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(54) **Title:** COMPOSITION, PREPARATION METHOD AND EVALUATION OF A COMPLEX IMMUNOGEN NAMED I-SPGA FOR PRODUCTION OF IMMUNOLOGICAL ACTIVE PROTEINS (IAP)

(57) **Abstract:** The present invention relates to the composition and method of preparing an immunogen designated as I-spga consisting of a complex antigen prepared from 18 to 26 species of pathogenic microorganisms isolated from patients, inactivated with binary ethyleneimine (BEI) and formalin, diluted in a SPGA immunopotentiator mixed with QS-21 adjuvant. By inoculating the hens with the I-spga immunogen, hyperimmune eggs (Imunospga) are obtained which contain immunologically active proteins specific to the 18-26 antigens used for immunization. The immune response of the hens is specific to the used antigens by amplification of the antigenic signal by the SPGA immunopotentiator and due to a special immunization program that allows the immune system to act complex and intense: The I-spga complex antigen contains 18-26 microorganisms isolated from patients, bacterial bodies, components from the bacterial bodies obtained by ultrasonography, cilia, exotoxins, endotoxins, spores, viruses, fungi or yeasts. This pathogenic material is inactivated with BEI and formalin. The I-spga antigen is of three types. The standard I-spga antigen is composed of 18 to 24 antibiotic-resistant bacterial species isolated from patients in Romania. The specific I-spga complex antigen is composed of the I-spga complex antigen containing a mixture of 7-9 strains from a single species of bacteria, fungi or yeasts isolated from patients in Romania mixed with SPGA and QS-21, used for inoculation of hens previously immunized with standard I-spga antigen. The personalized I-spga antigen is composed of patient-derived pathological material containing cellular debris and pathogenic germs inactivated with BEI and formalin and mixed with SPGA and QS-21 and is used to immunize hens previously immunized with the standard I- spga antigen. This now patented technology encompasses a new generation of biological products in which the immune response of the hens to different groups of parenterally inoculated antigens at different time intervals is overlapping. Chicken response is uniform and additional administration of immunogens and SPGA as an immunopotentiator amplifies the antigenic signal and immune response. The I-spga immunogen as well as the immune response contain two markers, G and A, which identify the I-spga antigen used for immunization against the antigens used to produce the Imunoinstant group bio-preparations or similar products. The I-spga immunogen is used to immunize the hens for obtaining immunologically active proteins that can be used to treat immune deficiencies, psoriasis, epidermolysis bullosa, other dermatitises, nosocomial infections, antibiotic-resistant infections in the urinary system of children and grownups.

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COMPOSITION, PREPARATION METHOD AND EVALUATION OF A COMPLEX IMMUNOGEN NAMED I-SPGA FOR PRODUCTION OF IMMUNOLOGICAL ACTIVE PROTEINS (IAP)

Technical field

Human medicine, veterinary medicine, immunopathology, polyclonal antibodies, preventive and curative treatments by passive immunization, control of antibiotic resistance.

DESCRIPTION OF THE INVENTION

Presentation of the state of the art including bibliography

During 2012-2014, studies have been conducted concerning the production of the hyperimmune egg and the use of hen immunoglobulins (IgY). These studies have led to several patents (16-20). The above mentioned products were the first generation of immunoglobulins and immunomodulators obtained by a different technology cocerning the immunogen used to immunize the hens. The Imunoinstant brand comprise biological products prepared from conventional laying hens in which the polyvalent antigen was prepared from bacteria, yeasts, viruses using for inactivation a standard method with 3% formalin for two hours at 37 °C. This method does not allow the preservation of certain antigenic fractions and epitopes present in the antigenic structure of the bacteria, yeasts, viruses or fungi. In all bacterial species, the same method was used, inactivation with formalin and formation of the immunogen with QS-21 adjuvant. There are some bacterial strains for which the ultrasound treatment allows the increase of antigen contact surface. In this generation of biological products the antigenic mass used for immunization, was represented by a mix of antigens obtained from several antibiotic-resistant or -susceptible strains isolated from patients, which, after inactivation, were prepared as immunogen in admixture with QS-21, a purified saponin derivative.

The success of an immunization depends on several factors, including the time intervals between first, second and subsequent immunizations. For this purpose, numerous studies were conducted using 0, 14 and 28 days intervals (37, 26) or once weekly for 7 consecutive weeks (27). Several authors immunize chickens at 10-day intervals (27, 28), but the general recommendation is that the interval between the first and the second injection of the immunogen should be of at least 4 weeks. This reflects the immunological memory, which must be given time to develop (31).

In hens immunized at day 0, week 10 and week 15, very high titres of antibodies were obtained, in the range of 1:160,000 (30). These results were reproducible, were similar, and application of a later impulse was recommended (31, 55). It has been demonstrated that a very high titer can be obtained by prolonging the pulse interval from 14 to 42 days (32). There is evidence that frequent immunizations over short periods of time lead to immunological depression instead of stimulating the production of antibodies (29).

For preparation of Imunoinstant, the first generation of products, the hens were immunized at short intervals of 10 days, respectively. Instead, in the new generation of biological products which is the subject of this patent, immunization was performed at 0, 30 and 45 days, respectively, leaving time to restore the immunological memory of the hen.

Influence of immunization on laying capacity.

Hen's performance is normally easily influenced by a simple antigen inoculation (40, 34, 35), but the antibody titer may decrease depending on the type of immunogen injected. For example, in hens immunized with solutions containing somatic, secreting-excreting antigens from an intestinal parasite (*Ascaris suum*), the immune response of the inoculated hens decreased or stopped completely after 3 weeks, probably due to the sub-toxic position which originates from para-intestinal sites (35, 36).

In addition, the Freund complete (FCA) and incomplete (FIA) adjuvants influence the stability of antibody (Ab) titers. It was found that the use of FCA caused a significant decrease in the titer of Ab in SPF Rhode Island hens (39), but had only a minor and transient effect (38) or even failed to significantly affect the stability the Ab titre in Leghorn white hens (40). The difference between these results was probably caused by differences in the experimental conditions.

