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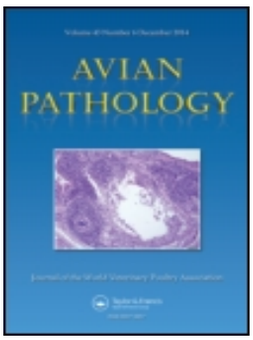
Devaney, R., Trudgett, J., Trudgett, A., Meharg, C., & Smyth, V. (2016). A metagenomic comparison of endemic viruses from broiler chickens with runting stunting syndrome and from normal birds. Avian Pathology. DOI: 10.1080/03079457.2016.1193123

Published in:
Avian Pathology

Document Version:
Peer reviewed version



Avian Pathology

ISSN: 0307-9457 (Print) 1465-3338 (Online) Journal homepage: http://www.tandfonline.com/loi/cavp20

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To cite this article: Ryan Devaney, James Trudgett, Alan Trudgett, Caroline Meharg & Victoria Smyth (2016): A metagenomic comparison of endemic viruses from broiler chickens with runting stunting syndrome and from normal birds, Avian Pathology

To link to this article: http://dx.doi.org/10.1080/03079457.2016.1193123

Accepted author version posted online: 23 May 2016.
Published online: 23 May 2016.

Publisher: Taylor & Francis & Houghton Trust Ltd

Journal: *Avian Pathology*

DOI: 10.1080/03079457.2016.1193123

Cavp-2016-0036.R1

A metagenomic comparison of endemic viruses from broiler chickens with runting stunting syndrome and from normal birds.

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Running head: Metagenomic comparison of endemic viruses

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Received: 14 April 2016

Abstract. Runting-stunting syndrome (RSS) in broiler chickens is an enteric disease that causes significant economic losses to poultry producers worldwide due to elevated feed conversion ratios, decreased body weight during growth, and excessive culling. Of specific interest are the viral agents associated with RSS which

have been difficult to fully characterise to date. Past research into the aetiology of RSS has implicated a wide variety of RNA and DNA viruses however, to date, no individual virus has been identified as the main agent of RSS and the current opinion is that it may be caused by a community of viruses, collectively known as the virome. This paper attempts to characterise the viral pathogens associated with 2 – 3 week old RSS-affected and unaffected broiler chickens using next-generation sequencing and comparative metagenomics. Analysis of the viromes identified a total of 20 DNA & RNA viral families, along with 2 unidentified categories, comprised of 31 distinct viral genera and 7 unclassified genera. The most abundant viral families identified in this study were the *Astroviridae, Caliciviridae, Picornaviridae, Parvoviridae, Coronaviridae, Siphoviridae,* and *Myoviridae.* This study has identified historically significant viruses associated with the disease such as chicken astrovirus, avian nephritis virus, chicken parvovirus, and chicken calicivirus along with relatively novel viruses such as chicken megrivirus and sicinivirus 1 and will help expand the knowledge related to enteric disease in broiler chickens, provide insights into the viral constituents of a healthy avian gut, and identify a variety of enteric viruses and viral communities appropriate for further study.

Keywords: Metagenomics, Virome, Next Generation Sequencing, Runting-Stunting Syndrome, Poultry, Picornavirus

Introduction. The overall performance of a poultry flock is dependent on various factors such as feed quality, poultry house management, and the presence of pathogenic microorganisms. One of the largest contributing factors to thriving flocks is the development and proper functioning of the gastrointestinal (GI) tract. The avian

GI tract represents a site of nutrient absorption and contains many different types of microorganisms (collectively known as the microbiome) and plays host to a variety of commensal and pathogenic microbes including viruses, bacteria, and fungi. Sub-optimal functioning of the GI tract can result in poor performance and can lead to production problems such as a poorer feed conversion ratio (FCR; the efficiency of converting feed to body mass), uneven flock growth, and can be caused by enteric diseases such as runting-stunting syndrome (RSS) in broiler chickens.

RSS, also known as malabsorption syndrome, was reported in broiler flocks as early as the 1970s by Kouwenhoven *et al.* (1978a,b) who described symptoms such as proventriculitis, poor FCR, and runting, which is defined as undersized at hatch. Further research described a wide variety of clinical symptoms including growth depression, irregular feathering (helicopter feathering, abnormal colouring), the presence of watery diarrhoea, other enteric problems such as intestinal lesions and pale intestines, and in severe cases mortality (Smart *et al.,* 1988; Shapiro *et al.,* 1998; Otto *et al.,* 2006; de Wit *et al.,* 2011). Globally, the incidence of RSS and uneven flock growth can cause substantial economic losses to poultry producers due to culls of stunted, undersized birds that are too small to pass through the processing plant.

Of specific interest is the enteric viral population associated with RSS of which many RNA and DNA viruses have been implicated and co-infections of multiple viruses such as rotavirus, chicken astrovirus (CAstV), avian nephritis virus (ANV), **and reoviruses** have been detected in birds affected with RSS or with poor performance (Reynolds *et al.,* 1986; Guy, 1998; Kang *et al.,* 2012). Metagenomic research into a similar disease in turkeys, known as poult enterits complex (PEC), has helped shed some light into the range of enteric pathogens associated with avian malabsorption

diseases (Day *et al.,* 2010; Day & Zsak, 2013) with Day *et al.* (2010) describing at least 16 different RNA viral genera from a pool of affected birds. Other enteric pathogens associated with avian malabsorption diseases include reoviruses, parvoviruses, and members of the family *Caliciviridae;* many of which have also been observed in broiler chickens (Spackman *et al.,* 2005; Smyth *et al.,* 2007; Pantin-Jackwood *et al.,* 2008, Wolf *et al.,* 2011, Zsak *et al.,* 2013). Pantin-Jackwood *et al.* (2008) demonstrated the presence of avian nephritis virus (ANV) in chicken flocks but also in turkey flocks for the first time indicating that some level of cross-species interaction can occur. Additionally, Day *et al.* (2007) demonstrated that both turkeys and chickens exhibit species-specific viruses such as turkey astrovirus (TAstV) and chicken astrovirus (CAstV).

