Research Note: Molecular surveillance of Avian Paramyxovirus type-1 in nonvaccinated village chickens in Central Rift Valley of Oromia, Ethiopia

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ABSTRACT Newcastle disease (**ND**) is a major infectious disease of poultry caused by a virulent strain of Avian Paramyxovirus type-1 (**APMV-1**). It is a major threat to the poultry industry in many countries of the world including Ethiopia. The aim of this study was to conduct molecular surveillance of ND Virus and identify potential risk factors for nonvaccinated village chicken in Central Rift Valley of Oromia, Ethiopia. A total of 84 pooled swab samples, each made from pools of 5 swabs for analysis, from cloacal and tracheal sites of chickens in the Central Rift Valley were collected, and RNA was extracted to carry out real-time quantitative polymerase chain reaction. Out of the 84 pooled swab samples tested for M-gene, 13 (15.48%) samples were found positive for APMV-1. The prevalence of ND in males was found to be 16.10% and that in females was 14.67%. Although the overall ND prevalence was 15.48% (13/84), the highest prevalence was recorded in Adama, 42.86% (6/14), and no positive case was observed in Bote and Bishoftu ($P < 0.05$), while intermediate prevalence was obtained from Batu, Arsi-negele, and Shashemene ($P > 0.05$). In general, the present study provides important information on the epidemiology of ND based on M-gene assay in Central Rift Valley of Oromia, Ethiopia, and highlights the importance of implementing molecular surveillances practice in live poultry markets and village chickens.

Key words: central rift valley, molecular surveillance, Avian Paramyxovirus type-1, village chicken

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INTRODUCTION

Newcastle disease (**ND**) is an acute, mild to severe, highly infectious and pathogenic disease of domestic poultry, caged, and aviary birds as well as wild birds caused by specified viruses of the Avian Paramyxovirus type 1 (**APMV-1**) (Amarasinghe et al., 2017). APMV-1 is an enveloped, nonsegmented, single-stranded negative-sense RNA virus with a helical morphology whose genome has 6 open reading frames which encode for the following proteins: nucleoprotein, phosphoprotein, matrix protein, fusion protein, hemagglutinin-neuraminidase, and RNA-dependent RNA polymerase. ND causes high mortality in nonvaccinated chickens. The sub-clinical forms of ND in vaccinated or APMV-1 exposed flocks have synergistic effect with other bacterial or viral infections (Perozo et al., 2012).

In Ethiopia, similar to other African countries, ND causes devastating losses to the poultry industry where it has been reported that backyard poultry management favors the existence and spread of ND virus by allowing free interaction of different poultry species and wild birds as well as the frequent introduction of birds from markets (Chaka et al., 2012; ACIAR, 2018). In many countries, matrix (**M**) gene– and fusion (**F**) gene–based real-time quantitative polymerase chain reaction (**RT-qPCR**) assays are often used as standard methods for APMV-1 screening and pathotyping directly from clinical samples. The RT-qPCR assay not only is faster and less cumbersome than the conventional diagnostic techniques but also provides equal or even greater sensitivity of virus detection than the gold standard virus isolation method. The choice of M gene detection in APMV-1 screening is due to its highly conserved nature in the APMV-1 genome that enables to detect most of APMV-1 isolates (Bello et al., 2018).

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Despite the wide spread problem of ND in Ethiopia in general and Oromia in particular (Chaka et al., 2012; Terefe et al., 2015; Miressa et al., 2016), there is no information on M gene–based study about the status of APMV-1 circulating in backyard poultry in Central Rift Valley of Oromia, Ethiopia. Therefore, the aim of this study was to conduct molecular surveillance of ND virus based on M gene assay and identify potential risk factors for nonvaccinated village chicken in central rift valley of Oromia, Ethiopia.

