Title: Molecular detection and characterization of human gyroviruses identified in the ferret fecal virome

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Abstract

The recently described novel gyroviruses may infect chickens and/or humans, however, their pathogenic potential is unknown. In our metagenomic investigation we detected many of the novel gyroviruses in the fecal viromes of ferrets with lymph node and organ enlargement. The whole genomic sequences of selected gyrovirus strains showed 90.7-99.4% similarity with homologous reference gyrovirus strains. This study does not elucidate an association between gyrovirus shedding of ferrets and the observed background disease; however, it provides evidence for genetic diversity within gyroviruses and raises the possibility that pet ferrets may transmit gyroviruses to heterologous hosts, e.g. humans.

Several new members of the genus *Gyrovirus* (family *Circoviridae*) have been described during the past three years. Chicken anaemia virus (CAV) causes anaemia, bone marrow atrophy and severe immunosuppression in poultry, and represents the prototype species within the genus. Newly described members include human gyrovirus (HGyV), avian gyrovirus 2 (AGV2), GyV3, GyV4, GyV5 and GyV6 [1-5]. Of interest, some of these new gyrovirus types have been identified in human skin (HGyV), blood (HGyV) and stool (all gyroviruses) specimens, although the etiologic role of these viruses in a human disease has not been revealed [1-7]. Furthermore, in addition to CAV, AGV2, HGyV and GyV4 have been also detected in chicken serum, meat and skin [1-3].

In this study, 23 diarrheic stool specimens were collected from 20 pet ferrets *(Mustela putorius furo)* housed in a shelter. The animals had a background disease characterized by lymph node and spleen enlargement. The majority of samples (21 samples of 18 ferrets) were subjected to viral metagenomics. In brief, the genomic RNA was extracted using the Viral RNA Mini kit (QIAGEN) according to the manufacturer's instructions. The RNA sample was denatured at 97°C for 5 min in the presence of 10 µM random hexamer tailed by a common PCR primer sequence [8]. Reverse transcription reaction mixture containing 400 µM dNTPs, 1X AMV RT buffer and 1 U AMV reverse transcriptase (Promega) was added and then incubated at 42°C for 45 min. 5 µl cDNA was amplified in a final reaction volume of 50 µl including 500 µM PCR primers, 200 µM dNTP mixture, 1.5 mM MgCl2, 1X Taq DNA polymerase buffer and 0.5 U Taq DNA polymerase (Thermo Scientific). The reaction conditions consisted of a denaturation step at 95°C for 3 min, 40 cycles of amplification (95°C for 30 sec, 48°C for 30 sec, 72°C for 2 min) and a final extension step at 72°C for 8 min.

Enzymatic fragmentation was carried out from 100 ng of the amplified cDNA using the reagents of the NEBNext® Fast DNA Fragmentation & Library Prep Set for Ion Torrent™ kit (New England Biolabs) according to the manufacturer's instruction. The adaptor ligation was performed with reagents from the same kit, whereas barcoded adaptors were retrieved from the Ion XpressTM Barcode Adapters (Life Technologies). The barcoded library DNA samples were column extracted using the Gel/PCR DNA Fragments Extraction kit kit (Geneaid) and then run on 2% precast gel (Life Technologies). Products between 300 and 350 bp were directly used without further purification in the PCR mixture of the NEBNextR Fast DNA Fragmentation & Library Prep Set for Ion Torrent^{TM} kit (NEB). Library DNA was subsequently amplified (initial denaturation at 98° C for 30 sec, followed by 12 amplification cycles of 98°C for 10 sec, 58°C for 30 sec and 72°C for 30 sec, and terminated at 72°C for 5 min). The amplified library DNA was purified by the Gel/PCR DNA Fragments Extraction kit