Increase of IgY titre

After immunization of a rabbit, the immune system produces a primary response followed by a secondary response caused by the injection of the second dose from the same immunogen. The two phases have different characteristics. During the primary response, IgM is synthesized but only at a low and transitory level for raising the antibody titres, while after secondary immunization, IgG antibodies appear at high titers. Antibody titres last for several weeks. IgY antibodies appear in the hen serum approximately 4 days after inoculation of the antigen, reach a maximum titre at 6 to 8 days and decline after that. Ab titre may be increased by booster

immunizations. There appear to be two different mechanisms of the secondary immune response. According to the results, some chickens respond with mammalian-like antibody kinetics, while others respond to the same immunization protocol with an increase in the Ab titer after the first immunization, but fail to make significant changes after the second inoculation.

Most frequently, the antibody titer kinetics in hens after the first immunization show a transient IgY increase in the first phase, while the second phase (booster immunization) is characterized by an initial increase in the Ab titer for approximately 10 days, followed by a plateau for another 10 days and a drop after that. Increase of antibody titre is probably a consequence of the relatively short half-life of IgY as compared to IgG. On the contrary, some authors have shown that the Ab titres remain high and stable for several weeks (41, 42, 43, 44). Until now, the reasons for these differences in the kinetics of antibody production remain unclear.

To increase the antibody titer, the solution presented in this patent, will relay on new formulations for immunopotentiators (SPGA) and adjuvants (QS-21), and will use a modified immunization program.

Imunoinstant products (16-19) have not been grouped according to their medical intended use (oral, cutaneous, mucosal, respiratory), nor was a program set up to control and differentiate these products.

No immunopotentiators have been used to increase the antigenicity in direct relationship with the immune system of the immunized hen and no immunopotentiator was used as final diluent. For this immunogen, in combination with QS-21, Tween 20 was used as an emulsifier to increase superficial tension on the antigenic proteins (16, 19).

Our studies in IMUNONOMEDICA-SH laboratories aimed at improving the preparation conditions of the immunogens, in respect to antigenic content, complexity of antigens, selective inactivation, use of an immunopotentiator and Tween 20 as an emulsifier. Preclinical and clinical studies on humans support the need for preventive and curative use of immunologically active proteins (IAP) as well as other products containing immunomodulators, contact proteins, peptides and radicals present in the hyperimmune egg. Concerns about the hyperimun egg, as set forth in this patent, have been aimed at improving the quality of immunogens prepared from bacteria, yeasts and viruses and increasing their immunogenicity. To this purpose, the use of binary ethyleneimine (BEI) and formalin as inactivatants, the fragmentation of bacterial bodies by ultrasonography and gamma radiation inactivation were tested.

The antigenic stimulus was amplified by the use of a SPGA immunopotentiator, studied by the author since 1970 and used in the production of vaccines after 1972. SPGA has been shown to have protective activity on herpes viruses (cell-free) extracted from infected cells (cell-associated), as well as potentiator of antigenic stimulus in the body of the immunized hens (1-8). SPGA is used in the production of new immunogens for humans and animals (6-9).

We shall continue to use hens for immunization and hyperimmune eggs from which immunological active proteins (IAP) will be extracted. Antibody response in hens (*Gallus domesticus*) is more pronounced due to a longer phylogenetic distance between it and the product used for immunization. This phenomenon also refers to the relationship between IAP prepared from the hyperimmune egg and humans, as organisms that come into contact with the IAP. The accessibility of IAP in the human body is due to oral or on other mucosae administration and to the ancestral relationships between birds and humans. Mammals and birds have a common ancestor with an evolutionary drift of 350 million years inbetween. For this reason, it is possible that IAPs to be recognized as self in the human body.

The diversity of hen antibodies is mainly achieved by gene conversion and, additionally, addition, by the V-J flexibility play and the somatic point mutations as in mammals. In contrast to mammals, there is only one VH or VL gene in chickens, but in addition there are about 25 so-called pseudo-V-genes (which have lost the usual sequences for transcription regulation and signal recognition). Hens can frequently produce antibodies that will recognize more epitopes than mammalian antibodies (9-11). In these immune processes, by administering immunologically active proteins, the network of lymphocytes and molecules possessing variable regions (V) is invoked. The immune system is therefore seen as a network with components interconnected by V-V interactions. The T helper and suppressor cells, the role of accessory cells in non specific immune responses (74-78).

The use of glutamate, which acts on hen's T lymphocytes, increases the antigenic signal and makes the mature lymphocyte to take it over, store and transfer it from its V variable onto the variable V of hyperactive B lymphocyte, which will start the production of immunoglobulins with higher affinity to the antigens that originally produced the information.

Until this date, sodium glutamate has not been used as an immunomodulator in the immunogen prepared from 18-26 antigens from bacteria, viruses, fungi or yeasts. Recent studies have shown that glutamate is not only a neurotransmitter, but has also an important immunomodulator role. In

this regard, several glutamate receptors have recently been described on the surface of T cells, while glutamate transporters have been expressed in antigen presenting cells, such as dendritic cells and macrophages. This opinion integrates and summarizes the various findings in this emerging field. It is necessary to study the role of glutamate as a key immunomodulator in initiating and developing the immunity of T cells mediated in peripheral tissues as well as in the central nervous system (13).

The immunogen called I-spga is of three types: standard, specific and custom. Using this type of immunogen, a stronger immune response is obtained in the immunized hen and the antibodies exhibit a higher reaction capacity. These IgY antibodies are present in the yolk and the vitelline membrane. In the egg white, active immunological proteins are present, such as apo and holo ovotransferrin, ovomucine, ovalbumin and lysozyme. Besides egg white, these products are also found in the cochlear membrane and in the egg shell still attached to the shell membrane. These immunologically active proteins potentiate immunologically the whole hyperimmune egg, allowing a diversification of the derived biological products as well as the forms of treatment.

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SUMMARY OF THE INVENTION

Brief formulation of the technical solutions

The objective

To develop a technology for preparation of immunogens that preserve the antigenic structures as close to the original structures as possible, which will generate a complex immune response, an immune response against several epitopes, with increased antibody coupling power including validity, amplification of the avidity of immunologically active proteins, increasing the combination between affinity and avidity.

The I-spga immunogen inoculated in Rhode Island Red or Leghorn or SPF laying hens produces an adequate immune response for a period of over 40 weeks. The specific immune response is equal for each antigen, among a complex of up to 24 antigens. Each antigen is a blend of at least 7 strains of the same microorganism species, all germs being isolated from patients in Romania during 2016-2017. The immunogen administrated to hens should have the ability to form biologically active molecules at a pre-embryonic stage. These protein molecules from the egg have not been recognized in the literature and have not been studied until this technology has been applied.

