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ORIGINAL RESEARCH

Molecular Detection and Serological Investigation of Newcastle Disease in Intensive, Semi-Intensive, and Backyard Production Systems in Central and Southwestern Areas of Ethiopia

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Purpose: The purpose of this research is to detect Newcastle disease virus and to assess the seropositivity among backyard, semi-intensive, and intensive farms located in central and southwestern areas of Ethiopia.

Material and Methods: A total of 239 oropharyngeal and cloacal swab samples were collected from symptomatic birds found in Holeta, Burayu, Jimma towns as well as Seka Chekorsa and Nadhigibe woredas of Jimma Zone. In addition, ninety blood samples were collected from wing veins of unvaccinated birds found in the study areas of Jimma zone. Side-by-side information related to risk factors estimated to contribute to the susceptibility of the disease was collected by interviewing owners of sampled birds. Reverse transcription polymerase-chain reaction (RT-PCR) was conducted to detect NDV. Likewise, Enzyme-linked immunosorbent assay (ELISA) was performed to determine the seropositivity of ND.

Results: The proportion of samples where NDV was detected was 24.6%. Similarly, 68.9% of the sampled birds were seropositive. It was observed that adult birds were more likely to encounter the disease than youngs (OR = 11.6; 95% CI: 4.0–33.3; P = 0.000). Birds owned by respondents who leave diseased birds in the flock were more likely infected (OR = 6.2; 95% CI: 1.8–21.2; P=0.004) as compared to those isolated and mode of disposal of dead chicken significantly affect exposure (OR = 0.13; 95% CI: 0.10–4.88; P = 0.044). Likewise, access to veterinary services highly likely reduces susceptibility to the disease (OR = 12.4; 95% CI: 3.2–46.9; P = 0.000). It was also found that birds farmed intensively were the most at risk (OR = 2.8; 95% CI: 0.58–13.71; P = 0.199).

Conclusion: Detection of ND from a significant proportion of sampled birds and their high seropositivity percentage revealed the circulation of the virus in the study areas.

Keywords: Newcastle disease, RT-PCR, ELISA, susceptibility

Introduction

Newcastle disease (ND) is one of the of the most important infectious diseases of poultry caused by Newcastle disease virus (NDV) which is also known as avian paramyxovirus type 1 (APMV-1). The virus is classified under the genus Avulavirus belonging to the family Paramyxoviridae.[1] Due to its highly contagious nature, the disease remains one of the listed diseases[2] and it is characterized by greenish diarrhea, dullness, respiratory distress (gasping, coughing, sneezing) and nervous symptoms (paralyzed wings and legs) including twisted necks[3] The symptoms and signs depend on the strain of the virus, host species, age of the host, co-infection, environmental stress, and immune status of the infected bird.[4] During postmortem examination, affected birds indicate hemorrhage at their trachea, proventriculus, and intestine. In addition, enlargement of the spleen and liver as well as thickening of the air sacs can also be observed. However, the lesion development depends on the predilection site of the specific strain.[5]

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ND remains still a major problem in the world[6–8] and in Africa.[9,10] In Ethiopia, it has been reported long ago,[11,12] and different reports indicated that the disease is endemic to the country.[13–16] However, studies usually focus on some areas and production system (i. e commercial farms in the Rift Valley) and the distribution and the effect of the disease in semi-intensive and backyard production systems is less studied. Meanwhile, in many African countries, studies indicated that the disease is prevalent even in backyard poultry.[17,18]

Despite the wide distribution of backyard or semi-intensive poultry production systems in Ethiopia, it can be predicted that the disease might be widespread in many rural villages because of low veterinary supply, low diagnostic coverage, and lack of thermostable vaccines that suit the hot tropical weather.[19] In addition, in rural areas, little or no biosecurity measures are practiced which could favor the spread of the disease and serve backyard poultry as a potential reservoir of different pathogens including NDV that could threaten the development of the sector at commercial or industrial scales in the country.

Thus, the objective of this research is to detect the virus and measure the seropositivity among different production systems namely; backyard, semi-intensive, and intensive using a molecular technique and ELISA respectively with the associated risk factors.

