Comparative viral metagenomics from chicken feces and farm dust in the Netherlands

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Running Head: Virome of Dutch farmed chicken feces and farm dust

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1 ABSTRACT (250 words, word limit = 250 words)

2 Livestock animals housed in close proximity to humans can act as sources or intermediate hosts
3 facilitating animal-to-human transmission of zoonotic diseases. Understanding virus diversity in livestock
4 is important for identifying potential zoonotic threats and for ensuring animal health and safe livestock
5 production. Here, we report viral metagenomic characterization of chicken feces (N=51) and paired farm
6 dust samples (N=13) using metagenomic deep sequencing. Samples were collected at 4-5 time points in
7 three broiler farms in the Netherlands. Viruses in the *Parvoviridae* and *Picornaviridae* families were the
8 most prevalent, detected in all feces and dust samples and in all feces and 85% of dust samples,
9 respectively. Virus composition found in chicken feces and corresponding dust samples were similar.
10 Great genomic diversity was identified in *Picornaviridae* and 46 sequences from five picornavirus genera
11 (*Siccinivirus*, *Megrivirus*, *Anatvirus*, *Gallivirus* and *Avisvirus*) were detected. For calicivirus, *Basovirus* and
12 an unclassified novel chicken calicivirus were identified in 13 fecal and 1 dust samples. Two distinct
13 types of chicken astroviruses were identified. Phylogenetic analyses of identified virus sequences from
14 *Picornaviridae*, *Astroviridae* and *Caliciviridae* suggested that viral sequences obtained from different
15 farms are often more closely related to each other than global reference sequences, and sequences
16 from feces and paired dust samples also clustered together. Importantly, our sequencing methodology
17 enabled the recover viral genome sequences from farm dusts, allowing the tracking of virus chatter
18 between livestock animals and their farm environment. This study, albeit relative sample size, does
19 expand current knowledge of virus communities in chickens and surrounding dusts.

20

21 IMPORTANCE (147 words, word limit = 150 words)

22 Chickens may harbor various zoonotic pathogens, some of which can cause severe clinical outcomes in
23 animals and humans. Farm dust can act as vector to facilitate zoonoses transmission. Here, we report
24 the metagenomic characterization of virus communities of chicken feces and paired farm dust samples
25 collected at multiple time points during the production cycle in broiler farms in the Netherlands.
26 *Parvoviridae* and *Picornaviridae* were most frequently detected. We also identified novel astrovirus and
27 calicivirus sequences that would inform future virus taxonomy classification. This is the first study to
28 characterize virus communities in farmed chickens and paired farm dust samples. We also describe a
29 dust sequencing strategy that can be adapted for future dust metagenomic characterization. Our study

could help setting up a surveillance baseline for tracking virus flow between chickens and their farm environment which could guide zoonotic outbreak preparedness and health risk assessment of farm exposure.

1. INTRODUCTION

Most emerging infectious diseases are of zoonotic origins (1). While 71.8% of zoonoses originated from wildlife, the emergence of MERS-CoV (2) and Nipah virus (3) has shown that livestock can act as intermediate hosts and/or reservoirs which can sustain zoonotic transmission at the wildlife-livestock-human interface. In addition to passing viruses from wildlife to humans, livestock is also reservoir of zoonotic pathogens, such as bacteria *Coxiella burnetii* (*C. burnetii*) that causes Q-fever in goats, sheep and cattle (4), and avian influenza (5, 6). Additionally, epidemiological studies have found evidence for increased risk of respiratory disease in persons with exposure to large scale animal farming (7, 8). In part, this can be attributed to farm dust exposure for which adverse health effects have been demonstrated (9–11), and the potential of zoonotic infections in these cases could be important yet under-reported. For instance, a Q-fever outbreak affecting approximately 4,000 people was reported in the Netherlands in 2007-2010 that was linked to circulation of *C. burnetii* in local dairy goat farms and dairy sheep farms (12). Q-fever was not notifiable in small ruminant farms in the Netherlands before the outbreak, making early detection of zoonotic transmission of *C. burnetii* challenging (13). Similarly, MERS-CoV had been circulating among dromedary camels long before it was recognized as a cause of severe respiratory disease in humans (14). These livestock-associated zoonotic disease outbreaks emphasize that continued surveillance of health risks due to livestock exposure is essential, especially when the intensification of livestock production is widely practiced to keep up the increasing food demand for the growing population (15).

