PLAQUE ASSAY OF NEWCASTLE DISEASE VIRUS

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ABSTRACT

The Newcastle disease virus (NDV) was isolated from a 3 months-old indigenous chicken (buras or kampung chicken) which showed clinical signs of Newcastle disease (ND). For viral isolation a small part of the spleen and lung were inoculated into 10 days-old embryonated chicken eggs. The physical characteristics of the isolate (A/120) were studied. The hemagglutination of chicken red blood cell showed slow elution, thermostability of hemagglutinin at 56^0C was 120 minutes. The virus was able to agglutinate horse erythrocytes but not those of sheep. The biological characteristics on mean death time (MDT) of embryonated chicken egg and plaque morphology on chicken embryo fibroblast (CEF) primary cell cultures were studied. The MDT was 56 hours, the isolate was velogenic NDV. There were three different plaque morphologies on CEF : 2 mm clear plaques, 1 mm clear plaques, and minute clear plaques which were visible only with microscopic examination.

INTRODUCTION

The plaque has been applied to many investigations ofvirus cell systems. Although several variations have been developed, the basic procedure essentially remains the same as developed by Dulbecco and Vogt[1]. Monolayer cell cultures are grown in suitable vessels, petri dishes, glass bottles or disposable plastic, until a confluent monolayer is obtained. The fluid medium is then removed and the confluent monolayer cell cultures are washed in a balanced salt solution to remove traces of residual growth medium and serum. A serial dilution of viral suspensions are inoculated on the cell monolayer in enough volume to just cover the monolayer cell. A predetermined period of time is allowed for maximum virus adsorbtion. The nonadsorbed viruses are removed by several washings with balanced salt solution. The infected monolayer cell culture is then covered with an overlay medium consisted of maintenance medium added with agar or other solidifying agents. After a period of time which varies with different virus-cell systems, the infecting virus replicates. The progeny viruses are released from the infected cell and infect the contiguous cells. The overlay medium allows the diffusion of viruses to the contiguous cells, but it prevents the convection of viruses to the other region of the monolayer cell culture. The primary infection which might be by a single virion on a cell results in a group of infected cells which undergoes degeneration, physiological alteration and/or lysis, which is called a plaque. In order to visualize the plaques, a vital dye, neutral red, is either incorporated into the primary solidified overlay, secondary solidified over-

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lay, or added through the solidified overlay after the plaques have developed. The plaque will be easily visualized as a defined clear area against a stained background of monolayer cells.

The morphology and the size of the plaque vary with the virus - cell system. The appearance of the plaque on monolayer cell culture after virus inoculation depends on the viruses and the cell used for the plaque assay. The plaque can develop as early as 24 hours or as late as 6 days after virus inoculation.

Newcastle disease virus belongs to the genus paramyxovirus, together with mammalian parainfluenza viruses type 1-5 and mumps virus. Paramyxovirus isolated from birds has been effectively regarded as a subgenus of paramyxovirus, and NDV is grouped in avian paramyxovirus-1. There are many isolates and strains of NDV, but they do not show antigenic differences by regular serological tests to classify them in serotypes[2]. The strains differ in their virulence for day-old chickens, 6-to-10-week-old chickens, and for chicken embryos. Primarily on the basis of virulence, NDV strains are differentiated into lentogenic, metogenic, velogenic neurotropic and velogenic viscerotropic pathotypes[3], which are avirulent to the most virulent respectively.

There are some markers which can be used to differentiate NDV strain, plaque morphology[4], elution time of the virus from hemaglutinate[5], heat stability of the hemaglutinin, and the ability of NDV strain to hemagglutinate mammalian erythrocyte[2]. Schloer and Hanson[4] recognized 6 plaques morphologies out of 14 NDV strains on chicken embryofibroblast (CEF) cell culture. The virulence of NDV strains can be determined from the size and heterogenicity of the plaques.

