Outbreak investigation of Newcastle disease virus from vaccinated chickens in Eritrea

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Newcastle disease (ND) is one of the most important infectious viral diseases of poultry in Eritrea. Diagnosis of ND virus infection in Eritrea has been mainly based on clinical examination and post mortem lesions. This study describes ND diagnosis using reverse transcription polymerase chain reaction PCR (RT-PCR), virus isolation and serological techniques (haemaglutination inhibition test) from two times vaccinated 40 days old chicks in Keren poultry farm. Generally clinical signs and postmortem lesions were characterized by rapid onset, death without visible lesions, ruffled plumage, dyspnea, in appetite, weakness and green colored diarrhea. Clinical samples collected were cloacal swabs. Observed postmortem lesions were tracheal hemorrhage, hemorrhage in proventriculus especially at the junction between esophagus and proventriculus and haemorrhage in caecal tonsils. Samples collected after postmortem include spleen, intestine and caecal tonsils. High titer of specific ND virus (NDV) antibody was detected indicating the occurrence of recent infection. ND virus was isolated from aseptically collected samples using embryonated chicken eggs. RT-PCR assay described was able to detect ND virus from cloacal swabs, spleen, intestine and caecal tonsils. Therefore, the described RT-PCR assay can be adopted for rapid and confirmative detection of NDV in Eritrea. Additionally, prevention of ND should not depend only on vaccination.

Key words: Newcastle disease virus, reverse transcription polymerase chain reaction (RT-PCR), haemagglutination inhibition, virus isolation, vaccination.

INTRODUCTION

Newcastle disease (ND) is a highly contagious and fatal viral disease that affects over 250 species of birds of all age groups (Aldous and Alexander, 2001; Al-Habeeb et al., 2013). It is also called avian paramyxovirus serotype 1 (APMV-1), is a single stranded, enveloped, negative sense RNA virus belonging to the genus Rubulavirus of subfamily Paramyxovirinae and family Paramyxoviridae (Swayne, 2011; Mayo, 2002; OIE, 2012). The disease is present worldwide and affects many species of birds causing severe losses in the poultry industry (Cattoli et
In developing countries, where the majority of chickens are reared under backyard conditions, ND can drastically limit the amount of dietary protein as well as damage the micro economy due to loss of ability to sell extra chickens or eggs. Eritrea is one of the countries facing the devastating effects of ND (OIE, 2014). Most countries where poultry are raised commercially and where the disease is endemic rely on vaccination to keep the disease under control. However, outbreaks have been reported in vaccinated populations despite the fact that vaccination is widely applied (Alexander and Senne, 2008; Van Bovan et al., 2008; Tsegaw et al., 2014). This report is detailed on ND outbreak in vaccinated commercial layer poultry farm.

Historically, diagnosis of NDV relies on the detection of the infectious agent by virus isolation in embryonated chicken eggs and identification by haemagglutination inhibition (HI) test (Aldous and Alexander, 2001; Smietanka et al., 2006). However, diagnosis based on these techniques is laborious and time consuming. The speed of the diagnosis can be considerably increased by using methods based on molecular biology for example reverse-transcription polymerase chain reaction (Gohm et al., 2000; Creenlan et al., 2002). Reverse transcription polymerase chain reaction (RT-PCR) methods are applied in many laboratories of the world for the detection and identification of ND virus (Aldous and Alexander, 2001). Using a reverse transcriptase, the RNA genome is transcribed into a DNA copy, which can be used as the template in PCR. Amplification of a specific gene region has been achieved using: (1) universal primers (Jestin and Jestin, 1991; Gohm et al., 2000); (2) pathotype specific primers (Kant et al., 1997).

In Eritrea, ND is an economically important disease in both commercial farms and backyard small holder units due to high mortality and morbidity nature of the disease. However the diagnosis of ND virus was limited within the recording of clinical signs and gross lesions. Reports detailing laboratory diagnosis of the virus are relatively few with the frequent outbreaks of the disease in the country. This report describes the isolation and molecular detection (RT-PCR) of NDV in one layer poultry farm ND outbreak. The birds in the farm were intensively managed and vaccinated two times against ND. Clinical characteristics of the disease included 100% morbidity, sporadic mortality which reached 15%.

