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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′ – TCGTCGGC AGCGTCAGATGTGTATAAGAGACAGGAATTGCCGTTTAGGCAAGA – 3′), GyV10_R1 (5′ – ATCGTCCTCGTC(T/G)GAAGGTG – 3′), GyV10_F2 (5′ – TCGTCGGCAGCGTCAGAT – 3′) and GyV10_R2 (5′ – GTCTCGTG GGCTCGGAGATGTGTATAAGAGACAGTCGGAATGAGGAACAGGAAC – 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

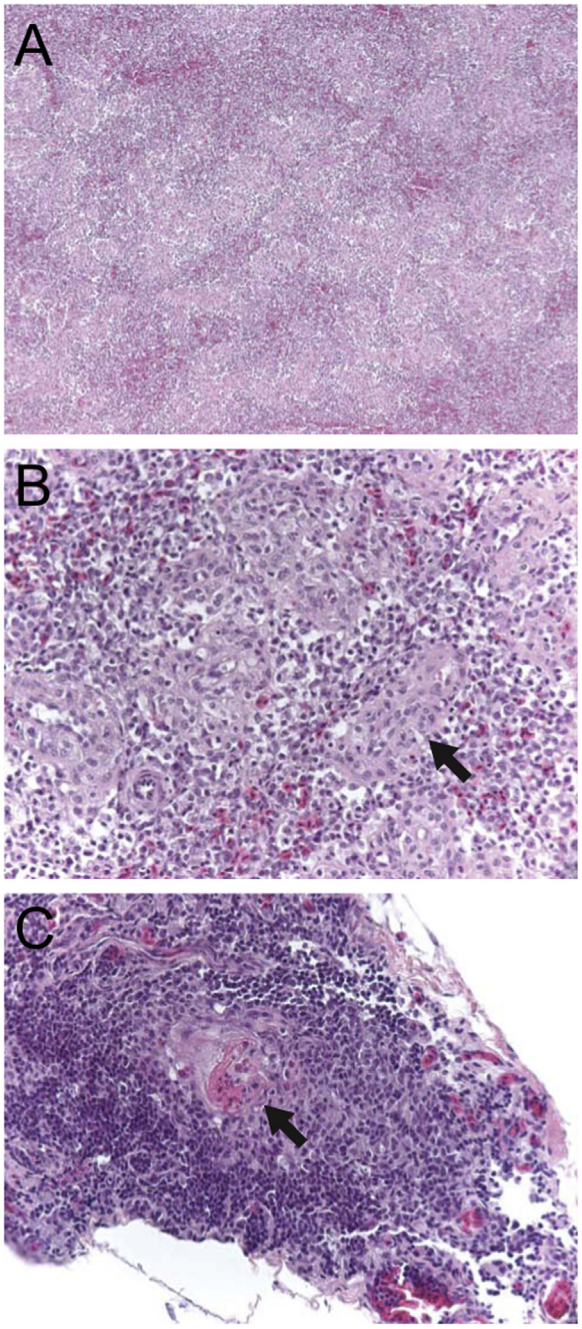

Fig. 1. Histopathology of tissues from a crested screamer chick co-infected with three variants of a novel *Gyrovirus* lineage, GyV10. A: Spleen 10×. There is marked lymphoid depletion and prominence of the sheathed capillaries (ellipsoids), and germinal follicles are not evident. B: Spleen 40×. This higher magnification view of the spleen reveals ellipsoids (arrow) with reduced numbers of surrounding lymphocytes. C: Thymus 40×. The thymic tissue shows atrophy with loss of zonal architecture and prominent depletion of cortical lymphocytes, and a Hassall's corpuscle (avian variant) is visible in the medulla (arrow).

total, 1451 reads (0.39%) mapped to this contig with an average depth of coverage of 107. The other two contigs, designated GyV10.2 (1525 bases, representing full VP2 and VP3 genes and a partial VP1 gene; GenBank accession number MH016741) and GyV10.3 (441 bases, representing partial VP1, VP2 and VP3 genes; GenBank accession number MH016742) shared only 82.1% and 77.7% nucleotide identity with GyV10.1, with 1348 (0.36%) and 33 (0.01%) reads mapping to each, respectively. A phylogenetic tree (Fig. 2) shows GyV10 to be distant sister taxon to GyV8 from a northern fulmar and outgroup to the clade containing CAV and other viruses from human feces and chickens. Across the coding genome, GyV10 is 50.9 ± 1.1% different at the nucleotide level and 58.6 ± 2.1% different at the amino acid level from the type virus, CAV.

