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RESEARCH PAPER

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Metagenomic characterization reveals virus coinfections associated with Newcastle disease virus among poultry in Kenya

Philip M. Panyako[1] | **Sheila C. Ommeh**[1] | **Stephen N. Kuria**[1] | **Jacqueline K. Lichoti**[2] | **Johns Musina**[3] | **Venugopal Nair**[4] | **Vish Nene**[5] | **Samuel O. Oyola**[5] | **Muhammad Munir**[6]

[1]Institute for Biotechnology Research, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya

[2]State Department of Livestock, Ministry of Agriculture, Livestock and Fisheries, Nairobi, Kenya

[3]Department of Zoology, National Museums of Kenya, Nairobi, Kenya

[4]Nuffield Department of Medicine, The Jenner Institute, University of Oxford, Oxford, UK

[5]International Livestock Research Institute (ILRI), Nairobi, Kenya

[6]Department of Biomedical and Life Sciences, Lancaster University, Lancaster, UK

Correspondence
Sheila C. Ommeh, Institute for Biotechnology Research, Jomo Kenyatta University of Agriculture and Technology, PO Box 62000, City Square 00200, Nairobi, Kenya.
Email: sommeh@jkuat.ac.ke

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Abstract

Newcastle disease (ND) is an endemic viral disease affecting poultry and causing massive economic losses. This cross-sectional purposive study detected coinfections that are associated with the Newcastle disease virus 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 nucleotides 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. Several known and unknown representatives of Coronaviridae, Picobirnaviridae, Reoviridae, Retroviridae, and unclassified Deltavirus were identified in the cloacal swab samples. However, no Newcastle disease virus (family Paramyxoviridae) was detected in the cloacal swabs, although they were detected in the oropharyngeal swabs of chickens sampled in Nairobi, Busia, and Trans Nzoia. Additionally, sequences representative of Paramyxoviridae, Coronaviridae, and Retroviridae were identified in the oral–pharyngeal swab samples. Infectious bronchitis virus and rotavirus were chickens' most prevalent coinfections associated with the Newcastle disease virus. The detection of these coinfections suggests that these viruses are significant threats to the control of Newcastle disease as the Newcastle disease virus vaccines are known to fail because of these coinfections. Therefore, this study provides important information that will help improve disease diagnosis and vaccine development for coinfections associated with the Newcastle disease virus.

KEYWORDS
chicken, coronavirus, infectious bronchitis virus, orthoavulavirus, paramyxovirus, rotavirus

Abbreviations: APMV, avian paramyxovirus; BLAST, basic local alignment search tool; NDV, Newcastle disease virus; PCR, polymerase chain reaction.

1 | INTRODUCTION

Newcastle disease (ND) is an infectious viral disease affecting poultry that is endemic in Africa and other parts of the world [1]. The disease is caused by an enveloped, nonsegmented, negative-sense, single-stranded RNA virus belonging to the family Paramyxoviridae [1]. Newcastle disease virus (NDV) belongs to the Avian orthoavulavirus 1 (avian paramyxovirus 1 [APMV-1]) serotype. APMV-1 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 [2]. The importance of coinfections has been demonstrated in humans [3, 4], wild birds [5, 6], and poultry [7–9]. 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 the uncontrolled spread of the virus [10]. This is especially evident in backyard poultry systems where the vaccine usually fails because of coinfections with other viruses such as infectious bronchitis virus (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 [11]. Hence, there is a need to control these coinfections if the vaccination efforts against highly pathogenic NDV are to be achieved.

Several approaches have been employed to study the epidemiology of viral infections affecting poultry, including pathogen surveillance and tracking the molecular epidemiology of pathogens [12]. Previous methods employed polymerase chain reaction (PCR)-based methods, especially reverse transcription PCR [12]. Using this strategy, several viruses have been implicated in poultry diseases, including astroviruses [13], parvoviruses [14], reoviruses [15], rotaviruses [16], paramyxoviruses [17], and coronaviruses [18, 19]. These methods often require prior knowledge of the target virus, which poses a challenge when investigating multiple pathogens in one or more hosts, especially from different viral families. Additionally, they cannot be adequately used for the surveillance of pathogens of other poultry diseases, as well as novel viruses [12].

Currently, metagenomics-based detection methods are being applied successfully to characterize viral populations in different hosts and environments [20]. This has facilitated the discovery of many novel viral agents from different types of tissues, including the gastrointestinal tract and the oral–pharyngeal regions in poultry and other livestock [12, 20–25]. The popularity of these techniques is largely due to their high sensitivity and wide coverage as they target the entire genome [12, 25]. 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 microbiomes (including viral communities) have been carried out on poultry reared under controlled and regulated feeding regimes. To the best of our knowledge, only one study investigated the microbial community profiles of indigenous backyard chickens on a scavenging feeding system in Ethiopia [26]. Metagenomics analysis of backyard poultry raised under a free-range scavenging feeding system is therefore required to explore the impact of local feed (plants, insects, and other small animals) on poultry health [26]. Our study, therefore, focused on the characterization of coinfections associated with NDV among mostly free-ranging poultry populations in selected regions in Kenya.

