

Isolation and Identification of Newcastle Disease Virus from Outbreak Cases and Apparently Healthy Local Chickens in South West Shewa, Ethiopia

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Abstract: This study was conducted in South West Shewa, Ethiopia to isolate and identify Newcastle disease virus from outbreak cases and apparently healthy chicken. A total of six and thirty samples were taken from outbreak cases and apparently healthy chicken respectively. All samples were inoculated in to fertile eggs and the allantoic harvests were subjected to haemagglutination and haemagglutination inhibition. Finally three samples from outbreak cases and three samples from apparently healthy chickens were subjected to RT-PCR. All samples taken from outbreak cases were able to kill embryo at the first passage within 48 hours. Allantoic harvest from the dead embryos agglutinated chicken erythrocyte with a different haemagglutination titer. None of the samples taken from apparently healthy chicken can lead to death of the embryo even at the second passage. All the samples from outbreak cases subjected to RT-PCR were amplified but none were amplified from apparently healthy chicken. Newcastle disease virus was isolated from outbreak cases of chicken in the study site where there is no vaccination program. Thus, it is recommended that there should be routine vaccination program in the study site.

Key words: Isolation • Identification • Newcastle Disease Virus • Local Chicken • RT-PCR • Haemagglutination Inhibition

INTRODUCTION

Poultry industry in Ethiopia is dominated by the traditional sector. Free-range poultry keeping is most common in the country. The chickens reeled under traditional or “backyard” conditions accounts for 99%, while only 1% are kept under intensive management system in commercial farms [1]. As far as the Ethiopian poultry farming system is concerned, it is apparent that village chickens are more important than those kept under intensive management system with regard to total numbers, egg and poultry meat production [2]. They provide livelihood and supply 100% of eggs and chicken meat consumed in rural areas, where 85% of the population live. Therefore, free-range chickens have an important role in economic and nutritional needs of the Ethiopian people especially in the rural areas.

Diseases and especially the devastating Newcastle disease (ND) is one the main constraints [3- 5] in poultry production in Ethiopia. ND has long been known to be

endemic in Ethiopia [3, 5, 6]. It has caused huge economic losses in the country. Farmers are discouraged to rear poultry because of this disease. It is caused by Newcastle disease virus (NDV) which is grouped under *Avulavirus* genus, subfamily *Paramyxovirinae* and family *Paramyxoviridae* [7]. The virus contain negative sense, single stranded RNA genome with six gene that codes for six structural and two non-structural proteins.

There have been frequent outbreak cases in the backyard chicken in different parts of the country which is suspected to be to be Newcastle disease. However, confirmations of cases were rare and there has been limited information available on the type of viruses responsible for these outbreaks. Therefore the objective of this work was to isolate and identify the type of the virus responsible for outbreak cases of suspected Newcastle disease in the study site. It was also aimed at isolation and identification of NDV from apparently healthy local chickens.

MATERIALS AND METHODS

Samples for Virus Isolation: Samples were collected from Southwest Shewa zone, Ethiopia in December 2009. There were outbreak cases in local chicken in the area which was suspected to be Newcastle disease. Six samples were taken from recently dead and sick chicken from this outbreak. At the same time 30 samples were taken from apparently healthy chicken.

Samples were collected according to the protocol of OIE terrestrial manual, [8]. Briefly, Tracheal and cloacal swabs were taken from sick and apparently healthy chickens. Cloacal swabs were visibly coated with faecal materials. Then the samples were placed in a labeled cryovials having isotonic phosphate buffered saline (PBS), pH 7.0-7.4 and containing antibiotics. From recently dead chickens, we took tracheal and cloacal swab which were collected separately as well as samples collected from lung, kidney, intestine (including contents), gizzard, proventriculus, spleen, brain, liver and heart tissues. These were collected as pool, although intestinal, gizzard, proventriculus and liver samples were taken and processed separately from other samples. The samples were put into labeled universal bottles containing phosphate buffered saline, pH 7.0-7.4 and containing antibiotics. Finally, the samples were kept cool during transport to the laboratory, where they were kept at -20°C until the isolation was carried out.

