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Characterization of a novel gyrovirus in human stool and chicken meat

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Abstract

Background—Sequence-independent amplification of clinical specimens can lead to the identification of novel pathogens.

Objectives—To identify novel viruses in human stool specimens from patients with diarrhea and to investigate the ecology and clinical significance of such viruses.

Study Design—Nucleic acid extracted from stool specimens from patients with diarrhea with no known aetiology were subjected to random PCR amplification and Roche/454 pyrosequencing. Novel viruses identified were genetically and epidemiologically characterized.

Results—Four gyroviruses, chicken anemia virus (CAV), human gyrovirus (HGV) / avian gyrovirus 2 (AGV2), gyrovirus 3 (GyV3) and a novel gyrovirus (tentatively designated as gyrovirus 4 (GyV4)) were identified in human stool specimens. GyV4, as well as CAV and AGV2/HGV were also detected in chicken skin and meat used for human consumption.

Conclusions—A novel gyrovirus (GyV4) was identified in human stool and in chicken meat sold for human consumption. This virus was phylogenetically distinct from previously reported gyroviruses in chicken and humans (chicken anemia virus, human gyrovirus, avian gyrovirus 2

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and recently reported gyrovirus 3). The epidemiology and pathogenesis of this virus in humans and in chicken needs to be further investigated.

Keywords

diarrhea; virus; gyrovirus; virus discovery; human; chicken; chicken anaemia virus

Background

Gyroviruses are non-enveloped DNA viruses with an icosahedral symmetry and circular single stranded DNA of around 2.3kb. They are currently classified in the family *Circoviridae*.⁽¹⁾ While other genera of the same family (i.e. Circoviruses and Cycloviruses) have a circular ambisense DNA genome, gyroviruses have a negative sense circular DNA genome with a genomic organization that resembles viruses within the family of *Anelloviridae*. It has been recently proposed that gyroviruses are reassigned to the family of *Anelloviridae*.⁽²⁾ Until recently, chicken anemia virus (CAV) was the only known representative of the gyrovirus genus. In the last two years, three novel gyroviruses have been reported; avian gyrovirus 2 (AGV2) in chickens⁽³⁾, a human gyrovirus (HGV) detected on human skin⁽⁴⁾ and gyrovirus 3 (GyV3) in human faeces and chicken meat.⁽⁵⁾

CAV is an important pathogen in the poultry industry.⁽⁶⁾ 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.⁽⁶⁾ AGV2 has only around 40% nucleotide similarity with CAV.^(3,7) The virus was detected in the feathers and brain of diseased as well as healthy chicken. It has been suggested that AGV2 detected in sick birds may be phylogenetically distinct from those found in healthy birds and that there may be pathogenic and apathogenic variants.⁽⁷⁾ A similar virus designated human gyrovirus (HGV) was identified by analysis of a skin swab from a healthy human. HGV was further detected in non-lesional human skin swabs from 5 of 115 other humans, but not in 92 bronchoalveolar lavages or nasopharyngeal aspirates or in 92 faecal samples.⁽⁴⁾ A new gyrovirus (provisionally designated gyrovirus 3, GyV3) was recently reported from human faeces.⁽⁵⁾ The epidemiology and pathogenesis of these novel gyroviruses in humans and in poultry remains to be elucidated.

Objectives

Our objective was to identify novel viruses in stool of humans with diarrhoea using sequence-independent amplification of partially purified stool samples and to investigate the ecology and clinical significance of identified viruses.

Study Design

Clinical specimens

Anonymised retrospective faecal specimens submitted for virological investigation of diarrhoea to the virology laboratory at the Queen Mary Hospital (QMH), Hong Kong in 2004 and 2005 (n=435) and in 2011 (n=191) were used for preliminary testing. The 435 specimens collected in 2004/5 had been previously screened for multiple viral agents causing diarrhea including norovirus, rotavirus, adenovirus, sapovirus and astrovirus while specimens collected in 2011 were only tested for rotaviruses.

Stool specimens and serum were collected from children undergoing renal, liver, lung, heart or haematopoietic stem cells transplantation between October 2008 and April 2010 at St. Louis Children's Hospital. Informed consent was obtained from parent or guardian.

Skin swabs were collected into virus transport medium from healthy volunteers.