2 | MATERIALS AND METHODS

2.1 | Sample collection

This study was carried out from 2016 to 2019 both retroactively and actively across six counties with varying agroecological conditions in Kenya (Figure 1). The study received institutional clearance from the Jomo Kenyatta

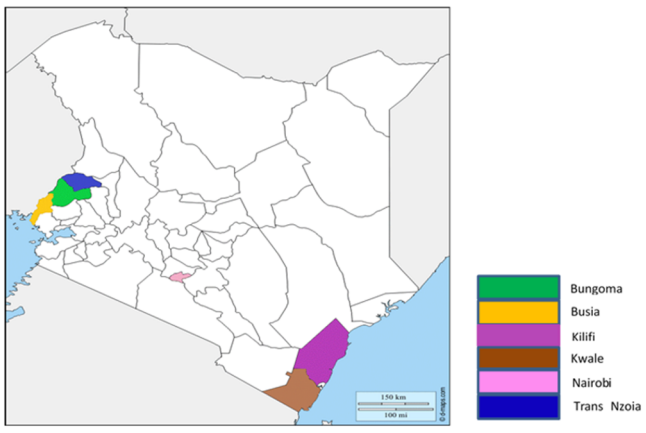


FIGURE 1 Map of Kenya showing the main sampling sites with varying geographic and climatic conditions for cloacal and oropharyngeal swab samples. (Source: d-maps.com).

University of Agriculture and Technology to conduct animal research. Clearance was also sought from the Director of Veterinary Services from the State Department of Livestock, Ministry of Agriculture and Livestock Development, Kenya to carry out the study on farm animals. The research 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.

2.2 | 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 (Supporting Information: Tables S1 and S2). The nucleic acids of each pooled sample were used to prepare the viral metagenomic libraries.

2.2.1 | RNA extraction

Viral RNA was extracted from the cloacal and oral–pharyngeal swab samples using the standard TRIzol reagent as described by Rio et al. [27]. The extracted RNA pellet was then resuspended 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.

2.2.2 | 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.). The resulting first strand cDNA was used as a template to synthesize the second strand, generating double-stranded cDNA (dscDNA) using the same kit. The dscDNA preparation was used as a template to prepare the Illumina sequencing library following the Illumina DNA prep kit protocol (Illumina Inc.). Indexed multiplexed samples were pooled and reconstituted to 4 nM before diluting to 12 pM for loading into MiSeq instrument (Illumina Inc.) for a 2×200 paired-end sequencing run at the ILRI Genomic platform. The number of reads obtained from each library is shown in Supporting Information: Tables S3 and S4.

2.3 | Bioinformatics analysis

Poor quality sequencing reads with a Phred quality score <20 and adaptors were trimmed using Trimmomatic version 0.39 [28]. The paired-end sequence reads were de novo assembled into contigs using Megahit version 1.0.2 [29]. The assembled contigs were analyzed by basic local alignment search toolx (BLASTx) against a viral protein database and visualized using Megan version 5.5.3 [30]. Sequences with the best BLAST scores ($E \leq 10^{-3}$) were selected and assigned to known viral families. Overall taxonomic similarities between metagenomes were examined by performing hierarchical clustering and heatmap analyses using the ggplot2 package in R version 4.3.0 [31].

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 [32]. These were used to generate maximum-likelihood phylogenetic trees using PhyML [33] with best-fit substitution models determined by Smart Model Selection [34]. Statistical significance analyses of tree topologies were performed with the approximate likelihood branch support test or the bootstrap method using 100 replicates [35].

3 | RESULTS

3.1 | 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 nucleotides (nts), were generated (Supporting Information: Tables S3 and S4, respectively). Using

TABLE 1 Coinfections 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%)

Abbreviations: ALV, avian leucosis virus; HDV, hepatitis D virus; IBV, infectious bronchitis virus; LDV, lymphoproliferative disease virus; REV, reticuloendotheliosis virus.

TABLE 2 Coinfections 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%)

Abbreviations: IBV, infectious bronchitis virus; REV, reticuloendotheliosis virus.

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 Tables 1 and 2, respectively.

3.2 | Cloacal and oral–pharyngeal viral abundances

The family and species level viral abundances and the relative frequencies in each pooled sample are shown in Supporting Information: Figures S1 and 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, the unclassified Deltavirus, and Picobirnaviridae. 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 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 hepatitis D virus (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

