

## OBTAINING, PURIFICATION OF ANTIBODIES (IGY) FROM HYPERIMMUNE EGG YOLK WITH ACTIVITY AGAINST VIRAL PATHOGENS AND FORMULATION OF SOME THERAPEUTIC PRODUCTS

### OBȚINEREA, PURIFICAREA ANTICORPILOR (IgY) DIN GĂLBENUȘ DE OU HIPERIMUN CU ACTIVITATE ÎMPOTRIVA UNOR AGENȚI VIRALI PATOGENI LA OM ȘI FORMULAREA UNOR PRODUSE TERAPEUTICE

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**Cuvinte cheie:** Imunoglobulina Y, Ou hiperimun, Virusul Herpes simplex tip I, Papilomavirus uman (HPV), Virusul varicelo-zosterian, Rotavirus

#### Abstract

In recent decades, avian antibody technology has become more popular in the medical field, successfully replacing the immunoglobulins obtained from mammals. Y antibodies (IgY) are extracted from the yolk of hyperimmune eggs harvested from hens (*Gallus domesticus*) immunized with different antigens and have an already proven applicability in various medical fields such as the manufacture of diagnostic kits and the development of passive antibody therapy protocols for various infectious diseases. It is well known that avian immunoglobulin therapy is effective in the prevention and treatment of bacterial diseases in humans and animals, especially with gastrointestinal localization. In the present study, the production of avian immunoglobulins with antiviral activity, their extraction and purification, as well as the formulation of therapeutic products for administration to human patients exhibiting clinically symptomatic infections with rotavirus, Herpes simplex virus type 1, human papillomavirus or Varicella zoster virus. These are highly pathogenic viruses, leading to significant human morbidity and mortality, for which the therapeutic options are currently quite limited.

#### Rezumat

În ultimele decenii, tehnologia pe bază de anticorpi aviari a câștigat tot mai mult teren în domeniul medical, înlocuind cu succes imunoglobulinele obținute de la mamifere. Anticorpii Y (IgY) se extrag din gălbenușul ouălor hiperimune recoltate de la găini (*Gallus domesticus*) imunizate cu diferite antigene și au aplicabilitate deja dovedită în diferite arii medicale precum fabricarea de kituri de diagnostic și elaborarea protocoalelor de terapie pasivă a diferitelor afecțiuni de natură infecțioasă. Este binecunoscut faptul că terapia pe bază de imunoglobuline aviare este eficientă în prevenția și tratamentul unor afecțiuni bacteriene la om și animale, în special cu localizare gastro-intestinală. În studiul de față s-au urmărit obținerea de imunoglobuline aviare cu activitate antivirală, extracția și purificarea acestora, precum și formularea unor produse terapeutice în vederea administrării la pacienți umani cu infecții clinic simptomatice date de Rotavirus, Virusul Herpes Simplex tip 1, Papilomavirusul uman sau Virusul varicelo-zosterian. Acestea sunt virusuri înalt patogene, responsabile de numeroase cazuri de morbiditate și mortalitate, pentru care, în momentul de față, opțiunile terapeutice sunt destul de limitate.

#### Introduction

Immunoglobulin Y (IgY) represents the

functional counterpart of mammalian immunoglobulin G (Guevarra et al., 2012).

IgY is the main antibody (Munhoz et al., 2014) synthesized by reptiles, birds, amphibians (Guevarra et al., 2012) as well as some species of fish (Munhoz et al., 2014).

In birds, it is released into the bloodstream (Munhoz et al., 2014), from where it is stored in egg yolk to provide passive immunity to the embryo.

It is used in various areas of the medical field, such as the diagnosis of diseases and the induction of passive immunity.

IgY technology has a number of advantages over production and application techniques of mammalian IgG, such as much lower costs of obtaining, ensuring the welfare of the animals used and the absence of reactivity to rheumatoid factor (Guevarra et al., 2012).

### **Herpes simplex virus type I (HSV-1)**

causes worldwide oro-facial infections in human patients.

It is a neurotropic alpha-herpes virus with rapid replication cycle.

HSV-1 may be a major cause of morbidity and mortality; the spread of the virus is maintained by the worldwide existence of clinically healthy carriers.

Although the main clinical manifestation is oropharyngeal infection, HSV-1 can cause genital, cutaneous and ocular infections and a number of rare diseases such as herpes encephalitis or herpes hepatitis.

Although several antiherpetic drugs have been discovered and released on the market over the past decades, there is, however, the risk of developing drug-resistant strains and an effective vaccine against this virus has not yet been discovered (Arduino et al., 2008).

**Human papillomaviruses (HPV)** are integrated into a family of over 130 strains with pathogenicity in the skin and mucosa.

