

IMMUNE ANALYSIS ASSESMENT OF IMUNIZED HENS WITH A COMPLEX OF ANTIGENES BY QUALITATIVE AND QUANTITATIVE TESTING OF IMMUNOGLOBULINES OBTAINED FROM THEIR HIPERIMUNE EGGS

EVALUAREA RĂSPUNSULUI IMUN AL GĂINILOR IMUNIZATE CU UN COMPLEX DE ANTIGENE PRIN TESTAREA CALITATIVĂ ȘI CANTITATIVĂ A IMMUNOGLOBULINELOR Y OBȚINUTE DIN OUĂ HIPERIMUNE PROVENITE DE LA ACESTEA

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Cuvinte cheie: Imunoglobulina Y, ou hiperimun, răspuns imun, titru, inoculare, antigen multiplu, test ELISA

Abstract

Immunoglobulins Y (IgY) are the main antibody synthesized by the body of birds, reptiles, amphibians and some fish species. In the last decades, IgY has been receiving a growing attention from medical specialists. It presents a number of structural differences from mammalian IgG and multiple benefits compared to mammalian antibodies. IgY can be applicable in the diagnosis, prevention and therapy of infectious diseases of pathogenic bacteria, fungi or viruses. This study aimed to evaluate the specific antibody titer against 18 antigens for a period of 13 months. Throughout the experiment, ELISA assays were performed to determine the total immunoglobulin concentration in the obtained hyper immune eggs and the specificity of the extracted IgG to each antigen.

Rezumat

Imunoglobulinele Y (IgY) reprezintă principalul anticorp sintetizat de către organismul păsărilor, reptilelor, amfibienilor și unor specii de pești. În ultimele decenii, IgY se bucură de o atenție tot mai mare din partea specialiștilor din domeniul medical. Acesta prezintă o serie de diferențe structurale față de IgG de la mamifere și multiple beneficii comparativ cu anticorpii mamiferi. IgY prezintă aplicabilitate în diagnosticul, prevenția și terapia unor afecțiuni de natură infecțioasă date de bacterii, fungi sau virusuri patogene. Studiul de față a avut ca scop evaluarea titrului de anticorpi specifici față de 18 antigene pe o perioadă de 13 luni. Pe întreaga perioadă a experimentului s-au efectuat testări de tip ELISA pentru determinarea concentrației imunoglobulinelor totale din ouăle hiperimune obținute și a specificității IgY-ului extras față de fiecare antigen în parte.

Introduction

Immunoglobulin Y (IgY) is the main antibody synthesized by laying hen (*Gallus domesticus*).

It is continuously synthesized, secreted into blood and transferred to egg yolk, where it accumulates (Young, 2007).

IgY is the evolutionary ancestor of IgG synthesized by mammals and is the main mechanism of defense against systemic infections (Paula, 2011).

In contrast to mammalian newborns that, after birth, have maternal antibodies in colostrum, in chickens, all maternal

immunoglobulins that must protect newly-hatched chickens are incorporated into the egg during ovulation.

Transfer of IgY from hen to chicken takes place in two stages; initially IgY is transferred from the hen's blood to the ovary follicle (egg yolk) and then to the embryo (Hamal, 2006).

IgY production is a non-invasive alternative to current methods and has the following advantages: maintenance of the hens is accessible, the animals are easy to handle, the production of the antibodies does not require repeated bleeding and does not cause pain to concerned animals.

In addition, IgY titer from egg yolk from immunized hens remains high for a long time (Carlander D., 2000).

In one egg yolk there is a higher amount of antibodies compared to the isolated medium in the blood of the immunized rabbits (Tini, 2002; Kovacs-Nolan, 2005; Chiurciu și col., 2017; Topilescu și col., 2014).

An additional advantage of using IgY involves obtaining a more effective immune response to mammalian antigens due to phylogenetic distance between hens and mammals (Schade et al., 2005).

To obtain IgY antibodies with specificity to an antigen of interest, laying hens are inoculated with the antigen; then, the antibodies are purified from the harvested eggs.

This non-invasive method of obtaining antibodies is one of the advantages of using IgY in immunodiagnosics (Kovacs-Nolan, 2012).

