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MOLECULAR AND SEROLOGICAL IDENTIFICATION OF NEWCASTLE DISEASE VIRUS PROPAGATED IN EMBRYONATED CHICKEN EGGS

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ABSTRACT

The aim of this study is to propagate the non-virulent Newcastle disease virus in the laboratory, determination the cytopathic effects in the inoculated chicken embryos, and confirmation of virus growth by serological and molecular techniques by performing haemagglutination and reverse transcriptase polymerase chain reaction (RT-PCR) tests, respectively. LaSota virus strain which is a live vaccine was used for this purpose. Nine-day-old embryonated chicken eggs were inoculated with the virus and further incubated for 48 hours; and the allantoic fluid was collected for further processing. Petechial haemorrhages and congestions were observed in the inoculated embryos while in the un-inoculated eggs; the embryos were normal and did not show any lesion. Virus growth in the allantoic fluid was confirmed by performing haemagglutination and RT-PCR tests. These results support the isolation of other viruses in our laboratories, which will contribute to perform other experiments such as studying virus characteristics and observation of its pathological effects on the embryos, preparation of viral antigens, sequencing of viral genome, and possibly discovering new viruses.

INTRODUCTION

Newcastle disease has been one of the most important poultry diseases worldwide. The causative agent of this disease is Avian Paramyxoviruses type-1 which is classified under the genus *Avularius*, family *Paramyxoviridae* (1). Newcastle disease viruses (NDV) are divided into three main groups: virulent (Velogenic), moderately virulent (Mesogenic) and non-virulent (Lentogenic). These strains differ in the number of amino acids at the site of cleavage of the fusion protein (2,3). Clinical signs of the infected birds are vary greatly according on the species of bird, strain of virus and preexisting immunity (4,5). The non-virulent strain mainly LaSota has been approved to be used as a live vaccine throughout the world, which gives good protection against the virulent and moderately virulent strains if they administered correctly to health birds (6).

Diagnosis of the disease is usually done in the laboratory using some techniques such as reverse-transcriptase polymerase chain reaction (RT-PCR), haemagglutination (HA), haemagglutination inhibition (HI), and Enzyme Linked Immunosorbent assay (ELISA). In addition, virus inoculating the allantoid cavity of the embryonated eggs is widely used (7). The HA assay is the most common indirect method for estimating the quantities of virus particles, generated from allantoic fluid or cell culture supernatants. This assay is based on the fact that several viruses contain a protein that can bind and clump red blood cells (8).

The aim of this study is to propagate Newcastle disease virus (non-virulent strain) in embryonated chicken eggs and to detect virus growth by serological and molecular techniques.

MATERIALS AND METHODS

VIRUS CULTIVATION AND HARVESTING

Fertile hen's eggs were used to propagate the viruses used in this study. LaSota virus strain. Working virus concentration of LaSota strain was prepared by diluting the virus stock 1:3000 in PBS containing 1% antibiotics (100 U/ mL penicillin and 100 ug/ mL streptomycin).

The fertilized eggs were first incubated at 37.5˚C for 9 days. During the period of incubation, the embryos were candled in a dark room using an egg-candling box. To determine the site of injection which is the opposite site of the embryo head, the air sacs were highlighted with a pencil. All the eggs that did not have developing embryos were discarded. The egg

surface was sterilized by wiping with ethanol. A small hole was made at the injection site using a special drill without any damage in the shell membrane. A small syringe (1 ml) was used to inoculate the eggs with the virus. The needle was passed through the chorionic membrane. The virus (0.1 ml) was injected into the allantoic cavity filled with allantois fluid. The hole was carefully closed with tape, and eggs were incubated at 37.5 ° C for 48 hours, and the allanoic fluid was then collected. Embryos were collected and moved in plastic Petri dishes to observe the cytopathic effects. Photos were then taken for documentation.

DETECTION OF VIRUS BY HEMAGGLUTINATION

Virus growth was confirmed by processing allantoic fluid to hemagglutination assay. Six well ceramic agglutination plate was prepared for the experiment. One drop purified RBC was mixed with another drop of allantoic fluid in the well. The negative control was represented by mixing one drop of RBC with another drop of PBS. After a few minutes of incubation at room temperature, the reaction was visualized and photos were taken for documentation.