MATERIALS AND METHODS

Description of Study Area, Populations, and Design

The study was conducted in selected Central Rift Valley areas of East Shoa (Bishoftu, Adama, Bote, Batu) and West Arsi Zone (Shashemene and Arsi Negelle) of Oromia Regional State (Figure 1). East Shoa Zone extends between 70′33′50″ N and 90′08′56″ N and from 380′24′10″ E to 400′ 05′ 34″ E. The temperature of the area ranges from 10°C in uplands to over 30°C in rift valley depressions with the mean temperature of 20°C. As the large portion of the zone is located along the rift valley system, rainfall varies from 600 mm to 1,000 mm with mean annual rainfall of 816 mm. The livestock population of East Shoa zone is estimated to be 1,090,091 cattle, 319,598 sheep, 568,761 goats, 10,644 horse, 7,039 mules, 284, 583 donkey, 6,818 camels, 14,627 beehives, and 1,250, 059 poultry. Out of a total 1,250,059 poultry, around 94% (1,169,710) are indigenous or village poultry, whereas only 6% (69,562) are hybrids. The livestock population of West Arsi Zone is estimated to be 1, 957, 066 cattle, 946, 595 sheep, 404, 118 goats, 214, 744 horses, 6, 304 mules, 210, 339 donkeys, 555 camels, and 1, 105, 688 chicken (CSA, 2013).

The study populations were nonvaccinated village chickens from East Shoa and West Arsi Zones in Central Rift Valley of Oromia regional State, Ethiopia. Study animals were nonvaccinated village chickens from randomly selected districts in East Shoa and West Arsi zones. Village chickens were sampled in respective of