Although previous studies detected many of these enteric viruses in affected flocks, and in different combinations of co-infections, some of these same viruses have been found in healthy flocks making it difficult to determine which viruses are implicated in the disease. For example, Reynolds *et al.* (1987) demonstrated that avian astrovirus and rotavirus were detected in 'normal' turkey poults as well as affected poults and Pantin-Jackwood *et al.* (2006) reported the presence of CAstV and ANV in healthy flocks as well as affected.

Some of the viruses associated with RSS are difficult to cultivate in the laboratory which makes them difficult to study using conventional techniques (Todd *et al.,* 2010). Furthermore, a conventional approach such as viral cultivation and Sanger sequencing would make it difficult and time consuming to study the community of viruses in an affected flock especially novel viruses. In order to study the complete enteric virome, and provide an unbiased comparison of affected and unaffected birds, a more comprehensive approach should be applied and high-throughput

sequencing is an ideal method to examine the community of viruses inhabiting the enteric tracts of broiler flocks (Day *et al.,* 2010).

High-throughput, next generation sequencing (NGS) is a tool that has been successfully used to characterise microbial communities from a variety of complex environmental samples (Mardis, 2008; Patterson *et al.,* 2009; Li *et al.,* 2015). Using current NGS technologies it is possible to achieve deep sequencing coverage from samples that permits comparative metagenomic analysis between samples to identify key pathogens related to avian enteric disease. Bacterial metagenomics via amplicon sequencing is a well researched area due to the availability of the highly conserved 16S ribosomal RNA (rRNA) sequence found in bacteria (Riesenfeld *et al.,* 2004; Gill *et al.,* 2006; Wang & Qian, 2009). As there is no viral equivalent of the 16S rRNA gene it is more appropriate to use a shotgun sequencing approach to discover novel viruses from environmental samples.

One of the major advantages of certain, newer NGS platforms is the ability to obtain longer reads from a single run; for example up to 800 base pairs (bp) for a single read using Roche's FLX+ system or 600 bp from a 300 bp, paired end read by the MiSeq system from Illumina, which are comparable with read lengths obtained from conventional Sanger sequencing. This increases accuracy of sequence assembly in regards to RNA viruses due to their high mutation rates leading to a high degree of variability and their ability to form quasispecies (Todd *et al.,* 2011; Smyth *et al.,* 2012). This paper describes the application of next generation sequencing to characterise the DNA and RNA viral communities present within samples from broiler birds affected and unaffected by RSS.

Materials & Methods

Sample preparation. Broiler chickens of between 13 and 21 days of age were received from UK farms. The gut contents of between 2 and 7 of these birds were pooled from RSS affected flocks displaying growth depression (VF13-188 E, VF14-91 A1, VF14-91 A2, VF14-92 A1, VF14-92 A2) and from two unaffected flocks of healthy birds (VF14-181 A1, VF14-181 B1). From each pool 2 g of gut content was removed from the intestinal tract of the chicken and added to 18 mL phosphate buffered saline (PBS), pH 7.2, in a 50 mL centrifuge tube and was homogenised with sterile glass beads and vortexed thoroughly for 10 minutes followed by centrifugation for 30 minutes at 2400 x *g*, 4°C. The supernatant was transferred to a fresh 50 mL centrifuge tube and centrifuged at 5000 x *g*, 4°C for 15 minutes. The supernatant was removed and filtered through sterile 0.22 µm syringe filters (Merck-Millipore, Billerica, MA, USA). The filtered supernatant was then ultracentrifuged (Sorvall WX Ultra Series, Thermo Scientific, Waltham, MA, USA) for 5 hours at 113,000 x *g*, 4°C using the SW40-Ti rotor (Beckman-Coulter, Brea, CA, USA). The supernatant was removed and the pellet formed was resuspended in 1 mL sterile PBS buffer, pH 7.2.

Removal of exogenous nucleic acids. RNase A (2 µg, Thermo Scientific) and 100 U DNase 1 (Thermo Scientific) were added as per the manufacturer's protocol to the enriched viral suspension and mixed by gently tapping the aliquot. The suspension was incubated in a water bath at 37°C for 30 minutes followed by inactivation by adding ethylenediaminetetraacetic acid (EDTA, Thermo Scientific) supplied with the DNase 1 kit, as per the manufacturer's instructions, and incubating at 65 °C for 10 minutes. Samples were divided into two fractions for DNA and RNA extraction and placed on ice.

DNA & RNA extraction. Total RNA was extracted from samples as prepared above using the Ribopure RNA extraction kit (Life Technologies, Carlsbad, CA, USA), according to manufacturer's instructions and collected in a 1.5 mL tube. Total DNA was extracted using the Viral RNA Mini Kit (Qiagen, Manchester, UK) to manufacturer's instructions and collected in a 1.5 mL tube. The Viral RNA mini kit was shown to extract DNA just as efficiently as it extracted RNA and more efficiently than other DNA extraction kits (unpublished results).

Whole transcriptome & whole genome amplification. Extracted DNA and RNA were subjected to whole genome amplification (WGA) and whole transcriptome amplification (WTA) respectively to produce enough genomic material for sequencing. WGA and WTA reactions were carried out in parallel using the Repli-g Cell WGA & WTA Kit (Qiagen) according to manufacturer's guidelines using random primers. The WTA reaction converted RNA to cDNA.

Library preparation. Genomic material was fragmented via sonication to achieve a 900 - 1200 bp range suitable for sequencing by the FLX+ system (Roche, Penzberg, Upper Bavaria, Germany). Total WGA and WTA material (60 µL) from each sample was diluted in 940 µL sterile PBS, pH 7.2, and sonicated at full power using the Soniprep 150 (MSE, London, UK) using an exponential probe. Samples were sonicated for 7 cycles each alternating between 10 seconds sonication followed by 10 seconds cooling on ice.

For the GS Junior system (Roche) genomic material was fragmented via sonication to achieve a 600 – 900 bp range suitable for sequencing. This was achieved via the same method as the FLX+ using 9 sonication cycles instead of 7.

Sonicated samples were purified using the QiaQuick PCR Purification Kit (Qiagen) according to the manufacturer's guidelines.