(Geneaid) and was quantified fluorimetrically on a Qubit® 2.0 equipment using the Qubit® dsDNA BR Assay kit (Life Technologies). Approximately equimolar aliquots of the individually barcoded products were mixed in a single tube and this library mixture was used in subsequent emulsion PCR according to the manufacturer's protocol using the Ion PGM Template kit on an OneTouch v2 instrument. Enrichment of the templated beads (on an Ion One Touch ES machine) and further steps of pre-sequencing set-up were performed according to the 200 bp sequencing protocol of the manufacturer (Life Technologies). The sequencing protocol recommended for Ion PGM™ Sequencing Kit on a 316 chip was strictly followed. Raw sequence data were mapped onto reference sequences of the GenBank in the CLC Bio software (http://www.clcbio.com/). Sequence alignments and distance matrixes using the *p*-distance algorithm were prepared in MEGA 6 using the MUSCLE program [9].

Viral metagenomics generated an average 67,737 sequence reads (range, 36,337-87,870) from each barcoded cDNA library. The metagenomic assemblage identified coronaviruses and a great diversity of gyroviruses (including HGyV, AGV2, GyV3, GyV4 and CAV; Table 1) in 50% (9/18) and 44% (8/18) of the animals, respectively. Although detection of chicken origin gyroviruses (such as CAV and AGV2) was not surprising in the fecal specimens, the identification of putative human gyroviruses in the ferret fecal virome was somewhat unexpected; therefore, we systematically screened by PCR all available ferret stool samples for different gyroviruses, including those that were previously found exclusively in humans, in chicken or both.

The PCR primers targeted the VP1 gene of HGyV/AGV2, GyV3, GyV4 and CAV, and the VP2 genomic region of HGyV/AGV2 and GyV3 [3-4]. When the results of targeted PCR and metagenomic analysis were merged, the stool samples of 14 animals were found to contain traces of the gyrovirus genomic DNA. The main type was HGyV (identified in 13 of 14 animals). Eleven animals shed a mixture of gyroviruses; CAV was a commonly seen strain in specimens containing multiple gyroviruses (11/11), whereas GyV3, GyV4 and AGV2 were amplified by PCR from two, one and one ferrets, respectively. Of interest, genomic DNA of another GyV3 and three GyV4 was identified by viral metagenomics in specimens negative by gene-specific PCR. Sequence variation in the primer binding region provided a possible explanation for PCR failures in some instances and suggested limited sensitivity of published GyV3 and GyV4 specific PCR primers [3-4].

Subsequently, PCR primers were designed for the amplification of representative full-length gyrovirus genomes. PCR assays with back-to-back primers were performed in a reaction volume of 20 µl containing 250 µM of each dNTPs, 250 nM of each primers, 1X Phusion Green HF Buffer and 0.3 U Phusion DNA polymerase (Thermo Scientific). Amplification conditions was as follows: denaturation at 98 ºC for 30 s denaturation, 40 cycles of 98 ºC for 10s, 53 or 59 ºC for 30s (for GyV4 or HGyV/AGV2, respectively) and 72 ºC for 90 s, and a