The virulent strain usually shows heterogenicity in size of its plaques, varies from small to large plaques, and the virulent strain correlates with the larger plaques[6]. Physical characteristics of the strains, elution time of the chicken erythrocyte, heat stability of the hemagglutinin, and the ability to hemagglutinate mammalian erythrocytes, do not have correlation with the virulence, but can be used to differentiate strains[2,7,8]. Studies using monoclonal antibody in hemagglutination inhibition (HI) test, and ELISA test showed that strains can be differentiated : lentogenic from mesogenic and velogenic strains[9,10]. Since 1926 when ND was first reported in Indonesia, the disease persists enzootically, sometimes epizootically. ND imposes a big hazard and produces high economic lash to poultry industries in Indonesia.

This study tries to characterize an NDV strain isolated from buras or kampung chicken, as the beginning of further studies on antigenic differences of Indonesian NDV strains, vaccine development, looking for lentogenic strains through biotechnology.

MATERIALS AND METHODS

Eggs

Fertile eggs for embryonic inoculation and chicken embryo fibroblast cell cultures were obtained from a farmer raising harcow layers with buras chicken roaster.

Virus isolation

Specimen for virus isolation was spleen and lung taken from 3 months old buras chicken showing clinical signs of ND. A 10% tissue suspension was prepared in sterile phosphate-buffered saline (PBS). The

suspension was clarified by centrifugation at 3000 rpm for 10 minutes; the supernatant was removed, and 10.000 u penicillin and 10 mg streptomycin (per ml supernatant) were added, and incubated at 37^0C for 3 hours. After incubation, the supernatant was inoculated (0,2 ml) into the allantoic sac of five 10-day-old embryonating eggs. The eggs were candled everyday for 6 days, the eggs with a dead or moribund embryo were removed from the incubator and kept in the refrigerator overnight. The next day the allantoic fluid was harvested, and rapid HA and HI tests were performed on glass slides to check the presence of the virus.

Cell culture

Primary chicken embryofibroblast (CEF) cell cultures were prepared from 10-day-old decapitated, eviscerated, crushed, and trypsinized embryos. The cell suspension was washed 3 times with PBS, cells which were alive were counted (day exclusion method with typhan blue), and the cell suspension was adjusted to 2×10^6 cells/ml in growth medium. Minimum essential Eagle medium supplemented with 10 % fetal calf serum (FCS), 100 μ/ml penicillin, 100 mcg/ml streptomycin, and 0,25 mcg/ml fungizon was used. In each 60 x 13 mm petri dish, 5 ml cell suspension was seeded, and then the petri dishes were incubated in a CO_2 incubator.

Plaque assay

Plaque assay was done. In brief, confluent CEF cell cultures were washed 3 times with PBS, 0,1 ml virus dilutions were inoculated to each petri dish; 60 minutes were allowed for virus adsorption, CEF cell cultures were washed again for unadsorbed virus, and 5 ml of melted overlay medium was poured in to each petri dish. The overlay medium was allowed to solidify at room temperature for 15 minutes. Before that the petri dishes were incubated in a CO_2 incubator for 72 hours. Virus dilutions 10^{-4} - 10^{-8} were used, 5 plates for each dilution. Secondary overlay medium was poured to each plate, 5 ml each, and after 24 hours of reincubation of the plate, plaques were observed.

Overlay medium

The first overlay medium consisted of MEM, 3 % FCS, 100 μ/ml penicillin, 100 mcg/ml streptomycin, 0,25 mcg/ml fungizone, 1 % purified agar. The composition of the second overlay medium was the same as the first overlay medium, but 0,001 % neutral red was added.

Hemadsorbtion and hemadsorbtion inhibition test

The overlay medium was removed carefully, and the monolayer cell culture was washed 3 times with PBS, and 1 ml 2.5 % chicken erythrocytes (CE) in PBS was added. After 15 minutes, the monolayer cell was washed again carefully and thoroughly 3-5 times with PBS, and 1 ml medium without serum was added. In the hemadsorbtion inhibition test, before the addition of CE, 1 ml chicken serum containing a high titer of anti ND Virus was added, after 15 minutes the monolayer cell culture was washed 3 times with PBS. The hemadsorbtion and hemadsorbtion inhibition was observed under the inverted microscope[2].

Elution pattern

This test was carried out as described[5], a microplate was used instead of a tube. Serial dilution of the virus suspension, 2^n was car-

ried out with a micro diluter, and 0.5 % CE in PBS was used. Hemagglutination was observed at 1,2,4,8,12, and 24 hours after the addition of CE. After 24 hours the hemagglutinate was resuspended and the presence of hemagglutination was recorded. Negative hemagglutination means rapid elution, and positive means slow elution.

