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Selection of staphylococcal strains and obtaining polyclonal immune serum

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Abstract
30 strains with stable characteristics (Staphyl.aureus -24, Staphyl.epidermidis-6) have been chosen from 102 laboratory and clinical cultures of staphylococci. Morphological, cultural and enzymatic activity, susceptibility to novobiocin and staphylococcal bacteriophage, ability to produce microcapsule, proteolytic, hemolytic characteristics, mannitol fermentation and plasmocoagulation values of strains have been studied. Producer animals were vaccinated with produced immunogens (thermoinactivated staphylococcal culture – 5 bn/ml; alpha-anatoxin, PV-leukocidin, hyaluronidase) according to the planned schedule. Immune serum has been obtained with highly specific activity. Antibody titer in Lh (Limes-hemolysis) reaction of alpha-toxin was - 150 IU/ml; antibacterial antibody titer in passive hemagglutination test - 1:6400-1:12800; anti-leukocidin antibodies - -1:320-1:1280; and antibody titer against hyaluronidase – 1:160-1:320; Normal and anti-staphylococcal immune serums were investigated with immunoenzyme method. Mean data from normal (control) strains was 0,081 for alpha-toxin, and antibody positivity index against α-toxin was 10,08 in immune serum, positivity index for antibacterial antibodies – 2, 3287.

Keywords: staphylococcus, antibodies, α-anatoxin, PV-leukocidin, hyaluronidase.

Introduction
Infections caused by antibiotic-resistant strains of staphylococci are a global threat given the mortality rate, which is characterized by trend towards increasing (Deng J. et al, 2019). Antibodies produced against wide range of staphylococcal antigens in sick patients, indicate diversity of Staphylococcus aureus pathogens (P. Colque-Navarro et al, 2010). Investigation of staphylococcal infections led scientists to the conclusion that the urgent task is to develop protective antigen vaccines for the disease prevention. In this regard, multiple groups of investigators have been focused on the development of prophylactic vaccines (L. Thomer et al, 2016; Broker B.M., Mrochen D., and Peton, 2016; Cohen T. et al, 2016).
S.aureus virulence is determined by several factors, including cell wall protein, which determines the ability of microbe to adhere to eukaryotic cell membrane; capsular polysaccharide, which protects bacteria from opsonophagocytosis; also few exotoxins (alpha, beta, gamma and delta hemolysin), which result in red blood cell hemolysis, skin necrosis and cytokine induction, the latter in turn results in shock syndrome. Enzyme coagulase binds to prothrombin, activates thrombin, which promotes conversion of fibrinogen to fibrin.
Panton–Valentine (PV) leukocidin synthesized by staphylococci causes destruction of neutrophils and other phagocytes (Tromp A, T, et al, 2018), and hyaluronidase – damage to the connective tissues of the body (Ibberson C.B, et al, 2016). Mortality caused by S. aureus infections is 30%. Sepsis and mortality caused by S. epidermidis has become more frequent event (Falahee P.C, et al, 2017).
Against the background of antibiotic resistance, specific bacteriophages are increasingly used to treat bacterial infections, which unlike antibiotics, are not characterized by side effects. Given the leading role of toxic factors in pathogenesis of complicated infections, it's appropriate to use highly active immune preparations for therapeutic purposes. The unique property of antibodies is to neutralize damaging actions of bacteria and toxins on cells and

tissues. Currently, homologous (donor's) anti-staphylococcal immunoglobulin is used for the treatment of staphylococcal infections, which contains only antitoxic (alpha-toxin) antibodies (activity – 30 IU/ml). According to the literature, therapeutic properties of the immunoglobulin are quite low. Therapeutic use of monoclonal antibodies is still less promising (Croze M., 2009), which is partly due to the technological complexity and high cost of the production, compared to the classic method (polyclonal antibodies). Treatment of a complicated infection is thoroughly problematic, as in many cases antibiotics and homologous immunoglobulin do not give the desired result. (Ohlsen K.,Lorenz U.2010; Lu B.et al,2018). This situation requires development of a safe, highly effective therapeutic immune preparation (Larkin E.A. et al.2010). It should be noted that staphylococcal toxins and enzymes (alpha-toxin, PV-leukocidin, hyaluronidase and other) play a leading role in the disease pathogenesis, which can only be neutralized with appropriate antibodies.

Given the problem, production and use of polyvalent anti-staphylococcal immunoglobulin is promising given the market potential. Use of immunoglobulin intended for the treatment of humans and animals is based on the successful test results of horse anti-staphylococcal (antibacterial, anti-alpha-toxin) immunoglobulins in National Anti-Sepsis Center, which was developed in G. Eliava Institute (Rigvava S..et al,2001). The preparation was used in more than 300 patients, suffering from generalized and chronic forms of severe staphylococcal infection. 88% of patients were completely cured with immunoglobulin, against the background of low, adverse effect of antibiotics and other therapeutic agents. Also, positive effect was obtained in 60% of patients with chronic staphylococcal septicemia (Bochorishvili T.V, 1982).

