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Article

Preparation of Hemolyzin from Blood Samples

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Abstract: In the process of preparing hemolysin from blood samples, biological materials were collected in accordance with veterinary-serological requirements and carried out in laboratory conditions. The steps of obtaining hemolysin were carried out by isolating erythrocytes from blood samples and immunobiologically processing them.

Keywords: Blood, Hemolysin, Complement Binding Reaction, Sheep, Bacteria, PBS, Erythrocyte, Plasma

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Introduction

Currently, one of the main fundamental areas of modern microbiology and medicine in the world and in our Republic is the study of microbial pathogenicity. It is known that pathogenic bacteria produce various substances that directly damage or kill macro and microorganism cells, as well as facilitate their entry into the body by overcoming the defense systems of bacteria. These substances play a major role in the development of diseases caused by bacteria.

Hemolysins are one of the biological substances that actively participate in immunological reactions, they have the property of ensuring the destruction of erythrocytes. Hemolysin complexes are widely used in the complement fixation reaction, hemolytic tests and other serological tests. The quality of these preparations is one of the main factors determining the accuracy and reliability of diagnostic tests. Therefore, improving the technology for preparing hemolysin complexes, increasing their biological activity and standardization is of particular importance today.

According to the current scientific literature, bacterial toxins are a group of bacterial products that have the ability to harm or kill living organisms when exposed to relatively small amounts. Today, it is difficult to give a precise definition due to the diversity of bacterial toxins in their structure, mechanisms of action, immunogenicity, and other properties. As a rule, toxins are relatively

high-molecular substances in the form of proteins, peptides, or polysaccharides. However, some bacterial toxins with molecular weights of less than 10,000 Da are also known [1]

One of the most important questions in the study of toxins is the question of their role in pathogenesis. This question is most easily answered in cases where the disease is a direct consequence of intoxication, for example, in food poisoning. In such cases, living bacterial cells are often destroyed when exposed to a toxic substance, and experimental reproduction of the disease can occur as a result of direct exposure to the toxin. When the production of this substance is carried out by the multiplication of bacteria in the host organism, it is somewhat more difficult to determine the role of the toxin in pathogenesis. In such cases, a number of conditions are necessary for the "successful" production of the toxin. Such conditions include additional factors produced by the microbe, which are necessary for its entry into the host organism and its maintenance. When the bacterium multiplies in the host organism, it is much more difficult to assign a specific role to a particular toxin. Nevertheless, it can be argued that all diseases caused by bacteria are caused directly or indirectly by the action of toxins [2][3][4].

Materials and Methods

In the preparation of hemolysin, the research material and object consists of biological components selected for immunological experiments. Usually, erythrocytes are used as antigens, and laboratory animals are used as objects of immunization.[5]

Blood samples from 10 healthy sheep, rabbit immune serum, guinea pig complement, physiological solution and PBS buffer are used as research materials.

Sheep 10 sheep erythrocytes were used because they have good antigenic properties in the immune reaction. Blood serum Serum obtained after immunization contains hemolysin. Guinea pig serum was often used as a supplement. It causes a hemolysis reaction. Cell media such as PBS (phosphate-buffer solution), physiological solution, RPMI-1640 are used from physiological solutions and nutrient media. Laboratory reagents, anticoagulants, dyes, chromatographic materials, filters, sterile containers were used. SDS-PAGE, chromatography and filtration are also used in biotechnological methods.[6]

In the preparation of hemolysin, immunological, serological and biotechnological research methods are used. These methods serve to obtain hemolytic antibodies, determine their activity and evaluate their quality.

In this research work, the preparation of hemolysin based on the complement binding reaction is carried out on the example of 10 sheep erythrocytes, its testing in laboratory conditions and biotechnological processing. Hemolysin is an immune antibody that destroys erythrocytes and is widely used in serological diagnostics and immunological research.[7]

In this research, the process of preparing hemolysin from blood samples taken from the farm "Zayniyeva Zamira Safarovna", laboratory analyzes based on the complement binding reaction, and stages of biotechnological processing were studied in detail. During the study, the interaction of erythrocyte antigens, immune serum and complement system was analyzed.[8]

Isolation of erythrocyte antigens from blood samples taken from 10 sheep from the farm, preparation of hemolysin based on them and determination of hemolytic antibody activity using the complement binding reaction.

Figure 1. Pictures of the process of blood collection in sheep on the "zayniyeva zamira safarovna" farm

Blood was collected from healthy sheep on the farm under sterile conditions. Sodium citrate was used to prevent blood clotting. The following processing was performed on the blood samples: centrifugation; - separation of plasma; - washing of erythrocytes 3–5 times in physiological solution; - preparation of a 5% erythrocyte suspension.

