Molecular Investigation for Matrix Gene of Newcastle Disease Virus in Non-vaccinated Village Chickens in Central Rift Valley of Oromia, Ethiopia

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Keywords: Central rift valley, M-gene, Newcastle disease, RNA, qRT-PCR, Village chicken

Posted Date: March 30th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-18290/v1>

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Abstract

Background Newcastle disease (ND) is a major infectious disease of poultry caused by a virulent strain of Avian Paramyxovirus – 1. It is a major threat to the poultry industry in many countries of the world including Ethiopia. Newcastle Disease Virus (NDV) is an enveloped, non-segmented, single-stranded negative-sense RNA virus with a helical morphology whose genome has six open reading frames (ORF) which encode for the following proteins: nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN) and RNA-dependent RNA polymerase (L). The aim of this study was to detect matrix gene (M-gene), for NDV by molecular tools and identify its risk factors in non-vaccinated village chicken in Central Rift Valley of Oromia, Ethiopia.

Methods A total of 84 pooled in five swab samples from 420 cloacal and tracheal chickens were sampled and RNA was extracted from the 84 pooled samples to carry out real-time quantitative polymerase chain reaction (qRT-PCR). A real-time reverse transcriptase polymerase chain reaction (rRT-PCR) along with the quantification was also done for 10 positive qRT-PCR samples that were having high concentration of viral load Ct. value.

Results Out of the 84 pools of five swab samples tested for M-gene using qRT-PCR, 16.7% (14/84) samples were detected which included 13 positives and 1 negative for NDV. 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 score was recorded in Adama, 42.86% (6/14), and no positive case was detected in Bote and Bishoftu ($p < 0.05$) while intermediate scores were obtained from Batu, Arsi-negelle and Shashemene.

Conclusions In general, the present study provides important information on the epidemiology of NDV based on M-gene assay in Central Rift Valley of Oromia, Ethiopia, and highlights the importance of implementing surveillances and biosecurity practices in live poultry markets and village chickens.

Background

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) [1, 2]. Newcastle Disease Virus (NDV) is an enveloped, non-segmented, single-stranded negative-sense RNA virus with a helical morphology whose genome has six open reading frames (ORF) which encode for the following proteins: nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN) and RNA-dependent RNA polymerase (L). ND is caused by Newcastle disease virus (NDV) which is a highly infectious agent capable of causing high mortality in non-vaccinated chickens. The sub clinical forms of ND in vaccinated and/or NDV exposure flocks have synergistic effect with other bacterial or viral infections [3, 4].

In Ethiopia, like other African countries, ND causes devastating losses to the poultry industry [5]. In backyard poultry, the disease is endemic but recurs as frequent epidemic outbreaks with high mortality

and thus affects the livelihoods of poor rural households who depend on poultry for food and income [6]. Backyard poultry management favors the existence and spread of diverse NDV strains by allowing free interaction of different poultry species and wild birds as well as the frequent introduction of birds from markets [7]. In many countries, matrix gene and fusion gene based qRT-PCR assays are often used as standard methods for NDV screening and pathotyping directly from clinical samples [8]. The qRT-PCR assay is not only 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 [9]. The choice of matrix gene detection in NDV screening is due its highly conserved nature in the NDV genome where matrix gene assay is able to detect most of NDV isolates [10]. Despite the wide spread problem of ND in Ethiopia in general and Oromia in particular [7, 11, 12], there is limited information about the genetic profile of NDVs circulating in backyard poultry in Ethiopia. Therefore, the aim of this study was to detect the existence of Newcastle Disease virus by molecular tool in non-vaccinated village poultry in central rift valley of Oromia, Ethiopia.

Materials And Methods

The study was conducted in selected Central Rift Valley areas of East Shoa (Bishoftu, Adama, Bote, Batu) and West Arsi Zone (Shashemene and ArsiNegelle) of Oromia Regional State (Fig. 1). East Shoa Zone extends between 70°33'50" N – 90°08'56" N and from 38°24'10" E – 40°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. Since the large portion of the zone is located along the rift valley system, rainfall varies from 600 mm to 1000 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, 10644 horse, 7039 mules, 284, 583 donkey, 6818 camels, 14627 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 [13]. 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 [14].

Study Populations

The study populations were non-vaccinated village chickens from East Shoa and West Arsi Zones in Central Rift Valley of Oromia regional State, Ethiopia.