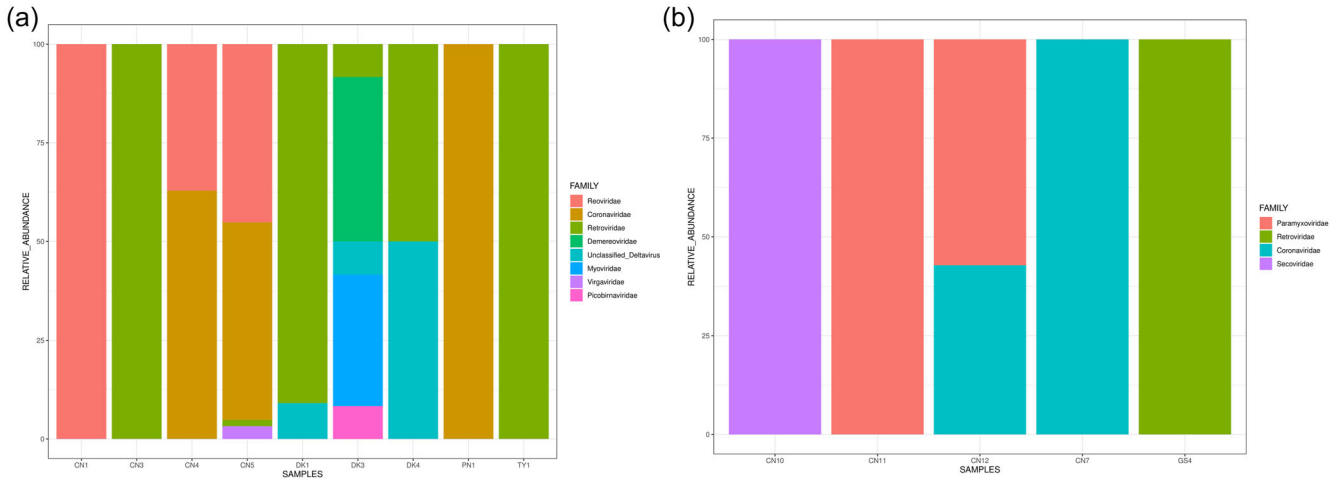


FIGURE 2 Classification of viral contigs detected from six poultry species (a) cloacal swab samples and (b) oral–pharyngeal swab samples. A stacked column chart with taxonomic relative abundances (*y*-axis) by sample (*x*-axis). The height of each bar chart relates to the taxonomic relative abundances in a sample. Only samples with detectable viruses are shown. For each poultry species and sample type. CN, chicken; DK, duck; GF, guinea fowl; GS, goose; PN, pigeon; TY, Turkey.

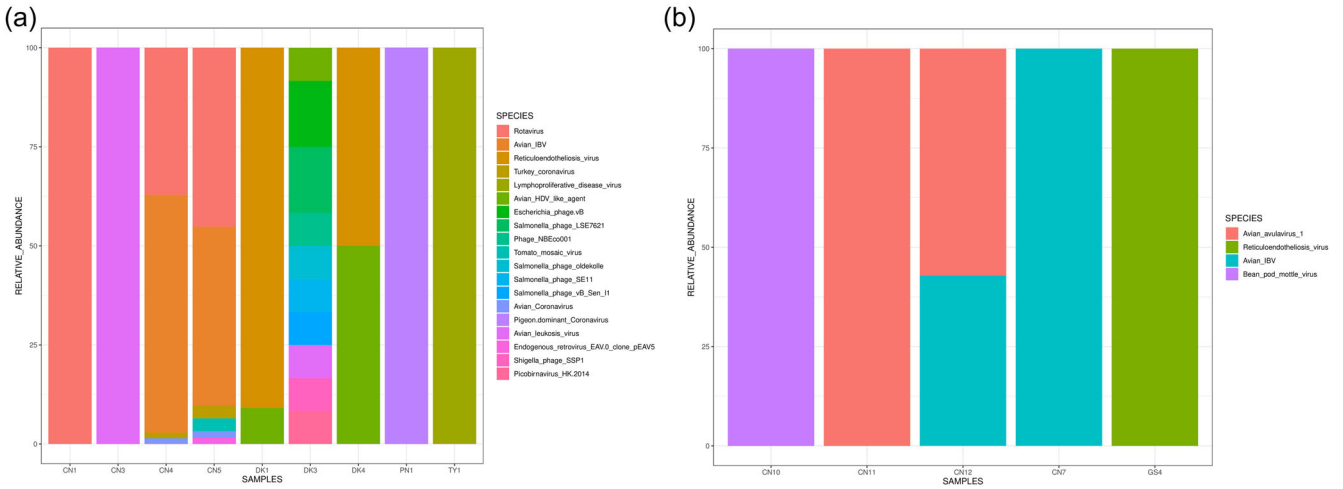


FIGURE 3 Bar graph showing species level relative viral abundance in each sample plotted using ggplot2 in R Studio version 4.0.3; (a) cloacal samples and (b) oropharyngeal samples. A stacked column chart with taxonomic relative abundances (*y*-axis) by sample (*x*-axis). The height of each bar chart relates to the taxonomic relative abundances in a sample. Only samples with detectable viruses are shown. For each poultry species and sample type. CN, chicken; DK, duck; GF, guinea fowl; GS, goose; PN, pigeon; TY, Turkey.

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 the 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%). The most abundant viral families in oral–pharyngeal chicken samples were Paramyxoviridae (57.14%–100%) and Coronaviridae (6%–100%).