Purified samples were subject to end repair, followed by sequencing adaptor ligation, and removal of small fragments using the Rapid Library Preparation Reagents & Adaptors Kit (Roche) according to manufacturer's guidelines. When preparing a single sample the RL adaptor from the kit was used. When multiplexing samples each sample utilised a different MID adaptor from the MID Adaptor Kit (Roche). Samples VF14-91 A1, VF14-91 A2, VF14-92 A1, VF14-92 A2, VF14-181 A1, and VF14-181 B1 were multiplexed in a single run. This was followed by a quality check on the 2100 Bioanalyser (Agilent, Santa Clara, CA, USA) using the High Sensitivity DNA kit (Agilent) to ensure optimum base pair range was achieved. Quantification for sequencing was performed on the QuantiFluor fluorometer (Promega, Madison, WI, USA). Due to the 200 µL minimum volume required by the QuantiFluor Fluorometer (Promega) a modified protocol was used. A dilution series was prepared using the RL Standard from the Rapid Library Preparation Reagents & Adaptors Kit (Roche).

Each standard was vortexed and centrifuged briefly at each stage of the dilution. 50 µL of each sample was made up to 200 µL with TE buffer in order to obtain an accurate measurement using the fluorometer. The fluorometer was set to raw readings and utilised the blue channel. RFU values were input into the Rapid Library

Calculator (Roche, www.my454.com) which calculated the dilutions required to bring each sample to a working stock of 1 x 10^7 molecules/μL suitable for emulsion PCR (emPCR). Once quantified, libraries were pooled together and 2 μL was used for emPCR, which was carried out to manufacturer's guidelines as found in the emPCR Amplification Method Manual – Lib-L (Roche).

For MiSeq (Illumina, San Diego, CA, USA) library preparation the Nextera XT library preparation kit (Illumina) was used as per manufacturer's instructions.

Sequencing. Sequencing on the GS Junior system was performed to standard protocol as found in the GS Junior Sequencing Method Manual (Roche). Sequencing on the FLX+ system was performed in Mannheim, Germany by Roche.

Sequencing on the MiSeq was performed to standard protocol as found in the MiSeq System User Guide (Illumina) using 300 bp paired end reads.

Data analysis. 454 sequencing reads were demultiplexed and assembled separately into contigs using the Newbler version 3.0 assembly software (Roche). MiSeq (Illumina) sequencing reads were assembled via BaseSpace (Illumina) using the Velvet assembly tool v1.0.0 (Zerbino & Birney, 2008). Contigs were input into the Basic Local Alignment Search Tool (BLAST, Geer *et al.,* 2010) using the non-redundant (nr) nucleotide (nt) database searching against highly similar sequences (megablast). Resulting XML files were input into Metagenome Analyser v5.7.1 (MEGAN, Huson *et al.,* 2011) for taxonomic analysis using default settings. Multiple alignments were performed using Geneious v6.1.8 (http://www.geneious.com, Kearse *et al.,* 2012) against reference viral genomes indicated by MEGAN analysis.

Sequencing data. Sequence data was uploaded to MG-RAST (http://metagenomics.anl.gov/, Meyer *et al.,* 2008) and made publically available at the following link: http://metagenomics.anl.gov/linkin.cgi?project=17287. Accession numbers: 4689857.3 (VF13-188 E), 4689861.3 (VF14-91 A1), 4689859.3 (VF14-91 A2), 4689858.3 (VF14-92 A1), 4689855.3 (VF14-92 A2), 4689860.3 (VF14-181 A1), 4689856.3 (VF14-181 B1).

Results & Discussion

Enteric virome analysis. Sequencing results produced 2,036,415 high quality reads which were assembled into 64,232 contigs ranging between 61 – 3,176 bp with the majority of contigs ranging between 250 – 500 bp. **Short contigs were visually inspected for repetitive and low complexity sequences and removed where appropriate.** A total of 2,533 contigs were assigned to 20 DNA & RNA viral families (Table 1) along with 2 unclassified categories and were comprised of 31 distinct viral genera and 7 unclassified categories (Fig. 2). The remaining contigs were assigned to cellular organisms (bacteria, fungi, and avian species) or produced no hits against the BLAST nucleotide database.

Table 1. Number of contigs assigned to viral families from MEGAN taxonomic analysis.

	Viral Sequence Hits						
Name	**VF13-188 E**	**VF14-91 A1**	**VF14-91 A2**	**VF14-92 A1**	**VF14-92 A2**	**VF14-181 A1** [a]	**VF14-181 B1** [a]
Picornaviridae	232 (62.03%)	27 (3.03%)	16 (11.27%)	9 (1.60%)	63 (21.80%)	8 (12.31%)	20 (9.48%)
Caliciviridae	2 (0.53%)	52 (5.84%)	21 (14.79%)	0 (0%)	25 (8.65%)	13 (20.00%)	0 (0%)
Astroviridae	73 (19.52%)	192 (21.55%)	42 (29.58%)	0 (0%)	114 (39.45%)	7 (10.77%)	5 (2.37%)
Coronaviridae	0 (0%)	0 (0%)	4 (2.82%)	0 (0%)	8 (2.77%)	6 (9.23%)	2 (0.95%)
Reoviridae	0 (0%)	0 (0%)	0 (0%)	0 (0%)	3 (1.04%)	9 (13.85%)	2 (0.95%)
Parvoviridae	12 (3.21%)	2 (0.22%)	20 (14.08%)	1 (0.18%)	2 (0.69%)	16 (24.62%)	52 (24.64%)
Circoviridae	0 (0%)	0 (0%)	0 (0%)	2 (0.36%)	0 (0%)	0 (0%)	0 (0%)
Retroviridae	8 (2.14%)	3 (0.34%)	0 (0%)	45 (8.02%)	8 (2.77%)	1 (1.54%)	0 (0%)
Polyomaviridae	2 (0.53%)	0 (0%)	0 (0%)	0 (0%)	3 (1.04%)	0 (0%)	0 (0%)
Herpesviridae	41 (10.96%)	18 (2.02%)	4 (2.82%)	34 (6.06%)	49 (16.96%)	2 (3.08%)	6 (2.84%)
Leviviridae	0 (0%)	1 (0.11%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Bunyaviridae	0 (0%)	4 (0.45%)	0 (0%)	1 (0.18%)	2 (0.69%)	0 (0%)	0 (0%)
Siphoviridae	0 (0%)	173 (19.42%)	16 (11.27%)	157 (27.99%)	6 (2.08%)	1 (1.54%)	122 (57.82%)
Myoviridae	0 (0%)	419 (47.03%)	16 (11.27%)	212 (37.79%)	2 (0.69%)	2 (3.08%)	1 (0.47%)
Podoviridae	0 (0%)	0 (0%)	0 (0%)	28 (4.99%)	1 (0.35%)	0 (0%)	1 (0.47%)