Study Animals

Study animals were non-vaccinated village chickens from randomly selected districts in East Shoa and West Arsi zones. Village chickens were sampled in respective of districts, sex and age. The age was determined based on history from the owners as young 3–6 months, adult 7–16 months and older more than 16 months [15].

Study Design

A cross-sectional study was conducted in non-vaccinated village chickens found in the study areas for the detection of Newcastle Disease 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 [11], using 95% confidence level and 5% marginal error. A formula according to [16] was used to determine the required sample size as indicated below:

$$n = \frac{z^2 P_{exp} (1 - P_{exp})}{d^2}$$

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 non-vaccinated 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 non-vaccinated and healthy live chickens were sampled using random sampling technique from poultry markets and villages by equal distribution in the study areas 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 (VTM). During sampling, all the samples were labeled, placed in ice pack (4 °C) and transported to molecular laboratory of National Animal Health Diagnostic and Investigation Center (NAHDIC), Sebeta, Ethiopia and stored at -80 °C until processing. Sample collection and transportation were conducted according to the standard techniques recommended by OIE [17].

RNA extraction

Viral RNA extractions from tracheal and cloacal swabs were conducted using Qiagen® viral RNA Mini kit according to manufacturer's instruction (Qiagen, 2014) as follows: - Swab samples collected with viral transport medium were centrifuged briefly at 8000 rpm for 1 minute in order to get cell free supernatant. Then, 140 µl of the supernatant were lysed by adding 560 µl of buffer AVL containing carrier RNA. After ten minutes' 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 8000 rpm for 1 minute and old collection tube was replaced by a new collection tube. A 500 µl wash buffer (AW1) was added and centrifuged at 8000 rpm for 1 minute 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 minutes in order to remove any unwanted protein and others. Again, new collection tube was used and centrifuged at full speed of 14,000 rpm for 1 minute without adding anything. A new and sterile 1.5 µl of micro centrifuge tube was used followed by adding 60 µl of elution buffer (AVE) to mini spin column and incubated for 3 minutes at room temperature. Finally, the eluted RNA was kept at -80 °C for storage.

Real –Time Quantitative Polymerase chain reaction (qRT-PCR)

One step qRT-PCR for conserved region of M-gene assay was performed using an Applied Biosystems 7500 fast thermocycler using the Forward Primer M + 4100- 5'-AGT GAT GTG CTC GGA CCT TC-3', 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' were used to specifically detect pathogenic strains of NDV (IDT). 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 (dNTPs), 1.25 µl of 25 mM MgCl₂ 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 to applied biosystem plate depending on number of samples to be tested. Finally, 8 µl of extracted RNA were added to each plate to get total volume of 25 µl reaction. For controls, NDV known positive field isolates and RNase free water as positive and negative controls, respectively, were used. The plates were sealed with a sealer in order to avoid evaporation and placed in to the thermal cycler or rRT-PCR 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 done 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 second denaturation at 94 °C, 30 second annealing at 52 °C, and extension at 72 °C for 25 second (Wise et al., 2004). 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 considered as negative for M genes based on qRT-PCR [18].

Real-time reverse transcriptase polymerase chain reaction (rRT-PCR),

rRT-PCR was performed using primer sequences with forward primer NOH- 5'-TAC ACC TCA TCC CAG ACA GG-3' and reverse primer NOH- 5'-AGT CGG AGG ATG TTG GCA GC-3' (Europhins MWG operon) used to specifically detect pathogenic strains of NDV. The following amounts of reagents per 25 µl reaction were used: 5 µl of kit supplied PCR buffer (5x), 0.25 µl of each primer (20 µM), 0.5 µl of kit supplied deoxynucleoside triphosphates (dNTPs) of 10 Mm, 0.1 µl of 40 U/µl of RNase inhibitor, 0.5 µl of one step RT-PCR enzyme mix and 13.4 µl Rnase free water (QIAGEN). All the reagents in a single reaction tube (master mix) were mixed and in to 20 µl of the mixed samples 5 µl of extracted RNA or template were added to get a total volume of 25 µl to each respective tube and were capped tightly in order to avoid evaporation and the tube were labeled. For controls, NDV known positive field isolates and RNase free water as positive and negative controls, respectively were used. After placing tubes, the thermal cycler or PCR machine was programmed for reverse transcription (RNA to cDNA) step and incubated for 30 min at 50 °C, followed by 15 min at 94 °C. The cycling conditions consisted 35 cycles and each cycle were having 30 second for denaturation at 94 °C, 1 min for annealing at 55 °C, and extension at 68 °C for 1 min and additionally one cycle at 68 °C for 7 min final extension [18].

