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Production of functional antibodies from hyperimmune duck eggs for neutralization of new coronavirus SARS CoV-2

M. D. Melnychuk¹, O. V. Sereda², W. Yu³, V. G. Spyrydonov³

¹ Vinnytsia National Agrarian University,
3, Sonyachna Str., Vinnytsia, Ukraine, 21008

² Research Station of Medicinal Plants, IAE NAAS of Ukraine
Vil. Berezotocha, Lubny distr., Poltava reg., Ukraine, 37535

³ Shanghai Gene Era Bio-Science, Co, Ltd.
No.211, Huancheng East Road, Fengxian distr., Shanghai, China
melnynchuk.maks@gmail.com

Aim. To develop a technology for producing coronavirus-neutralizing antibodies in duck eggs (*Anas platyrhynchos*) immunized with a recombinant fragment of the S-antigen of the SARS CoV-2 virus, and to analyze the possibility of their use as an active substance in the development of local inhalation drugs to prevent the aerosol spread of respiratory viral infections, including a new coronavirus. **Methods.** Recombinant antigen production in the yeast expression system, production of antibodies from duck eggs, affinity purification, ELISA. **Results.** We describe an efficient method for obtaining functional yolk antibodies (IgY) from the hyperimmune duck eggs. We have shown that the average yield of total yolk antibodies from duck eggs is 0.25 ± 0.05 grams per yolk. The obtained antibodies showed the virus neutralization activity at 70.6 % inhibition (PI) in blocking ELISA, which was comparable to the human serum antibodies after vaccination with coronavirus vaccine PI at 87 % ($p \leq 0.05$). **Conclusions.** The results obtained indicate that S-specific yolk antibodies can be used as an active substance for the development of topical inhalation drugs that prevent coronavirus infection.

Keywords: COVID-19, SARS CoV-2, yolk immunoglobulin, virus neutralizing antibodies, blocking ELISA, coronavirus vaccine

Introduction

The new coronavirus pandemic causing acute respiratory syndrome in late 2019 (SARS CoV-2) had a huge impact on the public health and economy of most countries worldwide. According to WHO more than 775 million laboratory-confirmed cases of COVID-19 have

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been reported worldwide in March 2024, about 7 million of which were fatal.

A vaccination program has now begun and about 2.5 billion vaccine doses have been used to prevent the spread of coronavirus infection. The main task of effective vaccination is to form virus-neutralizing antibodies that prevent the virus from entering the target cells. Development of the locally active drugs based on virus-neutralizing antibodies can effectively prevent the aerosol spread of respiratory viral infections, including the new coronavirus, since airborne transmission is the main route of transmission of coronavirus infection [1].

The immune response to viral infection is characterized by a cascade of protective reactions of the body, which leads to the formation of cellular and humoral immunity. Virus-specific antibodies are the basis of the humoral immune response. However, not all antibodies produced by the body against viruses are able to prevent the infection of new cells. Only the pool of antibodies directed to viral receptor proteins has virus neutralizing activity, such antibodies block viral receptors and prevent new cells from being infected. The main goal of obtaining an effective vaccine against the viral infections is precisely the production of virus-neutralizing antibodies by the vaccinated organism [2].

In the case of a new coronavirus infection (COVID-19), the main objective in obtaining an effective vaccine and its use is the production of specific antibodies by the organism directed against the fragment of the coronavirus spike protein (S-RBD), which is responsible for binding to the cellular ACE2 receptor, which is the key of virus penetration into the cell [3].

The antibodies derived from egg yolk (IgY) are equal in function to the mammalian IgG

class antibodies and have several outstanding advantages over the latter. These advantages related to the development and use of IgYs, which include better immune response to the mammalian antigens, higher affinity with constant antibody titers, non-invasive selection, simple and inexpensive antibody isolation process, high yield and scalable production [4]. One laying hen can provide up to 30–40 grams of antibodies per year, which can be compared to the average monthly yield of characteristic products of a small biotechnology company [5]. The market value of such amount of antibodies can range from 10 to 100 thousand dollars. Therefore, we believe that antibodies obtained naturally in immunized eggs are a good alternative to the antibodies obtained under laboratory conditions with expensive equipment and consumables.

