An ELISA for the quantification of chicken immunoglobulin (IgY) in various liquid media*

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Summary
The development of an assay for the quantification of chicken immunoglobulin (IgY) and data evaluation is being demonstrated. An enzyme immunoassay was established to perform the quality control of IgY purifications from the egg yolk and to control IgY producing cells in vitro. Each step of the test procedure and possible alternatives were described. The assay was characterized by special test parameters (sensitivity, detectability, accuracy, precision, specifity). Moreover, this simple and fast enzyme immunoassay is a special supplement to the alternative antibody production from egg yolk.

Zusammenfassung: ELISA zur Quantifizierung von Hühner-IgY in verschiedenen flüssigen Medien
Die Entwicklung eines Enzymimmunoassays zur Quantifizierung von IgY in Antikörperpräparationen aus dem Hühnerei sowie zur Kontrolle von Antikörper-produzierenden Zellen in vitro wird beschrieben. Die Etablierung der einzelnen Schritte des Testablaufes sowie insbesondere die Charakterisierung des ELISA durch spezifische Testparameter wird gezeigt. Im Ergebnis stellt der schnell und einfach durchzuführende Enzymimmunoassay einen spezifischen Beitrag zur alternativen Antikörpergewinnung über das Hühnerei dar.

Key words: enzyme immunoassay, quantification, chicken immunoglobulin, IgY

1 Introduction

The aim of our study was the establishment of an assay for the quantification of chicken IgG or the so-called IgY. Special requirements for this test were a high reproducibility, a high detectability and accuracy of the assay.

IgY is the major fraction of antibodies (ab) in chicken serum and it occurs in egg yolk as well (Warr et al., 1995). The function of this immunoglobulin class is comparable to that of mammalian IgG. On the other hand the structure of the IgY was identified as IgA-like (Hädge and Ambrosius, 1983, 1984, 1988 ; Hädge,1985).

IgY has many special properties. Egg yolk antibodies have no interactions with rheumatoid factors in serum of mammalians, and the risk of getting false-positive results in immunoassays is therefore small. IgY does not bind to protein A and G , to mammalian Fc receptors and mammalian complement . Anti-mammalian IgG antibodies which cross-react to IgG from various mammalian species show no or very little cross reaction to IgY. Because of these properties and the large evolutionary distance from chicken to mammals, the egg yolk antibodies offer a number of applications which are not feasible using mammalian antibodies (Brandt et al., 1981; Larsson and Sjönquist, 1990).

Furthermore, the stability of IgY against physical and chemical influences is much higher than that of mammalian IgG (Lösch et al., 1986).

Due to these specifities the IgY finds increased interest in laboratory practice. There is a growing number of publications on the use of polyclonal antibodies extracted from egg yolk (Lösch et al., 1986 ; Schade et al., 1991 ; Gassmann et al., 1990).

Another benefit is the efficiency of the production of egg yolk antibodies. The quantity of immunoglobulin separated from egg yolk of immunized chickens is in most cases higher than that obtained from the serum of rabbits. Lösch et al. (1986) described that one egg yolk can provide 40 – 500 mg IgY. This amount of IgY contains 2 – 10 % specific antibodies (Schwarzkopf, 1994). Kowlaczyk et al.(1985) showed an amount of IgY in the egg yolk of 15.7 mg/ml with a range from 5.3 mg/ml to 43.3 mg/ml. That means, in theory an amount of 120 mg Ig can be obtained from one egg.

Normally, laboratory mammals such as rabbits, goats, or sheep are used for antibody production. This method includes two procedures causing distress to the animals: The first is the immunisation itself, whereas the second one consists in the bleeding of the animal as a prerequisite for antibody preparation. In using chickens for antibody production the painful step of blood collecting can be replaced by egg collecting and IgY extraction from egg yolk. Therefore, the so-called IgY-technology is a refined method for animal welfare.

* *This work was supported by grants from the Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie.*

But a central problem of this alternative antibody production is the preparation of the antibodies from the egg yolk and the quantification of IgY (Fischer et al., 1996).