Thermostability of the hemagglutinin

Chorioallantoic fluid containing NDV, which has been cleared by centrifugation, was distributed in 7 tubes, 1 ml each. One tube as a control, the other tubes were put in a 56^0C waterbath. After 5,10,15,30,60 and 120 minutes, one tube was removed and put in ice water for 15 minutes[11]. Hemagglutination test was carried out on a microplate.

Erythrocytes

Chicken blood was taken from the brachial vein, horse and sheep blood were taken from the jugular vein, Alserver's solution was used as anticoagulant. Erythrocytes were washed 3 times with PBS, and resuspended to 0.5% (v/v) or 2.5% erythrocyte suspension in PBS.

RESULTS

Plaque morphology

Confluent monolayer CEF cell cultures were obtained in 24 hours after cell seeding. Newcastle disease virus, isolate A/120, produces a clear plaque in three different sizes, 72 hours after virus inoculation. The clear plaques have a sharp margin, and are 2 mm, 1 mm, and less than 1 mm in diameter. The smallest plaque can be observed only under the microscope. If the hemadsorbtion and hemadsorbtion inhibition tests are positive, the plaques are due to NDV replication. As a control, La sota vaccine strain was used; it did not produce plaques when the overlay medium did not contain DEAE-dextran and Mg^{++}.

Elution pattern

Isolate A/120 showed slow elution, 24 hours after hemagglutinat (HA) test, the hemagglutinate was resuspended and hemagglutination was still positive. As a control La sota strain and B1 strains were used. They were slow and rapid eluters respectively.

Thermostability of hemagglutinin

Isolate A/120 showed thermostability in 120 minutes. As a control, La sota and B1 strains showed thermostability in 5 minutes only.

Mammalian erythrocyte hemagglutination

Isolate A/120 showed positive hemagglutination with horse erythrocytes, and negative with sheep erythrocytes. As a control, La sota strains showed positive hemagglutination with horse and sheep erythrocytes; and B1 strains did not show hemagglutination with neither horse erythrocytes nor sheep erythrocytes.

DISCUSSION

Newcastle disease virus varies in producing plaques in primary cell culture or cell line, CEF primary cell culture is used as a standard for NDV plaque morphology[2]. On CEF primary cell culture 6 types of plaques has been shown, clear plaques with a sharp margin, clear plaques with a red margin, clear

plaques with some viable cells in the center, dark red plaques, turbid red plaques, and plaques alternating red and clear with a red color in the center[4]. The size of the plaques varies, small plaques < 1 mm, medium plaques >1.5 - 2.5 mm, and large plaques 2.5 mm. The heterogenicity of the plaques and the presence of large plaques are usually associated with velogenic strains[6]. Isolate A/120 shows three different sizes of plaques, so it might be categorized as velogenic. Kawamura et al.[7] isolated NDV from caged birds, *Cacatua Sirphurea,* which was imported from Indonesia. The isolate showed biological characteristics, 1 - 1.5 mm clear plaques, mean death time of chicken embryo 45 hours, intra cerebral index 2.0, which are the characteristics of velogenic NDV.

Table 1. Physical characteristics of A/120, La sota, and B1

Isolate Strain	Thermostability of hemagglutinin (minutes)	Mammalian erythrocyte hemagglutination		Elution pattern
		horse	sheep	
La sota	5	+	+	slow
B1	5	-	-	rapid
A/120	120	+	-	slow

