HIPERIMMUNE EGG:PREPARATION, CHARACTERIZATION AND ALTERNATIVE IMUNOTERAPEUTHIC ACTIVITY

OUL HIPERIMUN: PREPARARE, CARACTERIZARE ȘI ACTIVITATE IMUNOTERAPEUTICĂ ALTERNATIVĂ

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Abstract

The research carried out describes one preparation procedure of the monovalent and polyvalent hyper immune eggs, obtained from hens immunized with a bacterial and fungal antigen complex, pathogenic for humans, resistant or sensitive to antibiotics. It was prepared a monovalent antigen of *Staphylococcus aureus* MRSA strain, isolated from a patient with urinary infection. Lots of laying hens have been formed, at the beginning of the laying period, which were immunized on days, 0, 14 and 28. From the hyper immune eggs obtained the specific immunoglobulin Y (IgY) was extracted and purified by polyethylene glycol precipitation (PEG 6000). Purity analysis was performed by polyacrylamide gel electrophoresis in a denatured system (SDS-PAGE) obtaining a single fraction with highlighting of heavy chains (molecular weight=75KDa) and light chains (molecular weight=25KDa). By the agar gel immune-diffusion assay (IDGA), the identity between analyzed IgY and standard IgY (MyBiosurse) was highlighted. The specificity of IgY antibodies obtained from hyper immune egg yolk was proved by the ELISA immune enzymatic assay, obtaining high titers for each antigen present in the inoculums. The hyper immune eggs were used for preparation of biologically active products from the range IMUNOINSTANT, recommended in the therapy of diseases caused by multi resistant germs.

Rezumat

Cercetările efectuate descriu un procedeu de preparare a ouălor hiperimune polivalente și monovalente (personalizate) obținute de la găini imunizate cu un complex de antigene bacteriene și fungice, patogene pentru om. rezistente sau sensibile la antibiotice. S-a preparat un antigen monovalent din tulpina de *Staphylococcus aureus MRSA* izolată de la un pacient cu infecție urinară. S-au format loturi de găini ouătoare, aflate la începutul perioadei de ouat, care s-au imunizat în zilele 0, 14 și 28. Din ouăle hiperimune obținute s-a extras și purificat imunoglobulina Y (IgY) specifică prin obținerea fracțiunii solubile în apă și precipitare cu polyethylene glycol 6000 (PEG). Analiza purității s-a efectuat prin elecroforeză în gel de poliacrilamidă în sistem denaturant (SDS-PAGE), obținându-se o singură fracțiune cu evidențierea lanţurilor grele (masa moleculară = 75KDa și a lanțurilor ușoare (masa moleculară=25KDa). Prin testul de imunodifuzie în gel de agar (IDGA) s-a pus în evidență identitatea între IgY analizat și IgY standard (MyBiosource). Specificitatea anticorpilor IgY obținuți din gălbenușul de ou hiperimun s-a efectuat prin testul imunoenzimatic ELISA, obținându-se titruri ridicate pentru fiecare antigen prezent în inocul. Din ouăle hiperimune s-au preparat produse biologic active din gama Imunoinstant, utilizate în terapia unor afecțiuni determinate de germeni multirezistenți.

Introduction

Since 1893, F. Klemperer has shown that a hen infected with a pathogen has the ability to produce a specific immunoglobulin, immunoglobulin Y (IgY), which is transferred from blood to the egg, in order to protect the future chicken [1].

Starting from this discovery, researchers around the world began to consider the hyper immune egg as a pharmaceutical product [8].

Immunoglobulins from the yolk belong to the IgY class; IgA and IgM are also present, but in small amounts.

IgY plays the same role for birds, as IgG for mammals [2, 3].

Scientists have found a very interesting way to provide a natural immunological support using the biologically active proteins from the hyper immune egg [5].

In addition to immunoglobulin Y (specific antibodies) against certain pathogenic germs, the hyper immune egg also contains immunomodulators, transfer factors, lysozyme, ovotransferrin, ovomucin, etc., of particular importance in restoring and stimulating the immune response of the whole organism [4].

The hyper immune egg is obtained by immunizing the hens with antigens from bacterial, viral or fungi strains, resistant to antibiotics, harvested from human patients; its content is a valuable source of antibodies that help to strengthen the body's immunity.

