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Presence of Newcastle Disease Virus in Vaccinated Indigenous Chicken in Selected Regions in Kenya — A Cross-Sectional Study

Auleria Ajiambo Apopo[1, 2, *], Jane Ngaira[2], Jacqueline Kasiiti Lichoti[1], Henry Athiany[4], Yatinder Binepal[3], Leonard Ateya[3]

[1]Directorate of Veterinary Services, State Department for Livestock, Ministry of Agriculture, Livestock and Fisheries, Nairobi, Kenya

[2]Department of Biochemistry, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya

[3]Biotechnology Research Institute, Kenya Agricultural, and Livestock Research Organization, Kaptagat Road, Loresho, Nairobi, Kenya

[4]Department of Statistics and Actuarial Sciences, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya

Email address:
aajiambo@gmail.com (A. A. Apopo), jmngaira@gmail.com (J. Ngaira), kasiiti.orengo@gmail.com (J. K. Lichoti),
henry.athiany@jkuat.ac.ke (H. Athiany), ybinepal@yahoo.com (Y. Binepal), leonard.ateya@kalro.org (L. Ateya)
[*]Corresponding author

Abstract: Vaccination of flocks against Newcastle disease virus (NDV) outbreaks is the main approach for controlling the spread of Newcastle disease (ND). Nevertheless, NDV outbreaks have been reported in vaccinated chickens. In this study, we determined the prevalence of NDV among vaccinated indigenous chickens (ICs) and examined the relationship of the disease with the weather (temperature, rainfall, humidity, and wind speed) at the time of sample collection, production system, and the presence of other species. The genetic diversity of the NDV matrix and fusion genes was also inferred. A total of 1,210 swabs were collected between 2017 and 2018 from ICs that were vaccinated or unvaccinated against NDV in free-range and semi-free-range production systems. We collected 650 swabs each from the oropharynx and cloaca of ICs in 68 households within the Bomet, Baringo, Kilifi, Nakuru, Kakamega, and Machakos counties in Kenya. NDV matrix genes were detected using reverse transcription-polymerase chain reaction, and amplicons of matrix and fusion genes were sequenced using a capillary sequencer from the pooled samples. Among the vaccinated ICs, the prevalence of NDV was 78.5% (p=0.045). There were significant relationships between the presence of NDV and vaccination history of the ICs (p=0.034), the type of production system for ICs (p=0.004) and the months of sample collection (p < 0.0001). However, no significant relationship was found between the presence of NDV and the interaction between ICs and other birds. The presence of matrix and fusion genes in samples from vaccinated flocks indicated the presence of both virulent and low-virulence strains of NDV. These findings highlight the significant presence of NDV among vaccinated ICs and suggest the possibility of inadequate vaccination and viral shedding post-vaccination as the drivers of infections.

Keywords: Newcastle Disease, Vaccination, Indigenous Chicken

1. Introduction

Poultry farming accounts for approximately 30% of the agricultural activities that contribute to Kenya's gross domestic product (GDP) [1]. There are approximately 30 million chickens in Kenya, with the majority kept as free-ranging indigenous chickens (ICs) [2]. IC production is an important component of urban and rural households as a source of food, income, nutrition, insurance against emergencies and has the potential to reduce poverty. The rearing of ICs is also linked to different ceremonial roles among communities in Kenya such as gifts, spiritual cleansing, funeral rights, and entertainment, namely

cockfighting [3].

Several challenges, including slow growth and maturity rate, poor feeding, high feed cost, and high chicken mortality due to poultry disease are constraints in ICs farming. Newcastle disease (ND) is one of the most important infectious diseases in poultry globally and a constraint in poultry production in rural areas [4, 5].

In Kenya, different local communities have adopted various names for ND because of a perceived relationship between the disease and wind flow. As a result, ND is referred to as "Muyekha" in the western region, "Kidere" at the coastal lines, "Kihuruto" in the central area, "Amalda" in the south rift, "Chepkinuch" in the north rift, and "Mavui" in eastern Kenya. The endemicity of the virus has been attributed to weather, agro-ecological zoning, disposal of infected carcasses, vaccination processes, interspecies farming, and restocking of birds from markets [6, 7]. The prevalence of ND as monitored by sero-surveillance in parts of Kenya has been reported to be higher in dry hot zones (17.8%) than in cool wet zones (9.9%) and cold seasons [7].

Vaccination of healthy chickens against ND with a designed regime has been used to control the spread of NDV. In Kenya, the ND vaccine used is either a live attenuated viral vaccine prepared from the "F" strain (AVIVAX-F^{TM}) or La Sota strain (AVIVAX-L^{TM}), or a "I-2" thermostable vaccine. Nevertheless, there have been reported cases of vaccination failures among the bird population [8], which has been attributed to several factors including virus shedding, environmental contamination after the previous outbreak, improper vaccination, vaccine neutralization, paternal effects on passive immunity, and the evolution of the Newcastle disease virus (NDV) genotypes [8-10]. In an experiment to evaluate the effect of vaccination on the transmission of highly virulent vaccines [11], virus shedding was observed in most vaccinated birds compared to those receiving booster vaccination. Other species of birds, such as ducks that are reared alongside chickens, are known reservoirs of ND and can transmit the virus to non-infected birds [4].

NDV, a single-stranded RNA virus of the genus avulavirus [13], is the causative agent of ND [12]. The virus is also called avian ortho-avulavirus 1 (AOAV-1), formerly known as avian paramyxovirus virus-1 (APMV-1) [14, 15]. The AOAV-1 virions are pleomorphic in shape and consist of single-stranded, non-segmented, negative-sense RNA genomes. Its genome contains six major genes that encode structural proteins: nucleocapsid (N), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and RNA-dependent RNA (large) polymerase [10, 16].

Reverse transcription- polymerase chain reaction (RT-PCR) using gene-specific primers has been used to detect the presence of NDV nucleic acids [17, 18]. To detect the lentogenic (low virulence), mesogenic (moderate virulence), and velogenic (highly virulent) strains of AOAV-1, target

detection of the nucleic acids of the conserved matrix and the fusion gene have been performed [5, 19]. Although there are several molecular detection techniques for NDV, little is known about the distribution, spread, and risk factors associated with NDV among vaccinated ICs in Kenya. In addition, there is a knowledge gap on variants of NDV based on matrix and fusion genes that are in circulation in the vaccinated ICs population in Kenya.