Virus Culture: Virus culture or virus isolation was performed following the protocol of OIE terrestrial manual, [8]. Briefly, the supernatant fluids of swabs or homogenized tissue suspensions, obtained through clarification by centrifugation at 1000 g for 10 minutes, were inoculated in 0.2ml volumes in to the allantoic cavity of embryonating eggs of 10 days incubation. After inoculation, the eggs were incubated at 37°C for five days. The eggs were candled every 12 hours. Eggs containing dead embryos and all eggs remaining at the end of the incubation period, were chilled at +4°C for 24 hours and allantoic fluids were harvested and tested for its ability to haem agglutinate chicken RBCs. Fluids that gave a negative reaction were passed in to one further batch of eggs. Each sample were tested in three eggs.

Haemagglutination and Haemagglutination Inhibition Tests: For identification of viruses, haemagglutination and haemagglutination inhibition tests were conducted on the allantoic harvest based on the recommendations of

the OIE terrestrial manual, [8]. For haemagglutination test, a 0.025ml of PBS was dispensed in to each well of V-bottomed microtiter plate and the same amount of the allantoic fluids were placed in the first well. After that two-fold dilution of 0.025ml of the allantoic fluid were made across the plate. Then a further 0.025ml of PBS was dispensed to each well to make the final volume 0.075ml after dispensing 0.025ml of 1% (v/v) chicken RBCs to each well. The solutions were mixed by tapping the plates gently. The RBCs were allowed to settle for about 40 minutes at room temperature when controls RBCs were settled to a distinct button. Haemagglutination was determined by tilting the plate and observing the presence or absence of tear shaped streaming of the RBCs. The titration was read to the highest dilution giving complete haemagglutination (no streaming); this was taken as 1 Haemagglutination units (1HAU). Haemagglutination activity detected in bacteriologically sterile fluids harvested from inoculated eggs was confirmed by the use of specific antiserum for Newcastle disease virus in a haemagglutination inhibition (HI) test based on again OIE terrestrial manual, [8].

Extraction of RNA: For RNA extraction, three samples from outbreak cases and another three samples from apparently healthy chicken were used and extracted using GenElute™ Mammalian Total RNA Miniprep Kit. Extraction was done according the procedure recommended by the manufacturer. Briefly, 500µl of lysis solution and 5µl mercaptoethanol was added in to the eppendrofs containing the sample (140 µl) and vortexed thoroughly. Then, the lysate was transferred to filtration column and centrifuged at 14,000 g for 2 minutes. After this, 500µl of 70% ethanol was added to the filtrated lysate and vortexed again thoroughly. Then, 700µl of the lysate /ethanol mixture was loaded in to Genelute binding column and centrifuged at 14,000 g for 15 seconds before discarding the flow-through liquid. The next step was to remove the contaminants by washing. 500µl of wash solution 1 was pipetted in to the column and centrifuged at 14,000 g for 15 seconds. Then, the binding column was transferred into a new collection tube. Again another 500µl of wash solution 2 was added in to the column and centrifuge at 14,000 g for 15 seconds. 500µl of wash solution 2 was added in to the column for the second time and centrifuged at 14,000 g for 2 minutes after discarding the flow through of first wash solution 2. Finally, the binding column was transferred to a new collection tube and 50µl of the elution solution was added in to the center of the binding column before centrifuging

at 14,000 g for 1 minute. Then the RNA extract was stored at a temperature of -20°C until the real time RT-PCR was carried out.

Primers for RT-PCR: 5'-AGTGATGTGCTCGGACCTTC-3' and 5'-CCTGAGGAGAGGCATTGCTA-3' were used as forward and reverse primers respectively for the partial amplification of matrix gene of Newcastle disease virus.

Real time RT-PCR: QIAGEN one-step RT-PCR Kit was used for Reverse transcription and PCR. Reverse transcription and PCR was carried out sequentially in the same tube. All components required for both reactions were added during setup and there was no need to add additional components once the reaction had been started. The master mix was prepared according to the protocol of the kit. The reaction components were template RNA, primer solutions, dNTP Mix, 5x QIAGEN OneStep RT-PCR buffer, RNase-free water, probe and enzyme mix.

The thermal cycler (platform miniopticon with soft ware opticon monitor 3) was programmed as outlined below. A reverse-transcription reaction temperature was set at 50°C for 30 minutes. Then it was set at 95°C for 15 minutes to activate the Taq DNA Polymerase and inactivate Reverse Transcriptase. After this, denaturation temperature was set at 94°C for 10 seconds, annealing temperature at 56°C for 30 seconds and extension temperature at 72°C for 10 seconds. Number of cycles was 39.