The efficacy of hyper immune eggs therapy in patients with various forms of arthritis, cardiovascular diseases, ulcerative disease, autoimmune diseases, cancer, inflammation, etc. has been demonstrated [7, 8].

Our studies aimed at obtaining polyvalent and monovalent (personalized) hyper immune eggs, as products which contain specific Y immunoglobulins against several pathogens or against a single microbial strain isolated from a particular patient, respectively.

1. Materials and methods

1.1. Animals.

The studies were conducted in healthy, conventional or specific pathogen free (SPF) Rhode Island Red hens, aged 20-23 weeks, divided in lots.

Birds were kept 2 per cage, at appropriate ambient temperature (20 ±2℃) and fed the standard R 21.5 diet.

During the experiment, the photoperiodic lighting program was 12 light / 12 dark.

1.2. Antigens

1.2.1. Polyvalent antigen preparation

The bacterial and fungal strains used in this study were obtained from hospital patients with clinical signs of disease, which were proven to be resistant to antibiotics: *Streptococcus pneumoniae, Staphylococcus aureus, Klebsiella pneumoniae, Salmonella* sp., *Pseudomonas aeruginosa, Clostridium difficile* - bacteria and toxins, *Acinetobacter baumannii, Escherichia coli, Enterococcus faecalis, Streptococcus* group B, *Candida albicans, Candida glabrata, Candida krusei* and the collection of the Microbiology Laboratory of Romvac Company: *Streptococcus mutans* ATTC 55670, *Salmonella typhimurium* R005TL3 / *enteritidis* TL 248 and *Helicobacter pylori* ATCC 49503.

For preparation of the polyvalent antigen used for inoculation, bacterial and fungal strains were grown on selective culture media, washed three times with sterile PBS, pH = 7.2 ±0,2 and inactivated with 0.5% formaldehyde for 18 h.

1.2.2. Personalized monovalent antigen preparation

The strain of methicillin resistant *Staphylococcus aureus* (MRSA) was isolated from a patient with urinary infection.

From the 24-hour bacterial culture obtained on BHI medium (Oxoid), the antigen was prepared according to the technique described in subchapter 1.2.1.

1.3. Immunization of the hens.

The chickens were inoculated at the beginning of the laying period, intramuscularly with the polyvalent or the monovalent antigen, representing 3-4 mg protein/ml of each strain re-suspended in sterile PBS pH = 7.2±0.2.

The mixture was emulsified in Vet-Sap (Desert King) adjuvant by calculating 0.045 ml adjuvant per 1 ml dose.

The antigen was injected at two different points in the chest muscles (0.5 ml/point). Antigen administration was repeated 14 days and 28 days, respectively, after the first inoculation.

Hyperimmune eggs were collected daily, two weeks after the last inoculation, and kept at 4 C until processing.

1.4. Extraction and purification of immunoglobulin Y (IgY)

The white was automatically separated from the yolk by means of an egg breaker (Ovo-Tech).

The yolk was diluted with cold (+4℃) deionised water at a 1:8 ratio; the suspension was homogenized with a turmix and adjusted to pH = 5.0 with 0.5 M HCl solution.

In the first step the mixture was frozen at -30 ℃ in an Arctisstore container.

For IgY extraction, the suspension was maintained at +20 ℃ for 48 hours.

The water-soluble fraction (IgY) was processed by pre-filtration and filtration and maintained at -4℃ until quantitative and qualitative tests were performed.

In the second step, the fraction composed of lipids and lipoproteins was removed by filtration and centrifugation.

For purification of IgY, the water-soluble fraction was precipitated with polyethylene glycol 6000 PEG (Sigma-Aldrich).

The mixture was stirred for 10 minutes, and then centrifuged at 10500 rpm for 20 minutes.

The supernatant was re-precipitated twice with 12% PEG and, after centrifugation at 10500 rpm, the deposit was re-suspended in PBS pH = 7.2±0.2.

The IgY purity was assayed by SDS-PAGE.

1.5 Denaturing polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis was performed using an Omni Page Electroblotter (Cleaver Scientific) equipment following the technique described by Laemmli [6].

IgY samples were diluted to a final concentration of 2 mg/ml protein using 2-mercaptoethanol (Sigma Aldrich) and bromophenol blue (Sigma Aldrich).