Advances in Next-Generation Sequencing (NGS) technology have allowed simultaneous and unbiased detection of all viruses and microbes in a single sample, offering great potential in catch-all infectious disease surveillance. This metagenomic NGS (mNGS) approach combines the detection of known target pathogens with identification of novel and/or rare viruses (16, 17) and characterization of complex viral communities (18, 19). Indeed, numerous reports of virus diversity of animals including bats (20–22), pigs (23–25), wild birds (26, 27), and chickens (28) have been published since the invention

of NGS. Our previously published systematic review of all available viral mNGS studies of common livestock (29) found that metadata of available mNGS studies is often patchy and majority of the studies only had a very small sample size ($N \leq 10$). Sequencing data of most studies was also not publicly available. Continued surveillance of virus communities in common livestock following the “FAIR” data principle (Findable, Accessible, Interoperable and Reusable) (30) could provide a better understanding of what is normal virus diversity in livestock that is an essential baseline for early detection of potential zoonotic and/or animal pathogens using mNGS.

Chickens (*Gallus gallus domesticus*) are the most abundant domestic livestock. According to the Food and Agriculture Organization of the United Nations (FAO), the global chicken population was >22 billion birds in 2017 (31). Chicken products include meats and eggs that are produced by broiler and layer chickens respectively, and spent layer chickens can be harvested for meats as well. Chicken farming ranges from backyard to large-scale commercial farms that house ten-thousands of chickens. The broiler production systems usually follow a pyramidal selective breeding structure, with four generations (top to bottom: great grandparents [GGPs], grandparents [GPs], parent breeders and broilers) (32). Briefly, GGPs are produced by a few breeder farms and their offspring are the GPs that produce parent breeders. Broilers are the final offspring that are grown for consumption. The Netherlands is the second largest agricultural exporter in the world (33, 34). There are > 1 billion housed chickens and 1,741 chicken farms in the Netherlands in 2020, in which one-third of them ($N=637$) are broiler farms (35).

Chicken can carry various pathogens such as *Salmonella* (36), *Campylobacter* (37) and avian influenza viruses including subtypes that can cause severe infection in humans like H5N1 (38) and H7N9 (5); and it has been found that avian influenza virus can be transmitted to humans as a result of close exposure to contaminated chicken (6). Monitoring virus diversity in chickens is a key component to setting the baseline for identifying potential zoonotic threats. Meanwhile, little is known about the virome composition of chicken farm dust although it has been suggested that farm dust might play a role in infectious disease transmission (39, 40). Farm dust consists of animal feed, bedding material, animal feces, dander, mites and microorganisms (43), as well as poultry feathers in the case of poultry farm dust; and farm dust exposure may cause adverse health effects (41, 42). Most studies on farm dust

have focused on measuring antimicrobial genes (44) and endotoxins (9, 11), yet limited has been done to document the viral content of farm dust samples.

Here, we document metagenomic characterization of virus diversity in a comprehensive sample set of chicken feces and paired farm dust samples longitudinally collected at multiple time points in three commercial broiler farms across the Netherlands. We show that chicken fecal virome and farm dust virome are highly comparable and stable over time, host age and farms. This study provides useful insights in understanding the virus flows between chickens and poultry farm environments and would contribute to the continued effort of One Health surveillance.

2. RESULTS

2.1 Comparative virus prevalence in chicken feces and farm dust

A total of 56 pooled chicken feces and 46 individual farm dust samples were collected. Five chicken fecal samples were excluded due to insufficient cDNA concentration for library preparation. Individual farm dust samples collected at the same sampling moment from the same farm were pooled together during sample processing for sequencing, making up a final set of 13 pooled farm dust samples. Metagenomic deep sequencing of pooled chicken feces (N=51) and pooled chicken farm dust samples (N=13) generated ca 1-2 million and 1-3 million reads per sample, respectively.

From the total of 64 chicken feces and farm dust samples analyzed, *Parvoviridae* was consistently detected in all samples, followed by *Picornaviridae* with a 100% and 85% prevalence in chicken feces and dust samples respectively (**Table 1**). *Astroviridae* was the third most prevalent family detected in 82% chicken feces and 85% of farm dust samples, followed by *Caliciviridae* with 78% (chicken feces) and 69% (farm dust samples) prevalence. The pattern of virus detection observed in dust samples was similar to that in chicken feces, suggesting that dust could play a role in the virus transmission of these animal viruses (**Table 1** and **Figure S1**).