Materials and Methods

Study Area

Samples were collected from Holetta, Burayu, and Jimma towns as well as Seqa Chekorsa and Nadhigibe woredas of the Jimma zone which are located 20 and 64 km away from Jimma town, respectively. Both Holeta and Burayu are high land areas with an altitude of 2391 and 2712 meters above sea level both with mean annual rainfall above 1000mm. Jimma town has an altitude of 1780 meters above sea level and Seqa Chekorsa with an average of 2070 and 2220 meters above sea level, respectively. The Jimma zone has an average rainfall of 2000mm per annum.

Sample Collection and Study Design

A total of 239 oropharyngeal and cloacal swab samples were purposively collected from symptomatic or dead birds in the period between Jan. 2020 and April 2022 from all three production systems namely backyard, small-intensive, and intensive. The samples were collected from all study areas by inserting sterile VTM-dipped cotton-tipped swabs into the oropharynx and/or vent of the bird and by gently rotating them against the walls. Then the swabs were kept in labeled sterile universal bottles containing VTM. After that, all the collected samples were transported to the National Agricultural Biotechnology Research Center, Animal Biotechnology Research facility via cold chain and preserved in -80^{O}C freezer. Side by side, a semi-structured questionnaire was prepared and farmers were interviewed to assess the associated risk factors.

RNA Extraction and Reverse Transcription Polymerase Chain Reaction

Samples were prepared for extraction after the removal of gross contaminants and debris. The cryovials containing the swab samples were first centrifuged at 10,000 g for 5 min. And the supernatant was collected in a separately labeled 1.5 mL Eppendorf tube. Similarly, the tissue samples were minced by scissors and centrifuged in the buffer they were kept and the supernatant was collected for each of the samples. Then, total RNA was extracted by taking 100µL of each on the sample using DaAn Gene RNA purification kit (Guangzhou, China) according to the manufacturer's instruction. Finally, the RNA was kept at -80^{O}C freezer.

A set of primers targeting the conserved region of the Fusion gene of Newcastle disease virus was designed using Primers 3 input (version 0.4.0; http://primer3.ut.ee) a free online primer designing software after extraction of the nucleotide of the virus from NCBI (https://www.ncbi.nlm.nih.gov/nucleotide/). The forward primer was 5' TCGCAAAATTATGGAGAAGC-3' and the reverse was 5'-AGCAAGGTCTTTTGTTGTGC-3' with an amplicon product size of 386 bp.

To determine the optimum annealing temperature of the primers, gradient RT-PCR was conducted within a range of 50–63°C using the HB1 vaccine strain purchased from National Veterinary Institute, Bishoftu, Ethiopia, as positive control. Finally, the reverse transcription and the polymerase-chain reaction were conducted in a single

reaction tube by using Accu power Dual-Hot start RT-PCR kit (Bioneer, Korea). To describe the process in brief, 1 µL of each of the forward and the reverse primers, 2 µL of the extracted RNA as template, and 16 µL of nuclease-free water which added up to 20 µL was dispensed into the master mix pellet and vortexed to create a homogenous mixture. Then, the mixture was placed in a PCR machine (Mastercycler® PCR thermal cycler, Germany) and the machine was set for thermal conditions of 51°C for 1 hour for reverse transcription followed by 95°C for 5 min, of initial denaturation, and 36 cycles of 94°C for 20 sec, 58°C for 30 sec, at 72°C for 30 sec with a final extension of 72°C for 10 min. In the end, products were visualized in 1% agarose gel stained with ethidium bromide under a gel documentation system for the presence of the expected amplicon product in comparison with the reference 100bp ladder (Biobasic, Canada).

Serology

Blood samples were collected from the wing veins of unvaccinated birds where swab samples had already been collected by using plain vacutainer tube. The blood samples were kept overnight tilted in one direction, and 200 µL of each of the serum samples were collected and preserved in a freezer.

Enzyme-linked Immunosorbent assay (ELISA) was conducted using ProFlock NDV ab kit (Zoetis, USA) according to the manufacturer's instruction. To describe in brief, the preserved serum samples were taken out and thawed. In the meantime, components of the kit were also thawed to room temperature and dilution buffer was dispensed into a separate 96-well microplate. Then, the serum samples, negative and positive controls were dispensed to their respective wells and mixed gently by multi-channel pipette. After that, all the mixture was transferred into the antigen-coated 96-well microplate with their corresponding wells. After incubation for 30 minutes at 25 °C, the entire content of the microplate was discarded and washed thrice to remove unbound antibodies. Next, horse radish peroxidase enzyme (HRP) conjugated secondary antibody was dispensed into each well and incubated for some time under darkness followed by repeated washing steps. At the end, substrate solution for the antibody-conjugated enzyme was added, incubated for 15 minutes, and finally the color change was quantified by using a spectrophotometer machine (Multiskan™ FC Microplate Photometer, Thermo Scientific) at 450 nm wavelength. The readings were further transformed into Excel format and the validity of the test, dichotomous values to determine seropositivity, and absolute amounts of the antibody titer of each of the serum sample were analyzed.