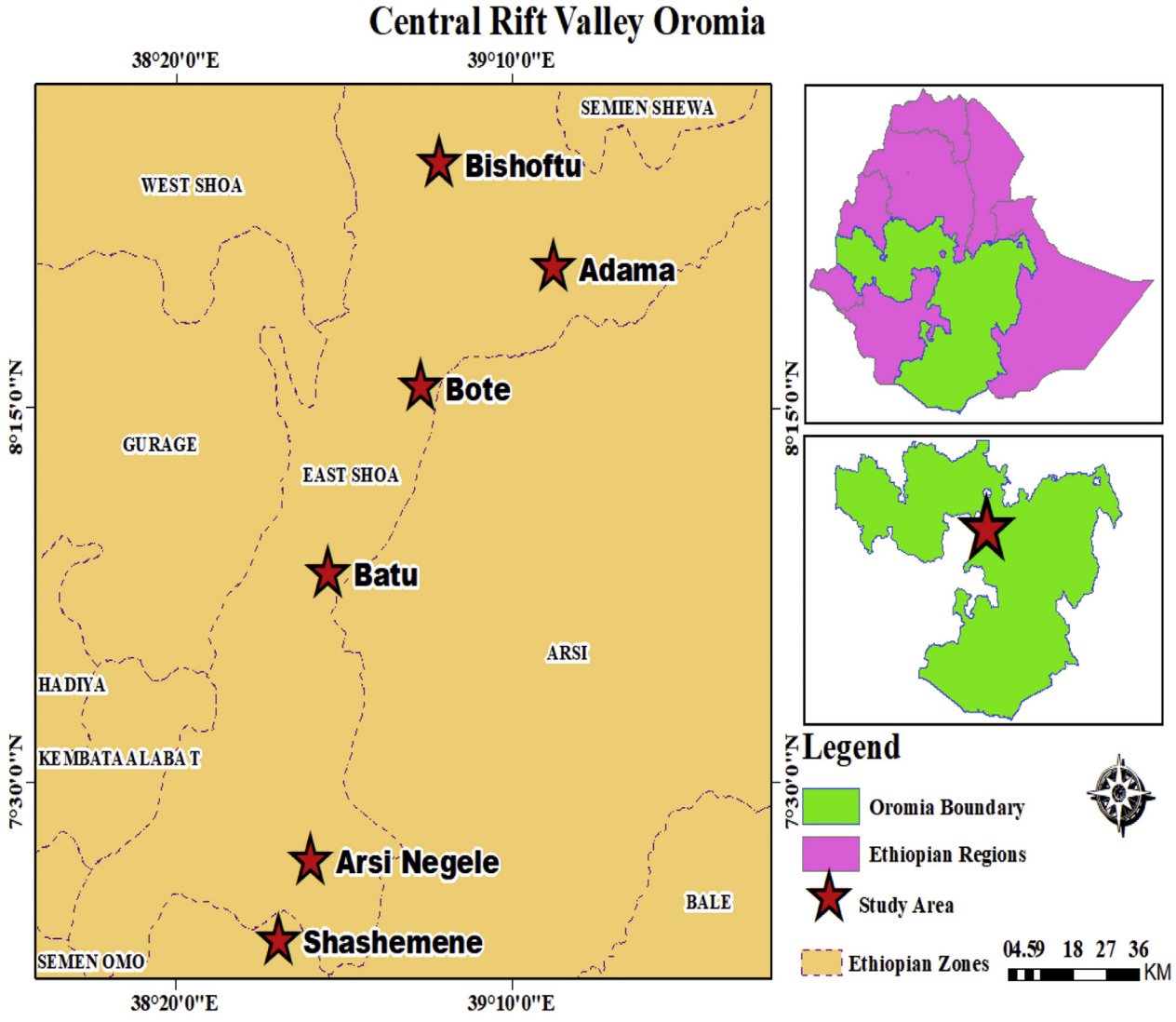

Figure 1. Map of study area, East Shoa and West Arsi Zones in Central Rift Valley of Oromia regional State, Ethiopia (by ArcGIS 10.4.1).

districts, sex, and age. The age was determined based on history from the owners as young 3–6 mo, adult 7–16 mo, and older more than 16 mo (Belayneh et al., 2014).

A cross-sectional study was conducted in nonvaccinated village chickens found in the study areas for the detection of ND during the period of January to May, 2019.

Sample Size Determination

Sample size was calculated based on the prevalence of 38.4%, as previously reported by Terefe et al. (2015), using 95% confidence level and 5% marginal error. According to Thrusfield (2005), the following formula was used to determine the required sample size:

$$n = \frac{z^2\ P \exp\ (P \exp)}{d^2}$$

where n = sample size, Z = statistic for a level of confidence, d^2 = required absolute precision, and P_{exp} = expected prevalence. Therefore, at 95% confidence level, the sample size was

$$n = \frac{1.96^2 P \exp\ (1-P \exp)}{d^2}$$

$$n = \frac{3.8416 \times 0.384\ (0.616)}{(0.05)^2}$$

$$n = \frac{0.909}{0.0025}$$

$$n = 364$$

Hence, a total of 364 nonvaccinated village chickens were required to be sampled. However, 420 domestic birds were randomly selected at poultry markets and villages in the study areas to increase the precision of the study. To calculate the prevalence, the following formula was used:

$$\text{Prevalence} = \frac{\text{Number of positive sample}}{\text{Total number of sample}} \times 100$$

Sampling and Method of Data Collection

A total of 420 nonvaccinated and healthy live chickens were sampled using random sampling technique from poultry markets and villages by equal distribution in the study areas (a total of 70 samples from each location) on consecutive market days. Tracheal and cloacal swab samples were collected from live chickens by inserting sterile cotton tipped swab into the trachea gently swabbed its wall and cloacal swab deeply into the vent and gently swabbing the wall of the vent. Both the tracheal and cloacal swab samples were placed in sterile separate cryovial (2 mL volume) containing 500 µL of freshly prepared viral transport media. It has been demonstrated that analysis of pooled samples is a time- and cost-saving method for accurate diagnosis of infectious diseases (Quinn et al., 2000). Thus, during sampling, samples were pooled in 5 swab samples, labeled, placed in ice pack (4°C) and transported to molecular laboratory of National Animal Health Diagnostic and Investigation Center, Sebeta, Ethiopia, and stored at −80°C until processing. Sample collection and transportation were conducted according to the standard techniques recommended by OIE (2013).