Baculoviridae	0 (0%)	0 (0%)	0 (0%)	4 (0.71%)	1 (0.35%)	0 (0%)	0 (0%)
Inoviridae	0 (0%)	0 (0%)	0 (0%)	1 (0.18%)	0 (0%)	0 (0%)	0 (0%)
Phycodnaviridae	0 (0%)	0 (0%)	0 (0%)	4 (0.71%)	0 (0%)	0 (0%)	0 (0%)
Closteroviridae	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (0.35%)	0 (0%)	0 (0%)
Poxviridae	0 (0%)	0 (0%)	0 (0%)	8 (1.43%)	0 (0%)	0 (0%)	0 (0%)
Unclassified Phages	1 (0.27%)	0 (0%)	3 (2.11%)	55 (9.80%)	1 (0.35%)	0 (0%)	0 (0%)
Unclassified Viruses	3 (0.80%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Total	374	891	142	561	289	65	211

Percentage values were calculated for each sample according to the total number of viral contigs assigned per sample found at the bottom of each sample column.

[a]VF14-181 A1 & B1 represent unaffected samples.

Across all samples the most abundant viral families identified were the *Picornaviridae, Astroviridae*, *Caliciviridae, Parvoviridae, Herpesviridae, Siphoviridae,* and *Myoviridae* (Table 1). Within the affected samples the most abundant groups included the *Picornaviridae, Astroviridae, Siphoviridae,* and *Myoviridae.* The most abundant family identified in sample VF13-188 E was the *Picornaviridae* with over half of the total amount of contigs (62.03%) being assigned to this family and the second most abundant family being the *Astroviridae* with 19.52% of contigs being assigned (Table 1). This sample differed from all others due to the absence of any viral contigs assigned to the bacteriophage families *Siphoviridae, Myoviridae,* and *Podoviridae*. Sample VF14-91 A1 displayed the *Myoviridae* as the most abundant

family (47.03%) followed by the *Astroviridae* (21.55%) and the *Siphoviridae* (19.42%). The most abundant family associated with sample VF14-91 A2 was the *Astroviridae* (29.58%) followed by an even spread across the families *Picornaviridae* (11.27%), *Caliciviridae* (14.79%), *Parvoviridae* (14.08%), *Siphoviridae* (11.27%), and *Myoviridae* (11.27%). Sample VF14-92 A1 differed compared to all other samples with no viral contigs assigned to the *Astroviridae* and *Caliciviridae* and only a small number of viral contigs associated with the *Picornaviridae* (1.60%). Within this sample the most abundant families were the *Myoviridae* (37.79%) and the *Siphoviridae* (27.99%). Additionally, this sample had the largest amount of *Retroviridae* contigs associated with it (8.02%) compared to all other samples. Sample VF14-92 A2 showed a similar viral profile to that of sample VF13-188 E as the most abundant viral families were the *Astroviridae* (39.45%) and the *Picornaviridae* (21.80%) closely followed by the *Herpesviridae* (16.96%).

Within the unaffected sample set, sample VF14-181 A1 displayed the *Paroviridae* as the most abundant family with 24.62% of the viral contigs being thus assigned. This was followed by the *Caliciviridae* (20.00%) which was most abundant in this sample compared to all others (Table 1). This sample also displayed a relatively even spread across the families *Picornaviridae* (12.31%), *Astroviridae* (10.77%), *Coronaviridae* (9.23%), and *Reoviridae* (13.85%). The most abundant family identified in sample VF14-181 B1 was the *Siphoviridae* with over half the viral contigs being assigned to this family (57.82%). This sample was also very similar to the other unaffected sample, VF14-181 A1, in regards to the *Parvoviridae* family with 24.64% of the viral contigs being associated with this family (compared to 24.62% associated with sample VF14-181 A1). The unaffected samples displayed a larger abundance of *Parvoviridae* contigs compared to the affected samples while the affected samples

displayed a larger abundance of *Picornaviridae* and *Astroviridae* compared to the unaffected samples (Table 1). A higher abundance of the bacteriophage families was observed in the affected samples compared with the unaffected samples.

Noticeably, there was an absence or low abundance of *Reoviridae* contigs across all samples even though reoviruses have been strongly associated with RSS. Otto *et al.* (2006) described the presence of reoviruses via RT-PCR from 32/34 (94%) of RSS-affected broiler chicks tested and also from 2/7 (29%) of 'healthy' control birds tested. The birds tested in the 2006 study ranged from 5 – 14 days of age compared to the present study which tested 2 – 3 week old birds. As reoviruses are quite commonly detected in young broiler chicks it may be possible that any reovirus infection has largely cleared from these older birds and is present in relatively low abundance accounting for the absence of *Reoviridae* contigs in the majority of samples (Table 1).

RNA virome analysis.

