FIRST DETECTION OF CHICKEN ANEMIA VIRUS AND NOROVIRUS GENOGROUP II IN STOOL OF CHILDREN WITH ACUTE GASTROENTERITIS IN TAIWAN

Meng-Bin Tang[1,2#], Hung-Ming Chang[3#], Wen-Chih Wu[4], Yu-Ching Chou[4] and Chia-Peng Yu[4,5]

[1]Department of Family Medicine and Department of Hospice Palliative Medicine, Wei-Gong Memorial Hospital, Toufen City, Miaoli County; [2]Department of Public Health and Department of Health Services Administration, China Medical University, Taichung; [3]Department of General Surgery, Wei-Gong Memorial Hospital, Toufen City, Miaoli County; [4]School of Public Health, National Defense Medical Center, Taipei; [5]Department of Bioengineering, Tatung University, Taipei, Taiwan

Abstract. To date, there has been no report of co-infection of chicken anemia virus (CAV) with enteric virus in patients with acute gastroenteritis (AGE). CAV has been recently detected in various types of human samples including stool, indicating pathogenicity in gastrointestinal tract. Examination by PCR-based methods of CAV and norovivus genogroup II (NV GII) in stool of 110 children with AGE at a hospital in Taiwan revealed for the first time of co-infection in two cases. This is the first description of CAV infection in children with AGE in Taiwan. Systematic surveillance and evidence-based studies are required to determine the transmission pathways and spread of CAV in Taiwan.

Keywords*:* acute gastroenteritis, co-infection, chicken anemia virus, norovirus, Taiwan

INTRODUCTION

Viral gastroenteritis is one of the most frequently encountered illnesses in children and adults worldwide (Eckardt and Baungart, 2011). It is estimated that viral gastroenteritis is the cause of 30-40% of gastroenteritis cases in developed countries (Hodges and Gill, 2010). For instance, around 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) is associated frequently with AGE (Hall *et al*, 2012).

Based on antigenic and genetic properties, NV can be classified into seven genogroups (GI-GVII) (Vega *et al*, 2014; Vinjé, 2015), but only GI, II and IV 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 and non-enveloped single-stranded RNA virus of positive sense with a genome of approximate 7.5 kb (Green *et al*, 2000), coding

Correspondence: Chia-Peng Yu, School of Public Health, National Defense Medical Center, No 161, Sec 6, Minquan E. Road, Neihu District, Taipei City 114, Taiwan.
Tel: +886 (2) 87923100 ext 18437
E-mail: yu6641@gmail.com

#Contributed equally to the work.

three open reading frames (ORFs), with ORF1 encoding six nonstructural proteins, ORF2 major capsid protein VP1, and ORF3 minor capsid protein VP2 (Thorne and Goodfellow, 2014). The highly conserved region in the NV genome is located at the ORF1/ORF2 junction and has been used as a preferred target location for detection of NV by RT-PCR method (Stals *et al*, 2012). Although NV is associated with both sporadic and epidemic AGE infection 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).

Gyroviruses (GVs) are non-enveloped icosahedral shaped viruses containing a circular single-stranded DNA genome of around 2.3kb (Chu *et al*, 2012) currently classified in the family Circoviridae (Biagini *et al*, 2011; Zhang *et al*, 2014b). Whilst other genera of the same family (*eg*, Cycloviruses and Circoviruses) have a circular ambisense DNA genome, gyroviruses contain a negative-sense circular DNA genome with a genomic organization that resembles viruses within the family Anelloviridae. It has been proposed that gyoviruses be reassigned to the family Anelloviridae (Biagini *et al*, 2011). Until recently, chicken anemia virus (CAV) was the only known representative of the *Gyrovirus* genus, but during the past five years, nine novel GVs have been reported: avian gyrovirus 2 (AGV2) in chicken (Rijsewijk *et al*, 2011), human gyrovirus (HGV) on human skin (Sauvage *et al*, 2011), GyV3 and phylogenetically distinct GyV4 in chicken meat and human feces (Phan *et al*, 2012), GyV5 and GyV6 in stool of Tunisian children with diarrhea (Smuts, 2014), GyV7 in chicken (Zhang *et al*, 2014), GyV8 in spleen and uropygial gland tissue of a diseased northern fulmar (*Fulmarus glacialis*), a pelagic bird beached in San Francisco, California, USA (Li *et al*, 2015), and GyV9 in feces of a French adult with diarrhea (Phan *et al*, 2015). The genomes of AGV2, HGV and GyV3 harbor genetic organizations similar to that of CAV, despite having a high genetic divergence (49-65%) (Biagini *et al*, 2013).

CAV is an important pathogen in the poultry industry. The virus can be detected in feathers long after the acute phase of the infection, indicating that the virus may also be present in a latent or persistent state (Schat, 2009). CAV infection results in severe anemia and immunosuppression in young susceptible chickens (Snoech *et al*, 2012). Chickens are considered the only natural host of CAV, although anti-CAV antibodies have been detected in Japanese quail but not in other domestic or wild bird species (McNulty *et al*, 1988; Farkas *et al*, 1998). CAV can be transmitted either vertically from hen to offsprings or horizontally through oral-fecal route (van Santen *et al*, 2004). Epidemiology and pathogenesis of CAV in humans remain to be elucidated (Phan *et al*, 2013). CAV genome contains three partially overlapping ORFs, coding for VP1, VP2 and VP3 proteins (Noteborn *et al*, 1991).