RNA Extraction

Viral RNA extractions from tracheal and cloacal swabs were conducted using the Qiagenviral RNA Mini kit according to manufacturer's instruction (QIAGEN) as follows: Swab samples collected with viral transport media were centrifuged briefly at 8,000 rpm for 1 min to get cell-free supernatant. Then, 140 µL of the supernatant were lysed by adding 560 µL of buffer AVL containing carrier RNA. After 10 min incubation at room temperature, the sample was mixed, and 560 µL of 96% ethanol was added to filtrate and again mixed thoroughly. Samples containing 630 µL of lysis buffer and absolute alcohol were transferred for binding to mini spin column and centrifuged at 8,000 rpm for 1 min, and old collection tube was replaced by a new collection tube. A 500-µL wash buffer (AW1) was added and centrifuged at 8,000 rpm for 1 min, and a new collection tube was used. Again, a 500-µL wash buffer (AW2) was added and centrifuged at full speed of 14,000 rpm for 3 min to remove any unwanted protein and others. Again, a new collection tube was used and centrifuged at full speed of 14,000 rpm for 1 min without adding anything. A new and sterile 1.5-µL micro centrifuge tube was used followed by adding 60 µL of elution buffer to mini spin column and incubated for 3 min at room temperature. Finally, the eluted RNA was kept at −80°C for storage.

Real-Time Quantitative Polymerase Chain Reaction

One-step RT-qPCR for conserved region of M-gene assay was performed using an Applied Biosystems 7,500 fast thermocycler using the forward primer M + 4100-5′-AGT GAT GTG CTC GGA CCT TC-3′ and reverse primer M-4220-5′CCT GAG GAG AGG CAT TTG CTA-3'. Probe M + 4100-5′FAM-TTC TCT AGC AGT GGG ACA GCC-TAMRA-3′ was used to specifically detect pathogenic strains of APMV-1. For M-gene assay, the following amounts of reagents per 25-µL reaction were used: 5 µL of kit supplied PCR buffer (5x), 0.5 µL of each primer (10 pmol), 1 µL of probe (6 pmol), 0.8 µL of kit supplied deoxynucleoside triphosphates, 1.25 µL of 25 mM $MgCl_2$ and 0.5 µL of 13.3 u/µL of RNase inhibitor, and 1 µL Qiagen enzyme mix and 6.45 RNase-free water (QIAGEN). All the reagents in a single reaction tube (master mix) were

mixed to ensure a homogenous distribution of the reagents. Aliquots of 17 μL of prepared reagents were distributed in an applied biosystem plate depending on the number of samples to be tested. Finally, 8 μL of extracted RNA was added to each plate to get total volume of 25-μL reaction. For controls, APMV-1 known positive field isolates and RNase-free water as positive and negative, respectively, were used. The plates were sealed with a sealer to avoid evaporation and placed into the RT-qPCR machine that was connected to computer with its own software (Applied biosystems sequence detection software version 1.4.0), and the program was adjusted for each specific reaction or set accordingly. The reverse transcription (RNA to cDNA) step was performed for 30 min at 50°C, followed by 15 min at 95°C incubation. The cycling conditions for the M-gene assay were programmed at 40 cycles of 10-s denaturation at 94°C, 30-s annealing at 52°C, and extension at 72°C for 25 s. The reporter dye (FAM) and quencher dye (TAMRA) signals were measured at the extension step of each cycle, and the threshold cycle (Ct.) for each sample was calculated. The samples that have a Ct. value < 35 were considered as positive and >35 Ct. value were considered as negative for M genes based on RT-qPCR (OIE, 2013).

RESULTS AND DISCUSSION

Quantitative real-time PCR (RT-qPCR) Based Molecular Detection of APMV-1 in Village Chickens

Out of the 84 pooled swab samples tested by RT-qPCR for M gene, 13 (15.48%) samples were identified as positive for APMV-1 in nonvaccinated village chickens in the study area ranging from 0% at Bishoftu and Bote to 42.86% at Adama. The overall prevalence of 15.48% (13/84) APMV-1 revealed in the present study (Table 1) supports the view that ND is endemic in Central Rift Valley of Oromia.

Prevalence of ND in Village Chickens Using RT-qPCR for M Gene Test by Age and Sex

The prevalence of ND based on M gene assay was higher in male (16.10%) than in female (14.67%) chicken even though there was statistically no significance difference ($P > 0.05$) by sex (Table 1). This finding corroborates with the study reported by Zeleke et al. (2005) in the Southern and Rift Valley districts of Ethiopia where a higher sero-prevalence rate among males than females was observed. The higher prevalence of the disease observed in the present study could be due to higher number of male chickens involved by chance in the study area during sampling than females.