Picornaviridae. Members of the family *Picornaviridae* (order: *Picornavirales*) were detected across all 7 (100%) samples with a total of 375 viral contigs assigned to this family (Table 1). The majority of viral contigs (293) in this metagenomic analysis were assigned to the unclassified *Picornaviridae* category with the remaining contigs (82) assigned to 3 recognised genera – *Gallivirus, Kobuvirus,* and *Megrivirus* (Fig. 2). Within these genera, viral contigs showed similarity to multiple species such as chicken gallivirus 1 (*Gallivirus*), aichivirus C (*Kobuvirus*), and chicken megrivirus (*Megrivirus*). A total of 217 contigs characterised as unclassified *Picornaviridae* showed similarity to the species sicinivirus 1, a novel picornavirus isolated from

commercial broiler chickens in Cork, Ireland (Bullman *et al.,* 2014) and recently reported in mainland China (Zhou *et al.,* 2015). Sicinivirus 1 contigs were almost exclusively associated with RSS-affected samples with only one sicinivirus 1 contig associated with unaffected sample VF14-181 A1. Sicinivirus 1 was commonly found alongside chicken megrivirus, chicken picornavirus 1, 4, and 5, and aichivirus C however the pathogenic potential of sicinivirus 1 is yet to be determined. The remaining viral contigs (76) associated with the unclassified *Picornaviridae* displayed similarity to chicken picornavirus 1, 4, and 5, picornavirus chicken/CHK1/USA/2010, pigeon picornavirus B, and aichivirus C. The species chicken picornavirus 1, 4, 5, chicken megrivirus, and aichivirus C were only associated with the affected samples compared to picornavirus chicken/CHK1/USA/2010, pigeon picornavirus B, and chicken gallivirus 1 which were found in both affected and unaffected samples. Members of the *Picornaviridae* are characterised by non-enveloped icosahedral virions, around 30 nm in diameter, containing a single-stranded positive-sense RNA (ssRNA(+)) genome, 7 – 9 kb in length (Stanway, 1990; LeGall *et al.,* 2008). Picornaviruses have been linked to disease across multiple species (such as sheep, cattle, humans, felines, and birds) and have been implicated in enteric disease (Knox *et al.,* 2012; Tapparel *et al.,* 2013). Previous metagenomic studies into the avian gut microbiome have identified multiple picornavirus species in disease-affected and healthy birds, findings which have been echoed in the present study. However, the pathogenic potential of these viruses in avian species remains relatively unknown due to the large amount of genetic variation observed among these viruses (Day *et al.,* 2010; Farkas *et al.,* 2012; Bullman *et al.,* 2014; Day *et al.,* 2015a,b; Zhou *et al.,* 2015). In the present study picornaviruses were commonly found alongside the *Astroviridae, Caliciviridae, Parvoviridae,* and bacteriophage families (Table 1) and

seem to be prevalent in growth-stunted birds. Due to the large number of picornavirus strains in circulation it is perhaps unsurprising that picornavirus contigs were also detected in the unaffected samples and is possible that non-pathogenic picornavirus strains may be constituents of a healthy avian gut virome.

Coronaviridae. Members of the family *Coronaviridae* (order: *Nidovirales*) were detected in 4/7 samples and were found in both affected (2/5) and unaffected (2/2) samples although in relatively low numbers with a greater number of contigs found in samples VF14-92 A2 and VF14-181 A1 (Table 1). All viral contigs (20) assigned to the *Coronaviridae* family belonged to the genus *Gammacoronavirus* (Fig. 2) with all 20 of the viral contigs displaying high similarity (98 – 100% nucleotide identity) to infectious bronchitis virus (IBV). Although both unaffected samples were observed to also have IBV contigs, this may be due to the small sample set tested. Members of this family are characterised by enveloped, positive-stranded RNA genomes, 26 – 32 kb in length (Brian & Baric, 2005; Gorbalenya *et al.,* 2006). Coronaviruses have been detected in a variety of wild animals and are responsible for mild to severe respiratory symptoms, central nervous system diseases, and gastrointestinal diseases (Gallagher & Buchmeier, 2001; Weiss & Navas-Martin, 2005; Weiss & Leibowitz, 2011). The main coronavirus affecting chickens is IBV which can cause respiratory disease and can also replicate in non-respiratory areas such as enteric tissues. Although IBV is not typically associated with enteric disease it may contribute to enteritis in combination with other microbial factors (Cavanagh & Gelb Jr., 2003; Cavanagh, 2007; Jackwood *et al.,* 2012).

Caliciviridae. A total of 113 viral contigs belonging to the *Caliciviridae* family were detected in 5/7 of the tested samples and were found in 4/5 affected samples and 1/2 unaffected samples. A total of 98 viral contigs displayed similarity to calicivirus

isolates calicivirus chicken/V0021/Bayern/2004 (85 – 96% nucleotide identity), calicivirus chicken/V0027/Bayern/2004 (90 – 93% nucleotide identity), and calicivirus chicken/V0013/Bayern/2004 (89% nucleotide identity) with the remaining contigs (15) showing similarity to chicken calicivirus isolates chicken/L11038 polyprotein gene (90 – 98% nucleotide identity), chicken/L11041 polyprotein gene (91 – 96% nucleotide identity), and caliciQ45/2013 polyprotein gene (81 – 93% nucleotide identity). The *Caliciviridae* family is comprised of 5 recognised genera (*Lagovirus, Nebovirus, Norovirus, Sapovirus, and Vesivirus*) and an unclassified *Caliciviridae* genus with the *Caliciviridae* contigs in this study belonging to the unclassified *Caliciviridae* genus. Virions belonging to the *Caliciviridae* family are typically non-enveloped, around 30 – 40 nm in diameter, and with an RNA genome of around 7.5 kb in length (Thiel & König, 1999). The *Caliciviridae* family can infect a variety of host organisms including humans, birds, pigs, cattle, and avian species and have been associated with gastroenteritis in humans as early as the 1970s (Kapikian *et al.,* 1972; Smith *et al.,* 1977; Chen *et al.,* 2006; Wolf *et al.,* 2009). Calicivirus has been detected and characterised from both healthy chickens and chickens displaying growth retardation via electron microscopy, reverse transcriptase polymerase chain reaction (RT-PCR) and next-generation sequencing (Day *et al.,* 2010; Wolf *et al.,* 2011; Day *et al.,* 2015a). A previous metagenomic analysis of SPF and SPF sentinel birds performed by Day *et al.* (2015a) described the majority of viral contigs (11,309) associated with the SPF flock being assigned to the *Caliciviridae* family (99.05%) with the virus appearing to clear from sentinel birds placed on commercial farms with enteric and respiratory problems although testing of a backyard sentinel flock, with a history of enteric disease, identified 7819 contigs (25.55%) assigned to the *Caliciviridae* family. Conversely, in the present study, members of the *Caliciviridae*

were more commonly associated with RSS-affected samples with the most common isolate being calicivirus chicken/V0021/Bayern/2004, the same isolate described by Day *et al.* (2015a) in the SPF and backyard flocks, this isolate was also detected in unaffected sample VF14-181 A1. Contigs displaying similarity to the isolates L11038, L11041, Q45, and calicivirus chicken/V0027/Bayern/2004 were only found in RSS-affected samples possibly suggesting and association with enteric disease while isolate calicivirus chicken/V0013/Bayern/2004 was only associated with unaffected sample VF14-181 A1 and not in affected samples. Interestingly, there were no *Caliciviridae* contigs associated with affected sample VF14-92 A1 (Table 1).

