Virus Research 261 (2019) 9–20

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

The intestinal virome of malabsorption syndrome-affected and unaffected broilers through shotgun metagenomics

Diane A. Lima[a,*], Samuel P. Cibulski[b], Caroline Tochetto[a], Ana Paula M. Varela[a], Fabrine Finkler[a], Thais F. Teixeira[a], Márcia R. Loiko[a], Cristine Cerva[c], Dennis M. Junqueira[d], Fabiana Q. Mayer[c], Paulo M. Roehe[a]

[a] Laboratório de Virologia, Departamento de Microbiologia, Imunologia e Parasitologia, Instituto de Ciências Básicas da Saúde (ICBS), Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil
[b] Laboratório de Virologia, Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil
[c] Laboratório de Biologia Molecular, Instituto de Pesquisas Veterinárias Desidério Finamor (IPVDF), Eldorado do Sul, RS, Brazil
[d] Centro Universitário Ritter dos Reis - UniRitter, Health Science Department, Porto Alegre, RS, Brazil

ARTICLE INFO

Keywords:
Enteric disorders
Virome
Broiler chickens
High-throughput sequencing

ABSTRACT

Malabsorption syndrome (MAS) is an economically important disease of young, commercially reared broilers, characterized by growth retardation, defective feather development and diarrheic faeces. Several viruses have been tentatively associated to such syndrome. Here, in order to examine potential associations between enteric viruses and MAS, the faecal viromes of 70 stool samples collected from diseased (n = 35) and healthy (n = 35) chickens from seven flocks were characterized and compared. Following high-throughput sequencing, a total of 8,347,319 paired end reads, with an average of 231 nt, were generated. Through analysis of *de novo* assembled contigs, 144 contigs > 1000 nt were identified with hits to eukaryotic viral sequences, as determined by GenBank database. A number of known and unknown representatives of *Adenoviridae*, *Anelloviridae*, *Astroviridae*, *Caliciviridae*, *Circoviridae*, *Parvoviridae*, *Picobirnaviridae*, *Picornaviridae* and *Reoviridae*, as well as novel uncharacterized CRESS-DNA viruses, were identified. However, the distribution of sequence reads of viral genomes identified in diseased or healthy birds revealed no statistically significant differences. These findings indicate no association between the occurrence of MAS and enteric viruses. The viral genomes reported in the present study, including a variety of novel viruses, seem part of the normal intestinal microbiota of chickens.

1. Introduction

Malabsorption syndrome (MAS), also known as runting-stunting syndrome, is an economically important disease of broilers characterized by growth retardation, defective feather development and diarrhea (Songserm et al., 2002). The condition has been reported in chickens worldwide since the seventies (Sellers et al., 2010). Its economic impact is primarily related to inefficient food conversion, decreased meat production, immune dysfunctions and increased mortality rates (Day and Zsak, 2013).

For decades, efforts have been made in attempting to identify the aetiology of MAS. However, to date, the causative agent (or agents) remains undetermined (Zsak et al., 2013). Although bacteria, raising conditions and environmental factors seem associated to disease development, viruses have been pointed out as major players in MAS aetiology (Day et al., 2010; Kang et al., 2012). Among these, astroviruses (Baxendale and Mebatsion, 2004; Pantin-Jackwood et al., 2008, 2011), parvoviruses (Day and Zsak, 2010; Koo et al., 2015; Zsak et al., 2009, 2008), reoviruses (Chen et al., 2014; Songserm et al., 2000) and rotaviruses (Otto et al., 2006; Spackman et al., 2010) have, on different occasions, been linked to the occurrence of MAS. However, such viruses can be detected in both healthy and diseased birds, making it difficult to establish direct causal relationships (Devaney et al., 2016; Palade et al., 2011).

More recently, viral metagenomics has been successfully applied to characterize viral populations in different hosts and environments (Bexfield and Kellam, 2011; Rosario and Breitbart, 2011). These studies have allowed discovery of a impressive numbers of previously unknown agents in various tissues and organs, including the gastrointestinal tract of broilers and other animals (Cheung et al., 2015, 2013; Day et al., 2010, 2015; Kim et al., 2012; Lima et al., 2017; Ng et al., 2015a; Shah et al., 2014; Shan et al., 2011; Zhang et al., 2014). Metagenomics has

* Corresponding author.
E-mail address: diane.lima@yahoo.com.br (D.A. Lima).

https://doi.org/10.1016/j.virusres.2018.12.005
Received 8 August 2018; Received in revised form 6 December 2018; Accepted 8 December 2018
Available online 10 December 2018

also been employed to investigate the etiology of diseases where no previously defined causative agents had been reported (Cox-Foster et al., 2007; Mihalov-Kovacs et al., 2014; Victoria et al., 2008, 2009). Here, metagenomics was employed to characterize the intestinal virome of chickens with and without MAS, in a case-control study. Faecal samples were collected from diseased and healthy birds, raised under the same conditions, and examined comparatively.

2. Results

2.1. Viral metagenomics

Faecal samples from MAS-affected chickens (n = 35) and healthy controls (n = 35) were pooled according to the farm of origin and clinical condition (named G1 to G7 "R", runt, or "H", healthy), resulting in 14 pool. The randomly enriched nucleic acids of each pooled sample were used to prepare the viral metagenomic libraries. A total of 8,347,319 paired end reads, with an average of 231 nt, were generated using the Miseq sequencing platform (Table S1, available in the online Supplementary Material). Through analysis of *de novo* assembled contigs, 144 contigs > 1000 nt were identified with hits to eukaryotic viral sequences, as determined by BLASTx analysis. The percentage of eukaryotic virus reads detected in each group was 22.1% in the MAS-affected against 14.5% in the healthy group. Sequences identified were assigned to nine known viral families, including *Adenoviridae*, *Anelloviridae*, *Astroviridae*, *Caliciviridae, Circoviridae*, *Parvoviridae*, *Picobirnaviridae*, *Picornaviridae* and *Reoviridae*. In addition, a range of novel CRESS-DNA viruses was also identified. Their relative frequencies in each faecal pooled sample from MAS-affected and healthy chickens are shown in Fig. 1a. In addition, Fig. 1b shows the distribution of the viral reads related to members of the *Picornaviridae* family, which were detected in greater numbers in the present study.

2.2. Evaluation of associations between viruses and MAS

In order to determine the numbers of reads corresponding to the sequence of a specific viral genome (or genome fragment), viral contigs were used to match reads in each pool. For this, contigs were identified with hits to eukaryotic viral sequences following BLASTx analysis. Next, very closely related contigs were concatenated and used to enumerate matching raw reads in samples from diseased and healthy birds.

Differences in distributions of viral reads matching the different eukaryotic viruses found in this study were compared using Mann-Whitney tests (Fig. 2). In the MAS-affected group, higher numbers of viral reads were identified for megrivirus and sicinivirus, followed by rotavirus D, astroviruses, galliform aveparvovirus, avian adeno-associated virus, chicken-associated cyclovirus 2 (ChCyV 2) and fowl aviadenoviruses (blue bars in Fig. 2).

In healthy animals, a greater amount of CRESS-DNA virus and DuCyV reads were detected, as well as gyrovirus, chicken picobirnavirus, rotavirus F and chicken calicivirus (red bars in Fig. 2). However, no statistical significance (p-value < 0.05) was detected when comparing the amount of reads for each of the viruses between MAS-affected and healthy broilers (Fig. 2).

In order to assess the relatedness and the overall taxonomic similarities between genomic sequences identified in diseased and healthy animals, hierarchical clustering analyses was performed. The hierarchical clustering analysis on both groups resulted in a dendrogram with intermingled branches, evidenced no clear separation between samples from either diseased or healthy groups of birds. Additionally, a heat map data generated with the relative amount of reads did not reveal any distinct pattern that could be - positively or negatively - associated to either diseased or healthy groups of birds (Fig. 3).

2.3. Complete and partial viral genome sequences

Sequences corresponding to complete or partial genomes of putative viruses identified in diseased and healthy birds were selected for further analysis. The selection of viral genomes was based on sequences that either represent novel genotypes not previously been detected in the country, or sequences with a significant divergence (< 90% aa identity) to previously described viruses. Next, a brief report is provided on the sequences recovered from such selected viruses identified in MAS-affected and healthy birds.

2.3.1. Adenoviridae

Ten contigs > 1000 nt matched to different regions of fowl aviadenovirus D (FAVD) genome were identified in this study. These contigs ranged from 1017 to 2422 nt in length and displayed among 93 to 100% aa identity to other FAVD deposited in GenBank, as shown in Table S2. Sequence analysis of DNA polymerase confirms its close relationship with other FAVD sequences (Fig. S1).

In addition, forty-six contigs < 1000 nt-long related to FAV E (FAVE) were found (data not shown). These contigs were concatenated and used to measure the number of matching reads.

2.3.2. Anelloviridae

Four gyrovirus-related contigs > 1000 nt were recovered (Fig S2a and Table S2). The first contig corresponds to a complete genome of chicken anemia virus (CAV); this was shown to share 99% deduced aa identity to the VP1 protein of a CAV genome of Chinese origin (GenBank no. ac. KU645522). The second contig represents the full genome of avian gyrovirus 2 (AGV2) with 99% identity to the putative nucleocapsid of the prototype sequence (GenBank no. ac. NC_015396). Two other contigs matched to gyrovirus 4 (GyV4) and showed 97–99% identity to VP1 protein of a GyV4 recently reported in chicken faeces (GenBank no. ac. KY024580). Phylogenetic analysis of the VP1 nucleotide sequences confirmed the close relationship between gyroviruses reported here and its previously described counterparts (Fig. S2b).

