Metagenomic Characterization of Cloacal and Oral-Pharyngeal Swabs Reveals Virus Coinfections Associated with Newcastle Disease Virus among Poultry in Kenya

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Abstract

Newcastle disease is an endemic viral diseases affecting poultry and causing massive economic losses. This cross-sectional purposive study detected coinfections that are associated with the Newcastle disease virus (NDV) among poultry from selected regions in Kenya. Cloacal (n=599) and oral-pharyngeal (n=435) swab samples were collected and pooled into 17 and 15 samples, respectively. A total of 17,034,948 and 7,751,974 paired-end reads with an average of 200 nt were generated from the cloacal and oral-pharyngeal swab samples respectively. Analysis of the *de novo* assembled contigs identified 177 and 18 cloacal and oral-pharyngeal contigs respectively with hits to viral sequences, as determined by BLASTx and BLASTn analyses. A number of known and unknown representatives of *Coronaviridae*, *Picobirnaviridae*, *Reoviridae*, *Retroviridae*, and unclassified Deltavirus were identified in the cloacal swab samples. However, no NDV (family *Paramyxoviridae*) were detected in the cloacal swabs, although they were detected in the oropharyngeal swabs of chicken sampled in Nairobi, Busia, and Trans Nzoia. Additionally, sequences representative of *Paramyxyviridae*, *Coronaviridae*, and *Retroviridae* were identified in the oral-pharyngeal swab samples. Infectious bronchitis virus (IBV) and rotavirus were the most prevalent

coinfections associated with NDV among chickens. The detection of these coinfections suggests that these viruses are significant threats to the control of Newcastle disease as the NDV vaccines are known to fail because of these coinfections. This study therefore provides important information that will help in improving disease diagnosis and vaccine development for coinfections associated with NDV.

Keywords: infectious bronchitins virus, orthoavulavirus, rotavirus

Introduction

Newcastle disease (ND) is an infectious viral disease affecting poultry that is endemic in Africa and other parts of the world (Rabalski et al., 2014). The disease is caused by an enveloped, non-segmented, negative-sense, single-stranded RNA virus belonging to the family *Paramyxoviridae* (Rabalski et al., 2014). ND virus (NDV) belongs to the Avian Orthoavulavirus 1 (avian paramyxovirus 1, APMV-1) serotype. Its genome contains 15,186, 15,192, or 15,198 nucleotides depending on the genotype and encodes six proteins, which include the nucleocapsid protein (NP), phosphate polymerase cofactor protein (P), membrane associated matrix protein (M), fusion surface glycoprotein (F), hemagglutinin-neuraminidase surface glycoprotein (HN), and large RNA dependent RNA-polymerase (L) (Rabalski et al., 2014). Paramyxovirus virions usually enter the host organism through the respiratory or gastrointestinal epithelial cells.

Backyard poultry are usually exposed to a wide range of viral pathogens; unfortunately, most studies tend to focus on single infections (Bettridge et al., 2014). The importance of coinfections has been demonstrated in humans (N. Kumar et al., 2018; Aghbash et al., 2021), wild birds (Torrontegi et al., 2019; Musa et al., 2020) and poultry (Xuan et al., 2015; Naguib et al., 2017; Gowthaman et al., 2019). However, there is limited information about coinfections associated with NDV in backyard poultry In Kenya.

Although vaccines based on low pathogenic variants of NDV protect against clinical signs and mortality; however, they do not prevent infection with highly pathogenic strains, leading to uncontrolled spread of the virus (Nakamura et al., 2013). This is especially evident in backyard poultry systems where the vaccine usually fails because of coinfections with other viruses such as IBV and rotaviruses. It has also been previously reported that NDV normally prepares a conducive environment for several respiratory/immunosuppressive pathogens to complicate the

outcome of the disease process (Gowthaman et al., 2017). Hence, there is need to control these coinfections if the vaccination efforts against highly pathogenic NDV are to be achieved.

A number of approaches have been employed to study the epidemiology of viral infections affecting poultry, including pathogen surveillance and tracking the molecular epidemiology of pathogens (Qiu et al., 2019). Previous methods involved extracting and subsequently amplifying the viral nucleic acids using conventional polymerase chain reaction (PCR) methods, espoecially reverse transcription PCR (RT-PCR) (Qiu et al., 2019). Using this strategy, a number of viruses have been implicated in poultry diseases, including astroviruses (Pantin-Jackwood et al., 2008, 2011), parvoviruses (Koo et al., 2015), reoviruses (Chen et al., 2014), rotaviruses (Spackman et al., 2010), paramyxoviruses (Ogali et al., 2018), and coronaviruses (Chamings et al., 2018; Woo et al., 2012). These methods often require prior knowledge of the target viral pathogen, which poses a challenge when investigating multiple pathogens in one or more hosts, especially from different viral families. Additionally, these methods are unable to monitor and provide an early warning system for pathogens of other poultry diseases, as well as emerging viruses that are yet to be characterized (Qiu et al., 2019).

Currently, metagenomics-based detection methods are being applied successfully to characterize viral populations in different hosts and environments (Lima et al., 2019). This has facilitated the discovery of a high number of novel viral agents from different types of tissues, including the GIT and the oral-pharyngeal regions in poultry and other livestock (Amimo et al., 2016; Cheung et al., 2013; Day et al., 2010, 2015; Kim et al., 2011; Lima et al., 2017, 2019; Ng et al., 2015; Qiu et al., 2019; Shah et al., 2014; Vibin et al., 2020). The popularity of these techniques has largely been due to their high sensitivity and wide coverage as they target the entire genome (Qiu et al., 2019; Vibin et al., 2020). They can therefore be used to detect all pathogens found in the sample of interest. Furthermore, these methods can discover novel viruses whose sequence information is unknown. Metagenomics is therefore an important tool for detecting coinfections among poultry or any other host.

Most metagenomic studies on poultry microbiome (including viral communities) have been carried out on poultry reared under controlled and regulated feeding regimes. To the best of our knowledge, the only exception is a study that investigated the microbial community profiles of

indigenous backyard chickens on a scavenging feeding system from two geographically and climatically distinct regions of Ethiopia (H. Kumar et al., 2019). Metagenomics analysis of backyard poultry raised under a free-range scavenging feeding system is therefore required in order to explore the impact of local feed (plants, insects and other small animals) on poultry health (H. Kumar et al., 2019). In addition, this aids the understanding of the virome compositional structure in relation to the environment in free-ranging poultry. Our study therefore focused on the characterization of coinfections associated with NDV among mostly free-ranging poultry populations in selected regions in Kenya.

Materials and Methods

Sample Collection

This study was carried out from 2016 to December 2018 both retroactively and actively across six counties with varying agroecological conditions in Kenya (Figure 1). The study received institutional clearance from the Jomo Kenyatta University of Agriculture and Technology (JKUAT) to conduct animal research. Clearance was also sought from the Director of Veterinary Services from the National Veterinary Laboratories, State Department of Livestock, Ministry of Agriculture, Livestock, Fisheries and Co-operatives, Kenya to carry out the study on farm animals. Permisions were also granted by the respective county governments of Busia, Bungoma, Kilifi, Kwale, Nairobi, and Trans Nzoia to carry out the research. The research used a employed a participatory approach and prior informed consent from the farmers was also sought and given before collecting samples. In this cross-sectional purposive study, cloacal swab samples (n=599) and oral-pharyngeal swab samples (n=435) were collected from selected regions in Kenya with distinct geographic and climatic conditions. The targeted regions included counties bordering Uganda (Bungoma, Busia and Trans Nzoia), maritime borders (Kilifi and Kwale), and urban areas of Nairobi (Figure 1). Information on flock condition or performance was also collected. The collected cloacal and oral-pharyngeal swab samples were then immediately frozen in dry ice and later placed in liquid nitrogen in the field. They were then processed in preparation for downstream analysis or permanently preserved at -80°C until processing.

