The Characteristic of Egg Drop Syndrome Virus of Medan Isolate

Gusti Ayu Yuniati Kencana[1*], I Nyoman Suartha[2], Arini Nurhandayani[3], Syamsidar[3]

[1)]Laboratorium of Virology, [1)]Laboratorium Internal Medicine,
Faculty of Veterinary Medicine, Udayana University,Telp/Fax (0361)223791 Jln. PB. Sudirman, Denpasar
[3)]PT. Sanbio Laboratories.
Jln. Melati Rt.02/09 Ds. Wanaherang, Kec. Gunung Putri , Bogor 16965
*Corresponding author: yuniati_kencana@unud.ac.id

Abstract. Egg Drop Syndrome (EDS) is a poultry disease marked by a decrease of egg production up to 40% with a declined quality of eggs. In Indonesia, EDS is astrategically infectious disease that must be eradicated. Until now, EDS vaccines are available, vaccinations on egg-laying chicken have been conducted nevertheless the case of EBS are still found. This research collaborates with PT Sanbio Laboratories (Poultry vaccine producer in Bogor) aimed to prepare EDS seed vaccines from local isolates. Isolate samples are collected from ten egg-laying chicken farmswith signs of declined production and egg quality as well as unevenly shaped and thinning of egg shells. Virus isolation was conducted on embryonated duck eggs at the age of 11 days through the allantoic space. Embryonated duck eggs are then incubated for 3 days in the incubator at 37^0C and are observed daily. On the third day, the eggs are taken out of the incubator and then inserted in the cooler overnight. The allantoic fluid is harvested on the third day post inoculation; it is identified with haemaglutination test and Polymerase Chain Reaction (PCR). Six isolates are positively identified as EDS virus: two from the Medan isolates, there from the Rumpin isolates, and one from Surabaya isolate. One isolate is chosen; which was the EDS isolate of Medan, with the highest titer for passaging and characterization. The content of the virus is calculated with Reed and Muench formula and expressed in a unit of Egg Lethal Dose 50 (ELD_{50}). Research results shows titer of Medan EDS virus isolate after the second passage was 10^{12} HA Unit, with virus content of $10^{9,5}$ EID_{50}, length base product of PCR that was successfully amplified as 500bp. Therefore the Medan isolate is a recommended candidate for EDS vaccine seeds.

Keywords: **isolation, characterization, biological test, local EDS virus**

I. INTRODUCTION

Egg-laying farms often experience losses due to the drastic declining of egg production and decreased quality of the eggs. One of the causes is a viral disease called Egg Drop Syndrome (EDS), of which is a poultry viral disease marked by declining production and quality of the eggs. EDS cases were first reports in 1976 in the Netherland [1], therefore called Egg Drop Syndrome-1976abbreviated as EDS-76. The agent causing the disease was a type A Duck Adenovirus, of the Atadenovirus genus, and the Adenoviridae familiae. EDS virus are grouped as non-enveloped double strand DNA virus, with a diameter of 74-80nm, 33.2kb genome, with only one serotype; duck adenovirus serotype 1 [2][3]. The EDS-76 virus attacks egg-laying chicken at the age of production peak (25-26 weeks). Clinical symptoms of EDS are marked by decreased egg production by 22,56% with a declined in quality of eggs (smaller egg size, unevenly shaped, thin and fragile shell, and paler color of the egg) [4]. Economic losses due to EDS are high which can directly influence the Food-product stability in Indonesia.

Clinical signs of EDS in egg-laying chicken are difficult to observe because chicken do not show signs of illness. EDS are only recognized when there is a decrease in production with a declining quality of eggs with paler shell, smaller in size with a very soft shell. Decreased egg production can reach up to 40% [5] and occur for 4 to 10 weeks. EDS disease causes high economic losses in poultry farms around the world. The EDS virus infects breeding farms, and spreads to other farms through infected eggs which are found almost all around the world.

Prevention of EDS is done through vaccination. Vaccination could protect egg-laying chicken from EDS up

to 96.5% which are marked by resistance of decrease in production and quality of the eggs [6]. There is a relation of the immune system with the quality and quantity of chicken eggs [7]. Currently, EDS vaccines have been used to prevent EDS diseases, egg-laying chicken are being vaccinated but EDS cases remains throughout the world. The failure of vaccination is one cause of the EDS diseases. Various factors contribute to the vaccine failure, one of them is the product of vaccine being used. Vaccine that is not properly attenuated can be a cause of the vaccine failure, such example of the Infectious Bursal Disease vaccine failure in egg laying chicken [8].