2.3.3. Astroviridae

Nine contigs were detected with similarity to genomes of members of the *Avastrovirus* genus, specifically to avian nephritis virus (ANV) and chicken astrovirus (CAstV), which currently comprise the *Avastrovirus* 1 species (Smyth, 2017). One complete ANV genome (RS/BR/15/6R, 6,890 nt-length) was recovered, with three open reading frames (ORFs) putatively coding for a nonstructural protein, a RdRp and a capsid protein (Fig. S3a). Such viral genome (ANV RS/BR/15/6R) showed 78% aa identity to the non-structural polyprotein of pigeon avian nephritis virus (GenBank no. ac. HQ889774). Two shorter sequences, corresponding to the partial CDS of ANV non-strutural and capsid proteins, were also identified (Table S2, Fig. S3a).

The CAstV-related contigs (n = 6) were 1425 to 3204 nt long and consist of full or partial CDS of capsid and non-structural proteins. The genetic maps of these sequences are displayed in Fig. S3a.

Contigs showing identity to the capsid protein of CAstV were selected to build a phylogenetic tree. The ML phylogenetic analysis supports the classification of the avastroviruses reported in this study at the species level (Fig. S3b).

2.3.4. Caliciviridae

A total of thirteen contigs of chicken calicivirus were found (Table S2). Two of these encompassed a complete genome of two novel genotypes of chicken calicivirus (ChCaV RS/BR/15/1R-1 and 6R), resulting in 8374 and 8,187-bases sequences, respectively (Fig. S4a). The genome deduced from these contigs revealed typical features of previously reported ChCaV genomes, with a major ORF encoding the putative polyprotein and a minor ORF encoding the putative VP2 protein. ML phylogenetic analysis based on the RdRp aa sequence revealed that

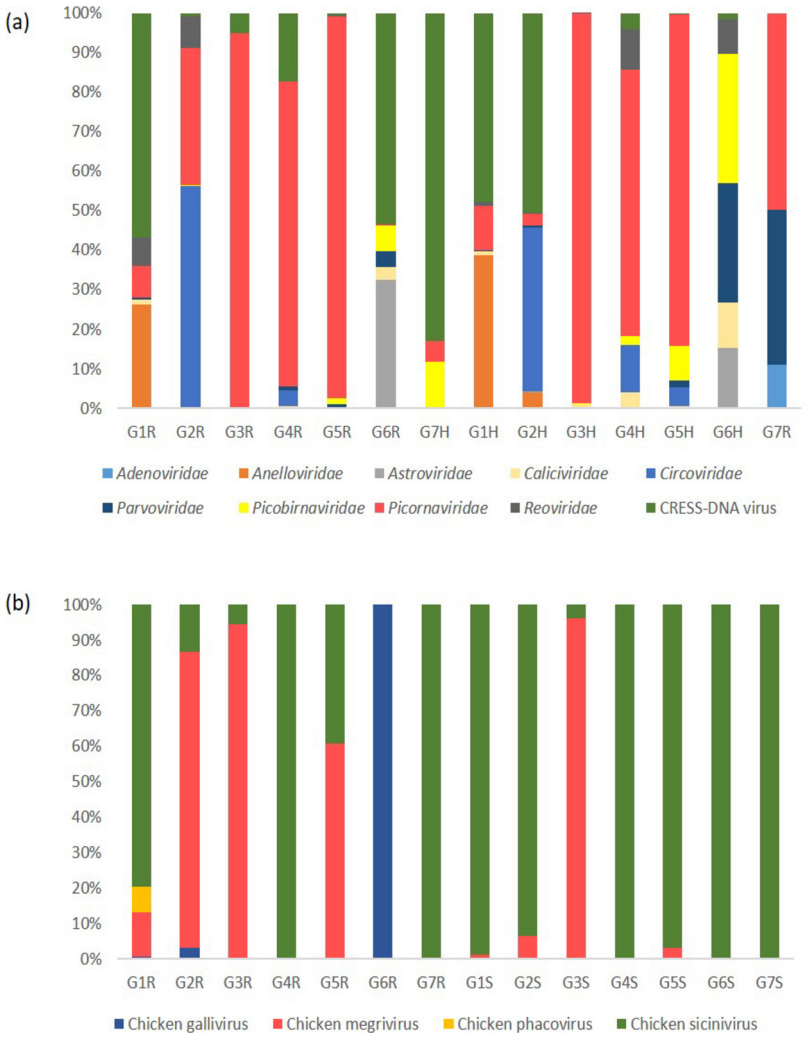


Fig. 1. Virus family assignment of sequence reads detected in stool samples from MAS-affected and healthy chickens. (a) Bar charts represent relative amounts of reads assigned to different eukaryotic viral families. (b) Bar charts showing the distribution of the viral reads related to members of the *Picornaviridae* family. MAS-affected (G1 to G7R) and healthy (G1 to G7H) chickens.

these two genomes clustered in the proposed *Bavovirus* genus along with other ChCaV reported in Brazil (Lima et al., 2017), Fig S4(b).

2.3.5. *Circoviridae*

Five full genomes of cycloviruses (1778 nt to 1905 nt-long) were recovered in this study (Fig. 4a). The major ORFs (putative Rep and Cap proteins) are arranged in opposite directions and are separated by an intergenic region that contains the putative origin of replication. Four sequences (named duck associated cyclovirus, DuCyV_RS/BR/15/4R, 2S, 4S and 5S) shared 98–99% deduced aa identity to the Rep protein of duck associated cyclovirus (GenBank no. ac. NC_034977). The other cyclovirus contig showed only 48% aa identity to the putative Rep of dragonfly associated cyclovirus 6 (GenBank no. ac. KC512918) and was provisionally named chicken associated cyclovirus 2 (ChCyV 2 RS/BR/15/4R).

The phylogenetic analysis of the cyclovirus Rep protein mentioned here is shown in Fig. 5. A maximum-likelihood phylogenetic analysis confirmed that sequences DuCyV described here are closely related to duck associated cyclovirus, with which it forms a monophyletic cluster. On the other hand, ChCyV 2 remained in a separated branch, suggesting that this may be the genome of a divergent cyclovirus.

2.3.6. *CRESS-DNA viruses*

Seven complete circular genomes of CRESS-DNA viruses were detected. Such genomes encode a Rep and a Capsid protein bidirectionally organized and varying in size from 2161 nt to 3173 nt. The genome characteristics are shown in Fig. 4(b). Table S2 presents the deduced aa similarity with their matches deposited in GenBank.

A maximum-likelihood phylogenetic tree was reconstructed based on alignments of the putative Rep aa sequences (Fig. 5). One Rep sequence (chicken stool-associated gemycircularvirus, ChSGmV, RS/BR/15/5S) clustered with members of the recently described *Genomoviridae* family (Krupovic et al., 2016), while other four sequences (chicken associated smacovirus, ChSmV, RS/BR/15/1R-1 to 4) were more closely to members of the *Smacoviridae* family (Ng et al., 2015b; Varsani and Krupovic, 2018). The two remaining sequences (chicken stool associated circular virus, ChSCV, RS/BR/15/1R-1 and 2) were more closely related a distinct cluster of CRESS-DNA viruses reported in faecal samples from Peruvian patients with diarrhea (Altan et al., 2017).

2.3.7. *Parvoviridae*

2.3.7.1. Aveparvovirus. Three contigs corresponding the full CDS of galliform aveparvovirus (GaPV/RS/BR/15/1R, 4R and 6S) genomes were assembled (Fig. 6a). The overall genomic organization of these sequences is similar to other GaPV, with three predicted ORFs, which encode the putative non-structural (NP1 and NS1) and capsid (VP1) proteins. In addition, GaPV/RS/BR/15/1R (5265 nt-long) and 4R

Virus	Mean difference MAS-affected - Healthy *	P-values (Mann-Whitney tests
Chicken megrivirus	69,828	0.5495
Chicken sicinivirus	24,401	1.0000
Chicken phacovirus	119	0.7221
Chicken gallivirus	71	0.7772
CRESS-DNA VIRUS	-33,977	0.8478
Duck associated cyclovirus	-12,348	0.4364
Chicken associated cyclovirus 2	487	1.0000
Chicken anemia virus	-534	1.0000
Avian gyrovirus 2	-1,385	0.4770
Gyrovirus 4	-6,287	0.9362
Rotavirus D	2,364	1.0000
Rotavirus F	-2,117	0.7221
Rotavirus A	-318	0.4770
Avian orthoreovirus	-87	0.3369
Chicken picobirnavirus	-4,415	0.1994
Avian nephritis virus	1,872	0.7221
Chicken astrovirus	242	0.8103
Chicken calicivirus	-1,157	0.3603
Galliform aveparvovirus	733	0.6077
Chicken chapparvovirus 1	3	1.0000
Chicken chapparvovirus 2	-192	0.4770
Avian adeno-associated virus	628	0.9435
Fowl aviadenovirus D	8	0.7221
Fowl aviadenovirus E	75	0.7658

Fig. 2. Distribution of numbers of sequence reads matching eukaryotic viruses in MAS-affected and healthy birds. Mean differences* refers to the average of reads from diseased minus the average of the healthy group of animals. Color bars represent group mean differences: Blue bars represent those above average and red bar those below average of read numbers in MAS-affected or healthy chickens. Mann-Whitney test p-values indicate whether the red or blue values are statistically significant ($p > 0,05$).