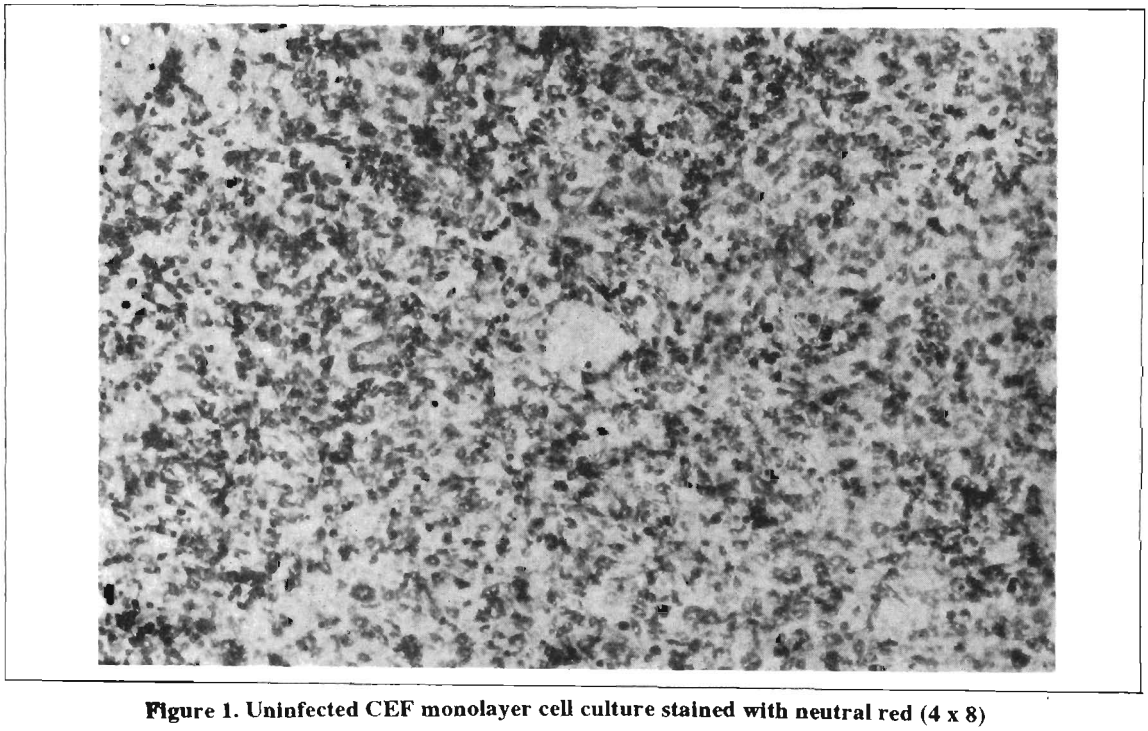

Figure 1. Uninfected CEF monolayer cell culture stained with neutral red (4 x 8)