The aim of the work was to develop a technology of producing the coronavirus neutralizing antibodies in eggs of ducks (*Anas platyrhynchos*) immunized with the recombinant fragment S of the new coronavirus antigen and to analyze the possibility of their use as an active substance in the development of local inhalation drugs preventing the spread of the new coronavirus.

Materials and Methods

Recombinant S-RBD antigen production in yeast expression system

The yeast cells used in the study were *S. cerevisiae* INVSc1 (MATa, his3D1, leu2, trp1-289, ura3-52, MAT, his3D1, leu2, trp1-289, ura3-52) from Invitrogen (USA). Synthesis of the gene encoding the fragment of the spike antigen S (318–541 a.s.) was custom-made by Ruibiotech

(PRC). The gene fragment was cloned into the yeast expression vector pYES2 (Invitrogen, USA) at the EcoRI and XhoI restriction sites. The protocol for yeast cell transformation and recombinant protein expression is described in [6].

The transformed yeast cells were cultured in liquid YPD medium (1 % yeast extract, 2 % peptone, 2 % D-glucose), while the expression of the target protein was performed in synthetic minimal medium without uracil (SC-U) with 0.01 % galactose as an expression inducer, for 72 hours at 30 °C.

The yeast cells after fermentation were centrifuged and suspended in lysis buffer (40 mM NaH₂PO₄, 0.3 M NaCl, 5 mM Imidazole, 1 mM PMSF, 5 mM β-ME) and destroyed using a Bead Beater cell disintegrator (BioSpec, USA).

Extraction and purification of S antigen from the yeast lysate were performed on Ni-Sepharose using an Acta Prime Plus chromatographic system (GE Health Care, USA). The peak fractions of the target protein were pooled and dialyzed against 2 liters of PBS solution for 24 hours at 4 °C. After dialysis, S antigen was concentrated in centricones (MWCO 3000, Merk) by centrifugation for 3 hours at 5,000 rpm.

Yeast S-RBD serological validation

To validate the S-antigen serological identity, indirect ELISA was performed using human sera before and after vaccination with CoronaVac coronavirus vaccine (Sinovac Life Sciences, PRC), which is an inactivated SARS CoV-2 virus produced on Vero cells culture [7]. Purified S antigen was adsorbed at a concentration of 0.1 µg/ml in wells of polystyrene High binding microplate strips (Costar, USA) in 0.05 M CBB (pH 9.6) for 24 hours at 4 °C.

The immune antibodies (IgG) were detected by indirect ELISA with serum samples at a dilution of 1:25. Goat anti-human IgG antibodies labeled with horseradish peroxidase, Goat anti-human IgG (H+L)-HRP Cat. No. SH-0041 (DingGuo, PRC) was used as an immunoassay conjugate. The ELISA procedure was performed under standard conditions [8]. After each assay steps, the wells of the plate were washed with 0.05 % PBS-Tween-20 solution using a HydroFlex microplate washer (Tecan, Switzerland). At the last stage, the hydrogen peroxide substrate solution with TMB chromogen (DingGuo, PRC) was added to the wells and incubated at room temperature in the dark for 10 min. The enzymatic reaction was stopped by adding sulfuric acid solution (2 M), and the optical density was measured at 450 nm using an Infinite F50 microplate reader (Tecan, Switzerland). The obtained data were analyzed using Microsoft Excel; the statistical processing of the results was performed using the paired Student's t-test for dependent populations. Differences were considered statistically significant at P≤0.05.

Ducks S-RBD immunization and dIgY-S extractions

The Immunization of 6-month-old Shaoxing ducks (N=5) was carried out under the conditions of Guowei Poultry Industry farm (Zhuji, PRC). An S-antigen suspension (50 µg) of Al(OH)₃ (0.25 mg) per dose was used for immunization by injecting into the duck breast in 2 ml. Immunization was repeated twice with 10-day intervals. The immunization schedule was approved by the Institutional Ethical Committee (protocol No 8, from 2.09.2020). Duck eggs were collected on day 30 after the first immunization.

