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Severe neurologic disease and chick mortality in crested screamers (*Chauna torquata*) infected with a novel *Gyrovirus*

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ABSTRACT

Gyroviruses are small, single stranded DNA viruses in the family *Anelloviridae*. In chickens, the type virus (chicken anemia virus; CAV) causes epidemic disease in poultry flocks worldwide. In 2007 and 2008, young crested screamers (*Chauna torquata*) at a zoo in Wisconsin, USA, died of neurologic disease with clinical and pathological features resembling CAV infection. Conventional diagnostics were negative, but molecular analyses revealed coinfection of an affected bird with three variants of a novel *Gyrovirus* lineage, GyV10. Analysis of ten additional screamers from this and another zoo revealed infection in all but one bird, with co-infections and persistent infections common. The association between GyV10 ("screamer anemia virus," provisionally) and the disease remains unproven, but certain immunological and neurologic features of the syndrome would expand the known pathologic consequences of *Gyrovirus* infection. To control the virus, autogenous vaccines, environmental decontamination, and management strategies to limit vertical and horizontal transmission might prove effective.

1. Introduction

Gyroviruses (family *Anelloviridae*, genus *Gyrovirus*; Rosario et al., 2017) are an economically significant cause of poultry disease worldwide. The type species, chicken anemia virus (CAV), was first isolated in 1974 during an epidemic of delayed growth, anemia, abnormal feathers, and leg paralysis in chickens (*Gallus gallus domesticus*) in Japan, traced to a vaccine for Mareck's disease (caused by an alphaherpesvirus; family *Herpesviridae*, genus *Mardivirus*) contaminated with reticuloendotheliosis virus (family *Retroviridae*, genus *Gammaretrovirus*) (Taniguchi et al., 1977; Yuasa et al., 1979, 1976). CAV is immunosuppressive and remains a major cause of morbidity and mortality in chickens, alone and in combination with other agents, and it is also a persistently problematic contaminant of poultry vaccines (Amer et al., 2011; Balamurugan and Kataria, 2006; Marin et al., 2013; Varela et al., 2014).

Since its discovery, CAV has been found in chickens on every continent except Antarctica: Africa (Ducatez et al., 2006; Oluwayelu et al., 2008; Smuts, 2014; Snoeck et al., 2012); Asia (Bhatt et al., 2011; Eltahir et al., 2011; Islam et al., 2002; Kim et al., 2010; Kye et al., 2013; Nayabian and Mardani, 2013); Australia (Brown et al., 2000); Europe (Bougiouklis et al., 2007; Chettle et al., 1989; de Wit et al., 2004; Krapez et al., 2006); North America (Eregae et al., 2014; Ledesma et al., 2001; Toro et al., 2006; van Santen et al., 2001); and South America (Craig et al., 2009; Simionatto et al., 2006). Phylogenetic studies demonstrate CAV variants to be monophyletic within the genus *Gyrovirus*, with sub-clades showing little to no geographic clustering, probably indicating global spread through the international poultry trade.

Recently, other gyroviruses distinct from CAV have been found in tissues of a northern fulmar (*Fulmarus glacialis*; Li et al., 2015) and in the feces of domestic cats (*Felis domesticus*; Zhang et al., 2014) and ferrets (*Mustela putorius*; Feher et al., 2015). Gyroviruses have also been found on human skin (Sauvage et al., 2011), in human blood (Biagini et al., 2013), and in human feces worldwide (Chu et al., 2012; Oude Munnink et al., 2014; Phan et al., 2013, 2015, 2012; Smuts, 2014; Zhang et al., 2012). It is currently unclear whether gyroviruses in mammals represent active infections, passive dietary transit, or environmental contamination (Phan et al., 2015).

In 2007 and 2008, the Milwaukee County Zoo (MCZ; Wisconsin, USA) experienced episodes of mortality in crested screamers (*Chauna torquata*, order *Anseriformes*, family *Anhimidae*), a bird native to wetland habitats of southern South America (Stonor, 1939). Affected birds

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were young chicks that displayed severe neurologic signs, including ataxia and paresis, with resulting disorientation, stumbling, inability to eat, and subsequent death ("staggers," colloquially). Here, we describe the results of an investigation into this condition yielding a new *Gyrovirus* lineage, with co-infections and persistent infections frequent in two populations of captive birds. The study expands our knowledge of *Gyrovirus* host range, diversity, and pathogenesis.

2. Materials and methods

2.1. Clinical features, pathology, and diagnostics

On October 23, 2007, a two-month-old screamer chick born to experienced parents at the Milwaukee County Zoo became severely ataxic with left side paresis. The bird was anemic, with an elevated white blood cell count, monocytosis, lymphocytosis, and increased serum levels of alkaline phosphatase and creatine phosphokinase. Various treatments were attempted, but euthanasia was ultimately necessary due to progressively worsening disease. Differential diagnoses included avian encephalomyelitis (caused by avian encephalomyelitis virus, family *Picornaviridae*, genus *Tremovirus*), aspergillosis (caused by fungi of the genus *Aspergillus*), various bacterial and hemoparasitic infections, and neural larval migrans from the parasitic nematode *Baylisascaris procyonis*, which has been documented in screamers in zoological settings where raccoons (*Procyon lotor*, the definitive host) occur (Thompson et al., 2008).