All the immunologically active molecules such as ovalbumin, ovotransferrin, apo and holo forms, lysozyme and ovomucine, react immunologically specific for each of the antigens with which the hen was immunized, similar to IgY.

The immunogen called I-spga can be identified by two markers, G and A, using the techniques mentioned in this patent. No other similar product, at this date, uses biological markers for identification.

Following preclinical and clinical studies in hens and humans which responded to several technical and therapeutic needs, the following were obtained:

1. **The I-spga standard immunogen** comprising antigens prepared from 18-26 types of pathogens isolated from patients. I-spga is used to immunize hens which will lay standard hyperimmune eggs that are used in the production of immunologically active proteins (IAP).

2. **The specific I-spga immunogen** which consists of an antigen prepared from at least seven strains of the same species of bacteria or yeasts. This S-antigen is prepared in admixture with SPGA as immunopotentiator and QS 21 as adjuvant. It is used to reimmunize hens which were immunized with the standard immunogen. Finally, a complex immune response, consisting of the

standard and the specific immune response, is obtained. It is recommended for the treatment of *Clostridium difficile* infections, candidosis, etc.

3. **The personalized/custom I-spga immunogen** prepared from the pathological material collected from a patient, which is mixed with SPGA as immunopotentiator and QS-21 as adjuvant. This P-immunogen is inoculated into hens already immunized with the standard immunogen.

4. **Standard hyperimmune eggs** originating from hens inoculated with the standard immunogen and used for the treatment of immune deficiencies, including psoriasis and epidermolysis bullosa, in prevention and control of nosocomial infections, etc.

5. **Specific hyperimmune eggs** originating from hens inoculated initially with the standard immunogen and, after minimum 30 days, inoculated with the S-immunogen.

6. **Personalized/Custom hyperimmune eggs** originating from hens inoculated initially with the standard immunogen and, after minimum 30 days, inoculated with the P-immunogen.

The I-spga immunogen contains a complex antigen whose composition depends on the purpose for which the I-spga hyperimune egg is prepared.

- **The standard antigen** contains a blend of 18-26 antigens prepared from whole bacteria and/or yeasts, fractions of bacteria or yeasts obtained by ultrasonography, viruses and fungi. Bacterial and yeast antigens are in turn a blend of at least 7 antibiotic -resistant or -susceptible bacterial strains, and 5-7 yeast strains isolated from patients, resistant or susceptible to antibiotics.
- **The specific antigen** is a component that is administered supplementary and consists of 5-7 bacterial strains representative for each species of interest. This antigen, prepared as immunogen, is inoculated 30-40 days after the immunization of the hens with the standard immunogen.
- **The custom antigen** is made up of cell debris and microbial content collected as pathological material from a patient, from infected wounds, from psoriatic plaques, from mucous membranes or from urine. The bacteria isolated from the pathological material are cultivated in the laboratory, identified and inactivated, forming part of the customized antigenic content. The immunogen for inoculation is obtained by addition of SPGA and QS-21, and will be injected to standard immunized hens, at 30-40 days after the primary immunization.

The standard, specific, and customized immunogens are a microbial antigen mixture, 20 mg of each, diluted in 0.5 ml SPGA and 0.5 ml QS-21, respectively, for each hen.

- The **standard immunogen** is administered to Rhode Island Red, Leghorn conventional or SPF hens, aged 18-20 weeks, 2 ml immunogen by intramuscular route. Immunization is repeated after 30 and 44 days, respectively.

- Control of the immune response is performed 8 days after the first inoculation on blood samples, by quantitative and qualitative assessment of immunoglobulins, using ELISA and rapid and slow seroagglutination tests.

- **The supplementar immunization with monospecific or custom antigens** is done by the same procedure.

- Upon controls at days 30, 44, and 68, respectively, the total number of immune-responsive hens is determined, as well as the specificity of the immune response. At 68 days after the first immunization, quantitative and qualitative controls for all antigens used for immunization are also performed.

- To control the immune response after the first immunization, IgY is extracted and tested qualitatively and quantitatively using ELISA and rapid seroagglutination and slow seroagglutination.

The immunogen is stored at +4 °C and tested for the specific immune response in the inoculated hens at 8, 44 and 58 days after the first inoculation.

The immunization program is valid and the I-spga hyperimmune eggs can be used in production if the immune response is uniform in all inoculated birds and the amount of IgY is at least 300 mg/egg.

In the case of custom I-spga production, the eggs may be used even 8 days after the inoculation of the custom immunogen, if the immune response is positive, specific and in the I-spga-P hyperimmune egg contains at least 200 mg of IgY.

DETAILED DESCRIPTION OF THE INVENTION

OBTAINING THE STANDARD, SPECIFIC AND PERSONALIZED I-SPGA IMMUNOGEN

The standard immunogen. The standard I-spga biological product is prepared from 20-24 microbial antigens used for immunization of the laying hens. The antigen is represented by bacteria and yeasts isolated from hospitalized patients between 2016 and 2017. This product is used for immunization of the hens which will produce the standard hyperimmune eggs.

The specific I-spga immunogen. To the immunogen prepared from 16-20 germs, an immunogen specific to a certain bacterial or yeast species is added, inactivated under the same conditions, with binary ethyleneimine (BEI) and formalin. This specific immunogen is added to the standard immunogen or is used to additionally immunize birds already immunized with the standard I-spga (containing 16-20 types of antigens in its structure).

The personalized I-spga immunogen is obtained from a pathological material collected from a patient whose treatment with the standard I-spga immunogen was not successful, which is inactivated with BEI and formalin and, after purification, mixed with SPGA, QS-21 adjuvant and Tween 20. This immunogen is blended with the standard immunogen and the mixture used to immunize a new lot of hens, or is used by itself to immunize already standard immunized hens.

In accordance with the present invention, the preparation of the standard I-spga, specific I-spga and custom I-spga immunogens comprises several steps, among which: preparation of the antigens, preparation and control of I-spga immunogens.

PREPARATION OF THE ANTIGEN

In accordance with the present invention, 18-26 antigens are prepared from different pathogenic germs isolated from patients. The bacterial and yeast strains used for this purpose may be susceptible or resistant to antibiotics. These strains are part of the laboratory collection and originate from hospitals in Bucharest or from patients who requested treatment in the IMUNOMEDICA-SH medical clinic. For the control of the strains isolated from patients, strains from international collectionsare are used, officially purchased, with quality certificates and scientific guarantees.

