Detection of Newcastle Disease Virus from Domestic Chickens (*Galus domesticus*) in Surabaya Traditional Markets

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Abstract

Newcastle Disease is considered as one of the important diseases in the poultry sector caused by the Newcastle Disease virus (NDV) strain. NDV infects the nervous, digestive, respiratory, and reproductive systems. ND is one of the most reported poultry diseases in Indonesia. This study aims to determine the number of Newcastle disease cases in Surabaya. Samples were 30 oropharyngeal swabs from native chickens taken from 5 traditional markets in Surabaya. Samples were identified by the RT-PCR method using a specific forward primer encoding the Hemagglutinin-neuraminidase gene 5'-CAGAGATCACTCACATTCAT-3' and amplification with a target of 542 bp. The results obtained were 1 positive sample from the Darmo Market, Surabaya.

***Keywords**: Gallus domesticus, Hemagglutinin-neuraminidase, Newcastle Disease, PCR*

1. Introduction

The poultry industry is a major sector for the Indonesian national economy [1]. The poultry industry plays a role in providing employment for 10% of the national workforce [2]. In addition, the poultry industry also plays a role in providing 65% of the supply of animal protein [2]. The rapid development of the poultry industry in Indonesia is due to the increasing population and awareness of fulfilling nutritional needs [3]. One source of animal protein that is useful for fulfilling nutrition comes from domestic chickens (*Gallus domesticus*) [3]. Domestic chickens *(Gallus domesticus)* have the advantage of being able to form superior local chicken seeds that can adapt to the environment quickly [3].

Newcastle Disease is considered as one of the important diseases in the poultry sector. ND disease outbreaks often occur in groups of chickens that do not have immunity or groups that have low immunity due to late vaccination or failure of the vaccination program. Losses caused by ND disease include chicken death, decreased egg production in laying hens, growth disorders and decreased body weight in broiler chickens. Losses due to ND disease are caused by very high morbidity and mortality rates in poultry. Mortality and morbidity can reach 50-100% due to ND virus infection. Therefore, ND cases are a serious threat to the livestock industry in Indonesia [4].

OIE data 2015, in East Java there were 100 to 1500 chickens infected with the ND virus each month and jumped to 14,000 cases in February 2011 [5]. The greatest potential for transmission of Newcastle disease (ND) is the traditional poultry market [6]. Transmission of this disease is caused by various species of poultry being marketed together in one location [4]. The condition of traditional poultry markets with poor sanitation contributes to the spread of the ND virus in Indonesia. The market has indications that it is very susceptible to the spread of the ND virus, including the market in the city of Surabaya. The lack of information about the origin and health status of poultry is one of the causes of easy transmission of the virus both inside and outside the market [5]. Transmission of Newcastle disease (ND) can occur from one animal to another through contact with sick animals and carcasses of sufferers. Transmission from one place to another can occur through transportation, barn workers, dust, wind, insects and contaminated food. This transmission can be caused by a less intensive maintenance system, making it difficult to control [7]. Therefore, ND disease is a serious threat to the livestock industry in Indonesia [4].

Newcastle Disease (ND) is a disease caused by infection with a virus from the genus Avulavirus and the species avian avulavirus 1, commonly known as Newcastle Disease Virus (NDV) and abbreviated as avian paramyxovirus 1 (APMV 1) [8]. NDV contains six structural proteins and two nonstructural proteins with the sequence nucleoprotein (NP), phopspoprotein (P), matrix protein (M), fusion protein (F), haemagglutinin-neuraminidase protein (HN), and large polymerase protein (L) [9]. The HN protein of NDV is a multifunctional protein that has receptor recognition and neuraminidase (NA) activities associated with the virus. The HN protein recognizes sialic acid-containing receptors on the cell surface, enhances the fusion activity of the F protein, allowing the virus to penetrate the cell surface and acts as NA by removing sialic acid from progeny virus particles to prevent self-agglutination of progeny viruses. Thus, HN protein plays an important role in viral infection. Cleavage of F protein alone does not convert nonpathogenic strains into highly virulent pathotype strains. Fusion protein (F) and Haemagglutinin-neuraminidase protein (HN) have a collective role in the NDV infection process [10]. The terminal globular head of HN protein contains the active site involved in virus attachment and NA activity. Specific interactions between the HR2 domain of F protein and the proximal membrane ectodomain of HN protein spanning amino acids 124 to 152 occur to promote fusion. Only F and HN proteins play a role in the immune system because they can stimulate protective antibodies [11]. Hemagglutinin-neuraminidase protein (HN) is an important protein in determining the virulence of Newcastle Diseases Virus, it is known that NDV virulence is multigenic and the ability to cleave F protein alone does not determine the virulence of a strain. Cleavage of F protein alone does not change

nonpathogenic strains into highly virulent pathotypes. The HN protein of virulent strains provides the ability to spread systemically and increases the virulence of previously avirulent NDV [12].