After incubation for 10 minutes at 96 ℃, 5 µl of each sample was added to the 10% migration and 4% concentration gels. The molecular marker (Protein Marker VI,

Applichem) containing a mixture of 12 proteins with molecular weights ranging from 10-254 KDa was used as control.

Electrophoresis was performed at 90 V and 185 mA for 90 minutes.

The gel was stained with Coommassie Briliant Blue R 250 (Sigma Aldrich).

1.6. The agar gel immunodiffusion assay (AGID)

It was performed in 1% Agar Noble prepared in borate buffer pH = 8.6; 17 ml of warm agar at 45-60 ℃ was poured into 90 mm diameter Petri dishes.

Seven wells (one central and six peripheral) of 6 mm diameter and a distance of 3 mm between them were punched in the gel.

For identity determination, standard IgY (MyBiosource) was dispensed in wells 3 and 5, IgG obtained from the hyper immune egg in wells 2 and 6, while in the central well and wells 1 and 4, 40 µl of rabbit IgG anti-IgY (Romvac) were distributed.

Binary dilutions (1/2 - 1/2048) were performed in order to establish the optimum working dilution for the test IgY samples.

The precipitation reactions were read after 24 hours.

1.7. Direct ELISA for quantification of IgY

It was performed using a Spectra Max 190 microplate reader, at a wavelength of 450 nm, and a standard kit (MyBiosource).

The ELISA was prepared "in-house" for each individual determination.

96-well microplates (Greiner Bio-One) were coated with 150 µl/well rabbit IgG anti-IgY at a 3.75 µg/ml concentration in carbonate-bicarbonate buffer (0.05M, pH = 9.6).

After incubation the plates were washed with 300 µl/well PBS-Tween. 1% bovine serum albumin (BSA) (Merck) was used as a blocking agent to prevent non-specific binding. 150 µl of each sample diluted in PBS pH 7.4, of the positive control (IgY standard Sigma) and the negative control (IgY-SPF) were dispensed per well. Plates were incubated for 90 minutes at 37 ℃.

After washing, 150 µl of peroxidase-labeled IgG anti-IgG IgG (MyBiosurce) was added to each well at a 1:5000 dilution.

TMB was used as chromogenic substrate, and the reaction was stopped with 1N HCl.

1.8. Determination of specific IgY content by indirect ELISA.

For the quantification of antibodies in the hyper immune egg yolk, an "in house" developed ELISA was applied.

The 96 well microtiter plates (Greiner Bio-One) were coated with the bacterial suspension containing 10 µg/ml protein in carbonate-bicarbonate buffer (0.05M, pH = 9.0). After refrigerating the plate at 4 ℃ for 12 h and washing with PBS-Tween, the reaction was blocked with 300 µl/well fixation buffer and incubated for 30 minutes at room temperature.

After washing, 100 µl of a 1:1000 IgY suspension in binary dilutions was added to each well.

The microplate was incubated 2 h at 37 ℃ and washed with PBS-Tween; 100 µl rabbit IgG anti-IgY conjugate diluted 1:5000 was added to each well. Following incubation and washing, 100 µl / well TMB were dispensed,

and the reaction stopped with 100 µl/well stop solution. The plate was read on a spectrophotometer (Spectra Max 190) at 450 nm.

2. Results and discussions

Yolk processing for obtaining IgY comprised two steps: in the first step the water-soluble fraction and a lipid and lipoprotein layer were obtained. In the second step, the water-soluble fraction was precipitated three times with 3.5% and 12%, respectively, polyethylene glycol 6000. IgY purity was assessed by SDS-PAGE compared to a standard IgY (MyBiosource).

The results revealed two precipitation bands representing the heavy chains (HC) and the light chains (LC) (Figs. 1, 7 and 8).

Based on molecular marker migration, heavy chains with a molecular weight of 75 kDa and light chains of 25 kDa were identified. It should be noted that the purified IgY obtained by PEG precipitation shows the same two fractions (HC and LC) as the standard IgY (MyBiosource).