Egg yolk contains 8-10 mg IgY / mL and from one egg approximately 100-200 mg of total IgY can be extracted, of which 2-10% is

antigen-specific (Davison, 2008).

Specific viruses have been successfully synthesized against a wide variety of antigens, such as proteins, peptides, lipid hormones, viruses, bacteria, fungi (Hamal, 2006; Spillner E., 2012; Chiurciu și col., 2017; Topilescu și col., 2014).

1. Materials and methods

Animals included in the study

The study was conducted within the **Romvac S.A. Imunoinstant Research and Development Department**. All procedures have been performed in accordance with EU Directive 63/2010 on the handling of animals used for scientific purposes. The study was approved by the Ethics Committee of Romvac Company S.A.

To produce specific Y immunoglobulins, clinically healthy laying hens (*Gallus domesticus*), 19 weeks old, with a body weight of 2.5 kg, were included in the study.

They were housed in a battery-growing system in halls with controlled temperature, humidity, brightness and noise parameters. Birds were fed with standard diet, *ad libitum*.

Antigens used in inoculum preparation

The microbial strains used in the study were purchased either from the microbiological laboratories of the hospitals with which the Department collaborates or isolated from human patients treated within the Imunoinstant Complementary Medicine Cabinet.

A multiple inoculum was prepared based on the following 18 bacterial and fungal strains: *Pseudomonas aeruginosa*, *Staphylococcus*

aureus, *Klebsiella pneumoniae*, *Enterococcus faecalis*, *Escherichia coli*, *Salmonella spp.*, *Salmonella enteritidis*, *Salmonella typhimurium*, *Streptococcus mutans*, *Streptococcus Grup B*, *Acinetobacter baumannii*, *Clostridium difficile-corpi bacterieni*, *Clostridium difficile-anatoxina*, *Proteus mirabilis*, *Helicobacter pylori*, *Candida albicans*, *Candida krusei*, *Candida glabrata*.

Bird inoculation

The chickens were inoculated 3 times, intramuscularly, at 2 different points in the chest muscles (0.5 ml at each point).

Booster inoculations were performed 14 days and 28 days after initial inoculation.

The collection of hyper immune eggs was performed daily, beginning on the 14th day after the last inoculation.

They were stored at 2°-8°C.

For comparative tests, 20-weeks-old SPF hens, housed in insulators, were housed in our own halls.

IgY extraction

For the extraction of the immunoglobulin Y, a physicochemical procedure was applied.

The yolk was separated from the white; 1 ml of yolk was removed, which was then diluted with MilliQ water in a dilution of 1:8.

The mixture was homogenized and then the pH was adjusted to 4.5-5.

The samples were incubated for 24 hours at -20°C.

After thawing, the samples were centrifuged at 10500 rpm and 4°C for 20 minutes.

Samples of the supernatant which were

filtered through 0.45 µm filters were extracted.

Until testing, samples were stored at 2-8°C.

Qualitative and quantitative testing of Y immunoglobulins

ELISA, qualitative and quantitative methods, were performed to identify and quantify Y immunoglobulins in hyperimmune eggs.

Testing was done at 30 days, for a period of 13 months, starting on the 14th day after the last inoculation.

The qualitative determination of the immunoglobulin Y contained by the hyperimmune eggs against the inoculated antigens was performed by the *in-house* indirect ELISA test.

Quantitative determination of the overall immunoglobulin Y content was performed by the direct ELISA, *in-house* method.

2. Results and discussions

Total IgY concentration assay by direct ELISA

For IgY samples extracted from hyperimmune eggs, optical density (OD_{450nm}) values ranging from 2.000 to 3.000 were obtained.

These values were used to calculate the total immunoglobulin concentration using a straight-line equation (Fig. 1.) obtained on a standard IgY.

Concentrations ranging from 250 to 400 mg of total IgG / hyperimmune were obtained.

In a comparative manner, determinations of IgY concentration and SPF eggs were obtained for which 100-150 mg IgY / egg

values were obtained.