Chicken meat and eggs

Three fresh chicken wings sold for human consumption were purchased at each of two local markets in Hong Kong. Eight frozen chicken-wings originating in Brazil were purchased from a third market. The skin and muscle from each chicken-wing was removed using fresh sets of sterile scissors and forceps for each specimen. The skin and muscle was homogenized separately in virus transport medium.

Ninety unfertilized eggs sold for human consumption at local markets, originating from mainland China, Thailand and Netherlands, were purchased. The yolk and egg white was mixed and an aliquot was stored for extraction of virus DNA.

Nucleic acid extraction

Total nucleic acid was extracted from 140 µl of sample in viral transport medium by a QIAamp viral RNA mini kit (Qiagen) following the protocol provided by the manufacturer. Purified DNA was eluted in 60 µl of elution buffer provided in the extraction kit.

Roche 454 pyrosequencing

Extracted nucleic acids from stool specimens were subjected to random PCR amplification as described.⁽⁸⁾ Each sample was barcoded with a unique sequence tag and then samples were pooled. Libraries for Roche/454 pyrosequencing were prepared using standard protocols. Sequence reads were analyzed and those with similarity to known viruses identified using a customized bioinformatic pipeline as described.⁽⁹⁾

PCR for chicken anaemia virus (CAV), avian gyrovirus 2 (AGV2)/human gyrovirus (HGV), gyrovirus 3 (GyV3) and the novel gyrovirus 4 (GyV4) detected in this study

Primers targeted at the VP1 region of CAV, AGV2/HGV and GyV3 were designed to detect and differentiate these three groups of viruses in clinical specimens. The primers for CAV were forward (5'-3') GGAGACAGCGGTATCGTAG and reverse (5'-3') GTTCATTGACGCTAGGAGGAA; for AGV2/HGV forward (5'-3') CGCGTAGAAGATCCTTTGATC and reverse (5'-3') CTGCAAGTGCTGAGGATAAGG; and for GyV3 forward (5'-3') GACACAGACTGCGACGAAGA and reverse (5'-3') ATGCTCCTGGCTGTCTAGAT. The annealing temperature for these three primers sets was 52°C and the size of the amplicons for CAV, AGV2/HGV and GyV3 was 248bp, 293bp and 412bp respectively. A specific PCR for the detection of the novel gyrovirus 4 (GyV4) discovered in this study (see results, below) was designed targeting the VP1 region; the primers being forward (5'-3') GTGGTATCGAAGTGGAAGTACC and reverse (5'-3') CCCCCTGATACATACTGTACATA. The annealing temperature was 48°C and the expected size of the amplicon was 285 bp.

A commercial competitive ELISA assay for CAV (Flockchek, Idexx Laboratories, Maine, USA) was performed according to the manufacturer's protocol.

Results

Viral genetic detection and characterization by high-throughput deep-sequencing methods

Roche 454 high throughput sequencing was applied to nucleic acid extracted individually from 96 faecal samples from patients with diarrhoea from whom no other viral pathogen had been identified. In one sample, a single sequence read of 330 nucleotides was identified that shared only 40% similarity to the nucleocapsid gene (VP1) of CAV by BlastX suggesting

that a novel virus was present. Other sequence fragments with high similarity (>93%) to AGV2 and to CAV were also identified.

To sequence the complete genome of the putative novel gyrovirus, nucleic acid extracted from the original sample was subjected to rolling circle DNA amplification using Phi29 DNA polymerase (New England BioLabs, Massachusetts, USA). Inverse PCR was performed on the rolling circle amplified viral DNA using Platinum Pfx DNA polymerase (Invitrogen, California, USA). The complete genome of 2034 nucleotides was deduced from the original fragment and one inverse PCR product. Two overlapping open reading frames with sizes of 1059 and 654 nucleotides in the same orientation (Figure 1) (GenBank accession number XXXXX) were predicted to encode the viral capsid protein and the viral protein phosphatase. The putative capsid protein (VP1) had nucleotide sequence similarities of 33% to that of CAV and 31% to AGV2, HGV and GyV3 (Table 1). The putative protein phosphatase (VP2) similarly displayed nucleotide sequence similarities of only 26% to CAV and 28% to AGV2, HGV and GyV3. A third ORF present in CAV, AGV2/HGV and GyV3, which encodes for the apoptin gene, was not found.