final extension at 72 ºC for 10 min. Sequencing of the amplified genomes was performed with the 'primer-walking' approach and Sanger sequencing by ABI PRISM® 3100-Avant Genetic Analyzer using BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies). Primers used for amplification and sequencing are presented in Table 2. The full-length genomes of five ferret origin gyrovirus strains (three HGyV, one AGV2, one GyV4) were determined and three representative genome sequences were deposited in the GenBank (accession numbers: KJ452213 for AGV2 G17, KJ452214 for HGyV G13 and KJ452215 for GyV4 G14). Short fragments (~15-25 nucleotides in length) in the non-coding region could be sequenced only by a modified sequencing protocol due to the high GC content of the predicted hairpin structure just downstream the VP1, although, back-to-back and other primers designed to amplify smaller products (559 bp for HGyV and AGV2 and 599 bp for Gyv4) spanning the putative hairpin structure worked well in our hands. Sequencing issues concerning these non-coding fragments even after cloning was reported by other research groups [5]. In the present study PCR assays and sequencing reactions in the presence of different concentrations of DMSO, betaine and Q-solution (Qiagen) were tested alike, but early termination of the sequencing reaction was always noticed (Fig. 1, Panel A). A modified PCR using 7-deaza-dGTP (New England Biolabs) in a reaction volume of 20 µl contained 250 nM of the primers specific to the GC-rich region (Table 1), 25 µM of dATP, dCTP, dTTP and 7-deaza-dGTP (New England Biolabs), 1X DreamTaq Green Buffer and 0.5 U DreamTaq DNA Polymerase (Thermo Scientific). The cycling profile was as follows: denaturation at 95 ºC for 3 min, 40 cycles of 95 ºC for 30 s, 54 ºC for 30 s and 72 ºC for 1 min, and a final extension step at 72 ºC for 10 min. Similarly to previous attempts these sequences were also terminated before reaching the GC-rich region or yielded ambiguous sequencing results (Fig. 1, Panel B). The only effective way to obtain high quality sequences for the hairpin region was when 2.5-5 µM 7-deaza-dGTP was included in both PCR and sequencing reaction mixtures (Fig. 1, Panel C). As a result alternating reiterations of homo-G-polymer and homo-C-polymer regions, 61 nt (HGyV/AGV2) and 42 nt (GyV4) in length, were revealed in the missing genomic region.

The three HGyV genomes were 2375 nt long; the genome sequences were identical to each other, and were closely related (94.5%) to the AGV2 G17 strain having a genome of 2378 nt. The Hungarian HGyV G13 whole genomic sequence was 61 nt longer than that of the reference HGyV (accession number: FR823283) which latter lacked the GC-rich region identified in our HGyV and AGV2 genomes as well as in the AGV2 genomes deposited in the GenBank (accession numbers: JQ690763, HM590588). The complete genomes of HGyV G13 and AGV2 G17 showed 99.4% and 94.2-95.9% similarities along the overlapping regions of the whole genomes of homologous reference HGyV and AGV2 strains, respectively. Although the genome of the

G17 strain had a codon insertion affecting the potential VP2 and VP3 proteins, the whole genome size and the structure of the non-coding region was comparable with the G13 and the AGV2 reference genome sequences.

The full-length genome of the Hungarian GyV4 strain, G14, was 2028 nt long, i.e. six bases shorter than the single known GyV4 reference genome available in the GenBank (accession number: JX310702). These two genome sequences showed 90.7% nt similarity to each other. The nt and amino acid similarities along the VP1 and VP2 regions showed an inverse pattern (86.7% and 94% nt and 92.6% and 88.9% aa similarities, respectively) to the reference. This lower sequence similarity between GyV4 strains suggests a greater diversity within this group of gyroviruses than seen among HGyV and AGV2 strains, which latter are supposed to be minor sequence variants of the same virus [3].

PCR results obtained by the diagnostic primers were systematically confirmed by sequencing of the amplicons. Briefly, the short HGyV sequences (251-429 nt long) amplified by the diagnostic primers [3-4] shared 100% identity among themselves. A 392 nt long stretch [3-4] of two GyV3 strains shared 98% nt sequence identities between themselves and 99% to the reference sequence (acession number: JQ308210). The only GyV4 sequence (239 nt long) amplified by diagnostic primers [3] showed 93.3% and 94.6% nt similarity with our (G14) and the reference GyV4 strain, respectively. CAV strains showed 99-100% similarities to each other and 100% similarity to the reference strains found in the GenBank. No traces of GyV5 and GyV6 were observed in the metagenomic analyses; thus the study was not extended to the examination of these novel gyroviruses. Unfortunately, determination of whole genomic sequences of additional gyroviruses was not possible because of the small amount of stool samples and/or the poor amplification results.