This study aimed to investigate the prevalence of NDV among vaccinated ICs in Kenya using RT-PCR and genetic analysis of the matrix and fusion genes. Further, the study also sought to establish risk factors, and elucidate the relationship between the presence of NDV in ICs and weather (temperature, rainfall, humidity, and wind speed), interspecies interactions, and the production system of the ICs.

2. Materials and Methods

2.1. Study Location

This was a cross-sectional study of ND surveillance in Kenya. The study was undertaken in six counties during the months of May 2017, June 2017, September 2017, and March 2018, within two agro-ecological zones (AEZ) III and V. These six areas are key to IC farming, trade, and use in cultural events. Kakamega, Bomet Nakuru, and Kilifi are within AEZ III, whereas Baringo and Machakos are within AEZs V and IV, respectively (Figure 1).
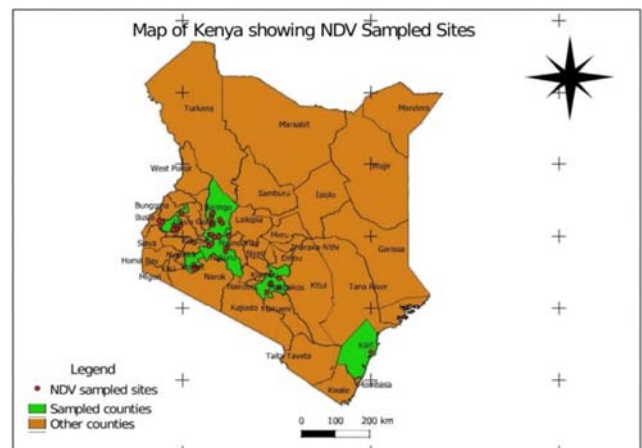
Figure 1. *Map of Kenya showing the sample collection sites. The map indicates specific areas where samples were collected in the different counties.*

2.2. Study Design and Sample Collection

The samples were collected during the dry months of the year with varying weather conditions (Table 1).

*Table 1. Weather conditions at the counties during the sample collection. Courtesy Kenya Meteorological Department and *climate-data.org.*

County/Month/Year	Max temp.	Min temp.	Total rainfall	RH 9am	RH 3PM	Wind run in km/day
Kakamega May 2017	27.9	16	217.5	81%	65%	56.2km/day
Kilifi (Mtwapa) March 2018	31.9	24	98.3	80%	69%	80.5km/day
Machakos September 2017	25.5	12.1	5.7	71%	43%	124.2km/day
Nakuru June 2017	26.6	11.1	36.7	73%	41%	104.3km/day
Bomet*(May 2017)	24.4	13	147			
Baringo*(May 2017)	32.4	17.5	100			

A simple random sampling method was used to identify farms in the sub-counties within the counties to be sampled. The coordinate readings were collected using a portable GPS reader and the study site map was developed using the QGIS-3® 3.12.2 Bucureti (https://download.qgis.org/) (Figure 1). The samples were collected after obtaining verbal consent from farmers, as written consent was not a requirement for sample collection in Kenya (KALRO/BIO/KAB/42/36).

The distance from one household to the next was greater than 1 km to ensure that samples in a given region were from unrelated households in terms of the interaction of their ICs, especially among the free-range ICs. The total number of birds sampled within a given village or ward was dependent on the total number of samples to be collected in that region. Data collected from the households were collated using a laboratory card. The production systems in homesteads are either free-range chicken or semi-free-range production systems. Oropharyngeal (OP) and cloacal (CL) swabs were taken from live, three weeks old domestic IC of both sexes. The collection was performed on farms that had ICs interacting with other poultry or were singly reared. Within the selected household, we sampled healthy chickens, those with NDV-like symptoms, as well as vaccinated and unvaccinated chickens against NDV.

Disposable sterile swabs (polyester or rayon) were used to collect OP and CL swabs [20]. The swabs were immediately placed into appropriately labeled cryovials containing 1 mL of viral transport media. Samples were then immediately placed in a cool box transferred to a liquid nitrogen tank, transported to the labs and stored at −80°C until processing [21].

2.3. Sample Size

The sample size was calculated using the formula given below [22]) which was inclusive of 10% non-responsive cases.

$$\text{Sample size} = \frac{Z_{1-\alpha/2}{}^2\, p(1-p)}{d^2} \qquad (1)$$

Where p is the expected proportion of the population=17.8% [7].

Z score at 95% confidence interval (CI)=1.96, and d (margin of error)=0.05.

$$\text{Sample size} = \frac{1.96^2 \times 0.178(1-0.178)}{0.05^2} = 225 \qquad (2)$$

A total of 605 chickens were included in this study and 1,210 samples were collected from the oropharynx (650) and cloaca (650) of ICs from farms across the country.

2.4. Sample Preparation

Sample processing was performed at the Biotechnology Research Institute of the Kenya Agricultural Livestock Research Organization (KALRO) following the regulations of the Care and Use of Animals Committee of KALRO. Samples from the same region, household, and type (OP or CL) were pooled in labeled cryovials, resulting in 260 pooled samples (~5 samples per pool) (OP: 130, CL: 130). Pooling was performed by centrifuging the cryovials containing the swabs at 1000 × g for approximately 10 min in a refrigerated centrifuge (4°C). Centrifugation ensured the release of the virus into the viral transport medium by dislodging the particles from the swab. After gently shaking the vial, 100 μL of the sample was transferred to a sterile cryovial [20].

2.5. Virus Isolation

We used the allantoic route of nine-day-old embryonated specific antibody negative (SAN) eggs for viral isolation. The eggs were obtained from Kenchic® hatcheries (Nairobi) on day eight and allowed to settle at 36°C ± 1°C. The pooled samples were gently vortexed for 15 s to mix the sample. The eggs were surface disinfected using iodine solution before puncturing the inoculation site on the eggshell. Next, 0.2 mL of the pooled sample was inoculated into the allantoic cavity of five labeled embryonated SAN eggs. [21].

2.6. RNA Extraction and Amplification Using Real Time RT-PCR

Total RNA was extracted from the allantoic fluid using TRIzol™ LS reagent (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions. The extracted RNA was amplified by one-step RT-PCR machine (QuantStudio™ 5 System) using matrix gene primers (Table 2). The La Sota vaccine strain was used as a positive control. The cycling conditions for real-time RT-PCR (qRT-PCR) were 40 cycles of denaturation at 94°C for 10 s, annealing at 56°C for 30 s, and extension at 72°C for 10 s [17].