Taken together, these findings suggested that this is a novel member in the genus of gyrovirus, which we tentatively designate as gyrovirus 4 (GyV4), pending formal taxonomic decisions by the ICTV. Remarkably, a region of 121 nucleotides in the untranslated region of the novel gyrovirus genome has a nucleic acid sequence similarity of 95% to that of the untranslated region of AGV2 and HGV but in the reverse direction (Figure 1).

Epidemiology of GyV4 in humans

Two panels of human faecal specimens from Hong Kong and one from USA were screened for the presence of CAV, AGV2/HGV and GyV4. One set of specimens from Hong Kong was additionally tested for GyV3 (a virus reported after this manuscript was initially submitted for publication) (Table 2). The positive rates for detecting CAV, AGV2/HGV, GyV3 and GyV4 in 191 faecal specimens from patients with diarrhoea collected in 2011 were 13.1%, 16.8%, 5.8% and 3.7% respectively (Table 2). The age-stratified positive rates for CAV, AGV2/HGV, GyV3 and GyV4 are shown in Table 3. Two patients had faecal specimens repeatedly positive for CAV one day apart, and the PCR amplicons in each pair were genetically identical. Specimens positive for one gyrovirus were more likely to be positive for other gyroviruses (Figure 2).

None of 33 skin swabs collected from healthy individuals was positive for CAV, AGV2/HGV or GyV4.

Data on immunocompromised patients

We tested 122 stool samples collected from transplant recipients in St. Louis, USA and 23 (18.9%), 18 (14.8%) and 17 (13.9%) were positive for CAV, AGV2/HGV and GyV4 respectively. The rate of co-detection was very high with 12 specimens being positive for all three viruses. Of the 28 specimens that were positive for one or more gyroviruses, seventeen were asymptomatic. Those with symptoms at the time of specimen collection had diarrhoea (n=1); upper respiratory symptoms (n=5); upper respiratory symptoms and diarrhoea (n=2); lower respiratory symptoms (n=2) and fever with rash (n=1) (Table 4). Three of these patients who had multiple specimens collected at different times had stool samples positive for GyV4 on 4, 2 and 2 sampling occasions. Paired specimens from these three patients were PCR amplified and sequenced over 220 nucleotides in VP1 gene and 500 nucleotides in the VP2 gene. One patient had genetically identical viruses from two samples 3 months apart. Of 9 other individual specimens from different patients, three had identical sequences with each other.

To determine whether an antibody response is generated to CAV, we analyzed 34 serum samples collected at different time points from 5 individuals who had stool samples that were CAV positive by PCR. The serum samples were collected between 1 month prior to the episode that was CAV PCR positive to 11 months after the episode. A commercial, competitive binding CAV serological ELISA assay was used. None of the samples were found to contain antibodies against CAV. As no serological assays are currently available for AGV2 or GyV4, we could not assess the immune response to these viruses.

Prevalence of gyrovirus DNA in poultry products

We tested for the presence of DNA of CAV, AGV2/HGV and GyV4 in 14 skin samples and 14 muscle samples removed from chicken-wings sold for human consumption at food markets in Hong Kong. CAV and AGV2 were detected in all 14 and GyV4 in 10 of the homogenized chicken skin samples. CAV and AGV2 were detected in 12 of the homogenized meat samples while GyV4 was detected in 4 of the samples. No gyrovirus DNA was detected from the 90 eggs tested.

Phylogeny

Viral sequences of the PCR amplicons of the VP1 gene were analyzed. They grouped into 4 distinct virus groups, each with high bootstrap support; CAV-like viruses, AGV2 or HGV-like viruses, GyV3 and GyV4-like viruses (Figure 3), with >91% nucleotide identity within each group and < 60% nucleotide similarity between the four groups of viruses. Thus GyV4 viruses are a clearly distinct and novel group of viruses sharing <45% nucleotide identity with CAV-like or AGV2/HGV or GyV3 viruses.

It is notable that AGV2 and HGV viruses are closely related to each other both in phylogeny (Figure 2) and genome organization (Figure 1) and are likely variants of the same virus species. Within each group of gyroviruses, viruses of human and animal origin are closely related with each other and do not form separate sub-clades (Figure 3).