The identification of genetically related, actually indistinguishable, gyroviruses in the fecal viromes of humans and ferrets is an interesting finding [2-7]. Ferrets of the Hungarian shelter were feed regularly with chicken meat. This could be an explanation why a well known chicken pathogen, CAV, and a newly described member of the genus, AGV2, were detected in the feces of these animals. Similarly, food-associated acquisition and passive transfer through the gut could explain the detection of gyroviruses in the feces of humans and cats as reported in other studies [3-5, 10]. However, gyroviruses were also detected in the human blood; thus, the possibility that certain gyroviruses are capable of infecting a mammalian host species can not be ruled out [6-7].

Of note is that ferret coronaviruses were also detected in the fecal specimens by high throughput sequencing. Coronavirus is a common cause of diarrhoea in a variety of animal species and may induce systemic disease [11]. Novel gyroviruses have been already detected in diseased humans and chickens [1, 3-5] and it seems plausible that, similarly to CAV [1], these novel members of the genus may also induce immune

suppression in a different host species, such as ferrets. However, this assumption awaits supporting epidemiological and experimental data.

In summary, the finding that genetically identical gyroviruses can be detected in humans and ferrets warrants the need for close monitoring of these newly described viruses. The causative role and the host range of different gyroviruses must be explored to help better understand the findings of this study.

Acknowledgments

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Conflict of interest

The authors declare that they have no conflict of interest.

References

1. Rijsewijk FA, Dos Santos HF, Teixeira TF, Cibulski SP, Varela AP, Dezen D, Franco AC, Roehe PM (2011) Discovery of a genome of a distant relative of chicken anemia virus reveals a new member of the genus Gyrovirus. Arch Virol 156(6):1097-100
2. Sauvage V, Cheval J, Foulongne V, Gouilh MA, Pariente K, Manuguerra JC, Richardson J, Dereure O, Lecuit M, Burguiere A, Caro V, Eloit M (2011) Identification of the first human gyrovirus, a virus related to chicken anemia virus. J Virol 85(15):7948-50
3. Chu DK, Poon LL, Chiu SS, Chan KH, Ng EM, Bauer I, Cheung TK, Ng IH, Guan Y, Wang D, Peiris JS (2012) Characterization of a novel gyrovirus in human stool and chicken meat. J Clin Virol 55(3):209-13
4. Phan TG, Li L, O'Ryan MG, Cortes H, Mamani N, Bonkoungou IJ, Wang C, Leutenegger CM, Delwart E (2012) A third gyrovirus species in human faeces. J Gen Virol 93(6):1356-61
5. Gia Phan T, Phung Vo N, Sdiri-Loulizi K, Aouni M, Pothier P, Ambert-Balay K, Deng X, Delwart E (2013) Divergent gyroviruses in the feces of Tunisian children. Virology 446(1-2):346-8
6. Maggi F, Macera L, Focosi D, Vatteroni ML, Boggi U, Antonelli G, Eliot M, Pistello M (2012) Human gyrovirus DNA in human blood, Italy. Emerg Infect Dis 18(6):956-9

7. Biagini P, Bédarida S, Touinssi M, Galicher V, de Micco P (2013) Human gyrovirus in healthy blood donors, France. Emerg Infect Dis 19(6):1014-5

8. Djikeng A, Halpin R, Kuzmickas R, Depasse J, Feldblyum J, Sengamalay N, Afonso C, Zhang X, Anderson NG, Ghedin E, Spiro DJ (2008) Viral genome sequencing by random priming methods. BMC Genom 9:5

9. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol 30: 2725-2729

10. Zhang X, Liu Y, Ji J, Chen F, Sun B, Xue C, Ma J, Bi Y, Xie Q (2014) Identification of a chicken anemia virus variant-related gyrovirus in stray cats in China, 2012. BioMed Res Int 2014:313252

11. Provacia LB, Smits SL, Martina BE, Raj VS, Doel PV, Amerongen GV, Moorman-Roest H, Osterhaus AD, Haagmans BL (2011) Enteric coronavirus in ferrets, the Netherlands. Emerg Infect Dis 17(8):1570-1