Although the detection frequency of *Parvoviridae* was slightly higher than *Picornaviridae*, the number of *Picornaviridae* contigs detected was the highest (**Figure 1**). Within the *Picornaviridae* family, *Sicinivirus* was consistently detected in all chicken feces and 69% of farm dust samples (**Table 2**), followed by *Anatvirus* (chicken feces: 70%; farm dust samples: 61%), and *Megrivirus* (chicken feces: 43%; farm dust samples: 46%). Of note, the number of contigs detected in different picornavirus genera

corresponded to the prevalence pattern (**Figure 2 & Figure S2**). In particular, *Anatvirus*, *Gallivirus*, *Megrivirus* and *Sicinivirus* were detected in all three farms, while *Orivirus* was only identified in farm F_01 and F_03 and *Avisivirus* was only found in farm F_03 (**Figure S2**). When looking at virus family level, we did not observe any substantial differences in overall viral contents (**Figure 1**) and virus-specific prevalence (**Figure S1**) of samples from different farms and from different production cycles (age) within farms.

2.2 Genomic diversity in viruses identified from chicken feces and farm dust

2.2.1 *Picornaviridae*

Phylogenetic construction was performed to compare the reported *Picornaviridae* sequences from 5 genera (*Avisivirus*, *Anatvirus*, *Gallivirus*, *Megrivirus* and *Sicinivirus*) with global sequences. Forty-six picornavirus sequences with $\geq 80\%$ genome coverage (chicken feces: 38; farm dust samples: 8) were included in the phylogenetic analysis of the polyprotein region. The maximum-likelihood (ML) phylogenetic tree (**Figure 3**) showed that the sequences identified from farm F_03, albeit small number (N=12), belonged to all 5 different genera *Avisivirus*, *Anatvirus*, *Gallivirus*, *Megrivirus* and *Sicinivirus*, suggesting a high viral diversity circulating among chicken in this farm. Interestingly, farm F_02 with the highest number of picornavirus contigs identified (N=31) has a moderate level of viral diversity, with these contigs belonging to 3 genera *Anatvirus*, *Sicinivirus* and *Megrivirus*. Only 3 picornavirus genomic sequences identified were from farm F_01, belonging to *Sicinivirus* (N=1) and *Anatvirus* (N=2). *Sicinivirus* and *Anatvirus* are the most prevalent virus genera found with virus sequences detected in all 3 farms, while sequences of *Gallivirus* and *Avisivirus* were less prevalent, found in only 1 farm, farm F_03.

Genome sequences detected from farm dust samples belonged to *Sicinivirus* and *Megrivirus* from farm F_02 that clustered with sequences identified in chicken feces from the same farm (**Figure 3**). Viruses in the *Sicinivirus* genus were detected in all chicken feces samples and 69% of dust samples with the highest number of viral contigs identified in each sample as compared to other genera in the *Picornaviridae* family. Slightly longer branches in the *Sicinivirus* sequences in the ML tree indicates higher diversity within this *Sicinivirus* clade (**Figure 3**). We further reconstructed a ML tree for all identified *Sicinivirus* polyprotein nucleotide sequences (N=15; chicken feces: 11; farm dust samples: 4) in

this study and compared them with global reference sequences (**Figure 4**). Two sequences from chicken feces (V_M_005_picorna_5 from farm F_01 and V_M_034_picorna_3 from farm F_03) were most distinct from the rest of the identified sequences (sharing only 69.4-72.3% nt identity) and were most closely related to strain JSY previously identified in China (78.9-79.0% nt identity). Other *Sicinivirus* sequences from the same farm often formed a monophyletic lineage, suggesting within-farm *Sicinivirus* sequences are highly genetically similar. Viruses identified from chicken feces and farm dust samples consistently clustered with each other forming their own sub-clusters within major clades in the ML trees, indicating high similarity between sequences from feces and dusts and that they are more closely related as compared to global reference sequences.