VIRAL RNA EXTRACTION AND QUANTIFICATION

Extraction of viral RNA from allantoic fluid was performed using a QIAamp viral RNA purification kit (Qiagen, Germany) according to the manufacturer's protocol. The concentration of the extracted RNA was determined using NanoDrop spectrophotometer by UV absorption. Eluted viral RNA samples were kept at –20˚C for further use.

REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION

One Step RT–PCR kit (Bioneer) was used this purpose. A forward primer 5'-TAC AAC AGG ACA TTG ACC ACT TTG CTC AC-3' and reverse primer 5'-TGC ATC TTC CCA ACT GCC ACT GC-3' were used to amplify 299 base pare (bp) fragment. Gene specific primers were used for cDNA synthesis and PCR amplification in a single tube. Starting material of viral RNA used in cDNA synthesis was 100 ng/µl. The PCR conditions were: cDNA synthesis at 45˚C for 30 min, initial denaturation at 95˚C for 2 min followed by 30 cycles of: denaturation at 95˚C for 10 s, annealing at 58˚C for 20 s, and extension at 72˚C for 30 s. The reaction was then held at 72˚C for 5 min, and then cooled down at 4˚C for 5 min. The amplified PCR product was detected using 1.5% agarose gel prepared with agarose (Promega) in TAE buffer. The amplicon size was estimated by comparison with a standard DNA ladder.

RESULTS

DETECTION OF CYTOPATIC EFFECT IN CHICKEN EMBRYOS

Cytopathic effects represented by represented by petechial haemorrhages and embryo distortion were observed on the embryos following inoculation with the virus. In comparison, the uninfected embryos (control group) showed no sign of pathological lesions (Figure1).

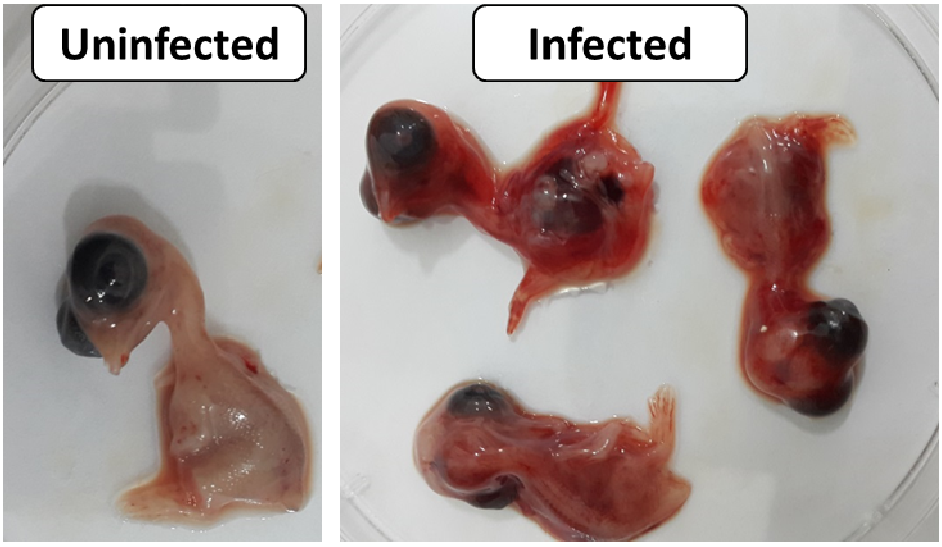

Figure 1: Chicken embryos inoculated with the virus.

DETECTION OF VIRUSES BY HEMAGGLUTINATION

Positive haemagglutination results were visualized after mixing allantoic fluid with RBCs, which confirm the growth of the virus. A negative result (no evidence of haemagglutination) was shown in the control group (Figure 2).