For the specific I-spga, an S-antigen is designes for each microorganism, which contains the most representative and most commonly encountered species/strains in patients as subtypes. For example, for Staphylococcus spp., the blend consists of *S. aureus, S. lugdunensis, S. haemolyticus, S. warneri, S. schleiferi, S. intermedius, S. saprophyticus*. The choice of bacterial strains or yeasts is in line with international standards, but they come from patients in Romania. The structure of an I-spga immunogen includes: *Escherichia coli, Staphylococcus aureus, Fusobacterium nucleatum, Staphylococcus epidermidis, Enterococcus spp., Enterococcus faecium, Enterococcus faecalis, Corynebacterium spp., Corynebacterium diphteriae, Klebsiella spp, Klebsiella pneumoniae, Proteus mirabilis, Proteus spp. , Candida glabrata, Candida*

albicans, Streptococcus pneumoniae, Salmonella enteritidis, Salmonella typhimurium, Acinetobacter baumani, Clostridium difficile vegetative form, *Clostridium difficile* sporulated form, Clostridium difficile exotoxins A and B, *Pseudomonas aeruginosa, Neisseria meningitidis, Haemophilus influenzae, Helicobacter pylori*, the composition depending on the frequency of infections with antibiotic resistant germs or being demand. For the custom I-spga, a P-antigen is assambled consisting of cellular debris and the pathogenic group of pathogens opresent in psoriatic plaques, other wounds without scarring tendency, or pathological products collected from mucosae, sputum, and/or urine. The bacterial cells, cultivated and identified, are washed 3 times in phosphate buffer (PBS) and centrifuged at 4000 rpm for 15 minutes at 20 °C, inactivated with either BEI or formalin or gamma radiation and freeze-dried in 10 ml vials, 4 ml of bacterial suspension in each vial. After lyophilization, the vials are stored at -20 °C. 50 mg of bacterial bodies are resuspended in phosphate buffer (PBS) at the concentration represented by an optical density OD_{600} = 1.00 and mixed with 45 µl QS-21 adjuvant.

PREPARATION OF IMMUNOGENES

Immunization is the most important step in the production program of the immunologically active proteins, when in the body of the inoculated hens, a complex antigenic stimulus is developed which allows the transfer of the antigenic information to the V variable of immature T lymphocyte V which subsequently, by transformation form naive lymphocyte into mature lymphocyte, stores the information in its DNA and starts to clonate immediately. The mature T-lymphocyte transfers the information about the structure of antigens to the B lymphocyte, which, in turn, produces immunoglobulins. These immunoglobulins are transferred into the yolk which finally is integrated into the egg.

The only places in the egg where the immunoglobulins are found (IgY) is the yolk and the vitelline membrane. In the white, the shell membrane and the egg shell no IgY was found (Pătraşcu I.V. unpublished data).

Inactivation of pathogenic germs is performed differently, depending on each microorganism, with formaldehyde, or bromoethylamine-2-bromohydrate (BEA) and sodium thiosulfate, purchased from Sigma-Aldrich. The used inactivation methods are:

Inactivation with formalin and binary ethyleneimine (BEI)

This method of inactivation is used for various bacteria including *Clostridium difficile*, vegetative form and spores. This test is based on a kinetic inactivation assessment. Under optimal conditions, BEI inactivation rates are 0.5-1.0 log_{10} per hour. Generally, final inactivation occurs after 48 hours. Formaldehyde, the "classic" inactivation agent, inactivates bacteria at a rate of only 0.3 log_{10} per hour. In our used inactivation program, formalin is added at the start of the inactivation process as soon as BEI was added to the bacterial suspension. Under these conditions, the rate of inactivation is higher than 2-3.5 log_{10} per hour. This procedure allows inactivation overnight at high levels of protection of the antigens. It is known that formaldehyde also makes cross-links between the proteins present in the inactivated material, stabilizing thus the antigen (15). Following inactivation, the suspension was stored at +4 °C and a representative sample was taken and tested for completion of inactivation. If inactivation was complete, the antigenic mass was separated by centrifugation at 4000-6000 rpm, excess formaldehyde removed by tangential filtration and the antigen resuspended in SPGA to a concentration of 20 mg protein/ml. Afterwards the antigen is supended in SPGA and mixed with the QS-21 adjuvant. The immunogen is stored at +4 °C until use.

BEI was prepared by cyclization of 0.1 M bromoethylamine hydrobromide (BEA) (Sigma) in 0.1 M NaOH 5 M solution for one hour at room temperature according to the Bahnemann method (13). Cyclization of 0.1 M BEA in active form was confirmed by BEI, by changing the pH from 12 to 8. The solution was freshly prepared before each experiment. Controls of inactivation with BEI were performed using formaldehyde as inactivation control and an untreated culture as negative control.

BEI virus inactivation

The Flury rabies virus and CVS suspension are inactivated with BEI 6 hours at 37 °C, under constant shaking and pH correction to 7.6. After inactivation, BEI is neutralized with BEA.

Mixing the antigen with SPGA as immunopotentiator

Bacterial and levurian antigens, standard, specific or custom preparations, stored at + 4 °C, after confirmation of inactivation, are centrifuged at 4000 rpm for 30 minutes, and the sediment resuspended in SPGA and homogenized at room temperature for 30 minutes by shaking.

Mixing the antigen with SPGA and QS-21 adjuvant

Over the antigen and SPGA mixture, add the same volume of 0.2% diluted QS-21 adjuvant and shake for 15 minutes to homogenize. Transfer to sterile storage containers and store at +4 °C until use.

USE OF I-SPGA PRODUCTS FOR THE IMMUNIZATION OF LAYING HENS AND CONTROL OF IMMUNIZATION

Immunization of conventional or SPF laying hens

The immunization of laying hens with the selected antigen is well known technique. The present invention may employ any method for immunization of hens which allows administration of the given antigen by any route: intramuscular, subcutaneous, intracutaneous, intramuscular, intravenous.

For immunization and immunization control, conventional Rhode laying or Leghorn white or SPF laying hens, aged 18-20 weeks, are used. The standard, specific or personalized I-spga immunogen, is administered by intramuscular route, 2 ml per hen, 0.5ml in four different points of the chest muscles. Immunization of the hens is continued and I-spga administration is repeated 30 and 44 days, respectively, after the first inoculation.

The immune response is controlled directly from the hyperimmune egg or serum. The control is performed at 8, 30 and 44 days, respectively, after the first inoculation. The final control is done 14 days after the last immunization and periodically throughout the use period of the immunized hens.