Yolk antibody extraction was performed according to the protocol described earlier [9] with slight modifications. The egg yolks were carefully separated from the egg whites and homogenized in twice the volume of PBS on a magnetic stirrer. Polyethylene glycol (PEG6000) was added to a final concentration of 3.5 % w/v to precipitate the lipids. The mixture was centrifuged at 8,000 rpm and the supernatant was filtered through two layers of filter paper. PEG6000 was added to the filtrate to a final concentration of 12.5 % w/v on a magnetic stirrer to precipitate the yolk antibodies. After repeated centrifugation at 8,000 rpm, the precipitate was dissolved in 1/2 of the original yolk volume in PBS. An equal volume of 95 % ethanol was then added to the yolk antibodies solution. The alcohol-antibody mixture was incubated overnight in the freezer at minus 20 °C. At the next day, the antibodies were precipitated by centrifugation at 5,000 rpm, washed twice with isopropanol and dried at 45 °C. The resulting antibody powder was weighed and stored in the refrigerator at 4 °C until used.

dIgY-S titration in indirect ELISA

Purified duck yolk antibodies (dIgY-S) were titrated by indirect ELISA with the S-antigen adsorbed at 0.1 µg/ml in wells of polystyrene High binding microplate strips (Costar, USA) under standard conditions described above. Duck antibodies were titrated by two-fold dilution, starting with a dilution of 1:50 and ending with a dilution of 1:6400. Bound antibodies were detected by protein A conjugate with horseradish peroxidase Cat. No. 10600-P07E-H (SinoBiological, PRC). The results of the ELISA were used to build a titration curve as a function of the optical density ($A_{450\text{ nm}}$) and antibody dilution.

dIgY-S blocking ELISA

In order to determine the functional characteristics of the obtained antibodies, a blocking ELISA was performed using a commercial reagent kit SARS-CoV-2 Neutralization Antibody Test Kit Cat № E-ScoV2-NAB (Hangzhou Zheda Dixun Biological Gene Engineering Co., PRC) according to the manufacturer's instructions. Human serum samples and yolk antibodies were tested at a dilution of 1:10. The results of the assay were presented in percentage of inhibition (PI), which was calculated according to the formula:

According to the instructions, if the PI value is $\geq 20\%$, such sample is considered as positive, containing coronavirus neutralizing antibodies.

All samples were tested in replicates and the obtained data were statistically calculated using Microsoft Excel program; differences were considered statistically significant at $P \leq 0.05$.

Results and Discussion

We obtained the recombinant S-antigen fragment of the new coronavirus (SARS CoV-2) in the yeast expression system. For this purpose, we cloned a fragment of the gene encoding 318–541 aa. (receptor binding domain) S protein of the new coronavirus (Gen Bank No. MN908947.3) into the yeast expression vector pYES2 under the control of the GAL1 promoter and CYC1 transcription terminator in a single reading frame with a sequence that encodes 6 a.s. histidine. The obtained genetic construct (pYES-RBD) was transformed into *S. cerevisiae* INVSc1 yeast cells, the selection of the transformed clones was performed on the selective synthetic medium SC-Ura, since the genetic construct contains the auxotrophic selection marker URA3, which allows the

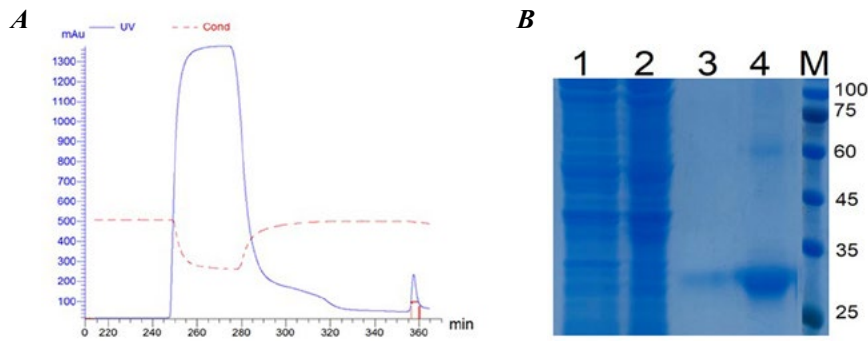


Fig. 1. Chromatographic profile of S-antigen purification from yeast lysate on Ni-sepharose (*A*) and PAGE of S-antigen purification fraction (*B*): 1 — lysate before column loading; 2 — lysate fraction flow trough; 3 — S-antigen elution peak fraction; 4 — S-antigen after concentration; *M* — marker

transformed cells to survive on minimal medium without uracil.

