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Production of *Staphylococcal anatoxin* and development of a diagnostic test system

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A B S T R A C T

In three small series a non-toxic, immunogenic form of staphylococcal alpha-hemolysin (α-toxin) was obtained. The purified anatoxin was precipitated and a diagnostics test system developed. Its activity, specificity, and other characteristics were studied. The test system is designed for rapid serological diagnosis of staphylococcal infection, as well as for the post-vaccination evaluation and quantification of antitoxic antibodies. In passive (indirect) hemagglutination reaction the normal titer of *Staphylococcal antitoxic* antibodies (IgG) in human serum varied among different individuals between 1:5 – 1:40. In clinical material the titer of antitoxic antibodies for infections caused by *Staphylococcus aureus* ranged from 1:160 to 1:1280. In animals, this titer is normally 1:40 - 1:160.

Keywords: Non-toxic, Immunogenic form, Infections, *Staphylococcal anatoxin*, Clinical material, Rapid serological diagnosis.

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Introduction

A staphylococcal infection is a complex pathological process that may result in an asymptomatic carrier state, severe intoxication, or formation of purulent inflammatory foci. Staphylococci cause both nosocomial and community-acquired diseases [1]. According to the World Health Organization (WHO), *Staphylococcus aureus* tops the list of bacteria that most commonly cause infections in healthcare facilities. *S. aureus* accounts for 31% of all nosocomial infections. Due to the rise of antimicrobial resistance (AMR), staphylococcal infections have become one of the greatest problems of modern medicine [2, 3]. In Georgia, according to the Vakhtang Bochorishvili Clinic, more people die from staphylococcal infections than from diphtheria, salmonellosis, hepatitis, and typhoid fever combined [4].

The virulence of *S. aureus* is associated with its adhesion to receptors of sensitive cells [5]. *S. aureus* synthesizes a number of toxins that differ by their mechanisms of action. The toxic substances are divided into three groups: (1) PTSAgs, superantigens that induce toxic shock syndrome (TSS); (2) exfoliative toxins (EF) implicated in staphylococcal scalded-skin syndrome (SSSS), which occurs most commonly in infants and young children; and (3) staphylococcal toxins that act on cell membranes and include alpha-toxins and several bi-component toxins (*e.g.,* PV leukocidin associated with severe necrotizing pneumonia in children [6, 7]. Of these toxins, the staphylococcal alpha-toxin (α-hemolysin) is attracting much attention for its role in disease pathogenesis.

This study is aimed at obtaining the staphylococcal alpha-toxin, purifying the anatoxin, and developing a diagnostic test system for the evaluation and quantification of staphylococcal antitoxic antibodies in human and animal blood serum.

Materials and Methods

Obtaining and purifying the staphylococcal anatoxin involved bacteriological, biochemical, and serological methods. The following microbiological growth media were used: brain-heart broth and agar, nutrient agar, blood agar (Biolife, Milano, Ita-

ly; Eliava Bacteriological Media Production, Tbilisi, Georgia).

To obtain the alpha-toxin (α-hemolysin), a known producer strain, *S. aureus* 0-15 was selected. Particular attention was paid to the preparation of a casein enriched nutrient medium (broth and agar). For this purpose, we prepared our own yeast extract (100 ml distilled water added to 25 g dry yeast powder, boiled for 30 min, filtered through double filter papers, and stabilized by the addition of 1% chloroform), casein hydrolysate (20 g casein powder (Sigma, USA) dissolved in 1 L distilled water and stored at 4 °C), and wheat bran extract (200 g wheat bran added to 1 L distilled water with 1% chloroform, incubated at 45 °C for 12-14 h, and filtered through 0.8-μm filter). The final medium contained 20 g casein, 120 g wheat bran, and 5 ml yeast extract in 1 L distilled water. It was autoclaved at 110 °C for 30 min.

The alpha-toxin producing staphylococcal strain was grown overnight in culture tubes. The inoculum was transferred into 5-liter flasks containing 1 liter of the casein medium and incubated at 37 °C for 5 days. At the end of the growth period, the suspension was treated with formaldehyde for 10 days, and the anatoxin purified in 3 steps. Purity of the anatoxin was checked *via* electrophoresis.