The relative abundance of poultry viruses in each cloacal and oral–pharyngeal sample at the 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%). 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

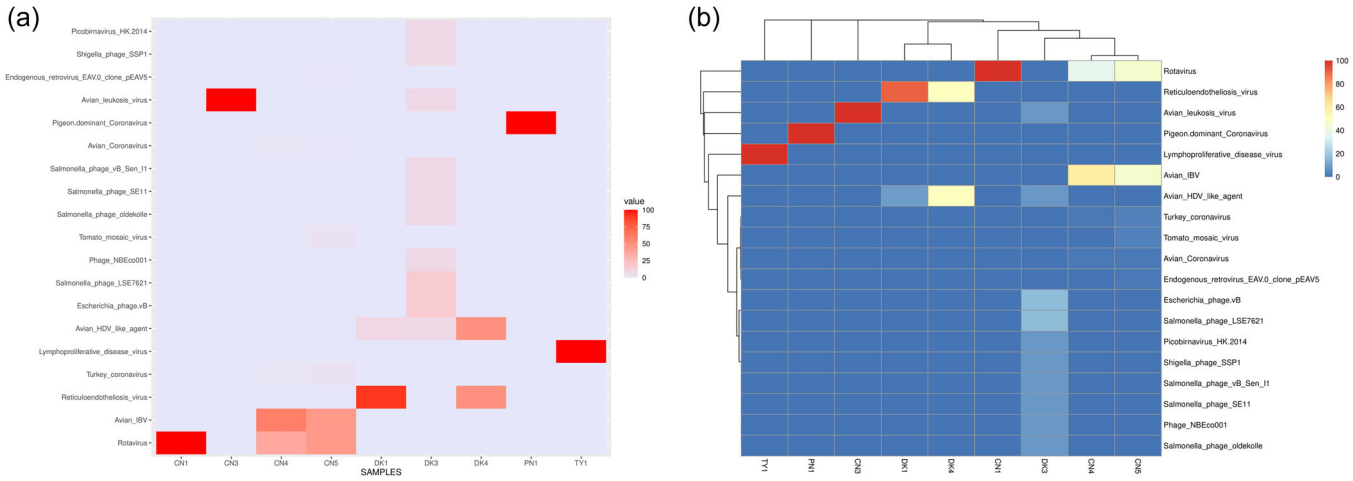


FIGURE 4 Taxonomic abundances heatmap and cluster map based on log-transformed relative abundance values: (a) heatmap and (b) cluster map of cloacal viral abundance in each species (*y*-axis) in all samples (*x*-axis) plotted using ggplot2 and pheatmap, respectively, in R version 4.3.0. Only samples with detectable viruses are shown. Colors scale from red (high abundance) to lavender/blue (low abundance) represent log-transformed relative abundance. For each poultry species and sample type. CN, chicken; DK, duck; GF, guinea fowl; GS; goose; PN, pigeon; TY, Turkey.

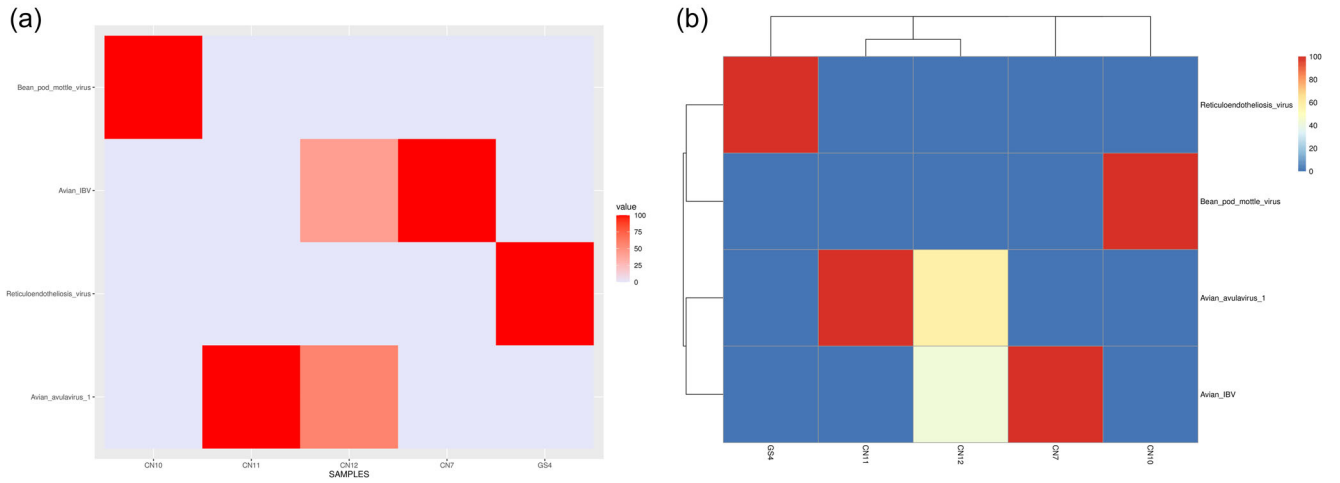
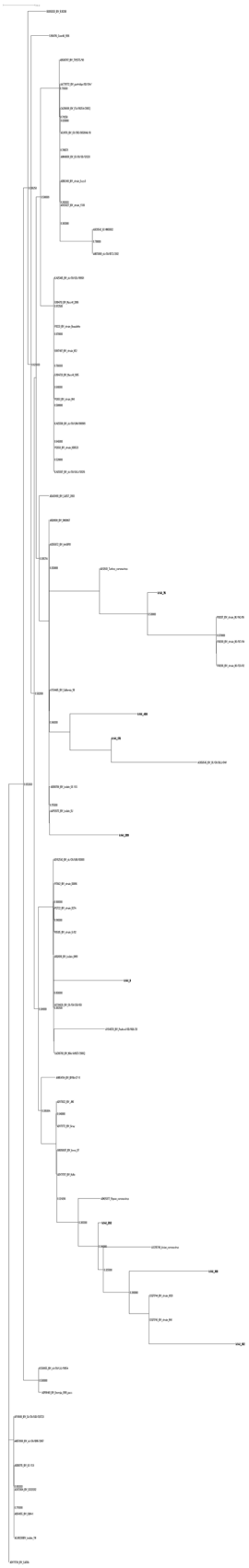