Discussion

We have identified and characterized GyV4 as a novel gyrovirus in human stool and chicken. It has <38% nucleotide sequence similarity to the previously known gyroviruses CAV, AGV2, HGV and GyV3. Demarcation of species in the sub-family Gyroviridae requires nucleotide divergence of >35%⁽²⁾ and thus GyV4 qualifies to be designated as a novel gyrovirus species. GyV4 also appears ubiquitous in chicken sold for human consumption, more commonly found in chicken skin than in chicken meat. GyV4 does not possess a homologue of the apoptin gene which may be important for pathogenesis of CAV, AGV2/HGV and GyV3. Apoptin in CAV encodes a protein that induces apoptosis of T-lymphocytes in chicken.⁽⁶⁾ Furthermore, it is important for future studies to investigate whether GyV4 in chicken meat and skin is in a latent form or is in a persistently replicating state, and to determine whether it causes disease in either poultry or humans.

GyV4 can be detected at variable prevalence in human stool specimens at all age groups (Tables 2 and 3). The lower prevalence of gyrovirus DNA in human specimens collected in 2004–2005 may be related to the repeated freeze-thaws these specimens have undergone over many years as part of testing for a wide range of diarrhoeal viruses which may have led to degradation of viral DNA. One or more gyroviruses were also detected in 28 of 122 specimens collected from US patients undergoing liver, kidney, lung, heart or haematopoietic transplants (table 4). Eleven had respiratory, gastro-intestinal or febrile rash symptoms at the time of specimen collection while seventeen were asymptomatic. Diarrhoea may not be the only disease associated with viruses found in the faeces and associations with systemic

disease may also need to be considered. Adequately powered case-control studies are needed to establish any associations with disease in humans.

The observation that all three viruses were detected in chicken skin and meat sold for human consumption suggests that humans may be often ingesting gyroviruses. It is important to establish whether GyV are replicating in the human gastrointestinal tract or whether viral DNA from ingested foods is being passively excreted in the faeces.⁽¹⁰⁾ Although we failed to detect antibodies against CAV in patients who were positive for CAV DNA, mucosal infections may not necessarily elicit a systemic antibody response, especially so in immunocompromised patients. HGV virus DNA has been reported in the plasma of some immunocompromised patients.⁽¹¹⁾ CAV detected in two pairs of faecal samples from two patients on consecutive days. In each case, the virus in the second sample was genetically identical to the first. One immunocompromised patient had a genetically identical virus detected three months apart. While there is some genetic heterogeneity within GyV, it is not high enough to conclude that detection of genetically identical viruses from the same patient indicates persistent replication rather than re-infection / re-ingestion from different sources. Of specimens from 9 individuals from this immunocompromised cohort genetically sequenced across these two regions, three have identical sequences with each other.

The high rate of codetection of the three gyroviruses in human specimens may indicate common dietary exposure to foods that contain all three viruses (as we have shown is the case with chicken skin and meat) or may imply some undefined dependency or co-dependency for successful proliferation. Although previous studies implied that HGV is a human gyrovirus distinct from avian gyroviruses, our findings suggests that HGV and AGV2 are essentially the same virus with nucleotide homology >92%. We find no phylogenetic distinction between viruses of human (including human skin) or chicken origin suggesting that HGV and AGV2 are indistinguishable from each other.

In summary, we detected a novel gyrovirus (GyV4) in chicken skin and in human stool. The pathogenic role of this virus in humans and chicken requires further investigation. We demonstrate that CAV, AGV2/HGV and GyV3 are also commonly found in human stool specimens, often codetected with GyV4. Future studies should investigate whether these gyroviruses are replicating in humans. Additional studies on the epidemiology and pathogenesis of gyroviruses in humans and of the novel GyV4 in chickens are warranted.

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Ethical approval: The study was approved by the Institutional Review Board of The University of Hong Kong and Hospital Authority Hong Kong West Cluster, Hong Kong and the Human Research Protection Office of Washington University in St. Louis.

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Abbreviations

(HGV)	human gyrovirus
(CAV)	chicken anaemia virus
(AGV2)	Avian gyrovirus 2
(GV3)	gyrovirus 3

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Figure 1. Schematic diagram of the gyrovirus genomes. Chicken anemia virus (CAV); avian gyrovirus 2 (AGV2); human gyrovirus (HGV); gyrovirus 3 (GyV3); gyrovirus 4 (GyV4).

Figure 2.

Venn diagrams showing the co-detection of gyroviruses in stool samples in a) Diarrhoeal specimens from Hong Kong 2004–5; b) Diarrhoeal specimens from Hong Kong 2011, and c) symptomatic and asymptomatic transplant recipients in the United States of America. Chicken anemia virus (CAV); avian gyrovirus 2 (AGV2); human gyrovirus (HGV); gyrovirus 3 (GyV3); gyrovirus 4 (GyV4).