The anatoxin was tested for safety and toxicity. Safety testing aimed to determine the general and local reaction of vaccinated animals in accordance with existing technical regulations. The experiment was performed on 3 clinically healthy chinchilla rabbits with a live weight of 1.5–2.0 kg. The animals were provided with proper nutrition and care under zoological hygiene conditions. The study drug was administered intravenously in the amount of 5 ml. We observed the experimental animals for 10 days. The anatoxin was deemed harmless if all three animals remained alive and clinically healthy for 10 days.

Toxicity of the purified staphylococcal anatoxin was studied according to the European Pharmacopoeia (European Pharmacopoeia, 5.0; 01/2005:20609, p. 153). We tested 5 clinically healthy white mice with a live weight of 17-22 g. One hundred microliters of anatoxin (warmed to 28–30 °C) were administered intravenously and the animals observed for 24 h. According to Pharmacopoeia, the animals should remain healthy without lethality during the observation period.

To increase the stability and shelf-life of the developed diagnostic test system, we lyophilized the drug in a gelatin-sucrose protective medium (7 % sucrose and 5 % gelatin). The semi-finished product was frozen at -40 °C and then freeze-dried. Testing the lyophilized drug no change was observed in 4 months in humidity, rehydration time, specificity, and sensitivity compared to the initial levels. The products will be retested after 1 year and 2 years.

Results and Discussion

Staphylococcal anatoxin has been successfully used in Ukraine and Russia for preventive (*e.g.,* during a pre-operative period) and therapeutic (furunculosis, hordeolum, or other relatively mild infectious diseases) purposes. The drug is not produced in Georgia. Actually, it is included in the list of deficient drugs. Imported anatoxin is expensive. Therefore, it is extremely important to improve the technology and introduce the drug in healthcare and veterinary practices.

For anatoxin production, selection of nutrients and the proper culture conditions for the producer strain, *S. aureus* s 0-15 are critically important. For culturing, we used the enriched casein hydrolysate medium (broth or agar) with an amine nitrogen concentration of 1.3-1.5 mg/ml, determined as the amount of alkali consumed (about 0.5 ml of 0.2 N NaOH). Once the amine nitrogen was determined, the medium was heated to boiling point. The mixture then was let to cool, and the pH adjusted to 7.2 -7.4. Then, 0.05 % Na_2HPO_4 and 0.05 % KH_2PO_4 were added and the mixture filtered through a 0.45-μm Millipore filter. One liter of medium was poured into a 5-liter container and sterilized in an autoclave at 112 °C for 30 min.

It is also important to select the clone with the highest hemolytic activity. Cells of strain 0-15 were grown for 18–20 h and then transferred onto blood agar to obtain isolated colonies. After an additional 20–22 h, colonies with the largest hemolysis zone (diameter 7–8 mm) were selected and grown in culture tubes containing 8-10 ml of casein broth for 18–20 h. The seed culture was transferred to a 5-liter container with 1 L of pre-filled and sterilized casein medium and incubated at 37 °C for 5 days. Twice a day, in the morning and in the evening, the culture was aerated through sterile filters with 10-12 L of oxygen (O_2) for 5-6 min and 2 L of carbon dioxide (CO_2) for 1 min. After 5 days, the cloudy suspension was separated by centrifugation at 3,000 x *g* for 20 min in a refrigerated centrifuge. The toxin-containing supernatant was sterile filtered through a 0.22-μm filter. The crude alpha-toxin was tested for

activity. In the hemolysis reaction, the minimum hemolytic dose of the toxin was determined to be 1:100.

To obtain the harmless form of the anatoxin, we added 0.4 % of formalin to the sterile liquid and stored it in a thermostat for 10 days. Then, the solution was kept for 5 more days at room temperature to convert the toxin into the anatoxin. To further purify the anatoxin, predetermined amounts of a 25 % sodium chloride solution and a 1 N trichloroacetic acid solution were added to the native anatoxin mixture. After holding for 1 h in a refrigerator, the mixture was centrifuged at 2-5 °C for 15 min. The resulting precipitate was dissolved in 100 ml of sterile saline (pH 6.8-7.0). The final step of anatoxin purification was ethanol precipitation with 2.5 volumes of pre-cooled (-20 °C) 96 % alcohol. The mixture was centrifuged at 2–3 °C. The resulting precipitate was dissolved in ice-cold saline (pH 6.8-7.0), filter sterilized (0.22 μm), and filled into ampoules.