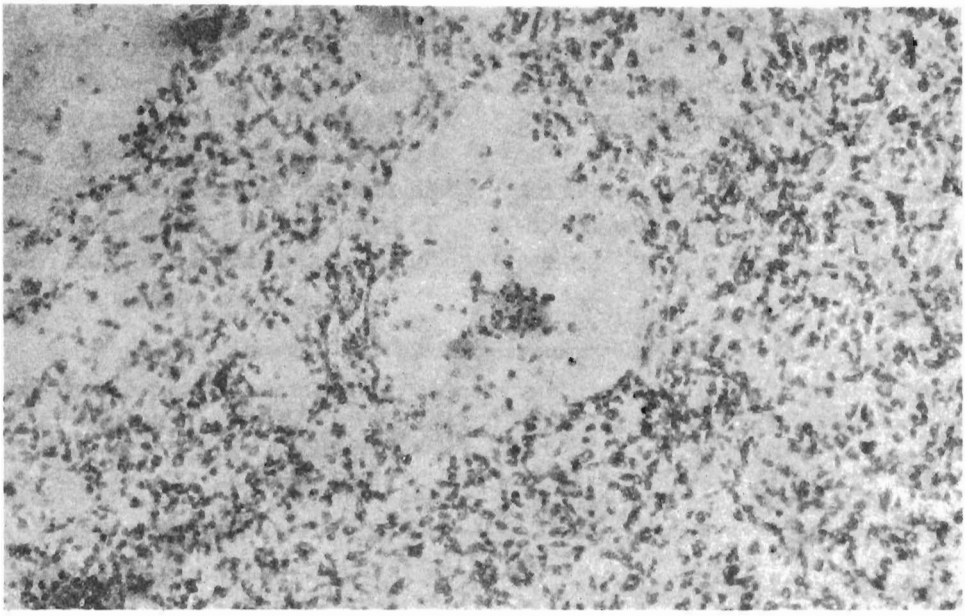

Figure 2. Plaque size of 1 mm, showing positive hemadsorbtion

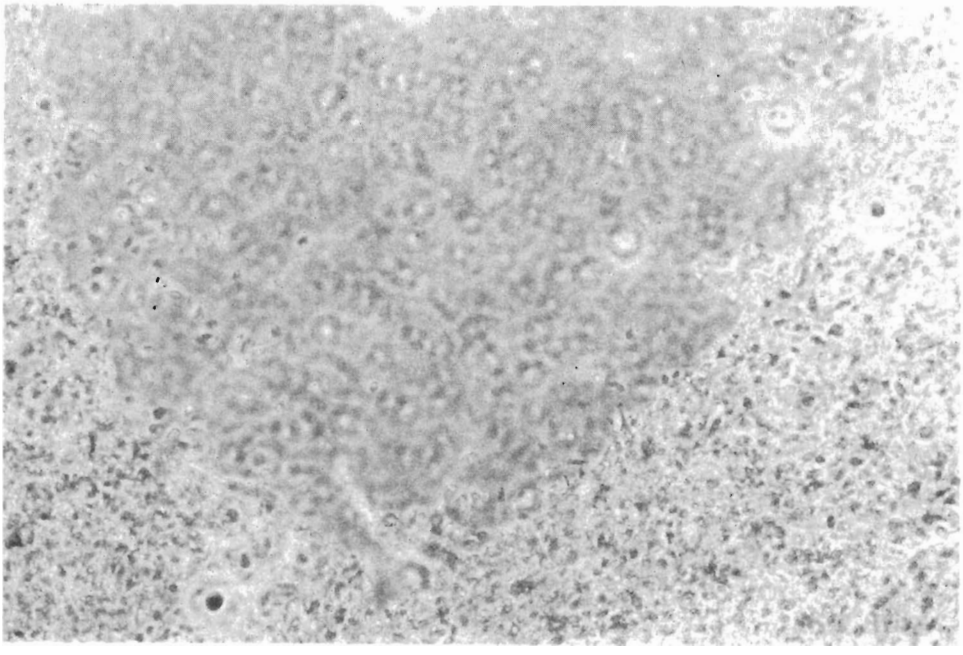

Figure 3. Plaque size 1 mm, positive hemadsorbtion

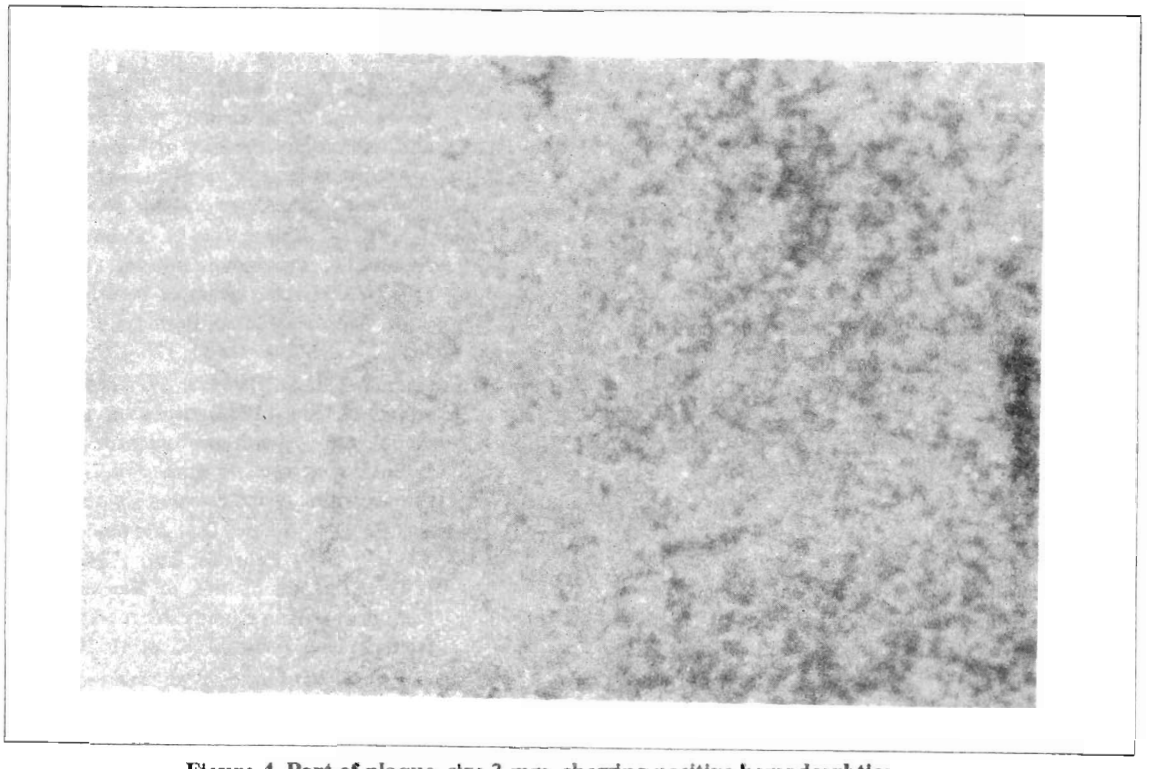

Figure 4. Part of plaque, size 2 mm, showing positive hemadsorbtion

Based on the physical and biological characteristics NDV strains or isolates can be differentiated. Schloer et al.[12] using a plaque assay, hemagglutinin and neuraminidage activity, and kinetic neutralization, differentiated old established NDV strains in California and velogenic viscerotropic NDV isolated in the ND out break in 1971-1973. Rosenberger et al.[13] also differentiated NDV isolated from migratory ducks, *Anas platyrincos*, in the Atlantic flyway, and NDV isolated from chickens. Their differentiation was based on physical and biological characteristics of the isolates. This differentiation is cumbersome and not every laboratory has the facility and expertise to do it.

Another method to differentiate NDV strains or isolates is using monoclonal antibodies in ELISA and HI test.

Srinivasappa et al.[9] developed monoclonal antibodies used in ELISA, which could differentiate lentogenic vaccine strains from mesogenic and velogenic strains. Peason et al.[10] also used monoclonal antibodies in identifying NDV isolated from pigeons. They used HI and indirect immunoperoxidase test using Madden Darby bovine kidney cells infected with the isolates. It seems more simple, the use of monoclonal antibody, when it is available commercially, but the technics used are still out of reach by Indonesian laboratories. To catch up with cur-

rent technics, Indonesian researchers need more training and facilities.

CONCLUSION

1. Newcastle disease virus isolate A/120 plaque morphology is heterogenic; it produces three plaques, different in size, < 1 mm, 1 mm, and 2 mm in diameter. The isolate could be considered as velogenic pathotype.