Table 1 Results of the metagenomic and gyrovirus PCR analyses of ferret fecal samples. Two samples were taken at different dates from ferrets labelled as G8, G10 and G20. Accession number of the references and nt similarities of those to our sequences are indicated in the parenthesis. HGyV: human gyrovirus, AGV2: avian gyrovirus 2, GyV3 and GyV4: gyrovirus 3 and 4, CAV: chicken anaemia virus. HGyV/AGV2: HGyV and/or AGV2 sequences were detected in the sample. N.D.: not done. **W**: whole genome sequenced strain

Table 2 Primer sequences designed for the amplification and sequencing of gyrovirus sequences of ferret fecal samples. Primer positions without parenthesis refer to human gyrovirus and values in the parenthesis label primer positions in the avian gyrovirus 2

Fig. 1 Sequencing of the GC-rich non-coding region of the human gyrovirus strain G13. Panel A: Early termination after amplification and Sanger sequencing. Panel B: Sequences gained by PCR containing 7-deaza-dGTP in the reaction mixtures. Panel C: Sequences after the utilization of 7-deaza-dGTP in both PCR and sequencing reaction mixtures

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The identification of genetically related, practically indistinguishable, gyroviruses in the fecal viromes of humans and ferrets is an interesting finding [2-7]. Ferrets in the Hungarian shelter were feed regularly with chicken meat. This could be an explanation for

why a well-known chicken pathogen, CAV, and a newly described member of the genus, AGV2, were detected in the feces of these animals. Similarly, food-associated acquisition and passive transfer through the gut could explain the detection of gyroviruses in the feces of humans and cats as reported in other studies [3-5, 10]. However, gyroviruses were also detected in human blood, and thus the possibility that certain gyroviruses are capable of infecting a mammalian host cannot be ruled out [6-7].

It is worth noting that ferret coronaviruses have also been detected in fecal specimens by high-throughput sequencing. Coronaviruses are a common cause of diarrhoea in a variety of animal species and may induce systemic disease [11]. Novel gyroviruses have already been detected in diseased humans and chickens [1, 3-5], and it seems plausible that, like CAV [1], these novel members of the genus may also induce immune suppression in a different host species, such as ferrets. However, this assumption awaits supporting epidemiological and experimental data.

In summary, the finding that genetically identical gyroviruses can be detected in humans and ferrets warrants close monitoring of these newly described viruses. The causative role and the host range of different gyroviruses must be explored to help better understand the findings of this study.

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Conflict of interest

The authors declare that they have no conflict of interest.

References

1. Rijsewijk FA, Dos Santos HF, Teixeira TF, Cibulski SP, Varela AP, Dezen D, Franco AC, Roehe PM (2011) Discovery of a genome of a distant relative of chicken anemia virus reveals a new member of the genus Gyrovirus. Arch Virol 156(6):1097-100
2. Sauvage V, Cheval J, Foulongne V, Gouilh MA, Pariente K, Manuguerra JC, Richardson J, Dereure O, Lecuit M, Burguiere A, Caro V, Eloit M (2011) Identification of the first human gyrovirus, a virus related to chicken anemia virus. J Virol 85(15):7948-50
3. Chu DK, Poon LL, Chiu SS, Chan KH, Ng EM, Bauer I, Cheung TK, Ng IH, Guan Y, Wang D, Peiris JS (2012) Characterization of a novel gyrovirus in human stool and chicken meat. J Clin Virol 55(3):209-13
4. Phan TG, Li L, O'Ryan MG, Cortes H, Mamani N, Bonkoungou IJ, Wang C, Leutenegger CM, Delwart E (2012) A third gyrovirus species in human faeces. J Gen Virol 93(6):1356-61
5. Gia Phan T, Phung Vo N, Sdiri-Loulizi K, Aouni M, Pothier P, Ambert-Balay K, Deng X, Delwart E (2013) Divergent gyroviruses in the feces of Tunisian children. Virology 446(1-2):346-8
6. Maggi F, Macera L, Focosi D, Vatteroni ML, Boggi U, Antonelli G, Eliot M, Pistello M (2012) Human gyrovirus DNA in human blood, Italy. Emerg Infect Dis 18(6):956-9
7. Biagini P, Bédarida S, Touinssi M, Galicher V, de Micco P (2013) Human gyrovirus in healthy blood donors, France. Emerg Infect Dis 19(6):1014-5