FIGURE 5 Taxonomic abundances heatmap and cluster map based on log-transformed relative abundance values: (a) heatmap and (b) cluster map of oral–pharyngeal viral abundance in each species (*y*-axis) in all samples (*x*-axis) plotted using ggplot2 and pheatmap, respectively, in R version 4.3.0. Only samples with detectable viruses are shown. Colors scale from red (high abundance) to lavender/blue (low abundance) represent log-transformed relative abundance. For each poultry species and sample type. CN, chicken; DK, duck; GF, guinea fowl; GS, goose; PN, pigeon; TY, Turkey.

endogenous 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%).

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 cluster maps and heatmaps of species-level viral abundances in each cloacal and oral–pharyngeal sample are shown in Figures 4 and 5, respectively. The hierarchical cluster maps for both groups had dendrograms with intermingled branches, implying no clear separation between samples from the different poultry species. Moreover, the 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.

3.3 | 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.

3.3.1 | Coronaviridae

Seventy-six coronavirus-related contigs (75 from chicken and one from pigeon) were recovered from the poultry cloacal swab samples (Supporting Information: Table S5). These contigs ranged from 316 to 5516 nt in length and displayed between 83.46% and 100% amino acid identity to other coronavirus sequences deposited in the NCBI Genbank database (data not shown). The majority of the coronavirus sequences belonged to avian IBV (70 contigs). TCoV, 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%). Contigs showing identity to the S protein were selected to construct a phylogenetic tree. The maximum likelihood phylogenetic analysis supports the classification of the coronavirus sequences reported in this study at the species level (Figure 6).

3.3.2 | Reoviridae

Sixty-four rotavirus-related contigs were recovered from chicken cloacal swab samples (Supporting Information: Table S5). These contigs ranged from 316 to 5516 nt in length and displayed between 83.46% and 100% amino acid identity to other rotavirus sequences deposited in the NCBI Genbank database (data not shown). The

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.