### MATERIALS AND METHODS

#### Case description
This report describes an outbreak of Newcastle disease in Keren poultry farm located in Keren, Anseba region, Eritrea. The birds were vaccinated two times against ND at 7 days with B1 strain through eye drop and at 21 days with Lasota strain through drinking water. The origin of the birds was from Hungary (Tetra Harco), imported for commercial dual purpose breeds. The outbreak started on 8th April 2016. The age of the birds at the time of the outbreak was 40 days. The total number of birds exposed to the outbreak was 12,500. The duration of the outbreak proceeded for 13 days with 100% morbidity and sporadically increasing mortality which finally reached 15% (Table 1). Sick birds exhibited dyspnea, swollen face, nasal discharge, ruffled feathers, weakness and greenish diarrhea. Clinical diagnosis was made first by the animal health technician in the farm. 30 sick and dead chicks sent to the laboratory were observed for clinical signs and postmortem lesions. On 13th April 2016, a total of 34 samples were collected from the 30 chicks sick and dead submitted to the laboratory for case confirmation. This includes cloacal swabs, spleen, caecal tonsils and whole intestine.

#### Sample preparation
For virus isolation, spleen, caecal tonsils and whole intestine were selected. These were homogenized using mortar and pestle with sterile sand and suspended in sterile phosphate buffered saline (PBS) with antibiotic (10,000 IU penicillin and 10 mg streptomycin) in 1:10 (w/v) dilutions, centrifuged at 3000 rpm for 10 min at 4°C, and the supernatants were used for inoculation of embryonated chicken eggs and RNA extraction.

Swab samples were soaked in one ml PBS with antibiotic (1000 IU penicillin, 1 mg streptomycin) for RNA extraction for overnight in a 2 ml eppendorf tubes. After vortexing for 15 s, the swabs were squeezed in the wall of the tube several times and discarded. The tubes were centrifuged at 5000 rpm for 10 min and the supernatant used.

#### Laboratory diagnosis

### Reference viruses
Lasota strain used as reference virus obtained from Veterinary Laboratory Agency New Ham Addlestone, surrey kt15 3NB, UK.

### Source of reference antiserum
The source of ND virus reference antiserum was from Veterinary Laboratory Agency New Ham Addlestone, surrey kt15 3NB, UK.

### Table 1. Number of dead chicks recorded versus days of the outbreak.

<table>
<thead>
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<th>Day</th>
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Isolation of virus

Specific antibody negative (SAN) from ten day old embryonated chicken eggs was inoculated through the allantoic cavity route using 0.2 ml inoculums per egg. The inoculated eggs were incubated at 37°C set incubator with humidity and were candled twice daily. Embryos started death after 24 h and all inoculated eggs were dead by the end of 60 h. Dead eggs were chilled at 4°C twice daily. Incubated at 37°C set incubator with humidity and were candled twice daily. Allantoic fluid was collected and tested by rapid slide hemagglutination (HA) test.

Micro haemagglutination test

Hemagglutination test in microtiter plates was done to define 4 HA unit for hemagglutination inhibition (HI) test according to the method of Terregino and Capua (2009). A volume of 0.025 ml PBS was dispensed into each well of V bottom microtiter plate. A 0.025 ml of virus suspension placed in the first column wells and two fold dilutions made from 1:2 to 1:4096, 0.025 ml PBS was dispensed into each well. 0.025 ml of 1% chicken RBC was added, mixed by gentle tapping and read after 30 to 40 min incubation at room temperature.

Serological methods

Hemagglutination inhibition (HI) test

HA positive allantoic fluid samples were subjected for HI test according to Terregino and Capua (2009). 0.025 ml of PBS was dispensed into all wells of a plastic V bottom microtiter plate and 0.025 ml of reference antisera was added to the first columnwells. Then serial two fold dilution of sera was made from 1:2 to 1:4096. A 0.025 ml of 4 HA unit antigen (allantoic fluid) added to all wells except the test antigen row (H1-H12). In the test antigen row, 0.025 ml of diluted allantoic fluid containing 4 HA unit dispensed and two fold dilutions made from H1-H6, the rest of the wells contain only PBS. A volume of 0.025 ml of PBS added to all wells and incubated at room temperature for 30 min. A volume of 0.025 ml 1% chicken RBC added to all wells mixed by gentle tapping and read after 30 to 40 min incubation at room temperature.

