Production of virus specific egg yolk antibodies for the diagnosis of Newcastle disease, infectious bronchitis and Gumboro disease by the direct fluorescent antibody technique


Key words

Summary
The suitability of egg yolk antibodies (IgY) for the detection of the Newcastle disease virus (NDV), infectious bronchitis virus (IBV) and Gumboro disease virus (IBDV) by the direct immunofluorescent antibody test was examined. Laying hens were immunized with commercially available vaccines containing inactivated NDV, IBV and IBDV. High titters of specific antiviral egg yolk antibodies were detectable from 4 to 8 weeks after initial immunization for a period of about 20 weeks. IgY was precipitated from the yolk with ammonium sulfate and consecutively purified by affinity chromatography, attaining 1.46 mg specific IgY per yolk on average. After labeling with fluorescein-isothiocyanate (FITC) the sensitivity and specificity of the conjugates were evaluated on coverslip cell cultures. In the direct fluorescent antibody test the 1:4 or 1:8 in PBS diluted conjugates reacted specifically with their homologous antigen in impression smears prepared from organs of experimentally infected chicks and in coverslip cell cultures. Reactions with heterologous antigens did not occur and non-specific fluorescence was successfully suppressed by absorption of conjugates with liver powder. It is concluded that FITC-labeled egg yolk antibodies can be produced in a simple and economical way on a large scale and therefore present an interesting alternative to the common practice of producing fluorescent antibodies for the diagnosis of NDV, IBV and IBDV from the serum of chickens or rabbits, particularly for laboratories with limited financial resources.

INTRODUCTION

Poultry production is of growing economical importance in many African and Asian countries. At the same time, diseases caused by infections with the Newcastle disease virus (NDV), Gumboro disease virus (IBDV) or infectious bronchitis virus (IBV) impair the health of flocks considerably. Control of such diseases is crucial for increasing the rentability of poultry production. The diagnosis of viral poultry diseases, forming the basis of every control program, is often done through isolation of the infective agent. Other diagnostic means, that have been applied successfully...
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for several years, are fluorescent antibodies (1, 2, 4, 7). They offer the opportunity to detect virus antigen directly in infected cells, making the laborious isolation of virus on tissue cultures or embryonated eggs dispensable in many cases. Fluorescent antibodies for the diagnosis of the main poultry diseases are commercially available, but at a price that prohibits their routine use in investigative laboratories with limited financial resources.

A method for the production of diagnostic antibodies that is simple and yields large quantities of specific antibodies in an economically and ecologically sound way would represent an interesting alternative to the purchase of commercial diagnostics. Recently, polyclonal antibodies from egg-yolk of immunized hens, IgY, have been discussed as a potential alternate to mammal antibodies (3, 10). IgY equals mammal IgG in its immunological properties (10) and can be employed in various serological tests such as precipitation, agglutination and neutralization techniques as well as in enzyme-linked immunosorbent assays (3, 10, 11).

The present study aimed at clarifying whether IgY can be used to produce antiviral fluorescent antibodies, that are suitable for the direct detection of virus antigen in specimens from infected poultry.

Materials and Methods

Chicken embryo fibroblast-cultures

Chicken embryo fibroblasts (CEF) were prepared from 11-day-old specific pathogen free (SPF) chicken embryos as previously described (5). CEF-cultures on coverslips were prepared for the evaluation of the FITC-conjugates. A suspension of 10⁶ cells per ml was seeded onto tissue culture petri dishes containing coverslips. The covered petri dishes were incubated at 37°C in a moist atmosphere containing 5% CO₂ until confluency of cells was observed. For the propagation of virus antigen for use as antigen in affinity chromatography, a suspension of 8 x 10⁵ CEF containing 5% CO₂ per ml was seeded onto Roux-bottles and incubated at 37°C until confluency was reached.

Infection of chicken embryo fibroblast cultures with NDV, IBV and IBDV

For infection of confluent cell cultures, the growth medium was removed. Several flasks of cell cultures were each infected with 100 ml minimum essential medium with Earle’s salts (MEME) containing either 1 ml of a suspension of NDV Hitchner B1 or 1 ml of a suspension of IBV Beaudette 222 or 1 ml of a suspension of IBDV D 78 (Institute for Poultry Diseases, Freie Universitat Berlin). Incubation was continued at 37°C in a moist atmosphere containing 5% CO₂. When the first cells showed cytopathic effects, the coverslip cultures were washed with PBS after removal of the medium, air dried and fixed in cooled acetone for 10 min at -20°C. After fixation they were once again washed with PBS, thoroughly dried and stored at -20°C. The cultures in Roux bottles were incubated until cytopathic effects were pronounced and detachment of cells was observed on a large scale. The supernatant of these cultures was harvested and stored at -20°C. After thawing at 4°C, the supernatant was centrifuged at 7000 x g for 30 min to spin off cell debris and subsequently centrifuged in a Sorvall RC-5B refrigerated superspeed centrifuge for 120 min at 50,000 x g (supernatant of NDV-/IBDV-infected cultures) or 30,000 x g (supernatant of IBV-infected cultures). The resulting sediment was resuspended in 0.05 M carbonate/bicarbonate buffer, pH 9.6, and diluted to 1/100 of the initial volume.