8. Djikeng A, Halpin R, Kuzmickas R, Depasse J, Feldblyum J, Sengamalay N, Afonso C, Zhang X, Anderson NG, Ghedin E, Spiro DJ (2008) Viral genome sequencing by random priming methods. BMC Genom 9:5
9. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol 30: 2725-2729
10. Zhang X, Liu Y, Ji J, Chen F, Sun B, Xue C, Ma J, Bi Y, Xie Q (2014) Identification of a chicken anemia virus variant-related gyrovirus in stray cats in China, 2012. BioMed Res Int 2014:313252
11. Provacia LB, Smits SL, Martina BE, Raj VS, Doel PV, Amerongen GV, Moorman-Roest H, Osterhaus AD, Haagmans BL (2011) Enteric coronavirus in ferrets, the Netherlands. Emerg Infect Dis 17(8):1570-1

Table 1 Results of metagenomic and gyrovirus PCR analyses of ferret fecal samples. Two samples were taken at different dates from ferrets labelled as G8, G10 and G20. Accession numbers of the references and nt similarities of those to our sequences are indicated in parentheses. HGyV, human gyrovirus; AGV2, avian gyrovirus 2; GyV3 and GyV4, gyrovirus 3 and 4; CAV, chicken anaemia virus. "HGyV/AGV2" indicates that HGyV and/or AGV2 sequences were detected in the sample. N.D., not done. W, whole genome sequence

Table 2 Primer sequences designed for the amplification and sequencing of gyrovirus sequences from ferret fecal samples. Primer positions without parentheses refer to human gyrovirus, and values in parentheses indicate primer positions in avian gyrovirus 2

Fig. 1 Sequencing of the GC-rich non-coding region of the human gyrovirus strain G13. Panel A: early termination after amplification and Sanger sequencing. Panel B: sequences determined using PCR with 7-deaza-dGTP in the reaction mixtures. Panel C: sequences after the utilization of 7-deaza-dGTP in both PCR and sequencing reaction mixtures