The vaccine potential to produce antibody titers post-vaccination is influenced also by the virus content of the vaccine. The basis of viral content is conducted through virus titration and continued by inoculation in embryonated egg or in cell cultures [9][10]. The content of virus is stated in Egg Lethal $Dose_{50}$ (ELD_{50}) with the embryonated eggs and is stated as Tissue Culture Infective Dose-50 (TCID50) with the cell culture production.

In Indonesia, EDS is categorized as a "Strategically Infectious Disease" which must be eradicated because it could influence the Food Welfare. EDS vaccines are successful if the post-vaccination antibody titer are high (≥ 16 HA Unit) with a high prolonged duration of immunity [10] that protects poultry farming from EDS. Vaccines are considered good if it is substituted from a free isolates from specific cases. Therefore it is needed to conduct a research of EDS seed vaccines using free isolates. An early stage of the research is to isolate and characterize EDS viruses from field cases to collect appropriate EDS seed vaccines. Biological test as well as free isolate molecular test is conducted based on the golden standard procedure [10].

Biological test towards EDS seed vaccines in embryonated eggs is useful to know the ability of the viral antigen to infect the host. Identification of EDS virus with haemaglutination (HA) has a purpose to know the ability of the virus to agglutinate 1% of chicken red blood cells and continued by Haemaglutination Inhibition (HI), the viral content of EDS is tested with Virus Content Test (VCT) through passage of an eleven day duck embryonated egg. The molecular characteristic of DNA construction can be found using a Polymerase Chain Reaction (PCR) continued by sequencing to see the relations of EDS viruses from the free isolates. With the characterization of the EDS viruses, it makes it possible to select the right seed to be used as EDS vaccine which matches the EDS virus found in the fields.

II. RESEARCH METHOD

Field-Free Samples

Field free isolates are collected from 10 egg-laying farms in some parts of Indonesia; Rumpin (Bogor), Medan, and Surabaya. Cases of EDS samples are collected form chicken egg farms that have a decrease in production and quality of the egg (weak and fragile egg shells). Chicken organs that are collected are reproduction system (uterus and oviduct).

Propagation of EDS Virus

Uterus and oviduct samples made intoinoculums with a 5% concentration with a sterile Phosfat Buffered Saline (PBS) pH 7,2. The inoculate is then inoculated in an 11 day old duck embryo through its allantoic space. As much as 0,2 ml of inoculate per eggs is injected through the allantoic space, then incubated in an 37oC incubator for 5 days in a daily candling. The deaths of the embryo ≤ 24 jam are ignord and considered to be contaminated. The egg with a dead embryo ≥ 24 jam are then collected and kept in a cool room of 2-8oC. Day 5 post inoculation, all eggs are removed from the incubator and kept in the cool room overnight.

Haemaglutination Test

As much as 25 μl PBS is filled into a micro-plated well (v-shaped) from 1 to 12. EDS viral suspension is added as much as 25 μl into well number 1 then homogenized with a micropipette. The homogenate from well 1 is moved 25 μl into the next well (well number 2). The same procedures are conducted in number 3 up to number 11. Beginning at well number 11 the suspension is thrown away as much as 25 μl, but well number 12 is not filled with suspension and is used as control. All the wells (from number 1 up to 12) are added 25 μl of PBS. A 1 % erythrocyte suspension is then added to all the well (number 1 to 12) as much as 25 μl. Homogenation is conducted with a shaker then samples are incubated for 30 minutes at room temperature. The HA test readings are done by tilting the v-bottomed micro plates into a 45^o angle. The HA test is positive if the micro plate well shows a sandy-like grains.

Haemaglutination Inhibition Test

Hemaglutination Inhibition test is used to define the agent of EDS disease. As many as 0,025ml of PBS are each filled into the v-shaped micro plate wells. The first well is filled with 0,025 ml serum then diluted in series every two folds from well 1 to 10, then at well number 10, 0,025 ml of suspension is thrown out. Each micro plate well is added with 0,025 ml of antigen suspension of EDS at 4 unit HA. Well number 1 to 11 is then shaken for 15 second with a micro shaker and left for 30 minutes in room temperature. The 1% red blood cell suspension is added to well number 1 to 12 as much as 0,025 ml then shook for 15 seconds, then incubated at room temperature for one hour while observing any blood sedimentation at the base of the wells. HI readings are conducted after well number 11 showed agglutination of erythrocyte and at well 12 that showed erythrocyte sedimentation. HI titers are read by tilting the micro plate at a 45 degree angle and observing if there is any drop-shaped red blood cell (tear-shaped). HI titer is

assessed by observing the highest serum dilution that is able to inhibit erythrocyte agglutination as a positive sign of the HI test.

