Preparation of an Inactivated Egg Drop Syndrome Antigen for Using in Hemagglutination Inhibition Test

Short Communication

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Summary

To prepare hemagglutination inhibition (HI) antigen the egg drop syndrome virus (EDSV) was propagated in 10-day-old embryonated duck eggs. The virus antigen was inactivated with two methods including heating (65°C) and adding 0.5% formaldehyde by considering low destroying effect on hemagglutination (HA) and HI titers. The EDSV HI titers of 310 sera and 100 yolks obtained from 23 chicken farms and specific pathogen free (SPF) chickens before and after EDS vaccination, in various ages were evaluated. Specificity, HA activity, stability and electron microscopic observations were similar in the prepared and standard antigens. The HI titers showed an equal sensitivity and specificity and, complete similarity (>99%) between the results obtained by prepared and standard antigens. Detection of antibody against EDSV using HI test showed slightly less sensitivity but more specificity compared with ELISA test. Upon the results it is suggested that inactivated EDS antigen prepared by either heat or formalin treatment could successfully be used in avian diagnosis laboratories for routine flocks monitoring, EDS vaccine controlling and also surveillance of EDS vaccination program in poultry industry.

Key words: egg drop syndrome virus, inactivated antigen, hemagglutination inhibition

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Introduction

Egg drop syndrome virus (EDSV), a hemagglutinating adenovirus, causes lost egg production in chicken flocks. Since initial description of egg drop syndrome (Van Eck *et al* 1976) EDS 76 has become a major cause of lost egg production in breeder and layer flocks and severe economic losses throughout the world (McFerran & Adair 2003). EDSV is different from other fowl adenoviruses because of agglutinating avian red blood cells (McFerran 1998). Although, shortly after the first description of the disease in Western Europe suspicious cases occurred in broiler parent flocks of Iran, but the virus infection that confirmed by hemagglutination inhibition (HI) test reported later (Aghakhan & Khodashenas 1990). To use in HI test, they produced EDS antigen without any inactivation. Pathogens inactivation is a necessary stage in antigen production because of the hazards of the infection spread. However, any effective method for complete disinfections might have some decrease in antigenicity of the virus. The HI test is the one of choice for diagnosis of EDSV infection and also for evaluation of vaccination and epidemiological surveillance of EDSV in poultry flocks (Baxendale *et al* 1980, Cook & Darbyshire 1981, Cook 1983, Adair *et al* 1986, McFerran & Adair 2003). Some laboratory host systems such as duck, goose, chick and turkey cell cultures, and embryonated duck or goose eggs have been used for EDSV propagation (McFerran 1998, McFerran & Adair 2003). Adair *et al* (1976) and Zsak *et al* (1982) reported the high titers of 1/16000-1/32000 by EDSV inoculation into the allantoic sac of embryonated duck or goose eggs but they observed no growth in the eggs. The purpose of this study was to prepare and evaluate an inactivated EDS antigen for usage in HI test.

Materials and Methods

Antigen preparation. EDSV was provided from Venzie-Padova Institute, Italy. Embryonated duck eggs were purchased from Couvoir de la Seigneurtier Co., France. The mean antibody HI titers of their yolks against EDSV were below than 6

based on $\log_2$. The virus was propagated in 10-day-old embryonated duck eggs inoculated via the allantoic cavity and after 2-7 days the allantoic and amniotic fluids were separately harvested. The eggs fluids were clarified by centrifugation at 1500*g* for 15min and the supernatants were collected and subjected to inactivation procedure. In order to inactivate the virus two methods including heat and formalin treatments were used. For heat treatment one ml aliquots of the virus pool were dispended into glass tubes and immersed in a water bath at different temperatures between 55-85°C for 30-60min. For formalin treatment different final concentrations of formaldehyde (Merck), 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 and 1% was prepared with PBS after adjusting to pH7.2. Each solution was mixed with an equal volume of the virus suspension containing known hemagglutination (HA) activity and incubated at 37°C for 30-60min. Inactivation of the virus pool was investigated by using injection of 0.2ml of it into the allantoic sac of 10-day-old embryonated duck eggs followed by two blind passages. The propagation of virus indicated an inadequate inactivation.

Evaluation of prepared antigens. To evaluate the prepared inactivated antigens some factors were considered as follows:

a) HA activity. The HA activity of harvested allantoic and amniotic fluids were determined using standard microtiter procedure. Serial two-fold dilutions of the antigens suspended in PBS were mixed with equal volume of 1% chicken erythrocyte suspension and incubated at 25°C for 30min. The reciprocal of the highest antigen dilution showing hemagglutination was taken as the titer (HAU/0.025ml). Before and after each inactivation treatment the HA activity was also tested and compared with standard HI antigen.

b) Specificity. Since common hemagglutinating agents including avian influenza virus (AIV), Newcastle disease virus (NDV) and some bacteria demonstrating HA activity, the responsible agent must be determined. In order to ascertain the presence or absence of NDV or AIV, antisera against these viruses were used in microtiter HI testes. Chicken embryos, 9-11 days old were also injected via the allantoic sac with

0.2ml of antigen before each treatment. A loopful of the antigen before and after each treatment was cultured for isolation of bacteria and fungi using standard media such as blood agar, PPLO broth and Sabouraud dexterose agar. Electron microscopy study of prepared antigens and standard HI antigen was carried out (Philips 400) using 2.5% phosphotungstic acid for negative staining.

c) Stability. The HA titers of antigens and diluted antigens (4HAU) were evaluated during 9 months at 4°C and-20°C.