To date, there has not been any report of co-infection of CAV and NV GII in children with AGE. As NV GII is not able to be cultured (Thorne and Goodfellow, 2014), coinfection cannot be demonstrated by *in vitro* studies. Therefore, this study was performed using molecular methods to determine CAV and NV GII coinfection prevalence in patients with AGE and to investigate phylogenetic characteristics of gastrointestinal viral strains among AGE patients in Taiwan, which could provide a greater understanding of the epidemiology among the viruses circulating in Taiwan.

Table 1
Primers used in detecting chicken anemia virus (CAV) and norovirus (NV) genogroup (G)II.

Primer	Sequence	Amplicon size (bp)
CAV		
CAV-F	5′-GGAGACAGCGGTATCGTAG-3′	248
CAV-R	5′-GTTCATTGACGCTAGGAGGAA-3′	
NV GII		
COG2F	5′-CARGARBCNATGTTYAGRTGGATGAG-3′	98
COG2R	5′-TCGACGCCATCTTCATTCACA-3′	

B= C, G or T; N = A, C, G or T; R= A or G; Y= C or T.

MATERIALS AND METHODS

Case definition and specimen collection

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, and vomiting. The study was conducted from August 2012 to July 2013 at Wei-Gong Memorial Hospital, Miaoli County, Taiwan. Patients were given a follow-up questionnaire in the week after enrolment to obtain epidemiological data, clinical symptoms and to ascertain that AGE had occurred. Stools of 110 AGE patients were collected and stored at -20°C while waiting to be transferred on ice to the Department of Bioengineering, Tatung University, Taipei City, where they then were stored as 10% suspension in a balanced salt solution (Medicaco, Uppsala, Sweden) at -70°C until used.

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

PCR detection of CAV

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 nucleic acid was stored in 50 µl of RNase-free H_20 at -20°C.

CAV DNA was detected by a PCR-based method using primers derived from CAV VP1 region (Table 1) (Chu *et al*, 2012). 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 mixture containing 10.5 µl of RNase-free H_20, 5 µl of template nucleic acid (DNA), 2.5 µl of 10 µM (stock) CAV-F and CAV-R primers, 1 µl of 10 mM (stock) each dNTP, 5 U *Taq* DNA polymerase (IT'S Science Corporation, 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 were performed in a Thermo Electron Corp thermal cycler (Waltham, MA) as follows: 95°C for 5 minutes; followed by 40 cycles of 95°C for 30 seconds, 52°C for 45 seconds and 72°C for 1 minute; with a final step at 72°C for 10 minutes. Clinical stool samples positive for CAV were provided by the Wei-Gong Memorial Hospital as positive

Table 2
Demographic and clinical features of AGE children, Wei-Gong Memorial Hospital, Miaoli County, Taiwan.

Parameter	AGE children (N = 110)		p-value[a]	CAV and NV GII co-infection (%) (n = 2)
	CAV-positive (%) (n = 2)	CAV-negative (%) (n = 108)		
Detection rate (%)	2 (1.8)			
Gender				
Male	0 (0)	58 (53.7)	0.221	0 (0)
Female	2 (100)	50 (46.3)	0.221	2 (100)
Setting				
Out-patient	0 (0)	11 (10.2)	1.000	0 (0)
Emergency	1 (50)	61 (56.5)	1.000	1 (50)
In-patient	1 (50)	36 (33.3)	1.000	1 (50)
Age (years)				
<2	2 (100)	35 (32.4)	0.111	2 (100)
2-10	0 (0)	53 (49.1)	0.496	0 (0)
10-18	0 (0)	20 (18.5)	1.000	0 (0)
Season				
Spring	0 (0)	22 (20.4)	1.000	0 (0)
Summer	1 (50)	17 (15.7)	0.302	1 (50)
Fall	0 (0)	19 (17.6)	1.000	0 (0)
Winter	1 (50)	50 (46.3)	1.000	1 (50)
Fever > 38°C				
Yes	0 (0)	54 (50)	0.496	0 (0)
No	2 (100)	54 (50)	0.496	2 (100)
Vomiting				
Yes	1 (50)	74 (68.5)	0.537	1 (50)
No	1 (50)	34 (31.5)	0.537	1 (50)
Stool type				
Watery	2 (100)	84 (77.8)	1.000	2 (100)
Bloody	0 (0)	2 (1.9)	1.000	0 (0)
Non-watery, non-bloody	0 (0)	22 (20.3)	1.000	0 (0)
Dehydration				
Moderate	0 (0)	1 (1)	1.000	0 (0)
Mild	1 (50)	31 (28.7)	0.499	1 (50)
No	1 (50)	76 (70.3)	0.512	1 (50)
Abdominal pain				
Yes	2 (100)	102 (94.4)	1.000	2 (100)
No	0 (0)	6 (5.6)	1.000	0 (0)

[a]Two-tailed chi-square, Fisher's exact test comparing CAV-positive with -negative cases. AGE, acute gastroenteritis; CAV, chicken anemia virus.

control, and nuclease-free water (Qiagen, Taipei City, Taiwan) was used as negative control. Both positive and negative controls were included in each series of PCR assay. Amplicons (248 bp) were separated by 2% agarose gel-electrophoresis and visualized by staining with ethidium bromide. DNA 100 bp size markers (Geneaid, New Taipei City, Taiwan) were used as gel calibration markers.