(5256 nt) contain an inverted terminal repeat (ITR) sequences at both the right and left genomic termini. These sequence shared among 99 and 100% identity to NS1 of other GaPV previously described (Table S2).

2.3.7.2. Chapparvovirus. Three contigs showed higher similarity to "Protoparvovirus HK-2014" (GenBank ac.no. KM254174) recovered from chicken faeces, which has been recently classified in the genus *Chapparvovirus*. Among these sequences, a 1930 nt-long contig (named chicken chapparvovirus, ChCPV 1 RS/BR/15/6S) contains a partial CDS of the NS1 protein and shared 99% aa identity to KM254174. Two other contigs correspond to the complete (ChCPV 2 RS/BR/15/2S, 4432 nt in length) and partial (ChCPV 2 RS/BR/15 5S, 4228 nt-long) coding regions of NS1 and capsid proteins, and showed 75 and 74% aa identity to NS1 of the KM254174 genome, respectively. The genomic organization of these sequences is shown in Fig. 6a.

2.3.7.3. Dependoparvovirus. A near-complete genome comprising the full coding region of a avian adeno-associated virus (AAAV/RS/BR/15/1R) were identified with 4062 nt in length and 94% aa identity to VP1 of the AAAV (GenBank ac.no.GQ368252) (Fig. 6a).

The ML phylogenetic analysis performed with NS1 nucleotide sequences from parvoviruses described in this study confirmed their genus classification. Interestingly, ChCPV 2 RS/BR/15/2S and 5S clustered on a separate branch, suggesting they form a distinct lineage from the other two chapparvovirus identified in chickens (Fig. 6b).

2.3.8. Picobirnaviridae

Twenty-four contigs of picobirnavirus (PBVs) were assembled. Complete or nearly complete CDS encoding a capsid protein were generated in twelve contigs (Table S2). These sequences showed identity at the aa level ranging from 32 to 56% to capsid protein of PBVs of swine, dromedaries, otarines and humans (Banyai et al., 2014; Woo et al., 2014, 2016). Other twelve contigs correspond to full or partial CDS of the RNA-dependent RNA polymerase (RdRp) protein and shared between 61 and 89% aa identity to the RdRp of PBVs identified in different mammals species (Fig. 7a, Table S2).

Through phylogenetic analysis of RdRp sequences, the PBV genomes identified here were classified in genogroups I and II. In view of this, RdRp-contigs here reported were used for maximum-likelihood (ML) phylogenetic analyses, which revealed that only two sequences belong to PBV genogroup II. The additional ten RdRp-sequences clustered to genogroup I and were widely scattered in the tree presented in Fig. 7(b).

2.3.9. Picornaviridae

2.3.9.1. Megrivirus. A total of eleven contigs ranging in size from 1226 to 9577 nt were identified based on protein similarity (Table S2). Complete or nearly complete genomes were generated in four contigs, named ChMGV/RS/BR/15 2R, 3R, 5R and 3S/2 (Fig. 8a). The ChMGV/RS/BR/15-2R, 3R and 3S/2 showed 96%, 93% and 98% identity at amino acid (aa) level to the polyprotein of chicken proventriculitis virus (Genbank ac. no.KJ690629), respectively. The ChMGV/RS/BR/15 5R displayed 96% aa identity to chicken megrivirus polyprotein (Genbank ac. no. KF961186).

2.3.9.2. Sicinivirus. Seven complete (9075 nt to 9874 nt) and one partial (8710 nt) coding sequence (CDS) of sicinivirus were detected (Fig. 8a). Fourteen shorter partial genomes were also identified (Table S2).

2.3.9.3. Phacovirus. Two phacovirus-related contigs were detected. One of these sequences (ChPhV/RS/BR/15/1R-1, 4,104-nt-long) contained 5′UTR and encode the first half of the phacovirus genome (Table S2). A second contig (ChPhV/RS/BR/15/1R-2, 3,925-nt-long) contained the 3′UTR corresponding to the final portion of the phacovirus genome (Fig. 8a). At the deduced aa level, ChPhV/RS/BR/15/1R-1 displayed 87% identity to polyprotein of chicken phacovirus (GenBank ac. no. KT880670), while ChPhV/RS/BR/15/1R-2 shared 99% aa identity to the same genome.

2.3.9.4. Gallivirus. A 2226 nt-long contig (ChGV/RS/BR/15/2R) of chicken gallivirus was detected (Fig. 8a). The sequence shows a 3′UTR and encoding a partial polyprotein which shares 99% aa identity to the prototype strain of chicken gallivirus 1 (GenBank ac. no. NC_024770).

The sequences described here were compared to sequences of picornaviruses from different genera and used for phylogenetic analysis performed using 3D polymerase amino acid sequences. The phylogenetic tree shown in Fig. 8b support its genera assignments into genera.

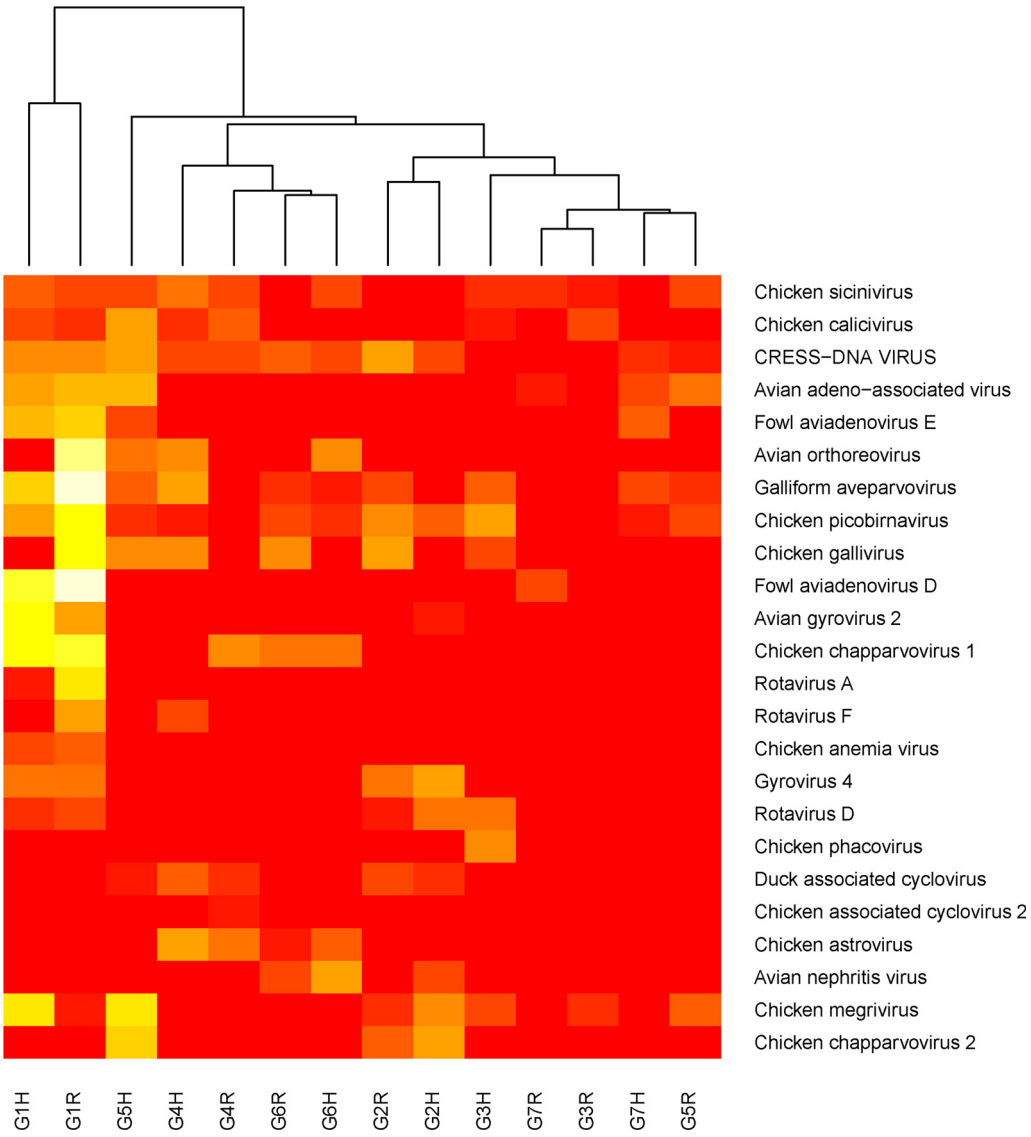


Fig. 3. Hierarchical clustering of MAS-affected and healthy animal groups. Hierarchical clustering of the number of sequence reads matching eukaryotic viruses in each sample. The columns represent the analyzed samples (G1-G7R and G1-G7S), while the rows represent the distribution of reads associated to each of the listed viruses. The clustering tree of samples is shown at the top. Colors refer to the amount of reads matching a particular virus, from white (higher number of maches) to red (lower number of matches).