The incubation period and clinical symptoms of ND disease in chickens vary, depending on the virus strain and the immune status of the chicken when infected. In lentogenic strain virus infections, the disease is subclinical, or characterized by mild respiratory disorders such as sneezing and nasal discharge. Mesogenic strain virus infections are acute, characterized by respiratory disorders and neurological disorders. Clinical symptoms in chickens are characterized by decreased appetite, cyanosis of the comb and wattles, swelling in the head area, sneezing, coughing, snoring, and greenish-white diarrhea. Velogenic strain virus infections are fatal, often followed by high mortality rates. These symptoms vary widely, starting with conjunctivitis, diarrhea, and followed by neurological symptoms such as tremors, torticollis, or paralysis of the neck and wings [13].

2. Material and Methods

2.1.Research Design

This study is included in descriptive observational research using an exploratory research design. Thirty samples were taken using a purposive sampling method. Samples were taken from native chickens suspected of Newcastle Disease Virus (NDV) and unvaccinated from the Wonokromo, Darmo, Pabean, Keputran and Tembok Dukuh markets. The sample was an oropharyngeal swab of native chickens.

2.2. Variable Operational Definition

In this study, Newcastle Disease virus was collected from the traditional Gallus domesticus market in Surabaya. Oropharyngeal swabs were taken from domestic chickens that had symptoms of colds, snoring, nerves and green diarrhea. HN protein plays a role in the attachment of virus particles to receptors on target cells, the release of viruses from infected cells and as an activation promoter. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) is a laboratory technique used to amplify and detect RNA in biological samples.

This study will be conducted at the Viral Diarrhea Laboratory, Institute of Tropical Diseases, Airlangga University.

2.3. Research Procedure

2.3.1. Samples Collection

The virus isolate samples used were oropharyngeal swab samples from native chickens at the Surabaya Market. Samples were collected in 2.5 ml containers with phosphate buffered saline (PBS) transport media, pH 7.0-7.4 containing antibiotics (2,000 IU penicillin/ml, 2,000µg streptomycin/ml 50µg/ml) and stored in a coolbox. Furthermore, the samples were taken to the laboratory using an ice box containing ice gel and stored in a freezer at -80ºC (Agricultural Quarantine Agency, 2010) at the Institute of Tropical Diseases, Airlangga University until analysis was carried out.

2.3.2. Samples Preparation

Gallus domesticus oropharyngeal swabs that were collected were then subjected to RNA extraction procedures. To obtain viral RNA, RNA extraction was carried out using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) extraction kit according to the Handbook protocol. A total of 140 µl of sample was added with 560 µl of AVL Buffer and 5.6 µl of Carrier RNA, pipetted and inserted into a 2 ml microctube then mixed using a vortex for 15 seconds. The mixture was placed at room temperature (15-250 C) for 10 minutes. The mixture was centrifuged briefly to remove liquid spots that adhered to the inside of the side or lid of the tube. Ethanol (96-100%) was added as much as 560 µl to the sample and mixed using a vortex for 15 seconds then centrifuged briefly. 630 µl of the

mixture was inserted using filter tips into the QIAamp mini column (in a 2 ml collection tube) without wetting the edge of the column. After that, it was closed and centrifuged at 8000 rpm for 1 minute. Place the QIAamp mini column into a clean 2 ml collection tube and discard the tube containing the filtrate. The QIAamp mini column was opened, and the previous steps were repeated. Next, 500 µl of Buffer AW1 was added. After that, it was closed and centrifuged at 8000 rpm for 1 minute. The QIAamp mini column was placed into a clean 2 ml collection tube and the tube containing the filtrate was discarded. then 500 µl of Buffer AW2 was added. After that, it was closed and centrifuged at full speed of 14,000 rpm for 3 minutes. The QIAamp Mini Column 2 ml collection tube was placed in a new 1.5 ml tube, and the old collection tube was discarded along with its filtrate. The new collection tube containing the QIAamp mini column was centrifuged at 8,000 rpm for 1 minute. RNA samples were stored at -800 C until used as templates for One Step RT-PCR.