2.2. Virus identification and characterization

Because conventional diagnostics yielded negative results (see below), we retrieved archived plasma samples from this case to identify potential viral pathogens using metagenomic methods (Kapgate et al., 2015). Briefly, we extracted total viral nucleic acids from plasma using the QIAamp MinElute virus kit (Qiagen, Hilden, Germany), omitting carrier RNA. We then performed sequencing for virus discovery on an Illumina MiSeq instrument (Reagent Kit v3, 600 cycles, 2 × 300nt paired-end, with 1% Phi-X control DNA; Illumina, Inc., San Diego, USA) as previously described (Toohey-Kurth et al., 2017). This approach has proven useful in our hands for detection of other unknown infectious agents, including at the MCZ (Goldberg et al., 2014; Lee et al., 2016). Following sequencing, we processed the resulting 413,418 sequence reads using CLC Genomics Workbench version 8.5 (CLC bio, Aarhus, Denmark) by trimming low-quality bases (phred quality score < 30) and discarding short reads (< 75 bp), then subjecting the remaining 375,948 reads to *de novo* assembly.

This effort resulted in the discovery of a novel *Gyrovirus* lineage, GyV10 (see below), including a full viral genome. To explore the relationship of GyV10 to known gyroviruses, we constructed a phylogenetic tree of GyV10 and other gyroviruses in GenBank using complete viral genomic coding sequences. We first aligned sequences using the Prank algorithm (Loytynoja, 2014) in Translator X (Abascal et al., 2010) and then analyzed the resulting alignment using the maximum likelihood method in PhyML (Guindon et al., 2010), with the model of nucleotide substitution (GTR + I + Γ) estimated from the data, and we displayed the resulting phylogeny using FigTree (Rambaut, 2016).

2.3. Characterization of co-infections and viral variants

To investigate GyV10 co-infection, we performed semi-nested PCR and amplicon sequencing of sera from five additional screamers (ages 1 month to 1.5 years, obtained between 1998 and 2015) from MCZ, where similar cases had occurred between June 29, 1998 and September 18, 2008. For comparison, we also tested archived sera from five screamers (ages 1–24 years, obtained between 2002 and 2015) from the Louisville Zoo (Kentucky, USA), where such signs had never been reported. We first designed primers GyV10_F1 (5′ – TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGAATTGCCGTTTAGGCAAGA – 3′), GyV10_R1 (5′ – ATCGTCCTCGTC(T/G)GAAGGTG – 3′), GyV10_F2 (5′ – TCGTCGGCAGCGTCAGAT – 3′) and GyV10_R2 (5′ – GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCGGAATGAGGAACAGGAAC – 3′) to amplify a 219 (predicted) nucleotide region of the VP2/VP3 gene and adjacent non-coding region, based on the newly generated *Gyrovirus* sequences and published sequences, where primers F1, F2 and R2 include full or partial adapter sequences (underlined bases) for indexing and direct paired-end sequencing on the Illumina MiSeq platform, which currently has a maximum read length of 300 bp. PCRs were performed using HiFi HotStart ReadyMix (Kappa Biosystems, Wilmington, MA, USA), with 0.3 μM of primers GyV10_F1 and GyV10_R1 (external PCR) and primers GyV10_F2 and GyV10_R2 (internal PCR), with cycling parameters as follows: initial denaturation and enzyme activation at 95 °C for 3 min, followed by 10 "touchdown" cycles of 98 °C denaturation for 20 s, 72–63 °C annealing (decrease of 1 °C per cycle) for 15 s, and 72 °C extension for 30 s, followed by an additional 35 cycles of 98 °C denaturation for 20 s, 63 °C annealing for 15 s, and 72 °C extension for 30 s, a final 72 °C extension for 3 min, and a terminal 12 °C indefinite soak. We then electrophoresed PCR products on 2% agarose gels, visualized gels under ultraviolet light, excised amplicons, and purified them using the Zymoclean Gel DNA Recovery Kit (Zymo Research Corporation, Irvine, CA, USA). Subsequently, unique DNA barcodes and Illumina flow-cell adapter sequences were added using index PCR (Nextera XT v2 Kit, Illumina, Inc., San Diego, USA), and products were sequenced on an Illumina MiSeq instrument, as described above.

We trimmed the resulting sequences as described above and truncated them to uniform aligned length (249 positions). To identify co-infecting viral variants, we collapsed sequences from the same bird at the same time point using usearch at a 95% sequence identity cutoff (Edgar, 2010). We aligned these sequences and performed phylogenetic reconstruction as described above (HKY85 model of nucleotide substitution).

3. Results

3.1. Clinical features, pathology, and diagnostics

Gross pathological findings on the screamer chick that died in 2007 included dirty and ragged feathers, brown mucoid exudate in the choana and nostrils, congestion of the lungs, fluid feces within the cloaca, hyperemia and hemorrhage of the colonic mucosa and ceca, lack of grossly observable thymus tissue, a small spleen, and dermatitis. Histopathologic findings included thymic atrophy and lymphoid depletion (Fig. 1), focal lympho-histocytic myocarditis, chronic active enteritis, focal hepatitis, pineal hypertrophy, focal degenerative encephalopathy with axon degeneration, pyogranulomatous cellulitis and ulceration of the wing skin (where external lesions were noted). Bacterial cultures were negative for *Salmonella*, *Shigella*, *Aeromonas*, *Plesiomonas*, and *Campylobacter* and yielded only normal, commensal taxa. Histopathology ruled out baylisascariasis and aspergillosis. Serologic assays for *Aspergillum* and avian encephalomyelitis virus were also negative, as were microscopic evaluations of blood and feces for eukaryotic parasites.

3.2. Virus identification and characterization

Unbiased sequencing and subsequent bioinformatics yielded three contiguous sequences ("contigs"), each showing significant similarity (blastx E-values 10^{-6} to 10^{-66}) to the VP1, VP2 and VP3 genes of viruses within the genus *Gyrovirus*. The largest of these, designated GyV10.1 (GenBank accession number MH016740), represented a complete circular genome of 2195 bases with three overlapping open reading frames (Fig. 2), showing the characteristic genomic architecture of viruses within the genus *Gyrovirus* (Rosario et al., 2017). In