Preparation of antiviral antibodies

White-Leghorn- and Brown-Leghorn-hens from a commercial Jordanian breeder were raised individually in cages and received feed and water ad libitum. They were not vaccinated until the beginning of the trial. At the age of 17 weeks three groups of layer chickens were each vaccinated with a commercial oil adjuvant vaccine containing either inactivated NDV, La Sota strain (TAD, Cuxhaven) or IBV, Mass 41 strain (TAD), or IBDV, D 78 strain (Intervet, Boxmeer). Each hen received 0.5 ml subcutaneously or intramuscularly following the manufacturer’s recommendations. The immunization was repeated at 2, 5 and 10 weeks after the initial immunization. Eggs were collected over a period of 6 months and stored at 4°C until processed for extraction of egg yolk antibodies.

Extraction and purification of egg yolk antibodies

Each egg yolk was tested for its content of specific antiviral antibodies by indirect ELISA (Flockchek, Idexx). Antibodies from yolk with high titers were isolated by ammonium sulfate precipitation as described by Jensenius et al. (7) and Wallmann et al. (12). After dialysis against PBS the antibody pool was purified by affinity chromatography following the method recommended by Pharmacia, Freiburg. NDV-, IBV- or IBDV-antigen, concentrated by ultracentrifugation, was immobilized on a matrix of CNBr-Sepharose (Pharmacia, Freiburg). The IgY-solution was added to the column (PD 10, 8 x 1.6 cm, Pharmacia) and the specific antiviral IgY was allowed to bind to the immobilized antigen, while IgY with other specificity was washed out. In the next step the retained specific IgY was eluted by adding 0.1 M glycine-HCl buffer, pH 2.5. Following dialysis against PBS the protein concentration was measured using Biuret reagent (13) and adjusted to 10 mg/ml.

Labeling of egg yolk antibodies with fluorescein-isothiocyanate

The antibodies were labeled with fluorescein-isothiocyanate (FITC) according to the method described by Goldman and Carver (6). Unbound FITC was removed by gel filtration on Sephadex G 25 equilibrated with PBS as described by Porath and Flodin (10).

Adjustment of FITC labeled egg yolk antibodies

The anti-NDV-, anti-IBV- and anti-IBDV-FITC conjugates were diluted in PBS after absorption with acetone-precipitated chicken liver powder (8). Titration was performed on CEF-coverslip cultures infected with homologous virus at dilutions between 1:2 to 1:32. Coverslip cultures infected with heterologous virus and uninfected cultures were included as controls. The optimal dilution of the conjugates resulting in a bright fluorescence of infected cells against a dark background was chosen as working dilution in the direct fluorescence test on smears of experimentally infected organs.

Experimental infection of chicks

Forty-eight Brown-Leghorn chicks bought from a commercial hatchery were divided into three groups and kept in separate housing units. At the age of 21 days, when maternal antibodies were no longer detectable in the serum by indirect ELISA, group 1 was infected intranasally with 7 x 10⁴ EID₅₀ of IBV MA 5 (Intervet), group 2 was infected intranasally with 7 x 10⁴ EID₅₀ of NDV La Sota (IVAZ Srl) and group 3 was infected perorally with 7 x 10⁴ EID₅₀ of IBDV D 78 (Intervet). Two chicks of each group were exsanguinated daily and smears were prepared from different organs. The specimens were fixed in acetone at -20°C for 10 min and stored at -20°C until tested in the direct fluorescent antibody test.
Direct fluorescent antibody test

The smears were thawed and stained for 30 min at 37°C with conjugate. After incubation the slides were washed with PBS three times, dried and mounted in glycerol with 10% PBS, covered with a coverslip and examined under a fluorescent microscope at a magnification of x 400. The severity of the infection was graded from 0 for no fluorescence to 3 for many fluorescent foci distributed over the entire smear (a focus being a cell or cell debris).