The quantification of IgY is also useful for the monitoring of immunoglobulin-producing cells in vitro or the documentation of the immunological status in chickens.

We developed a simple assay to measure IgY. The results of the evaluation procedure with special test parameters were satisfactory.

All elements of the assay are commercially avialable. The application of the test in other laboratories is easy to perform. It will be possible to establish this assay in a diagnostic or immunological lab without special equipment.

2 Material and Methods

2.1 The IgY samples

Probes containing the chicken antibodies were sera of specific pathogen free chickens and conventional housed chickens, cell culture supernatants of IgG-producing B-lymphocytes and several IgY preparations purified from egg yolk.

2.2 Tools

The ELISA was performed with microtiterplates (Nunc-Immuno Plate F 96, PolySorp). The rabbit anti-chicken IgG antibody, the rabbit POD-labeled anti-chicken antibody (conjugate), and the IgY standard preparation were obtained from SIGMA / Immunochemicals (Germany).

The results of the purity analysis of the IgY standard preparation were demonstrated in a previous study (Fischer et al. ,1996). We established a dilution curve of the IgY standard. The dilution steps (expected concentrations) of this standard curve are shown in Tab.1.

2.3 Devices

A SLT-reader system (SLT Labinstruments,Germany) with integrated software (EASY-FIT) was used to measure the extinction and the data evaluation.

2.4 Assay procedure

First, microtiterplates were coated with the catch antibody. A rabbit anti-IgY -antibody was diluted 1:10.000 in coating-buffer (1.59g Na_2CO_3 plus 2.94g $NaHCO_3$; ad 1000 ml Aqua dest., pH 9.6) and 50µl per well were added. The plates were incubated for 4 hours at room temperature and after that overnight at 4°C.

The plates were washed three times with washing buffer (8,0 g NaCl; 0,2 g KH_2PO_4; 2,9 g Na_2HPO_4 x 12 H_2O; 0,2 g KCL; 0,5 ml Tween 20; ad 1000,0 ml Aqua dest., pH 7,4). All further washing steps were carried out with this buffer. Samples containing the IgY were diluted in several different proportions in washing buffer plus 10 % Gelafusal (Serumwerk Bernburg; Germany) in a ratio 1:10. Following 90 min incubation at 37°C, the plates were washed three times, and 50 µl per well POD-labeled anti-chicken IgG were added (1:10.000 in washing buffer / 10 % Gelafusal). Plates were incubated for 90 min at 37 °C. After this time the plates were washed and 50 µl of substrate chromogen solution (5 mmol/l o-phenyldiamin; 5mmol/l H_2O_2 in citrate buffer; pH 5,0) were pipetted in each well. The reaction was stopped with 1M H_2SO_4 (50 µl/well) after 15 min. The absorbtion was measured at 492 nm using a SLT-reader system .

2.5 Assay parameters

The standardization of the assay was performed with the following parameters: sensitivity, detectability, accuracy, precision and specifity (Tijssen, 1987; Olechnowitz et al., 1990).

Sensitivity is the dose response curve corresponding to changes in the amount of the reactant . A high sensitivity signifies a stronger response to slight changes in the concentration.

Detectability defines the detection limit or the ability to detect small antibody or immunoglobulin levels.

Accuracy is the conformity of results to an accepted standard value (reference accuracy) or true value.

Precision is the calculated result with standard deviation or the number of digits (decimals) in which a result is expressed.

Specifity refers to the degree of discrimination of the assay between negative and positive samples.

Table: The theoretical IgY concentration according to weight and protein determination compared with the results obtained from the assay.

expected concentration (ng/ml)	found concentration (ng/ml)
200	198
100	102
50	50
25	25.2
12.5	12.8
6.25	6.54
3.125	3.207
1.563	1.643
0.781	0.8
0.381	0.405
0.195	0.186
0.097	0.117

3 Results

3.1 Test procedure

We developed an enzyme immunoassay to detect IgY in various fluids, especially in different antibody preparations from the egg yolk and in the supernatant of antibody-producing cells, in particular

B-lymphocytes. To isolate the IgY from the egg yolk fluid we used

different separation techniques, combinations and/or variants of these methods (Fischer et al., 1996). The results of these antibody extraction methods were used for the establishment of our assay.