Mucosal localization strains can cause cervical cancer in women and anogenital warts in children and adults of both genders (Mammas et al., 2009).

Human papillomavirus infection is the most common sexually transmitted viral infection worldwide.

Human papillomavirus is one of the most powerful carcinogens, being involved in cancers with various localizations. It only infects epithelial cells.

Although there is an effective vaccine against the major pathogenic strains causing cervical cancer, the success of a large vaccination campaign would be visible only over decades (Crosbie et al., 2013).

**Varicella-Zoster Virus**, also known as the Type 3 Human Herpes Virus, naturally infects humans only, targeting T lymphocytes, epithelial and glial cells.

Primary infection provokes varicella (chicken pox) (Gershon et al., 2015), with viral replication in various organs, mostly in the skin, where major clinical manifestations occur (Baird et al., 2013).

During varicella evolution, the virus is settled down in the neuronal glans, where it remains latent.

As immunity decreases with age or in case of immunosuppressed patients, the virus reactivates and triggers the herpes zoster (shingles)(Gershon et al., 2015).

This is manifested by rash and local pain, which can often become chronic (Baird et al., 2013).

**Human rotavirus** is an ubiquitous organism responsible for severe gastroenteritis manifested by severe diarrhea, vomiting and dehydration in children less than 5 years of age (Tagbo et al., 2014; Ramig, 2004), being one of the major agents causing mortality patients in this age group, especially in developing countries (Diez-Valcarce et al., 2019).

It spreads from person to person, especially by faecal-oral route (Anderson and Weber, 2004).

Rotavirus preferentially infects mature upper intestine enterocytes, but the pathogenetic mechanism is not known accurately (Vega et al., 2011).

In the **Research & Development Department Imunoinstant of Romvac Company S.A.**, specific avian immunoglobulins are obtained against a wide range of bacterial, fungal and viral agents, pathogenic to humans and to veterinary medicine patients (Chiurciu și col., 2017; Topilescu și col., 2014).

In the present study, standard viral strains were used to obtain avian immunoglobulin Y with specificity against 4 pathogenic viruses in humans.

Immunoglobulins obtained were used to prepare therapeutic products for oral or cutaneous application.

## 1. Materials and methods

### Animals included in the study

The study was developed within the Research & Development Department Imunoinstant of Romvac Company S.A.

All procedures complied with EU Directive no. 2010/63 on the handling of animals used for scientific purposes.

The study was approved by the Ethics Committee of Romvac S.A. 4 batches of 10 healthy chickens (*Gallus domesticus*), Rhode Island breed, 18 weeks of age and 2.5 kg body weight were made.

The birds were accommodated in the battery-growing system in rooms with controlled temperature, humidity, noise and light.

They were fed with standardized, ecological diet, on an *ad libitum* principle.

### Antigens

Standard viral strains were purchased: Herpes Simplex virus type 1 (HSV 1), the Oka strain of live attenuated varicella-zoster virus, Rotavirus and Human Papillomavirus (HPV) 9-valent - types 6, 11, 16, 18, 31, 33, 45, 52 and 58.

In the microbiology laboratory, these strains were used to prepare four separate antigens.

### Immunization of the hens and the collection of eggs

Each batch was administered with one of four prepared immunogens.

Three inoculations were performed intramuscularly with 0.5 ml antigen in two separate points in the chest muscles.

Egg collection was performed starting on the 14th day after the third immunization, when the yolk antibody titer reached a maximum level.

In addition, it is considered the ideal time

to collect the eggs when 100-250 mg IgY / egg can be extracted from the yolk.

The collected eggs are stored at 2 - 8 °C.

### **Obtaining antiviral specificity immunoglobulin Y-based products**

The hyperimmune eggs harvested from the four batches of chickens were stored under refrigeration conditions, depending on the four antigens used for inoculation.

Of the four categories of hyperimmune eggs were prepared products with therapeutic value, as follows:

Eggs containing anti-HSV-1 IgY were used to prepare two categories of products. Some of them were used for the extraction of immunoglobulins by physical methods, obtaining highly purified IgY in aqueous, sterile solution.

The solution was dispensed into vials of 80 mL for one daily use.

To prepare an ointment for local applications, whole eggs (yolk + white) were used, from which a freeze dried powder was obtained.

The ointment used on specific oro-facial lesions was prepared according to a standard Romvac recipe:

- Eggs containing anti-HPV IgY were used to prepare an aqueous, sterile IgY solution similarly to the previous point.
- Eggs containing anti-Varicela-Zoster Virus IgY were used similarly to were used similarly to IgY anti-HSV-1.
- Eggs containing anti-Rotavirus IgY were used to prepare an aqueous, sterile IgY solution, similarly to those from previous points. However, given the target patient

category for which it was conceived, children younger than 5 years, the solution was concentrated and dispensed into 2 ml bottles for single use.