Extraction of Nucleic Acids and Sequencing

Cloacal and oral-pharyngeal samples collected from poultry (chickens, ducks, guinea fowls, geese, pigeons, and turkeys) were pooled according to the species and region of origin. They were named CN1-CN12 for chickens, DK1-DK6 for ducks, GF1-GF2 for guinea fowls, GS1-GS4 for geese, PN1-PN6 for pigeons, and TY1-TY2 for turkeys, resulting in 17 cloacal and 15 oral-pharyngeal sample pools (Supplementary Materials Table S1 and Table S2). The nucleic acids of each pooled sample were used to prepare the viral metagenomic libraries.

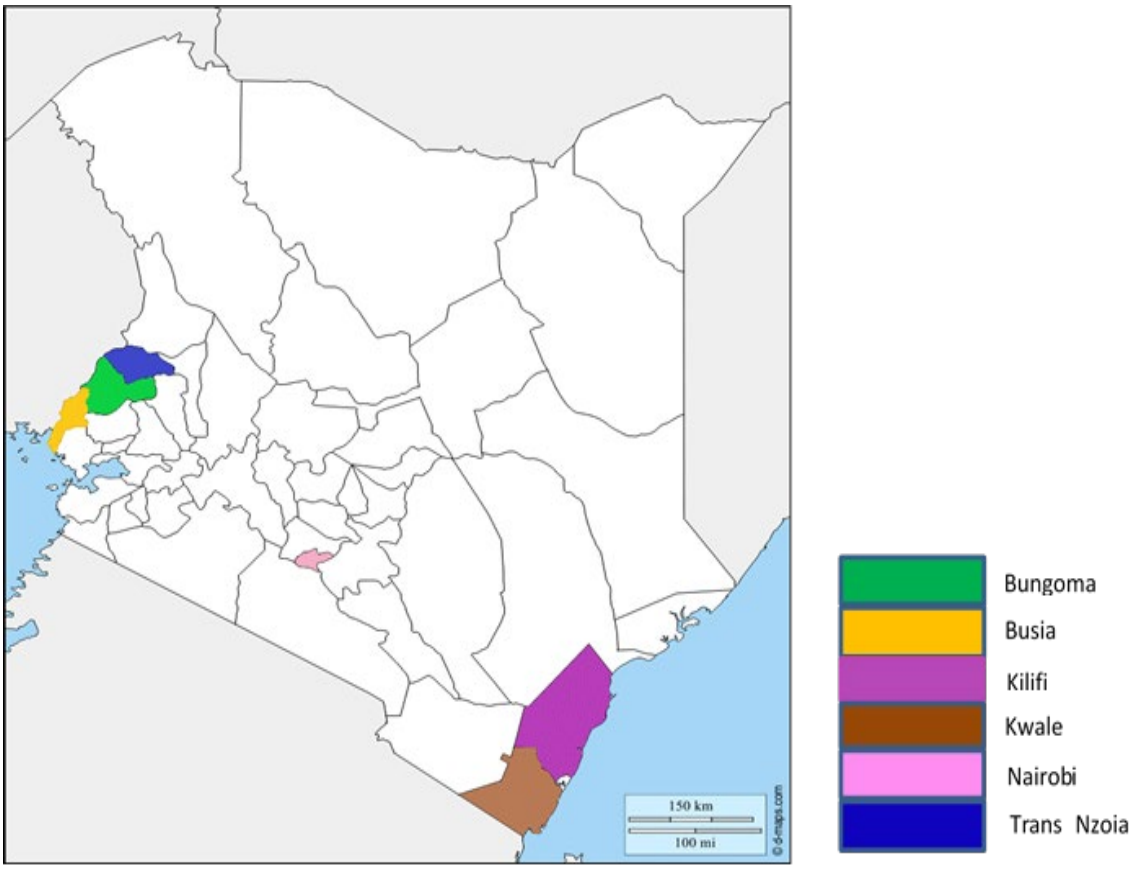

Figure 1: Map of Kenya showing the main sampling sites with varying geographic and climatic conditions for cloacal and oropharyngeal swab samples (Source:)

DNA Extraction: DNA was extracted from the pooled cloacal and oral-pharyngeal swab samples using the PureLink Genomic DNA Mini Kit (Invitrogen, Life Technologies) following the manufacturer's protocol. Briefly, the swab sample was placed into a micro-centrifuge tube to which 200 µl of phosphate buffered saline (PBS) and 20 µl of Proteinase K were added and

mixed well by pipetting. An equal volume (200 µl) of PureLink^{R} Genomic Lysis/Binding Buffer was then added to the lysate and mixed well by vortexing briefly before incubating at 55°C for at least 10 minutes. The lysate was briefly centrifuged and 200 µl of 99% ethanol added and mixed well by vortexing for 5 seconds before removing the PureLink^{R} Spin Column in the Collection Tube from the package. The lysate was then added to the PureLink^{R} Spin Column and centrifuged at 10,000 × *g* for 1 minute at room temperature. The collection tube was discarded and the spin column placed into a clean PureLink^{R} Collection Tube. To wash the extracted DNA, 500 µl of the Wash Buffer 1 prepared with ethanol was added to the column and centrifuged at room temperature at 10,000 × *g* for 1 minute. The collection tube was then discarded and the spin column placed into a clean PureLink^{R} collection tube. A second washing was done by adding 500 µl of Wash Buffer 2 prepared with ethanol to the column which was also centrifuged at maximum speed for 3 minutes at room temperature and the collection tube discarded. The spin column was finally placed in a sterile 1.5-ml micro-centrifuge tube and 50 µl of PureLink^{R} Genomic Elution Buffer added to the column which was then incubated at room temperature for 1 minute and centrifuged at maximum speed for 1 minute at room temperature. To recover more DNA, a second elution step using the same elution buffer volume as the first was performed in another sterile, 1.5-ml micro-centrifuge tube. The column was then removed and discarded. The purified DNA was stored at -20°C freezer before being sent to The African Genomics Centre and Consultancy (TAGCC) for library preparation and whole genome shotgun sequencing.

RNA Extraction: Viral RNA was extracted from the cloacal and oral-pharyngeal swab samples using the standard TRIzol reagent. The extracted RNA pellet was then re-suspended in RNase free water and stored at -80°C freezer before being sent to the International Livestock Research Institute (ILRI) laboratories for library preparation and sequencing.

