Tween 20). Thermocycling was performed as follows: 94°C for 5 minutes; 40 cycles of 94°C for 1 minute, 50°C for 1 minute, and 72°C for 2 minutes; with a final step of 72°C for 5 minutes. Amplicons (482 bp) were analyzed as described above. The second round PCR was performed as in the first round PCR except that primers Hex 1 (5'-TTCCCCATGGCICACTAACAC-3') and Hex 3 (5'-AGGAACCA(AG) TCCTTTAGGTCAT-3') (Ko *et al*, 2003), and 1 µl from the first round PCR reaction were used. Thermocycling was reduced to 30 cycles and amplicons (443 bp) were analyzed as described above.

Semi-nested PCR detection of TTV

Semi-nested PCR was performed in a 25 µl reaction volume containing 10 µl of RNase-free water, 5 µl of template DNA, 0.5 µl of 10 µM NG133 (5'-GTAAGTGCACTTCCGAATGGCTGAG-3') and NG147 (5'-GCCAGTCCCGAGCCCGAATTGCC-3') primers (Okamoto *et al*, 1999), 0.5 µl of 10 mM dNTPs, 2.5 µl of *Taq* DNA polymerase (5 U/µl, IT'S Science Corp, 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 was performed as follows: 95°C for 9 minutes; 35 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 45 seconds; with a final step of 72°C for 7 minutes. Amplicons (143 bp) were analyzed as described above. The second round PCR was performed as in the first round PCR except that primers NG134 (5'-AGTTTTC-

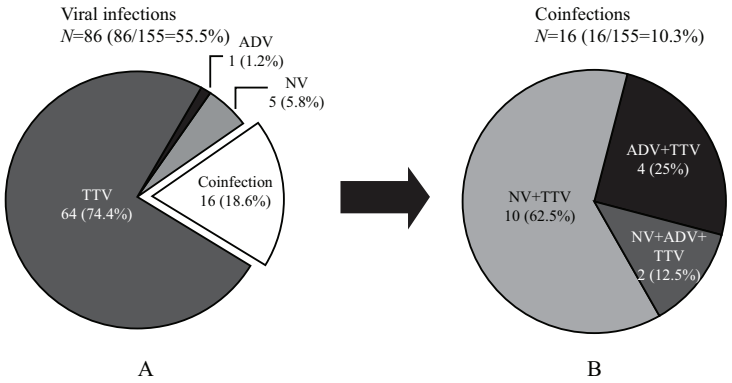

Fig 1–Distribution of single and co-infections of enteric viruses among 86 patients with acute gastroenteritis (A) and of different virus combinations among the 16 co-infected cases (B). Virus was detected from stool sample using PCR-based assay as described in Materials and Methods. ADV, adenovirus; NV, norovirus; TTV, torque teno virus.

CACGCCCGTCCGCAGC-3′) and NG132 (5′-AGCCCGAATTGCCCCTTGAC-3′) (Okamoto *et al*, 1999), and 2.5 µl from the first round PCR reaction were used. Thermocycling was reduced to 25 cycles and amplicons (110 bp) were analyzed as described above.

Statistical analysis

Chi-square test was used to examine differences in proportions between groups, and a $p < 0.05$ is considered statistically significant. Fisher's exact tests were used when the expected value was lower than 5. The odds ratio (OR) and 95% confidence interval (CI) were calculated when $p < 0.05$.

RESULTS

The 3 target enteric virus (NV, ADV and TTV) were detected in 86/155 (55.5%) of the stool samples, including 70 (45%) single viral infections and 16 (10.3%) viral co-infections (Fig 1). TTV was identified in 80 (51.5%) of the cases, NV in 17 (11%) cases, of which all were NV GII.4, and ADV in 7 (4.5%) samples. TTV was detected in all co-infections, together with NV in 10 (62.5%) samples, with ADV in 4 cases and with both NV, and ADV in 2 cases.