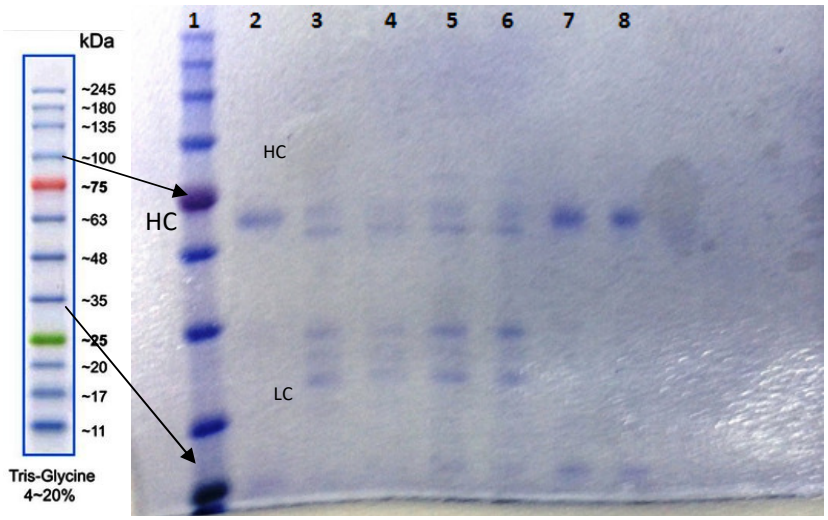
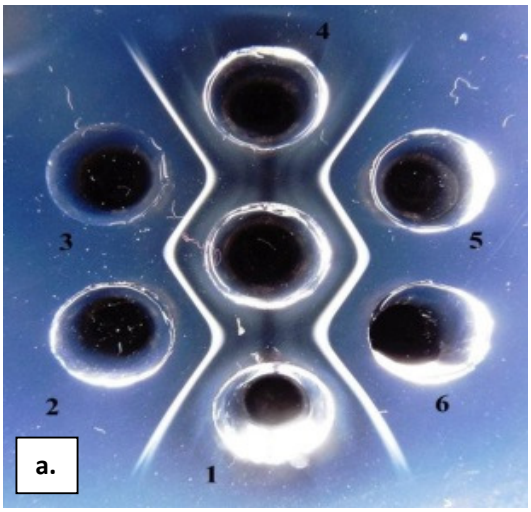


Figure 1. IgY purity analysis by SDS PAGE: 1. Molecular marker; 2. Standard IgY (MyBiosurce); 3, 4, 5, 6. IMUNOINSTANT MULTIPLU, 7, 8. PEG purified IgY, pointing out the presence of light and heavy chains (LC / HC)

Purified IgY obtained from hyperimmune eggs was tested against standard IgY (MyBiosource) by the AGID assay.

The results obtained demonstrated the identity between the standard IgY (wells 3 and 5) and the purified IgY (wells 2 and 6) against rabbit IgG anti-IgY (Fig. 2a)

It should be pointed out that the purified IgY precipitation line shows continuity with the one given by the standard IgY, being located halfway between the wells (Figure 2a).



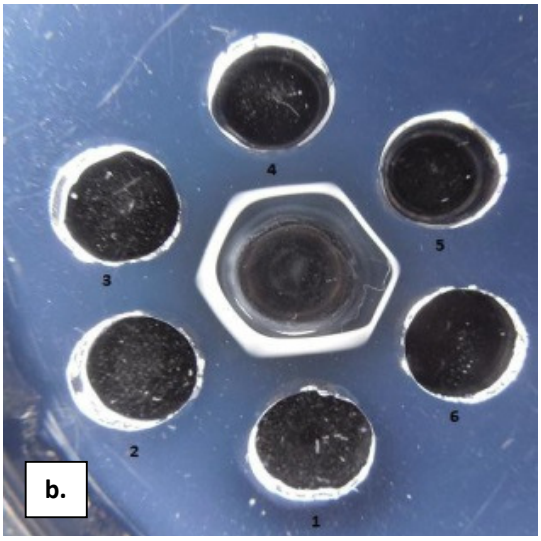


Figure 2: AGID test: a) identity assessment between standard IgY (wells 3 and 5) and purified IgY (wells 2 and 6); in wells 1, 4 and central: rabbit IgG anti-IgY; b) testing of purified IgY in binary dilutions (1/2 - 1/64): wells 1 to 6; in the central well: rabbit IgG anti-IgY

The purified IgY was tested in dilutions, ½ up to 1/2048, against rabbit IgG anti-IgY. Intense precipitation lines were obtained at 1/2, 1/4, 1/8, 1/16 dilutions, 1/32 being set as the optimal working dilution (Fig. 2b, well 5).