2.3.10. Reoviridae

A total of twenty-six contigs related to members of the family *Reoviridae* were found (Table S2). These include members of three rotavirus species: Rotavirus D (15 contigs, ranging in size from 1017 to 3302 nt), Rotavirus F (9 contigs, 1011 to 2726 nt) and Rotavirus A (1 contig, 1016 nt). The phylogenetic analysis of the VP2 nucleotide sequences confirms their species assignment (Fig. S5).

Another contig (1157 nt-long) was detected which mapped to avian orthoreovirus (GenBank no. ac. KF741696), with 99% amino acid identity to lambda-A protein (Table S2). The phylogenetic analysis of such sequence supports its relatedness to avian orthoreoviruses (Fig. S6).

3. Discussion

A case-control study was conducted here in search for viruses that might be involved in the pathogenesis of MAS. So far, to the knowledge of the authors, previous viral surveys in MAS-affected chickens (with the exception of virus culturing attempts before molecular biology became more widely available) were predominantly focused on PCR-based approaches (Day and Zsak, 2010; De la Torre et al., 2018; Koo et al., 2015; Pantin-Jackwood et al., 2008, 2011; Spackman et al., 2010). However, this depends on previous knowledge on the genome sequences of one or more virus(es), whose presence in samples could then be investigated. With such approach, no previously unidentified agents would be encountered. Previous studies using metagenomic approach, based on sequence-independent amplification and deep sequencing on a high throughput platform, have revealed novel and highly divergent viruses in the gut of poultry (Boros et al., 2016; Lima et al., 2017).

Among the enteric viruses detected in high numbers of reads in MAS-affected broilers (megrivirus, sicinivirus, chicken-associated cyclovirus 2 (ChCyV 2), rotavirus D, astroviruses, galliform aveparvovirus, avian adeno-associated virus and fowl aviadenoviruses — only rotaviruses, astroviruses, galliform aveparvovirus and fowl aviadenoviruses), have previously been associated to enteric disorders in chickens (Chen et al., 2014; Day and Zsak, 2010; Kang et al., 2012; Otto et al., 2006; Palade et al., 2011; Pantin-Jackwood et al., 2008, 2011). Here, no association could be made between any particular virus and MAS, since the distribution of reads related to such viruses in diseased

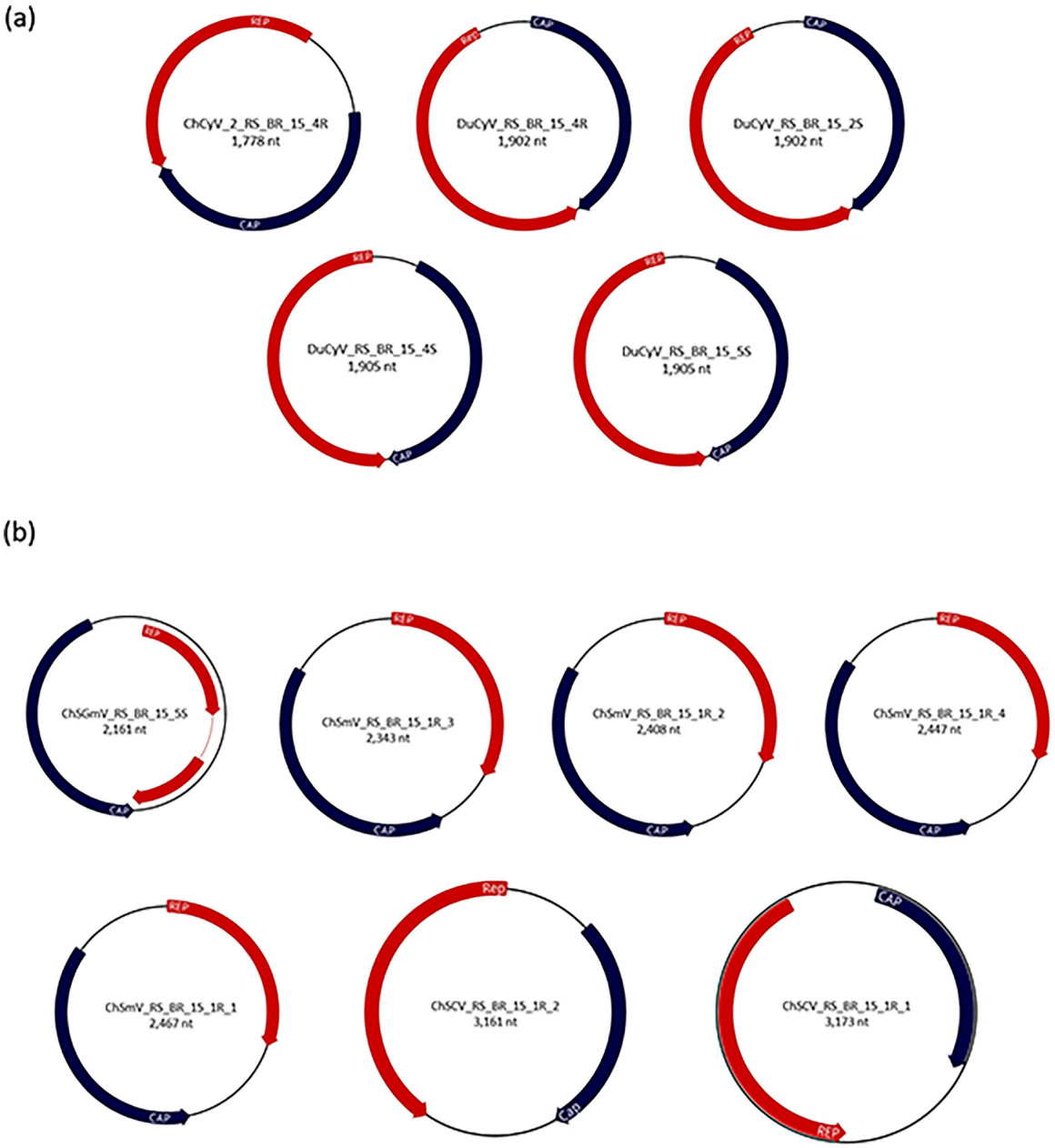


Fig. 4. Genome organization of cycloviruses and CRESS-DNA viruses identified in faeces of MAS-affected and healthy chickens. (a) Putative genome maps of gemycircularvirus, smacoviruses and chicken stool associated circular viruses. (b) Putative genome organization of chicken associated cycloviruses.

and healthy broilers revealed no statistically significant differences. Other authors have proposed associations between these viruses and MAS (Kang et al., 2012; Zsak et al., 2013). The finding reported here indicate no association between the occurrence of MAS and such agents, therefore providing evidence in support that these viruses are part of normal intestinal microbiota. On the other hand, one of the limitations of the study is that the data generated comes from pooled samples, rather than from individuals. The authors are aware that this reduces the strength of the study in its potential to evaluate different virus prevalences/viral loads, since the viral reads could have been biased by such a procedure. However, this approach allows access to diversity of viral genomes present in the feces of these populations, including hitherto unknown viruses.

In healthy animals, a great number of reads corresponding to genomes of CRESS-DNA viruses and DuCyV were detected, as were gyroviruses, rotavirus F, chicken picobirnaviruses and chicken caliciviruses. Similarly, when comparing the amount of sequence reads of these viruses, again no significant differences were found among MAS-affected and healthy animals. These findings support the hypothesis that there seems not to be a specific viral agent associated with the occurrence of MAS.

As a final comment, it must be mentioned that cycloviruses and CRESS-DNA genomes have been detected from a diversity of invertebrates and vertebrates specimens including insects (Dayaram et al., 2013), birds (Feher et al., 2017; Li et al., 2011; Lima et al., 2017), mammals (Li et al., 2015, 2011; Male et al., 2016; Rosario et al., 2017), as well as in plants and sewage (Kraberger et al., 2015; Krupovic et al., 2016; Phan et al., 2015). It seem likely that such agents may be commensals in the birds intestinal virome. However, as postulated previously, it is also possible that such viruses might be acquired through food consumption (Delwart and Li, 2012; Rosario et al., 2012).

