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

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Key words

Newcastle disease - Infectious bronchitis - Gumboro disease - Diagnosis - Egg yolk - Antibody - Fluorescence - Poultry - Layer chicken.

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 titers 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 10^6 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% CO_2 until confluency of cells was observed. For the propagation of virus antigen for use as antigen in affinity chromatography, a suspension of 8 x 10^5 CEF 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 Universität Berlin). Incubation was continued at 37°C in a moist atmosphere containing 5% CO_2.

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 glycin-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 homologue virus at dilutions between 1:2 to 1:32. Coverslip cultures infected with heterologue 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 10^4 EID_{50} of IBV MA 5 (Intervet), group 2 was infected intranasally with 7 x 10^7 EID_{50} of NDV La Sota (IVAZ SrI) and group 3 was infected perorally with 7 x 10^3 EID_{50} 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).

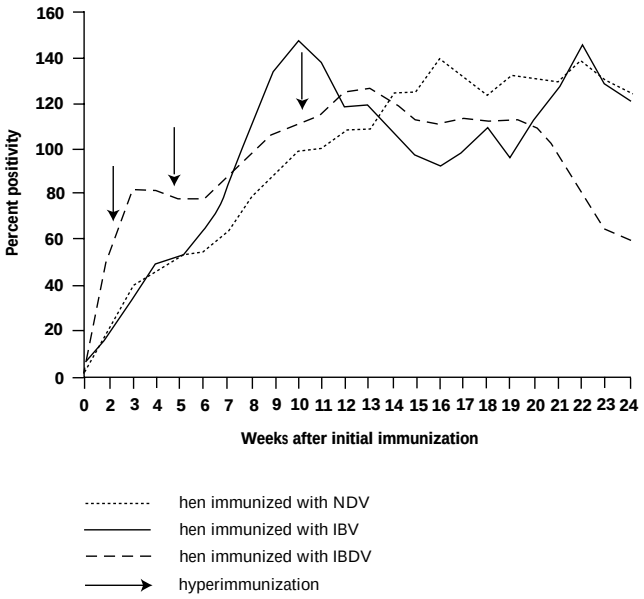

Figure 1: *Development of antibodies in the yolk of immunized hens measured by indirect ELISA (percent positivity = mean absorbance value of sample/mean absorbance value positive control serum).*

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.

Table I

Specificity and sensitivity of FITC-conjugates in the FAT

Antigen	Dilution of conjugate	Anti-NDV-FITC-conjugate	Anti IBV-FITC-conjugate	Anti-IBDV-FITC-conjugate
Homologous antigen	1:2	3+	3+	3+
	1:4	3+	2+	3+
	1:8	2+	+	2+
	1:16	+	+	+
	1:32	+	-	+
Heterologous antigen 1	1:2	-	-	-
Heterologous antigen 2	1:2	-	-	-
Uninfected cells	1:2	-	-	-
Working dilution		**1:8**	**1:4**	**1:8**

- = no fluorescence; + = weak fluorescence; 2+ = bright fluorescence; 3+ = very bright fluorescence

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

Table II

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

Days p.i.	Trachea	Lungs	Caecum	Spleen	Liver	Brain
1	1	1	0	1	0	0
2	1	1	1	1	0	0
3	1	1	1	2	1	0
4	2	1	2	2	2	0
5	2	2	2	2	1	1
6	2	2	1	1	1	1
7	1	2	1	1	0	0

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

Days p.i.	Trachea	Lungs	Kidneys	Spleen	Liver
1	1	0	0	1	0
2	1	1	1	1	0
3	2	1	2	1	2
4	2	1	1	1	1
5	2	2	1	2	2
6	2	2	1	1	1
7	1	1	0	1	0

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

Days p.i.	Bursa	Spleen
1	1	1
2	2	2
3	3	3
4	3	2
5	2	1
6	2	1
7	1	1

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

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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Résumé

Gervelmeyer A., Abu-Ajamijeh H., Bani-Younis E., Anasweh O., Bzour N., Akasheh R., Clausen P.-H., Staak C., Monreal G.
Production d'anticorps de jaune d'œuf antiviraux spécifiques pour le diagnostic de la maladie de Newcastle, de la bronchite infectieuse et de la maladie de Gumboro par le test d'immunofluorescence directe