RNA extraction

The genomic viral RNA was extracted from 140 µl of cloacal swabs, spleen and intestine homogenate (inocula), allantoic fluid and reference virus using QIAamp viral RNA mini kit (QIAGEN, Hilden, Germany) according to manufacturer’s protocol. The RNA was extracted in 60 µl of elution buffer and used as template for RT-PCR assay.

Oligonucleotide primer used

A set of universal oligonucleotide primers specific for Newcastle disease virus, forward (NDVF): 5’–CTG CAG GAA TTG TRG TAA CAG G–3’ and reverse (NDVR): 5’ – ACN TGG ACA CAG ACY CTT – 3’ were used for the amplification of 220 bp. The primers were manufactured by Eurofins (Eurofins MWG Operon, Austria).

RT-PCR

Synthesis of cDNA

A volume of 3 µl eluted RNA mixed with random hexamer primer (100 µM) (1 µl), RNase free water (8 µl), 5× reaction buffer (4 µl), RNase inhibitor (1 µl), 10 mM dNTP mix (2 µl) and M-Mulv reverse transcriptase (2 µl) were mixed in 0.2 ml individual PCR tubes. The tubes were then placed in a 96 wells thermocycler (Mastercycler, Eppendorf, Hamburg, Germany) and incubated at 25°C for 5 min followed by 60 min at 37°C with the final heating for termination of the reaction at 70°C for 5 min for the synthesis of cDNA from RNA.

Synthesis of DNA from cDNA

For the synthesis of DNA from cDNA of ND virus, reaction mixture was used as 20 µl volume comprising 10× PCR buffer 2 µl, 50 mM MgCl₂ 0.8 µl, 10 mM dNTP mix 1.6 µl, Taq DNA Polymerase (5 U/µl) 0.3 µl, specific primers NDVF (5 µM) 0.8 µl, NDVR (5 µM) 0.8 µl, cDNA 2 µl and RNase free water 11.7 µl were mixed properly by vortex and minispin in 1.5 ml microtube and aliquot to 0.2 ml PCR tubes. These tubes were subjected to the following PCR cycling conditions in the thermal cycler: Initial denaturation at 94°C for 2 min followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 60°C for 30 s, extension at 72°C for 30 s.

Agarose gel electrophoresis

To confirm the target gene, 6 µl of PCR product with 2 µl of 6× gel loading dye (Fermentas® × DNA loading dye) was electrophoresed on 1.5% agarose gel containing GelRed (Nucleic Acid Gel Stain, 10,000 × in water at 4 µl per 50 ml) at constant 100V for 50 min in 1× TAE buffer. A 4 µl DNA size marker (100 bp ladder, Fermentas) was loaded in one well. The amplified product was visualized under UV tranilluminator and documented by gel documentation system.

RESULTS

Clinical signs and gross lesions

The observed clinical signs were anorexia, ruffled plumage, weakness, swollen face, respiratory distress with gasping and sneezing (Figure 1) and green colored
diarrhea.

Gross lesions were absent in some dead birds. The observed lesions include fevered and dehydrated carcasses, hemorrhages in trachea, ecchymotic haemorrhage in the mucosa of proventriculus especially at the junction of esophagus and proventriculus (Figure 2), multifocal hemorrhages in serosa of intestine and caecal tonsils (Figure 3), spleen enlargement and mottling and petechia in thigh muscles.

**Laboratory diagnosis**

**Isolation of ND virus**

The selected samples for virus isolation were found to be positive for virus isolation in embryonated chicken eggs. All inoculated embryos died between 55 to 60 h post inoculations. Embryos which died within 24 h were discarded as nonspecific mortality. Harvested allantoic
Figure 4. RT-PCR products showing 220 bp specific bands on 1.5% agarose gel. Lane 1 reference ND virus, lanes 2,3,4 spleen, caecal tonsils and intestinal homogenates respectively, lanes 5,6,7 allantoic fluid of samples in lanes 2,3,4 after propagation in embryonated eggs. M = 100 bp DNA ladder.

fluid showed positive by rapid slide haemagglutination activity after 30-60 s which indicate that the isolates were haemagglutinating viruses. Micro HA test was performed from the allantoic fluid harvest to derive 4 HA unit for HI test.

**Haemagglutination inhibition test**

Haemagglutinating activity of the allantoic fluid samples were inhibited by reference mono specific hyperimmune ND serum which indicates the haemagglutination activity is due to ND virus.