The concentration of immunoglobulin Y (mg/100 ml) in polyvalent hyper immune eggs was compared to the one in conventional eggs by direct ELISA.

The results are summarized in Fig. 3. IgY concentration in the hyper immune eggs ranged between 630.47 - 686.13 mg/100 ml, while in the conventional eggs it varied between 297.45 - 402.7 mg/100 ml.

The statistical analysis of these values indicated a significant difference ($p < 0.001$) between the two egg categories.

This gives the hyper immune egg special qualities due to its rich content in specific immunoglobulins (antibodies) against certain pathogens.

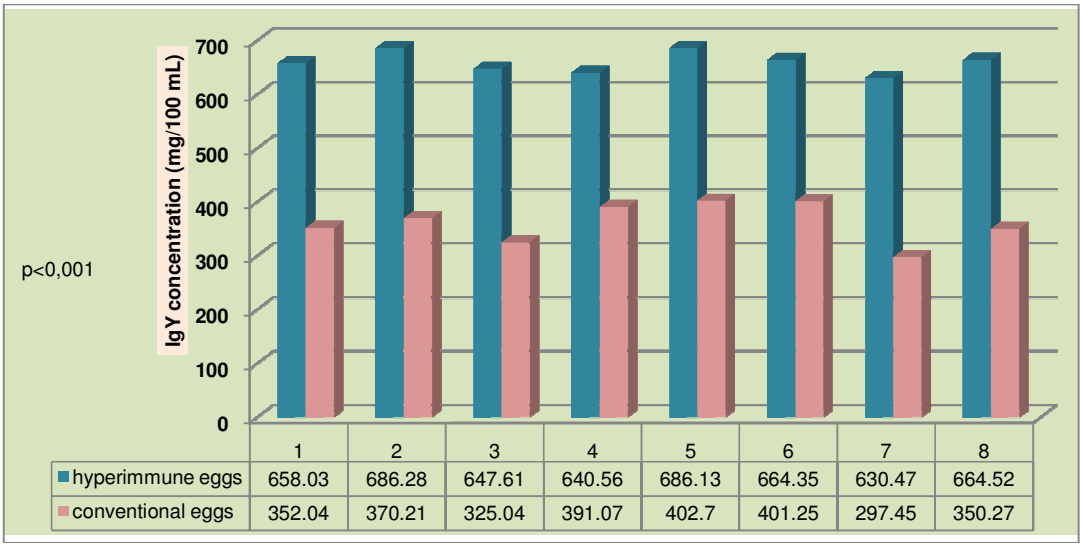


Figure 3. Average IgY concentration (mg/100 mL) in hyper immune and conventional eggs

Immunoglobulin Y obtained from polyvalent hyper immune eggs was tested for specificity by a qualitative ELISA technique (Fig. 5) as compared to conventional eggs (Fig. 4).

The test was performed against the antibiotic resistant bacterial strains contained in the inoculum: *Staphylococcus aureus, Escherichia coli, Acinetobacter baumannii* and *Clostridium difficile*.

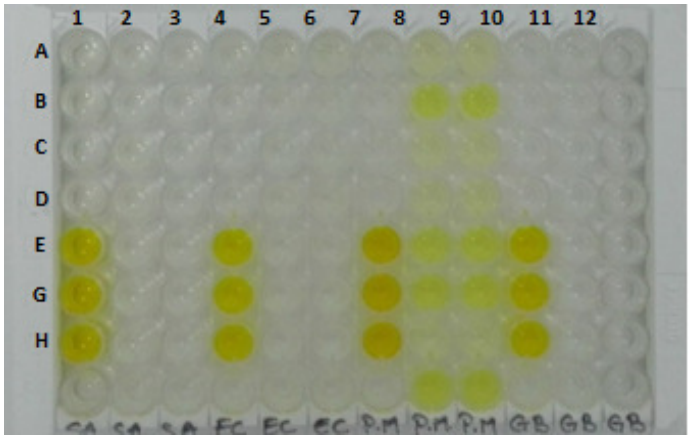


Figure 4. Specificity testing of IgY extracted from conventional egg yolk against four antigens (*Staphylococcus aureus, Escherichia coli, Acinetobacter baumannii, Clostridium difficile*) by ELISA