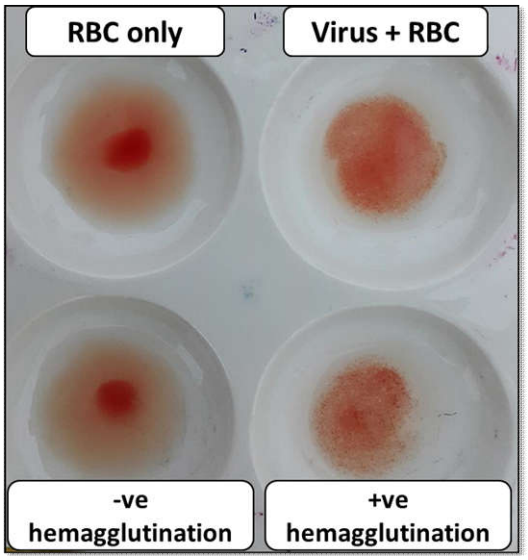

Figure 2: Confirmation of virus growth by haemagglutination test**.**

DETECTION OF VIRUSES BY RT-PCR

PCR product of partial polymerase (F) gene of Newcastle disease virus (LaSota) were separated on 1.5% agarose gel pre-stained with ethidium bromide. The results showed the amplification of 299 bp from allantoic fluid collected from inoculated eggs with the virus (Figure 3).

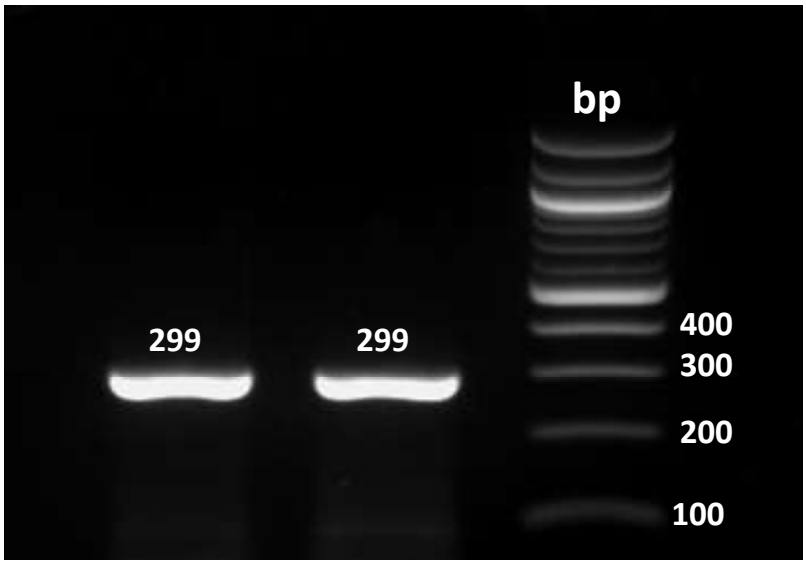

Figure 3: Detection of virus growth by RT-PCR.

DISCUSSION

Newcastle disease virus is a good example of studying determinants of viral pathogenicity. There are certain strains of this virus that play a role in causing important agricultural diseases in birds in general and poultry in particular with a high mortality rate. On the other hand, there are non-virulent strains (such as the LaSota strain) and are often used as vaccines. The use of this type of virus in the laboratory, and because of the safety of dealing with it, is of great importance in studying the pathogenicity of the virus on chicken egg embryos as well as cell cultures (9).

In this study, a non-virulent virus was successfully grown in embryonated chicken eggs to obtain a high titer of virus stock, which is necessary for performing further in vitro experiments. Among these experiments are studying the structure of viruses by determining the nucleotide sequence in the viral genome, as well as preparing viral antigens, which are important in performing various laboratory techniques such as ELISA, neutralization tests, haemagglutination and haemagglutination inhibition. In addition, amplification of viral genes

would be useful for the preparation of DNA probes to detect the full length of viral RNA by northern blotting technique, and for sequencing the viral genome to detect any nucleotide variations between virus strains (10).

The LaSota virus strain has been cultivated in chicken eggs for the production of vaccines (7). In this study, virus was not grown for virus production, but was for obtaining stock of viruses important to perform further studies. In this regard, it is highly recommended to prepare antigens from the new generation of viruses using various laboratory methods. Moreover, it is important to determine which viral antigen should be isolated (for example, the type of antigen used in the ELISA differs from that used in the haemagglutination test). Furthermore, it is also recommended to sequence the PCR product and align the sequences with GenBank to highlight differences between other strains and to study the effect of these differences.

The types of methods described above may lead to the potential new results, which will help in preparing the different types of antigens needed for a variety of *in vitro* techniques, and thus require further studies.

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