Induction of recombinant protein S expression was performed using galactose, which was added to the nutrient medium during fermentation of a selected yeast clone with a maximum expression level of 0.3 mg per liter of culture after 72 hours of incubation at 30 °C. After purification and S-antigen concentration, the purity of the obtained preparation was checked by electrophoresis in 12 % PAGE-SDS. As seen in Fig. 1 A, B, the purified protein is quite homogeneous and migrates in the gel with a size close to 28 kD compared to the protein size standard, which is consistent with

the theoretically calculated data during experimental design.

To validate the antigenic identity of the purified S-antigen, we performed ELISA using sera of healthy humans, after vaccination with the Chinese vaccine CoronaVac (Sinovac Life Sciences, PRC), which is an inactivated SARS CoV-2 virus produced on Vero cell culture [7]. For this purpose, we selected several volunteers (N=8) of different age group, sex and race [10]. Fig. 2 shows a diagram of the indirect immunoassay results using the S-antigen and serum samples before vaccination and on day 28 post-vaccination. The vaccination was done in two stages with an interval of 14 days. The

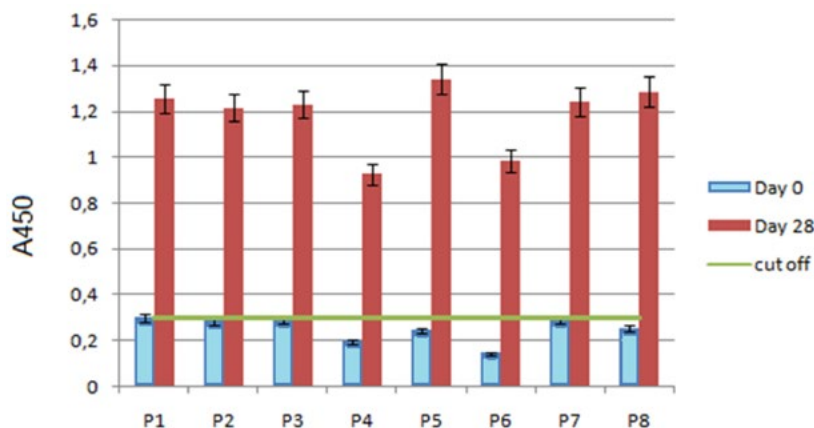


Fig. 2. Indirect ELISA human sera antibody titers before (day 0) and after vaccination CoronaVac (day-28)

level of antibodies (class IgG) was significantly (4.7 times) higher than before vaccination ($p < 0.05$). This confirmed that the yeast S-antigen has a similar antigenic structure to the native viral SARS CoV-2, and can be used to immunize ducks and produce the virus neutralizing antibodies in duck eggs.

We showed that the average yield of total yolk antibodies from duck eggs was 0.2 ± 0.05 grams per yolk. The obtained antibodies showed sufficiently high specific activity in indirect ELISA, with a titer of 1: 2500 (Fig. 3), indicating that the yeast recombinant S-antigen is a potent immunogen and induces the production of specific antibodies (class IgY) in eggs after immunization of ducks.

In order to test the functional activity of the yolk antibodies, namely the virus neutralizing activity, we performed a blocking immunoassay using yolk antibodies and human sera after vaccination as a control. The essence of the method is to simulate the interaction of S-antigen coronavirus that is labeled with an enzymatic tag (horseradish peroxidase, HRP) with cell receptor angiotensin converting enzyme 2 (ACE2), which is adsorbed on the surface of polystyrene plates. If virus-neutra-

lizing antibodies are present in the test sample, they compete with the receptor for interaction with the S-antigen and prevent its binding. According to the instructions for the corresponding reagents kit, if the tested sample shows a percentage of inhibition ($PI \geq 20\%$), this sample contains the virus neutralizing antibodies. We showed that yolk antibodies of the ducks immunized with S-antigen (dIgY-S) had reliably 70.6 % PI at 40 days after immunization, in contrast to 2.4 % PI of dIgY's isolated from non immune eggs, which confirmed functional activity of the obtained antibodies (Table 1, Fig. 4). At the same time, the virus neutralizing activity of the yolk antibodies was comparable with the virus neutralizing activity of human serum after vaccination with CoronaVac coronavirus vaccine — PI 87 %.