Results

From the total 239 swab samples collected, the proportion of NDV containing samples was 24.6% by RT-PCR. Similarly, the proportion of the samples in which the antibody level considered positive was 68.9%. The highest proportions of the virus were detected in Burayu, from Commercial farms, and from older birds (Table 1). Multivariable analysis of risk factors associated with the prevalence of ND was conducted (Table 2). It was observed that adult birds were more than 11 times more likely to encounter the disease than Youngs (OR = 11.6; 95% CI: 4.0–33.3; P = 0.000). The chance of acquiring the disease in birds owned by respondents who leave diseased birds in the flock was more than 6 and 3 times more likely compared to those who quarantine or sell them, respectively. (OR = 6.2; 95% CI: 1.8–21.2; P=0.004) (OR = 3.0; CI: 0.6–14.0; P=0.154). Again, the mode of disposal of dead chicken significantly affected the probability of getting infected and the lowest was among birds owned by individuals who responded that they would burry dead birds (OR = 0.13; 95% CI: 0.10–4.88; P = 0.044) in contrast to those who through into ditches whenever they die (OR = 1.38; 95% CI: 0.098–1.04; P = 0.741).

Access to Veterinary services significantly affects the distribution of the disease. The virus was found 12 times less likely among those who have access to veterinary services as compared to birds owned by those who do not have (OR = 12.4; 95% CI: 3.2–46.9; P = 0.000). The occurrence of the disease in comparison with different production systems indicated that chickens farmed intensively were more likely to acquire the disease (OR = 2.8; 95% CI: 0.58–13.71; P = 0.199) in comparison with birds raised in semi-intensive and backyard production systems.

Table 1 RT-PCR and ELISA results Across Different Factors

Factors		RT-PCR		Total	ELISA		Total
		Positive (%)	Negative (%)		Positive (%)	Negative (%)	
Age	Young	11(15.7)	59(84.3)	70(100)	28(60.8)	18(39.2)	46(100)
	Adult	48(28.4)	121(71.6)	169(100)	34(77.2)	10(32.8)	44(100)
Breed	Local	7(14.0)	43(86.0)	50(100)	34(70.8)	14(29.2)	48(100)
	Exotic	51(26.7)	140(73.3)	191(100)	28(66.6)	14(33.3)	42(100)
Production System	Semi-intensive	15(22.7)	51(77.2)	66(100)	-	-	-
	Backyard	33(23.2)	109(76.8)	142(100)	62(68.9)	28(31.1)	90(100)
	Intensive	11(35.4)	20(64.6)	31(100)	-	-	-
Site	Holeta	23(26.7)	63(73.2)	86(100)	-	-	-
	Burayu	22(35.4)	40(64.6)	62(100)	-	-	-
	Jimma	6(31.5)	13(68.5)	19(100)	14(15.6%)	76(84.4%)	90(100)
	Seqa	8(17.7)	37(82.3)	45(100)	31(34.4%)	59(65.6%)	90(100)
	Nadhigibe	3(11.1)	24(88.9)	27(100)	17(18.9%)	73(81.1%)	90(100)

Table 2 Multivariable Analysis of Factors Associated with Detection of the Virus Using RT-PCR