Table 2. *Primer sequence for isolation of the matrix gene using qRT-PCR.*

Primer	Primer sequence
M+4100 (Forward Primer)	5′-AGTGATGTGCTCGGACCTTC-3′
M+4169 (Matrix Probe)	5′-[FAM]TTCTCTAGCAGTGGGACAGCCTGC [TAMRA]-3′
M−4220 (Reverse Primer)	5′-CCTGAGGAGAGGCATTTGCTA-3′

2.7. Complementary DNA Preparation

Samples that were positive for the matrix gene (CT values<35) were passaged in nine-day-old embryonated SAN eggs. Total RNA was extracted from allantoic fluid using TRIzol^{TM} LS reagent according to the manufacturer's instructions. The extracted RNA was converted to cDNA by first-strand cDNA synthesis using a Thermo-Scientific^{TM} revert-aid first-strand cDNA synthesis kit (Waltham, Massachusetts, USA).

2.7.1. Amplification of cDNA Targeting the Matrix Gene

The cDNA was amplified using one pair of matrix gene universal primers targeting 232 bp [23] (Table 3). The PCR conditions for amplification of the NDV fusion gene were as follows: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 1 min, and a final extension at 72°C for 7 min. The amplicon quality was assessed using agarose gel electrophoresis and purified using a Millipore plate MSNU030 (Millipore SAS, Molsheim, France).

Table 3. *Matrix gene primer sequence for amplification of cDNA and subsequent sequencing of the matrix gene.*

Primer	Primer sequence	Position (bp)	Reference
MATRIX MF	5′-TCGAGTCTGTACAATCTTGC-3′	232	[23]
MATRIX MR	5′-GTCCGAGCACATCACTGAGC-3′	232	

2.7.2. Amplification of cDNA Targeting the Fusion Gene

The cDNA was amplified using five paired primers with different sequences (Table 4). The PCR conditions for amplification of the NDV fusion gene were as follows: initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and initial extension at 72°C, and final extension at 72°C for 45 s. The amplicon quality was assessed using 1% agarose gel electrophoresis prior to cleaning and sequencing.

Table 4. *Fusion gene primer sequences for amplification of cDNA and subsequent sequencing of the fusion gene.*

Primer Name	Primer Sequence	Position (bp)	Reference
NDVF1F1 (forward)	5′-GCAAGATGGGCYCCAAACC-3′	550	
NDVF1R1 (reverse)	5′-CATCTTCCCAACTGCCACT-3′	550	
NDVF2F2 (forward)	5′-GACCACTTTACTCACTCCTC-3′	642	
NDVF2R2 (reverse)	5′-GTAGGTGGCACGCATATTATT-3′	642	[24]
NDVF3F2 (forward)	5′-CGACTCACAGACTCAACTC-3′	685	
NDVF3R2 (reverse)	5′-TATARGTAATRAGRGCRGATG-3′	685	
NDVF4F2 (forward)	5′-GCAAGATRACAACATGTAGRTG-3′	709	
NDVF4R2 (reverse)	5′-CTTGGCTAACYGCRCGGTCCAT-3′	709	
NDVF5F (forward)	5′-ATGGGCYCCAGACYCTTCTAC-3′	535	[25]
NDVF5R (reverse)	5′-CTGCCACTGCTAGTTGTGATAATCC-3′	535	

2.7.3. Sequencing of Amplified cDNA

The cleaned amplicons were sequenced using Sanger sequencing. Forward and reverse sequence reads were obtained for each amplicon. Prior to sequencing, the PCR products were cleaned using the ExoSAP-IT^{TM} (Waltham, USA), which consists of exonuclease and alkaline phosphatase at a temperature of 37°C for 15 min. Fragments were sequenced using the BrilliantDye™ terminator cycle sequencing kit V3.1, BRD3-100/1000 (Nimagen, Nijmegen, Netherlands) according to manufacturer's instructions. The labelled products were then cleaned with the ZR-96 DNA sequencing clean-up kit (Zymo Research, Irvine, California, USA). The cleaned products were injected on a Genetic Analyser with a 50 cm array, using POP7 (Applied Biosystems ABI 3500XL). Sequence chromatogram analysis was performed using FinchTV analysis software (Geospiza, Inc.).

3. Data Analysis

3.1. Analysis of the Relationships from RT-qPCR Matrix Gene Results

The Statistical Package for Social Sciences software (SPSS$^{®}$ 2019) was used for data analysis. The chi-square and Fisher's exact tests were used to test the independence of the variables with a statistical significance of $p \leq 0.05$. The analysis was performed to examine the relationship between the presence of NDV in vaccinated flocks and other variables (vaccination history of the ICs, interaction with other domestic poultry, production system, and months of sample collection).

3.2. Analysis of Sequence Results for the Matrix Gene and Fusion Gene

The five raw M gene paired reads and seven raw F gene paired reads were trimmed and assembled using Geneious Prime version 2020.2 software (https://www.geneious.com/) by mapping to the reference sequence MN685356.1, which was retrieved from the National Centre for Biotechnology Information (NCBI). Consensus sequences were retrieved from the respective generated contigs and multiple sequence alignment was performed using the ClustalW algorithm within the Mega X version 10.1.6 software.

3.3. Phylogenetic and Haplotype Analysis

The matrix and fusion gene phylogeny was inferred using the maximum likelihood method by applying neighbor-joining and BioNJ algorithms on nucleotide pairwise distances, which were estimated using the maximum composite likelihood approach with a bootstrap of 1000 repeats [26]. Genetic indices were computed using DNA Sequence Polymorphism (Dna SP) version 6.0 software (http://www.ub.edu/dnasp/downloadTv6.html) and TCS network was computed using Population Analysis with Reticulate Trees (PopArt) version 1.7 (http://popart.otago.ac.nz/) [27].