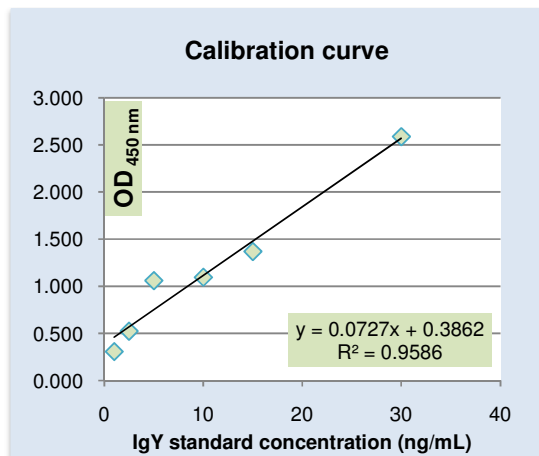


Figure 1. Calibration curve performed using standard IgY to calculate IgY concentration in hyperimmune eggs

Testing of immunoglobulin specificity in hyperimmune eggs by indirect ELISA

The results obtained by the ELISA test confirmed the presence of specific immunoglobulins in hyperimmune eggs harvested during the 13-month experiment.

The harvested hyperimmune eggs revealed an immunoglobulin content with specificity against all 18 antigens used in inoculum preparation.

Figure 2 highlights that IgY has specificity for 4 of the antigens used in hen immunization (*Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli*).

The specific antibodies titer is elevated for each antigen, thus revealing that the immune system of the inoculated hens responded to all inoculated antigenic stimulus.

To confirm the results, negative IgG reactive reagent isolated from eggs produced by SPF hens was used as negative control.

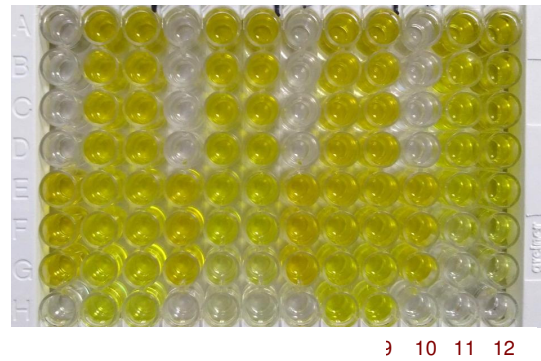


Figure 2. Qualitative ELISA for determining antibody specificity against 4 antigens, inoculum-immune components (*Pseudomonas aeruginosa* – strips 1-3, *Staphylococcus aureus* – strips 4-6, *Klebsiella pneumoniae* – strips 7-9, *Escherichia coli* – strips 10-12)

To determine the specificity of IgY antibodies, dilutions ranging from 1:100 to 1:64.000 were performed which were plated into ELISA plates coated with specific antigens (Figure 2.).

The results obtained over the 13 months of testing are highlighted in Figures 3-6.

In the first 8 months of testing, there were large variations in the values obtained for all 4 antigens.

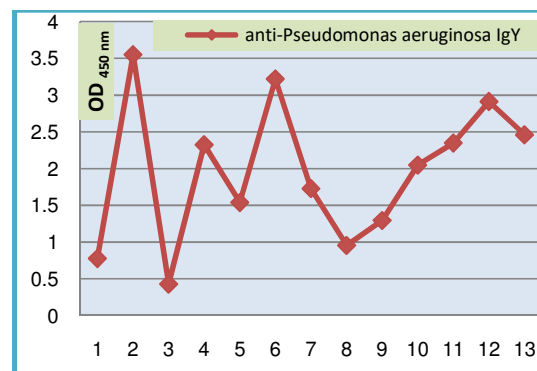


Figure 3. Variation of spectrophotometric readings at OD_{450nm} for 1:1000 titre; the values were obtained by indirect ELISA testing of IgY specificity extracted from hyperimmune eggs versus the *Pseudomonas aeruginosa* strain introduced into the multiple inoculum

Following the OD_{450nm} records obtained by specific IgY anti-*Pseudomonas aeruginosa* antibody testing, their values are

rising steadily over the last 5 months of testing, with a slight decrease over the last month. The results obtained are shown in Figure 3.