Variable	Categories	RT-PCR		P-value	OR	(95% CI)	
		Positive (%)	Negative (%)			Lower	Upper
Breed	Local	7(14.3)	42(85.7)		1		
	Exotic	51(26.8)	139(73.2)	0.948	1.04	0.282	3.873
Age	Young	33(45.2)	40(54.8)		1		
	Adult	25(15.1)	141(84.9)	0.000*	11.6	4.019	33.320
Site	Burayu	22(33.3)	44(66.7)	0.301	1		
	Holeta	23(27.4)	61(72.6)	0.371	2.5	0.342	17.833
	Jimma	3(16.7)	15(83.3)	0.877	0.9	0.145	5.196
	Nadhigibe	3(11.5)	23(88.5)	0.392	0.5	0.080	2.698
	Seka	8(17.8)	37(82.2)	0.553	1.8	0.262	12.216
Fate of chicken if diseased	Isolated	21(14.7)	122(85.3)	0.013	1		
	Sold	28(54.9)	23(45.1)	0.154	3.0	0.659	14.007
	Left as it is	9(20)	36(80)	0.004*	6.2	1.824	21.231
Fate of chicken If died	Burnt	3(25)	9(75)	0.030	1		
	Buried	4(14.3)	24(85.7)	0.044*	0.13	0.107	4.888
	Consumed by pets	11(12.2)	79(87.8)	0.427	0.44	0.016	0.560
	Thrown to ditches	38(35.5)	69(64.5)	0.741	1.38	0.098	1.048

(*Continued*)

Table 2 (Continued).

Variable	Categories	RT-PCR		P-value	OR	(95% CI)	
		Positive (%)	Negative (%)			Lower	Upper
Access to vet	Yes	9(9.3)	88(90.7)		1		
	No	49(34.5)	93(65.5)	0.000*	12.4	3.284	46.953
Production system	Backyard	34(23.9)	108(76.1)	0.103	1		
	Semi-intensive	58(24.3)	181(75.7)	0.165	0.356	0.083	1.531
	Intensive	10(30.3)	23(69.7)	0.199	2.821	0.580	13.718

Notes: 1=indicate for reference group, *Significant association at p-value < 0.05.
Abbreviations: ND, Newcastle disease; PCR, Polymerase chain reaction; ELISA, Enzyme-linked Immunosorbent Assay; OR, Odds Ratio.

Discussion

In Ethiopia, unlike serological studies, fewer molecular studies are conducted to detect NDV and there are only limited studies for comparison. The proportion of positive samples in the current study was 24.6% by detecting the conserved region of the Fusion(F) gene using RT-PCR. Similarly, by targeting the F and Polymerase(L) genes, the virus was detected from two woredas of East Shewa Zone of Ethiopia both in the dry and wet seasons of the year.[20] In 2015 in a study conducted at Bishoftu, Tikurwuha, and Ziway, the Matrix(M) gene was involved in detecting the viral family, and the F gene was used for strain identification.[21] Likewise, a significant proportion of the virus was detected from collected samples targeting the F gene in a study conducted in two open markets of Addis Ababa in 2022.[22] In two states of India, it was also reported that it was possible to determine the prevalence of the disease by detecting the M gene of the virus.[23,24]

In this study, antibody against ND was detected in a higher proportion of the samples (68%) even though the sampled birds were not vaccinated (Table 1). Similarly, antibodies were detected from unvaccinated birds located at Melekoza district of Gofa zone,[25] Sodo Zuria district;[26] Central Ethiopia;[27] Adama;[13] Buno Bedelle zone;[16] Eastern Shewa zone;[28] Wolaita zone[29] and Sebeta area.[15] This elicited the endemic nature of the disease over a wide area of the country which requires intervention to control.[30]

Moreover, in the backyard production system, which is the common production system in the rural areas of the country, biosecurity measures are less/none practiced and chickens are scavenging without confinement all over the surroundings. Since the serology part of this study was conducted solely on the backyard-farmed chicken, the high seropositivity might be related to the vast exposure of the chickens to any of the pathogens including NDV while scavenging. This was supported by finds which illustrate that, the biosecurity measures in medium, small scale[31] or even in commercial[32] poultry farms are poorly practiced in some areas of the country.

We do not identify any statistically significant difference in the susceptibility of the disease between the two breeds except for some level of proportion (14.3% vs 26.8%) as well as among the five study locations (Table 2). However, in a study comparing Fayoumi with Leghorn sublines, it was reported that interbreed differences in the immune response to ND were prominent and the innate immune response components play major roles.[33,34]

In the current study, it was found that adult birds were more than 11 times more likely to encounter the disease than Youngs (AOR = 5.24; 95% CI: 2.0–13.6; P < 0.001) (OR = 11.6; 95% CI: 4.0–33.3; P = 0.000). The reason could be related to the decreasing maternal antibody over time as indicated by earlier studies.[29,35] The other reason could be lengthy exposure to the virus as the age increases since biosecurity measures are considered less strict.[31]

