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TIME EVOLUTION OF IMMUNOGLOBULIN Y (IgY) TITER IN THE EGG YOLK HARVESTED FROM HENS AFTER THREE INOCULATIONS WITH MULTIPLE ANTIGENS

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Abstract

The present research focuses on the determination of the immunoglobulin Y (IgY) levels derived from egg yolks obtained from hens previously inoculated with a combination of bacterial and fungal antigens (multiple antigens).
The purpose of the work consists of establishing the frequency of the inoculations, in order to obtain a high level of antibodies throughout the duration of the experiment. The antigens were obtained from inactivated bacterial and fungal humans pathogenic strains. Egg-laying hen lots were formed out of individuals at the beginning of the egg-laying period. The hens were inoculated with multiple antigens, three times, at the first, 14th and the 28th day of the experiment. The control for the immune response was performed by sampling eggs on the 14th day from the third inoculation process. The IgY was extracted from the egg yolk in order to obtain the aqueous phase. The characterization of the IgY titers was performed every 30 days, for a period of 9 months, by using qualitative and quantitative ELISA assays.
Following the end of the 9 months period since the 3rd inoculation process, the specific IgY titers maintained at high levels, another (4th) inoculation shouldn't be necessary.

Key words: *ELISA, IgY, inoculation, multiple antigens.*

INTRODUCTION

Antibodies represent the main molecular effectors of the immune system, being glycol-proteins synthesized under the influence of antigens, with which they interact specifically both in vivo, as well as in vitro.
They are multiple chain proteins resulted from the combination of two multiple peptide chains, with different molecular weight and sequences of amino acids.
The multiple peptide chains are bound by disulfide bridges (-S-S-), and generally have the Y letter shape (Răpuntean G., 2010).
In 1983, Klemperer published the work that stated that there are virus neutralizing proteins in eggs. Between 2004 and 2006, worldwide experts have started, for the first time ever, to acknowledge eggs like a pharmaceutical product. The egg yolk was reviewed as an important source for immunoglobulins that can be used for prophylaxis, diagnosis and therapy purposes in humans and animals (Klemperer, 1893).
Leslie and Clem proved, in 1969, that IgY is different from IgG. IgY does not interfere with the activity of drugs in the digestive tract, nor with their blood circulation in the human body. IgY does not induce antimicrobial resistance and it does have remanence in the organism (Leslie et al., 1969).
The antibodies presently used for research, diagnosis and therapy purposes originate from mammals. These are monoclonal or polyclonal antibodies. In order to produce polyclonal antibodies, animals such as horses, sheep, pigs, rabbits, rats are used. For monoclonal anti-bodies, it is the case of rabbits or mice. Both technologies have advantages and disadvantages (Mojca N., 2003).
It has been noticed that the obtaining of mammalian antibodies requires complex technologies, with a modest yield. The technology used in mammals induces stress for

them both in the immunization stage, as well as when control blood is sampled in order to produce the antibodies (Larsson et al., 2003).
The past 25 years have shown an increase in the use of hens as a replacement of mammals in the production of antibodies. The foremost advantage is that the antibodies are obtained from the eggs, and not from the serum. At the same time, the quantity of produced antibodies by an egg-laying hen is considerably higher than that of a mammal of the same size (Carlander et al., 2002).
The purification of immunoglobulin from mammals requires considerable time and is an expensive process. Presently, hens are recognized as a cheap and convenient source for the production of antibodies. The quantity of immunoglobulin produced from a single egg is comparable to that prepared from 300 mL of blood sampled from rabbits (Wang et al., 2012).
Opposite mammalian antibodies, avian origin immunoglobulins can be produced in high quantities from the egg yolk and are an ideal source for medical and scientific applications (Chiurciu et al., 2017; Leslie et al., 1969).
The egg yolk antibodies have been intensely studied and IgY is considered to be ancestral when compared to mammalian IgG and IgE. IgY technologies possesses a series of advantages to those used in extracting antibodies from mammals, such as: bird husbandry is considerably cheaper, egg collection is non-invasive, and IgY is easily obtainable (Kim et al., 1998).
Furthermore, the phylogenetic distance between the species allows for avian antibodies to be more efficient in inducing immunity responses than those of mammalian origin (Larsson et al., 2003).
IgY technologies have been evaluated for the case of therapeutic application, by oral administration in the prophylaxis or treatment of various bacterial pathogens in humans. IgY differs both structurally, as well as functionally from the mammalian IgG and does not cross-react with mammalian IgG.
Due to the fact that hens can lay eggs almost on a daily basis, the IgY rich immunized egg yolks have become the main source of antibodies used for scientific research, both fundamental and applicative (Pauly et al., 2011; Scheett et al., 2007).
The present study focused on the evolution of specific IgY antibodies obtained after three inoculations, during a 9 months experimental period of time.