Gel electrophoresis

A 2 µl of loading dye and 8 µl of amplified PCR products were mixed up and loaded in to separate lane (wells on the gel) and the gel was run for 45–60 minutes. The gel was placed under UV transilluminator or Gel doc machine that was connected to the computer with its respective software (Image Lab software version 5.0) for imaging. Finally, the presence or absence of band were observed and the image was captured.

Results

Quantitative real-time PCR (qRT-PCR) based molecular detection of NDV in village chickens

The current findings in village chickens for pooled five swab samples obtained in the study area for M gene qRT-PCR test with their Ct. values are presented in Table 1. Out of the 84 pooled five samples tested by qRT-PCR for M gene, 14 (16.7%) samples were detected for NDV where 13 (15.5%) were identified as positive for NDV and 1 is having a Ct. value of 36.38 considered as negative for NDV in non-vaccinated village chickens.

Table 1: M gene qRT-PCR test Ct. values for NDV detection in non-vaccinated village chickens in Central Rift Valley of Oromia, Ethiopia.

Sample ID	Study areas	Swab type	Ct. value
A1	Adama	Cloaca	24.82
A2	Adama	Cloaca	26.42
A3	Adama	Trachea	30.82
A4	Adama	Trachea	33.87
A5	Adama	Trachea	27.20
A6	Adama	Trachea	31.23
A7	Adama	Trachea	36.38
AN1	Arsi Negelle	Cloaca	23.41
AN2	Arsi Negelle	Cloaca	34.85
AN3	Arsi Negelle	Trachea	28.78
BA1	Batu	Trachea	31.50
S1	Shashemene	Trachea	28.96
S2	Shashemene	Trachea	24.39
S3	Shashemene	Trachea	23.68
Mean			28.41

The present findings of both positive and negative controls (Figure 2) in non-vaccinated village chickens swab samples pooled five based on qRT-PCR test for M gene of NDV was indicated (Figure 3) when read from Applied Biosystems 7500 fast real time PCR thermo cycler.

Prevalence of ND in village chickens using qRT-PCR for M gene test by Age and Sex

The prevalence of ND based on M gene assay was higher in male (16.10%) than female (14.67%) chicken even though there was statistically no significance difference ($p > 0.05$) by sex (Table 2). On the other hand, the prevalence of ND was higher in Adult (16.59%) but lower in old chicken (11.11%) as shown in Table 2.

Table 2: Prevalence of Newcastle disease in village chickens by Sex and Age in the study area.

Risk factors	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	

Prevalence of ND using qRT-PCR for M gene test in village chickens in the study districts

The overall ND prevalence was 15.48% (13/84) in the study districts where the highest score was from Adama (42.86%) while none was detected in Bote and Bishoftu (Table 3). There was statistically no significance difference ($p > 0.05$) among the study districts.

Table 3: Prevalence of Newcastle disease in village chickens in the study Districts.

Districts	No. examined in pool of five	No. Positive	Prevalence %	p-value
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	

The current qRT-PCR test results also revealed a higher detection rate for M-gene from tracheal swab samples (TS), 21.43% (9/42), as compared to cloacal swab samples (CS), 9.52% (4/42), collected from village chicken from the study areas (Table 4).

Table 4: qRT-PCR test result for M-gene detection rate from swab samples from study area.

Swab type	No examined	No positive	Detection rate %	p-value
Trachea swab	42	9	21.43	> 0.05
Cloacal swab	42	4	9.52	
Total	84	13	15.47	

rRT-PCR- based molecular detection of NDV

Ten samples were tested by rRT-PCR assay for the detection of M-gene. Each band represents the extracted RNA from ten samples (Figure 4). Detection of M gene in the present study by rRT-PCR (Figure 4) is a demonstration of infection by the virus in these chickens.

Test agreement result between qRT-PCR and rRT- PCR detection technique

The kappa test agreement between qRT-PCR and rRT- PCR were having a value of 0.615 which indicates substantial result agreement between the two raters (Table 5).