4. Results

4.1. Sampled Households' Characteristics

The households had either a free-range or a semi-free-range production system. Most of the ICs were kept in the free-range system 435/605 (71.9%). A majority of the households had an average of 10 ICs of all ages and sexes. Vaccination of ICs was performed less than three months before sampling. Vaccination in this context means that the farmer, through their intervention or that of an animal service provider, had administered the NDV vaccine within three months, inclusive of the sample collection date. La Sota was the most administered vaccine, mainly through the oral route. Among all the households (n=68), only 17/68 (25%, 95% CI=14.7–35.3) had vaccinated their ICs (Table 5). The vaccination history per county indicates that all the counties had a higher number of unvaccinated ICs (435/605; 71.9%, 95% CI=68.3–75.5) than vaccinated ICs (170/435; 28.1%, 95%=24.5–31.7). The vaccinated ICs accounted for 67/175 (38.3%, 95% CI=31.1–45.5), 14/169 (8.3%, 95% CI=4.1–12.4), 35/35 (100%), 25/70 (31.3%, 95% CI=24.5–46.9), and 29/76 (33.3%, 95% CI=27.2–49.1) in Baringo, Kakamega, Kilifi, Machakos, and Nakuru, respectively (Table 6). None of the households in Bomet County had vaccinated their ICs. Six households (8.8%, 95% CI=2.1–15.6) had previous outbreaks of ND before sample collection (Table 5). Samples were collected from one household in Kilifi (Sample ID NDVKE-0303-3-F Bird # 17-51) that had an active case with signs and symptoms of suspected NDV despite having vaccinated their flocks (Figures 2 and 3). Symptoms were observed two days after the vaccination. One household in Kakamega (Sample ID NDVKE-0517-37-F Bird # 21-25) had ICs with symptoms similar to those of fowlpox disease.

Figure 2. *ICs exhibiting NDV symptoms two days after vaccination with NDV vaccine.*

Table 5. *Summary of household history with the number of households, those with previous outbreaks, and those with an existing poultry disease during sample collection.*

County	No. of households	Vaccination history	Previous outbreak	Presence of any other disease
Baringo	21	6	2	
Bomet	5	0	0	
Kilifi	1	1	1	0
Kakamega	20	4	1	1
Machakos	10	2	2	
Nakuru	11	4	0	
Total	68	17	6	1
Proportions (%, 95% CI)		17/68 (25, 14.7–35.3)	6/68 (8.8, 2.1–15.6)	

Table 6. *Number of vaccinated and unvaccinated ICs in the different counties.*

Sample source	Unvaccinated ICs	Vaccinated ICs	Total (N)	The proportion of unvaccinated ICs (%, 95% CI)	The proportion of vaccinated ICs (%, 95% CI)
Baringo	108	67	175	61.7 (54.5–68.9)	38.3 (31.1–45.5)
Bomet	80	0	80	100	0
Kakamega	155	14	169	91.7 (87.5–95.9)	8.3 (4.1–12.4)
Kilifi	0	35	35	0	100
Machakos	45	25	70	64.3 (53.1–75.5)	31.3 (24.5–46.9)
Nakuru	47	29	76	61.8 (50.9–72.8)	33.3 (27.2–49.1)
Total	435	170	605	71.9 (68.3–75.5)	28.1 (24.5–31.7)

Figure 3. IC exhibiting NDV symptoms two days after vaccination with NDV vaccine.

4.2. Virus Detection

The RT-PCR targeting matrix gene detected NDV nucleic acids in the pooled samples 184/260 (70.8%, 95% CI=65.2–76.3) obtained from the six counties in Kenya. The distribution of positive cases per county was 48/72 (66.7%, 95% CI=56.1–77.9), 25/34 (78.1%, 95% CI=63.8–92.4), 44/68 (64.71%, 95% CI=71, 53.3–76.1), 20/26 (76.9%, 95% CI=60.7–93.1), 23/30 (71.9%, 95% CI=55.8–88), and 24/30 (80%, 95% CI=65.7–94.3) for Baringo, Bomet, Kakamega, Kilifi, Machakos, and Nakuru, respectively. The positive samples in pooled CL were 93/130 (71.5%, 95% CI=63.7–79.3) and in the pooled OP were 91/130 (70%, 95% CI=62–78) (Table 7).

Table 7. The proportion of positive and negative NDV results from the pooled samples and sample type per county.

County	Total no. of samples (N)	Total no. of pooled samples (n)		The proportion of positive samples (%, 95% CI)		
		CL	OP	CL	OP	Total
Baringo	350	36	36	24 (67, 51.6–82.4)	24 (67, 51.6–82.4)	48 (66.7, 56.1–77.9)
Bomet	160	16	16	11 (68.75, 46.03–91.5)	14 (87.5, 71.3–100)	25 (78.1, 63.8–92.4)
Kakamega	338	34	34	24 (70.6, 55.3–86)	20 (58.8, 42.3–75.3)	44 (64.71, 53.3–76.1)
Kilifi	70	13	13	10 (76.9, 54–99.8)	10 (76.9, 54–99.8)	20 (76.9, 60.7–93.1)
Machakos	140	15	15	11 (68.75, 45.3–2.2)	12 (75, 53.1–96.9)	23 (71.9, 55.8–88)
Nakuru	152	15	15	13 (86.7, 69.5–100)	11 (73.3, 50.9–95.7)	24 (80, 65.7–94.3)
Total	1210	130	130	93 (71.5, 63.7–79.3)	91 (70, 62–78)	184 (70.8, 65.2–76.3)

4.3. Relationship Between IC Vaccination History and the Presence of NDV in Vaccinated ICs

NDV was detected in vaccinated and unvaccinated IC flocks. The prevalence of NDV in vaccinated ICs was 55/70 (78.5%, 95% CI=69–88, *p*=0.045), with the distribution in counties accounting for 19/28 (67.9%, 95% CI=51–85), 6/6

(100%), 11/14 (78.6%, 95% CI=57–100), 9/10 (90%, 95% CI=71.4–100), and 10/12 (83.3%, 95% CI=62.2–100) in Baringo, Kakamega, Kilifi, Machakos, and Nakuru, respectively (Table 8). There was a significant (*p*=0.034) relationship between the presence of NDV in the ICs and the vaccination history of the ICs.

Table 8. Prevalence of NDV in vaccinated samples as a percentage and 95% CI.

Sample source	Vaccinated ICs (N)	No. of pooled samples from vaccinated flocks (n)	Proportion of positive NDV cases (%, 95% CI)	Proportion of negative NDV cases (%, 95% CI)
Baringo	67	28	19 (67.9, 51–85)	9 (32.1, 14.8–49.4)
Kakamega	14	6	6 (100, 100–100)	0
Kilifi	35	14	11 (78.6, 57–100)	3 (21.4, 0.06–42.9)
Machakos	25	10	9 (90, 71.4–100)	1 (10, 8.5–28.6)
Nakuru	29	12	10 (83.3, 62.2–100)	2 (16.7, 4.4–37.8)
Total	170	70	*55 (78.5, 69–88)	15 (21.5, 11.8–31)

* *p*=0.045

4.4. Relationship Between the IC Production System and the Presence of NDV in Vaccinated ICs

The proportion of positive NDV cases in the production system with vaccinated IC flocks was 52/70 (74.3%, 95% CI=64.5–84.5). Vaccinated free-range ICs had 34/47 (72.3%,

95% CI=59.5–85.1) and 18/23 (78.2%, 95% CI=61.3–95.1) for the vaccinated semi-free-range ICs (Table 9). There was a significant (*p*=0.004) relationship between the production system used in rearing the ICs and the presence of NDV.