Sequencing: Viral RNA was reverse-transcribed into complementary DNA (cDNA) using random hexamers in a single step process (Illumina TRUSeq Stranded total RNA Kit, Illumina, Inc, USA). The resulting first strand cDNA was used as template to synthesise the second strand, generating double stranded cDNA (dscDNA) using the same kit. The dscDNA preparation was used as a template to prepare Illumina sequencing library following Illumina DNA prep kit protocol (Illumina, Inc, USA). Indexed multiplexed samples were pooled and reconstituted to 4 nM before diluting to 12 pM for loading in to MiSeq instrument (Illumina, CA, USA) for a 2 x

200 paired-end sequencing run at the ILRI Genomic platform, Nairobi Kenya. The number of reads obtained from each library are shown in Supplementary Materials Table S3 and Table S4.

Bioinformatics Analysis

Poor quality sequencing reads with a Phred quality score < 20 and adaptors were trimmed using Trimmomatic version 0.39 (Bolger et al., 2014). The paired-end sequence reads were *de novo* assembled into contigs using Megahit version 1.0.2 (Li et al., 2015). The assembled contigs were analyzed by BLASTx against a viral protein database and visualized using Megan version 5.5.3 (Huson et al., 2016). Sequences with the best BLAST scores ($E \leq 10^{-3}$) were selected and assigned into known viral families. Contigs > 1000 nt were confirmed by mapping reads and selected to perform ORF predictions using Artemis. Overall taxonomic similarities between metagenomes was examined by performing hierarchical clustering and heatmap analyses using the Seaborn package in Python version 3.7.

For phylogenetic analyses, sequences representative of known viral families were obtained from GenBank and aligned with the sequences identified in the present study using MUSCLE software (Edgar, 2004). 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) or the bootstrap method using 100 replicates (Guindon and Gascuel, 2003).

Results

General Overview of the Sequence Data

A total of 17,034,948 clean paired-end reads (from cloacal swab samples) and 7,751,974 clean paired-end reads (from oral-pharyngeal swab samples), with an average of 200 nt, were generated (Supplementary Materials Table S3 and Table S4 respectively). Using BLASTx and BLASTn analyses, a total of 177 and 18 *de novo* contigs were identified with hits to known viral sequences from cloacal and oral-pharyngeal swab samples, respectively. The distribution of viral sequences and their detection rate in the pooled poultry cloacal and oral-pharyngeal swab samples are shown in Table 1 and Table 2, respectively.

Cloacal and Oral-Pharyngeal Viral Abundances

The family and species level viral abundances and the relative frequencies in each pooled sample are shown in Supplementary Materials Figure S1 and Figure S2, respectively. Most of the detected potentially pathogenic viruses in cloacal samples belonged to the *Coronaviridae* (43.4%) and *Reoviridae* (36.6%) families. Other potentially pathogenic viruses detected in smaller proportions belonged to *Retroviridae* (11.4%), the unclassified Deltavirus (1.7%), and *Picobirnaviridae* (0.6%). No viruses belonging to the *Paramyxoviridae* family were detected. Among the oral-pharyngeal samples, the most abundant potentially pathogenic viral families were *Paramyxoviridae* (50.0%), *Coronaviridae* (38.9%), and *Retroviridae* (5.6%).

The most abundant viral species detected in the cloacal samples were avian infectious bronchitis virus (IBV) (42.6%) and rotavirus (35.0%). Other potentially pathogenic viruses detected were reticuloendotheliosis virus (REV) (6.0%), lymphoproliferative disease virus (LDV) (2.7%), turkey coronavirus (TCoV) (1.6), avian leucosis virus (ALV) (1.6), avian HDV-like agent (1.6%), avian coronavirus (1.1%), pigeon-dominant coronavirus (0.5%) and picobirnavirus strain HK-2014 (0.5%). The most abundant potentially pathogenic viruses in the oral-pharyngeal samples were avian orthoavulavirus 1 (50.0%), avian IBV (38.9), and REV (5.6%).

The relative abundance of poultry viruses in each cloacal and oral-pharyngeal sample at family level is shown below in Figure 2. The most frequently detected viral families in cloacal chicken samples were *Reoviridae* (37.14-100%), *Coronaviridae* (50-62.86%) and *Retroviridae* (1.61-100%). The most abundant viral families in ducks were *Retroviridae* (9.09-90.91%), unclassified Deltavirus (9.09-50%) and *Picobirnaviridae* (9.09%). *Coronaviridae* and *Retroviridae* were also detected in one pigeon and turkey sample each, respectively. The most abundant viral families in oral-pharyngeal chicken samples were *Paramyxoviridae* (57.14-100%) and *Coronaviridae* (6-100%). *Retroviridae* were also detected in one goose sample.

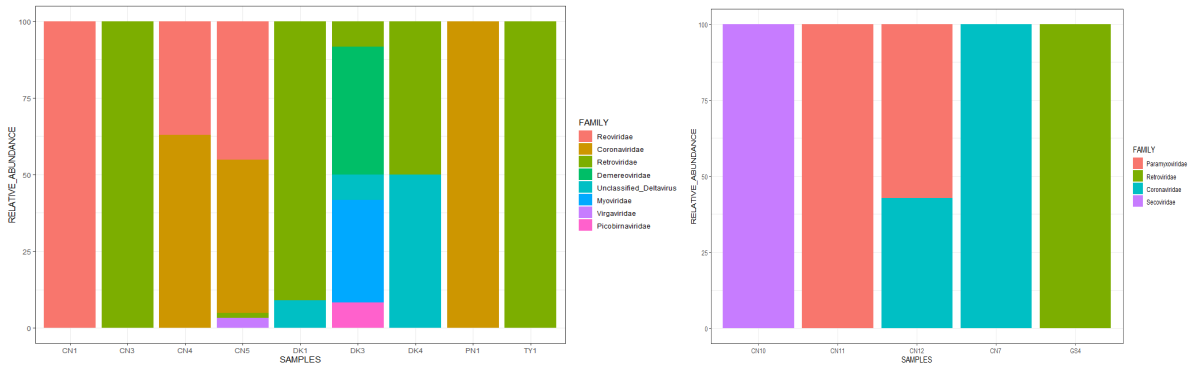

Figure 2: Classification of viral contigs detected from six poultry species a) cloacal swab samples and b) oral-pharyngeal swab samples. Only samples with detectable viruses are shown (CN= chicken, DK= duck, GF= guinea fowl, GS= goose, PN= pigeon and TY= turkey).

The relative abundance of poultry viruses in each cloacal and oral-pharyngeal samples at species level is shown below in Figure 3. The most frequently detected viral species in chicken cloacal samples were rotavirus (37.14-100%) and avian IBV (45.16-60%). Other coronaviruses such as TCoV and avian coronavirus were also detected at 1.49-3.23% and 1.43-1.61% respectively. ALV was likewise detected in one chicken sample. The most frequently detected viral species in duck cloacal samples were REV (50-90.91%) and avian HDV-like agent (8.33-50%). Other viruses detected in ducks were ALV (8.33%), picobirnavirus (6.33%) and endogeneous retrovirus strain EAV-0 (1.61%). LDV and pigeon-dominant coronavirus were also detected in turkey and pigeon cloacal samples respectively. The most frequently detected viral species in chicken oral-pharyngeal samples were orthoavulavirus 1 (57.14-100%) and avian IBV (42.86-100%). REV was the only virus detected in one goose oral-pharyngeal swab sample.