Molecular Detection

EDS virus samples from allantoic fluid of duck eggs are extracted with Trizol® to be assessed by PCT. PCR amplification is conducted with an enzyme SuperScript TM III onestep RT-PCR System with Platinum® Taq DNA Polymerase (Invitrogen). A PCR cycle is conducted with a temperature of 50° C for 1 hour, 95° C, for 7 minutes, 94° C for 45 seconds, 52° C for 45 seconds, and elongation at 72° C 1 minute and 30 seconds. The first cycle is repeated 44 times. The enzyme refinement is conducted at 72° C for 5 minutes to achieve perfect fragments using My Genie Thermal Blok. The front primer sequence was EDSH4F (CACCGATAAAGGTGTCACAGAG) while the back primer was EDSH5R (AGTACCACGTTGAGTTGCTGTG). Observation of PCR results was conducted with electrophoresis. As many as 4 µl of PCR product is added with a loading dye (bromphenol-blue and cyline cyanol) of 1 µl, then undergoes electrophoreses on the gel of 1% concentration (0,5 gram agarose dalam 50 ml TAE). Red Gel of 5 µl was used with 100-bp ladder (Invitrogen) as a marker. DNA Visualization is conducted with ultraviolet (UV) transluminatorwhich was documented with a Polaroid camera. PCR results are then read through its base length that was amplified.

Viral Content Test

Allantoic fluid was diluted in series every 10 folds with sterilePhosfat Buffer Saline (PBS). EDS virus is then titrated, starting a dilution of 10-4 up to 10-8, virus was inoculated each in 5 embryonated duck eggs at 11 days old through the allantoic space, then incubated for 4-5 days. After 5 days. All eggs with dead embryos more than 24 hours are taken out of the incubator and kept in a cool room at 2-8 ^{o}C overnight. The harvested allantoic fluid are then separated and centrifuged and inseerted into a sterile tube then are tested with the HA/HI tests. The viral content of EDS is tested with the Reed and Muench method to define the Egg Infectious Dose-50 (EID_{50}). Repeated passaging is done towards EDS virus until it has produced a titer of $\geq 2^{10}$ to be chosen as a EDS seed vaccine.

III. RESULTS AND ANALYSIS

Isolation and Virus Identification Results

As many as 10 free isolate samples that are propagated into the 11 day old embryonated duck eggs, it was found that six were positive of the EDS virus. It showed differences with the most of embryonated chicken eggs that are commonly used to isolate virus, instead the EDS virus growth was better when isolated in embryonated duck eggs of 10 days old through the allantoic space. [5].

Biological test are marked by the death of embryo on the 4th day after the inoculation of the free inoculate. Cha *et al*., (2013) stated that the death of embryos occurred 5 days after inoculation, but in this study, the death of the embryos occurred on the 4th day after the inoculation [11]. The test result of allantoic fluid through haemaglutination test (HA) showed a positive result of EDS virus which was marked by the formation of haemaglutinate (sandy-grains) when the harvested allantoic fluid was added with 1% of poultry red blood cells (as seen in Fig. 1).

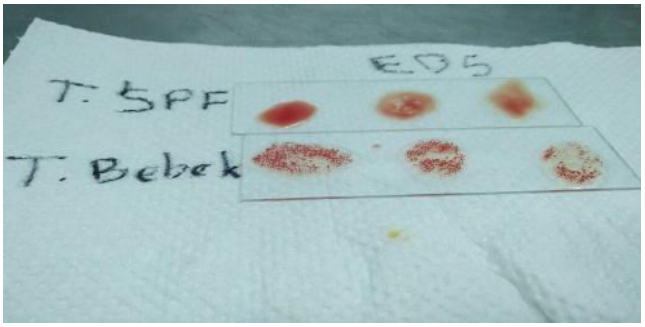

Fig. 1. Haemaglutination test (HA) of harvested allantoic fluid of embryonated chicken egg and duck eggs, 5 days post inoculation.

Antigen titer from free isolate as results of embryonated duck eggs propagation varies with the lowest titer of 2^4 and the highest of 27 HA Unit. A complete data of the free isolate are found in Table 1. These data includes: Isolate codes, place of origin of the isolates, organs collected, time of embryo death after inoculation, and the antigen titer.

TABLE 1.
CODES, ORIGIN OF FREE ISOLATES, ISOLATE SAMPLES, AND TIME OF EMBRYO DEATH POST INOCULATION OF FREE-FIELD EDS.