Newcastle disease analysis using Takara's One Step RT-PCR Kit (AMV). The primer used for amplification was the forward protein HN Newcastle disease 5'-CAGAGATCACTCACATTCAT-'3. RNA amplification was carried out using the One step RT-PCR technique where 1 µl of RNA sample was added with 6.25 µl Nucleasese free water, 1 µl forward primer, 1 µl reverse primer, 2.5 µl, MgCl2, 1.25 µl dNTP Mix, 10x one-step buffer 1.25 µl, AMV reverse transcriptase 0.25 µl, AMV optimized Taq 0.25 µl, and 0.25 µl RNAse inhibitor were inserted into the PCR tube and then inserted into a conventional PCR machine. The thermocycler machine is programmed as follows: reverse RNA to cDNA at a temperature of 50°C for one hour, pre-denaturation at a temperature of 95°C for seven minutes and denaturation at 94°C for 45 seconds. Furthermore, the annealing process at a temperature of 55°C for 45 seconds and the extension stage at a temperature of 72°C for 30 seconds. One reaction cycle, namely the denaturation, annealing, and extension stages, is repeated up to 40 times (cycles). The last stage is the refinement of enzyme work at a temperature of 72°C for five minutes. After the refinement stage is complete, the thermocycler is at a temperature of 20ºC (Putra et al., 2016).

The results of RT-PCR were then continued with electrophoresis, the desired amplification result was 542 bp. A total of 3 ul of RT-PCR product was added with 1 µl of loading dye (bromphenol-blue and cyline cyanol), then electrophoresed on a 1% agarose gel (1 gram of agarose in 100 ml of TAB) which was added with 2.5 µl of ethidium bromide on an electrophoresis machine with a voltage of 100 Volts for 30 minutes. The marker used as a reference is 100bp of DNA leadder fermentas with a lower limit of 100bp and an upper limit of 1000bp. The test results were visualized with ultraviolet light and documented with a camera (Kencana et al., 2012).

3. Research Result

Sampling from traditional markets in Surabaya, samples in the form of oropharyngeal swabs of native chickens. Samples obtained were 30 samples from 5 traditional markets in Surabaya. Samples were taken based on native chickens that showed symptoms of ND such as snoring, weakness, runny nose, and green feces.

Table 1. Orofaring Swab Domestic Chicken (Gallus domesicus) collected from Traditional Market Surabaya.

Location	Samples Code	Clinical Symptoms

Tembok Dukuh Market	TD1	Weak condition, hair loss, green feces, slight nasal mucus discharge, decreased appetite
Tembok Dukuh Market	TD2	weak condition, there is green nasal discharge, a bit of snoring, green diarrhea defecation
Tembok Dukuh Market	TD3	Very weak condition, only lying down, doesn't want to eat, there is green discharge in the oropharyngeal area, green feces
Darmo Market	D1	Very weak condition, torticollis, oropharynx shows thick discharge, green feces
Darmo Market	D2	Weak condition, torticollis, oropharynx has a slight discharge, greenish white feces
Darmo Market	D3	Semi-active condition, there is snoring, there is a cough, there is discharge in the oropharynx, decreased appetite, green feces
Darmo Market	D4	Condition is somewhat weak, there is a cough, oropharynx has mucus discharge
Darmo Market	D5	Condition: Weak, cough, oropharyngeal discharge mucus
Darmo Market	D6	Condition is somewhat weak, discharge of greenish nasal mucus, decreased appetite, hair loss
Darmo Market	D7	Very weak condition, decreased appetite, green nasal mucus discharge
Darmo Market	D8	Very weak condition, does not want to eat, discharges green nasal mucus, hair loss
Darmo Market	D9	Weak condition, cough, oropharynx has green mucus discharge
Darmo Market	D10	Weak condition, cough, oropharynx has green mucus discharge
Pabean Market	P1	Very weak condition, torticollis, fur looks rough, no appetite
Pabean Market	P2	Very weak condition, oropharynx appears to have green discharge, thick green feces
Pabean Market	P3	Very weak condition, oropharynx appears to have green discharge, green feces
Pabean Market	P4	Condition is somewhat weak, discharge of brownish green nasal mucus, decreased appetite, greenish white feces.
Pabean Market	P5	The chicken is weak, the chicken's body is thin, it appears to be coughing, there is a thick greenish discharge in the oropharynx

Pabean Market	P6	Weak condition, slight discharge in the oropharynx, normal colored feces
Pabean Market	P7	Weak condition, slight discharge in the oropharynx, normal colored feces
Wonokromo Market	W1	Very weak condition, there is green oropharyngeal discharge, dark green feces
Wonokromo Market	W2	weak condition, there is a slight discharge in the oropharynx, normal feces
Wonokromo Market	W3	Very weak condition, there is a lot of green oropharyngeal discharge, dark green feces
Wonokromo Market	W4	weak condition, decreased appetite, chicken feathers falling out
Wonokromo Market	W5	Weak condition, green nasal discharge, does not want to eat, chicken feathers fall out
Keputran Market	K1	Semi-active condition, there is discharge in the oropharynx, decreased appetite, green feces
Keputran Market	K2	Weak condition, slight nasal discharge, decreased appetite
Keputran Market	K3	semi-active condition, snoring, slight oropharyngeal discharge
Keputran Market	K4	weakness, snoring, slight nasal discharge, greenish feces
Keputran Market	K5	weak condition, abundant green oropharyngeal discharge, greenish feces