The chance of acquiring the disease in birds owned by respondents who leave diseased birds in the flock was more than 6 and 3 times more likely compared to those who quarantine or sell them, respectively. (OR = 6.2; 95% CI: 1.8–21.2; P=0.004) (OR = 3.0; CI: 0.6–14.0; P=0.154). This response of the respondents reflects the experience of the farmers on disease management directly affects the exposure of the birds to the virus at the time of study. The importance of quarantine is underlined by different reports and explained as segregation is the basis of most biosecurity measures.[36–38]

So, it is straightforward that if quarantine is not practiced, it favors the dissemination of the virus on the farm through aerosol or contaminated feed and water.[39]

Again, the mode of disposal of dead chicken significantly affected the probability of getting infected and the lowest was among birds owned by individuals who responded that they would burry dead birds (OR = 0.13; 95% CI: 0.10–4.88; P = 0.044) in contrast to those who through them into ditches (OR = 1.38; 95% CI: 0.098–1.04; P = 0.741). The habit of disposing of birds that died of disease in an open environment could contaminate the village and might create an infection cycle that might affect the disposing household itself. In support of this hypothesis, it is explained that carcass disposal is critical to control ND outbreaks because virulent NDV can remain viable in the tissue of infected birds for weeks and become a source of environmental contamination or direct infection of susceptible birds.[40] In addition to the supposition of viral dissemination via open disposal of carcasses, it was observed that disposal of dead chickens far away from poultry houses is significantly associated with low seropositivity for ND.[29] Similarly, a study conducted in Bangladesh also mentioned different dead chicken disposal habits had created variability in the amount of antibody levels measured.[41]

In the current study, the disease was 12 times less likely to occur in birds owned by farmers who have access to veterinary services (OR = 12.4; 95% CI: 3.2–46.9; P = 0.000) than those who do not have. Treatment against internal and external parasites; proper application of antibiotics and vaccination against different diseases could affect the occurrence of diseases due to the synergistic effect of the pathogens or deprivation of proteins for the synthesis of immunoglobulins as can be seen in blood-sucking parasites.[42] Supporting this observation, a study conducted in Kenya showed parasite control resulted in improved immune response to ND in experimental birds in which the reverse can be anticipated.[43] The co-existence of bacterial diseases like Escherichia coli can affect the immune response to ND indicated that their impacts can be alleviated by the use of antibiotics.[44] Thus, it is possible to say that access to veterinary services can be directly correlated with the prevalence of ND.

The occurrence of the disease in comparison with different production systems indicated that chickens farmed intensively were more likely to acquire the disease (OR = 2.8; 95% CI: 0.58–13.71; P = 0.199) in comparison with birds raised in semi-intensive and backyard production systems. A similar result was found in India that a lower risk of encountering the disease was observed with backyard or semi-intensive housing when compared to intensive housing of chickens.[23] Similarly, a higher proportion of virulent forms of ND were found in the commercial type of farms as compared to in the backyard in the study conducted in the same country.[24] Backyard production systems usually lack vaccination. However, it can be noted that the confinement of birds in intensive production systems increases the chance of exposure to the virus through bird-to-bird contact and/or discharges once the pathogen is introduced into the farm.

Conclusion

ND was detected in one out of the four (24.6%) samples collected from symptomatic birds by using RT-PCR. This indicates, that the circulation of probably the virulent form of the virus in the study areas. Similarly, although they were not vaccinated, more than 2/3rd (68.9%) of the sampled birds were found seropositive against ND by using ELISA. This is another piece of evidence supporting the conclusion that ND was well-established in the study areas. Moreover, molecular characterization of the circulating strains portrays the severity of the impact produced hence; the most feasible intervention measures can be forwarded. All in all, poultry production mainly intensification and industry-level production will fall under threat unless veterinary services including vaccination are improved and proper biosecurity measures are in practice.

Ethical Approval and Consent

This study was approved by the Animal Research Ethics Review Committee of NABRC. Ethical clearance was obtained from the Ethics Committee. The study animals were handled throughout the study period according to the World Organization for Animal Health (OIE) animal welfare and ARRIVE guidelines 2.0.

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Disclosure

The authors report no conflicts of interest in this work.

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