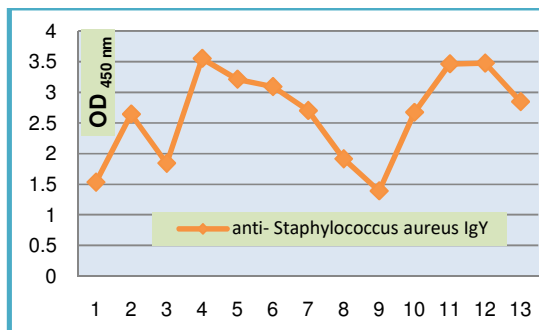


Fig. 4. Variation of spectrophotometric readings at OD_{450nm} for 1:1000 titer; the values were obtained by indirect ELISA testing of IgY specificity extracted from hyperimmune eggs versus the *Staphylococcus aureus* strain introduced into the multiple inoculum

Figure 4 shows the values obtained by testing IgY specificity extracted from eggs harvested against *Staphylococcus aureus* antigen.

In the last 4 months of testing, there was an increase in values with a slight decrease in the 13th month. Throughout the testing period, the numerical values recorded are greater than 1.000.

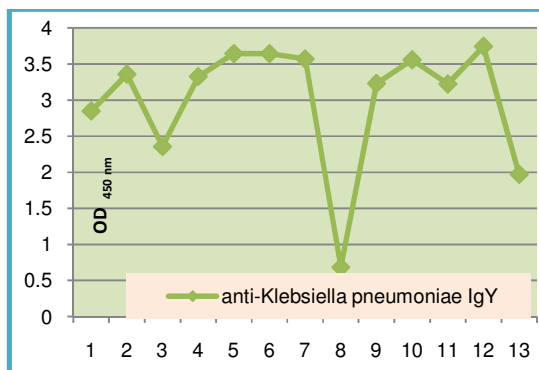


Figure 5. Variation of spectrophotometric readings at OD_{450nm} for 1: 1000 titre; the values were obtained by indirect ELISA testing of IgY specificity extracted from hyperimmune eggs versus the *Klebsiella pneumoniae* strain introduced into the multiple inoculum

Figure 5 reveals the values obtained by testing IgY specificity extracted from eggs harvested against *Klebsiella pneumoniae* antigen. Their evolution is similar to that of tests for *Staphylococcus aureus* antigen.

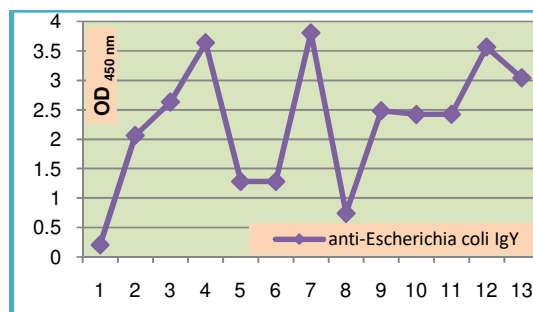


Fig. 6. Variation of spectrophotometric readings at OD_{450nm} for 1:1000 titre; the values were obtained by indirect ELISA testing of IgY specificity extracted from hyperimmune eggs versus the *Escherichia coli* strain introduced into the multiple inoculum

Figure 6 shows the values obtained by testing IgY specificity extracted from eggs harvested against *Escherichia coli* antigen.

For the last 5 months of testing, the evolution is similar to that of tests for *Pseudomonas aeruginosa* antigen.

3. Conclusions

- The study allowed the production of specific IgY antibodies from hyperimmune egg yolk over all 18 bacterial and fungal antigens used in hens immunization: *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Enterococcus faecalis*, *Escherichia coli*, *Salmonella spp.*, *Salmonella enteritidis*, *Salmonella typhimurium*, *Streptococcus mutans*, *Streptococcus grup B*, *Acinetobacter baumannii*, *Clostridium difficile – corpi bacterieni*, *Clostridium difficile - anatoxina*, *Proteus mirabilis*, *Helicobacter pylori*,

Candida albicans, *Candida krusei*, *Candida glabrata*.

- ELISA assays have demonstrated the specificity of IgY antibodies to the antigens used in hen immunization.
- Following ELISA assays, the antibodies titer (IgY) is maintained throughout the experiment.

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