2.2.2 *Astroviridae*

Sixteen astrovirus sequences with $\geq 80\%$ genome coverage were identified from this study, among which only 1 astrovirus sequence was found in farm dust (VE_7_astro_14; farm F_02). There was only 1 astrovirus genomic sequence from farm F_01. RNA-dependent RNA polymerase (RdRp) and capsid regions of these sequences were extracted for phylogenetic analyses (**Figure 5A and 5B**, respectively). ML trees of both regions showed that these 16 sequences belonged to two distinct lineages, A1 and A2 (**Figure 5**). Sequences from farm F_02 and F_03 were found in both lineages A1 and A2, suggesting co-circulation of different astrovirus strains in both farms. The 12 sequences from lineage A1 were most closely related to avian nephritis virus strains previously reported in China and Brazil, while the 4 sequences from lineage A2 were more closely related to each other and clustered with chicken astroviruses previously identified in Malaysia, Canada and the USA. The 12 sequences from lineage A1 shared 96-100% amino acid (aa) identity in the RdRp region while 4 sequences from lineage A2 shared a higher 99-100% aa identity. For the less conserved capsid region, three small sub-lineages can be observed within lineage A1 (**Figure 5B**). Amino acid identity matrix also supported this observation that intra-sub-lineage identity (range 87%-100%) is higher than inter-sub-lineage identity (range 64-74%). Notably, the only astrovirus sequence found in dust (strain VE_7_astro_14) was identical to astrovirus recovered from its paired chicken feces sample (strain V_M_038_astro_4) at the same farm.

174 2.2.3 *Caliciviridae*

175 Similar to astrovirus distribution in feces versus dusts, 13 out of 14 calicivirus sequences ($\geq 80\%$
176 genome coverage) identified were recovered from fecal samples and only 1 calicivirus sequence
177 identified from dust sample. Partial ORF1 polyprotein region of these sequences was extracted to
178 construct ML tree comparing the local calicivirus with global sequences. The ML tree showed that the
179 reported calicivirus sequences formed two distinct lineages (C1 and C2) (**Figure 6**). All calicivirus
180 sequences from farm F_02 and F_03 belonged to lineage C1, while sequences from F_01 belonged to
181 both lineages C1 and C2 suggesting higher diversity of calicivirus circulating in farm F_01. The 11
182 sequences in lineage C1 shared 98.7-100% aa identity despite being collected from different farms and
183 they shared 97.2-99.1% aa similarity with two partial ORF1 sequences identified in Germany in 2010
184 (F10026n and F10034n). BLAST searches and multiple sequence alignment indicated that these 11 local
185 calicivirus sequences are the first report of complete ORF1 sequence of that particular chicken calicivirus
186 lineage. The only calicivirus sequence from dust (VE_8_calici_66) clustered in lineage C1. The 3
187 sequences in lineage C2 are highly similar, sharing 99.9-100% aa identity and were all collected from
188 farm F_01 at two time points. These 3 sequences shared 93.8-96.8% aa similarity with reference
189 sequences from USA, Korea, Brazil and Germany (*Bavaria virus*, *Bavovirus* genus).

190

191 2.2.4 *Coronaviridae*

192 Three nearly-complete coronavirus genome sequences were identified in 3 chicken feces (V_M_001,
193 V_M_002 and V_M_003) from farm F_01. BLAST analyses showed that these sequences are Infectious
194 bronchitis virus (IBV) strains and share 99.92-99.95% identity at the nucleotide level with IBV vaccine
195 strain 4/91 (GenBank accession number KF377577), suggesting these IBV may possibly be viral shedding
196 following vaccination or environmental contamination as vaccines were given by drinking water.

197

198 3. DISCUSSION

199 Livestock kept in close proximity to humans can act as intermediate hosts and/or sources to amplify
200 zoonotic disease transmission (45). The surge of zoonotic disease outbreaks around the world has
201 prompted the need for more proactive surveillance strategy involving animal surveillance, which could
202 potentially guide zoonotic outbreak preparedness. Chicken is the most abundant livestock in the world

(31) and has been identified as reservoir for various zoonotic pathogens (37, 38). In this study, we employed viral metagenomic deep sequencing to document total viruses present in chicken feces and paired farm dust, data from which provide insights into virus flow between chickens and their farm environment as well as a platform for future surveillance in farm animals and environmental samples. To our knowledge, this is the first study characterizing the virome (viral metagenomics) of chicken feces and farm dust from the Netherlands.