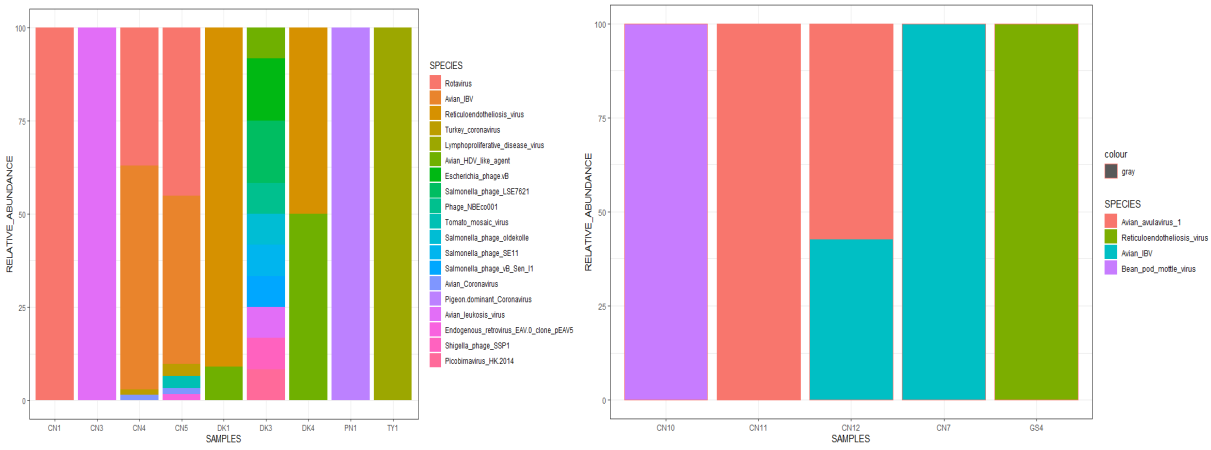

Figure 3: Bar graph showing species level relative viral abundance in each sample plotted using ggplot2 in R Studio version 4.0.3; a) cloacal samples and b) oropharyngeal samples. Only samples with detectable viruses are shown (CN= chicken, DK= duck, GF= guinea fowl, GS= goose, PN= pigeon and TY= turkey).

In order to assess the relatedness and overall taxonomic similarities between the identified sequences in the cloacal and oral-pharyngeal swab samples, a hierarchical clustering analysis was performed, augmented by heatmaps for both groups. The clustermaps and heatmaps of species level viral abundances in each cloacal and oral-pharyngeal sample are shown in Figures 4 and 5 respectively. The hierarchical clustermaps for both groups had dendrograms with intermingled branches, implying no clear separation between samples from the different poultry species. Moreover, heatmaps generated did not reveal any distinct pattern for the samples from the different poultry species. The heatmaps also corroborate the findings that show rotavirus and infectious bronchitis virus detected in higher numbers in some chicken cloacal samples, while APMV 1 and IBV are observed in higher numbers in some oral-pharyngeal chicken samples.

a) b)

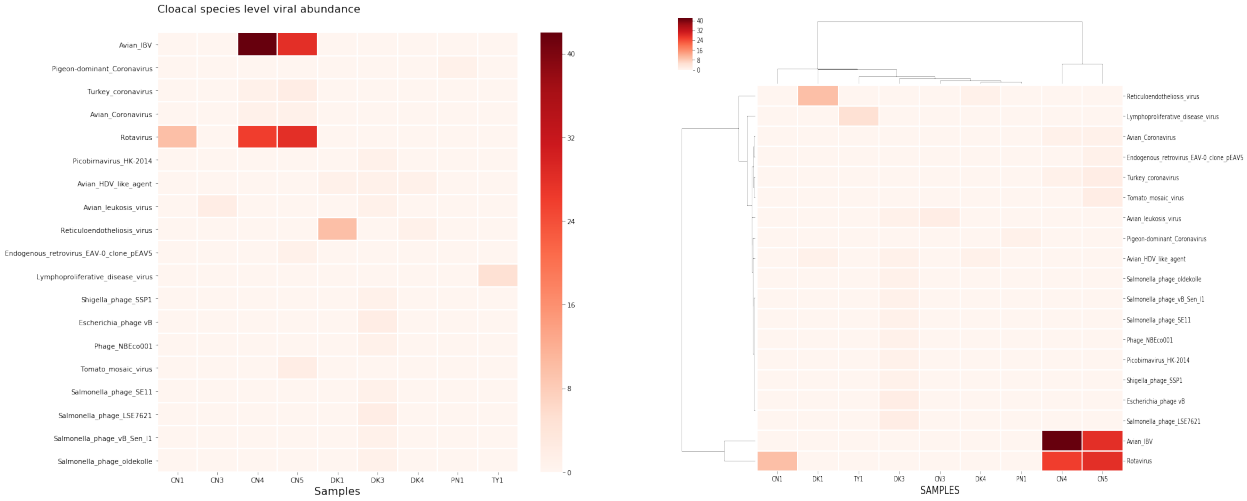

Figure 4: Heatmap (a) and clustermap (b) of fecal species level viral abundance in each sample plotted using Seaborn in Python version 3.7. Only samples with detectable viruses are shown (CN= chicken, DK= duck, GF= guinea fowl, GS= goose, PN= pigeon and TY= turkey).

a) b)

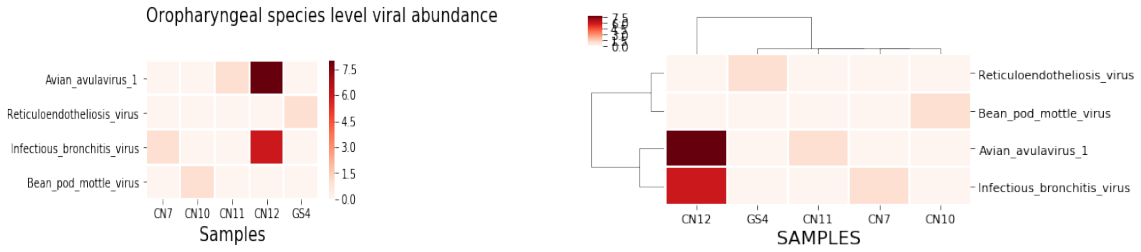

Figure 5: Heatmap (a) and clustermap (b) of oropharyngeal species level viral abundance in each sample plotted using Seaborn in Python version 3.7. Only samples with detectable viruses are shown (CN= chicken, DK= duck, GF= guinea fowl, GS= goose, PN= pigeon and TY= turkey).

Detected Coinfections in Cloacal Swab Samples

Sequences identified in cloacal samples were assigned to five viral families, including *Coronaviridae*, *Picobirnaviridae*, *Reoviridae*, *Retroviridae*, and the unclassified Deltavirus.

Coronaviridae: Seventy six coronavirus-related contigs (75 from chicken and 1 from pigeon) were recovered from the poultry cloacal swab samples (Supplementary Material Table S5). These contigs ranged from 316 to 5,516 nt in length and displayed between 83.46 to 100% amino acid identity to other coronavirus sequences deposited in the NCBI Genbank database (data not shown). Majority of the coronavirus sequences belonged to avian IBV (70 contigs). Turkey coronavirus, avian coronavirus, and pigeon-dominant coronavirus were also detected, albeit in lower numbers. Most of the coronavirus sequences were detected in chicken samples collected from Kwale, Nairobi and Trans Nzoia (detection rate 2/6 or 33.33%), with only 1 sequence being detected in a pigeon sample collected from Kilifi. Contigs showing identity to the S protein were selected to construct a phylogenetic tree. The ML phylogenetic analysis supports the classification of the coronavirus sequences reported in this study at the species level (Figure 6).