From the eggs harvested for control, IgY is extracted by the simplified method, at cold. For this purpose, the yolk is separated and processed individually by cold extraction of IgY in deionized water acidified with 0.1N HCl, pH 5.1, at a 1:8 ratio, and overnight incubation at +4 °C. IgY is separated from the water by centrifugation at 4000 rpm for 15 minutes. The supernatant, which contains the pH 5.1 water-extracted immunoglobulins, can be preserved by freezing in 1 ml cryotubes at -20 °C or -85 °C. For routine control activities it is recommended to use thimerosal to limit bacterial multiplication. Thimerosal is better than azide because azide containing products can not be tested by ELISA.

The egg white is processed separately for preparation of biological products that can be used in humans and animals. The shell membrane is separated from the egg shell and is used for different formulations of biological products for external use in humans and animals. The shell of the

hyperimmune egg can be used for preparation of calcium-containing products by drying and granulation. In a second variant, the egg shell and the shell membrane are mixed together generating another product.

IgY extraction

a) Separate the yolk from the white after breaking the vittelin membrane of the eggs collected from immunized hens, 8 or 30 days after the last administration of the given antigen.

b) Measure the volume of yolk and then mix with cold (+ 4 °C) distilled water, pH 4.5 at a 1:8 ratio, and blend with a turmix for 2 minutes at room temperature.

c) Adjust the pH to 4.5 and add Thimerosal 0.01% (Thimerosal is not added to some products).

d) Store the mixture for 6 hours or overnight at + 4 °C.

e) Collect the aqueous phase containing IgY.

f) Extract IgY by filtration at 20 °C using filters that allow the passage of proteins weighing less than 20 kDa to retain the water-insoluble biomolecules, mostly lipids and yolk granules.

g) Store IgY at +4 °C for 7 days or at -22 °C for 6 months. If freeze-dried, it can be stored at least 1 year.

Control tests for the Ispga immunogen

The control tests used for the I-spga immunogene are:

1. Microbiological sterility
2. Safety
3. Evaluation of the immune response of the inoculated hens
4. Identification of the G and A biological markers that differentiate these products from the similar ones in Romania or other countries.
5. Evaluation of the immune response of hens against I-spga immunogen as intensity, mg protein/egg, uniformity per immune response in time
6. Evaluation of the complex immune response against *Clostridium difficile* (model and case study)
7. Evaluation of I-spga capacity to transform protein molecules (OTf, OM, OA and LY) into biologically active molecules by ELISA and RSAR

1. Microbiological sterility

The method addresses the testing of the finished product for assessing the presence of microbial or fungal contaminants in the I-spga immunogen solution. The microorganisms may come from the production process or the materials used to prepare the immunogen.

Testing is performed in accordance with provisions of Eurepean Pharmacopoeia 8th edition.

Samples are taken from the finished product vials and inoculated into tryptic soy broth (TSB) and thioglycollate medium (FTM). The samples are incubated at +30-35 °C and +20-25 °C, respectively, for a period of 14 days.

The I-spga immunoglobulin batch is admitted for use if no contaminants are present.

2. Safety of the I-spga immunogen administered intramuscularly to laying hens

The safety studies for the I-spga immunogen administered intramuscularly to Rhode Island Red breed laying hens aged 20 weeks consisted of assessment of the reproductive function. Local and general reactions were studied, as well as the influence on the onset of laying and potential adverse effects at all check stages (60-70).

One of the main objectives of these tests was to obtain a prognosis for the effects of the I-spga immunogen administration in the hens, based on the reactions observed locally, generally and on the laying. The tests were performed to ensure the safety of I-spga immunogen (71). These effects may vary depending on the species and depend on the bioavailability and reactivity of the agent on the receptor called muscle tissue.

Safety test for I-spga immunogen

Parameters:

- Reaction at the inoculation site
- General reactions
- Egg production
- Quality of the eggs

Procedure:

Laying hens: Rhode Island Red aged 20 weeks

Number of lots: 2

- Lot 1: I-spga immunogen group where the amount of inoculum is twice as high as the recommended inoculum for intramuscular administration at four different points.

- Lot 2: control group inoculated with 4 ml sterile PBS, 1 ml per each inoculation point

Number of chickens per group: 10

Amount of immunogen used in the test: twice the recommended amount.

Route of administration: intramuscular

Number of doses: single dose

If complete or incomplete Freund's adjuvant is used: NO

Observation period: 30 days

Table 1: Safety assessment of the I-spga immunogen batch 3/2017[a)]

Specification	Local Reaction	General Reaction	Egg Production	Eggs Quality
Lot 1 (inoculated with I-spga)	0/10	0/10	8%	According to the age
Lot 2 (control)	0/10	0/10	8,1%	According to the age

[a)] Rhode Island red laying hens, 20 weeks. Number of hens/lot: 10. Amount of I-spga inoculum: 4 ml in 4 different points. Amount of PBS: 2 ml in 4 different points. Number of doses: 1. Observation period: 30 days

3. Evaluation of immune response of hens immunized with I-spga at 8 days and 44 days after first inoculation

To assess the immune response of chickens immunized with I-spga, laboratory techniques are used for the control of precipitins (ELISA, Mancini radial agar immunodifusion test and Ouchterlony double immunodiffusion test) and of agglutinins (rapid serum agglutination test and slow seroagglutination test). For assessment of the direct action of the immune response of the hens against bacterial exotoxins, the *in vitro* neutralization of exotoxins against Vero and BHK cells is used.

4. Identification of biological markers from the I-spga immunogen

Specification	Biological Markers[d)]	
	G Marker[a)]	A Marker[b)]
ELISA[c)]	present	present
Spectophotometric assay	present	ND

[a)] Spectrophotometric detection and ELISA

[b)] OD at 1:1000 dilution of IgY

[c)] ELISA test using Abcam kits

[d)] average of three replicates

The markers present in I-spga products prove that the biological products were prepared following the technology described in this patent. At the time of registration of this patent, there is

no other biological product containing immunologically active proteins which can be identified by biological markers added for this purpose.

RECOMMENDED MODELS FOR USE OF THE INVENTION

To assess the I-spga immunogens used for hen immunization, it is recommended to control the immune response at 8 days or 44 days after the first inoculation. For this purpose I-spga identification is performed by evaluating the immune response of the hens, in serum or egg, upon inoculation of the immunogen containing a biological marker (Example No. 1).

For evaluation of the IgY immune response the ELISA test is used.