In this study, we described a primer-independent viral metagenomic deep sequencing strategy to sequence viruses in dust samples that usually possess low viral load and are hence difficult to sequence. We further demonstrated that using this method, we were able to recover long viral sequences with $\geq 80\%$ genome coverage from farm dust samples. This method could be adapted for future metagenomic characterization of virus diversity in dust samples. Farm dust is associated with adverse respiratory effects observed in farm workers with prolonged exposure (41). There is a knowledge gap in virus diversity of farm dust. Characterizing farm dust virome could aid in investigating possible health effects of occupational and environmental exposure to virus-containing farm dust.

Phylogenetic analyses indicated that viruses identified from farm dust samples were genetically closely related to viruses identified from chicken feces collected from the same farm, suggesting that viruses identified in dust were attributed to the viruses circulating in the chicken feces that is also source of farm dust. This observation is in agreement with a previous study that reported the correlation found in the resistomes between animal feces and farm dust (46). More importantly, the detection of chicken-related virus sequences in dust samples raises the possibility of dust-borne transmission of viruses especially important in the cases of chicken and/or zoonotic pathogenic viruses (47), although infectivity of the viruses identified was not confirmed as no virus culture was performed in our study. Future investigation on the infectivity of viruses in farm dust could provide useful insights in mapping virus flows between and among chickens as well as chicken and surround environment including dust, which collectively would guide future outbreak control strategies in farms.

Viruses identified in chicken feces from different farms are phylogenetically closely related to each other and formed sub-lineages or monophyletic lineages when comparing to global related sequences. This could possibly be explained by the pyramid structure of broiler production system which chicks were bought from a few suppliers that were not bred by the broiler farmers; in other words, chicks

carrying similar viruses from a supplier were then distributed to different broiler farmers, and further spread within the farms, leading to similar viruses circulating in different broiler farms. Of note, the pyramid production structure was also suggested as a possible transmission routes for ESB/pAmpC producing bacteria between broiler farms (32). Future characterization of virus diversity of chickens from the top part of the pyramid is needed to confirm the speculation.

Viruses belonging to five genera of the *Picornaviridae* family were identified in our study, suggesting a great diversity of picornaviruses circulating in chickens, which is consistent with previous studies that detected various picornavirus genera in chicken respiratory samples (48, 49). Astroviruses identified in this study clustered in two distinct phylogenetic lineages; these astroviruses could present possible new genotypes. For astrovirus, all chicken astroviruses are grouped to one single species according to the current taxonomy assignment from International Committee on Taxonomy of Viruses (50). Current classification of avastrovirus is based on phylogenetic analysis of capsid (ORF2) amino acid sequences (51). More comprehensive assignment of genotypes is hindered by limited number of complete capsid sequences. A similar observation was seen in *Caliciviridae*, we identified two types of caliciviruses forming two distinct lineages in the ML tree. Lineage C2 is closely related to the *Bavovirus* genus, while the sequences from C1 are most related to an unclassified chicken calicivirus strain F10026n (52) that only possessed a partial ORF1 sequence. Our data have provided 16 astrovirus and 14 calicivirus sequences, which help expand the current knowledge of chicken astrovirus and calicivirus diversity and could potentially contribute to improve future taxonomy assignment.

We observed that viruses in the *Parvoviridae* and *Picornaviridae* families were consistently detected in chicken feces and farm dust samples at multiple time points in all three farms. These virus families could potentially present the “signature” viruses to indicate chicken exposure. By combining these viruses together, a chicken viral fingerprint profile may be built that can be used to map the virus flow between farms and surrounding areas. In case of any future avian-associated zoonotic outbreaks, these marker viruses could also be used to assess spatial transmission risk in poultry farm surrounding areas (29, 53). Additionally, these viruses could also be used as markers to assess the efficiency of disinfection procedures in farms and surrounding environments although stabilities of different viruses may differ due to different structures. Since this is the first study of its kind in chicken and farm dust in the Netherlands, further and perhaps larger scale of surveillance with the inclusion of samples from layer

hens and more environmental samples from surrounding areas are certainly necessary to confirm our observation and the applicability of using these identified viruses as signature virus fingerprint.

In conclusion, we provide a comprehensive dataset of virus diversity of chicken feces and paired farm dust samples. Highly prevalent viruses like parvoviruses and picornaviruses were detected and they could potentially serve as marker viruses to follow the virus flows between chickens and their surrounding environment. Identification of novel calicivirus and astrovirus sequences indicates that continued effort is needed for increasing understanding of virus diversity in chicken feces. Viral surveillance using the One Health approach is warranted for early detection of zoonotic viruses. In the long term, understanding virus flows at the animal-human-environmental interface is vital for identifying potential zoonotic threats and zoonotic outbreak control and preparedness.