MATERIALS AND METHODS

Animals. The study was developed within the Research & Development Laboratory of Romvac Company S.A., over a 9 months experimental period. All procedures were compliant with Directive EU 2010/63 relating to the manipulation of animals used in scientific purposes. The study was approved by the Ethics Committee of Romvac Company S.A.
Clinically healthy, 2.5 kg and 19 weeks old egg-laying hens (*Gallus domesticus*) were inoculated. The environmental conditions consisted of individual halls, battery-based, controlled temperature, humidity, noised and light. The birds were fed a standard *ad libitum* diet.

Antigens. The microbial strains used in the study were obtained in the laboratories of the Victor Babes Hospital for Infectious and Tropical Diseases in Bucharest, as well as from isolates from the patients that were consulted at the Alternative Therapies Medical Practice of ROMVAC Company S.A. The Victor Babes Hospital provided the following strains: *Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii* and *Escherichia coli.* The strains isolated and characterized in the ROMVAC microbiology laboratory were: *Pseudomonas aeruginosa, Clostridium difficile, Salmonella ssp., Enterococcus faecalis, Salmonella enteritidis, Salmonella typhimurium, Streptococcus mutans, Streptococcus group B, Proteus mirabilis, Helicobacter pylori, Candida albicans, Candida glabrata,* and *Candida krusei.*

Inoculum preparation. The bacterial strains were cultivated on nutrient broth, and the Candida strains on liquid Sabouraud medium. The cultures were incubated at 37°C for 24-48 hours, were washed twice every day using sterile PBS, pH=7,2 and were inactivated using formaldehyde 0.5% for 18 hours. The suspensions were adjusted for 0.05 at OD 600

by using a micro plate reader (Spectra Max 190), corresponding to a cellular density of approximately $1x10^5$ CFU/ml.

Inoculation of the hens. Egg-laying hens lots were formed, at the beginning of their egg-laying period, for which a mixture of antigens was prepared, previously described.
The hens were inoculated three times with the multiple antigens, in four different inoculation sites belonging to the chest muscles. The 2nd and 3rd inoculations were performed at 14 and 28 days difference from the initial one. The hyperimmune eggs were collected on a daily basis, starting with the 14th day from the last inoculation. The eggs were stored at 2^0 - 8^0C. For comparative testing, 30 weeks old hens SPF eggs were used. These hens were housed in individual halls.

IgY extraction. Presently, the literature highlights numerous methods for extracting and purifying immunoglobulins from the egg yolk (Kim et al., 1998; Pauly et al., 2011). The present study used the aqueous phase supernatant for the extraction of IgY. The yolk was separated from the white, 1 mL of yolk was sampled in 15 mL tubes. Each tube was added 7 mL of Milli Q water in order to achieve the 1:8 dilution. The mixture was homogenized, and the pH was adjusted at 4.5-5. The tubes were incubated for 20 hours at -20^0C, followed by defrosting, centrifugation at 10500 rpm for 20 minutes and Millex 0.45 µm filtering. The samples were mantained at la 2^0-8^0 C until the moment of testing.