Samples obtained were 30 samples from 5 traditional markets in Surabaya. Samples were taken based on native chickens showing symptoms of ND such as snoring, weakness, runny nose, and green feces. All samples were stored using transport media and then taken to the Viral Diarrhea Laboratory, Institute of Tropical Diseases, Airlangga University for RT-PCR examination. Extraction of viral RNA from 30 samples collected and continued with RT-PCR testing to detect the presence of ND virus based on the HN gene. The resulting PCR product was then electrophoresed. The results of this electrophoresis were analyzed by comparing the thickness of the band visually. The optimal band in question is a thick, single band and according to the target size (Setyawati and Zubaidah, 2021).

The PCR amplification results can be seen through the DNA bands that appear after the electrophoresis process. The RT-PCR results are declared positive if there is a DNA band through the electrophoresis process which can be seen in Figure 2. The PCR product amplification results show the presence of a DNA band (542 bp) in 1 of the 30 samples collected. The Newcastle disease virus sample that was detected positive for the 542 bp HN gene region was the D3 code sample (Darmo 3).

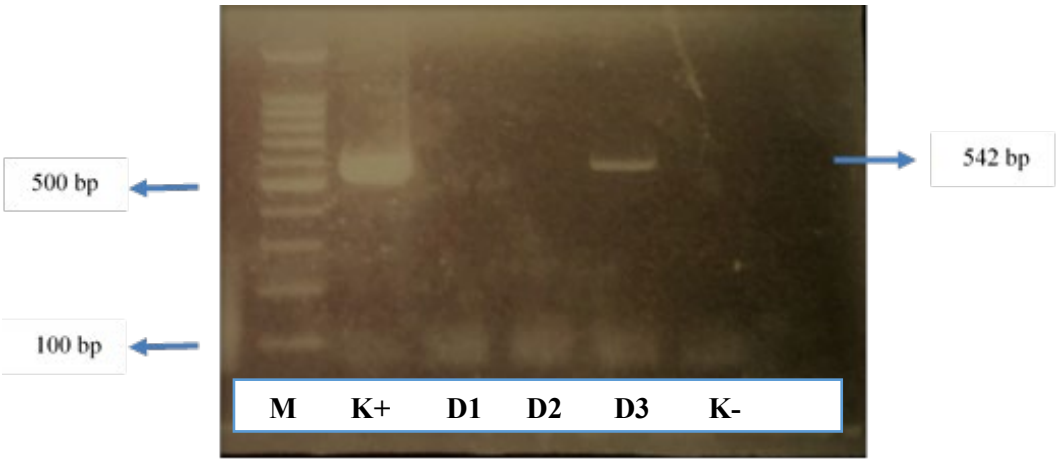

Figure 1. Results of PCR Product Electrophoresis with 2% Agarose Gel.

The samples collected were 30 samples of oropharyngeal swabs of native chickens at the Surabaya Traditional Market, there was 1 positive sample from the RT-PCR examination, namely in the D3-SBY sample. There was only 1 sample that had a positive result in the RT-PCR examination, possibly due to human error, where when performing the oropharyngeal swab, the sample was not taken deep enough or did not touch the oropharyngeal wall. In addition, there were clinical symptoms of Avian Influenza disease which were almost similar to the clinical symptoms of ND disease so that it was possible that the oropharyngeal swab sample of native chickens that was taken was infected with the Avian Influenza virus. The PCR results obtained from the D3-SBY sample were using the forward primer of the HN coding gene of the ND virus, 5' CAG AGA TCA CTC ACA TTC AT '3 with a length of 542 bp.

The spread of the ND virus isolate SBY D3 can occur through poultry trade and the movement of fomites between regions, because the geographical location of the Surabaya area with Kudus and Sragen is located in adjacent provinces, namely East Java and Central Java. Other factors that can cause the spread of the ND virus isolate SBY D3 is that Indonesia has a mixed poultry production system, where most poultry is raised traditionally, and many have markets selling live birds of various species and ages. Historically, traditional farms and live poultry markets have been considered the main point in the evolution of NDV strains because of the difficulty of vaccinating newly hatched birds. Unvaccinated commercial and domestic poultry, as well as wild birds, can act as reservoirs of NDV. Carrier animals or vectors such as waterfowl and wild birds from Kudus, Sragen and Banjarmasin that migrate to Surabaya can transmit the ND virus to native chickens because Surabaya is one of the bird migration destinations (Miller et al., 2015).

5. Conclusion

Based on the results of the RT-PCR test on samples of 30 oropharyngeal swabs of native chickens studied, the results showed that one sample of native chicken from the Darmo market was positive for infection by the ND disease virus. This study confirms that there are still cases of ND disease in Surabaya.

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