No.	Free Isolate Codes	Isolate Origin	Sample	Embryo death (post inoculation)	Virus Titer (HA Unit)
1	1793037	Surabaya	uterus and oviduct	Day - 4	2^6
2	1793038	Rumpin	uterus and oviduct	Day – 4	2^4
3	1793039	Rumpin	uterus and oviduct	Day - 4	2^5
4	1793040	Rumpin	uterus and oviduct	Day – 4	2^6
5	2145945	Medan 1	uterus and oviduct	Day – 4	2^6
6	2145946	Medan 2	uterus and oviduct	Day - 4	2^7

Haemaglutination test is a routine method to detect viruses that are able to agglutinate red blood cells including those to detect EDS virus. The haemaglutination process occurs due to protein activity of haemaglutinine on the virus envelope that reacts with 1% of chicken erythrocyte. The EDS virus has a haemaglutinine that enables haemaglutination of 1% of chicken erythrocyte. %. Rasool et al. (2005) stated that

EDS virus only agglutinates red blood cells of poultry but does not agglutinates red blood cells of mammals.

To confirm that the free isolates are EDS viruses, it is tested with the Haemaglutination Inhibition Test (HI) using a standard EDS serum from PT Sanbio. HI test results shows positive EDS virus with a titer of HI = 2^4 up to 2^7 HA unit). Rasool et al., (2005) reported that EDS viruses replicates well in embryonated duck eggs and are able to produce a high viral titer. Banani et al. (2007) also reported that virus titer after allantoic fluid harvest of embryonated duck eggs are 1/16000 – 1/32000. During the HI test, the viral characteristic that shows the ability to agglutinate red blood cells was detected, therefore the HA/HI test are the accurate test to be used to identify free EDS viruses.

The Medan isolate was able to be isolated and then passaged back into the 11 day old embryonated duck egg. The purpose of the passage is to increase the virus titer. Other than duck eggs, passages are also conducted on the 11 day old SPF chicken eggs. EDS virus titer of the Medan isolates as a result of the second passage on duck eggs was 1012 HA Unit, while SPF chicken of Medan isolate did not developed. This shows that duck eggs are a good media to develop EDS Virus [12], however the EDS virus did not developed in SPF chicken eggs (Fig. 1). Titer result of Medan isolate shows the viral content was $10^{9,5}$ EID^{50}.

The result of molecular detection with PCR showed all free isolates that was tested were positive to be EDS viruses. DNA electrophoresis result was marked by the HA fragment that was amplified and has a base length of 500 bp (Fig. 2).

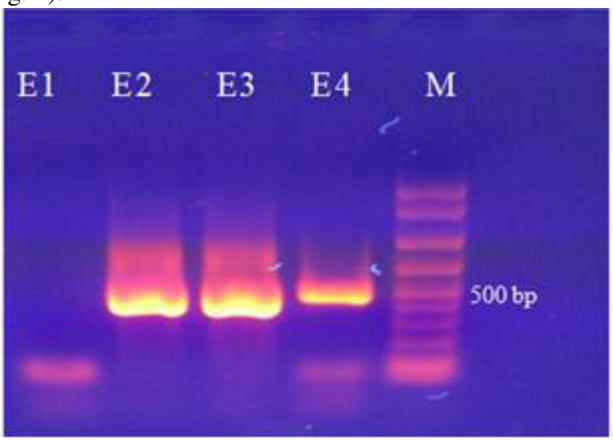

Fig. 2. Amplification results of Medan Isolate with EDS primer. M: marker, E1: negative control (aqubides), E2: positive control of EDS virus, E3: EDS virus after titration, E4: EDS virus after inactivation.

Characteristic of EDS Viruses can be molecularly tested using PCR [13][14]. PCR technique is used to detect EDS virus quickly and accurately because PCR has a sensitivity and specificity. PCR assay needs less time compared to virus isolation method and does not need any specific antiserum and live amplificated virus. Therefore the chance to be infected by the zoonosis virus can be prevented because PCR does not use live viruses. Other than safe, PCR are also sensitive because it only needs 1µ of antigen. PCR is an accurate test to confirm EDS viruses categorized as Adenovirus [15]. Compared to HA test, PCR testing are more sensitive because some samples that was shown negative by HA testing was actually positive of EDS virus when tested by PCR [16].

IV. CONCLUSION

Six free isolates have been isolated and were positive of EDS virus. The 11 day old embryonated duck eggs are preferred as a media to reproduce EDS viruses for Medan isolates while SPF chicken eggs was otherwise. Characterization of Medan isolate EDS virus shows a titer of 10^{12} HA Unit on the second passage, with virus content of $10^{9,5}$ EID_{50}, and PCR base length of 500 bp. Therefore EDS virus of Medan Isolate is recommended as a candidate of EDS seed vaccine.

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