The catch-antibody in the test system was a rabbit anti-IgY antibody coated on the microtiterplate. The other, POD-labeled, antibody was used to detect to bound IgY . Both of these mammalian antibodies were polyclonal.

We were able to use the catch-antibody coated microtiterplates after an storage at –20 °C over six months.

In pre-experiments, we examined different test conditions, e.g. the conditions of protein coating, the antibody concentrations , the necessity of a blocking step and the time regime of the separate test reactions (Hlinak et al., 1992; Minning et al., 1993). Following these experiments, we could define the described test procedure.

We established no blocking step in our test procedure. There was no improvement in minimizing the non specific reactions, neither by varying the blocking procedure in time and protein concentration, nor by using other proteins, like horse serum or milk protein(here not shown).

Different concentrations of the anti-IgY conjugate and the substrate solution were tested. The only effect found was a slight improvement of the assay sensitivity correlated with an increasing substrate concentration.

3.2 Test parameters

The assay had a good sensitivity to IgY concentration between 200 ng per ml and to 0.78 ng/ml. This meets exactly the linear part of the sigmoidal curve illustrated in fig. 1.

Our assay showed its detection limit at a concentration of 0.78 ng/ml. It has to be stressed that this means the limit for quantification only.

The table compares measured and proceeded data with the theoretical amount of IgY according to the weight and protein concentrations.

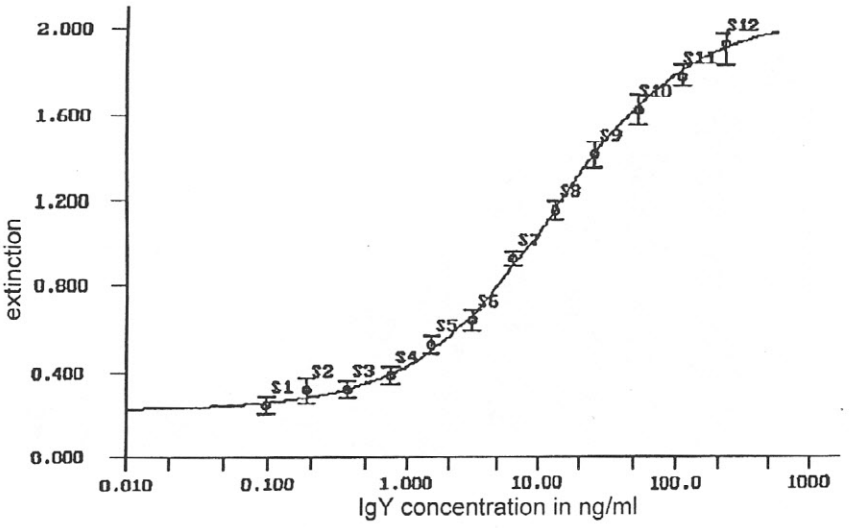

Figure 1: Standard curve simulated on the SLT-device (EASY-FIT SLT-system software) with the data of seven assays. The curve fitting has been performed by a four point interpolation in the lin-log modus.
The plot could be explained by: $OD=A+(D-A):(1+e^{B(C-\ln(KO))})$
OD = optical density measured
KO = concentration proceeded from the optical density
A and D = optical density of the asymptotic points to which the curve is convergating against on each side.
B and C = are two parameter proceeded by the software by linear regression.

The illustrated parameter is called accuracy (Tijssen, 1987).

The standard deviations as shown in fig. 1 and 2 show the precision of the assay .

Figure 3 illustrates the specifity of the assay.