d) Antibody detection. To detect antibody against EDSV, 270 serum samples from 23 chicken farms including broiler, layer and breeder farms with different ages from 2 days to 60 weeks old were collected. 40 serum samples from 6-week-old SPF chickens before (negative control) and 3 weeks after inoculation of EDS vaccine (Isbi Co., Italy) as positive control were also taken out. 100 yolks obtained from both farm and SPF chicken eggs, were also tested using two prepared antigens. The results were compared with HI EDSV strain BC 14 antigen (Ivaz Co., Italy) and ELISA EDSV antigen (Guildhay Co., England). The HI test was performed in U bottomed microplates, using two-fold dilution of sera, in 0.25ml volumes, 1% chicken red blood cells and 4HAU of antigens. Titers were expressed as reciprocals of the highest dilution of serum, which caused complete inhibition of hemagglutination. Positive and negative control sera were included in the tests. ELISA test were carried out based on the procedure of the ELISA kit. 180 sera and yolks tested with both HI and ELISA.

Results and Discussion

HA activity. The mean titers of allantoic and amniotic fluids were 15 and 8 based on $\log_2$, respectively. Only allantoic fluid was used for antigen preparation.

Specificity. No bacteria, fungi and other viruses were isolated from virus pool. The HI test with NDV and AIV antisera showed no inhibition but complete inhibition was done using EDSV antiserum. The virus pool before and after each

inactivation treatment fulfilled the morphological description of adenoviruses in electron microscopy. Prepared and standard antigens were completely the same in electron microscopy observations (Figures 1 and 2).

Figure *1. Negative contrast electron micrograph of the propagated EDS virus in allantoic sac of embryonated duck egg (X93600)*

Figure 2. *Electron micrograph of the EDS virus inactivated by 65°C heat (X57600)*

Stability. The HA activity of prepared antigens proved stable up to 9 months at -20°C and fall in titer from 14 to 12 (log_2) after 6 months at 4°C. The HA stability of a dilution of prepared antigens (4HAU) remained stable at 4°C up to 3 months. The HA stability of standard antigen was slightly less than prepared antigens at all temperatures and dilutions.

Inactivation. The results of various tests indicated inactivation of EDSV with 0.5% formalin at 37°C for 1h or 65°C heat for 1h completed with low destroying effect on HA and HI titers. After each treatment, the HA titer only decreased to 1/16000 or 14(log_2) equal to the HA titer of the standard HA antigen. It seams that 65°C heat treatment is slightly better.

Serological tests. Comparison of the HI test on 310 sera and 100 yolks at various titers showed over 99% similarity between the results of prepared and standard antigens. If the HI results considered as positive and negative, regardless of titers, the similarity between the results becomes completely equal. Statistical analysis showed that the sensitivity and specificity of HI test using by both prepared antigens are completely equal compared with HI standard antigen. Comparison of HI and ELISA tests to detect EDSV antibody in 150 sera and 30 yolks showed 96.6% similarity. ELISA test showed slightly more sensitivity and slightly less specificity compared with HI test.

Some laboratory host systems such as duck, goose, chick and turkey cell cultures, and embryonated duck or goose eggs have been used for EDSV propagation (McFerran 1998, McFerran & Adair 2003).We used embryonated duck eggs and produced the virus pool with the mean titer of 1/32000. Adair *et al* (1976) and Zsak *et al* (1982) reported the high titers of 1/16000-1/32000 using EDSV inoculation into the allantoic sac of embryonated duck or goose eggs but they observed no growth in embryonated chicken eggs. The EDSV pool propagated in CEL by Aghakhan and Khodashenas (1990), had a HA titer of 1/512. Our experiments before this study showed that the virus could grow in embryonated chicken eggs and chick embryo liver cells (CEL) with the mean titers of 1/64 and 1/256 respectively. The results of this study indicated that to produce a high titer of EDSV antigen for HI test compareable with standard antigen, embryonated duck eggs is the best laboratory host system. Embryonated goose eggs and duck or goose cell cultures can be used

instead (McFerran & Adair 2003), but they are not easily available or require more material and expertise in handling cell culture techniques.

Adair *et al* (1986) compared the results of HI, ELISA, serum neutralization (SN), fluorescent antibody (FA) and agar gel immunodiffusion (AGID) tests for EDSV antibody detection and concluded that only HI or SN should be used for detection of infection in commercial birds. In this study 4 of 45 HI negative sera tested were apparently positive by ELISA test. Cross-reaction between EDSV and the avian adenovirus group specific antigen was reported by McFerran *et al* (1978). Adair *et al* (1986) demonstrated that cross-reaction with avian adenovirus interferes with the specificity of ELISA, FA and AGID tests. Since multiple infections with adenovirus are known to be common in field conditions (Cowen *et al* 1978, Aghakhan *et al* 1994), it might therefore expected that a proportion of field sera would also be positive in ELISA test for EDS virus and that this proportion would increase with age. The results of this study showed that of 43 ELISA negative sera 2 were HI positive. Adair *et al* (1986) found out that with ELISA, some of HI positive tested sera were false negative. They suggested that hemagglutinating antigen is not major constituent of the ELISA antigen preparation. Comparison of the prepared and standard HI antigens to detect specific EDSV antibody showed complete similarity. In conclusion, it is suggested that the prepared HI antigens could successfully be used by avian diagnostic laboratories for routine flocks monitoring, EDS vaccine controlling and also surveillance of EDS vaccination program in poultry industry.

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