Reoviridae: Sixty four rotavirus-related contigs were recovered from chicken cloacal swab samples (Supplementary Material Table S5). These contigs ranged from 316 to 5,516 nt in length and displayed between 83.46 to 100% amino acid identity to other rotavirus sequences deposited in the NCBI Genbank database (data not shown). The rotavirus sequences were detected in chicken samples collected from Kilifi, Kwale, Nairobi, and Trans Nzoia (detection rate 3/6 or 50%). Contigs showing identity to the S protein were selected to construct a phylogenetic tree.

Phylogenetic analysis of the S protein amino acid sequences confirmed a close relationship between the detected rotavirus sequences and other previously described rotavirus sequences (Figure 7).

Retroviridae: Twenty retrovirus-related contigs (15 from duck and 5 from turkey) were recovered from the poultry cloacal swab samples (Supplementary Material Table S5). These contigs ranged from 316 to 5,516 nt in length and displayed between 83.46 to 100% amino acid identity to other coronavirus sequences deposited in the NCBI Genbank database (data not shown). Majority of the retroviral sequenced detected mapped to REV (11 sequences), followed by the LDV (5 sequences), and the ALV (3 viral contigs), with 1 contig mapping to the Endogenous retrovirus EAV-0_ lone pEAV5.

Other potentially pathogenic viruses recovered from poultry cloacal swab samples included Avian HDV-like agent (3 from duck samples) and Picobirnavirus HK-2014 (1 from duck sample). The rest of the viral sequences mapped to the Salmonella phage LSE7621 (2 from duck sample), Salmonella phage SE11 (1 from duck sample), Salmonella phage vB Sen I1 (1 from duck sample), Salmonella phage oldekoll (1 from duck sample), Escherichia phage Vb (2 from duck sample), Shigella phage SSP1 (1 from duck sample), Tomato mosaic virus (2 from chicken sample), and Phage NBEco001 (1 sequence from duck sample).

Detected Oral-Pharyngeal Viruses of Poultry

Oral-pharyngeal samples, on the other hand, were assigned to three potentially pathogenic viral families, including *Coronaviridae*, *Paramyxoviridae*, and *Retroviridae*. Nine orthoavulavirus 1-related contigs were recovered (Supplementary Material Table S6). These contigs ranged from 316 to 5,516 nt in length and displayed between 83.46 to 100% amino acid identity to other coronavirus sequences deposited in the NCBI Genbank database (data not shown). The ML phylogenetic analysis supports the classification of the Orthoavulavirus 1 (APMV 1) sequences reported in this study at the species level (Figure 8). Seven IBV-related contigs were detected (Supplementary Material Table S6). These contigs ranged from 316 to 5,516 nt in length and displayed between 83.46 to 100% amino acid identity to other coronavirus sequences deposited in the NCBI Genbank database (data not shown). Phylogenetic analysis of the S protein amino acid sequences confirmed a close relationship between the detected coronavirus sequences and

other previously described coronavirus sequences (Figure 9). Other viral contigs detected in the oropharyngeal swab samples belonged to REV and the bean pod mottle virus (1 contig each).

Table 1: Co-infections along with Newcastle disease virus in cloacal swabs of Kenyan poultry

Poultry species	Virus detected	County	Sample ID	Detection rate
Chicken	Rotavirus	Kilifi, Kwale, Nairobi, Trans Nzoia	CN1, CN4, CN5	3/6 (50%)
	Avian IBV	Kwale, Nairobi, Trans Nzoia	CN4, CN5	2/6 (33.33%)
	Turkey coronavirus	Kwale, Nairobi, Trans Nzoia	CN4, CN5	2/6 (33.33%)
	Avian coronavirus	Kwale	CN4	1/6 (16.67%)
Duck	Avian HDV-like virus	Kilifi, Kwale, Bungoma, Busia, Trans Nzoia	DK1, DK3, DK4	3/4 (75%)
	REV	Kilifi, Bungoma, Busia, Trans Nzoia	DK1, DK4	2/4 (50%)
	ALV	Kwale	DK3	1/4 (25%)
	Picobirnavirus	Kwale	DK3	1/4 (25%)
Pigeon	Pigeon-dominant coronavirus	Kilifi	PN1	1/3 (33.33)
Turkey	LDV	Kilifi	TY1	1/1 (100%)

Table 2: Co-infections along with Newcastle disease virus in cloacal swabs of Kenyan poultry

Poultry species	Virus detected	County	Sample ID	Detection rate
Chicken	Othoavulavirus 1	Busia, Nairobi, Trans Nzoia	CN11, CN12	3/6 (50%)
	Avian IBV	Kilifi	CN7	1/6 (16.67%)
Goose	REV	Bungoma, Busia	GS4	1/2 (50%)