Table 9. Proportion of positive NDV cases in different poultry production systems (% and 95% CI).

Production system	No. of ICs	No. of vaccinated ICs (n)	No. of pooled samples (n)	The proportion of positive NDV (%, 95% CI)	The proportion of negative NDV (%, 95% CI)
Free-range	435	124	47	34 (72.3, 59.5–85.1)	13 (27.7, 9.6–46.3)
Semi-free-range	170	47	23	18 (78.2, 61.3–95.1)	5 (21.7, 4.8–38.5)
Total	605	171	70	52 (74.3, 64.5–84.5)	18 (25.7, 15.5–36)

4.5. Relationship Between the Interaction of ICs with Other Birds and the Presence of NDV in Vaccinated ICs

The number of vaccinated ICs that interacted with other birds was 90/605 (14.9%). The proportion of positive NDV in the vaccinated ICs that interacted with other birds was 30/40 (75%, 95% CI=61.6–88.4) (Table 10). The analysis examined the relationship between the presence of NDV in vaccinated ICs interacting with other birds such as turkeys, ducks, wild birds, and geese. There was no significant (p=0.155) relationship between the interaction of ICs with other birds and the presence of NDV.

Table 10. *Positive cases of NDV in ICs that interacted with other domestic birds (% and 95% CI).*

County	No. of vaccinated ICs that interacted with other birds (N)	No. of pooled samples (n)	The proportion of positive NDV (%, 95% CI)	The proportion of negative NDV (%, 95% CI)
Baringo	20	12	7 (58.3, 30.4–86.2)	5 (42, 13.8–69.6)
Kilifi	35	14	11 (50, 23.8–76.2)	3 (21.4, –6–42.9)
Machakos	25	10	9 (90, 71.4–100)	1 (10, –8–28.6)
Nakuru	10	4	3 (75, 32.6–100)	1 (25, –17.4–67.4)
Total	90	40	30 (75, 61.6–88.4)	10 (25, 11.5–38.4)

4.6. Relationship Between the Presence of NDV in ICs and the Months of the Year

The proportion of positive NDV in vaccinated ICs was 11/14 (78.6%, 95% CI=57–100), 16/21 (76.2%, 95% CI=58–94.4), 21/29 (72.4%, 95% CI=56.2–88), and 9/10 (90%, 95% CI=79.1–100) for the months of March, May, June, and September, respectively (Table 11). The analysis to examine the relationship between the presence of NDV in ICs and month of the year revealed statistical significance ($p < 0.0001$).

**Table 11.** *Positive cases of NDV in vaccinated flocks during different months of the year.*

Month	No. of birds from vaccinated flocks (N)	No. of pooled samples (n)	The proportion of positive NDV cases (%, 95% CI)	The proportion of negative NDV cases (%, 95% CI)
March	35	14	11 (78.6, 57–100)	3 (21.4, –6–42.9)
May	47	21	16 (76.2, 58–94.4)	5 (23.8, 5.6–42.3)
June	59	29	21 (72.4, 56.2–88.7	8 (27.5, 11.3–43.9)
September	25	10	9 (90, 79.1–100)	1 (10, –0.9–20.9)
Total	166	74	57	17

4.7. Genetic Diversity of the NDV Matrix and Fusion Genes

4.7.1. Genetic Diversity of the NDV Matrix Gene

A total of six mutated sites were observed in the five analyzed sequences, with each sequence harboring at least one mutation. Two singleton variable sites (single nucleotide polymorphism) were observed at loci 62 and 77, whereas the remaining sites with mutations (loci 41, 66, 149, and 203) were informative for parsimony (occurred in more than one sequence). Parsimony informative sites 41, 66, and 203, where the nucleotide base guanine was substituted by adenine, adenine by guanine, and guanine by adenine, respectively, were observed in sequences of isolates (2C2, KF2C, and KF30), and mutation at locus 149 occurred only in sequences of isolates KF2C and KF30. The observed mutations across all sites (165.03) analyzed were non-synonymous with Pi (a), Jukes & Cantor model equaling 0.01972. Despite the presence of mutated sites, the matrix gene from the five isolates had high sequence conservation (C) with a C value of 0.974 out of 229 net sites analyzed. The five sequences exhibited low nucleotide diversity, with π=0.01475 and a high haplotype diversity (Hd) value of 0.900 (Table 12). Four haplotypes based on matrix genes were found to be circulating within Kenya (Figure 4).
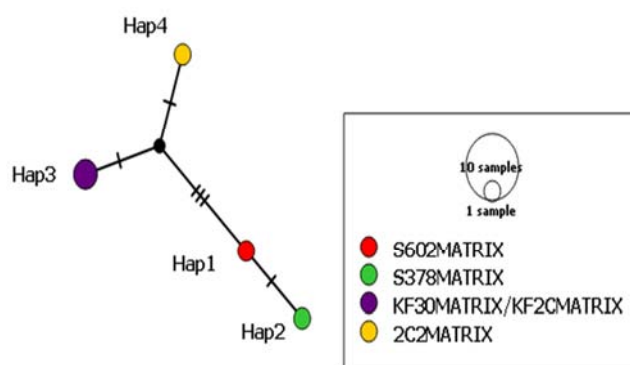
Figure 4. *TCS haplotype network estimating the four haplotypes as well as genealogies of NDV genotypes based on the matrix gene. The hatch marks represent the number of mutations that led to the emergence of a specific haplotype or genotype. The haplotypes are represented by the following colors: haplotype 1 (red), haplotype 2 (green), haplotype 3 (purple), and haplotype 4 (yellow). The size of the circles is proportional to the number of samples within the specific haplotype. Haplotype 3 was observed in two samples, thereby explaining the large size of the circle.*

Table 12. *Matrix gene segregation site, number of mutations, nucleotide diversity, and haplotype density.*

No. of Sequences	Segregating sites (S)	Total No. of Mutations (Eta)	Nucleotide Diversity (π)	π (per site) from Eta	Haplotypes (h)	Haplotype (gene) diversity (Hd)	Standard Deviation of Hd
5	6	6	0.01	0.21	4	0.90	0.16