Immunoenzymatic testing (ELISA). Quantitative and qualitative ELISA assays were performed in order to identify and quantify the IgY immunoglobulin from the hyperimmune eggs. The testing was performed every 30 days for the 9 months experimental period.

Qualitative determination. It was performed to identify the IgY immunoglobulins through the Elisa assay, by using the MyBioSource kit, the Elisa plate reader Spectra Max 190, an automatic ELISA plate washer (MultiWash III TRICONTINENT). As a negative control for the reaction, IgY from SPF hens was used. The reaction plates were padded with 100 µl of the antigens used for inoculation. The maximum positive dilution was considered that where OD is equal of higher than 0,200. The padded plates were kept overnight at 2^0-8^0C, and then washed three times with a PBS –Tween 20% washing solution. 300 µl/fixing pads were added, and the plate was maintained for 30 minutes at room temperature. The blocking liquid was removed and 100 µl of IgY suspension was added in dilution from 1:100 to 1:64000.
As a positive control, ROMVAC specific reference IgY was used. After the incubation of the plates for two hours at 37°C, 100 µl of 1:10000 anti-avian IgY IgG enzyme conjugate was added, followed by introducing 100 µl TMB for 5-15 minute, and then 100 µl of blocking solution.
The absorbance rate was read by using the plate reader at 450 nm wavelength. The reaction was validated when the blank control is lower than 0,060 OD, the negative control between 0,060 and 0,090, and the OD positive control is 1,400 to 1,800.

Quantitative determination. For the quantitative determination of IgY from hyperimmune eggs, the direct in-house ELISA assay was performed, by using International Reference IgY (Lampire Laboratories). We used ELISA plated (Falcon), reference IgY, enzyme conjugate anti-avian IgG (MyBioSource), diluted in stability buffer 1:10000, plate reader (Spectra Max 190), automated ELISA plates washer (MultiWash III-TRICONTINENT).
The samples represented the IgY obtained from hyperimmune eggs collected every 30 days for the 9 months experimental period. From these samples decimal dilutions were performed, from 1:100to 1:64000 and added on the plates.

RESULTS AND DISCUSSIONS

Based on the absorbance values measured for the Reference IgY, the calibration curve was obtained, having a straight equation of OD (450 nm) = 0,00667 C (ng/ml) – 0,0154 and a correlation coefficient of R^2 = 0,9785. By using this equation, we calculated the concentration of the IgY to be analyzed (Fig. 1).

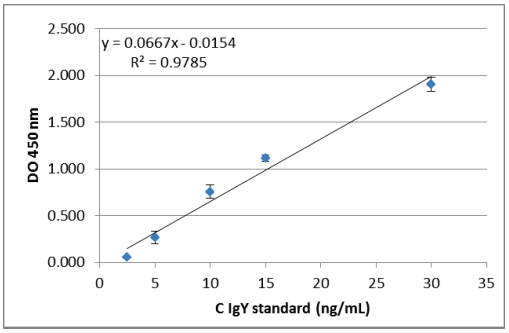

Fig. 1: Calibration curve for the Reference IgY used for the calculus of IgY concentration

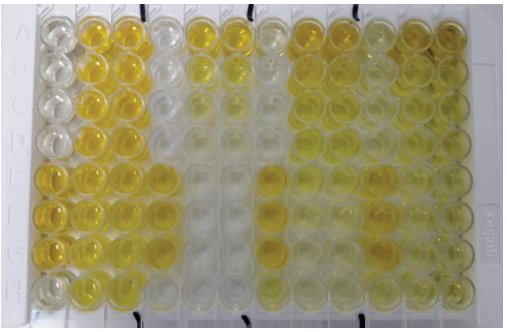

Figure 1: Qualitative ELISA test to establish the SPF IgY specificity to a component antigen from the multiple inoculum.