4. MATERIALS AND METHODS

4.1 *Sample collection*

Pooled chicken feces and farm dust samples were collected in three commercial broiler farms in the Netherlands at 4-5 time points from May to August 2019. The three commercial broiler farms are located in three different regions in the Netherlands (Western part: Noord/Zuid-Holland region; Northern part: Friesland/Groningen region; Eastern part: Gelderland region). Detailed sampling strategy and sample metadata is described in **Table 3**. Each pooled poultry fecal sample contains fresh fecal material from 3-4 chicks. Farm dust samples were collected using a passive air sampling approach using electrostatic dustfall collectors (EDCs) (54, 55). Electrostatic cloths were sterilized through overnight incubation at 200°C. Sterilized electrostatic cloths were then fixed to a pre-cleaned plastic frame. EDCs were exposed for 7 days at 1 meter above the floor with the electrostatic cloths facing up in broiler farms to enable sampling of settling airborne dust instead of resuspended dust from the floor. EDCs were contained in a sterile plastic bag before and after sampling. All samples were transported under cold chain management and stored at -20°C/-80°C before processing.

4.2 *Sample processing and metagenomic deep sequencing*

Chicken fecal samples were processed as previously described (56). Briefly, chicken fecal suspension was prepared in Phosphate-buffered saline (PBS) and treated with TURBO DNase (Thermo Fisher, USA)

at 37 °C for 30 minutes, and then subjected to total nucleic acid extraction using QIAamp viral RNA mini kit (Qiagen, Germany) according to manufacturer's instruction without addition of carrier RNA. For dust samples, electrostatic cloths were incubated in 3% beef extract buffer for 1 hour on rolling as previously published (57). After incubation, the suspension was collected and centrifugated at 4,000 g at 4°C for 4 minutes to pellet any large particles or debris. Total viruses in dust suspension were concentrated using polyethylene glycol (PEG) similar to virus concentration in sewage published previously (58, 59). Briefly, PEG 6000 was added to each dust suspension to make up a final 10% PEG 6000 (Sigma-Aldrich, USA) concentration, followed by pH adjustment to pH 4 and overnight incubation at 4°C with shaking. After overnight incubation, sample was centrifuged at 13,500 g at 4°C for 90 minutes. Supernatant was removed and the remaining pellet was resuspended in 500 µL of pre-warmed glycine buffer, and then subjected to 5-minute centrifugation at 13,000 g at 4°C. Supernatant was collected, and supernatants from EDC samples that were collected in the same farm at the same time point (N=1-4) were pooled together for further processing. Viral-enriched dust suspension samples were then treated with TURBO DNase as previously described (56) to remove non-encapsulated DNA, followed by total nucleic acid extraction using MagMAX™ viral RNA isolation kit (Thermo Fisher, USA) according to manufacturer' instructions but without the use of carrier RNA. Reverse transcription and second strand cDNA synthesis of chicken fecal samples and dust samples was performed as previously described (56), using SuperScript III reverse transcriptase and non-ribosomal hexamers (60) (reverse transcription) and Klenow fragment 3'-5' exo- (New England BioLabs, USA) (2nd strand cDNA synthesis), respectively.

Standard Illumina libraries were prepared using Nextera XT DNA library kit following the manufacturer's instruction. Final libraries were sequenced on the Illumina MiSeq platform (600 cycles; paired-end). Chicken fecal samples with a multiplex range of 16-19 samples. Dust samples and negative blank controls were sequenced with a multiplex of 12 samples per run.

4.3 Sequencing data analysis

Raw reads were subjected to adapter removal using Trim Galore/default Illumina software, followed by quality trimming using QUASR (61) with a threshold of minimum length of 125 nt and median Phred score ≤ 30. Remaining reads were *de novo* assembled using metaSPAdes v3.12.0 (62). *De novo* assembled contigs were classified using UBLAST (63) against eukaryotic virus family protein databases as

previously described (22, 25). We set a detection threshold of contig with minimum amino acid identity of 70%, minimum length of 300 nt and an e-value threshold of 1×10^{-10} when interpreting our contig classification results. Contig classification results were analyzed and visualized using R packages including dplyr, reshaped2 and ComplexHeatmap (64–66).