Although the evidences presented in this report suggest that viral genomes reported here most likely correspond to commensal viruses, its apparent non-pathogenicity must be examined with caution, since other

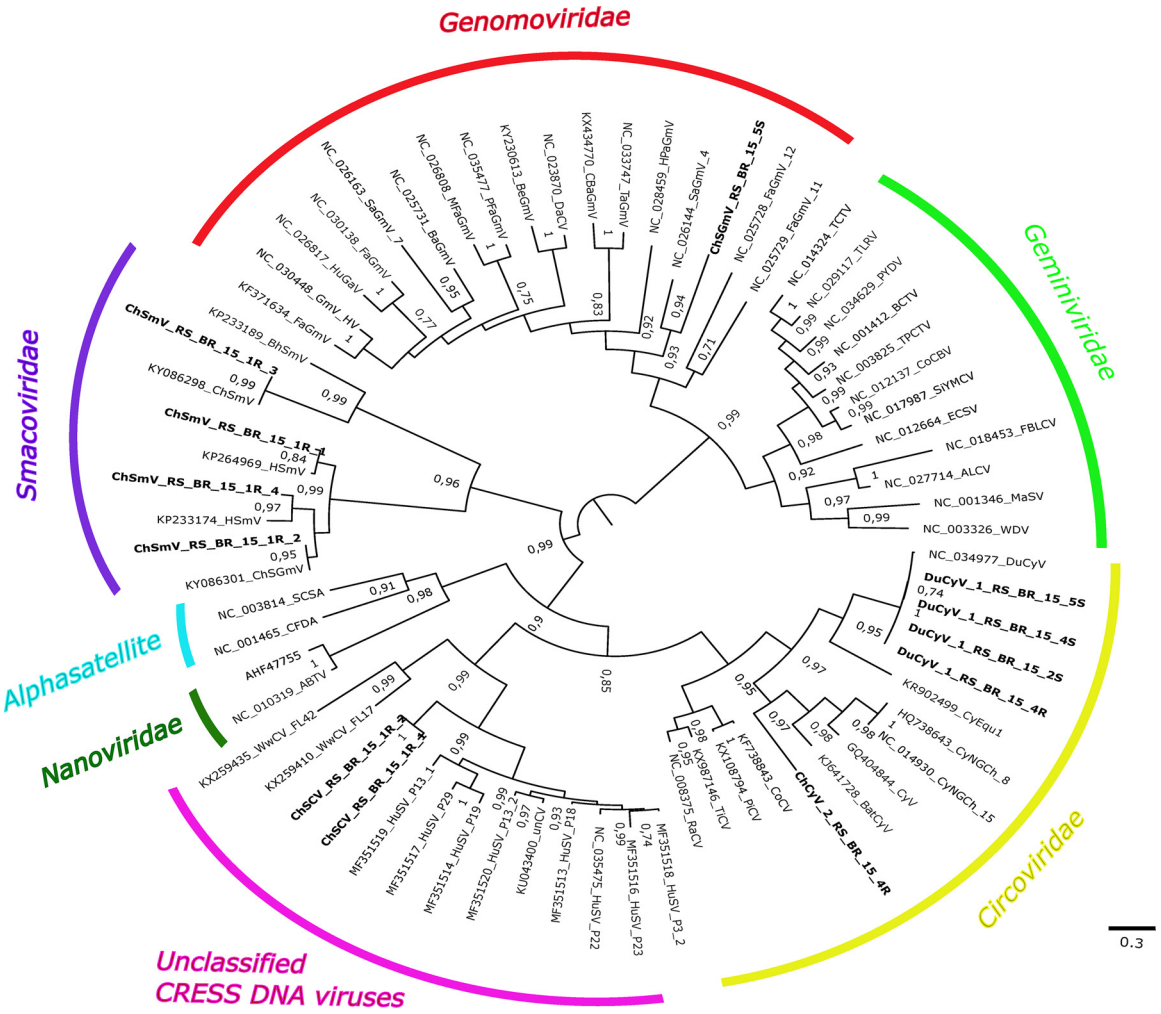


Fig. 5. Maximum likelihood phylogenetic tree reconstructed with the substitution model VT + I + G on Rep aa sequences from different CRESS-DNA viruses. The branch support is evidenced only for the clades with an aLRT greater than 70%. The CRESS-DNA viruses and cycloviruses identified here are highlighted in bold.

variables involved may play important roles in pathogenicity. Previous studies have demonstrated that genetic background may change susceptibility to MAS. The intensive genetic selection of poultry for fast body weight gain may result in unfavourable indirect selection, which might affect general resistance of birds leading to inadequate adaptation to food changes, intestinal disorders and/or disease susceptibility (Bayyari et al., 1997; Li et al., 2000). Differences in gene expression related to the immune system and nutrient absorption were identified in broiler lines that differ in MAS susceptibility. However, whether such genes could be used as markers to predict or prevent MAS susceptibility in chickens needs to be further investigated (Rebel et al., 2006).

4. Concluding remarks

In this study the faecal virome of MAS-affected and healthy chickens were analyzed comparatively. Genomes of a number of previously known and some as yet unreported viral genome sequences were identified in diseased and healthy birds. It was not possible to associate any particular virus or viruses with MAS, nor its relative abundancy (as revealed by the numbers of reads recovered) in diseased or healthy broilers. Future longitudinal studies may aid in attempting to define the aetiology of MAS.

5. Material and methods

5.1. Biological samples

Samples consisting of pooled chicken feces were collected in 2015 (May to December) from seven different commercial poultry farms in Rio Grande do Sul State, Brazil. Seventy, 3–5 weeks old chickens (35 from MAS- affected birds; 35 from healthy ones) were collected. Five birds presenting clinical signs suggestive of MAS (retarded growth, apathy, diarrhea and defective feather development) were selected in each flock. Control sample pools were collected from five randomly collected, clinically healthy broilers, at the same age and from the same flocks. The average weight of MAS-affected birds was 0.89 kg; this represents 491% less than the average weight of healthy broilers (1.75 kg). The birds were euthanized, the intestinal tracts removed and frozen at −80 °C until processing. All procedures were performed in compliance with the Brazilian College of Animal Experimentation (COBEA) and approved by the Commission of Ethics on Animal Use of the Veterinary Research Institute Desidério Finamor (CEUA - IPVDF) – No. 21/2014.

5.2. Sample preparation, nucleic acids enrichment and high throughput sequencing

The total intestinal contents of the birds were processed in pools. From each of the seven farms, one pool comprising five stool samples

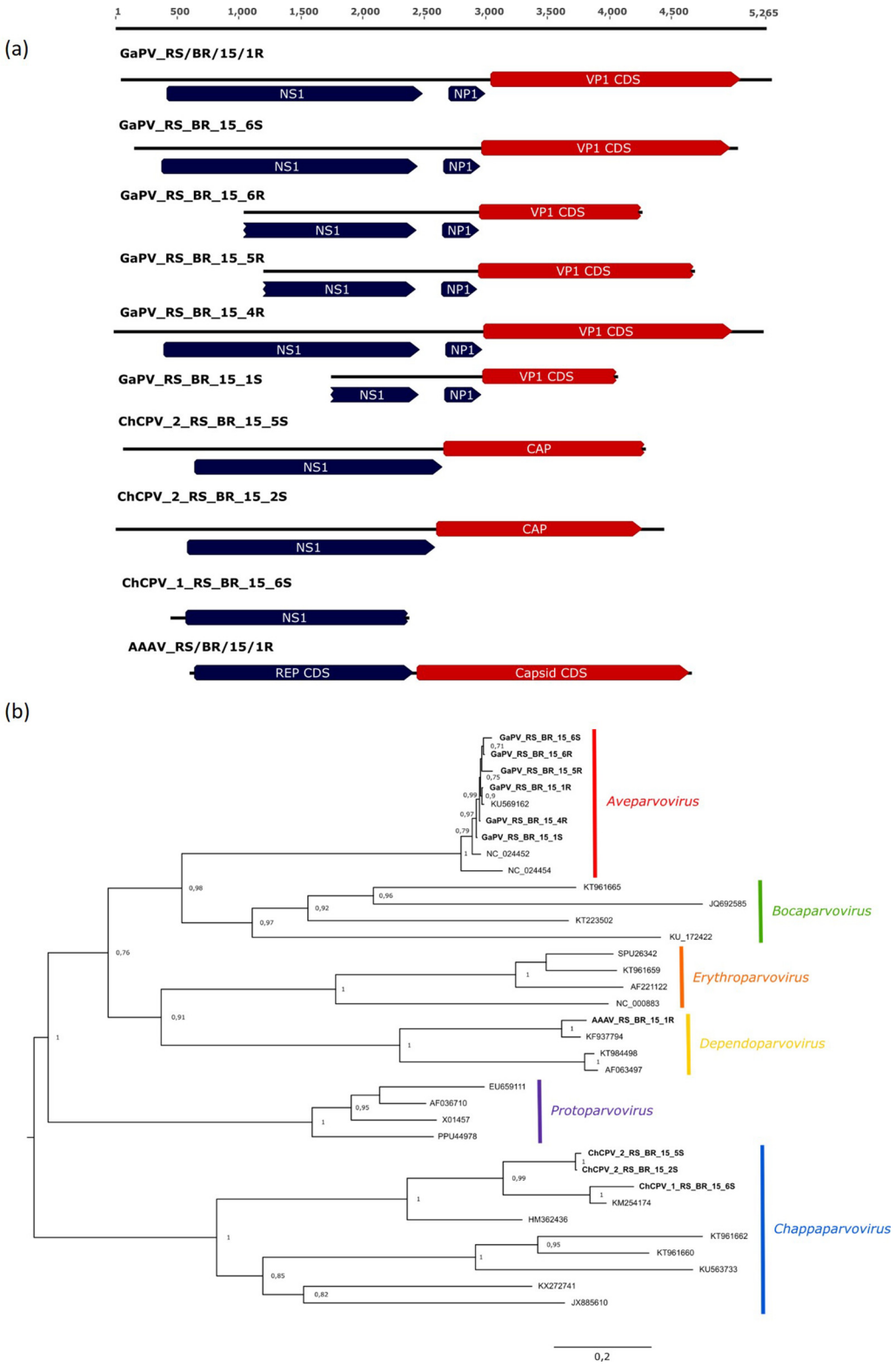


Fig. 6. Schematic representation and phylogenetic analysis of parvovirus genomes. (a) Putative genome organization of the parvovirus related contigs. (b) Phylogenetic tree based on the NS1 nucleotide sequences from representative members of subfamily *Parvovirinae*. Inferences carried out by maximum likelihood phylogenetic analysis with GTR + G + I substitution model. The branch support is evidenced only for the clades with an aLRT greater than 70%. The parvoviruses recovered here are shown in bold.