The results obtained via the qualitative ELISA testing have allowed the confirmation of the presence of IgY in the collected samples from immunized hens with multiple antigens over the 9 months experimental period. Figure 1 highlights that IgY manifests specificity for the antigens used in the immunization process. The tire of the antibodies is elevated for each individual antigen, indicating that the immune system of the hens reacts equally for the inoculated antigen stimuli. For the confirmation of the results, negative control SPF eggs were used (Figure 2).

Table 1 presents the optical densities (OD), read at 450 nm wavelength, for IgY antibodies against-*Streptococcus group B*, *Salmonella enteritidis*, *Proteus mirabilis*, *Salmonella sp*., *Candida krusei* and *Klebsiella pneumoniae* throughout the experimental period.

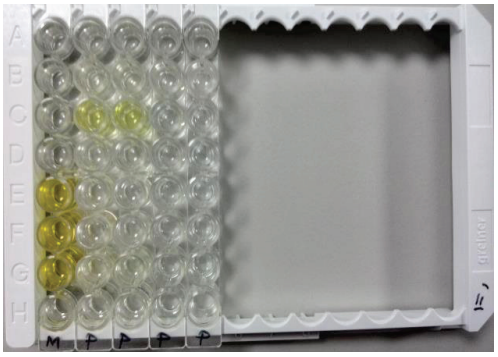

Figure 2: Qualitative ELISA test used for establishing the specificity of antibodies for the 4 component antigens of the multiple inoculum.

Table 1. ELISA test: IgY antibodies titer against -*Streptococcus group B, Salmonella enteritidis, Proteus mirabilis, Salmonella spp., Candida krusei* and *Klebsiella pneumoniae*

Antigen/ Dilution	**OD values of specific antibodies**								
	Months								
	Dec. 1	**Jan. 2**	**Feb. 3**	**Mar. 4**	**Apr. 5**	**May 6**	**Jun. 7**	**Jul. 8**	**Aug. 9**
Streptococcus group B	2,859	2,862	1,964	3,471	3,407	3,275	2,803	2,476	3,201
Salmonella enteritidis	3,143	3,160	2,116	3,598	3,468	3,696	1,624	1,333	2,221
Proteus mirabilis	1,496	2,654	1,731	3,130	3,137	3,323	2,488	1,639	3,077
*Salmonella sp*p.	3,456	2,834	3,196	3,757	3,551	3,701	3,009	1,574	3,005
Candida krusei	2,848	1,590	1,24	3,406	3,744	3,268	3,447	1,670	3,431
Klebsiella pneumoniae	Not tested	3,358	2,355	3,325	3,643	3,643	3,568	3,167	3,228

For the testing of IgY antibodies, 1:100 to 1:64000 dilutions were performed and then assigned to ELISA plates padded with specific antigens *(Streptococcus group B, Salmonella enteritidis, Proteus mirabilis, Salmonella spp., Candida krusei* and *Klebsiella pneumoniae).*
The titer of IgY anti *Streptococcus group B* is elevated in the first two months from the third inoculation (OD = 2,859 - 2,862), while noticing an increase of the OD values at 4,5 and 6 months (OD=3,471; OD=3,407; OD=3,275), and also maintaining at a constant level until the end of the experimental period (9th month OD=3,201); the results are highlighted in Table 1 and Fig. 3.