### **Qualitative and quantitative evaluation of IgY antibodies from eggs and prepared products**

In the Research and Development Department, the hyperimmune eggs are tested for the specificity of the immunoglobulins contained against the antigens used for immunization by the indirect *in house* ELISA test. In addition, the total IgY concentration was determined by the direct *in house* ELISA test.

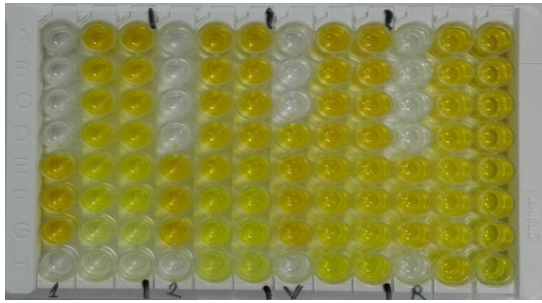
For products obtained from the hyperimmune eggs, the total Ig Y concentration is tested by the same technique and the total protein concentration by the Kjeldahl technique.

Microbiological controls aim the sterility for highly purified solutions with IgY, respectively contamination for ointments, according to the Romanian Pharmacopoeia, Xth edition.

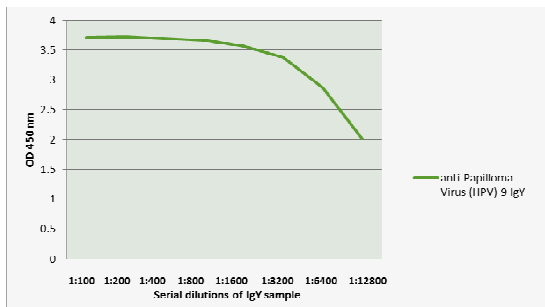
## **2. Results and discussion**

Hyperimmune eggs harvested 2 weeks after the last inoculation were tested for the specificity against the strains used to prepare immunogens by the indirect ELISA test (Figure 1).

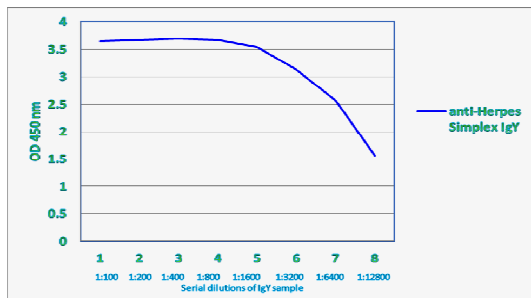
Spectrophotometric reading at OD<sub>450nm</sub> results in large, decreasing numerical values as the IgY sample dilutions tested on the ELISA plate increased (Figures 2, 3, 4, 5).



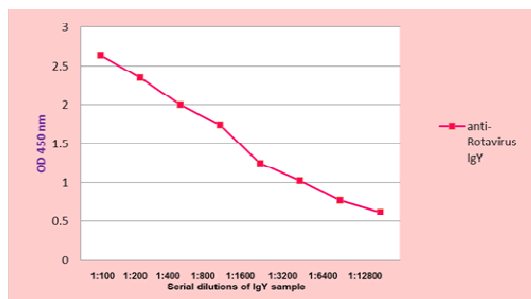
**Figure 1.** Specificity testing of IgY samples extracted from hyperimmune eggs harvested from hens immunized with Herpes simplex virus type 1 (1), 9-valent human papillomavirus (HPV) (2), Varicella zoster virus (V) and human rotavirus



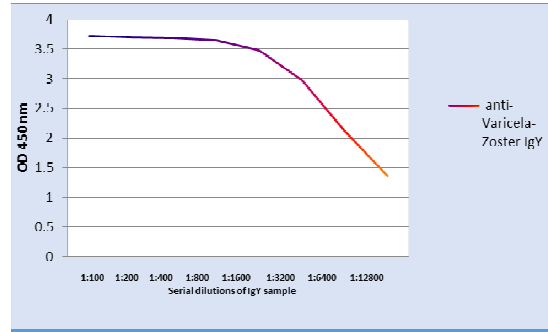
**Figure 2.** OD evolution depending on the dilutions of IgY anti-Papillomavirus containing sample, qualitatively tested by direct ELISA



**Figure 3.** OD evolution depending on the dilutions of IgY anti-Herpes virus type 1 containing sample, qualitatively tested by direct ELISA



**Figure 4.** OD evolution depending on the dilutions of IgY anti-Rortavirus containing sample, qualitatively tested by direct ELISA



**Figure 4.** OD evolution depending on the dilutions of IgY anti-Varicella zoster virus containing sample, qualitatively tested by direct ELISA

Therapeutic products were prepared containing purified IgY as sterile aqueous solutions for oral administration and ointments containing lyophilised whole egg yolk for local application, around the lesions.