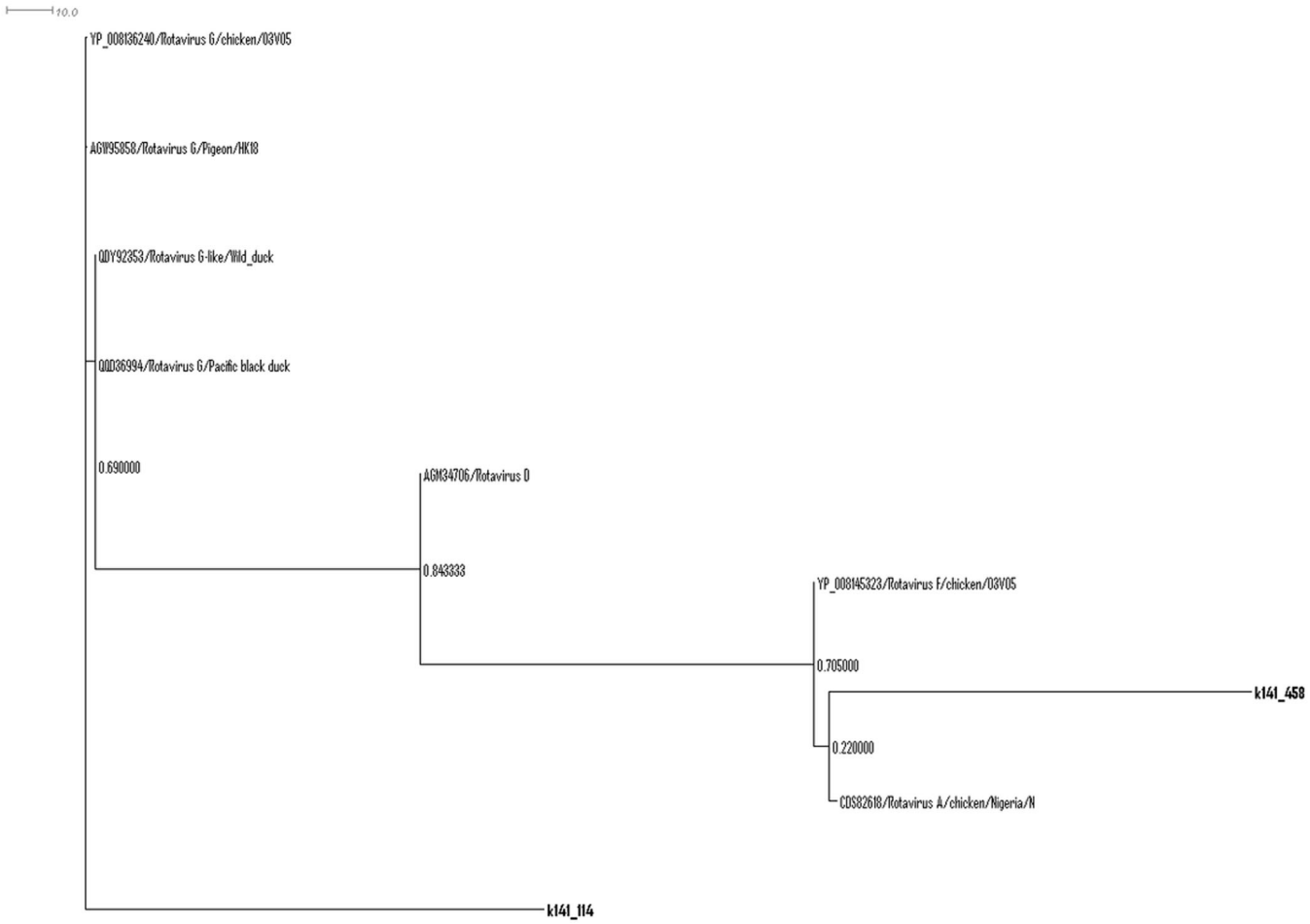


FIGURE 7 Phylogeny of the rotavirus sequences detected in poultry cloacal samples and other sequences downloaded from Genbank based on amino acid sequences of the S protein. Maximum likelihood tree generated using PhyML using the LG amino acid substitution model. Branch support was estimated by bootstrap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

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).

3.3.3 | Retroviridae

Twenty retrovirus-related contigs (15 from duck and five from turkey) were recovered from the poultry cloacal swab samples (Supporting Information: Table S5). These contigs ranged from 316 to 5516 nt in length and displayed between 83.46% and 100% amino acid identity to other coronavirus sequences deposited in the NCBI Genbank database (data not shown). The majority of the retroviral sequenced detected mapped to REV (11 sequences), followed by the LDV (five sequences) and the ALV (three viral contigs).

Other potentially pathogenic viruses recovered from poultry cloacal swab samples included Avian HDV-like agent (three from duck samples) and picobirnavirus HK-2014 (one from duck sample). The rest of the viral sequences mapped to several bacteriophages and the tomato mosaic virus.

3.4 | Detected oral–pharyngeal viruses in 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 (Supporting Information: Table S6). These contigs ranged from 316 to 5516 nt in length and displayed between 83.46% and 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 (Supporting Information: Table S6). These contigs ranged from 316 to 5516 nt in length and displayed between 83.46% and 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.

4 | 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 [26]. 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 [26].

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. [20] that the percentages of eukaryotic viral reads detected in the malabsorption 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 [21].

We identified 11 potentially pathogenic viruses from the cloacal swab samples (Supporting Information: Table S6). Three potentially pathogenic viruses and one plant virus were detected in oropharyngeal swab samples (Supporting Information: Table S6). Similar studies revealed the presence of pathogenic viruses in oropharyngeal swab samples [17, 36]. However, no orthoavulavirus 1-related sequences were detected in cloacal swab samples, implying that NDV was not shed in the poultry feces.

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 TCoV 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, and 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 that were done in other countries that identified these viruses as major pathogens in poultry [37-39]. It is noteworthy that most of the chickens 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 a continuous circulation of pathogenic viruses among backyard poultry species throughout the country as opined by Ogali et al. [17].

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 possible coinfection associated with NDV since the affected chickens were both sampled in Nairobi and Trans Nzoia. Coronaviruses have also been associated with inter-species spillover, for instance, severe acute respiratory syndrome coronavirus 1 and 2 and Middle Eastern respiratory syndrome coronavirus (SERS-CoV) [25, 40]. In a study conducted in Kenya, IBV was identified as a common cause of respiratory disease in poultry [38]. 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 these 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 cause diarrhea in poultry [25]. Previous studies have shown that rotavirus is one of the most common viruses detected in poultry [41].