The aim of the study is to obtain hyperimmune serum with highly specific activity by selecting virulent and opportunistic pathogenic strains with stable properties of clinical staphylococci (Staphylococcus aureus, Staphylococcus epidermidis) and using other staphylococcal immunogens (alpha-anatoxin, PV leukocidin, hyaluronidase) and to investigate serological parameters.

Material and methods

In order to achieve the goal set, standard strains obtained from the different clinics and bacteriological centers of Georgia (G. Eliava Institute of Bacteriophages, Microbiology and Virology, V. Bochorishvili clinic (Tbilisi), hospital in Kutaisi,,Medical City'', Tbilisi Iashvili Children's Hospital, Kutaisi Microbiological Center), as well as from USA and Germany (1), were investigated. In order to achieve the goal set, 102 pathogenic and opportunistic pathogenic clinical staphylococcal strains were collected and investigated. Morphological, cultural, biochemical, plasmocoagulative, toxigenic properties of strains have been investigated. Tests were performed using growth characteristics in culture media, differential API tests; Microbial susceptibility to antibiotics was investigated by disk-diffusion method, and phage susceptibility – in Petri dishes by pouring commercial staphylococcal bacteriophage (phage purified by chromatography, series P1-901, 2019) on microbial lawn culture on agar. We used standard liquid and solid growth areas in experiments. For investigation of plasmocoagulative activity of strains, we used rabbit plasma (Coagulase plasma, Biolife, Milano, Italia), and to determine antibiotic susceptibility – Novobiocin (Liverpool L9 7 AR,Abtek. Biological Ltd.UK).

We produced purified staphylococcal anatoxin and PV-leukocidin according to the existing methods (Rigvava S.et al, 2020). In order to evaluate antibodies against immunogens, we made diagnostic test-system, which is used in passive hemagglutination (PHA) assay. To load formalin-treated red blood cells for test-system preparation, we have established optimal sensibilization dose for individual antigen. After control test, we dissolved diagnostic reagent in protective solution (5.0% gelatose, 7.0% sucrose), subject to lyophilized drying. We used commercial staphylococcal hyaluronidase in experiments.

We identified levels of anti-staphylococcal antibodies in normal and immune serum of goat using immunoenzyme assay. Results were registered on immunoenzyme Reader (Sunostik SPR-960). Following reagent was used for immunoenzyme assay: Anti-Goat IgG (H&L) in rabbit Affinity Purified, Polysciences, Inc.exp.10.2022. According to normal values 50-390 ng/ml immunoenzyme assay has been performed based on common "Sandwich-ELISA" in accordance with the guidelines attached to the test-systems.

Obtained results and their discussion

Study activities were performed in compliance with the standard terms for working with bacteria (EUCAST) and using existing methods in laboratory practice (SOP).

In the study process 30 typical strains were selected from the investigated 102 strains, including 24 St. aureus and 6 St. epidermidis strains, considering main properties.

Staphylococcal cultures are characterized by similar morphological (table 1) signs, particularly, grape-like arrangements in smears, staining in bluish purple color (gram-positive), forming S-shaped colonies on agar; Although, significant difference is noted, which is determined by the formation of microcapsule by St. aureus, which St. epidermidis strains lack

Table 1: Morphological characteristics of staphylococci.

#	Microbe name	Number of cultures	Gram-stain	Arrangement	Colony shape	Microcapsule production
1.	St. aureus	24	+	Grape-like	S	+
2.	St. epidermidis	6	+	Grape-like	S	-

Significant difference is noted in the investigated strains in the values of enzymatic activity (table 2), which was reflected in red blood cell lysis by St. aureus, citrated rabbit plasma coagulation, mannitol fermentation and proteolytic properties. Mentioned property is not characterized for St. epidermidis strains.

Table 2: Enzymatic characteristics of staphylococcal strains.

#	Microbe name	Number of cultures	Plasmocoagulation	Mannitol fermentation	Hemolysis	Proteolysis
1.	St. aureus	24	+	+	+	+
2.	St. epidermidis	6	-	-	-	-

Study of staphylococcal susceptibility to novobiocin and staphylococcal bacteriophage revealed presence of resistant strains (table 3). For example, all St. aureus strains were found to be susceptible to novobiocin, 3 St. epidermidis strains from 6 susceptible, resistant – 3; 13 St. aureus strains from 24 were found to be susceptible to phages, non-susceptible – 11, and 1 St. epidermidis strain from 6 – susceptible, non-susceptible – 5.

Table 3: Susceptibility of staphylococci to antibiotics and phages.