To quantify the activity of our product, we determined its antibody-binding capacity (ABC). Results showed that the specific activity of anatoxin was 11 ABC, *i.e.,* it met the requirements of a standard drug. Similar result was obtained by examining the imported staphylococcal anatoxin (Medgamal, Moscow, Russia, 2019; anatoxin staphylococcal purified, C 174-0316, 1 ml; 11 ABC).

A second goal of the study was the development of a test system for post-vaccination immune response evaluation and rapid diagnosis of a staphylococcal infection in healthcare and veterinary practices. The passive (indirect) hemagglutination reaction is often used in diagnostic practices in Georgia and abroad [8-13]. It is easy to use and provides the test results in 1.5-2 h, but it requires expensive equipment.

One important step in the preparation of a highly sensitive and active diagnostic test system is the selection of the adsorbent (erythrocyte) stabilization method. Mostly, human Type I, sheep or goat erythrocytes, or latex particles are used. Formaldehyde was used to produce standard erythrocytes earlier [13]. The optimal number of formalin-treated erythrocytes in a 2.5 % suspension was 6 x 10^6 cells/ml. Erythrocytes were washed three times with saline, re-suspended in saline (pH 7.0-7.2), and counted. To increase the sensitivity and specificity of the diagnostic system (*i.e.,* to remove proteins, polysaccharides, and other impurities from the erythrocyte membrane surfaces), we treated in triplicates 5 ml of a 2.5% erythrocyte suspension with potassium dichromate ($K_2Cr_2O_7$) in different dilutions (1:1,000; 1:5,000; 1:20,000), and incubated these tubes at 37 °C for 20 min. Erythrocytes treated with potassium dichromate were washed with saline and the 2.5% suspension reestablished.

To further increase the sensitivity of the test system, 5 ml of the erythrocyte suspension were added to 5 ml of tannic acid (0,05 g/ml). The mixture was incubated at 37 °C for 20 min, washed twice with 1/15 M phosphate buffer (pH 7.2) and once with 1/15 M phosphate buffer (pH 6.4). After treatment, the erythrocytes were sensitized with the purified staphylococcal anatoxin. In terms of specific activity, the most sensitive test system was obtained by treating the erythrocytes with the 1:5,000 dilution of potassium dichromate. The optimal sensitizing dose of staphylococcal anatoxin is shown in Diagram 1 and Fig. 1.

Serial production of a sensitive test system depends on the optimal dose of anatoxin. This optimal dose is experimentally determined as the amount of anatoxin that detects antitoxic antibodies at a maximum dilution, while the control test is still negative. Table 1 shows that 30 μg of anatoxin detected low number of antibodies (11.42 ± 2.37). High dos-

Table 1. *Determination of optimal diagnostic dose for staphylococcal anatoxin*

№	Number of formalinized erythrocytes	Anatoxin dose [μg/ml]	Antibody titer range	Obtained result in PHAR (M±m) *	Probability of difference (P)
1	5.5x10^5	30	5–20	11.42±2.37	
2	5.5x10^5	80	16–640	457.1±88.7	<0.01
3	5.5x10^5	150	160–640	434.3±97.2	

** – Horse staphylococcal anti-toxin immunoglobulin (100 IU/ml) was used in the passive hemagglutination reaction.*

es resulted in higher titers, and the controls tended to be positive, risking over-diagnosis. The optimal amount of anatoxin was 80 μg/ml, which provided high titers of antibodies. Using the optimal dose, we prepared 3 small series of lyophilized drug, each in the amount of 35 - 40 ampoules (Fig. 2). This anatoxin diagnostic test system may now be used by experts for monitoring anti-staphylococcal immunoglobulin concentrations.