According to Rammensee (1984) and Rose et al.(1974), IgM and IgA have never been found in the egg yolk of not incubated eggs. Nevertheless, we tested the cross reactivity to IgA and IgM in our assay. The IgA was preparated from chicken

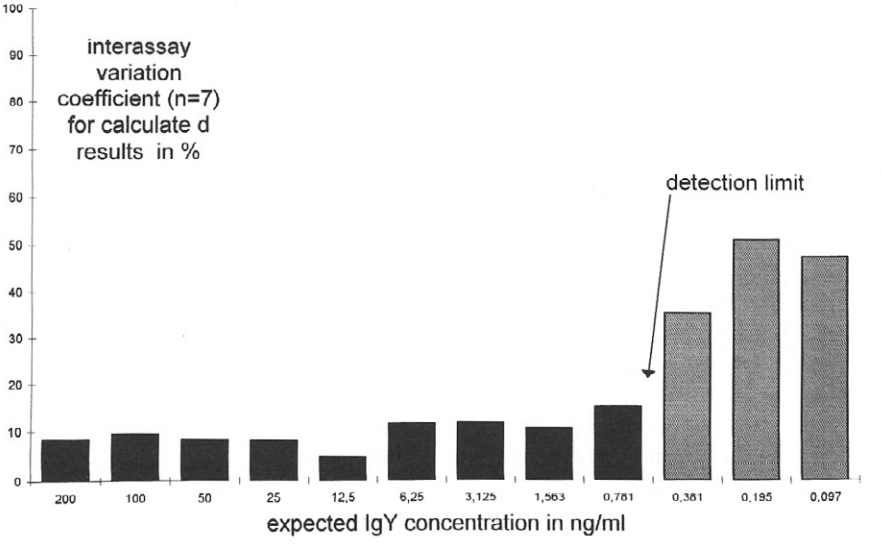

Figure 2: The between day quotient of variation (of seven assays) referring to the concentrations proceeded from the measured optical density (fourfold determination).

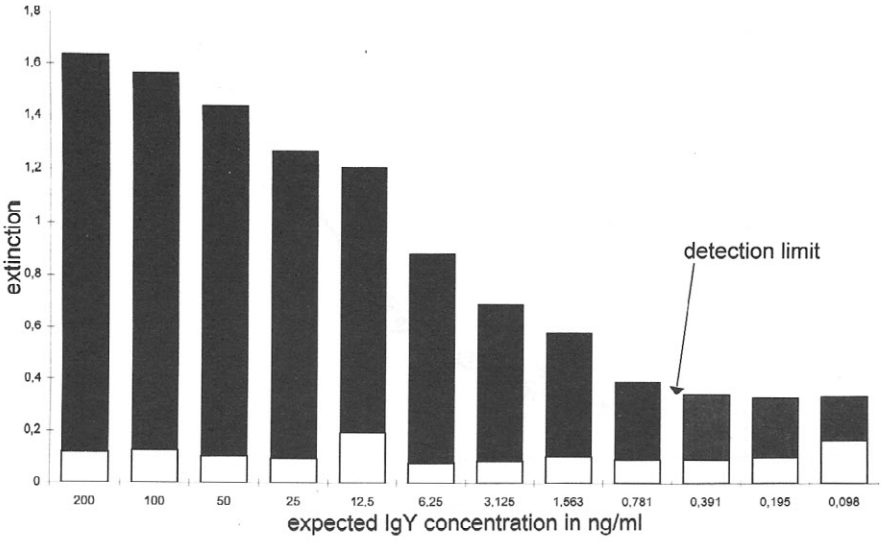

Figure 3: The specifity of the assay shown by fourfold determinations of IgY in different concentrations (visible in the diagram) and compared with a negative control (horse serum twofold determinated). The white sector of the columns shows the extinction determinated for the horse serum. The arrow marked with d.l. points to the detection limit or the lowest concentration where a positive-negative discrimination is still possible.

bile (Erhard et al.,1992) and the IgM was a generous gift of S. Khilkow (Institute of Agricultural Biotechnology, Moscow, Russia). We could not find any cross reactivities in this examination.

4 Discussion

The described assay was developed with the following requirements:

- inexpensive
- easy handling and establishment
- high detectability and accuracy.

We established a simple and fast test procedure to measure IgY in various liquid media. We used polyclonal antibodies as catch-antibody and as detection POD-labeled antibody in an enzyme immunoassay.