Figure 3.

Phylogenetic analysis of the VP1 gene (~300bp) of GV3, CAV and AGV2/HGV obtained from human stool and chicken samples in this study. Reference sequence of CAV (HQ872034), AGV2 (NC_015396), HGV (FR823283) and GyV3 (JQ_308210) were retrieved from GenBank. The origins of the chicken specimens tested in this study are denoted as follows, ChWA and ChWC were chickens imported from Mainland China; and ChWB were chickens imported from Brazil. Abbreviations: Chicken anemia virus (CAV); avian gyrovirus 2 (AGV2); human gyrovirus (HGV); gyrovirus 3 (GyV3); gyrovirus 4 (GyV4).

Table 1

Nucleotide homology of VP1 or VP2 sequences of chicken anemia virus (CAV); avian gyrovirus 2 (AGV2); human gyrovirus (HGV); gyrovirus 3 (GyV3); gyrovirus 4 (GyV4).

	AGV2	HGV	CAV	GyV3
VP1				
GyV4	0.314	0.312	0.332	0.307
AGV2		0.933	0.488	0.606
HGV			0.482	0.600
CAV				0.479
VP2				
GyV4	0.280	0.278	0.256	0.280
AGV2		0.971	0.539	0.710
HGV			0.549	0.716
CAV				0.514

Table 2

Detection of gyrovirus DNA by PCRs in human stool specimens.

Cohort	No. of samples	CAV [#] pos (%)	AGV2/HGV pos (%)	GyV3 pos (%)	GyV4 pos (%)	Overall positive samples* (%)
Diarrhoeal specimens Hong Kong (Yr 2004–2005)	435	16 (3.7%)	15 (3.4%)	-	4 (1.0%)	26 (6.0%)
Diarrhoeal specimens Hong Kong (Yr 2011)	191	25 (13.1%)	32 (16.8%)	11 (5.8%)	7 (3.7%)	37 (19.4%)
Immunocompromised patients USA (2008–2010)	122	23 (18.9%)	18 (14.8%)	-	17 (13.9%)	28 (23.0%)

[#]CAV - Chicken anemia virus; AGV2 - avian gyrovirus 2; HGV - human gyrovirus; GyV3 - gyrovirus 3; GyV4 - gyrovirus 4.

* Samples might be positive for more than 1 gyrovirus

Table 3

Detection of chicken anemia virus (CAV); avian gyrovirus 2 (AGV2); human gyrovirus (HGV); gyrovirus 3 (GyV3) and gyrovirus 4 (GyV4) DNA in faecal specimens from patients with diarrhoea, Hong Kong 2011.

Age group	No. of samples	CAV pos	AGV2/HGV pos	GyV3 pos	GyV4 pos	Overall positive samples*	Overall positive rate (%)
0-5	91	8	11	5	2	13	14%
6-10	37	1	1	2	1	2	5%
11-20	29	7	7	2	1	8	28%
21-30	3	0	1	0	1	1	33%
31-40	6	1	1	0	0	1	17%
41-50	5	1	0	0	0	1	20%
51-60	7	4	6	0	1	6	86%
61-70	4	1	2	1	1	2	50%
71-80	3	0	0	0	0	0	0%
81-90	5	2	3	1	0	3	60%
91-100	1	0	0	0	0	0	0%
Total:	191	25	32	11	7	37	19%

* Samples might be positive more than 1 gyrovirus

Table 4
Detection of gyroviruses chicken anemia virus (CAV); avian gyrovirus 2 (AGV2)/ human gyrovirus (HGV) and gyrovirus 4 (GyV4).

Symptoms	CAV	AGV2	GyV4	CAV+AGV2	CAV+GyV4	CAV+AGV2+GyV4	Total
Diarrhoea/gastro-intestinal	0	0	0	0	0	1	1
Upper Respiratory	1	0	2	0	1	1	5
Lower Respiratory	0	1	0	0	0	1	2
Upper respiratory and gastro-intestinal	1	0	0	1	0	0	2
Fever and rash	0	0	0	0	0	1	1
Asymptomatic	2	0	2	4	1	8	17
Total	4	1	4	5	2	12	28