The activity of anti-staphylococcal serum or immunoglobulin in the reaction (Passive Hemagglutination Reaction; PHAR) may be expressed in international units (IU/ml), as proposed by Varlakova and co-workers [14]. We used the reference immunoglobulin (26 IU/ml, Microgen, Moscow, Russian Federation) and the test serum diluted 1:10. We calculated the test serum activity in international units (IU/ml) with the following formula:

$$X = C \times B/A \quad \text{(Equation 1)},$$

where X is the staphylococcal antitoxic antibody activity in test serum in IU/ml; A indicates the reference serum back titer PHAR; B is the test antitoxic serum PHAR; and C is the reference serum activity in IU/ml.

Additionally, the test system detects in a passive hemagglutination reaction the minimum number of antitoxic antibodies in human or animal serum, and converts the titer (1:10 – 1:40) detected in healthy people into international units:

$$X = C \times B/A = 26 \times 40/1{,}280 = 0.8 \text{ IU/ml} \quad \text{(Equation 2)}.$$

This PHAR number is 10 or more times greater than the Lymes-hemolysis data calculated through the currently accepted method for antitoxic antibody determination. The advantage of PHAR is that results were obtained in 1.5–2.0 h and the test does not require significant material and labor costs. In a Passive Hemagglutination Reaction, unlike in other ELISA tests, hemolyzed serum can be examined.

In short, the diagnostic test system developed and adopted uses lyophilized antigen (anatoxin) and sensitized erythrocytes to detect and quantify staphylococcal antitoxic antibodies in human and animal blood serum. The test system can be used not only in research but also in healthcare and veterinary practices to diagnose staphylococcal infections, as well as to evaluate post-vaccination immunity.

The specificity of the proposed antigenic (anatoxin) diagnostics was tested against sera from clinically healthy humans, patients with a staphylococcal infection (complicated furunculosis and post-operative abscesses), other bacteriologically confirmed toxigenic strains, a commercially available immunoglobulin (Tetanus Gama 250 IU/ml; N 022488062, Paskoli, Barga, Lukka, Italy), and healthy bovine sera (heifers and cows; age 1.5–3 years). Conventionally, diagnostic antitoxic antibody titers in humans correspond to dilutions of 1:80 and higher. Results of this study are shown in Table 2.

The table shows that the number of antitoxic antibodies in healthy human serum is significantly smaller compared to the numbers in infected humans.

Results for healthy human serum antibody titers ranging from 1:5 to 1:20 were confirmed in an earlier study [13]. The titer for normal serum antitoxic antibodies in the blood of unvaccinated animals (cattle) was 1:40 - 1:160 in PHAR. Test antibodies were within the normal range against the anti-tetanus IgG (1:20), in spite that the amount of anti-tet-

Table 2. *Indicators of staphylococcal anatoxin diagnostic test system specificity*

№	Test serum	Number of sera	Titer range	Antibody titer (M±m)	Probability of difference (P)
1	Normal human serum	9	5–20	12.7±2.6	
2	Human serum with staphylococcal infection	17	40-320	98.82±10.41	<0.05
3	Tetanus Gama [250 IU/ml]	1	1:20		
4	Normal bovine serum	11	20–160	74.5±14.36	

anus antibodies was 250 IU (if converted in PHAR, it is equivalent to 1:200,000 dilution). The titer of anti-alpha-toxin antibodies in people infected with staphylococci was higher than normal; the difference is statistically significant.

We also tested the system specificity in the Passive Hemagglutination Inhibition Reaction. Purified anatoxin (0.25 ml; activity 10 ABC) was added to antitoxic serum applied in a polystyrene microtiter plate, and incubated at 37 °C for 20 min. Subsequently, the diagnostic drug was dripped on it. We placed the plate again in the thermostat for 40 min and finally, left it for 20 min at room temperature. The obtained results showed that the reaction in the last 5 wells was negative, which indicated inhibition of the hemagglutination reaction and its specificity.