from MAS-affected birds (named groups 1 to 7 "runt", or G1 to G7 R). Equivalent pools were prepared from control birds, collected from the same flocks (named G1 to G7 "healthy", or G1 to G7 H). Each pool was comprised by one gram of each stool sample was diluted q.s.p. 10 ml in PBS (pH 7.2) and each five (collected from a same farm) were pooled. Such pools were vigorously vortexed for five minutes and clarified at 3000 × g for 30 min at 4 °C. Subsequently, the supernatants were filtered through a 0.45 µm filter (Millipore) to remove bacteria and cell

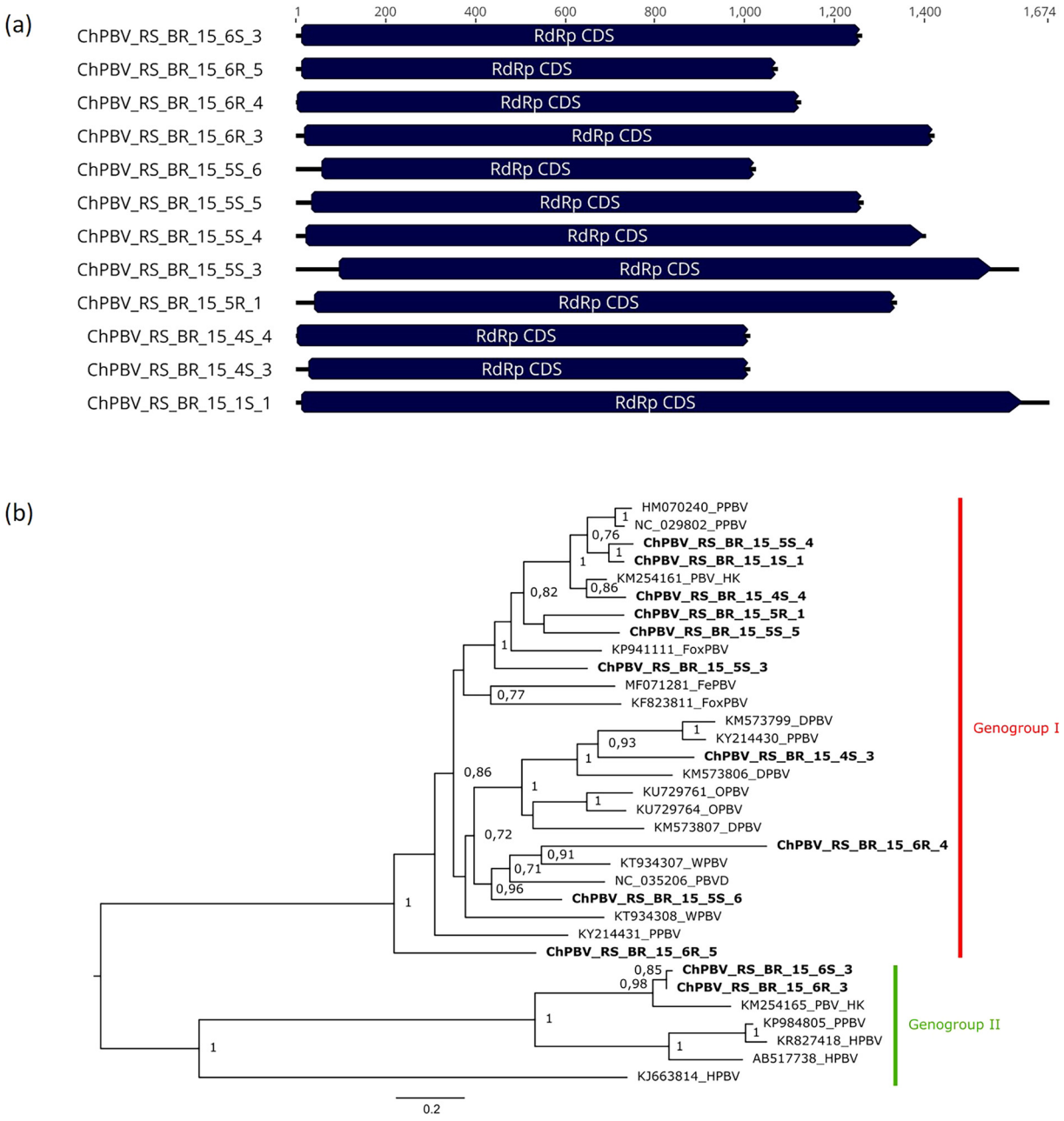
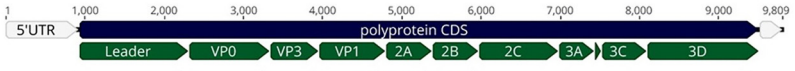


Fig. 7. Genomic organization and phylogenetic analysis of chicken picobirnaviruses genomes based on the nucleotide sequences coding for RdRp. (a) Schematic representation of the ChPBV-contigs recovered from faeces of MAS-affected and healthy chickens. (b) Phylogenetic inferences carried out by maximum likelihood phylogenetic analysis with the GTR + G + I substitution model. Branch support is highlighted only for clades with an aLRT greater than 70%. The ChPBV genomes reported here are shown in bold.

debris. These were then centrifuged at ~150,000 × g for 4 h at 4 °C (Sorvall AH629 rotor). The resulting pellets were resuspended in ultrapure water and treated with 2 µL DNase (2 Units/µL, Turbo DNase, Ambion), 5 µL RNase A (20 mg/mL), Invitrogen) and 0,5 µL benzonase (25 Units/µL, Novagen). Such mixes were incubated at 37 °C for 2 h to digest nucleic acids unprotected by capsids (Shan et al., 2011). Viral RNA was extracted with TRIzol LS (Ambion) and reverse transcribed to generate cDNA libraries with a commercial kit (SeqPlexRNA Amplification Kit; Sigma Aldrich), following manufacturer's instructions. Viral DNA extraction was performed with a standard phenol-chloroform protocol (Sambrook and Russel, 2001) and enriched by multiple displacement amplification (MDA) with φ29 DNA polymerase (Dean et al., 2002).

The randomly enriched nucleic acids of each original pools were mixed in equimolar amounts (enriched cDNA plus enriched DNA) and purified with a DNA-cleaning kit (Genomic DNA Clean & Concentrator; Zymo Research). The quality and quantity of DNA preparations were determined in a L-Quant spectrophotometer (Loccus Biotechnology) and in a Qubit fluorometer (Invitrogen). For high throughput sequencing, DNA fragment libraries were prepared with 50 ng of the purified viral derived, enriched nucleic acids, using a Nextera DNA sample preparation kit (Illumina). Sequencing was performed in an Illumina®MiSeq instrument using the Illumina v2 reagent kit (2 × 250 paired-end reads). Supplementary data (sample pools identification and number of reads obtained from each library) are presented in Table S1.

(a)



Virus	Genotype	5'UTR	Polyprotein CDS: Leader	VP0	VP3	VP1	2A	2B	2C	3A	3	3C	3D	3'UTR
Chicken megrivirus	RS/BR/15/2R													
Chicken megrivirus	RS/BR/15/3R													
Chicken megrivirus	RS/BR/15/5R													
Chicken megrivirus	RS/BR/15/3S-2													
Chicken sicinivirus	RS/BR/15/1R-1													
Chicken sicinivirus	RS/BR/15/3R													
Chicken sicinivirus	RS/BR/15/4R													
Chicken sicinivirus	RS/BR/15/5R													
Chicken sicinivirus	RS/BR/15/7S													
Chicken sicinivirus	RS/BR/15/2S-1													
Chicken sicinivirus	RS/BR/15/4S													
Chicken sicinivirus	RS/BR/15/5S													
Chicken phacovirus	RS/BR/15/1R-1													
Chicken gallivirus	RS/BR/15/2R													

(b)

Fig. 8. Putative genome organization and phylogenetic analysis of picornavirus genomes identified in faeces of MAS-affected and healthy chickens. (a) Schematic representation of the prototypic genome of chicken sicinivirus (NC_028380). Grey bars represent the coverage of genomes of megriviruses, siciniviruses, phacoviruses and galliviruses contigs identified in this study. Only contigs > 1000 nts are represented. (b) Maximum likelihood phylogenetic tree reconstructed with the substitution model LG of 3D polymerase amino acid sequences (dark shading) from representative members of the *Picornaviridae* family. The branch support is evidenced only for the clades with an aLRT support greater than 70%.The picornaviruses recovered in this study are shown in bold.

5.3. Bioinformatics pipeline

Low-quality sequencing reads with a Phred quality score < 20 were trimmed using PRINSEQ version 0.20.4 (Schmieder and Edwards, 2011). The paired-end sequence reads were *de novo* assembled in contigs using metaSPAdes 3.10.1 (Nurk et al., 2017). The assembled contigs and singlet sequences were analysed by BLASTx against a viral protein database. Sequences with the best BLAST scores (E values $\leq 10^{-3}$) were selected and assigned into known viral families and, where applicable, to the respective CRESS-DNA current classification. Contigs > 1000 nt were confirmed by mapping reads and selected to perform ORF predictions and genome annotations using the Geneious software (version 9.1.8). For the purposes of the present study and for sake of brevity, only contigs > 1000 nt are described, with the exception of those of fowl adenovirus E, for which no contigs > 1000 nt were detected.