3.3. Characterization of co-infections and viral variants

PCR amplification and sequencing yielded an additional 40 viral variants from nine birds (GenBank accession numbers MH016743-MH016783), with between 1 and 12 GyV10 sequences per individual and an average percent nucleotide and amino acid difference among sequences of 7.6 ± 1.1 and 8.1 ± 2.0, respectively. Phylogenetic analysis (Fig. 3) shows that the viruses from MCZ and the Louisville zoo do not form monophyletic groups; rather, certain viral variants appear in multiple birds from the same zoo (e.g. M1a07, M4a06 and M6b07) but never in different zoos. Moreover, some birds were clearly co-infected with divergent variants (e.g. L1a04 and L1h04, both obtained from the same bird on the same date). In several cases, identical sequences were recovered from the same bird years apart, most notably for M3a98 and M3c15, sampled in 1998 and 2015, respectively. Thus, a diverse population of GyV10 viral variants apparently circulates in captive screamers, and individual birds can be co-infected with divergent viruses that can persist for years.

4. Discussion and conclusions

Certain key clinical and pathologic features of the newly reported disease resemble those of chickens infected with CAV (Rosenberger and Cloud, 1998), including anorexia, enteritis, thymic atrophy and lymphoid depletion, secondary subcutaneous bacterial infections, and dermatitis. The presentation of this case also recalls features of chicken infectious anemia, which typically affects young chicks after maternal antibodies have waned (Miller and Schat, 2004; Schat, 2009). However, the neurologic signs and pathology of this syndrome were unique. Furthermore, not all infected chicks at MCZ were affected, MCZ kept screamers successfully from 1998 to 2008 (the zoo's breeding program was stopped in 2008 due to this disease), and variants of GyV10 were also present in apparently healthy, older birds from MCZ and the Louisville Zoo. If GyV10 (“screamer anemia virus,” provisionally) is causally associated with this disease, the association is probably complex and, as in chickens infected with CAV, may be limited to early life stages. Importantly, our data do not indicate definitively whether GyV10 is the cause of this disease in screamers. Making causal inferences from metagenomic data is fraught with difficulties, and experimental infection is usually necessary to confirm viral etiologies (Mokili et al., 2012). Experimental infection of screamers (or a suitable alternative model species) would be required to evaluate the clinical significance of GyV10. Nevertheless, GyV10 was the only infectious agent identified in this case, despite extensive diagnostics.

Our findings demonstrate a surprising degree of within-host viral diversity for GyV10. The affected screamer chick initially examined was infected with three distinct GyV10 variants differing by approximately 20% at the nucleotide level. Although little is known about evolutionary rates of gyroviruses, it seems unlikely that these three variants could have evolved *de novo* from a common ancestor in this chick during its short (2-month) life. Thus, the bird probably acquired a mixed infection from its parents or mixed/multiple infections from other birds in its enclosure. Alternatively, given that the barrier between exhibit animals and wild animals at zoos such as MCZ is “permeable,” GyV10 might also have originated from wild birds. Analysis of serum from nine other infected birds revealed a similar pattern of frequent co-infection with divergent viral variants, but also stability of infection over many years in some cases (Fig. 3). This observation also supports a scenario of mixed initial infection followed by limited subsequent evolution of viral variants within birds.