#	Microbe Title	Staph phage	Pyo phage	Novo biocin	#	Microbe title	Staph phage	Pyo phage	Novo biocin	#	Microbe title	Staph phage	Pyo phage	Novo biocin
1	S.aureus V39227	Ol	Ol	S	11	S.aureus 44	Scl	Cl	S	21	S. epid. 69	R	R	R
2	S.aureus A62758	Ol	Ol	S	12	S.aureus 121	R	R	S	22	S. epid. 793	R	R	S
3	S.aureus M67630	Ol	Scl	S	13	S.aureus 242	R	R	S	23	S. epid. 884	R	R	S
4	S.aureus 1007	Ol	Cl	S	14	S.aureus 0–15	R	R	S	24	S. epid. 5052	Scl	Scl	R
5	S.aureus 1009	Ol	Scl	S	15	S.aureus 234	R	R	S	25	St. Epid. 114	R	Cl	S
6	S.aureus 4701	Scl	R	S	16	S.aureus 91	R	R	S	26	S.aureus 889	R	Scl	S
7	S.aureus 017	Scl	Scl	S	17	S.aureus 72	Cl	Scl	S	27	S.aureus 1000	R	R	S
	S.aureus 956	Scl	Cl	S	18	S.aureus 42	-	Cl	S	28	S.aureus 4004	Cl	Scl	S
9	S.aureus 652	R	R	S	19	S.au DSMZ 18590	Ol	Ol	S	29	S.aureus 7007	Scl	Cl	S
10	S.aureus 875	Cl	Cl	S	20	S. epid. 62	R	R	R	30	S.aureus 8008	Cl	R	S

Notice: Cl- confluent lysis, Scl-semi-confluent lysis, Ol-opaque lysis
A zone-of-inhibition diameter of ≤15 mm was considered to indicate Novobiocin resistance

Complex investigations included: selection of producer-animals (goat) according to the high titer of antitoxic antibodies obtained as a result of initial anatoxin vaccination (1,0ml; 1,5ml); preparation of effective combination of immunogens; hyperimmunization of producers by planned schedule; obtaining anti-staphylococcal serum containing high-titer antibodies to immunogens. After 3 weeks from the initial vaccination – hyperimmunization of producers was conducted with increasing doses of immunogenes – staphylococcal thermoinactivated cultures, alpha-anatoxin, PV-leukocidin and hyaluronidase, subcutaneously (**Rigvava S**. et al.2021). Immunization schedule is given in the table (#4).

Table 4: Immunization Table of Producer-animals (Goat).

№	Immunogens	Antigen dose (ml)					Blood sampling		
		1 Injection ml	II Injection ml	III Injection ml	IV Injection ml	V Injection ml	Sample Day VI	First sampling, Day VIII	Second sampling, Day X
1	Staphylococcal anatoxin + 0.5% adjuvans –Kal$(SO_4)_2$	1	2	3,5	5				
2	Inactivated Staphyloc. culture (85-96°C)+ Kal$(SO_4)_2$	0,5	1	2	4				
3	PV - Leucocydin + 0.5% Kal$(SO_4)_2$		0,1mg	0,2 mg	0,4 mg				
4	Hyaluronidaza + 0.5% Kal$(SO_4)_2$			0,2 mg	0,4 mg				

Notice - antistaphylococcal antitoxic antibodies - >150 ME/ml,
-antistaphylococcal antibacterial antibodies - 1:6400 - 1:12800, PHR;
-PV –leukocidin antibodies - 1:320 – 1:640;
-Hyaluronidase antibodies - 1:160 - 1:320;

On 8th and 10th day after fourth vaccination blood was taken from producer-animals (250-300 ml from each) after control test. We investigated antibodies in normal and immune serum serologically. Antibody titer to α-toxin was

determined in Lh (limes-haemolisis) reaction, which was 150 IU/ml in serum mixture; range of antibacterial antibody titer in immune serum in passive hemagglutination test – 1:3200-1:12800; anti-leukocidin antibody titer – 1:640 – 1:1280, and to hyaluronidase – 1:160 – 1:320. These values ranged within 1:10-1:40 in normal serum. We investigated normal and immune serum of goat using immunoenzyme assay. Average data of normal (control) serums to staphylococcal toxin was 0,081; and positivity index of immune serum in relation to the toxin was – 10, 08. Positivity index of antibacterial antibodies - 2, 3287, positivity index of leukocidin - 4, 3968, and of hyaluronidase – 0,9214. At the same, we have identified some indicators of non-specific immunity in producers: particularly, phagocytic indicator, phagocytic index, tolerance, component of complement C3 and C4. E.g increase in complement values in #9 producer was found to be statistically authentic. C3 and C4 was found to be equal respectively 88,7 mg/dl and 15,1 mg/dl, and in immune serum analogous values were 183,8 mg/dl and 45,0 mg/dl

As a result of performed investigations, pathogenic (Staphyl.aureus) and opportunistic pathogenic (Staphyl.epidermidis) staphylococcal strains with stable properties have been selected; Staphylococcal alpha-toxin, PV-leukocidin has been produced and tested. By immunizing producer-animals with staphylococcal immunogens, anti-staphylococcal polyclonal immune serum has been obtained, which contains high-titer antibacterial, antitoxic (alpha-toxin, PV-leukocidin) and anti-enzyme (hyaluronidase) antibodies. Activities are planned in the near future, which involve release of immunoglobulin fraction and obtaining purified $F(ab')_2$-fragments of antibodies and investigation of therapeutic properties.

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