4.7.2. Phylogenetic Analysis of the Matrix Gene

Five GenBank retrieved sequences of isolates from Kenya and Tanzania and five matrix gene sequences (KF2C (MZ062109), KF3O (MZ062110), 2C2 (MZ062108), S378 (MZ062111), and S602 (MZ062112)) from the study were used to generate the tree (Table 13). Four genotypes (I, II, III, IV, and V) were identified from the tree (Figure 5).
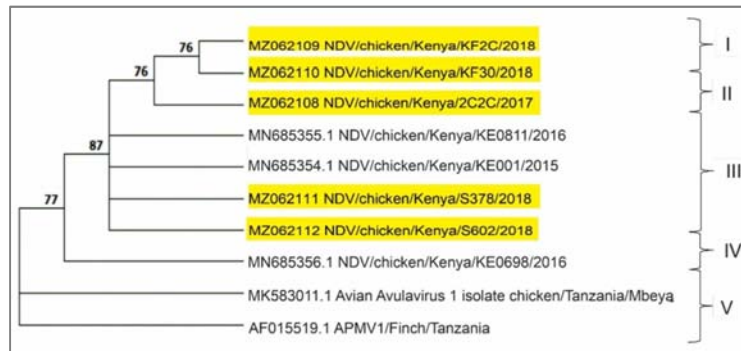
Figure 5. *Phylogenetic tree inferring evolutionary relatedness of NDV matrix gene and previously isolated NDV. The bootstrap values >75% are presented next to the node.*

Table 13. *Identity matrix for the matrix gene analysis.*

| Sample	AF015519.1_APMV1/Finch /Tanzania	MK583011.1_Avian_avul avirus_1_isolate_chicken/ Tanzania/Mbeya	MN685354.1_NDV/c hicken/Kenya/KE001 /2015	MN685355.1_NDV/c hicken/Kenya/KE081 1/2016	MN685356.1_NDV/c hicken/Kenya/KE069 8/2016
2C2	89.862	94.931	97.642	98.578	96.698
KF2C	89.912	95.197	97.768	98.578	96.861
KF30	89.912	95.175	97.758	98.578	96.861
S378	89.912	95.217	98.214	98.104	96.861
S602	89.912	95.197	98.661	98.578	96.861

4.7.3. Genetic Diversity of the NDV F Gene (Haplotype Analysis)

We identified 106 mutations across 103 segregating sites that showed differences (polymorphisms) between related genes in a sequence alignment on the 535 base pairs of the seven aligned sequences. Of the 103 sites with mutations, 102 sites were parsimony informative, as the mutation was observed in more than one sequence (Figure 6). The singleton variable site was only observed in one sequence, KF90NDV, at segregating site 447. A high nucleotide diversity (π) of 0.24639 was observed across the seven analyzed NDV sequences (Table 14). Five haplotypes (Figure 7) with a high Hd of 0.905 were identified among the seven analyzed sequences. Haplotypes one and five (H01 and H05) each had two samples, whereas the rest of the haplotypes (H02, H03, and H04) had a single sequence.

4.7.4. Phylogenetic Analysis of the Fusion Gene

Fifteen nucleotide sequences from other regions were retrieved from GenBank (accession number on the tree) and used together with the seven sequences S95NDV, BIICNDV, KF90NDV, S602NDV, S378NDV, 2CIINDV, and KF2CNDV (MZ062105, MZ062102, MZ062104, MZ062107, MZ062106, MZ062101 and MZ062103 respectively) from this study to compute the tree (Table 15) (Figure 7).
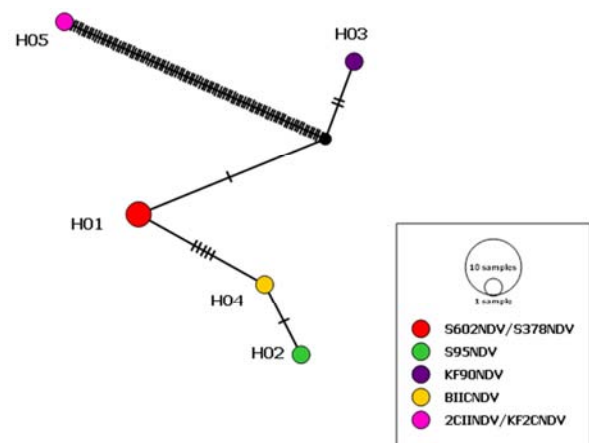
Figure 6. *TCS network estimating haplotypes as well as genealogies of the NDV genotypes from the seven analyzed samples. The hatch marks represent the number of mutations that led to the emergence of a specific haplotype or genotype. The haplotypes are represented by the following colors: haplotype 1 (red), haplotype 2 (green), haplotype 3 (purple), haplotype 4 (yellow), and haplotype 5 (pink). The size of the circles is proportional to the number of samples within the specific haplotype.*

Table 14. *Genetic diversity indices of seven NDV isolates from Kenya.*

No. of Sequences	Segregating sites (S)	Total no. of Mutations (Eta)	Nucleotide Diversity (π)	π (per site) from Eta	Haplotypes (h)	Haplotype (gene) diversity (Hd)	Standard Deviation of Hd
7	103	106	0.25	0.22	5	0.91	0.10
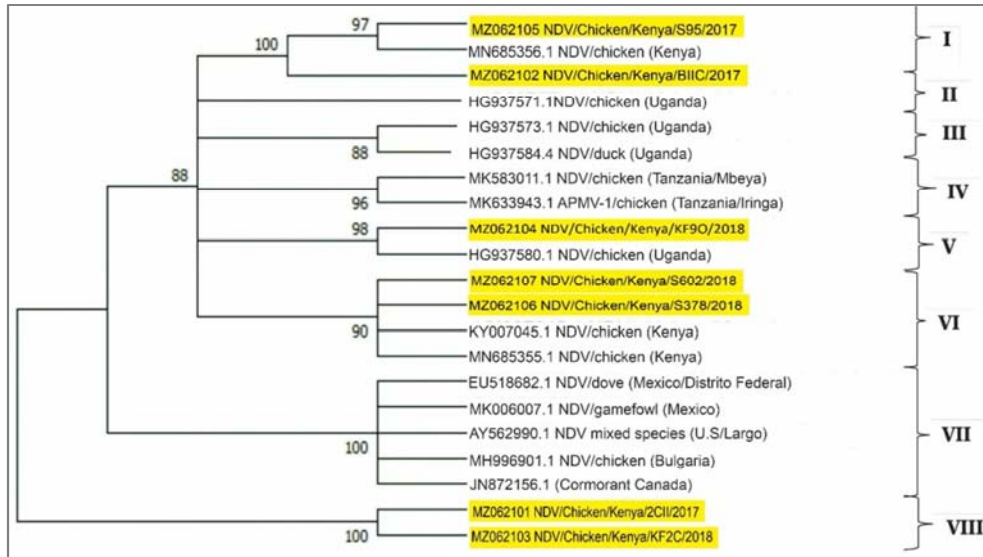
Figure 7. *Phylogenetic tree inferring evolutionary relatedness of the NDV fusion gene and previously isolated NDV. Each clade is designated by numbering numbers on the right, while the bootstrap values are shown at the nodes.*