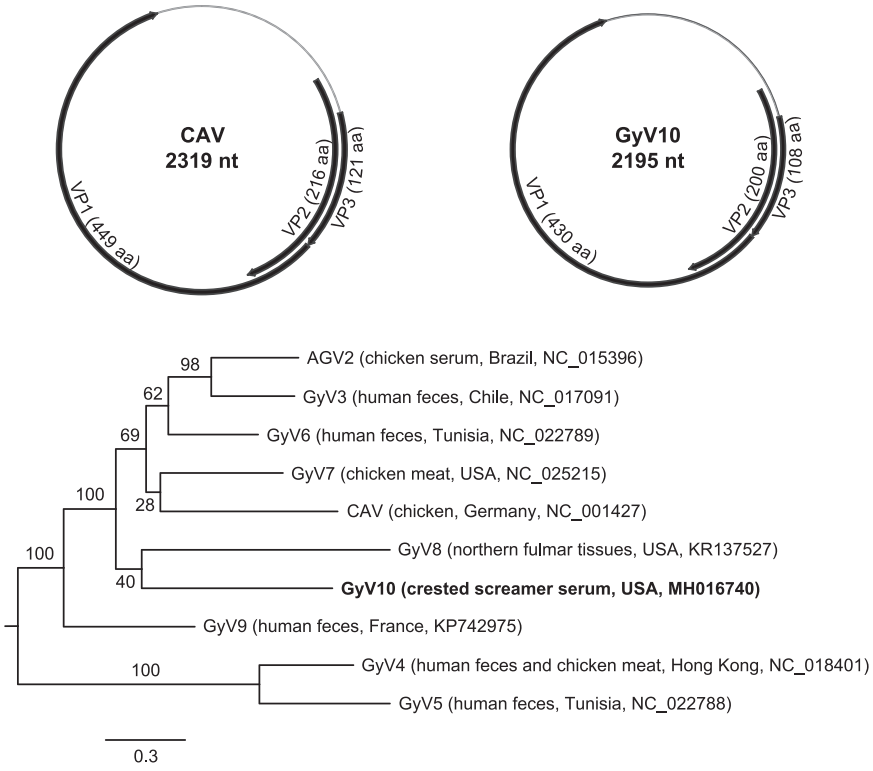

Fig. 2. Top: comparative genomic architecture of chicken anemia virus (CAV, the type virus) and GyV10 ("screamer anemia virus," the novel *Gyrovirus* discovered in crested screamers), showing genome sizes (nucleotides) and predicted sizes of viral proteins (amino acids). Bottom: maximum likelihood phylogenetic tree estimated from a 2655-position alignment of 10 full coding *Gyrovirus* genomes, including the novel GyV10 genome obtained from the serum of a crested screamer chick (bold). Values in parentheses indicate source of origin, country of origin, and GenBank accession number. Numbers above branches indicate statistical confidence based on 1000 bootstrap replicates of the data; scale bar indicates nucleotide substitutions per site. The tree is rooted at the midpoint of the longest branch.

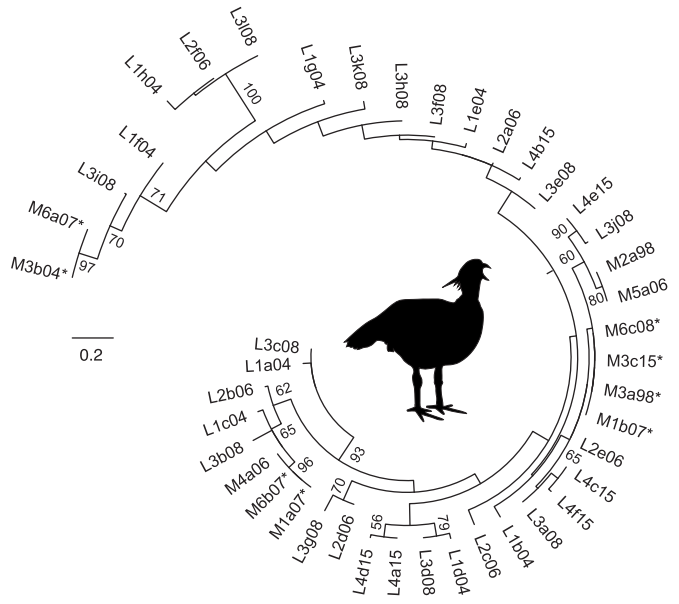

Fig. 3. Maximum likelihood phylogenetic tree of 43 GyV10 variants from six crested screamers at the Milwaukee County Zoo (Wisconsin, USA) and four crested screamers at the Louisville Zoo (Kentucky, USA) based on a 249-position alignment of the VP2 gene. Taxon names indicate the zoo of origin (M: Milwaukee County; L: Louisville), bird and viral variants assigned an arbitrary number and letter, and two digits to indicate the year of sampling (1998–2015). Variant M1a07 corresponds to GyV10 in Fig. 2. Asterisks indicate sequences from clinically affected birds. Numbers beside nodes indicate statistical confidence (percent) based on 1000 bootstrap replicates of the data (only values ≥ 50% are shown); scale bar indicates nucleotide substitutions per site. The tree is rooted on the midpoint of the longest branch.

CAV is notoriously difficult to manage because it is resistant to chemical and thermal inactivation, it can remain latent in gonadal tissues even in the presence of neutralizing antibodies, and it is readily transmitted vertically (Miller and Schat, 2004; Rosenberger and Cloud, 1998; Schat, 2009). Management options for gyroviruses in zoos would therefore appear to be limited. Approved CAV vaccines for use in chickens are available (Todd, 2000), although, to our knowledge, no information exists about their ability to induce cross-protective immunity or about their safety in birds other than chickens. Nevertheless, an autogenous vaccine based on the existing CAV system might be feasible, especially because CAV vaccines can be administered in drinking water (Todd, 2000). Control of gyroviruses in zoological settings would also likely hinge on management practices that reduce transmission, such as aggressive decontamination of exhibit spaces where gyroviruses are found or suspected, as well as early segregation of healthy chicks from affected nest mates and from parents with a history of producing affected offspring, to reduce post-natal horizontal transmission (Pope, 1991).

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Conflicts of interest

The authors declare that they have no competing interests.

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