Astroviridae. The present study has identified 433 viral contigs displaying similarity (81 – 100% nucleotide identity) to the *Avastrovirus* genus (Table 1, Fig. 2), specifically the species chicken astrovirus (CAstV) and avian nephritis virus (ANV) sero- or genotypes 1 (ANV 1), 2 (ANV 2), and 3 (ANV 3). Members of the *Avastrovirus* genus, especially ANV and CAstV, are strongly associated with RSS and the current study reports a much higher viral profile associated with this genus in RSS-affected samples with much lower profiles being observed in unaffected samples (Fig. 2). Contigs associated with the *Astroviridae* were commonly found in combination with the *Caliciviridae, Picornaviridae,* and *Parvoviridae* families (Table 1) which has been described in previous metagenomic studies in chickens and turkeys (Day *et al.,* 2015a,b). Viral contigs assigned to the chicken astrovirus and ANV 1 species were only associated with the affected sample set. Viral contigs associated with the ANV 2 and ANV 3 species were associated with both affected and unaffected sample sets with a notably larger ANV 2 profile associated with the affected samples. This may suggest greater ANV 2 strain diversity within the affected samples with certain strains exerting a greater pathogenic effect leading to stunted

growth. Day *et al.* (2015a) described a very low *Astroviridae* profile associated with a SPF flock (2 contigs, 0.02%). By contrast, Day reported increased levels of astrovirus contigs (1.14 – 98.97%) associated with sentinel SPF birds placed on commercial farms with enteric problems indicating that members of the *Astroviridae* family are more commonly found in flocks with growth problems and enteric disease. The *Astroviridae* family contains a group of enteric viruses which can infect multiple mammalian hosts (Genus: *Mamastrovirus*) and avian hosts (genus: *Avastrovirus*) causing a range of enteric disease symptoms such as diarrhoea, enteritis, interstitial nephritis and has been linked with visceral gout and RSS (Yamaguchi *et al.,* 1979; McNulty *et al.,* 1984; Herrmann *et al.,* 1991; Greenberg & Matsui, 1992; Moser & Schultz-Cherry, 2005; Spackman *et al.,* 2010; Benedictis *et al.,* 2011; Lee *et al.,* 2013). First identified in 1975 (Madeley & Cosgrove, 1975) *Astroviridae* are characterised as small, non-enveloped virions with a positive-sensed RNA genome around 7 kb in length (Jiang *et al.,* 1993; Carter, 1994; Willcocks *et al.,* 1994). The *Avastrovirus* genus contains at least 6 recognised species – avian nephritis virus (ANV), chicken astrovirus (CAstV), duck astrovirus types 1 & 2 (DAstV), and turkey astrovirus types 1 & 2 (TAstV). Previous studies have identified astroviruses in both growth stunted and healthy avian hosts (Reynolds *et al.,* 1987; Baxendale & Mebatsion, 2004; Pantin-Jackwood *et al.,* 2006; Kang *et al.,* 2012).

Reoviridae. Avian Orthoreoviruses (ARV) can be present without disease in broiler chickens however reovirus infection can lead to several disease symptoms such as tenosynovitis, growth suppression, and enteritis and can also cause immunosuppression leaving the host susceptible to other infections (Hieronymus *et al.,* 1982; Jones & Kibenge, 1984; Sharma *et al.,* 1994 Jones, 2000). Although

reoviruses are widespread among avian hosts, the present study reports detection of reovirus in only one sample (VF-14 181 A1) which represents a healthy flock. The low detection rate of ARV may be a consequence of the small sample set tested or may suggest their relative abundance is low compared to other viruses. Reovirus contigs obtained from sample VF14-181 A1 were assigned to the *Orthoreovirus* genus (Fig. 2, species: *Avian orthoreovirus*) and displayed homology to the L1, L2, L3, S1, and S4 genome segments and the *lambdaB* core protein gene. The family *Reoviridae,* consisting of 2 subfamilies comprising of 15 genera, contains a group of viruses with a wide host range including invertebrates, vertebrates, plants, and fungi and has been described in both RSS-affected broiler chickens and healthy chickens (Robertson *et al.,* 1984) and has been detected in previous avian enteric metagenomic studies (Day *et al.,* 2010, 2015a). Reoviruses are non-enveloped viruses that contain a segmented (10 – 12 segments) double-stranded RNA genome (Joklik, 1981; Gouet *et al.,* 1999; Forrest & Dermody, 2003).

Retroviridae. The *Retroviridae* family is comprised of 2 subfamilies (*Orthoretrovirinae* and *Spumaretrovirinae*) and contains 7 viral genera and has a large host range. This study has detected the presence of 1 viral contig from one sample, VF14-91 A1 (Table 1), displaying similarity (100% nucleotide identity) to avian erythroblastosis virus (AEV, genus: *Alpharetrovirus*) *erbA* and *erbB* genes. Additionally, the same sample had one viral contig displaying 98% nucleotide similarity to the avian endogenous retrovirus *gag* gene. Endogenous viral elements represent either entire viral genomes or fragments of viral genomes which have become integrated into the host germ line. The remaining viral contigs (63) from the MEGAN analysis displayed short sequence similarity, 30 – 90 bp (from contigs