More than 100 years of medical history show that the isolation and use of antibodies are generally accepted principle of the immunological approach against pathogens. Transmission of specific antibodies directed against certain disease antigens to infected people as passive immunization is a generally accepted therapeutic concept for many diseases such as diphtheria, rabies, tetanus, Ebola virus, *etc.* [11].

C. Shen *et al.* were the first to report that plasma from patients who have had COVID-19 can be a treatment option for the patients with severe disease course and respiratory failure. Passive immunization improved the clinical situation in 5 patients for whom antiviral drugs or steroids were not effective. The viral load of the patients decreased and became negative within 12 days after plasma transfusion [12]. In addition, the FDA has provided guidelines for collection and transfusion of reconstituted plasma: plasma should be collected in the re-

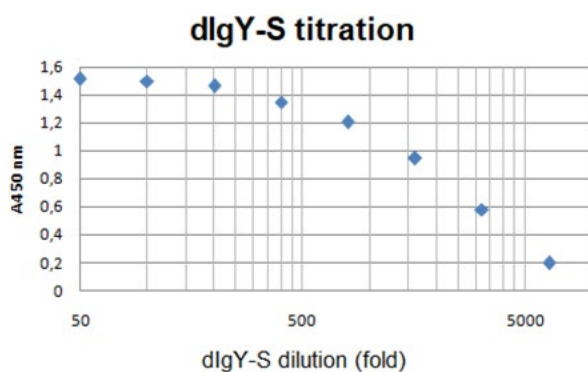


Fig. 3. Titration of duck yolk antibodies (dIgY-S) by indirect ELISA, antibody titer is 1:2500

Table 1. Results of blocking ELISA, mean values shown (coefficient of variation, CV ≤ 5 %)

№	Sample name	A _{450 nm}	PI, %
1	Positive control (PC)	0.02	97.6
2	Negative control (NC)	0.85	0
3	Human sera before vaccination (P1, 0)	0.79	7
4	Human sera day 28 after vaccination (P1, 28)	0.11	87
5	Yolk antibodies before immunization (dIgY-S, 0)	0.83	2.3
6	Yolk antibodies day 40 after immunization (dIgY-S, 40)	0.25	70.6

covered patients who can donate blood, who have been asymptomatic for 14 days, and who have tested negative for COVID-19 [13].

The use of virus-neutralizing antibodies in a prophylactic nasal spray adds to the arsenal of tools, which we have for controlling the spread of COVID-19, and the concept can be applied to other airborne diseases. The advantages of these topical preventive agents are ease of use, high efficacy, and availability at all times to provide self-protection in high risk environments such as crowded places.

Recently, a single use of a nasal spray against COVID-19, based on monoclonal antibodies, has been shown to protect mice against lethal doses of coronavirus for 10 hours in a model infection [1]. However, in our opinion, the use of monoclonal antibodies for the prevention of coronavirus carries the threat of the emergence of mutant strains that can evade neutralization due to mutations in the receptor region of the S-antigen, for example, the well-known South African strain of coronavirus (B.1.351) contains the mutation E484K which is responsible for evasion of virus neutralization by monoclonal antibodies against SARS CoV-2 [14]. The “excessive” specificity of monoclonal antibodies may reduce their effectiveness in contrast to polyclonal antibodies, which react with different epitopes on the surface of the target antigen. We have shown the successful use of chicken yolk polyclonal antibodies to solve this problem [15]. Therefore, our idea is to use polyclonal antibodies as the main active ingredient in the development of topical preventive agents against COVID-19. We proposed the use of yolk polyclonal duck antibodies (dIgY) as a source for isolating antibodies against corona-