The vials with sterile solution were tested by direct ELISA to determine the IgY concentration, respectively by the Kjeldahl technique to determine the total protein concentration.

Total IgY concentration values range from 57.12 mg / 100 mL to 540.20 mg / 100 mL and fit within the 50-250 mg IgY / 100 mL reference interval (Figure 6).

Total protein values ranged from 222.27 to 466.84 mg / 100 ml and fit within 200-500 mg / mL range, according to the internal guidelines (Figure 7).

Ointment products were tested by direct ELISA to determine IgY concentration.

The values obtained are between 122.23 mg / 100 g and 235.79 mg / 100g and fit within the reference interval of 50 - 250 mg IgY / 100 g.

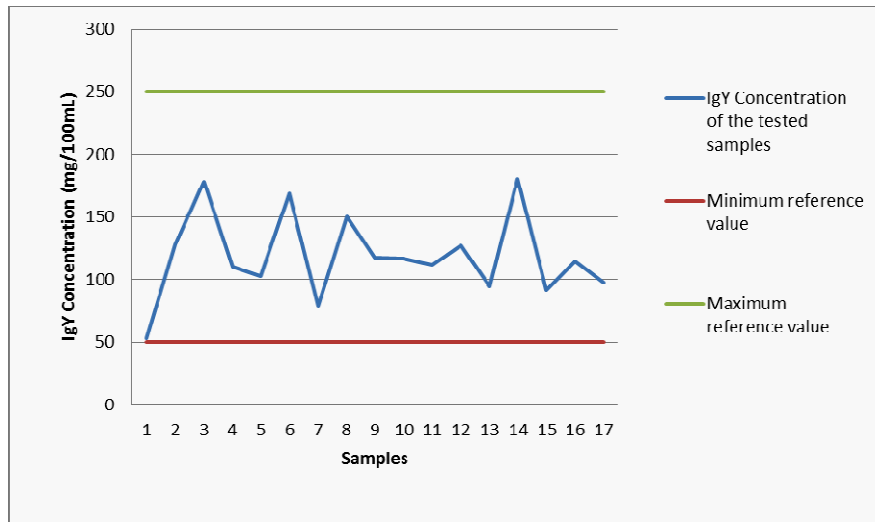


Figure 6. Variation of total IgY concentration in the obtained products

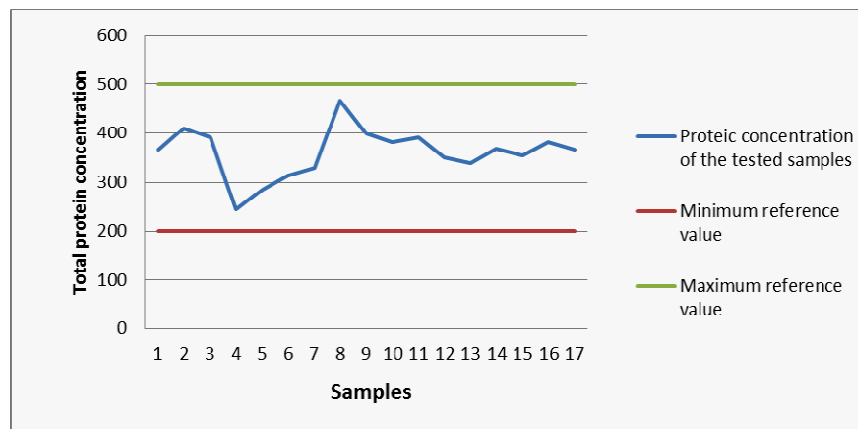


Figure 7. Variation of total protein concentration in the obtained products

### 3. Conclusions

Monovalent antigens were prepared using standard viral strains, with which four batches of laying hens were immunized.

From the harvested hyperimmune eggs, immunologically active proteins (IgY) were isolated and purified, based on which aqueous sterile preparations for oral administration were prepared.

In addition, ointments for local applications were prepared from eggs with specific IgY, anti-Herpes Simplex type I, respectively anti-Varicelo-Zosterian Virus.

The therapeutic products were administered to batches of human patients. Their clinical evolution was favorable.

Studies to identify the impact of specific anti-viral immunoglobulins on the patient's body, the histopathological evolution of lesions and cellular and humoral changes in the immune system's intimacy, will be developed.

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