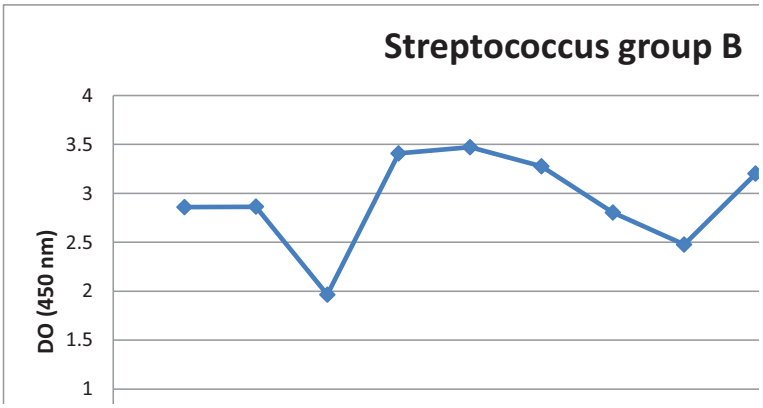

Fig. 3: The titer of IgY anti *Streptococcus group B*

Following the titration of IgY anti *Salmonella enteritidis* , we notice that the OD values are elevated for months 4,5 and 6 since the last inoculum (OD = 3,598; OD =3 ,468; OD = 3,696), and slowly decreasing at months 7 and 8 (OD= 1,624; OD = 1,333), followed by an increase at month 9 (OD = 2,221). The results are highlighted in Table 1 and Fig. 4.

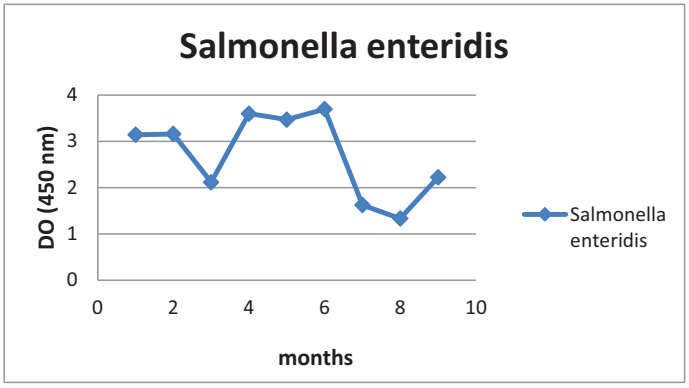

Fig. 4: The titer of IgY anti *Salmonella enteritidis*

The level of IgY anti *Proteus mirabilis* antibodies is relatively elevated for the first three months, offers higher OD values at months 4,5 and 6 (OD=3,130; OD=3,137; OD=3,323), and at 9 months from the start of the experiment (OD=3,077). The registered values are shown in Table 1 and Fig. 5.

By analyzing the results for the testing of the IgY anti *Salmonella spp*. antibodies, we notice that the 1:1000 titer is elevated throughout the experimental period (OD=3,456 for month 1 and OD=3,005 at month 9). The data are shown in Table 1 and Fig. 6.

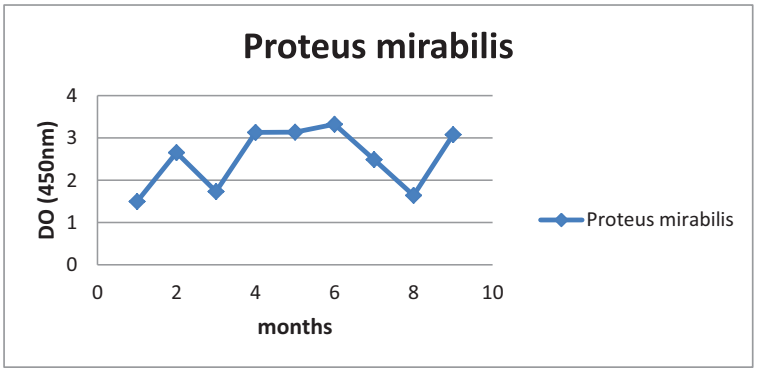

Fig. 5: The titer of IgY anti *Proteus mirabilis*

By analyzing the results for the testing of the IgY anti *Salmonella spp*. antibodies, we notice that the 1:1000 titer is elevated throughout the experimental period (OD=3,456 for month 1 and OD=3,005 at month 9). The data are shown in Table 1 and Fig. 6.