**RT-PCR**

Viral RNA was extracted from cloacal swabs, inocula (spleen, caecal tonsils and intestinal homogenates) and allantoic fluid. The extracted RNA was amplified by RT-PCR using specific ND virus primers. A clear and distinct band of RT-PCR product appeared at the position of 220 bp with the standard 100 bp DNA ladder passed through 1.5% agarose gel (Figure 4). Therefore the samples showed positivity for ND virus by RT-PCR using specific primers from crude samples and infected allantoic fluid.

**DISCUSSION**

The clinical signs and gross lesions observed strongly suggest Newcastle disease virus infection. The findings are similar with the findings of Alexander (2003), Terregino and Capua (2009), Susta et al. (2010), Swayne (2011), and Uddin et al. (2014). The present report shows the vaccination program applied could not protect the birds from clinical signs; however it protected from full 100% mortality characterized in fully susceptible flocks. Similar incidences have been reported by Musa et al. (2010) and Balachandran et al. (2014). The lentogenic B1 and Lasota vaccine strains of low virulence are commonly used worldwide, and can provide protection against virulent NDV if the vaccines are viable, administered correctly to healthy birds and time is allowed for an appropriate immune response to develop prior to exposure to the challenge virus (Cornax et al., 2012; Kapczynski and King, 2005). The possible reason for the
failure of the vaccination in Keren poultry farm could be due to inappropriate vaccine administration that can lead to lower immune response. Degefa et al. (2004) have also shown mass vaccination through drinking water route protects only 60% of the vaccinated flock. Additionally Dortmans et al. (2012) showed that inadequate application of NDV vaccines worldwide account for the current outbreaks and spreading of virulent NDV field stains. This shows the incidence of the outbreak could be related to inadequate application of the vaccine at the second time by drinking water, in which vaccination by drinking water route provokes lesser immunity than by eye drop method (Alexander et al., 2004).

Though signs and gross lesions associated with the virulent pathotypes will give rise to strong suspicion of the disease, they do not present a reliable basis for diagnosis of ND (OIE, 2012). Absolute identification is dependent upon the isolation and identification of the causative virus (Terregino and Capua, 2009; Susta et al., 2010). The preferred method for diagnosis of Newcastle disease involves mainly conventional viral isolation in embryonated chicken eggs with subsequent identification by haemagglutination inhibition test and molecular techniques like PCR (RT-PCR) (Alexander, 1991; Terregino and Capua, 2009). In Eritrea, published reports about isolation of ND virus and serological identification are rare. This report indicates successful isolation of the virus and serological identification by HI test. Virus isolation remains the method of choice for confirmatory diagnosis or as the gold standard method for the validation of other techniques (Alexander and Senne, 2008; Terregino and Capua, 2009). Therefore we also used virus isolation for the identification of ND virus and for the validation of RT-PCR for detection of ND virus.

Jestin and Jestin (1991) developed the first RT-PCR for the identification of NDV from isolates in embryonated eggs. Later the possibility of detecting NDV in tissues and feces samples using RT-PCR was investigated by Gohm et al. (2000). In our case we have applied RT-PCR for the detection of ND virus in allantoic fluids of infected embryonated eggs and from collected samples (cloacal swabs, spleen and whole intestine homogenate). The results in this report from the use of RT-PCR for rapid detection of ND virus from direct use of selected field samples show the reliability, simplicity and rapidity of the test in accordance with the goal of molecular based tests (Aldous and Alexander, 2001).

We selected spleen and intestine as best samples with the observed viscerotropic form of the disease based on the viewed gross pathological lesions. The early death of inoculated eggs less than 60 h shows the infecting virus could be possibly virulent strain. However, pathogenicity tests either intracerebral pathogenicity index (ICPI) or demonstration of multiple basic amino acids at the C-terminus of the F2 protein and phenylalanine at residue117, which is the N-terminus of the F1 protein are required to be performed for full assessment of the virus virulence (OIE, 2012).

Conclusion

Control of the disease depends mainly on accurate diagnosis. The two step RT-PCR technique described could be used for rapid detection of ND virus. We were not able to do pathogenicity tests to determine fully the virulence of the challenging virus. However, this report shows identification of the cause of the outbreak by virus isolation, serological identification and RT-PCR. Additionally the report shows, vaccination could not be relied on for full protection of ND virus infection.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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