Table 15. *Identity matrix for the fusion gene analysis.*

Reference sequence from NCBI	Sample Name						
	S602	S378	S95	KF90	BIIC	2CII	KF2C
MN685356.1_NDV/chicken_(Kenya)	96.545	96.353	98.566	95.652	98.686	43.922	39.92
HG937571.1_NDV/chicken_(Uganda)	97.505	97.313	97.133	96.86	97.969	43.922	39.92
HG937573.1_NDV/chicken_(Uganda)	96.929	96.737	96.774	97.222	97.372	43.922	39.92
MK583011.1_NDV/chicken_(Tanzania/Mbeya)	97.121	96.929	96.416	96.135	97.013	44.51	39.92
EU518682.1_NDV/dove_(Mexico/Distrito_Federal)	92.322	92.131	92.335	92.736	92.934	43.333	39.92
MH996901.1_NDV/chicken_(Bulgaria)	91.171	90.979	92.096	91.768	92.695	42.745	39.257
MK633943.1_APMV-1/Chicken_(Tanzania/Iringa)	97.036	96.838	96.047	95.842	97.036	45.673	37.62
MN685356.1_NDV/chicken_(Kenya)N	96.545	96.353	98.58	95.673	98.686	43.922	39.842
KY007045.1_NDV/chicken_(Kenya)	99.616	99.424	96.353	97.308	97.313	46.188	38.619
MK006007.1_NDV/gamefowl_(Mexico)	90.979	90.787	91.243	91.587	91.756	43.333	39.974
HG937580.1_NDV/chicken_(Uganda)	97.692	97.5	96.519	98.932	97.105	43.922	40.08
JN872156.1_(Cormorant_Canada)	91.346	91.154	91.236	92.052	91.797	43.529	40.483
AY562990.1_NDV_mixed_species/(U.S./Largo)	91.538	91.346	91.477	91.815	92.039	42.745	39.812
MN685355.1_NDV/chicken_(Kenya)	99.04	98.848	96.048	97.146	96.39	43.725	40.241
HG937584.1_NDV/duck_(Uganda)	97.972	97.769	97.395	97.543	97.88	44.118	40.334
2CIINDV	46.188	46.188	43.725	43.811	43.137		99.512
KF2CNDV	38.433	38.619	39.71	40	39.257	99.512	

5. Discussion

ND is a local and globally significant disease in poultry, and it is controlled by vaccination of healthy chickens [21]. Vaccination of chicken in Kenya is conducted by administering the La Sota vaccine (Avivax -L) from the Kenya Vaccine Production Institute (KEVEVAPI). However, vaccination is routinely performed on commercial exotic breeds rather than ICs [23]. Despite farmers' awareness of the importance of vaccinating ICs [28], the vaccine administration rate is low especially, for the free-range chickens. The low administration rate can be attributed to the small flock size reared by the farmers compared to the package dose of the vaccine, the lack of an appropriate cold chain to transport the vaccine, and the assumption that ICs are less susceptible to diseases [28, 29]. The NDV vaccines are packaged in vials of 50 doses whereas IC farmers rear a flock size of an average of 10 birds, mainly for domestic consumption. In comparison to a commercial farmer, a small-scale farmer is unlikely to follow the vaccination protocol. To mitigate this challenge, IC farmers in proximal neighborhoods can collectively vaccinate their flocks to control the spread of ND [29-32].

Previous studies on the seroprevalence of NDV in ICs in Kenya was estimated to be 17.8% in dry hot areas [7].

However, recent surveillance has indicated that NDV is widespread in Kenya [33]. The present study indicated high prevalence 78.5% [*p*=0.045] of the disease in vaccinated ICs. Our study indicates the possibility of an outbreak being transmitted by vaccinated chickens [23] and corroborates the findings of an experimental study on vaccinated chickens that detected NDV in 80% of La Sota vaccinated chickens [11].

The presence of virulent or avirulent NDV in chicken is an indication of infection or viral shedding. Viral shedding is a result of vaccinating poultry with NDV vaccine strains that are different from the circulating genotypes. This can lead to the subsequent transmission of the non-matched vaccine strain viruses to susceptible flocks [8, 11, 33]. Among the isolated NDV matrix genotypes, genotypes II and I had a close resemblance to the initially identified genotype MN685355.1 and MN685354.1 or genotype III from Kenya and are descendants of genotype IV. Isolates KF2C (MZ062109) and KF30 (MZ062110) from ICs in Kilifi were identified to be within low-virulence genotype I, which is a recent genotype thought to have emerged as a result of mutations within the matrix gene of isolate 2C2 (MZ062108) (genotype II) from Machakos. Class II genotype I has been identified as low-virulence NDV and is used for the development of NDV vaccines [10, 23]. However, the presence of various genotypes isolated from vaccinated birds in Kenya and few of the genotypes with dissimilarity from the commonly used vaccine La Sota genotype (genotype II), might indicate a failed vaccination due to incompatibility of the virus genotypes [8, 25, 34]. This can lead to the transmission of NDV to susceptible flocks [8, 11, 36]. The use of a vaccine that is phylogenetically divergent from the circulating NDV may result in reduced ability of the vaccine to prevent shedding, and continuous maintenance of NDV in the environment [10].

For laboratory diagnosis, detection of NDV is generally performed by targeting the virulent strains and is mostly performed in samples from unvaccinated ICs. This study shows that laboratory diagnosis of NDV should be complemented with the detection of matrix genes to examine shedding of the virus or failure of the vaccine [37].