TTV infection was more prevalent in summer (June to August) and autumn (September to November, whereas NV infection occurred more commonly in the autumn and winter (December to February) and ADV was detected sporadically throughout the year without any obvious seasonal trends (Fig 2).

NV and ADV single infections and TTV co-infection were more prevalent in children aged 2-10 years, whereas TTV co-infections were lowest in the 10-20 years group (Fig 3). There is no statistically significant differences in virus infection prevalence among females with single and co-infections (Table 1). Prevalence of AGE patients with co-infection is not statistically significant ($p < 0.05$) in the age group ≤ 10 years and drank spring water. AGE patients with co-infection have statistically significant ($p < 0.05$) higher fever (≥ 38°C) compared to those with single infection. However, both groups of patients did not differ in the occurrences of vomiting or abdominal pain.

Among the 16 patients with co-infection, 11 (69%) had fever, 15 (94%) were in-patients, 11 of whom were children (Table 2). Furthermore, co-infections in all age groups were observed in only 9 months of the year (the exceptions being May, July and August).

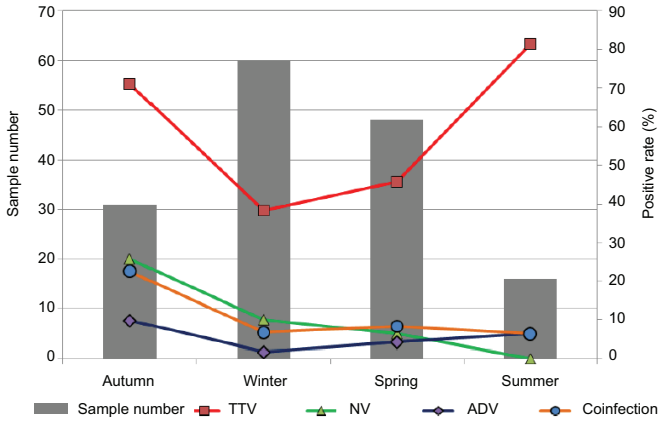

Fig 2–Seasonal distribution of ADV, NV and TTV infection in patients with acute gastroenteritis. Virus was detected from stool sample using PCR-based assay as described in Materials and Methods. ADV, adenovirus; NV, norovirus; TTV, torque teno virus.

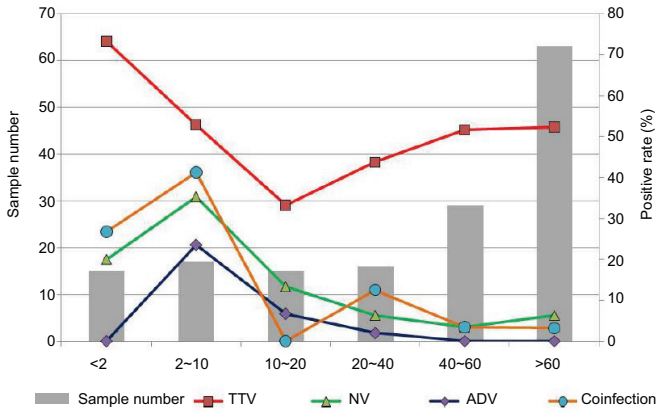

Fig 3–Age group distribution of ADV, NV and TTV infection in patients with acute gastroenteritis. Virus was detected from stool sample using PCR-based assay as described in Materials and Methods. ADV, adenovirus; NV, norovirus; TTV, torque teno virus.

DISCUSSION

To the best of our knowledge, this is the first description of triple co-infection of NV, ADV and TTV in AGE patients. Over 50% of patients with diarrhea in this study were infected with at least 1 enteric virus, mainly TTV or NV. TTV was the most common enteric virus detected similar to a previous study (Pinho-Nascimento *et al*, 2011). Braham *et al* (2009) have shown that TTV could be spread by fecal-oral transmission route, resulting in its spread among the population at large.