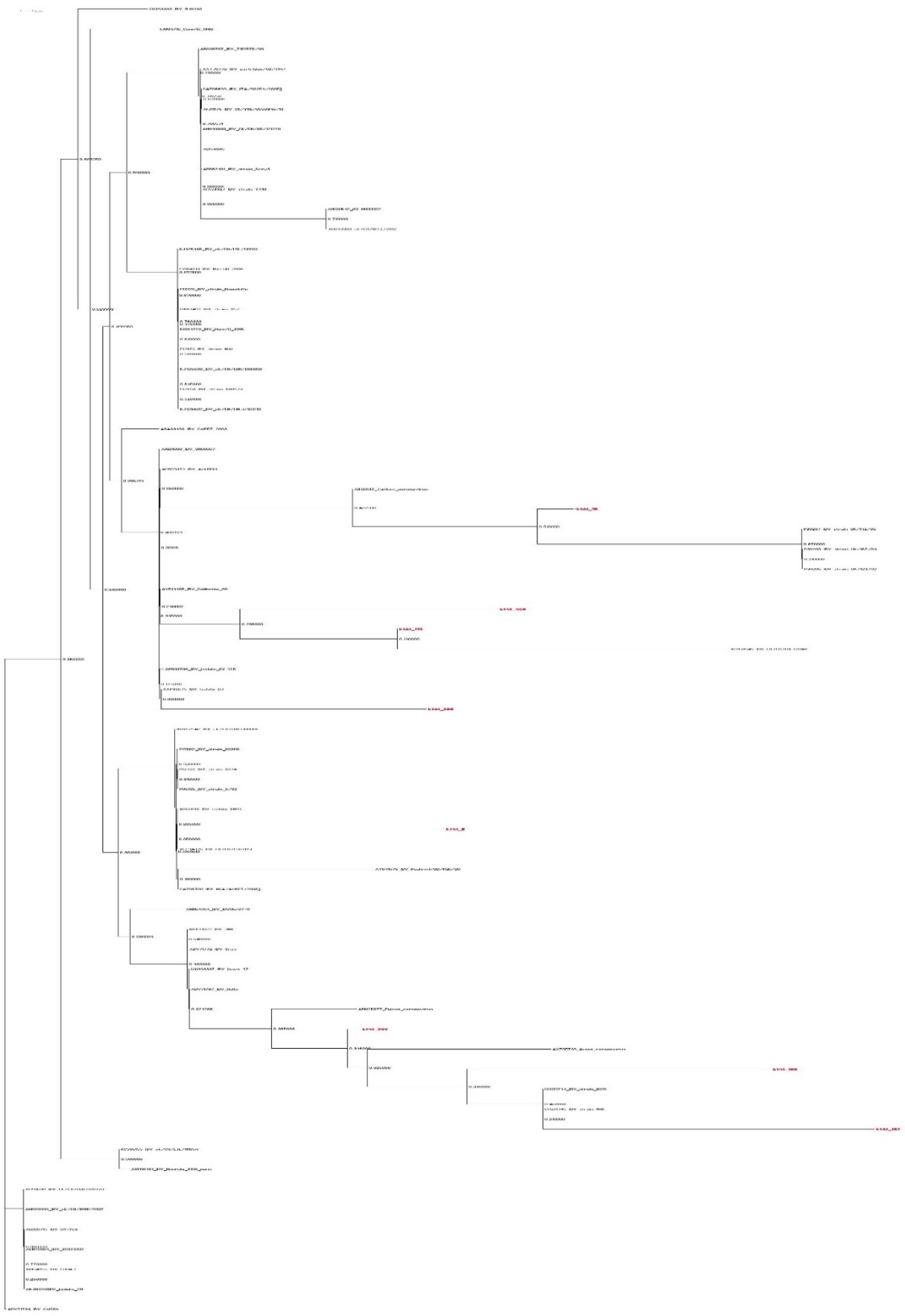

Figure 6: Phylogeny of the coronavirus sequences detected in poultry cloacal samples and other

sequences downloaded from Genbank based on amino acid sequences of the S protein. Maximum likelihood tree generated using PhyML using the LG amino acid substitution model. Branch support was estimated by bootstrap analysis with 100 replicates.

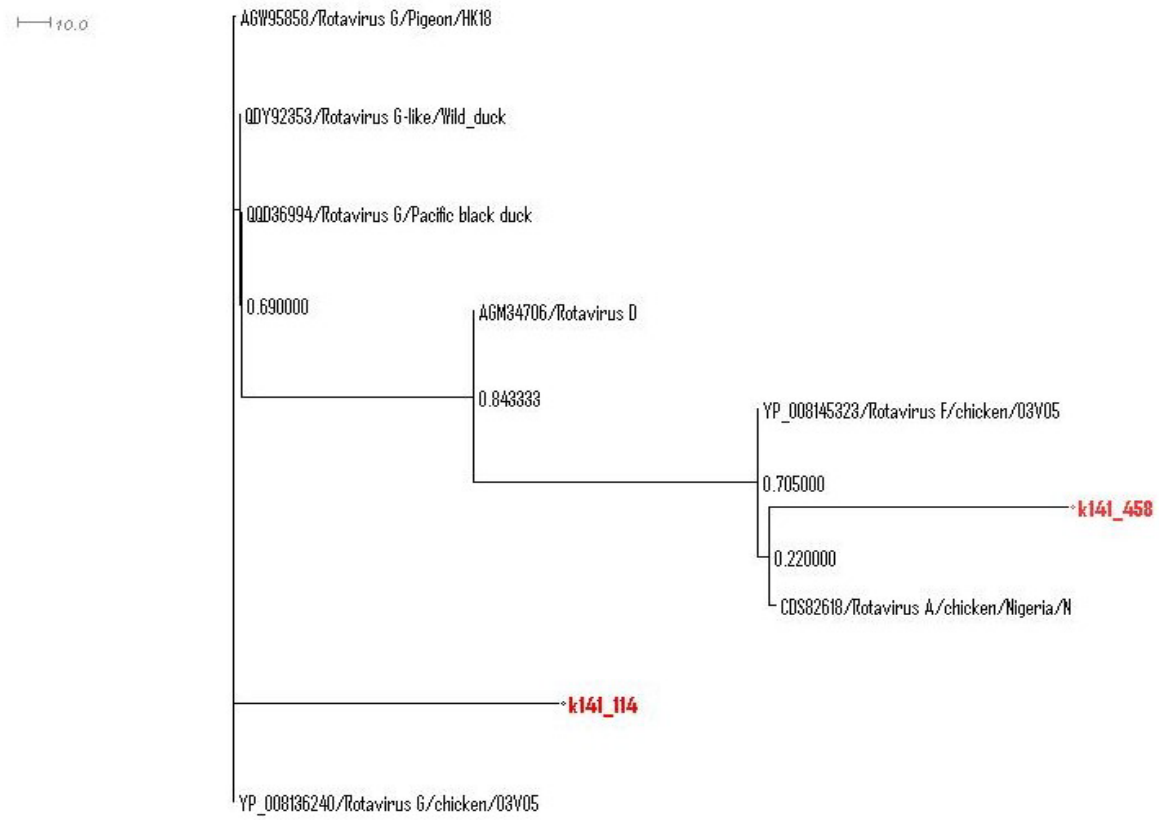

Figure 7: Phylogeny of the rotavirus sequences detected in poultry cloacal samples and other sequences downloaded from Genbank based on amino acid sequences of the S protein. Maximum likelihood tree generated using PhyML using the LG amino acid substitution model. Branch support was estimated by bootstrap analysis with 100 replicates.

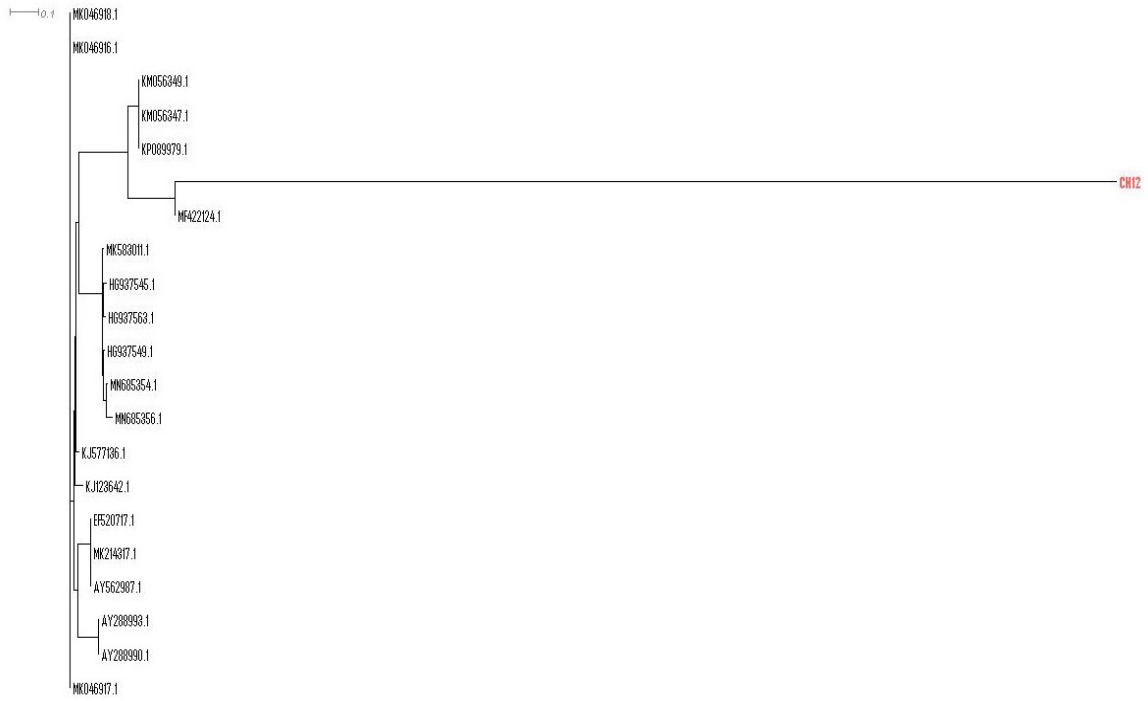

Figure 8: Phylogenetic tree of representative members of Avulavirus detected in poultry oral samples and other sequences downloaded from Genbank based on nucleotide sequences. Maximum likelihood tree generated using PhyML using the LG amino acid substitution model. Branch support was estimated by bootstrap analysis with 100 replicates.