5.4. Assessment of viral abundance

Numbers of raw reads matching selected viral sequences were measured with the Geneious software with all raw data output reads in medium-low sensitivity/fast mode. The amount of virus-specific reads detected in individual pooled sample were divided by the total number of reads generated from each sample, according to the methodology described by Kapusinszky et al (2017). The read numbers were expressed as percentages of viral reads.

Significant differences among the distribution of sequences matching viral contigs in MAS-affected and healthy groups were compared using Mann-Whitney tests (GraphPad Prism software version 5). In order to examine overall taxonomic similarities between metagenomes, hierarchical clustering and heat map analyses were performed using the Gplots package in the RStudio software.

5.5. Phylogenetic analyses

Sequences representative of known viral families, as well as CRESS-DNA genomes, were obtained from GenBank and then aligned with the sequences identified in the present study using either MUSCLE or MAFFT software (Edgar, 2004; Katoh and Standley, 2013). These were used to generate maximum-likelihood phylogenetic trees using PHYML (Guindon et al., 2010) with best fit substitution models determined by Smart Model Selection (Lefort et al., 2017). Statistical significance analyses of tree topologies were performed with the approximate likelihood branch support test (aLRT) (Guindon and Gascuel, 2003).

5.6. Nucleotide sequences accession numbers

Complete or partial viral genome sequences identified in this study were submitted to GenBank under the accession numbers presented in Table S2.

Funding information

Work supported by the FINEP (proj. SANIMARS no. 01.10.0783.04 and proj. MAIS ÁGUA no. 0112.0113), CNPq and CAPES. DAL has been supported by a CAPES Ph.D. grant. PMR is a CNPq 1A research fellow.

Conflicts of interest

The authors declare no conflicts of interest regarding this manuscript.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2018.12.005.

References

Altan, E., Del Valle Mendoza, J., Deng, X., Phan, T.G., Sadeghi, M., Delwart, E.L., 2017. Small circular rep-encoding single-stranded DNA genomes in peruvian diarrhea virome. Genome Announc. 5 (38).
Banyai, K., Potgieter, C., Gellert, A., Ganesh, B., Tempesta, M., Lorusso, E., Buonavoglia, C., Martella, V., 2014. Genome sequencing identifies genetic and antigenic divergence of porcine picobirnaviruses. J. Gen. Virol. 95 (Pt 10), 2233–2239.
Baxendale, W., Mebatsion, T., 2004. The isolation and characterisation of astroviruses from chickens. Avian Pathol. 33 (3), 364–370.
Bayyari, G.R., Huff, W.E., Rath, N.C., Balog, J.M., Newberry, L.A., Villines, J.D., Skeeles, J.K., Anthony, N.B., Nestor, K.E., 1997. Effect of the genetic selection of turkeys for increased body weight and egg production on immune and physiological responses. Poult. Sci. 76 (2), 289–296.
Bexfield, N., Kellam, P., 2011. Metagenomics and the molecular identification of novel viruses. Vet. J. 190 (2), 191–198.
Boros, A., Pankovics, P., Adonyi, A., Fenyvesi, H., Day, J.M., Phan, T.G., Delwart, E., Reuter, G., 2016. A diarrheic chicken simultaneously co-infected with multiple picornaviruses: complete genome analysis of avian picornaviruses representing up to six genera. Virology 489, 63–74.
Chen, W.T., Wu, Y.L., Chen, T., Cheng, C.S., Chan, H.L., Chou, H.C., Chen, Y.W., Yin, H.S., 2014. Proteomics analysis of the DF-1 chicken fibroblasts infected with avian reovirus strain S1133. PLoS One 9 (3), e92154.
Cheung, A.K., Ng, T.F., Lager, K.M., Bayles, D.O., Alt, D.P., Delwart, E.L., Pogranichniy, R.M., Kehrli Jr., M.E., 2013. A divergent clade of circular single-stranded DNA viruses from pig feces. Arch. Virol. 158 (10), 2157–2162.
Cheung, A.K., Ng, T.F., Lager, K.M., Alt, D.P., Delwart, E., Pogranichniy, R.M., 2015. Identification of several clades of novel single-stranded circular DNA viruses with conserved stem-loop structures in pig feces. Arch. Virol. 160 (1), 353–358.
Cox-Foster, D.L., Conlan, S., Holmes, E.C., Palacios, G., Evans, J.D., Moran, N.A., Quan, P.L., Briese, T., Hornig, M., Geiser, D.M., Martinson, V., vanEngelsdorp, D., Kalkstein, A.L., Drysdale, A., Hui, J., Zhai, J., Cui, L., Hutchison, S.K., Simons, J.F., Egholm, M., Pettis, J.S., Lipkin, W.I., 2007. A metagenomic survey of microbes in honey bee colony collapse disorder. Science 318 (5848), 283–287.
Day, J.M., Ballard, L.L., Duke, M.V., Scheffler, B.E., Zsak, L., 2010. Metagenomic analysis of the turkey gut RNA virus community. Virol. J. 7, 313.
Day, J.M., Oakley, B.B., Seal, B.S., Zsak, L., 2015. Comparative analysis of the intestinal bacterial and RNA viral communities from sentinel birds placed on selected broiler chicken farms. PLoS One 10 (1).
Day, J.M., Zsak, L., 2010. Determination and analysis of the full-length chicken parvovirus genome. Virology 399 (1), 59–64.
Day, J.M., Zsak, L., 2013. Recent progress in the characterization of avian enteric viruses. Avian Dis. 57 (3), 573–580.
Dayaram, A., Potter, K.A., Moline, A.B., Rosenstein, D.D., Marinov, M., Thomas, J.E., Breitbart, M., Rosario, K., Arguello-Astorga, G.R., Varsani, A., 2013. High global diversity of cycloviruses amongst dragonflies. J. Gen. Virol. 94 (Pt 8), 1827–1840.
De la Torre, D.I., Nunez, L.F., Astolfi-Ferreira, C.S., Piantino Ferreira, A.J., 2018. Enteric virus diversity examined by molecular methods in brazilian poultry flocks. Vet. Sci. 5 (2).
Dean, F.B., Hosono, S., Fang, L., Wu, X., Faruqi, A.F., Bray-Ward, P., Sun, Z., Zong, Q., Du, Y., Du, J., Driscoll, M., Song, W., Kingsmore, S.F., Egholm, M., Lasken, R.S., 2002. Comprehensive human genome amplification using multiple displacement amplification. Proc. Natl. Acad. Sci. U. S. A. 99 (8), 5261–5266.
Delwart, E., Li, L., 2012. Rapidly expanding genetic diversity and host range of the Circoviridae viral family and other Rep encoding small circular ssDNA genomes. Virus Res. 164 (1-2), 114–121.
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 Pathol. 45 (6), 616–629.
Edgar, R.C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32 (5), 1792–1797.
Feher, E., Kaszab, E., Forro, B., Bali, K., Marton, S., Lengyel, G., Banyai, K., 2017. Genome sequence of a mallard duck origin cyclovirus, DuACyV-1. Arch. Virol. 162 (12), 3925–3929.
Guindon, S., Gascuel, O., 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. 52 (5), 696–704.
Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W., Gascuel, O., 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst. Biol. 59 (3), 307–321.
Kang, K.I., El-Gazzar, M., Sellers, H.S., Dorea, F., Williams, S.M., Kim, T., Collett, S., Mundt, E., 2012. Investigation into the aetiology of runting and stunting syndrome in chickens. Avian Pathol. 41 (1), 41–50.
Kapusinszky, B., Ardeshir, A., Mulvaney, U., Deng, X., Delwart, E., 2017. Case-control comparison of enteric viromes in captive Rhesus macaques with acute or idiopathic chronic diarrhea. J. Virol. 91 (18).
Katoh, K., Standley, D.M., 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol. Biol. Evol. 30 (4), 772–780.
Kim, H.K., Park, S.J., Nguyen, V.G., Song, D.S., Moon, H.J., Kang, B.K., Park, B.K., 2012. Identification of a novel single-stranded, circular DNA virus from bovine stool. J. Gen. Virol. 93 (Pt 3), 635–639.
Koo, B.S., Lee, H.R., Jeon, E.O., Han, M.S., Min, K.C., Lee, S.B., Bae, Y.J., Cho, S.H., Mo, J.S., Kwon, H.M., Sung, H.W., Kim, J.N., Mo, I.P., 2015. Genetic characterization of three novel chicken parvovirus strains based on analysis of their coding sequences. Avian Pathol. 44 (1), 28–34.
Kraberger, S., Arguello-Astorga, G.R., Greenfield, L.G., Galilee, C., Law, D., Martin, D.P.,