The 11% NV-infected AGE patients in this study were similar to those previously reported in Taiwan (Yang *et al*, 2010) and Australia (Marshall *et al*, 2003). DNA sequences of the 443 bp amplicon from the RNA-dependent RNA polymerase gene revealed that 17 NV samples belonged to GII.4 strain, the same as in the previous AGE outbreak in Taiwan in 2009 (Lai *et al*, 2013). The 2006 epidemic strain in Taiwan was GII.4-2006b and that of 2010 pandemic GII.4-New Orleans strain. The current finding in our patients of NV GII.4 variant strain that is implicated in sporadic AGE worldwide (Ramani *et al*, 2014) indicates its global spread.

ADV is highly infectious and can easily become wide spread (Chen *et al*, 2013). However, until now, reports on ADV infections in Taiwan are limited (Tebruegge and Curtis, 2012). The prevalence of ADV infection in AGE patients in our investigation was similar to precious studies (Rodriguez *et al*, 1985; LeBaron *et al*, 1990; Huhulescu *et al*, 2009; Kittigul *et al*, 2013). ADV is a rare cause of AGE

Table 1
Demographical and clinical data of patients with AGE.

Demographical and clinical data	Coinfection	Monoinfection	*p*-value[a]	OR	CI
Females (%)	50% (8/16)	37.1% (26/70)	-	-	-
Age <10 years (%)	68.8% (11/16)	12.9% (9/70)	<0.0001	14.911	4.197-52.980
Autumn	43.8% (7/16)	24.3% (17/70)	-	-	-
Winter	25% (4/16)	31.4% (22/70)	-	-	-
Spring water (%)	25% (4/16)	4.8% (3/62)	0.029	6.556	1.297-33.143
Patients suffering of fever (%)	73.3% (11/15)	35.7% (25/70)	0.01	4.95	1.426-17.181
Patients suffering of vomiting (%)	31.3% (5/16)	23.5% (16/68)	-	-	-
Patients suffering of abdominal pain (%)	56.3% (9/16)	61.2% (41/67)	-	-	-

[a]Only significant *p*-value (<0.05) reported. [b]OR, odds ratio; [c]CI, confidence interval.

in adults, but we have detected ADV in stools from 5 children and 2 adult AGE cases. However, ADV can cause a variety of infectious diseases, such as respiratory disease, epidemic keratoconjunctivitis, hemorrhagic cystitis, and gastroenteritis (Shimada *et al*, 2004).

It still needs to be elucidated whether co-infection of TTV with other intestinal tract viruses play a role in the pathogenesis of viral AGE. The majority (62.5%) of co-infection was of NV and TTV, but there were 2 cases involving NV, ADV and TTV. Co-infections of NV GII.4 and enteric viruses (sapovirus and rotavirus) have been reported (Dai *et al*, 2011; Li *et al*, 2012), suggesting that NV GII.4 is a frequent strain co-infecting with other types of enteric viruses. 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), a situation that is similar to this study, in which the number of hospitalized AGE children with co-infection was higher than those who were out-patients. Furthermore, previous studies have suggested that TTV virus in particular is a co-infecting virus that promotes the pathogenic effects of other infectious agents (Okamoto *et al*, 2001; Maggi *et al*, 2003; Szladek *et al*, 2005). In our study TTV was present in all co-infections. Thus clinicians and researchers should consider the possible presence of viral co-infection in the etiology of AGE and monitor a broader range of enteric viruses.

A study has indicated that rotavirus infections were positive for NV or ADV in AGE patients are significantly associated with various clinical symptoms, such as low-grade fever, vomiting and depletion (Ferreira *et al*, 2012). We observed fever was the most frequent clinical symptom among the co-infected patients (OR = 4.95). Thus fever is a risk factor and may be used by clinicians for clinical diagnosis of co-infected AGE patients.

Climate change is likely to contribute to the burden of infectious diseases. In this study, most NV infections occurred during late autumn and winter, as has been observed in other countries (Dai *et al*, 2010; Inaida *et al*, 2013). On the other hand, TTV infections occurred more commonly in summer and autumn. ADV infections occurred more commonly in autumn. Thus

Table 2
Demographical, clinical and virological data of the norovirus ,adenovirus and TTV co-infected patients.