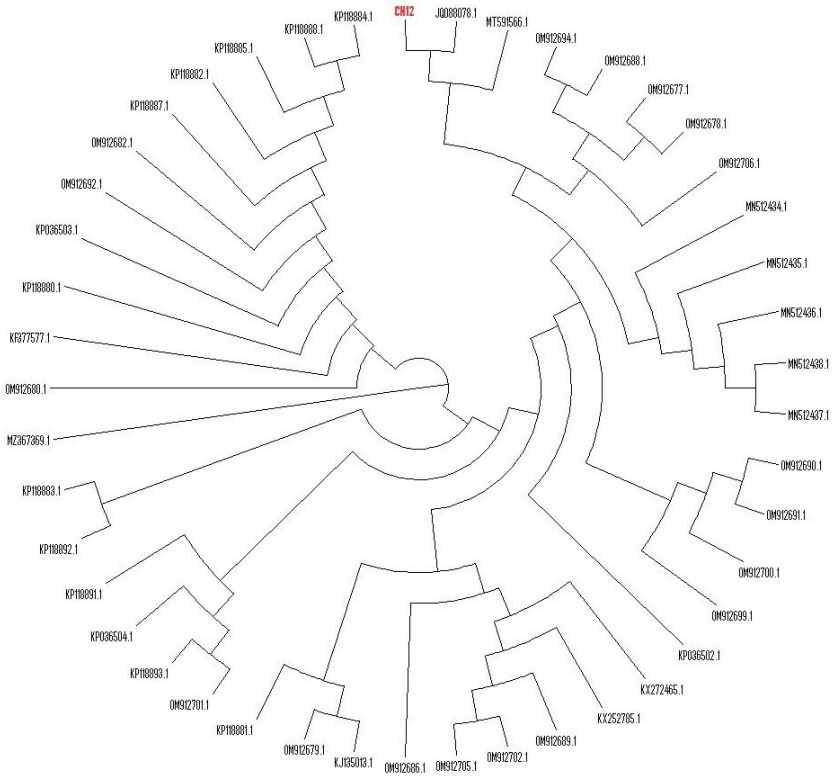

Figure 9: Phylogeny of the infectious bronchitis virus sequences detected in poultry oral samples and other sequences downloaded from Genbank based on nucleotide sequences. Maximum likelihood tree generated using PhyML using the LG amino acid substitution model. Branch support was estimated by bootstrap analysis with 100 replicates.

Discussion

To the best of our knowledge, most metagenomic studies on poultry microbiome have mainly focused on poultry reared under controlled and regulated feeding regimes, except for one study that investigated the microbial community profiles of indigenous backyard chickens on a scavenging feeding system from two geographically and climatically distinct regions of Ethiopia (H. Kumar et al., 2019). Metagenomics analysis of poultry raised under a free-range scavenging feeding system is therefore crucial in helping to explore the impact of local feed (plants, insects and other small animals) on poultry health (H. Kumar et al., 2019).

From the 254 viral contigs detected in cloacal swab samples, 77 viral contigs had no significant similarity to any sequences identified in Genbank, which was 30 % of the contigs that mapped to viral sequences. This was higher than the observations made by (Lima et al., 2019) that the

percentages of eukaryotic viral reads detected in the mal-absorption syndrome affected and healthy chicken were 22.1 % and 14.5 % respectively. Similarly, 57 % of the identified oropharyngeal viral contigs had no significant similarity to any sequences identified in Genbank. The proportion of viral contigs that had no significant similarity to any sequences identified in Genbank was also higher than the proportion of unclassified sequences reported from the metagenomic analysis of the fecal virome in asymptomatic pigs in East Africa (Amimo et al., 2016).

We identified 11 potentially pathogenic viruses from the cloacal swab samples (Supplementary Materials Table S6). Three potentially pathogenic viruses and one plant virus were detected in oropharyngeal swab samples (Supplementary Materials Table S6). Similar studies revealed the presence of pathogenic viruses in oropharyngeal swab samples (Ogali et al., 2018; Qiu et al., 2017). However, no orthoavulavirus 1-related sequences were detected in cloacal swab samples, implying that NDV were not shed in the poultry faeces.

The main potentially pathogenic viruses detected from chicken cloacal swab samples of Kenyan poultry under this study included Avian IBV (from samples collected in Kwale, Nairobi and Trans Nzoia) and Rotavirus (from samples collected in Kilifi, Kwale, Nairobi and Trans Nzoia). Other viruses that were detected in smaller numbers in chicken include turkey coronavirus and Avian coronavirus. The main potentially pathogenic viruses detected from duck cloacal swab samples were Avian HDV-like virus (from samples collected in Kilifi, Kwale, Bungoma, Busia, Trans Nzoia), REV (from Kilifi, Bungoma, Busia, Trans Nzoia), and ALV and Picobirnavirus from Kwale. Pigeon-dominant coronavirus and LDV were also detected in pigeons and turkeys respectively from Kilifi. IBV and rotavirus were therefore the most prevalent in chickens while retroviruses were predominantly detected in ducks. This is consistent with previous studies which were done in other countries that identified these viruses as major pathogens in poultry (Abd El Rahman et al., 2019; Rahman et al., 2020). It is noteworthy that most of the chicken sold in Nairobi are transported from Western Kenya, especially Trans Nzoia, and the coastal region, including Kilifi and Kwale counties. This implies that there is continuous circulation of pathogenic viruses between backyard poultry species throughout the country as opined by Ogali et al. (2018).

Avian IBV sequences detected compared well with the other previously described Avian IBV sequences. Avian IBV is a highly contagious avian coronavirus that causes respiratory disease in chickens, leading to economic losses in the poultry industry. Avian coronaviruses have been implicated in certain severe infections in poultry. The presence of coronaviruses therefore indicates a posibble coinfection associated with NDV since the affected chicken were both sampled in Nairobi and Trans Nzoia. Coronaviruses have also been associated with inter-species spill over, for instance, severe acute respiratory syndrome coronavirus (SARS-CoV) 1 and 2 and Middle Eastern respiratory syndrome coronavirus (SERS-CoV) (Ommeh et al., 2018; Vibin et al., 2020). In a study conducted in Kenya, IBV was identified as a common cause of respiratory disease in poultry (Kariithi et al., 2023). This also suggests that IBV is a significant threat to the poultry industry in Kenya and that further research is needed to understand the epidemiology and pathogenesis of the virus.