The choice of a polyclonal antibody indicated several advantages and disadvantages, which were evaluated. In contrast to polyclonal antibodies, the monoclonal antibody (mab) reacts with „one" specific epitope. This results in an assay, which is 100% specific and reproducible. On the other hand, due to the high specifity of the mab to a defined epitope, a lower amount of immune complexes is formed, which means a weaker amplification of the chromogenic reaction or a lower sensitivity. For the same reason the detectability of the assay increases with the use of polyclonal antibodies (Kiessig, 1991). Furthermore, the complicated way of production and characterization of monoclonal antibodies did not meet the requirements for our assay.

We found a detection limit of 0.78 ng/ml. It exceeds the sensitivity obtained by Erhard et al. (1992). They demonstrated a detection limit of 20 ng IgY per ml in an assay based on monoclonal antibodies.

Polyclonal antibodies can not be characterized in the same way as mab because they are a heterogenous fraction of antibodies to several epitopes. Therefore the assay has to be equilibrated to every new antibody charge (Kiessig, 1991). However, we did not find any differences when changing the IgY charge or preparation. The polyclonal antibodies proved to be a powerful and specific antibody as well as an inexpensive one.

The statistical work has been reduced to the determination of the standard deviation and the variation coefficient. The interpretation of the variation-coefficient (CV) represents simple statistical work. Nevertheless, it provides a lot of information.

In the statistical parameters, the assay meets the requirements of Porstmann and Kiessig (1992) who recommend a CV <10% for the intraassay variances and a CV <15% for a day to day imprecision.

According to McLaren et al.(1981), for measurements on different days a variation coefficient (interassay variation) below 10% is said to be in a good range. That means the figure is very tight to this upper level in our assay. This fact has to be considered in the interpretation of the results. Around the detection limit (0,78 ng/ml) the variation coefficient has increased more than 50%. Therefore we fixed the detection limit at this concentration. Nevertheless, the assay is able to detect IgY below this limit, as well as in a YES-or-NO-answer.

The parameters of the test system seem to be sufficient for a test with the use of polyclonal antibodies. The sensitivity is suitable to the effort of quantitation of IgY in laboratory practice. As mentioned before, we developed a more sensitive assay by using an indirect sandwich technique. Porstmann and Kiessig (1992) recommended the use of an antibody produced in the same species as the specific catch-antibody for the anti-species antibody. Nevertheless, the detectability dropped so much that a positive-negative discrimination was no longer possible.

In conclusion, we demonstrated a simple, fast and inexpensive test-procedure for the quantification of IgY, based on polyclonal antibodies in an enzyme immunoassay. This assay is characterized by very good test parameters and its establishment is possible without special laboratory equipment. Therefore this enzyme immunoassay is a special supplement to the alternative way of antibody production from egg yolk .

References

Brandt, C. D., Kim, H. W., Rodriguez, W. J., Thomas, L., Yolken, R. H., Arrobio, J. O., Kapikian, A. Z., Parrott, R. H. and Channot, R. M. (1981). Comparison of direct electron microscopic, Immun electron microscopic, and rota virus enzyme-linked immunosorbent assay for detection of gastroenteritis viruses in Chicken. *J. Clin. Micrbiol. 13* , 976–981.

Erhard, M. H., Quistorp, I. von, Schranner, I., Jüngling, A., Kaspers, B., Schmidt, P. and Kühlmann, R. (1992). Development of specific enzyme-linked immunosorbent antibody assay systems for the detection of chicken Immunglobulins G, M and A using monoclonal antibodies. *Poult. Sci. 71*, 302–310.

Fischer, M., Hlinak, A., Montag, T., Claros,M., Schade, R. and Ebner, D. (1996). Vergleich von Standardmethoden zur Präparation von Dotterantikörpern. *Tierärztl.Praxis 24*, 411–418.

Gassmann, M., Weiser, T., Thömmes, P. and Hübscher, U. (1990). Das Hühnerei als Lieferant polyklonaler Antikörper. *Schweiz. Arch. Tierheilk. 132*, 289–294.

Hädge, D. and Ambrosius, H. (1983). Evolution of low molecular weight immunglobulins. III. The immunglobulin of chicken bile - not an IgA. *Mol.Immunol. 20*, 597–606.