RESULTS

Inoculation of tissue and swab suspension from recently dead and sick chickens resulted in the death of all embryos within 48 hrs. Allantoic harvest from the dead embryos agglutinated chicken erythrocytes with a different HA titer (Table 1), which were subsequently inhibited by NDV antiserum. However, cloacal and

tracheal swab samples taken from 30 apparently healthy chicken revealed that, there was no haemagglutinating viral agent. None of the samples can lead to the death of the embryo even at the second passage.

Subjection of six samples to real time RT-PCR using specific primer for matrix gene resulted in the amplification of the samples taken from outbreak cases. Three of samples taken from outbreak cases were amplified but none from apparently healthy chicken. All the three samples, which were positive by HA and HI were also positive by real time RT-PCR. Again, all the three samples negative for HA were also negative for real time RT-PCR (Figure 1).

DISCUSSION

The haemagglutinating activity observed by the isolates and their subsequent haemagglutination inhibition with specific Newcastle disease virus antibody confirmed that all the six isolates from outbreak cases were Newcastle disease virus. The amplification of matrix gene with real time RT-PCR from outbreak samples, further confirmed the identity of the isolate as Newcastle disease virus. Nasir, [5] had also isolated and identified Newcastle disease virus as the causes of outbreaks of

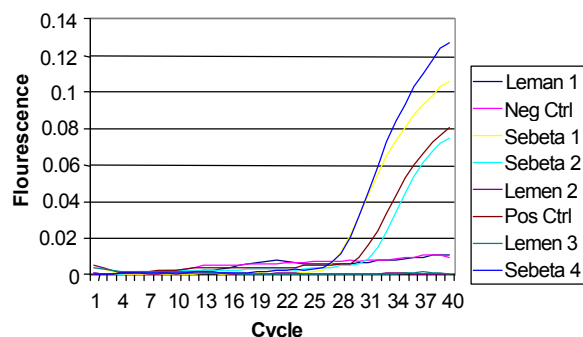


Fig. 1: Real time RT-PCR results of six samples taken from suspected cases of outbreak area and apparently healthy chickens

Table 1: Isolation and identification of Newcastle disease virus from outbreak of suspected Newcastle disease

Sample ID	Type of samples	Passages in embryos	Dead embryo /eggs inoculated (time)	Identification of viruses	
				HA end titer	HI-Test
Seb 1	Tissue	1	3/3 (2 days)	1:1024	+
Seb 2	Tissue	1	3/3 (2 days)	1:1024	+
Seb 3	Tissue	1	3/3 (2 days)	1:2048	+
Seb 4	Swab	1	3/3 (2 days)	1:256	+
Seb 5	Swab	1	3/3 (2 days)	1:512	+
Seb 6	Swab	1	3/3 (2 days)	1:512	+

HA: Haemagglutination HI: Haemagglutination inhibition

suspected Newcastle disease in Ethiopia. Killing of all the embryos by the samples taken from outbreak area may indicate velogenic strain of the virus is circulating in the study site.

All HA positive and negative samples were positive and negative respectively by real time RT-PCR which indicated that there is 100% test agreement between HA and real time RT-PCR in this case. Thus, HA and HI are good diagnostic option for identification of Newcastle disease virus in laboratory where there is no sophisticated lab equipments.

Newcastle disease virus was isolated from outbreak cases of chicken in the study area where there was no vaccination program. This indicating that the village chickens in this area would be highly susceptible to the pathogenic NDV infection. Belayneh *et al.*, [9] has also reported that the overall sero-prevalence of the disease in the study area was 5.6% which means the majority of the populations are susceptible for infection. Thus, it is recommended that there should be routine vaccination program in the study site.

The time of the outbreak at the study site was at Christmas time when there was mass mobilization of chickens to the market. Sa'idu *et al.* [10] and Nwanta *et al.* [11] reported that increased movement of sick and healthy chickens in anticipation of various festivals particularly of Christmas and New Year. These may be responsible for the occurrence of ND outbreak in December in the study site. Within the country, NDV may spread with animal movements, such as buying, selling and exchanging poultry. The risk of markets, however, has not been evaluated and thus their role in the spread of NDV is not known. Therefore, further study should be conducted to determine the role of markets for the transmission of the diseases in the country.

ACKNOWLEDGEMENTS

The authors are grateful to the National Animal Health Diagnostic and Investigation Center (NAHDIC) of Ethiopia, for allowing us to use all the facilities that we need for the work and providing us the kits that we used for the tests. We would also like to thank Abera Kebede, technicians at NAHDIC, for his help in laboratory work.

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