Rotaviruses (Rotavirus F and G) were also detected in high numbers in chicken samples. Although infection with this serotypes has not been considered fatal, infection from other Rotavirus serotypes can be lethal to poultry in extreme cases since it is one of the common enteric viruses that causes diarrhoea in poultry (Vibin et al., 2020). Previous studies have shown that rotavirus is one of the most common viruses detected in poultry (Cheung et al., 2013; Pinheiro et al., 2023). Interestingly, rotaviruses were also detected from samples collected in Kilifi, Kwale, Nairobi, and Trans Nzoia. This also indicates that rotaviruses too are possible coinfections associated with NDV since the affected chicken were sampled from the same regions.

Retroviruses are a group of RNA viruses that can cause a wide range of diseases in birds, including lymphomas and leukemias. It is noteworthy that a majority of the retroviral sequences detected were harbored by ducks. Ducks are a major source of pathogens, but interestingly, they mostly remain asymptomatic even as they transmit these viruses to other vulnerable poultry, especially chickens and turkeys. ALV and REV have previously been implicated as etiological agents of some immunosuppressive and neoplastic diseases in poultry (Xuan et al., 2015). ALV mainly infects chickens while REV infects chickens, turkeys, and other avian species (Fadly, 1997). In addition to causing tumors, both pathogens can reduce productivity and induce immunosuppression in affected flocks (Fadly, 1997). LPDV, a retrovirus associated with tumors

in wild and domestic turkeys, has also been described in turkey flocks in Europe, the Middle East and the United States (Thomas et al., 2015). A study conducted in Brazil also detected REV in Muscovy ducks, wild turkeys, and chickens at a relatively high prevalence rate of 16,8% (Caleiro et al., 2019). The impact of ALV on broiler chickens has also been reported in Malaysia (Bande et al., 2016).

Other viruses that were detected in smaller numbers, include avian HDV-like agent, turkey coronavirus, and picobirnavirus. These too have been identified in other studies, including a study conducted in Korea which identified picobirnavirus as a common virus in chicken faeces (Kim et al., 2020). The presence of Avian HDV-like agents in ducks has previously been described in ducks (Wille et al., 2018). Studies have also shown that in humans, coinfection with HDV and HBV causes more severe liver disease than is seen in individuals infected with HBV alone (Centers for Disease Control and Prevention, 2020). Hence the coinfection of these pathogens with NDV is of major economic importance to poultry farmers. It is equally noteworthy that most retroviruses and Avian HDV-like agents were detected in duck samples yet ducks are rarely affected by these retroviruses, which suggests that they may serve as major hosts, carriers, or transporters of viral pathogens, as earlier alluded by Tolf et al. (2013).

The majority of the potentially pathogenic viruses detected in oropharyngeal swab samples from poultry in Kenya are Orthoavulavirus 1 and IBV. Orthoavulavirus 1 is a genus of the family *Paramyxoviridae* that includes NDV, an important pathogen in poultry worldwide. IBV, on the other hand, is a coronavirus that causes respiratory and renal disease in chickens. A number of studies on the prevalence of avian viruses in Kenya have identified NDV and IBV as significant pathogens in poultry. A study by Ogali et al. (2018) and Kariithi et al. (2021) found that NDV was the most prevalent virus in backyard poultry in Kenya, while IBV was also detected, but at a lower prevalence. Similarly, a study by Umar et al. (2019) detected NDV and IBV in commercial poultry farms in Pakistan. Considering that these two viruses were detected from samples collected in Nairobi, Busia, and Trans Nzoia, this strongly implies that IBV is a major coinfection with NDV among poultry in Kenya. Understanding these coinfections will thus greatly boost the efforts being made to develop more viable vaccines against NDV.

Most of the non-avian host associated viruses that were identified in both cloacal and oropharyngeal swab samples were likely either part of the food eaten by the poultry, or bacteriophages affecting enteric bacteria, some of which are pathogenic. For instance, tomato mosaic virus and bean pod mottle virus detected in duck and chicken samples respectively, are likely to be from the food eaten. Bacteriophages detected were Salmonella phage LSE7621, Salmonella phage SE11, Salmonella phage vB Sen I1, Salmonella phage oldekoll, Escherichia phage Vb, Shigella phage SSP1, and Phage NBEco001. It is interesting to note that most of the identified phages infect enteric bacteria with pathogenic potential such as *Salmonella*, *Shigella* and *Escherichia*. *Salmonella* is associated with pullorum disease, fowl typhoid, and paratyphoid infections (Porter, 1998), while *E. coli* and *Shigella* are associated with colibacillosis, air sacculitis, and cellulitis (Gross, 1991). The presence of these bacteriophages therefore is a possible indication of the kind of bacteria colonizing the poultry gut and can be informative regarding bacterial coinfections associated with NDV in poultry. Interestingly, all the bacteriophages were detected in duck samples, which again strongly suggests that they may serve as major hosts, carriers, or transporters of both viral and bacterial pathogens.

A limitation of this study is that the data generated comes from pooled samples, rather than from individuals. This has the potential to reduce the epidemiological strength of the study as it affects the study's potential to evaluate different virus prevalences and/or loads because the reads may have been biased by such a procedure as suggested by Lima et al. (2019). However, this approach provides an opportunity to access diverse viral genomes that are present in the faeces and oral secretions of these populations, including novel viruses. There is also need to determine the proportion of the detected viruses that is commensal *vis a vis* the pathogenic viruses.

Conclusion

The present study has demonstrated the presence of a number of viruses that have previously been identified in cloacal and oropharyngeal swab samples in poultry species, especially IBV, other coronaviruses, rotaviruses, and retroviruses, which seem to be coinfections associated with NDV. To the best of our knowledge, this is the first study that detected coinfections associated with the Newcastle disease virus among poultry in Kenya. This study provides important information that will help in improving disease diagnosis and vaccine development for coinfections associated with NDV since NDV vaccines are known to fail because of these

coinfections.

Conflict of interest

The authors declare that they have no conflict of interest.

Data Availability

The sequencing data of the cloacal and oropharyngeal swabs of the Kenyan poultry under this study have been submitted in the NCBI Sequencing Read Archive (SRA) under the bio-project PRJNA972968.

Supplementary Material

Table S1: Pools and sampling regions for cloacal swab samples.

Table S2: Pools and sampling regions for oral-pharyngeal swab samples.

Table S3: Number of raw reads, clean reads, assembled contigs, viral contigs, and viruses identified per cloacal swab sample.

Table S4: Number of raw reads, clean reads, assembled contigs, viral contigs, and viruses identified per oral-pharyngeal swab sample.

Table S5: Number of contigs and detection rate of viruses in poultry cloacal swab samples

Table S6: Number of contigs and detection rate of viruses in poultry oral-pharyngeal swab samples

Figure S1: Cloacal (a) and oral-pharyngeal (b) family level viral abundances. Pie charts plotted using Seaborn in Python version 3.7.

Figure S2: Cloacal (a) and oral-pharyngeal (b) species level viral abundances. Pie charts plotted using Seaborn in Python version 3.7.

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