4.4 *Phylogenetic analysis*

Sequences with $\geq 80\%$ of genome coverage from the *Picornaviridae*, *Astroviridae* and *Caliciviridae* families were used for phylogenetic analyses, comparing them with global sequences retrieved from GenBank. Multiple sequence alignment was performed using MAFFT (67) and manually checked in Geneious v2021.0.3 (Biomatters, New Zealand). Best-fit models of evolution for phylogenetic tree reconstruction were estimated using ModelFinder module in IQ-TREE v1.6.11 (68), determined by Akaike Information Criterion (AIC). Maximum-likelihood trees were constructed using RAXML-NG v1.0.1 (69) with 100 pseudo-replicates. Resulting trees were visualized and annotated using R package ggtree (70).

4.5 *Data availability*

The raw reads are available in the SRA under the BioProject accession number [PRJNA670873](#) (chicken feces) and [PRJNA701384](#) (farm dust samples) (**Table S1**). All sequences in phylogenetic analysis have been deposited in GenBank under the accession numbers MW684778 to MW684847 (**Table S2**).

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348 **AUTHOR CONTRIBUTION**

349 K.T.T.K and M.V.T.P conceived the study and performed experiments. M.M.T.dR and I.M.W. designed
 350 sampling strategy. M.M.T.dR. and A.B.M. handled sample collection and collected associated metadata.
 351 K.T.T.K. and M.V.T.P. analyzed data. K.T.T.K. drafted the manuscript. M.V.T.P., M.P.G.K., L.A.M.S., I.M.W.,
 352 M.M.T.dR and A.B.M. edited the manuscript. M.V.T.P., M.P.G.K. and I.M.W. supervised the study.
 353 M.P.G.K., D.J.J.H. and L.A.M.S. acquired the funding.
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Figure legends

Figure 1. An overview of virus contigs detected in chicken feces and chicken farm dust samples. Each column represents one sample. Associated metadata is shown at the top panel. The color intensity of the heatmap (bottom panel) is determined by number of contigs with minimum length of 300 nt/bp, at least 70% identity and an e-value threshold of 1×10^{-10} when comparing with closest reference in our database. Sample order is assorted by farm ID, sampling time point. Time point is an arbitrary number. Samples were collected at the same time and place when both Farm ID and Time point match.

Figure 2. An overview of picornavirus contigs detected in chicken feces and chicken farm dust samples. Each column represents one sample. Associated metadata is shown at the top panel. The color intensity of the heatmap (bottom panel) is determined by number of contigs with minimum length of 300 nt, at least 70% identity and an e-value threshold of 1×10^{-10} when comparing with closest reference in our database. Sample order is assorted by farm ID, sampling time point. Time point is an arbitrary number. Samples were collected at the same time and place when both Farm ID and Time point match.

Figure 3. Maximum-likelihood phylogenetic tree of 76 (sequences from this study: 46; reference sequences: 30) picornavirus partial polyprotein amino acid sequences (3786 sites) reconstructed using a best-fit evolutionary model LG+FC+R10. For clarity, only bootstrap supports ≥ 70 are shown as a navy-blue node point. Tip labels of our samples are colour-coded by corresponding farm ID. Reference sequences are in grey. Tree was mid-point rooted for clarity.

Figure 4. Maximum-likelihood phylogenetic tree of 30 sicinivirus partial polyprotein nucleotide sequences (sequences from this study: 15; reference sequences: 15; 8672 sites) reconstructed using a best-fit evolutionary model GTR+F+R10. For clarity, only bootstrap supports ≥ 70 are shown as a navy-blue node point. Tip labels of our samples are colour-coded by corresponding farm ID. Reference sequences are in grey and tree was mid-point rooted for clarity.

Figure 5. A. Maximum-likelihood phylogenetic tree of 33 astrovirus partial RdRp amino acid sequences (sequences from this study: 16; reference sequences: 17; 548 sites) reconstructed using a best-fit evolutionary model LG+FC+I+G4m. B. Maximum-likelihood phylogenetic tree of 33 astrovirus partial capsid amino acid sequences (sequences from this study: 16; reference sequences: 17; 908 sites) reconstructed using a best-fit evolutionary model LG+FC+I+G4m. For clarity, only bootstrap supports ≥ 70 are shown as a navy-blue node point. Tip labels of our samples are colour-coded by corresponding farm ID. Reference sequences are in grey and tree was mid-point rooted for clarity.