To evaluate the transformation of the proteins from the egg white, which became immunologically active, the ELISA and the seroagglutination tests are used. These tests are used for assessing the immune response of ovotransferrin, ovomucine, ovalbumin and lysozyme.

Example #1

Identification of I-spga markers, which differentiate these second-generation products from the related ones obtained previously to this patent.

Identification of I-spga biological markers is made on egg samples harvested individually or in groups from I-spga immunized hens.

I-spga contains in its components the A and G markers, two proteins that can be confirmed in the laboratory using the ELISA test, the serum agglutination and the agar gel immuno-diffusion.

The G Marker

The G Marker indicates the presence of sodium glutamate in the I-spga biological products.

Glutamate (glutamic acid) is one of 20 proteinogenic amino acids. Carboxylated anions and glutamic acid salts are known as glutamates. Glutamate is an important neurotransmitter that plays a key role in potentiating immunologically active proteins in the long run and is important for learning and memory. Glutamic acid is the GABA precursor, but has somewhat the opposite function; it could play a role in the normal functioning of the heart and prostate. As one of the few nutrients that cross the blood-brain barrier, glutamic acid is used in the treatment of some diseases such as depression, ADD and ADHD, fatigue, alcoholism, epilepsy, muscular dystrophy, mental retardation and schizophrenia.

The presence of sodium glutamate in I-spga biological products acts as a potentiator of the immunologically active proteins and as a neurotransmitter.

The detection of sodium glutamate in I-spga biological products is performed by the colorimetric method and the Glutamate Assay Kit (ab83389)/Abcam, 330 Cambridge Science Park, Cambridge CB4 0FL UK. Sodium glutamate is found in the I-spga immunogen, in the immunologically active I-spga products and in the IgY extracted from the yolk of the eggs laid by hens that received the I-spga immunogen (72, 73).

The Glutamate Assay Kit (Fluorometric) (Ab138883)/Abcam provides a fast and sensitive method for measuring glutamate (glutamic acid) in different biological samples. In this test, the coupled enzyme system catalyse the reaction between L-glutamic acid and NADP+ to produce NADPH, which is specifically recognized by the NADPH sensor and recycled back to NADP+. During the reaction, a red fluorescence product is generated, which in turn can be detected with a microplate fluorescence reader at Ex/Em = 540/590 nm (Ex/Em range = 530-570 / 590-600 nm). This test can detect down to 1 µM glutamic acid. The signal can also be read by absorbance at OD 576±5 nm, but the sensitivity of the test is reduced by 10 times. The working protocol:

Preparation of reagents

⇩

Standard curve drawing

⇩

Sample preparation

⇩

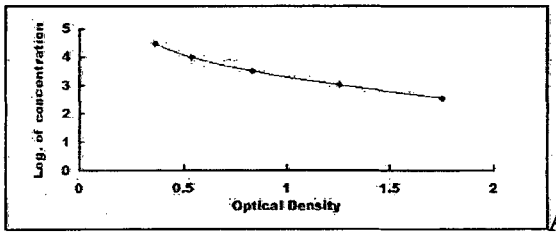
Adding the reaction mixture and incubation at 37 °C for 30 minutes, protected from light

⇩

Reaction assessment at OD 450 nm

The A Marker

The A Marker is the bovine albumin used to protect the proteins in liquid medium and lyophilized products. The identification assay for A Marker is ELISA for Bovine Serum Albumin (BSA), catalog number CEA248Ge, Wuhan USCN Business Co., Ltd.



Abcam

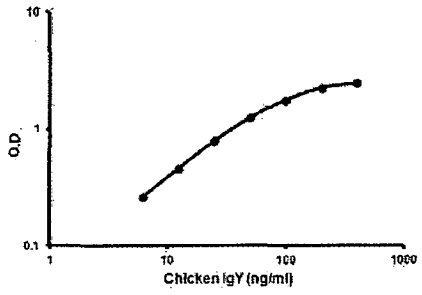
BSA is found in the I-spga immunogen, in the immunologically active I-spga biological products, as well as in the IgY extracted from yolk of the eggs laid by hens that received the I-spga immunogen.

Example #2

Determination of the IgY content in the hyperimmune egg

The content of IgY in the Imunogall hyperimmune egg is assessed by using the Abcam methodology and ELISA kit, Ab157693.

The IgY content in the Imunogall hyperimmune egg should be 400 ng.



Abcam

Example #3

Determination of specific IgY content by ELISA

The specific activity of IgY is determined qualitatively against the antigen or antigens used for immunization. Strips of different antigens from the I-spga immunogen are attached to the reaction plates and the specific IgY is tested in successive dilutions from 1:100 to 1:1000, respectively, in triplicate. The maximum positive dilution is considered dilution at which the reaction is equal to or greater than 0.200 OD or the mathematical value for dilution greater than 0.200 OD. At this dilution the positive reaction is produced by 5-10 ng of specific IgY per well (150µl).

Procedure:

a) Coat an ELISA plate with 150 µl of the desired antigen, lyophilized suspension at 1.67-1.70 µg per ml or 10 µg protein per ml in carbonate-bicarbonate buffer (0.05 M, pH 9.6);

b) The coated plate is stored for 12 hours (overnight) at +4 °C;

c) Discard the liquid and wash the plate 3 times with the PBS-Tween 20 wash solution (2%);

d) Block the reaction with 300 µl/well blocking buffer, and incubate 30 minutes at room temperature;

e) Discard the blocking fluid;

f) Dry the plate for 30 minutes in a desiccator;

g) Dispense 100 µl of 1:100 and 1:1000 diluted IgY suspension according to the plate configuration into each well. IgY under evalution will be tested in triplicate;

h) Keep wells A1, H1 and H2 as antigen controls, wells B1, C1 and D1 as negative reaction controls using IgY-SPF and wells E1, F1 and G1 as positive controls;

i) Incubate the plate 2 hours at + 37 °C;

j) Wash 3 times with washing solution;

k) Add 100 µl of 1:5000 diluted anti-bird IgG conjugate, using the dilution buffer as diluent;

l) Incubate the plate 2 hours at + 37 °C;

m) Wash plate 4 times with washing solution;

n) Add 100 µl TMB and leave at room temperature for 5-15 min.

o) Add 100 µl stop solution;

p) Read the reaction on a spectrophotometer at 450 nm.

q) The test is validated when the reactions in the blank wells A1, H1 and H2 are less than 0.060 OD, when the reactions in the B1, C1 and D1 wells with IgG-SPF negative control show values of 0.060-0.090 OD, and the positive control wells E1, F1, G1 exhibit values of 1,400-1,800 OD.