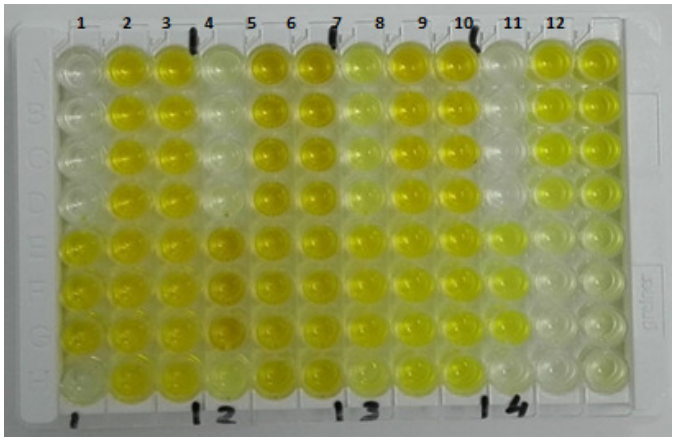
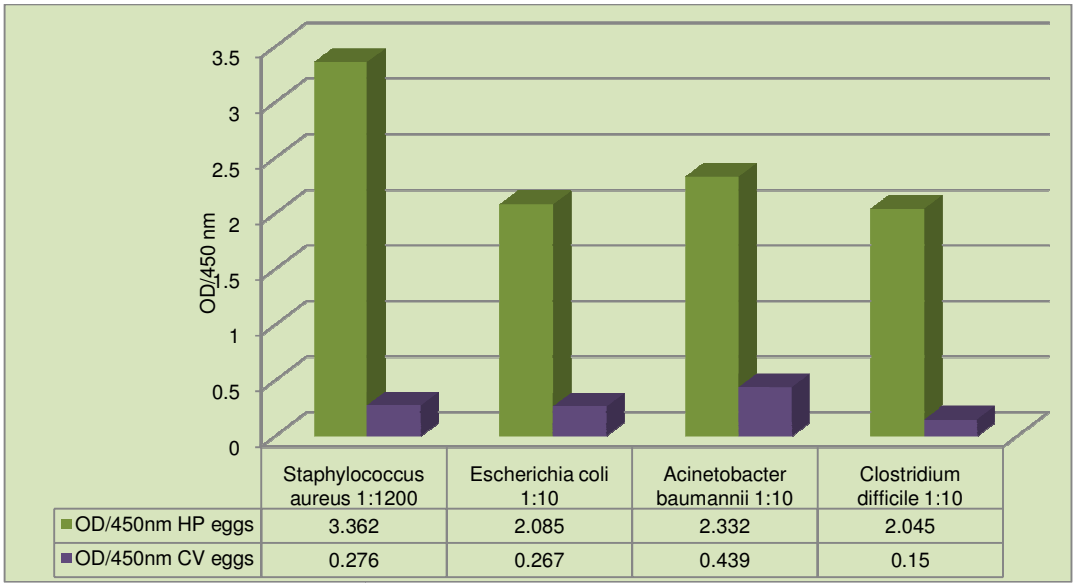


Figure 5. Specificity testing of IgY extracted from polyvalent hyperimmune egg yolk against four antigens (*Staphylococcus aureus, Escherichia coli, Acinetobacter baumannii, Clostridium difficile*) by ELISA



	Staphylococcus aureus 1:1200	Escherichia coli 1:10	Acinetobacter baumannii 1:10	Clostridium difficile 1:10
OD/450nm HP eggs	3.362	2.085	2.332	2.045
OD/450nm CV eggs	0.276	0.267	0.439	0.15

Figure 6. Specificity testing of IgY obtained from polyvalent hyperimmune eggs and conventional eggs against four antigens by ELISA

The data presented in Figure 6 revealed that IgY antibodies extracted from hyper immune eggs exhibit a high specificity against the tested bacterial antigens.

Optical density values are high, but different for each antigen:

- OD = 3,325 for *Staphylococcus aureus*;
- OD = 2,085 for *Escherichia coli*,
- OD = 2,322 for *Acinetobacter baumannii*
- OD = 1,500 for *Clostridium difficile*.