Table 3
Prevalence of sporadic CAV infection in children in different countries.

Country	Year	Stool sample		Prevalence (%)	PCR assay of CAV		Reference
		Source	Number		Genome region	Primer name	
Taiwan	2014	AGE	110	2	VP1	CAV-F, CAV-R	Present study
South Africa	2014	AGE	149	16	VP2	F1n, R1n	Smuts, 2014
		Non-AGE	49	51			
Chile	2012	AGE	100	32	VP2	ConGyF, ConGyR	Phan *et al*, 2012
China	2012	AGE	435	3.7	VP1	CAV-F, CAV-R	Chu *et al*, 2012

AGE, acute gastroenteritis; CAV, chicken anemia virus.

RT-quantitative (q)PCR detection of NV GII

RT-qPCR was performed using a Step One™ real-time PCR system (Applied Biosystems, Foster City, CA) employing SYBR green dye (PCR Biosystems, London, UK) and NV GII primers COG2F and COG2R (Table 1) (Tajiri-Utagawa *et al*, 2009). NV GII-positive stool sample provided by Wei-Gong Memorial Hospital was used as positive control and sterile deionized water as negative control. A standard curve was created using 10-fold dilutions of NV GII RNA stock solution (Wei-Gong Memorial Hospital), the concentration of which was determined spectrophotometrically (UV-1700 spectrophotometer, Shimadzu, Japan) at $A_{260\,nm}$ versus Ct values ($R^2 > 0.99$; slope of curve: -3.1 to -3.6; PCR efficiency: 90-110%). Samples with Ct values of ≤ 36 are considered to be NV GII-positive.

Statistical analysis

Samples were classified as positive or negative for viral infection. For categorical variables, chi-square test was used to examine differences in proportion between groups. A $p < 0.05$ is considered statistically significant. Fisher's exact test was used when the expected value for a cell was < 5.

RESULTS

CAV prevalence among AGE patients is not statistically significant (Table 2). CAV was detected in 2/110 (2%) of the stool samples, both samples being co-infected with NV GII (Table 2). Clinical symptoms of the two female co-infected AGE patients included vomiting, watery stool, dehydration and abdominal pain. These symptoms were not different from those of NV GII-positive AGE patients.

DISCUSSION

To the best of our knowledge, this is first description of CAV and NV GII co-infection in stools of AGE children. CAV in stool of children with AGE have previously been reported from Chile, China and South Africa (including children without AGE) (Table 3). The presence of CAV in stools is indicative of a fecal-oral route of transmission. The prevalence of CAV infection in our survey was lower than those observed in stool samples of patients with AGE from the other countries including Chile and South Africa (Phan *et al*, 2012; Smuts, 2014). The two children with co-infection of CAV and NV did not

present any clinical symptoms different from those of other NV-associated AGE, but it must be borne in mind the very limited number of such patients. This study reports the first detection of CAV together NV in children with AGE in Taiwan, a finding that differs from other studies in the literature.

It is worth noting that co-infections of NV GII and enteric viruses, *viz*, sapovirus and rotavirus have been reported (Dai *et al*, 2011; Li *et al*, 2012), suggesting that NV GII is a frequent strain associated with other types of enteric viruses. During co-infection, pathogenic potential of each virus type could to be enhanced leading to a possible worsening of the patients' clinical manifestation (Liu *et al*, 2006; Bhavnani *et al*, 2012). Thus clinicians should consider the possible presence of viral co-infection in the etiology of AGE and monitor a broader range of enteric viruses.

The CAV strains in Taiwan are closely related to the epidemic strain in China, Japan, Korea and Thailand. Thus it is crucial to emphasize the global spread of CAV implicated in sporadic gastroenteritis.

There are at least three strong points in this study. First, all clinical departments of the study hospital adopted a uniform case definition to enrol AGE patients and to collect stool samples, and the same formatted questionnaire was used to record patients' demographic and clinical data. Second, two types of enteric virus were analyzed by molecular methods, allowing detection of co-infection. Third, phylogenetic analysis was conducted in the virus samples. On the other hand, the study was qualitative and thus mossed the opportunity to measure vial load, which could have allowed information regarding correlation of epidemiological with clinical data.

In summary, this study is the first to identify co-infection of CAV and NV in stool of children with AGE. Furthermore, our results indicate that the CAV strains were similar to the epidemic strains reported in other parts of the world. Systematic surveillance and evidence-based studies are required to determine the transmission pathways and spread of CAV infection among the human population of Taiwan.

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