Hädge, D. and Ambrosius, H. (1984). Evolution of low molecular weight immunglobulins. IV. IgY-like immunglobulins of birds, reptils amphibians – precursors of mammalian IgA. *Mol. Immunol. 21*, 699–707.

Hädge, D. (1985). Zur Evolution der Immunglobuline. *All. Immunol. 31*, 597–605.

Hädge, D. and Ambrosius H. (1988). Comperative studies on the structure of bilary immunglobulins of some avian species, II. Antigenic properties of the biliary immunoglobulin of chicken, turkey, duck and goose. *Dev. Comp. Immunol. 12*, 319–329.

Hlinak, A., Dahms, H. and Kraus, G. (1992). Optimierung eines Enzymimmunoassay zum Nachweis von Antikörpern gegen aviäre Reoviren – unter besonderer Berücksichtigung der Ergebnisberechnung und Testinterpretation. *J. Vet. Med. 39*, 428–442.

Kiessig, S. (1991). Enzymimmunoassays. *BioTec 4*, 30–34.

Kowalczyk, K., Daiss, J., Halpern, J. and Roth, T. F. (1985). Quantitation of maternal-fetal IgG transport in the chicken. *Immunol. 54*, 755–762.

Larsson, A. and Sjöquist, J. (1990). Chicken IgY: Utilizing the evolutionary difference. *Comp. Immun. Microbiol. Infect. Dis. 13*, 199–201.

Lösch, U., Schranner, I., Wanke, R. and Jürgens, L. (1986). The chicken egg an antibody source. *J. Vet. Med. B 33*, 609–619.

McLaren, M. L., Lillywhite, J. E. and Au, A. C. S. (1981). Indirect enzyme linked immunosorbent assay (ELISA): practical aspects of standardization and quality control. *Med. Lab. Sci. 38*, 245–249.

Minning, P., Hlinak, A. and Klaczinski, K. (1993): Entwicklung eines Enzym-Immuno-Assay zum Nachweis von Antikörpern des Huhnes und der Maus gegen das Virus der Newcastle -Disease. *Berl. Münch. Tierärztl. Wschr. 106, 5*, 149–153.

Olechnowitz, A. F., Miko, A., Koepernik, H., Starick, E. and Fröbe, I. (1990): Die Validierung eines Enzymimmunoassays zum Nachweis von Antikörpern gegen das Rinderleukosevirus. *Arch. exper. Vet. med. 44, 2*, 279–288.

Porstmann, T. and Kiessig, S., (1992). Enzyme immunoassay techniques. An overview. *J. Immunol. Meth. 114*, 1–16.

Rammensee, M. (1984). Immunglobulinkonzentration in Serum, Sekreten, Ei und Embryo des Haushuhns. Universität München: Vet. med. Dissertation.

Rose, M. E., Orlans, E. and Buttress, N. (1974). Immunglobulin classes in the hens egg: their segration in yolk and white. *Eur. J. Immunol. 4*, 521–523.

Schade, R., Pfister, C., Halatsch, R. and Henklein, P. (1991). Polyclonal IgY Antibodies from Chicken Egg Yolk-an Alternative to the Production of Mammalian IgG Type Antibodies in Rabbits. *ATLA 19*, 403–419.

Schwarzkopf, C. (1994). *Gewinnung und immunologische Charakterisierung speziesspezifischer IgY sowie deren Einsatz zur Bestimmung der Wirtstierart aus dem Abdominalblut hämatogener Insekten*. Freie Universität Berlin: Vet. med. Dissertation.

Tijssen, P. (1987). Practice and theory of enzyme immunoassays. In R. H. Burdon and P. H. v. Knippenberg (ed.), *Laboratory techniques in biochemistry and molecular biology. Vol.15* (pp. 385–421). Amsterdam: Elsevier Science Pub.

Warr, G. W., Magor, K. E. and Higgins, D. A. (1995). IgY: clues to the origins of modern antibodies. *Immunol. Today 16, 8*, 392–398.

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