around 250 - 400 bp in length), to human endogenous retroviruses and the *Lentivirus* genus. Upon analysis the full contigs displayed homologies to various bacterial genera such as *Salmonella, Escherichia,* and *Bacteroides.* Retroviruses reverse transcribe their RNA genome into DNA using their own reverse transcriptase followed by integration into the host genome where they replicate using the host polymerase genes (Dahlberg, 1988; Coffin, 1992; Luciw & Leung, 1992; Gifford & Tristem, 2003). Previous studies have described the presence of endogenous avian retroviruses (EAVs) within domesticated chickens which are vertically transmitted through the host germ line following genomic integration (Frisby & Weiss, 1979). Although not normally linked to disease EAVs have been related to subgroup J avian leukosis virus (ALV), an exogenous avian retrovirus, which has been reported to induce myeloid leukosis, can cause mortality through the development of tumours, and can cause immunosuppression within the host (Purchase *et al.,* 1968; Friedman & Ceglowski, 1971; Fadly & Smith, 1998; Payne, 1998; Smith *et al.,* 1998). It is possible that the presence of avian retroviruses in RSS-affected samples is not directly contributing to enteric disease however the immunosuppression caused by these viruses may leave affected birds susceptible to infection from other viral species.

DNA virome analysis.

Parvoviridae. Parvoviruses were detected in 7/7 samples tested with the all of the viral contigs displaying similarities to either the *Aveparvovirus* genus (90 – 100% nucleotide identity), specifically chicken parvovirus strains, and the *Protoparvovirus* genus (88 – 100% nucleotide identity), specifically to the NS1, NP1, VP1, and VP2

genes of the *Protoparvovirus* genus, isolates ParvoD11/2007 and ParvoD62/2013, and the NS1 gene of chicken parvovirus Ch_114_10 and chicken parvovirus isolates Ch841_3/2009/HUN and Ch538/2009/HUN. Sample VF14-181 A1, representing a healthy flock, contained a combination of avian orthoreovirus (strains T1781, AVS-B, 1017-1 and 138) and chicken parvovirus ABU-P1 (Table 1) mirroring a study by Decaesstecker *et al.* (1986) in which chicken parvovirus ABU-P1 in combination with reoviruses failed to cause growth retardation in day old chicks following oral inoculation. Conversely, an earlier study isolated chicken parvovirus ABU-P1 from chickens with stunted growth and re-inoculation of embryos and day old SPF chicks with this isolate caused enteritis, a decrease in egg hatchability, and severe growth retardation (Kisary, 1985). All affected samples contained parvovirus contigs although a substantially larger *Parvoviridae* profile was observed in the unaffected samples compared to the affected samples. Both affected and unaffected samples contained a combination of sequences from members of the *Protoparvovirus* and *Aveparvovirus* genera however the affected samples contained more viral contigs assigned to the *Protoparvovirus* genus while the unaffected samples contained more viral contigs assigned to the *Aveparvovirus* genus (species: chicken parvovirus ABU-P1). The *Paroviridae* family is divided in to 2 sub families (*Densovirinae* and *Parvovirinae*) that contain 13 genera between them and are characterised as having a non-enveloped capsid containing a single stranded DNA genome around 5 kb in length (Berns, 1990; Cotmore & Tattersall, 1995, 2005).

Circoviridae. The *Circoviridae* family contains 2 distinct genera – *Gyrovirus* and *Circovirus.* They are characterised by a circular non-segmented single-stranded DNA genome, around 1.7 kb to 2.4 kb in length (Finsterbusch & Mankertz, 2009).

The present study detected 2 viral contigs exhibiting similarity (96 – 99% nucleotide identity) to the *Gyrovirus* genus in only one RSS-affected sample, VF14-92 A1, which specifically showed similarity to chicken anaemia virus (CAV, genus: *Gyrovirus*) while the unaffected samples displayed no *Circoviridae* contigs. The circoviruses can infect a range of birds and mammals such as chickens, pigs, dogs, geese, and pigeons (Yuasa *et al.,* 1979; Tischer *et al.,* 1982; Todd *et al.,* 2001; Kapoor *et al.,* 2012). . McNulty *et al.* (1991) described the effects of subclinical CAV infection in broiler chickens resulting in a decrease in profitability and production - specifically a decrease in the average weight per bird and an adverse effect on feed conversion ratio theorised to be caused by the immunosuppressive capabilities of CAV. CAV was later detected in 4 week old growth stunted birds in combination with *Cryptosporidium baileyi* (Dobos-Kovács *et al.,*1994) and has been detected in broiler chickens displaying lymphocyte depletion (Van Santen *et al.,* 2001) further indicating a role in immunosuppression. Rosenberger & Cloud (1998) also reported that the immunosuppressive aspects of CAV frequently lead to secondary infections with *Clostridium perfringens* and *Staphylococcus aureus.* This immunosuppression, in combination with other viral pathogens, may contribute to the development of RSS. Although CAV was only detected in one sample it is possible that the infection had largely cleared from these 2 – 3 week old birds and could have been detected if tested at an earlier age. Additionally, it is possible that CAV was present in samples in relatively low abundance. Throughout the virus enrichment process during sample preparation there were losses associated with each of the processing stages. It is possible that the less abundant CAV has been lost in these processing stages therefore not detected in the final sequencing results.

Caudovirales. The *Caudovirales* order contains tailed bacteriophages and is comprised of the families *Siphoviridae*, *Myoviridae*, and *Podoviridae* and were detected in all samples tested (Table 1). Additionally, the families *Leviviridae, Inoviridae,* and the unassigned phage category (Table 1) were comprised of phage sequences. Within the affected samples there was a large *Caudovirales* profile associated with samples VF14-91 A1 and VF14-92 A1 (over 50% of the viral contigs for each sample) and relatively little *Caudovirales* contigs associated with VF14-91 A2 and VF14-92 A2 (Table 1). Interestingly, affected sample VF13-188 E contained no *Caudovirales* contigs. Unaffected sample VF14-181 A1 had a very small number of contigs (3) associated with the *Caudovirales* while unaffected sample VF14-181 B1 had a large *Caudovirales* profile (over 50% viral contigs were assigned to the *Caudovirales*). In the present study the samples had an overall total of 1,165 viral contigs assigned to phage species with the majority of contigs being assigned to the families *Siphoviridae* (475 contigs) and *Myoviridae* (652 contigs) (Table 1) making the bacteriophages the most abundant species identified in the current study (45.99% of total viral contigs). Sample VF14-92 A1 had 28 viral contigs assigned to the *Podoviridae* family. Sample VF13-188 E displayed no similarity to any of the bacteriophage families but had one viral contig identified as an unclassified phage (Table 1). This was similar to samples VF14-92 A2 and VF14-181 A1 which displayed a very low amount of phage contigs (3.82% and 4.62% respectively) compared to the large abundance of phage contigs identified in samples VF14-91 A1 (67.01%), VF14-91 A2 (24.65%), VF14-92 A1 (81.64%), and VF14-181 B1 (58.76%). An increase in viral contigs associated with the *Caudovirales* may coincide with an overgrowth of specific groups of intestinal bacteria which are then infected by species specific bacteriophages helping regulate the intestinal microbiota. The