The positive reaction of this control test proves that the immunogen produced an adequate and specific immune response.

Example #4

Determination of IgY content using the Mancini simple radial immunodiffusion test.

Simple Radial Immunodeficiency (SRID) was accepted as an accurate test of quantifying an antigen and/or serum antibodies using a standard reagent (27, 28). Using SRID, it is possible to determine with great accuracy the protein content of IgY against anti-IgY rabbit IgG. For this purpose it is recommended to prepare the gel from gelose or agar and to carry the test out by the following steps:

a) Preparation of 1% agarose in PBSA containing 0.05% sodium azide: agarose is heated to 80-90 °C and let to cool to 45-50 °C; serum containing IgG anti-IgY is added at a rate of 0.33 ml / ml of agarose. Keep one milliliter of agarose at 45-50 °C.

b) Pour agarose into a disposable plate 4 ml/6 cm diameter and let it cool to room temperature.

c) Punch wells of 6 mm diameter at distances of at least 20 mm, and dispense 0.30 ml warm agar to each well to seal the bottom of the well and not allowing the liquid to pass under the agar. Cool the agar plate to room temperature.

d) Dispense 30 µl of whole IgY and in 1/1, 1/2, 1/4, 1/8 successive dilutions in PBS into the wells.

e) Store the reaction plates at room temperature in a humid environment and read the reaction at 24, 48 and 72 hours.

If necessary, consider to color the agar with Coomassie blue. Fix the gel with methanol containing 10% acetic acid for 30 minutes at room temperature. Remove the fixing solution. Color the gel with 10 volumes of Coommassie Blue dye for one hour or overnight at room temperature (the dye solution may be re-used). Discolor the gel in 10 volumes of decolorant at room temperature for 30 minutes, repeat the discoloration, and soften the gel for 15 minutes in 1-2% glycerol in deionized water. The gel is photographed and the diameter of each precipitation circle is measured. The concentration in milligrams of protein of IgY is assessed either by reference to a reference table or to a standard control.

Example # 5

Determination of IgY content using the Oucherlony double immunodiffusion test

The ID assay was performed following the Abcam protocol and is based on the migration in the agar gel of the IgY antigen and the antibodies (anti-IgY rabbit serum HAPL 97136 Abcam) which, at the site of contact, combine specifically and form a precipitation line. All tests performed using the AGID technique aimed to identify IgY against international standards. These tests are part of the first category of controls that have been used to identify IgY molecules.

The test is valid when the control sample precipitation line joins the standard IgY precipitation line and does not react with the negative control.

Example #6

Assessing the immune response 8 days after immunization

Control of immunologically active proteins extracted from the I-spga hyperimune egg.

Specification[c)]	IgY[a)]	OTf[b)]
IgY	++++	-
Ovotransfferin	-	++++

[a)] Chicken IgY ELISA Kit (ab189577)
[b)] Chicken Ovotransferrin ELISA Kit (ab157694)
[c)] Tests performed according to Abcam instructions
[d)] For each sample three replicates were considered

Example # 7

Immune response of hens immunized with I-spga immunogen

To control the immune response of laying hens immunized with the I-spga immunogen, the qualitative ELISA immunoassay was used. For testing the specificity of the antibodies present in the I-spga hyperimun egg, IgY was extracted from the yolk and used for evaluation. A positive reaction (3,000-3,200 OD) was recorded for each specific antibody within the composition of the I-spga polyvalent IgY.

Example #8

Admissibility conditions

Specimens obtained from the egg of hens hyperimmunized with I-spga must be tested by ELISA, Abcam's or in-house prepared. Tests can be done 8 days or 58 days after first inoculation.

IgY I-spga must contain the G and A markers that confirm the manufacturing technology and origin of the product.

IgY I-spga must contain antibodies that respond specifically to each antigen with which the hen has been immunized. In quantitative ELISA test, at 1:1000 IgY dilution, the optimal accepted response for each antibody is 3,000-3,200 OD. This uniform and equal response for each antigen is characteristic for the hen (*Gallus domesticus*).

CLAIMS

1. A method for preparing a complex immunogen called I-spga, characterized in that it is composed of a complex antigen obtained from 18 to 26 species of pathogenic microorganisms isolated from patients, inactivated with binary ethyleneimine (BEI) and formalin and mixed with SPGA as immunopotentiator and with QS-21 as adjuvant.

2. The method of preparation and composition of the Immunopabo hyperimmune egg characterized in that these hyperimmune eggs are obtained from hens (*Gallus domesticus*) inoculated parenterally with the I-spga complex immunogen.

3. A method for preparing a complex immunogen called I-spga according to claim 1 characterized in that the complex I-spga antigen prepared from 18 to 26 species of microorganisms contains a mixture of bacterial bodies, constituents of bacterial bodies obtained by ultrasonography, cilia, exotoxins, endotoxins, spores, viruses, fungi, or isolated from patients.

4. A method for preparing a complex immunogen called I-spga according to claim 1 characterized in that the I-spga complex antigen is diluted in SPGA as immunopotentiator containing 0.218 M sucrose, 0.0038 M monopotassium phosphate, 0.0072 M dipotassium phosphate, 0.0049 M monopotassium glutamate and 1% bovine albumin powder.

5. A method for preparing a complex immunogen called I-spga according to claim 1 characterized in that the I-spga complex antigen is diluted in SPGA as immunopotentiator and mixed with QS-21 as adjuvant.

6. A method for preparing a complex immunogen called I-spga according to claim 1 is characterized in that the I-spga complex antigen contains bacterial bodies components of bacterial bodies obtained by ultrasonography, cilia, exotoxins, endotoxins, spores, viruses, fungi or yeasts inactivated by BEI and formalin.

7. A method for preparing a complex immunogen called I-spga according to claim 1 wherein the I-spga immunogen containing bacterial bodies, constituents of bacterial bodies obtained by ultrasonography, cilia, exotoxins, endotoxins, spores, viruses, fungi or yeasts, may be standard I-spga, specific I-spga and custom I-spga.

8. A method for preparing a complex immunogen called I-spga according to claim 7 characterized in that the standard I-spga immunogen is composed of 18 to 24 antibiotic resistant or susceptible bacterial species isolated from patients.