Varsani, A., 2015. Characterisation of a diverse range of circular replication-associated protein encoding DNA viruses recovered from a sewage treatment oxidation pond. Infect. Genet. Evol. 31, 73–86.
Krupovic, M., Ghabrial, S.A., Jiang, D., Varsani, A., 2016. Genomoviridae: a new family of widespread single-stranded DNA viruses. Arch. Virol. 161 (9), 2633–2643.
Lefort, V., Longueville, J.E., Gascuel, O., 2017. SMS: smart model selection in PhyML. Mol. Biol. Evol. 34 (9), 2422–2424.
Li, Z., Nestor, K.E., Saif, Y.M., Luhtala, M., 2000. Flow cytometric analysis of T lymphocyte subpopulations in large-bodied turkey lines and a randombred control population. Poult. Sci. 79 (2), 219–223.
Li, L., Shan, T., Soji, O.B., Alam, M.M., Kunz, T.H., Zaidi, S.Z., Delwart, E., 2011. Possible cross-species transmission of circoviruses and cycloviruses among farm animals. J. Gen. Virol. 92 (Pt 4), 768–772.
Li, L., Giannitti, F., Low, J., Keyes, C., Ullmann, L.S., Deng, X., Aleman, M., Pesavento, P.A., Pusterla, N., Delwart, E., 2015. Exploring the virome of diseased horses. J. Gen. Virol. 96 (9), 2721–2733.
Lima, D.A., Cibulski, S.P., Finkler, F., Teixeira, T.F., Varela, A.P.M., Cerva, C., Loiko, M.R., Scheffer, C.M., Dos Santos, H.F., Mayer, F.Q., Roehe, P.M., 2017. Faecal virome of healthy chickens reveals a large diversity of the eukaryote viral community, including novel circular ssDNA viruses. J. Gen. Virol. 98 (4), 690–703.
Male, M.F., Kraberger, S., Stainton, D., Kami, V., Varsani, A., 2016. Cycloviruses, gemycircularviruses and other novel replication-associated protein encoding circular viruses in Pacific flying fox (Pteropus tonganus) faeces. Infect. Genet. Evol. 39, 279–292.
Mihalov-Kovacs, E., Feher, E., Martella, V., Banyai, K., Farkas, S.L., 2014. The fecal virome of domesticated animals. Virusdisease 25 (2), 150–157.
Ng, T.F., Kondov, N.O., Deng, X., Van Eenennaam, A., Neibergs, H.L., Delwart, E., 2015a. A metagenomics and case-control study to identify viruses associated with bovine respiratory disease. J. Virol. 89 (10), 5340–5349.
Ng, T.F.F., Zhang, W., Sachsenröder, J., Kondov, N.O., da Costa, A.C., Vega, E., Holtz, L.R., Wu, G., Wang, D., Stine, C.O., Antonio, M., Mulvaney, U.S., Muench, M.O., Deng, X., Ambert-Balay, K., Pothier, P., Vinjé, J., Delwart, E., 2015b. A diverse group of small circular ssDNA viral genomes in human and non-human primate stools. Virus Evol. 1 (1) vev017.
Nurk, S., Meleshko, D., Korobeynikov, A., Pevzner, P.A., 2017. metaSPAdes: a new versatile metagenomic assembler. Genome Res. 27 (5), 824–834.
Otto, P., Liebler-Tenorio, E.M., Elschner, M., Reetz, J., Lohren, U., Diller, R., 2006. Detection of rotaviruses and intestinal lesions in broiler chicks from flocks with runting and stunting syndrome (RSS). Avian Dis. 50 (3), 411–418.
Palade, E.A., Kisary, J., Benyeda, Z., Mandoki, M., Balka, G., Jakab, C., Vegh, B., Demeter, Z., Rusvai, M., 2011. Naturally occurring parvoviral infection in Hungarian broiler flocks. Avian Pathol. 40 (2), 191–197.
Pantin-Jackwood, M.J., Day, J.M., Jackwood, M.W., Spackman, E., 2008. Enteric viruses detected by molecular methods in commercial chicken and turkey flocks in the United States between 2005 and 2006. Avian Dis. 52 (2), 235–244.
Pantin-Jackwood, M.J., Strother, K.O., Mundt, E., Zsak, L., Day, J.M., Spackman, E., 2011. Molecular characterization of avian astroviruses. Arch. Virol. 156 (2), 235–244.
Phan, T.G., Mori, D., Deng, X., Rajindrajith, S., Ranawaka, U., Fan Ng, T.F., Bucardo-Rivera, F., Orlandi, P., Ahmed, K., Delwart, E., 2015. Small circular single stranded DNA viral genomes in unexplained cases of human encephalitis, diarrhea, and in untreated sewage. Virology 482, 98–104.
Rebel, J.M.J., Balk, F.R.M., Post, J., Van Hemert, S., Zekarias, B., Stockhofe, N., 2006. Malabsorption syndrome in broilers. Worlds Poult. Sci. J. 62, 17–30.
Rosario, K., Breitbart, M., 2011. Exploring the viral world through metagenomics. Curr. Opin. Virol. 1 (4), 289–297.
Rosario, K., Dayaram, A., Marinov, M., Ware, J., Kraberger, S., Stainton, D., Breitbart, M., Varsani, A., 2012. Diverse circular ssDNA viruses discovered in dragonflies (Odonata: epiprocta). J. Gen. Virol. 93 (Pt 12), 2668–2681.
Rosario, K., Breitbart, M., Harrach, B., Segales, J., Delwart, E., Biagini, P., Varsani, A., 2017. Revisiting the taxonomy of the family Circoviridae: establishment of the genus Cyclovirus and removal of the genus Gyrovirus. Arch. Virol. 162 (5), 1447–1463.
Sambrook, J., Russel, D.W., 2001. Molecular Cloning: a Laboratory Manual, Edited by, 3 ed. Cold Spring Harbor, New York, USA.
Schmieder, R., Edwards, R., 2011. Quality control and preprocessing of metagenomic datasets. Bioinformatics 27 (6), 863–864.
Sellers, H., Linneman, E., Icard, A.H., Mundt, E., 2010. A purified recombinant baculovirus expressed capsid protein of a new astrovirus provides partial protection to runting-stunting syndrome in chickens. Vaccine 28 (5), 1253–1263.
Shah, J.D., Baller, J., Zhang, Y., Silverstein, K., Xing, Z., Cardona, C.J., 2014. Comparison of tissue sample processing methods for harvesting the viral metagenome and a snapshot of the RNA viral community in a turkey gut. J. Virol. Methods 209, 15–24.
Shan, T., Li, L., Simmonds, P., Wang, C., Moeser, A., Delwart, E., 2011. The fecal virome of pigs on a high-density farm. J. Virol. 85 (22), 11697–11708.
Smyth, V.J., 2017. A review of the strain diversity and pathogenesis of chicken astrovirus. Viruses 9 (29).
Songserm, T., Pol, J.M., van Roozelaar, D., Kok, G.L., Wagenaar, F., ter Huurne, A.A., 2000. A comparative study of the pathogenesis of malabsorption syndrome in broilers. Avian Dis. 44 (3), 556–567.
Songserm, T., Zekarias, B., van Roozelaar, D.J., Kok, R.S., Pol, J.M., Pijpers, A.A., ter Huurne, A.A., 2002. Experimental reproduction of malabsorption syndrome with different combinations of reovirus, Escherichia coli, and treated homogenates obtained from broilers. Avian Dis. 46 (1), 87–94.
Spackman, E., Day, J.M., Pantin-Jackwood, M.J., 2010. Astrovirus, reovirus, and rotavirus concomitant infection causes decreased weight gain in broad-breasted white poults. Avian Dis. 54 (1), 16–21.
Varsani, A., Krupovic, M., 2018. Smacoviridae: a new family of animal-associated single-stranded DNA viruses. Arch. Virol. 163 (7), 2005–2015.
Victoria, J.G., Kapoor, A., Dupuis, K., Schnurr, D.P., Delwart, E.L., 2008. Rapid identification of known and new RNA viruses from animal tissues. PLoS Pathog. 4 (9), e1000163.
Victoria, J.G., Kapoor, A., Li, L., Blinkova, O., Slikas, B., Wang, C., Naeem, A., Zaidi, S., Delwart, E., 2009. Metagenomic analyses of viruses in stool samples from children with acute flaccid paralysis. J. Virol. 83 (9), 4642–4651.
Woo, P.C., Lau, S.K., Teng, J.L., Tsang, A.K., Joseph, M., Wong, E.Y., Tang, Y., Sivakumar, S., Bai, R., Wernery, R., Wernery, U., Yuen, K.Y., 2014. Metagenomic analysis of viromes of dromedary camel fecal samples reveals large number and high diversity of circoviruses and picobirnaviruses. Virology 471-473, 117–125.
Woo, P.C., Teng, J.L., Bai, R., Wong, A.Y., Martelli, P., Hui, S.W., Tsang, A.K., Lau, C.C., Ahmed, S.S., Yip, C.C., Choi, G.K., Li, K.S., Lam, C.S., Lau, S.K., Yuen, K.Y., 2016. High diversity of genogroup I picobirnaviruses in mammals. Front. Microbiol. 7, 1886.
Zhang, B., Tang, C., Yue, H., Ren, Y., Song, Z., 2014. Viral metagenomics analysis demonstrates the diversity of viral flora in piglet diarrhoeic faeces in China. J. Gen. Virol. 95 (Pt 7), 1603–1611.
Zsak, L., Strother, K.O., Kisary, J., 2008. Partial genome sequence analysis of parvoviruses associated with enteric disease in poultry. Avian Pathol. 37 (4), 435–441.
Zsak, L., Strother, K.O., Day, J.M., 2009. Development of a polymerase chain reaction procedure for detection of chicken and turkey parvoviruses. Avian Dis. 53 (1), 83–88.
Zsak, L., Cha, R.M., Day, J.M., 2013. Chicken parvovirus-induced runting-stunting syndrome in young broilers. Avian Dis. 57 (1), 123–127.