Table 5: Kappa test agreement between qRT-PCR and rRT- PCR of Newcastle disease in the study area.

qRT-PCR * rRT-PCR Cross tabulation				
		rRT-PCR		Total
		Negative	Positive	
qRT-PCR Negative	Count	1	0	1
	% within qRT-PCR	100.0%	0.0%	100.0%
	% within rRT-PCR	50.0%	0.0%	10.0%
Positive	Count	1	8	9
	% within qRT-PCR	11.1%	88.9%	100.0%
	% within rRT-PCR	50.0%	100.0%	90.0%
Total	Count	2	8	10
	% within qRT-PCR	20.0%	80.0%	100.0%
% within rRT-PCR		100.0%	100.0%	100.0%

Symmetric Measures				
	Value	Asymptotic Standard Error ^a	Approximate T ^b	Approximate Significance
Measure of Agreement Kappa	.615	.337	2.108	.035
N of Valid Cases	10			

Discussion

The present study on ND from non-vaccinated village chicken using qRT-PCR for M gene assay revealed an overall prevalence of 15.48% in the study area ranging from 0% at Bishoftu and Bote to 42.86% at

Adama. The Detection of NDV at 15.48% obtained in this study (Table 3) supports the view that ND is endemic in Central Rift Valley of Oromia. Detection of M gene in the present study by RT-PCR (Fig. 4) is a demonstration of infection by the virus in these chickens. The occurrence of positive result supports the use of the technique to identify NDV. Positive samples obtained in this study can further be subjected to pathotyping and phylogenetic studies which is an advantage that goes beyond the previous serological study. Even though, the distribution of the disease differs among the study areas in Eastern Shoa Zone using qRT-PCR analysis with statistically significance difference ($p < 0.05$), [12] reported an overall prevalence of 26.7% by using rRT-PCR for fussion (F) gene assay from Adama and Bishoftu which is in a close resemblance with our current findings (21.42%) when it was analyzed only for Adama and Bishoftu. In addition, using a rRT-PCR for M gene study conducted by [11] in three selected districts (Batu, Bishoftu and TikurWuha) in rift valley areas revealed an overall prevalence of 8.4% which is less than the current finding and this could be due to the small area coverage. Even though the negative results from the qRT-PCR test in Bishoftu and Bote seem strange, a molecular and serological study conducted for NDV from Iran in non-vaccinated village chicken also showed negative result on qRT-PCR for M gene assay but positive reaction on HI test [19]. They concluded that the serological finding may imply a circulation of NDV antibody on the native chickens in the area but by molecular test no virus was detected since there was no occurrence of active viral infection.

The result of the present study (15.48%) was higher than the findings of other researches done by serological detection method in Eastern Shoa and Kersana Kondality district with an overall sero-prevalence of 5.9% and 5.6%, respectively [7, 20]. In another serological study conducted in selected areas in rift valley an overall sero-prevalence of 5.6% was reported [15] and 11.6% [11] from which one can discern out that the results under the present study, molecular tool, outweighs the results of the former studies by serological (ELISA) method. The overall discrepancy of the findings might be due to variations on management system that may serve as a stress factor that favor infection or the diagnostic tools used.

The present higher proportion (42.86%) of qRT-PCR positive result for NDV in chickens from Adama district might indicate chickens have been previously infected by the non-virulent strains or survived outbreaks of the virulent NDV strains [21]. Moreover, the detected high prevalence of ND in this study may mean that natural infection could have occurred since all the chickens were non-vaccinated. However, clinical signs of ND were not observed on the examined chickens during sample collection. Furthermore, the higher prevalence recorded in Adama district cannot be attributed to more chicken had been sampled (70 chicken sampled) compared to other study districts (70 chicken sampled), because as the number of sample size increase the probability of having higher prevalence's also increase. The present study also revealed significant variation with almost closer agro-ecology between East Shoa and West Arsi Zones.

The findings in village chickens of the present study also found out to be higher than the results reported from North Eastern Coast and Amazon Biome of Brazil from 1022 cloacal/tracheal samples collected from domestic and wild birds tested by qRT-PCR targeting the M gene segment of APMV-1, 7 (0.7%) were

positive [22]. These variations could have been attributed to seasonal differences during sampling periods, geographical location and type of birds employed for the studies.