Les possibilités d'utilisation d'anticorps de jaune d'œuf (IgY) pour la détection des virus de la maladie de Newcastle (NDV), de la bronchite infectieuse (IBV) et de la maladie de Gumboro (IBDV) par le test d'immunofluorescence directe ont été examinées. Des poules pondeuses ont été immunisées avec différents vaccins commerciaux à NDV, IBV et IBDV inactivés. L'immunisation a donné des titres élevés d'anticorps spécifiques dans le jaune d'œuf à partir de 4 à 8 semaines après l'immunisation initiale, et ce durant une période d'environ 20 semaines. Les IgY ont été extraites du jaune d'œuf par précipitation au sulfate d'ammonium, puis purifiées par la méthode de chromatographie d'affinité, donnant 1,46 mg d'IgY spécifique par jaune d'œuf en moyenne. Après marquage avec l'isothiocyanate de fluorescéine (FITC) la sensibilité et la spécificité des conjugués ont été évaluées sur des cultures cellulaires sur des lamelles couvre-objets. Dans le test d'immunofluorescence directe, les conjugués dilués au 1:8 ou au 1:4 avec du PBS réagissaient spécifiquement avec l'antigène homologue dans des calques d'organes de poussins infectés expérimentalement et dans des cultures cellulaires sur des lamelles couvre-objets. Aucune réaction n'a été observée avec des antigènes hétérologues. La fluorescence non spécifique était facilement éliminée par une absorption avec de la poudre de foie. En conclusion, ces résultats montrent que les IgY marquées avec FITC peuvent être produites en grandes quantités de manière facile et économique. Pour le diagnostic de NDV, IBV et IBDV, les anticorps de jaune d'œuf constituent donc une excellente alternative à la production d'anticorps fluorescents à partir du sang de lapins ou de poules, notamment pour les laboratoires ayant des moyens financiers limités.

Mots-clés : Maladie de Newcastle - Bronchite infectieuse - Maladie de Gumboro - Diagnostic - Jaune d'œuf - Anticorps - Fluorescence - Volaille - Poule pondeuse.

Resumen

Gervelmeyer A., Abu-Ajamijeh H., Bani-Younis E., Anasweh O., Bzour N., Akasheh R., Clausen P.-H., Staak C., Monreal G.
Producción de anticuerpos de yema de huevo específicos para virus, para el diagnóstico de la enfermedad de Newcastle, bronquitis infecciosa y enfermedad de Gumboro, mediante la técnica de inmunofluorescencia directa de anticuerpos

Se examinó la posibilidad de utilización de anticuerpos de yema de huevo (IgY) para la detección del virus de la enfermedad de Newcastle (NDV), el virus de la bronquitis infecciosa (IBV) y el virus de la enfermedad de Gumboro (IBDV), mediante la prueba de inmunofluorescencia directa de anticuerpos. Se inmunizaron gallinas ponedoras con vacunas comerciales, conteniendo NDV, IBV y IBDV inactivados. Se detectaron altos títulos de anticuerpos de yema de huevo específicamente antivirales, de 4 a 8 semanas después de la inmunización inicial, durante un período de alrededor de 20 semanas. La IgY se precipitó a partir de la yema, con sulfato de amonio y se purificó seguidamente mediante cromatografía de afinidad, alcanzando un promedio de 1,46 mg de IgY específica por yema. Después de marcar con isotiocianato-fluoresceína (FITC), se evaluó la sensitividad y la especificidad de los conjugados en cultivos celulares. Durante el test de inmunofluorescencia directa de anticuerpos, los conjugados diluidos a 1:4 y 1:8 en PBS reaccionaron específicamente con los antígenos homólogos en frotis preparados a partir de órganos de pollos infectados experimentalmente y en cultivos celulares. No se presentaron reacciones con antígenos heterólogos y la fluorescencia no específica se suprimió exitosamente mediante absorción de conjugados con polvo de hígado. Se concluye que los anticuerpos de yema de huevo marcados con FITC pueden producirse en larga escala, de manera simple y económica y por lo tanto representan una alternativa interesante a la práctica usual de producción de anticuerpos fluorescentes para el diagnóstico de NDV, IBV y IBDV, a partir de suero de pollos o conejos, particularmente en laboratorios con recursos financieros limitados.

Palabras clave: Enfermedad de Newcastle - Infecciosa bronquitis - Enfermedad de Gumboro - Diagnóstico - Yema de huevo - Anticuerpo - Fluorescencia.