Figure
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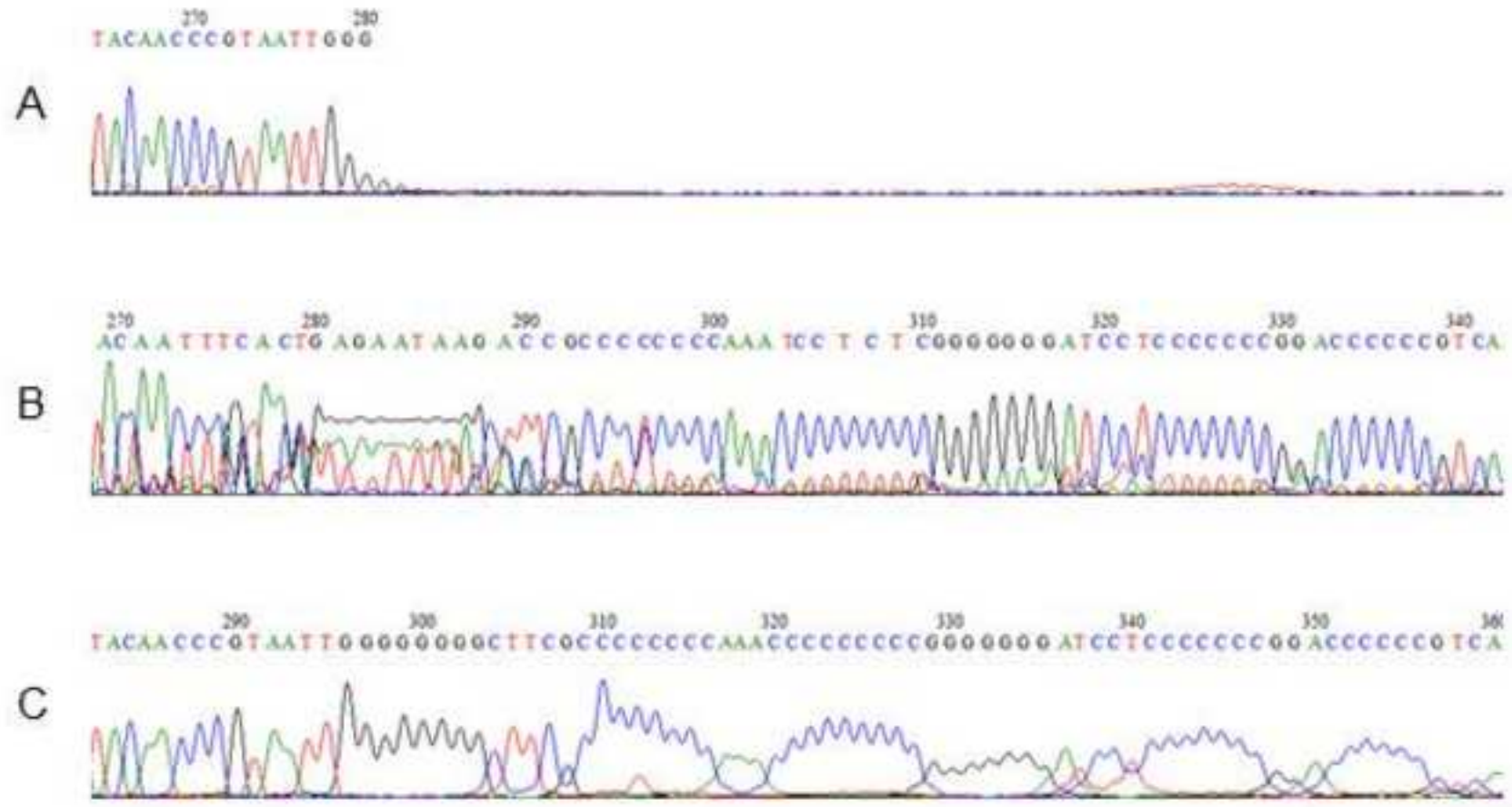

Table 1

Sample name	Metagenomic analysis				PCR			
	HGyV/AGV2	**GyV3**	**GyV4**	**CAV**	**HGyV/AGV2**	**GyV3**	**GyV4**	**CAV**
G1	+	-	+	-	+ (FR823283, 100%)	-	-	+ (FJ360724, 100%)
G2	N.D.				+ (FR823283, 100%)	-	-	+ (FJ360724, 100%)
G3	+	-	+	+	+ (FR823283, 100%)	-	-	+ (no sequence)
G4	-	-	-	-	-	-	-	-
G5	-	-	-	-	-	-	-	-
G6	N.D.				-	-	-	-
G7	-	-	-	-	+ (FR823283, 100%)	-	-	-
G8	-	-	-	-	-	-	-	-
G8A	-	-	-	-	+ (FR823283, 100%)	-	-	+ (no sequence)
G9	-	-	-	-	+ (FR823283, 100%)	+ (JQ308210, 99.0%)	-	+ (FJ360725, 100%)
G10	-	-	-	-	-	-	-	-
G10A	-	-	-	-	-	-	-	-
G11	-	-	-	-	+ (FR823283, 100%)	+ (JQ308210, 99.0%)	-	+ (no sequence)
G12	-	-	-	-	-	-	-	-
G13	+	-	-	-	**W** (FR823283, 99.4%)	-	+ (JX310702, 94.6%)	+ (FJ360724, 100%)
G14	+	-	**W** (JX310702, 90.7%)	-	**W** (FR823283, 99.4%)	-	-	+ (FJ360724, 100%)
G15	-	-	-	-	+ (FR823283, 100%)	-	-	+ (FJ360725, 100%)
G16	-	-	-	-	-	-	-	-
G17	+	-	-	-	**W** (JQ690763, 95.9%, HM590588, 94.2%)	-	-	-
G18	+	-	-	-	+ (FR823283, 100%)	-	-	+ (FJ360724, 100%)
G19	+	+	-	-	**W** (FR823283, 99.4%)	-	-	+ (FJ360724, 100%)
G20	+	-	-	-	+ (FR823283, 100%)	-	-	-
G20A	-	-	-	-	-	-	-	-
Positive samples	8/21 (38.1%)	1/21 (4.8%)	3/21 (14.3%)	1/21 (4.8%)	14/23 (60.9%)	2/23 (8.7%)	1/23 (4,3%)	11/23 (55%)
Positive animals	8/18 (44.4%)	1/18 (5.6%)	3/18 (16.7%)	1/18 (5.6%)	14/20 (70%)	2/20 (10%)	1/20 (5%)	11/20 (47.8%)