This demonstrates that the immune system of the hens responds to each inoculated antigenic stimulus.

The results obtained show that the polyvalent hyper immune egg contains specific antibodies (IgY) against the bacterial strains present in the inoculums.

The hyper immune egg contains high antibody titres as compared to the normal egg for consumption which possesses IgY only against the pathogens with which the hen came in contact either by chance, or by vaccination.

The hyper immune egg also contains immunomodulators and transfer factors of particular importance in stimulating the immune response of the entire organism, as well as specific ovotransferrin.

Since the products containing polyvalent IgY specific against the antigens inoculated into the chickens cannot cover the entire range of bacterial, fungal or viral infections with which patients are confronted, personalized monovalent hyper immune eggs have been produced, which contain antibodies to a particular microbial strain that affects a particular patient.

Personalized hyper immune eggs were obtained using the MRSA strain isolated from a patient with an old, recurrent urinary infection, rebellious to the classical treatment.

The patient was given hyper immune eggs and a multivalent immunoglobulin solution, but clinically, the response was reduced; so we decided to set up a personalized therapy based on monovalent immunoglobulin against that given MRSA.

The therapy consisted in the administration of personalized eggs and of a solution containing customized monovalent immunoglobulin.

The anti-MRSA antibodies were tested for specificity against the strain used for inoculating the hens (Table 1).

Table 1.

ELISA test: OD values of the anti-*Staphylococcus aureus* (MRSA) IgY antibodies (personalized treatment)

Wells	Controls (OD)		IgY anti *Staphylococcus aureus* (MRSA)		
			Dilutions	1 (OD)	2 (OD)
A	Blank	0.035	1:100	3.704	3.709
B	IgY negative control	0.057	1:200	3.708	3.709
C	IgY negative control	0.050	1:400	3.685	3.694
D	IgY negative control	0.056	1:800	3.626	3.671
E	IgY positive control	3.670	1:1600	3.496	3.519
F	IgY positive control	3.896	1:3200	3.013	3.081
G	IgY positive control	3.933	1:6400	2.304	2.379
H	Blank	0.057	1:12800	1.534	1.617

The results in Table 1 show high OD values of anti-*Staphylococcus aureus* (MRSA) antibodies diluted:

- 1:100 (OD = 3,704);
- 1:200 (OD = 3,708);
- 1:400 (OD = 3,685);
- 1:800 (OD = 3.626);
- 1:1600 (OD = 3.496);
- 1:3200 (OD = 3,013).

Application of the personalized therapy had a favourable result, the microbiological examination of the urine samples scoring negative.

The advantage of monovalent hyper immune eggs is that they can be personalized depending on the patient and/or infection, so that they act strictly upon the microbial strain that affects a particular patient.

Our results are consistent with studies by Lesli [2] and Peng Wei [3], which demonstrated that the hyper immune egg differs from the conventional egg due to the high content of specific immunomodulator antibodies, transfer factors, and ovotransferrin [4], exhibiting antimicrobial, anti-inflammatory and immunomodulator activity against pathogens that affect humans.

The hyper immune egg and the biologically active products from our IMUNOINSTANT brand (solutions, powders, sprays, gels, cosmetics, and suspensions) contain immunoglobulin Y as active substance, and help maintain the healthy status of the organism.

Immunoglobulin Y present in these products is a highly effective therapeutic agent against antibiotic-resistant pathogens.

The products are 100% natural, being used in the therapy of diseases caused by multi resistant germs (*e.g.* gastric, respiratory, and urinary infections, periodontal conditions, various forms of dermatites, autoimmune diseases, etc.)

3. Conclusions

- The polyvalent and monovalent personalized hyperimmune eggs have showed immunological activity and can be used as alternative biological products in the prevention and treatment of infections caused by sensitive and antibiotic resistant pathogens.
- The obtained results demonstrated the specificity of immunoglobulin Y derived from the hyper immune eggs, which reacts with the epitopes of the antigens used for immunization of the hens.
- The hyperimmune egg is a natural deposit of biologically active proteins, with protective capabilities, a product that can be used safely in immunotherapy, formulated as solution, powder, spray, ointment, gel, suspension, etc.
- The biologically active proteins ensure the passive immunization of the organism, with an overall stimulating effect upon the functions of the immune system.

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