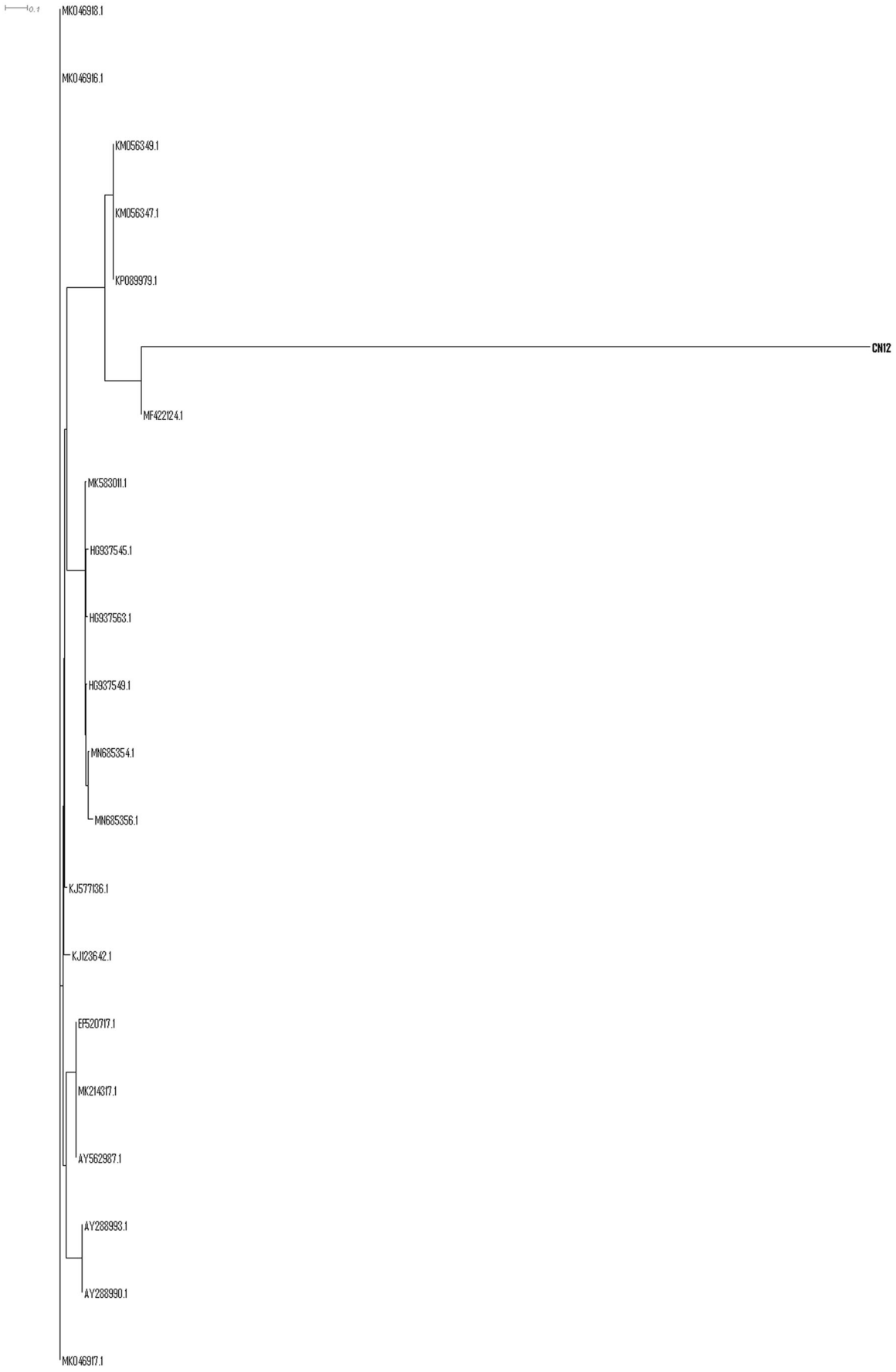


FIGURE 8 (See caption on next page).