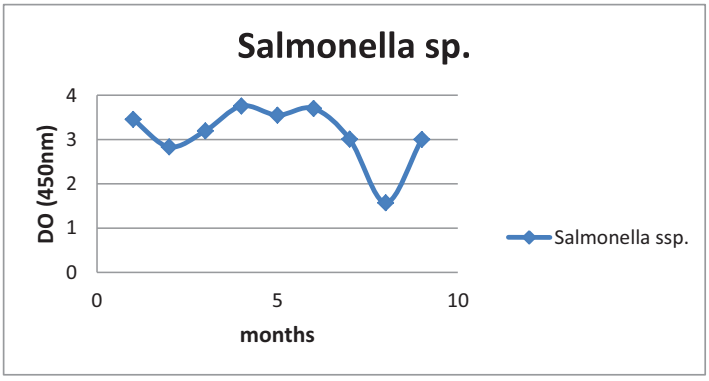

Fig. 6: The titer of IgY anti *Salmonella sp.* antibodies

The evaluation of IgY anti *Candida krusei* antibodies has shown elevated titers for months 4-7 (OD= 3,406; OD= 3,744). We notice that even at 9 months since the last inoculum the titer is high (OD= 3,431). The results are shown in Table 1 and Fig. 7.

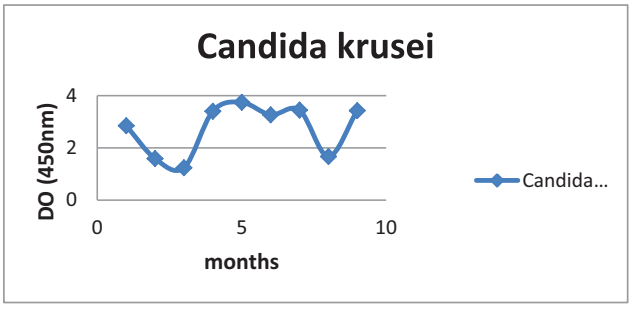

Fig. 7: The titer of IgY anti *Candida krusei*

Following the analysis of the results for the levels of IgY anti *Klebsiella pneumoniae* antibodies, we notice elevated levels throughout the experiment (OD between 2,355 – 3,643). The results are shown in Table 1 and Fig. 8.

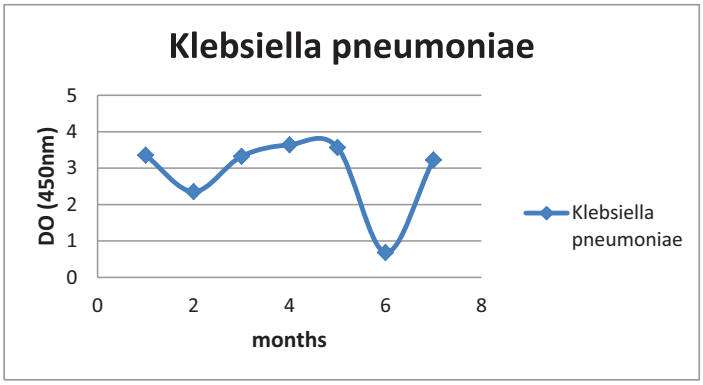

Fig. 8: The titer of IgY anti *Klebsiella pneumoniae*

Gathered data suggests that IgY antibodies extracted from hyperimmune eggs show specificity for the utilized antigens in the hens' immunization process. For the entire array of studied antigens, a decrease of the antibody levels was noticed during the 8th month, followed by an increase in month number 9.

CONCLUSIONS

Igy antibodies have attracted a significant degree of attention from the researchers, due to the structural differences from mammalian IgG and reactivity in the human body. The characteristics that differentiate IgY from IgG make it a hope for medicine, due to its potential use in several specialties.

The experiments performed have proven that Immunoglobulin Y extracted hyperimmune eggs show specificity to the epitopes of bacterial and fungal antigens used for the immunization of hens.

The determination of the titer for IgY antibodies, by using ELISA assays, has shown elevated levels over a 9 months period from the last inoculation process.

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