No.	Sex	Age group (y)	Month of collection	Patient status	Type of drinking water	Gastrointestinal symptoms	Adenovirus	TT Virus	Norovirus
10	M	2-10	Sep	Inpatient	Tap water	Fever	+	+	+
25	F	2-10	Oct	Inpatient	Spring water	Fever	+	+	
27	F	2-10	Oct	Inpatient	Spring water	Fever, vomiting, AP		+	+
31	F	<2	Oct	Inpatient	Spring water	Fever		+	+
40	M	2-10	Nov	Inpatient	Spring water	Fever, AP	+	+	+
44	M	40-60	Nov	Inpatient	Tap water	AP		+	+
45	F	2-10	Oct	Inpatient	Spring water	Fever		+	+
49	M	>60	Dec	Inpatient	Tap water	Fever, AP		+	+
56	M	<2	Dec	Inpatient	Tap water	Fever		+	+
84	M	>60	Jan	Inpatient	Tap water	-		+	+
107	F	2-10	Feb	Inpatient	Tap water	Vomiting, AP		+	+
122	M	2-10	Mar	Inpatient	Tap water	Fever, vomiting, AP		+	+
161	F	20-40	Apr	Outpatient	Tap water	AP	+	+	
171	F	<2	Apr	Onpatient	Tap water	Vomiting, AP		+	+
197	F	<2	Apr	Inpatient	Tap water	Fever, vomiting	+	+	
229	M	20-40	Jun	Inpatient	Tap water	Fever, AP	+	+	

[a]M male; F, female; AP, abdominal pain; +, positive.

the most frequent viral infection, single or co-infection occurred in the autumn.

Among children and adults with TTV infection, 73% were younger than 2 years of age and there are a peak in NV and ADV positive cases in children between 2 and 10 years, suggesting that the immature immune system in children makes them more susceptible to enteric virus infections than adults. That AGE-related co-infection is statistically more common in children < 10 years of age compared to other age groups (OR = 14.9) indicates that young children are at greater risk of enteric virus co-infections. Furthermore, previous co-infection in AGE is associated with a more severe clinical course in children (Valentini *et al*, 2013). As noted above, there were more in-patient children with co-infection than out-patient children. Therefore, viral co-infected children represent a subgroup of AGE patients who need to be paid attention by attending clinicians for appropriate treatment.

Tang *et al* (2013) have indicated that AGE-related NV infections are significantly related to the consumption of spring water. In this study, viral co-infection in AGE patients is significantly associated with the consumption of spring water (OR = 6.6), indicating that spring water is a potential source for transmission of AGE-related NV.

There are at least two important features of this study. Firstly, all clinical departments of the study hospital adopted a uniform case definition by which AGE patients were enrolled and a common questionnaire was used to record the patients' history. Secondly, all three common enteric virus were analyzed in each stool sample, allowing detection of single and co-infections. However, the study has a number of weaknesses. Firstly, the total proportion of viral co-infection was likely underestimated because other pathogens of AGE were not analysed and a small number of AGE fecal samples were investigated. Secondly, some important clinical and virological information, such as dehydration, duration of diarrhea episode (day), length of hospitalization (day) and viral concentration were lacking, which made it difficult to calculate the disease severity of patients in greater detail.

In summary, this study is the first report of NV, ADV and TTV co-infection in children and adults with AGE. Such co-infection was found more among hospitalized than out-patient AGE children. Risk factors for AGE-related co-infection included age, drinking of spring water and fever. As TTV co-infection can promote the pathogenic effects of other infectious agents, it is suggested that investigations of enteric virus co-infection and viral load in patients with gastrointestinal symptoms could be useful to better identify viral agents responsible for AGE, to understand the route of transmission and the interactions among these viruses.

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