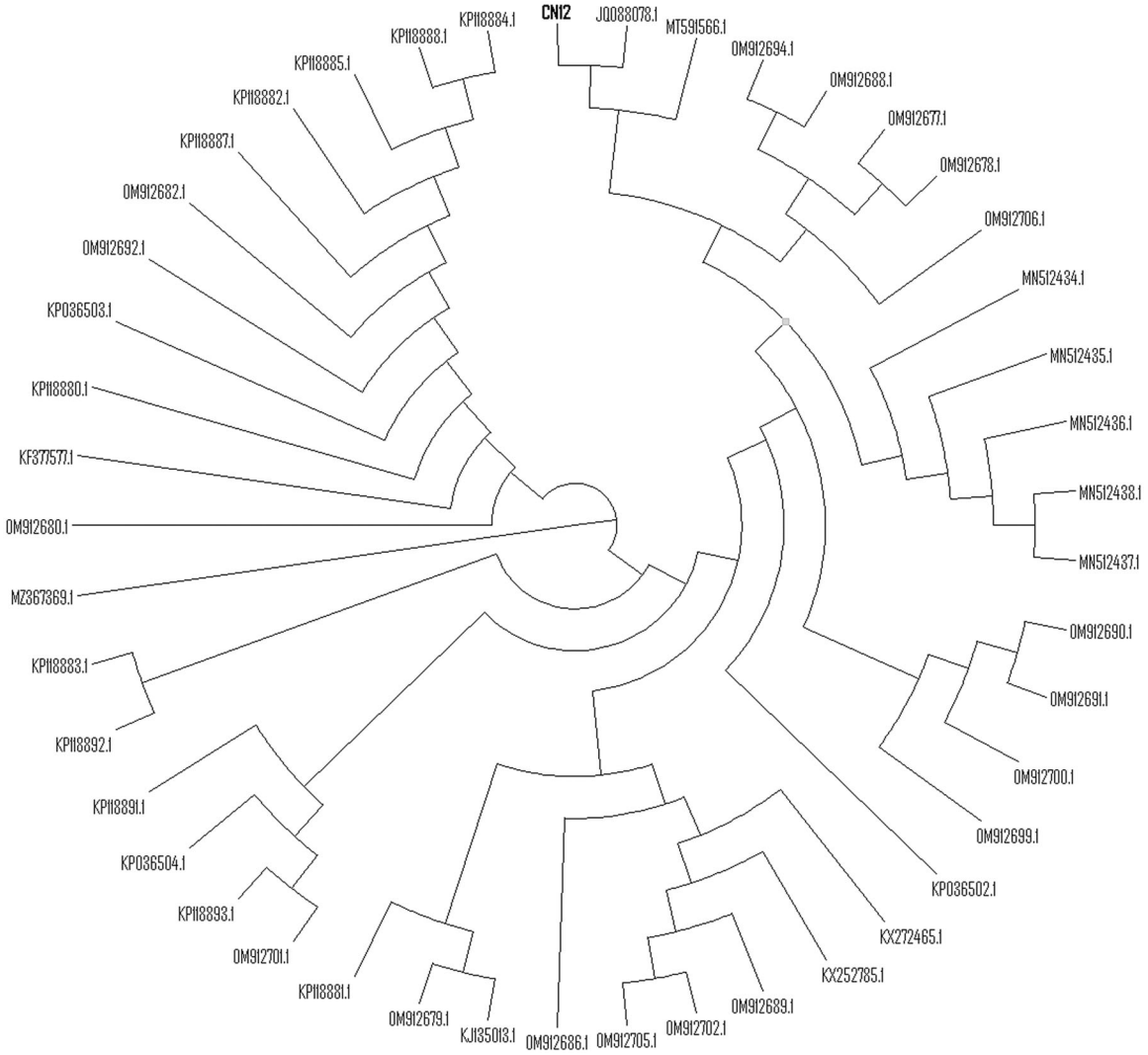


FIGURE 9 Phylogeny of the infectious bronchitis virus sequences detected in poultry oral samples and other sequences downloaded from Genbank based on nucleotide sequences. Maximum likelihood tree generated using PhyML using the LG amino acid substitution model. Branch support was estimated by bootstrap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

Interestingly, rotaviruses were also detected from samples collected in Kilifi, Kwale, Nairobi, and Trans Nzoia. This also indicates that rotaviruses are possible coinfections associated with NDV since the affected chickens 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 [7]. ALV mainly infects chickens while REV infects chickens, turkeys, and other avian species [42]. In addition to causing tumors, both pathogens can reduce productivity and induce immunosuppression in affected flocks [43]. Limphoproliferative disease virus, a retrovirus associated with tumors in wild and domestic turkeys, has also been described in turkey

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.

flocks in Europe, the Middle East, and the United States [42]. A study conducted in Brazil also detected REV in Muscovy ducks, wild turkeys, and chickens at a relatively high prevalence rate of 16.8% [44]. The impact of ALV on broiler chickens has also been reported in Malaysia [45].

Other viruses that were detected in smaller numbers include avian HDV-like agent, TCoV, 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 feces [46]. The presence of Avian HDV-like agents in ducks has previously been described in ducks [47]. Studies have also shown that in humans, coinfection with HDV and hepatitis B virus (HBV) causes more severe liver disease than is seen in individuals infected with HBV alone [48]. 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. [49].

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. Several 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. [17] and Kariithi et al. [50] 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. [39] 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 nonavian 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 [51], while *E. coli* and *Shigella* are associated with colibacillosis, air sacculitis, and cellulitis [52]. 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. [20]. However, this approach provides an opportunity to access diverse viral genomes that are present in the feces and oral secretions of these populations, including novel viruses. There is also a need to determine the proportion of the detected viruses that is commensal *vis avis* the pathogenic viruses.

The present study has demonstrated the presence of several 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 NDV 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.

AUTHOR CONTRIBUTIONS

Philip M. Panyako: Investigation; methodology; visualization; writing—original draft; writing—review and editing. **Sheila C. Ommeh**: Conceptualization; data curation; funding acquisition; methodology; project administration; resources; software; supervision; validation; writing—review and editing. **Stephen N. Kuria**: Investigation. **Jacqueline K. Lichoti**: Conceptualization; project administration; supervision; writing—review and editing. **Johns Musina**: Data curation; project administration. **Venugopal Nair**: Conceptualization; funding acquisition; project administration; resources; writing—review and editing. **Vish Nene**: Conceptualization; funding acquisition; project administration; resources; writing—review and editing. **Samuel O. Oyola**: Conceptualization; funding acquisition;

methodology; project administration; resources; writing—review and editing. **Muhammad Munir**: Conceptualization; funding acquisition; methodology; project administration; resources; writing—review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Additionally, the sequencing data of the cloacal and oropharyngeal swabs of the Kenyan poultry under this study has been submitted to the NCBI Sequencing Read Archive (SRA) under the bio-project PRJNA972968.

ORCID

Sheila C. Ommeh http://orcid.org/0000-0002-0780-0399

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SUPPORTING INFORMATION

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