Conclusion

1. Staphylococcal anatoxin (harmless, immunogenic form of alpha hemolysin) was produced and studied. Its activity (11 ABC) meets the required standard.
2. The staphylococcal anatoxin is a treatment and prevention drug, which may be used in healthcare and veterinary practices.
3. A rapid diagnostic test system for staphylococcal infection (formalin- and tannic acid-treated sheep erythrocytes sensitized with staphylococcal anatoxin) was designed and produced. The diagnostic system is used in a Passive (Indirect) Hemagglutination Reaction (PHAR). Its sensitivity is equivalent to a standard ELISA test.
4. The test system is designed to detect and quantify antitoxic antibodies in human and animal sera, as well as to assess the post-vaccination immunity.

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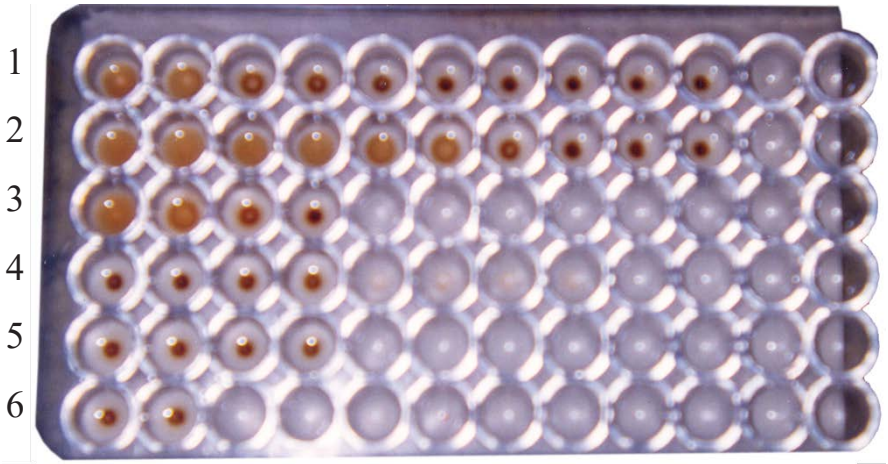

Fig. 1. *Determination of staphylococcal antitoxin antibodies in a Passive Hemagglutination Reaction (PHR). (Row 1: healthy cattle serum; Row 2: serum of a human with a staphylococcal infection; Row 3: healthy human serum; Rows 4, 5, and 6 are controls)*

Fig. 2. *Ampuls with the diagnostic test system*

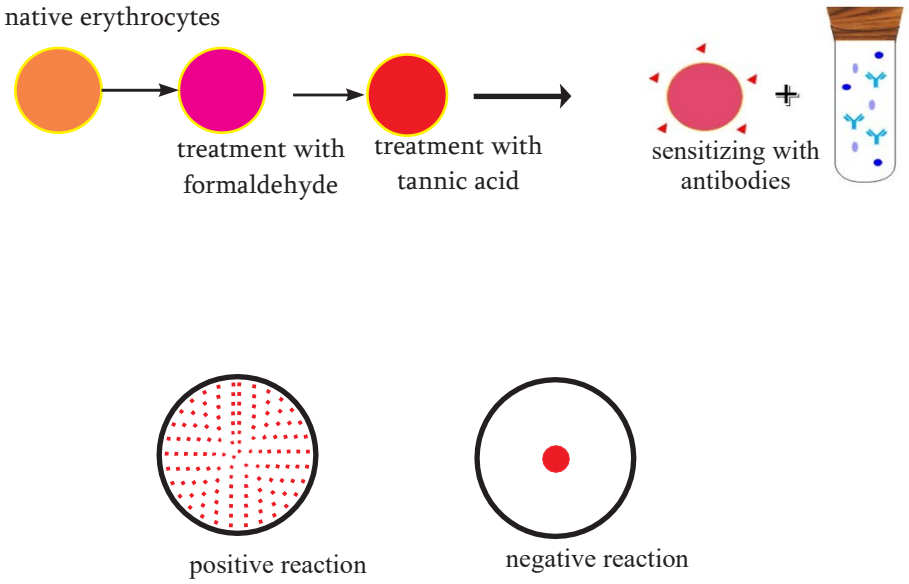

Diagram 1. *Passive (indirect) hemagglutination reaction*