majority of the viral contigs assigned to the *Caudovirales* were identified as enterobacteria phages, enterococcus phages, and bacteroides phages which relate to normal bacterial constituents of the chicken gastrointestinal tract such as *Escherichia coli*, *Enterococcus* species, and *Bacteroides* species (Devriese *et al.,* 1991; Lu *et al.,* 2003; Amit-Romach *et al.,* 2004). These viruses are widespread and co-exist with bacterial populations across a large range of hosts and environments and aid in regulating the diversity, population, and function of microbial communities (Riesenfeld *et al.,* 2004). The role of bacteriophages in relation to RSS is unclear and represents an interesting group for further study since previous metagenomic studies have also detected members of the *Caudovirales* order as part of the broiler chicken gut microbiota in growth retarded flocks (Kim & Mundt, 2011; Day *et al.,* 2015a).

Herpesviridae. Of the remaining viral families 154 contigs were assigned to the *Herpesviridae* family. Samples VF13-188 E and VF14-92 A2 displayed a higher *Herpesviridae* abundance (10.96% and 16.96% respectively) compared to all other samples which displayed a more even spread (2.02 – 6.06%). Contigs assigned to this family displayed similarity to the *Cytomegalovirus* genus (Fig. 2), with 143 contigs displaying similarity to the *Ceropithicine herpesvirus 5* species. Within this species all contigs assigned displayed greatest similarity (77 – 100% nucleotide identity) to stealth virus 1. Additionally, the small number of contigs in the 'unclassified virus' category (Table 1, sample VF13-188 E) contained 3 contigs displaying high similarity to the species stealth virus 4 and 5 (95 – 100% nucleotide identity). Stealth virus 1 has previously been isolated from human hosts displaying symptoms such as chronic fatigue (Martin *et al.,* 1995) and encephalopathy (Martin,

1996) however no studies have been conducted in relation to avian hosts but it has been shown to be transmissible to dogs and cats (Martin & Anderson, 1997). This study appears to be the first study to report the presence of stealth virus in RSS-affected broiler chickens however further studies must be undertaken to understand the role this virus plays in disease.

Most previous investigations into the enteric viruses associated with poultry have been limited to culture-dependant methods and molecular assays targeting previously known viruses. The use of high-throughput next generation sequencing in this study, and a small number of other studies, presents a viable technique for the characterisation of the complex viral communities present in the gastrointestinal tract of disease-affected and unaffected broiler chickens. This study presents preliminary data on the viral communities present in the poultry gut from a small number of samples and has identified multiple viral families historically associated with RSS co-infecting broiler guts, along with the broad characterisation of other viral families such as the *Siphoviridae, Myoviridae,* and *Podoviridae* in relation to the disease. Additionally, this study attempts to characterise the virome associated with unaffected broiler chickens with the results showing a difference in the viral contigs assigned in the unaffected samples – typically many of the same families are observed in unaffected samples but with a lower number of viral contigs assigned to these families when compared to affected samples. The main viral families related to RSS-affected samples from this study included *Astroviridae, Caliciviridae, Picornaviridae, Parvoviridae, Coronaviridae, Siphoviridae,* and *Myoviridae* **although many of these viral families were also found in unaffected samples indicating that certain strains within these families may be constituents of a normal**

broiler gut virome. One of the advantages of the sequencing platforms used is their ability to be semi-quantitative; the sequencing reads and contigs output by the platforms are assumed to be representative of the microbial contents within a tested sample. However, the use of whole genome and whole transcriptome amplification steps in the sequencing procedures have previously been shown to introduce bias to sequencing results (Pinard *et al.,* 2006) resulting in a non-quantitative analysis. The present study had to use whole genome and whole transcriptome amplification methods to generate sufficient viral genomic material from samples for library preparation and as such these results cannot be considered quantitative however the kit used to perform these procedures was shown to generally minimise bias when compared to other similar kits (Pinard *et al.,* 2006). Furthermore it might be assumed that any bias from whole genome amplification methods may apply equally across all samples although further bias may be subsequently introduced by library preparation methods specific to each platform or kit. **Subsequent quantitative studies currently underway via quantitative real-time PCR (qPCR)** will help clarify the roles these viruses play in RSS and would aid in understanding the differences in viral load associated with each virus. **These studies** will also help characterise the differences between affected and unaffected birds leading to a greater knowledge of the key viral agents associated with RSS.

Acknowledgements

The authors would like to acknowledge the Department of Agriculture and Rural Development for Northern Ireland (DARD) for funding this study.

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Figure captions.

Figure 1. MEGAN taxonomic analysis displaying a viral family comparison between all 7 samples. VF14-181 A1 & B1 (*) represent unaffected samples. The “Viruses” and “dsDNA viruses, no RNA stage” categories contained viral contigs from the families *Siphoviridae, Myoviridae, Podoviridae, Herpesviridae, Reoviridae, Retroviridae, Polyomaviridae, Inoviridae, Baculoviridae, and Poxviridae.* These unassigned contigs were accounted for in Table 1. Bars located next to each taxon are proportional to the total number of contigs assigned to each category from sequencing runs.

Figure 2. MEGAN taxonomic analysis displaying a viral genera comparison between all 7 samples. VF14-181 A1 & B1 (*) represent unaffected samples. Bars located next to each taxon are proportional to the total number of contigs assigned to each category from sequencing runs.