Figure 6. Maximum-likelihood phylogenetic tree of 30 calicivirus partial polyprotein amino acid sequences (sequences from this study: 14; reference sequences: 16; 2794 sites) reconstructed using a best-fit evolutionary model LG+FC+R10. For clarity, only bootstrap supports ≥ 70 are shown as a navy-blue node point. Tip labels of our samples are colour-coded by corresponding farm ID. Reference sequences are in grey and tree was mid-point rooted for clarity.

Table 1. Prevalence of different eukaryotic virus families in chicken feces and farm dust samples

Sample types		Chicken feces (N=51)	Farm dust (N=13)
Virus family	<i>Astroviridae</i>	42 (82%)	11 (85%)
	<i>Caliciviridae</i>	40 (78%)	9 (69%)
	<i>Coronaviridae</i>	19 (37%)	3 (23%)
	<i>Parvoviridae</i>	51 (100%)	13 (100%)
	<i>Picobirnaviridae</i>	8 (16%)	2 (15%)
	<i>Picornaviridae</i>	51 (100%)	11 (85%)
	<i>Reoviridae</i>	7 (14%)	3 (23%)

Table 2. Prevalence of picornavirus genera in chicken feces and farm dust samples

Sample types		Chicken feces (N=51)	Farm dust (N=13)
Picornavirus genera	<i>Anatvirus</i>	36 (70%)	8 (61%)
	<i>Avisivirus</i>	3 (5%)	0 (0%)
	<i>Gallivirus</i>	16 (31%)	4 (30%)
	<i>Megrivirus</i>	22 (43%)	6 (46%)
	<i>Orivirus</i>	8 (15%)	0 (0%)
	<i>Sicinivirus</i>	51 (100%)	9 (69%)

Table 3. Detailed sampling strategy

Farm #	Sampling moment identifier	Time point	No. of pooled fecal sample(s) collected	No. of pooled EDC sample(s) collected (no. of individual EDC sample[s] collected)	Week production cycle
F_01	F_01_1	1	4	1 (4)	3
	F_01_2	2	4	1 (4)	4
	F_01_3	3	4	1 (4)	5
	F_01_4	4	4	1 (1)	4
	F_01_5	5	4	0 (0)	5
F_02	F_02_1	1	4	1 (2)	4
	F_02_2	2	4	1 (4)	5
	F_02_3	3	4	1 (4)	2
	F_02_4	4	4	1 (4)	4
	F_02_5	5	4	1 (4)	5
F_03	F_03_1	1	4	1 (3)	4
	F_03_2	2	4	1 (4)	5
	F_03_3	3	4	1 (4)	4
	F_03_4	4	4	1 (4)	5

Supplementary materials legends.

Figure S1. An overview of virus family prevalence in chicken feces and farm dust samples. Associated metadata is shown at the top panel. The color intensity of the heatmap (bottom panel) is determined by number of contigs with minimum length of 300 nt, at least 70% identity and an e-value threshold of 1×10^{-10} when comparing with closest reference in our database. Sample order is assorted by farm ID, sampling time point. Time point is an arbitrary number. Samples were collected at the same time and place when both Farm ID and Time point match.

Figure S2. An overview of picornavirus genera prevalence in chicken feces and farm dust samples. Associated metadata is shown at the top panel. The color intensity of the heatmap (bottom panel) is determined by number of contigs with minimum length of 300 nt, at least 70% identity and an e-value threshold of 1×10^{-10} when comparing with closest reference in our database. Sample order is assorted by farm ID, sampling time point. Time point is an arbitrary number. Samples were collected at the same time and place when both Farm ID and Time point match.

Table S1. A list of corresponding BioProject, BioSample and SRA accession numbers for all samples.

Table S2. A list of corresponding GenBank accession numbers for sequences used in phylogenetic analyses.

Farm ID

F_01

F_02

F_03

Sicivirus

Gallivirus

Megrivirus

Anativirus

Avisivirus

Farm ID

F_01 bioRxiv preprint doi: <https://doi.org/10.1101/2021.03.09.434704>; this version posted March 10, 2024. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

F_02
F_03

0.2 nt subs/site

A

B

Farm ID

bioRxiv preprint doi: <https://doi.org/10.1101/2021.03.09.434704>; this version posted March 10, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

F_01
F_02
F_03