In the process of hemolysin preparation, high-quality washing of erythrocytes is one of the most important stages of laboratory work. Correct and complete washing of erythrocytes directly affects the accuracy, sensitivity and reliability of subsequent serological reactions. If erythrocytes are not washed sufficiently, serum proteins, complement residues and other biological substances can negatively affect the reaction. For this, blood samples were collected from 10 healthy sheep under sterile conditions for the experiment. The samples were collected in test tubes with a special anticoagulant added to prevent clotting. The obtained blood was delivered to the laboratory and separated into plasma and erythrocyte fractions using centrifugation. The separated erythrocyte sediment was prepared for the next stage of washing. Sterile physiological solution was used to wash erythrocytes. In practice, a 0.85–0.9% sodium chloride (NaCl) solution was mainly used. This solution creates an isotonic environment for erythrocytes and maintains the osmotic stability of cells.[9]

Washing erythrocytes was carried out in several stages:

Stage 1 — adding physiological solution 5–10 ml of sterile physiological solution was added to the separated erythrocyte sediment and mixed carefully.

Stage 2 — centrifugation

The resulting mixture was centrifuged at 1500–2000 rpm for 5–10 minutes. As a result, the erythrocytes settled to the bottom of the test tube.

Stage 3 — separation of the upper layer After centrifugation, the upper liquid was carefully removed using a pipette. Plasma residues and excess proteins were present in this layer.

Stage 4 — re-washing The washing process was repeated 3–5 times. Washing was continued until a bleached and clear erythrocyte sediment was formed.

In most of the 10 sheep samples, high-quality washing of erythrocytes was noted. This allowed obtaining accurate and stable results in the subsequent hemolysin preparation stage.

High-quality washing of erythrocytes reduced nonspecific hemolysis and background reactions.

During the study, blood samples from healthy sheep were used to determine the antigenic properties of erythrocytes. The erythrocytes in the obtained blood were separated from the plasma by

centrifugation and washed several times with 0.85% saline. The main purpose of washing erythrocytes was to remove plasma proteins, complement components, and other foreign substances while preserving the antigens on their surface.

A 5–10% suspension was prepared from washed erythrocytes. The prepared erythrocyte suspension was used as an antigen in the immunization of laboratory animals, mainly rabbits. During the immunization process, erythrocytes were injected into the rabbit body according to a certain scheme and the formation of an immune response was observed at certain intervals.

To assess the antigenic properties, an antigen-antibody reaction was used. In this method, serum obtained from immunized rabbits was mixed with a erythrocyte suspension. The observation of agglutination or hemolysis of erythrocytes as a result of the reaction indicated the presence of an interaction between the antigen and the antibody. The strength of the reaction was taken as a criterion for assessing the antigenic activity of erythrocytes.[10]

According to the results of the study, it was found that washed erythrocytes retained high antigenic properties. In rabbits immunized with erythrocytes, specific antibodies were formed, and subsequently their high activity in the hemolysin composition was noted. The obvious manifestation of the hemolysis reaction confirmed the effective recognition of erythrocyte antigens by the immune system.

The effect of the number of erythrocyte washings on the antigenic properties was also evaluated. In erythrocytes washed three to four times, antigenic activity was high, and the reactions were clear and distinct. In erythrocytes that were not washed sufficiently, nonspecific reactions were observed in some cases due to the retention of plasma residues.

Results and Discussion

The results obtained showed that the antigenic properties of erythrocytes are one of the main factors in the process of preparing hemolysin. The use of erythrocytes with a high antigenicity level made it possible to obtain high-titer and biologically active hemolysin. This subsequently served to increase the accuracy and reliability of the complement fixation reaction, hemolysis reaction, and other serological studies. Stage 1 - preparation of erythrocyte suspension A 2–5% suspension is prepared from pre-washed erythrocytes. Erythrocytes were mixed in sterile physiological solution at the same concentration. In the preparation of the antigen, the antigen used for the experiment was prepared at a certain concentration. The quality and biological activity of the antigen were checked in advance. A certain volume of immune serum was added to the test tube for adding immune serum. This serum contained antibodies formed against the antigens. The antigen solution was added to the test tube storing the immune serum. The mixture was mixed carefully. During incubation, the test tubes were kept in a thermostat at a temperature of 37 °C for 20–30 minutes. During this period, antigen-antibody binding occurred. In the addition of complement, after incubation, a complement solution was added to the reaction mixture. The complement bound to the antigen-antibody complexes. In the addition of erythrocyte suspension, erythrocyte suspension was added in the next step and the test tubes were placed in the thermostat again. When evaluating the results, the degree of hemolysis in the test tubes was observed at the end of the reaction.[11]

Table 1. The results are evaluated as follows.

Condition	Result
No hemolysis	Positive reaction
Complete hemolysis	Negative reaction
Partial hemolysis	Doubtful or weak reaction

If the antigen and antibody bind, the complement binds to the immune complex and cannot break down the erythrocytes. In this case, hemolysis is not observed and the reaction is considered positive.

If antigen-antibody binding does not occur, the complement remains free and breaks down the erythrocytes. As a result, hemolysis occurs and the reaction is considered negative.