9. A method for preparing a complex immunogen called I-spga according to claim 7 characterized in that the specific I-spga immunogen is composed of a monospecific antigen prepared from 6-8 strains from a single strain of bacteria, fungi or yeasts isolated from patients in Romania inactivated with BEI, mixed with SPGA as immunopotentiator and with QS-21 as adjuvant, used to immunize hens previously immunized with the standard I-spga immunogen.

10. A method for preparing a complex immunogen called I-spga according to claim 8 characterized in that the personalized I-spga immunogen is composed of patient-derived pathological material containing cell debris and pathogenic germs inactivated with BEI and formalin mixed with SPGA as immunopotentiator and with QS-21 as adjuvant used to immunize hens previously immunized with standard I-spga immunogen.

11. A method for preparing a complex immunogen called I-spga according to claim 4 characterized in that, that in the I-spga immunogen, each antigen specific for a microorganism species is a protein mixture of bacterial bodies, cilia, spores, exo- and endotoxins prepared from several bacterial strains of the same antibiotic resistant or susceptible species.

12. A method for preparing a complex immunogen called I-spga according to claim 1, characterized in that the I-spga hyperimmune egg, obtained from hens immunized with I-spga as immunogen, can be identified by the particular content of an immunoglobulin (IgY) specific for the target antigen and designed with the generic name I-spga.

13. A method for preparing a complex immunogen called I-spga according to claim 2, characterized in that the hyperimmune egg, obtained after the immunization of the hen, called IMMUNOSPGA, contains in the egg white, egg membranes and egg shell immunologically active proteins of the apo and holo ovotransferrin groups, ovalbumin and ovomucin .

14. A method for preparing a complex immunogen called I-spga according to claim 1, characterized in that the hyperimmune egg obtained after immunization of hens, called IMUNOPABO egg, contains immunologically active proteins from the IgY group in the vitelline membrane.

15. The I-spga hyperimune egg as well as its components (white, yolk, vitelline and shell membranes, and egg shell) originates from hens hyperimmunized with standard, specific or customized I-spga immunogen.

16. The biologic products of the I-spga group mentioned at claim 2 which are not harmful to humans and are administered orally or topically, on the skin and mucosae, as sterile solution, granules, milk or juice solutions, ointment, spray, tablets or capsules, according to the instructions for use.

17. The biological products in the I-spga group contain two markers G and A which allow their differentiation from Imunoinstant or similar products.

18. A method for preparing a complex immunogen called I-spga according to claim 1 according to which immunization of the hens with the I-spga immunogen can produce hyperimmune eggs from which immunologically active proteins can be prepared, powders from egg shell and membranes, creams and ointments from yolk and whites, sterile solutions containing immunologically active proteins from yolk and white, which can treat infections with antibiotic-resistant germs.

19. A method for preparing a complex immunogen called I-spga according to claim 1 according to which immunization of the hens with the I-spga immunogen can produce hyperimmune eggs from which immunologically active proteins can be prepared, powders from egg shell and membranes, creams and ointments from yolk and whites, sterile solutions containing immunologically active proteins from yolk and white, which can be used for the treatment of immune deficiencies such as epidermolysis bullosa, vulgar psoriasis.

20. A method for preparing a complex immunogen called I-spga according to claim 1 according to which immunization of the hens with the I-spga immunogen can produce hyperimmune eggs from which immunologically active proteins can be prepared, powders from egg shell and membranes, creams and ointments from yolk and whites, sterile solutions containing

immunologically active proteins from yolk and white, which can be used for the treatment of infections with *Staphylococcus aureus* MSSA and MRSA.

21. A method for preparing a complex immunogen called I-spga according to claim 1 according to which immunization of the hens with the I-spga immunogen can produce hyperimmune eggs from which immunologically active proteins can be prepared, powders from egg shell and membranes, creams and ointments from yolk and whites, sterile solutions containing immunologically active proteins from yolk and white, which can be used for the treatment of infections with *Clostridium difficile*.

22. A method for preparing a complex immunogen called I-spga according to claim 1 according to which immunization of the hens with the I-spga immunogen can produce hyperimmune eggs from which immunologically active proteins can be prepared, powders from egg shell and membranes, creams and ointments from yolk and whites, sterile solutions containing immunologically active proteins from yolk and white, which can be used for the treatment of urinary infections in children and adults.

23. A method for preparing a complex immunogen called I-spga according to claim 1 according to which immunization of the hens with the I-spga immunogen can produce hyperimmune eggs from which immunologically active proteins can be prepared, powders from egg shell and membranes, creams and ointments from yolk and whites, sterile solutions containing immunologically active proteins from yolk and white, which can be used for the treatment of digestive infections with antibiotic-resistant or susceptible germs.

24. A method for preparing a complex immunogen called I-spga according to claim 1 according to which the use of pathogens isolated from the country and the geographic area, respectively, where the immunogen is to be used, increases the therapeutic value.

INTERNATIONAL SEARCH REPORT

International application No
PCT/RO2018/000008

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/00 A61K39/02 A61K39/12 C07K16/02 C07K16/08
C07K16/12 C07K16/14
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	RO 132 299 A0 (PATRASCU IONEL VICTOR [RO]) 29 December 2017 (2017-12-29) abstract, 6th full paragraph of p. 7, paragraph bridging p. 7 to p. 8, paragraph bridging p. 8 to p. 9-p. 10 4th paragraph, example 1 p. 14 and claims ----- -/--	1-24

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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Date of the actual completion of the international search	Date of mailing of the international search report
30 January 2019	14/02/2019

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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X	IONEL-VICTOR PATRASCU ET AL: "Specific activity of immunological active proteines I-spga. The specific reaction against Colistin resistance Klebsiella Pneumoniae strains", ONE HEALTH INTERNATIONAL JOURNAL, vol. 3, no. 2, 1 May 2017 (2017-05-01), pages 120-126, XP055549440, abstract,p. 121 right-hand column first full paragraph - p. 122 left-hand column second full paragraph, results and discussion	1-24
A	WO 2016/114677 A1 (ROMVAC COMPANY SA [RO]) 21 July 2016 (2016-07-21) the whole document	1-24
A	WO 2017/065626 A1 (ROMVAC COMPANY SA [RO]) 20 April 2017 (2017-04-20) the whole document	1-24
A	US 5 853 765 A (STOLLE RALPH J [US] ET AL) 29 December 1998 (1998-12-29) the whole document	1-24
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Information on patent family members

International application No
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