2. The physical characteristics of the isolates are : slow eluter, heatstability of hemagglutinin 120 minutes, positive hemagglutination with horse erythrocytes and negative with sheep erythrocytes[5].

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QUESTIONS AND ANSWERS :

1. Question: What reason do you think that NDV do not show serotypes ? Is this a kind of exception to wellknown presence of group specificity in virology ? Can monoclonal antibodies dicriminate the NDV strains (serotypes) ?

   Answer : The regular/conventional serological tests are not sensitive enough to differentiate small antigenic differences among NDV strains. Two kinds of monoclonal antibodies have been developed, one which reacts only with avirulent lentogenic strains, the other which reacts only with velogenic virulent strains.

2. Question: Are there strains of New Castle Disease virus which do not produce plagues in CEF cell culture ? Thank You.

   Answer : Yes there are. Lentogenic vaccine strains, F.B., La sota do not produce plagues, using regular overlay medium. When Mg^{++} and DEAE - dextran is added in the overlay medium, these vaccine strains produce small plagues. (DEAE = diethylaminoethyl).

3. Question: - What is the source of antibody used in HI tests ?
   - What is the property of the monoclonal antibody :
   - Hemagglutinating or
   - Neutralizing
   - Others ?

   Answer : The source of antibody used in HI test is chicken immunized with NDV vaccine.
   The property of the monoclonal antibody is neutralizing and inhibiting hemagglutination, since the monoclonal antibody is directed to epitope on hemagglutinating antigen or HA on the surface of the virus.
   The HA antigen is neutralizing antigen, and an antigen responsible in hemagglutination.