According to the analysis of the research results, freshly obtained erythrocytes showed high antigenicity, while the risk of hemolysis increased in erythrocytes stored for a long time; it was observed that the optimal environment is pH 7.2–7.4. Immunologically, sheep erythrocytes were considered a strong antigen.

The process of immunization of rabbits was carried out. In order to obtain hemolysis, rabbits were injected with a suspension of erythrocytes. Immunization was carried out with 3 injections: 1st injection - 2 ml; 2nd injection - after 4 days; 3rd injection - a booster dose. Stage 1 - primary immunization. Rabbits were injected with a certain dose of antigen subcutaneously or intramuscularly. At this stage, the body first became acquainted with the antigen. During primary immunization, macrophages absorbed the antigen, T and B lymphocytes were activated, and the initial immune response occurred. Stage 2 - re-immunization After 7–10 days, the antigen was re-administered. This stage was carried out to enhance the immune response and increase the antibody titer. As a result: the amount of immunoglobulins increased, immunological memory was formed, and sensitivity to antigens increased. Stage 3 - observation and control After immunization, the general condition of the rabbits was regularly monitored. During the control, body temperature, nutrition, motor activity, the condition of the injection site, and allergic reactions were observed.

The process of immunization of rabbits was observed in our studies: IgM antibodies were formed in the primary response, IgG levels increased during repeated immunization, and a significant increase in hemolysin titer was observed.

A complement fixation reaction was performed. Immune serum was placed in test tubes; erythrocyte antigen, complement were added. The mixture was incubated at 37°C. Then the indicator system: sheep erythrocytes; hemolytic serum was added.[12]

The results were considered as positive reaction if hemolysis was not observed;

- if hemolysis was observed - negative reaction.

In our experiments, hemolysin titer was determined. In determining the hemolysin titer, serum was serially diluted to assess hemolysin activity: 1:10 → 1:20 → 1:40 → 1:80 → 1:160 → 1:320.

In this experiment, a complement fixation reaction was performed based on erythrocyte antigens prepared from blood samples taken from 10 sheep. The main purpose of the reaction was to determine the presence of hemolysin and assess the level of immunological activity. Each sample was examined separately, and the state of hemolysis and the level of complement fixation were analyzed.

Sheep sample 1 The reaction was positive. Hemolysis was not observed. Complement was fully bound. High hemolysin titer - 1:320 Sheep sample 2 Partial hemolysis was observed. Part of the complement was bound. The reaction was assessed as weakly positive. Titer - 1:160. Sheep sample 3

Hemolysis was not observed. A strong antigen-antibody complex was formed. Complement activity was high. Titer - 1:320. Sheep sample 4 Complete hemolysis was observed. The reaction was assessed as negative since the complement remained free. Titer - low - 1:80. Sheep sample 5

A positive reaction was recorded. Erythrocyte fragmentation was not observed. Titer - 1:160. Sheep sample 6 Hemolysis was not observed. Complement binding was high. A strong immunological response was observed. Titer — 1:320. Sheep sample 7

Partial hemolysis was noted. The reaction was assessed as moderately positive. Titer - 1:160. Sheep sample 8 The highest result was observed in this sample. Complement was maximally bound. There was no hemolysis. Titer — 1:640. Sheep sample 9

Hemolysis was almost completely observed. Due to the weak antigen activity, complement binding was low. Titer — 1:80. Sheep sample 10

The reaction was clearly positive. Hemolysis was not observed. The biological material was assessed as high quality. Titer — 1:320. General conclusion. General analysis results: - A clear positive reaction was observed in 7 out of 10 samples; - Partial hemolysis was noted in 2 samples; - A negative reaction was observed in 1 sample. The highest complement binding was determined in sheep sample 8. Low results were associated with a decrease in erythrocyte quality or low antigen activity. During the study, it was confirmed that: - 37°C is the optimal temperature; - the importance of using fresh complement; - the need for high-quality washing of erythrocytes.[13]

In the results of hemolysin activity dilution, some samples were active up to 1:160, high-quality samples showed a titer of 1:320, and partial hemolysis was observed in weak sera.[14]

In this experiment, erythrocyte antigens were prepared from blood samples taken from 10 sheep, and hemolysin was produced in rabbits on their basis. The hemolysin titer in each sample was determined separately using the complement fixation reaction and immunological analysis was performed

According to the results of the general analysis: 4 out of 10 samples showed high titers (1:320 and above). 4 samples showed medium titers (1:160); 2 samples showed low titers (1:80). During the experiment, the quality of erythrocytes, complement activity, and sterile conditions significantly affected the results. The highest result was observed in the 8th sheep sample.[15]

Conclusion

In conclusion, in the process of preparing hemolysin from blood samples taken from the farm "Zayniyeva Zamira Safarovna", biological materials were collected in accordance with veterinary-serological requirements and examined in laboratory conditions. During the study, erythrocytes were isolated from blood samples and hemolysin was obtained by immunobiological treatment.

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