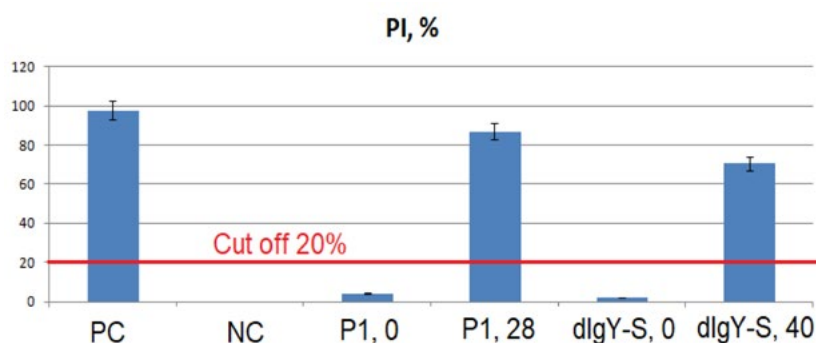


Fig. 4. Percent Inhibition (PI), in human serum and duck antibody samples in blocking ELISA: PC and NC, positive and negative controls, respectively; P1,0 and P1,28, patient No 1 samples before vaccination and on day 28 after vaccination with CoronaVac vaccine, respectively; dIgY-S, 0 and dIgY-S,40 samples of yolk duck antibodies before immunization and on day 40 after immunization with recombinant S-antigen, respectively

virus. It is already well known that yolk antibodies against coronavirus produced in SPF chick eggs have virus neutralizing activity against SARS CoV-1 in a titer of 1:640 using Vero E6 cell culture [16].

Duck yolk antibodies have advantages over chicken antibodies in terms of performance, the presence of a heavy chain isoform with a lost Fc domain (ΔFc) [17], and the ability to interact with protein A [18], which can be used to purify and detect dIgY. That is why we chose the domestic duck *Anas platyrhynchos* as the main producer of yolk antibodies in eggs. In this study, we successfully immunized ducks with the S-antigen of a new corona virus, then purified yolk immunoglobulins with a high titer against SARS corona virus CoV-2, and showed their neutralizing activity (PI of 70.6 %) under *in vitro* conditions. At the same time, the data of competitive ELISA (*in vitro*) have recently been shown to coincide by 86 % with the data of virus neutralizing test on Vero E6 cell culture (*in vivo*) using human sera after inoculation with coronavirus vaccine [19]. Moreover, recent studies have shown that chicken IgY against Spike antigen of SARS CoV-2 showed strong and significant inhibition of S1/ACE2 binding interactions, and significantly inhibited viral replication at a concentration of 16.8 mg/ml [20]. It encourages us that dIgY-S can be also effectively used to neutralize SARS-CoV-2 in topical inhalation preparations for self-administration.

Conclusions

We have shown that the developed technology of immunization of ducks with recombinant S-antigen makes it possible to obtain sufficiently high levels of specific and functional

yolk antibodies, which have coronavirus neutralizing activity (PI 70.6 %), that was confirmed in the *in vitro* model. We believe that the yolk antibodies may be a powerful candidate in the development of topical inhalation drugs, for self-administration use, preventing the spread of new coronavirus.

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Отримання функціональних антитіл з гіперімунних качиних яєць для нейтралізації нового коронавірусу SARS CoV-2

М. Д. Мельничук, О. В. Серeda, В. Йу, В. Г. Спиридонов

Мета. Розробити технологію отримання коронавірус-нейтралізуючих антитіл з яєць качок (*Anas platyrhynchos*), імунізованих рекомбінантним фрагментом S-антигену вірусу SARS CoV-2, та проаналізувати можливість їх використання як діючої речовини в розробці інгаляційних препаратів локальної дії для запобігання аерозольного поширення респіраторних вірусних інфекцій, у тому числі нового коронавірусу.

Методи. Виробництво рекомбінантного антигену в системі експресії дріжджів, отримання антитіл з качиних яєць, афінна очистка, ІФА. **Результати.** Описаний ефективний метод отримання функціональних антитіл (IgY) з жовтка гіперімунних качиних яєць. Ми показали, що середній вихід загальних жовткових антитіл з качиних яєць становить $0,25 \pm 0,05$ г на жовток. Отримані антитіла показали віруснейтралізуючу активність 70,6 % інгібування (PI) у методі блокуючого ІФА, порівнянню з активністю антитіл сироватки людини після вакцинації вакциною проти корона вірусу — PI87 % ($p \leq 0,05$). **Висновки.** Отримані результати свідчать про те, що S-специфічні жовтківантитіла можуть бути використані як діюча речовина для розробки місцевих інгаляційних препаратів, що запобігають зараженню коронавірусом.

Ключові слова: COVID-19, SARS CoV-2, жовтковий імуноглобулін, віруснейтралізуючі антитіла, блокуючий ІФА, вакцина проти коронавірусу

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