In the present study a higher detection rate by qRT-PCR for M gene assay was obtained from pooled tracheal 21.43% (9/42) than cloacal 9.52% (4/42) swab samples with statistically insignificance difference ($p > 0.05$). This is in agreement with the finding of [7] who reported higher positivity of tracheal than cloacal pooled swab samples by rRT-PCR for F gene assay speculating the presence of viscerotropic velogenic virulent NDV in the study areas. On the other hand, Haque and his colleagues detected NDV from pooled oropharyngeal and cloacal swabs and suggested that the chickens are in the early or advanced stage of the disease since NDV is transmitted through aerosol and ingestion or the chickens are on the shedding stage of the infection [23].

The difference in the prevalence between adult, young and old age was statistically significant ($p < 0.05$), which agrees with the finding of [24] who stated that the young had a significantly lower ND prevalence than the adult. Furthermore, the result of the current study corroborates with the findings of [25] from Alamata District, Southern Tigray, Ethiopia with sero-prevalence of 17.3% and 29.7% for young and adult, respectively. They hypothesized that, more frequent exposure of Adult birds to field virus, which might have survived the disease at an earlier age.

The present study revealed a higher prevalence rate of ND among the male 16.1% (38/236) compared to female chickens 14.67% (27/184) with statistically insignificance difference ($p > 0.05$). This finding corroborates with the study reported by [26] in the Southern and Rift Valley districts of Ethiopia where a higher sero-prevalence rate among males (21.74%) than females (19.16%) was observed. In contrary to this finding, [27] reported a slightly higher sero-prevalence among female (32.63%) chickens when compared with male (31.63%) chickens in Ethiopia. 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.

Conclusion

The present study provides important information on the epidemiology of NDV based on M-gene assay in Central Rift Valley of Oromia, Ethiopia, and highlights the importance of implementing molecular surveillances and biosecurity practices in live poultry markets and village chickens. Because the current study revealed the presence of NDV among village chickens that represents a continual threat to newly hatched susceptible chicks and to commercial poultry, we recommend the immediate vaccination of village chickens in order to raise the immune status of village chickens to prevent the likelihood of ND outbreaks in Central Rift Valley of Oromia. In addition we recommend a detail molecular characterization study of the virus 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 virus strains that circulates in the country that plays a great role in designing appropriate control strategy of ND.

Abbreviations

APMV	Avian Paramyxovirus
cDNA	Complementary Deoxyribonucleic acid
CSA	Central Statistical Agency
Ct	Cycle Threshold
ELISA	Enzyme Linked Immuno Sorbent Assay
F	Fusion Protein
HI	Hemagglutination Inhibition
HN	Hemagglutinin Neuraminidase
IDT	Integrated DNA Technologies
L	Large RNA Polymerase
M	Matrix Protein
NAHDIC	National Animal Health Diagnosis and Investigation Center
ND	Newcastle Disease
NDV	Newcastle Disease Virus
NP	Nucleoprotein
OIE	Office International des Epizooties
ORF	Open Reading Frames
P	Phospho-protein
PCR	Polymerase Chain Reaction
qRT-PCR	Real – Time Quantitative Polymerase chain reaction
rRT-PCR	Real Time Reverse-transcriptase Polymerase Chain Reaction
VTM	Virus Transport Media

Declarations

Ethics Approval

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

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on request.

Competing interests

The authors declare that there is no conflict of interest.

Funding

Small amount of financial support was obtained from Adama Science and Technology University.

Authors' contributions

AM and HD participated in the design of the study, sample collection, performed M-gene assay, data analysis, and drafted the manuscript. RB and AA participated in the optimizing laboratory works. All authors read and approved the final manuscript.

Acknowledgments

We would like to acknowledge both Adama Science and Technology University and National Animal Health Diagnostic and Investigation Center (NAHDIC) for the overall technical and logistic support they provided us during the research work. The authors also would like to acknowledge poultry owners for their cooperation during sample collection.

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Figures

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).

Figure 2

Positive and negative controls from qRT-PCR for M gene test.

Figure 3

Amplification plot result of qRT-PCR test for M gene with their Ct. value in village chickens where PS, PC, NS were positive sample, positive control and negative sample, respectively.

Figure 4

Gel electrophoresis in 2% Agarose gel for rRT-PCR amplified product of Newcastle disease virus (270bp) specific products. Lanes 4, 6, 7, 9, 10, 11, 12, 13 indicates positive for NDV and lane 5, 8 negative bands for NDV. Lane 1 for marker or ladder of 100bp; while lane 2 and 3 as positive and negative controls, respectively.