On the other hand, the prevalence of ND was higher in adults (16.59%) but relatively lower in old chicken (11.11%) as shown in Table 1. The difference in the prevalence between adult, young, and old age was statistically significant ($P < 0.05$), which agrees with the finding of Vui et al. (2002) who revealed that the young had a significantly lower ND prevalence than the adult. Furthermore, the result of the present study corroborates with the findings of Getachew et al. (2014) from Alamata District, Southern Tigray, Ethiopia, who reported more frequent exposure of adult birds to field virus, which might have survived the disease at an earlier age.

Prevalence of ND Using RT-qPCR for M Gene Test in Village Chickens in the Study Districts

The present study revealed significant variation with agro-ecology between East Shoa and West Arsi Zones.

Table 1. Prevalence of Newcastle disease in village chickens by sex, age, swab type, and districts in the study area.

Categories	Number examined	Number positive	Prevalence %	*P* value
Sex				
Male	236	38	16.1	>0.05
Female	184	27	14.67	
Age				
Young	146	23	15.75	<0.05
Adult	211	35	16.59	
Old	63	7	11.11	
Swab type				
Trachea	42	9	21.43	>0.05
Cloacal	42	4	9.52	
Total	84	13	15.47	
Districts				
East Shoa Zone				
Adama	14	6	42.86	<0.05
Batu	14	1	7.14	
Bishoftu	14	0	0	
Bote	14	0	0	
West Arsi Zone				
Arsi Negelle	14	3	21.43	>0.05
Shashemene	14	3	21.43	
Total	84	13	15.48	

In East Showa Zone, the highest prevalence was from Adama (42.86%), while none was detected in Bote and Bishoftu (Table 1) with statistically significance difference ($P < 0.05$) among study sites, whereas there was no statistically significance difference ($P > 0.05$) in the West Arsi Zone district. The high prevalence of ND in the East Showa Zone district might be due to natural infection that could have occurred because all the chickens were nonvaccinated. However, clinical signs of ND were not observed on the examined chickens during sample collection.

Even though the distribution of the disease differs among the study areas in Eastern Shoa Zone using RT-qPCR on M gene assay with statistically significance difference ($P < 0.05$), Miressa et al. (2016) reported an overall prevalence of 26.7% by using real-time reverse transcription polymerase chain reaction for F gene assay from Adama and Bishoftu. While the negative results from the RT-qPCR test in Bishoftu and Bote seem strange, a molecular and serological study conducted for APMV-1 from Iran in nonvaccinated village chicken also showed negative result on RT-qPCR for M gene assay but positive reaction on hemagglutination inhibition test (Rezaeianzadeh et al., 2011).

In the present study, a higher detection rate by RT-qPCR for M gene assay was obtained from pooled tracheal, 21.43% (9/42), than cloacal, 9.52% (4/42), swab samples with statistically insignificant difference ($P > 0.05$) (Table 1). This is in agreement with the finding of Chaka et al. (2012) who reported higher positivity of tracheal than cloacal pooled swab samples by real-time reverse transcription polymerase chain reaction for F gene assay speculating the presence of viscerotropic velogenic virulent APMV-1 in the study areas.

The present study provides important information on the epidemiology of ND based on M-gene assay for APMV-1 in Central Rift Valley of Oromia, Ethiopia, and highlights the importance of implementing molecular surveillances practices in live poultry markets and village chickens. Because the present study revealed the presence of APMV-1 among village chickens which represents a continual threat to newly hatched susceptible chicks and to commercial poultry, we recommend the immediate vaccination of village chickens to raise their immune status to prevent the likelihood of ND outbreaks in Central Rift Valley of Oromia. In addition, we recommend a detail molecular characterization study of APMV-1 in Central Rift Valley of Oromia and in Ethiopia in its larger context to better understand the molecular epidemiology of the disease and to characterize the viral strains circulating in the country that plays a great role in designing appropriate control strategy for ND.

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Ethics approval: Ethics approval was obtained from Adama Science and Technology University Ethical Review Committee.

DISCLOSURES

The authors declare that there is no conflict of interest.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.psj.2020.12.053.

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