Table 2

Primer name Position	5'-3' primer sequence	Function
human gyrovirus/avian gyrovirus 2		
GGyV1-F1 203-231	AGTAAACTGAGACTCATACCGGTACAGGG	back-to-back PCR, sequencing
GGyV1-R1 202-172	TACATATCGTTGGTTACCACGCCTTGTG	back-to-back PCR, sequencing, modified PCR of GC-rich region
GGyV1-F2 1009-1031 (1012-1034)	ACGATGGCACTGGAGACACAGAC	back-to-back PCR, sequencing
GGyV1-R2 1008-986 (1011-989)	CCTCTGTGGTAGAAGCCAAAGCG	back-to-back PCR, sequencing
GGyV1-F3 1098-1124 (1101-1127)	GCGTTAAGAGGAGGATCTTCAACCCAC	back-to-back PCR, sequencing
GGyV1-R3 1097-1074 (1100-1077)	GGCGATCAAAGGATCTTCTACGCG	back-to-back PCR, sequencing
GGyV1-F4 1494-1519 (1497-1522)	GAGCACCTGGCAGATTTTACAATGCC	back-to-back PCR, sequencing
GGyV1-R4 1493-1472 (1496-1475)	TAGGGTGCATCACCAGGAGAGC	back-to-back PCR, sequencing
GGyV1-F/1 685-710	GAGATCCAAATTGGTATCGGGTCAAC	sequencing
GGyV1-F/2 1683-1706 (1686-1709)	TAGAGGGCTTCCCAGTTAAAGGTG	sequencing
GGyV1-F/3 1946-1968 (1949-1971)	GGTGCACCCTGGTCATTTCCTAG	sequencing
GGyV1-F/4 2039-2060 (2042-2063)	CGACGGTGGTTAACACTTGTGC	sequencing, modified PCR of GC-rich region
GGyV1-R/1 539-518	AAGTTGCCCCTCTTGCCTAAGC	sequencing
GGyV1-R/2 662-639	GGAGACGAACTTGTTGGATGCTCG	sequencing
gyrovirus 4		
GGyV4-F (974-992)	TCAGATTCAGACGGAGACC	back-to-back primers
GGyV4-R (973-954)	CCATTCCACAAAACTGTCCC	back-to-back primers
GGyV4-F/1 1446-1469	TGCAAATGGAACTAGGCCGACAG	sequencing
GGyV4-F/2 277-299	ATGGCAGGGTACTTATTGCCGTG	sequencing
GGyV4-F/3 1835-1862	TCGGTACATTGTTTCTCGCGGAATTTAG	sequencing, modified PCR of GC-rich region
GGyV4-R/1 538-517	ACTAAAGCGATGGTCAGGGCTG	sequencing
GGyV4-R/2 405-384	AGCAAAAATTCACTGCCCCGAG	sequencing, modified PCR of GC-rich region
GGyV4-R/3 1256-1232	TCAGCCTGTCCAGACTGATATACGG	sequencing