RESULTS

Development of specific antiviral egg yolk antibodies

For a period of 26 weeks after initial immunization yolk samples were tested by ELISA for their antibody content. The results, expressed as the ratio of the mean absorbance value of the sample and the mean absorbance value of the positive control serum (percent positivity), are shown in figure 1. The animals responded to the initial immunization and the following booster injections in week 2 and 5 with a marked rise in antibody production with a peak reached around 10-16 weeks post immunization (p.i.) and the formation of a subsequent plateau lasting approximately 7-10 weeks (figure 1).

Extraction and purification of egg yolk antibodies

Yolk samples with reasonable antibody titers were chosen for isolation of globulins. Up to 59 mg of protein per egg yolk were extracted by ammonium sulfate precipitation. After purification by affinity chromatography 1.46 mg of specific anti-NDV-, anti-IBV- and anti-IBDV-γ-globulins were recovered on average from one egg yolk.

Sensitivity and specificity of FITC labeled NDV-, IBV- and IBDV-specific egg yolk antibodies

The sensitivity of antiviral conjugates was tested on coverslip cultures of CEF infected with the homologous virus. A bright, cytoplasmic fluorescence against a dark background was observed, when the conjugates were applied at dilutions of 1:8 or 1:4 (table I). No fluorescence was observed when the conjugates were applied to specimens infected with a heterologous antigen or on uninfected coverslip cultures.

Demonstration of viral antigen in organs from experimentally infected chicks with FITC-labeled egg yolk antibodies

The results of the direct fluorescent antibody test on smears made from organs of experimentally infected chicks are shown in tables II to IV. All smears prepared before experimental infection and from chicks of the control group tested negative. Fluorescence was only observed on smears incubated with the homologous conjugate.

DISCUSSION

The fluorescent antibody test has been used for several years for the diagnosis of Newcastle disease, infectious bronchitis and Gumboro disease (1, 2, 7). The antibodies used in this test have usually been obtained from the serum of immunized chickens or rabbits, which were exsanguinated for this purpose. The quantities of antibodies that can be attained by this method vary according to the blood volume of the animal sacrificed. Gehringer (4) isolated 50 mg γ-globulins from 10 ml chicken serum by batch preparation using DEAE-cellulose; Schwarzkopf (11) reports that an amount of 10-50 mg of specific antibodies can be achieved by bleeding one rabbit.

These amounts are opposed to the quantities of egg yolk antibodies attainable from immunized laying hens every day. Gassmann et al. (3) report gains of 3 mg specific IgY per egg that can be obtained daily from day 30-50 p.i. onwards. In the present study...
FITC-labeled IgY for diagnosis of NDV, IBV and IBDV

Table II
Results of the FAT on impression smears of organs from 14 chicks experimentally infected with NDV

<table>
<thead>
<tr>
<th>Days p.i.</th>
<th>Trachea</th>
<th>Lungs</th>
<th>Caecum</th>
<th>Spleen</th>
<th>Liver</th>
<th>Brain</th>
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0 = no fluorescence detected on the slide; 1 = sporadic fluorescent foci on some sites; 2 = sporadic fluorescent foci on many sites

Table III
Results of the FAT on impression smears of organs from 14 chicks experimentally infected with IBV

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<tr>
<th>Days p.i.</th>
<th>Trachea</th>
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0 = no fluorescence detected on the slide; 1 = sporadic fluorescent foci on some sites; 2 = sporadic fluorescent foci on many sites

Table IV
Results of the FAT on impression smears of organs from 14 chicks experimentally infected with IBDV

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0 = no fluorescence detected on the slide; 1 = sporadic fluorescent foci on some sites; 2 = sporadic fluorescent foci on many sites; 3 = many fluorescent foci on all sites

REFERENCES


reasonable titers of antiviral antibodies appeared four to eight weeks after initial immunization, with 1.46 mg specific IgY on average in the egg yolk of immunized hens. These quantities were produced for a period of about 20 weeks. Labeled with FITC, the egg yolk antibodies reacted specifically with the homologous virus in infected tissue cultures as well as in smears from experimentally infected chicks.

CONCLUSION

From the results of this study it can be concluded that FITC-labeled IgY is suitable for use as a diagnostic reagent for the detection of poultry virus infections in different clinical specimens. It can be produced in large amounts in a simple and cheap manner. The need for isolators or specific-pathogen-free units for housing the laying hens is circumvented by removal of antibodies with unwanted specificity by affinity chromatography. These properties of FITC-labeled IgY make the described method for the production of fluorescent egg yolk antibodies a useful method for providing reagents for the diagnosis of poultry diseases especially for diagnostic laboratories with limited financial resources.


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