To understand the determinant of virulence of the NDV, this study characterized the fusion gene that codes for virulent F protein among the isolated NDVs [38]. Initial studies on the partial characterization of the NDVs in Kenya showed that the Kenyan genotype is genotype V and was isolated from non-vaccinated ICs, which showed 83% similarity with the La Sota vaccine, derived from the La Sota virus genotype II of avian paramyxoviruses [25]. The findings of the present study indicated that isolated genotypes in Kenya belong to genotypes VIII, VI, V, and I. Genotype VI is associated with doves and pigeons and predominantly found in the world with other genotypes, such as V, VI, VII, and VIII [10, 39]. Genotype VI has been isolated in wild doves and pigeon in Ethiopia [40] and in Sudan [41]. The presence of genotype VI in Kenyan ICs could be a result of the introduction of ancestral class II genotype VI Columbidae birds (pigeons and doves), low biosecurity, trade practices in the markets [39], and interaction of the local ICs with other

birds before purchase. Some genotypes within clade VII are from wild birds and are closely related to 2CIINDV (MZ062101) and KF2CNDV (MZ062103) [42]. Genotypes S602NDV (MZ062107) and S378NDV (MZ062106) (genotype VI) share the same lineage as isolates KY007045.1 and MN685355.1, which are previously identified NDV isolates in Kenya. Genotype V was found in isolate KF90NDV (MZ062104) and shared lineage with isolate HG937580.1 (genotype V) which was initially identified to have infected chickens from Uganda [24]. Genotype V was isolated from the ICs of unvaccinated Kenya birds IC [25]. None of the genotypes sequenced in this study shared close ancestry with isolate MK583011.1 and MK633943.1 (genotype IV) from Mbeya and Iringa, respectively, in Tanzania. However, the phylogeny results showed clade IV to have arisen from a common ancestry with genotype HG937571.1 which is also shared by clades I, II, III, IV, V, and VI. Mutations within genotype BIICNDV (MZ062102) led to the emergence of recent genotypes, such as S95NDV (MZ062105) and MN685356.1 of genotype I. Similarities of certain isolated genotypes from Kenya ICs to genotypes identified in wild birds indicate that the transmission of NDV could have occurred from a known source of NDV [13]. This reaffirms that the interaction between wild birds and ICs can result in the cross-species transmission of NDV [43].

Free-range and semi-free-range poultry production systems are the preferred systems for rearing ICs. In this study, there were more vaccinated ICs in the semi-free-range production system than in the free-range production system possibly due to the commercial aspect of semi-free-range farming [29]. The presence of NDV in the vaccinated flocks in the two production systems is indicative of possible challenges in the effective administration of the NDV vaccine. ICs are mainly vaccinated via drinking water, which is a more effective method if the birds are fed and deprived of water. For free-range ICs, the possibility of effectively vaccinating all the birds with the required dosage is minimal [44] because they source their feed and water away from the homesteads. The ICs should be monitored for the presence of other infections before vaccination. Chickens infected with low-virulence viruses exhibit mild respiratory symptoms [5], which cannot be easily detected in free-range ICs. The presence of other diseases before vaccination reduces the effectiveness of the vaccine compared with vaccination conducted on healthy birds [44]. The history of IC vaccination was observed to significantly influence the presence or absence of NDV, and could mainly be attributed to factors such as human culture and bird immunity. Traditionally, practices in Kenya revolve around "gifting" and "lending" of ICs to neighbors and guests [3]. The new chicken is introduced into the existing flock with no history of NDV infection and the possible presence of maternal antibodies against NDV [9]. These new chickens may transmit the disease through shedding of the virus if they were previously vaccinated or if the new farm had no history of vaccination. In many farms in Kenya, cockerels are "borrowed" from neighbors to sire chickens on farms. In this case, the cockerel, through paternal inheritance, may genetically

influence the ability of the offspring to maintain maternal antibody levels against ND [9]. This may then affect the immune response of the new flock toward the existing vaccine [8, 44].

Weather conditions vary during different months of the year in Kenya. Typically, there are two seasons, wet and dry, which were found in this study to have a significant effect on the spread of NDV. During extreme weather conditions such as strong winds, high temperatures, and high humidity, ICs are stressed, and their immunity is low. This is due to hunger or limited access to food, particularly for the free-range ICs, resulting in lowered immunity and increased cases of NDV [7, 25, 43], as well as the reduced success of ND vaccination [34]. The directional flow of wind is known to disperse the NDV viral particles, thereby transmitting the virus to the ICs [45]. Most farmers were aware that the disease occurred just before the rainy season and attributed it to the strong winds. This resulted in the origin of the local names. Therefore, this period should be considered an appropriate time for vaccination of the ICs [42, 45].

The integration of ICs and other species of birds, ducks, turkeys, and feral birds is a common practice in most households. However, the other species are also known to be a source of NDV. Birds have the potential to spread the virus as they feed and drink from the same source [42]. Ducks are known to be resistant to the disease, exhibiting no clinical signs, and can be a source of virus shedding [4, 46]. Thus, in a mixed-species farm, other birds can harbor the virus, which in turn can affect an existing flock, new flock, or any immunocompromised flock. In our study, there was no significant relationship between the presence of NDV and the extent of interaction with other species. However, this does not imply that transmission is absent and further analysis should be performed on samples collected from other domestic birds to determine what strain (s) they harbor, and their similarities to strains circulating in the country.

6. Conclusion

The purpose of this research was to determine the prevalence of NDV among the vaccinated flocks and the possible risk factors, as well as determine the circulating genotypes of NDV in Kenya. Based on this study, we conclude that ND is an endemic disease in Kenya, and ICs, vaccinated and unvaccinated, are prone to infection. To control the spread of disease, a vaccination regime for ICs should be developed. The prevailing weather condition and production system should be considered as possible risk factors to determine the type of vaccine to be used and the time of vaccination. To ensure proper diagnosis of NDV, detection should not only focus on the virulent strain, but also the low-virulence and avirulent strains. Therefore, laboratory detection of NDV should always be complemented by targeting the matrix genes, which targets all the NDV strains. The presence of genotypes isolated from the ICs that are dissimilar to vaccine genotype but similar to genotypes isolated in wild birds should encourage further

research to identify and characterize circulating strains of NDV in other species of birds in Kenya. Together, these advances will establish the existence of other reservoirs of certain observed genotypes and facilitate the development of an effective NDV vaccine in Kenya.

Data Availability

The NDV sequences obtained in this study have been deposited in the GenBank database with accession numbers MZ062101 to MZ062112.

Conflict of Interest

The authors declare that they have no competing interest.

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