Results of studies on the development of an immuno-enzymed test system for diagnosing infectious enterotoxemia in animals

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Abstract. Research materials on the development of an enzyme-linked immunosorbent assay for the determination of specific antibodies to Cl. perfringens bacteria were presented. Strains of Cl. perfringens were used as production strains: No. 28 (type A), LD-1 (type B), No. 392 (type C), No. 213 (type D). To obtain antigen, a daily suspension of Cl perfringens bacteria, containing 20 billion microbial cells in 1 cm³ (5 billion microbial cells of each serotype), was sounded on an ultrasonic disintegrator at a frequency of 20 MHz for 15 minutes at 4 °C, then the endotoxin was precipitated with ammonium sulfate and purified by differential dissolution of the precipitate in 0.02 M phosphate buffer, followed by dialysis against tap water. Control positive serum with specific antibodies of at least 1:12800 to antigens of Cl. perfringens bacteria was obtained by hyperimmunization of cattle with corpuscular antigens and toxoids. The basic conditions of the ELISA reaction were standardized. Determination of the concentration of sorption of the antigen was carried out by parallel testing of various dilutions of the conjugate. The most acceptable titer of the blood serum of animals was found at a dilution of the conjugate of 1:2500 and a concentration of the sorbed antigen of 5-8 μg/cm³. The production test of the enzyme immunoassay test system was carried out in cattle farms in three regions of the Russian Federation, unfavorable for anaerobic enterotoxemia in calves.

1 Introduction

Infectious enterotoxemia of young farm animals caused by toxins of the spore-forming anaerobic bacteria Clostridium perfringens is widespread in the Russian Federation. The disease occurs when disorders of the gastrointestinal tract function, accompanied by rapid multiplication of the pathogen in the intestine [1–3]. The name Clostridium perfringens

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unites a group of microorganisms that are similar in cultural and morphological properties, but differ in the antigenic structure of the toxins produced, and therefore they are divided into six types: A, B, C, D, E, F. In lambs, anaerobic enterotoxemia is caused by pathogens of types B and D; in calves - types A, C, D; in piglets - mainly of type C, less often others [4–11]. The diagnosis of infectious enterotoxemia of animals is considered established in the case of:

- detecting a toxin in the filtrate of the contents of the small intestine by a biological method and determining its type in a neutralization reaction with type-specific sera;
- isolation from the contents of the small intestine of a culture with characteristic properties of a given pathogen, producing a toxin, the type of which is established in the neutralization reaction with type-specific sera.

The disadvantages of the laboratory method are the laboriousness and duration of the research, which requires up to 8 working days for the diagnosis, as well as the high cost of the research [6]. It is quite obvious that a modern diagnostic service should rely on highly specific and sensitive diagnostic methods that would provide reproducible, reliable results within the method, and should be recognized as suitable for large-scale research in veterinary laboratories. In the foreign literature there are numerous data on the use of the enzyme-linked immunosorbent assay for these purposes, which allows, by the level of titers of specific antibodies in the blood serum to bacteria *Cl. perfringens*, to judge the tension of post-vaccination immunity, as well as the circulation of the pathogen among the unvaccinated livestock of animals [12–18].

It was also found that the ELISA method allows quantitative determination of various types of toxins, which can be useful for monitoring the activity of *Cl. perfringens* bacteria [19]. It is emphasized that the method has high reproducibility and sensitivity. The aim of this study was to develop an enzyme-linked immunosorbent assay for the determination of specific antibodies to *Cl. perfringens* bacteria in the blood serum of animals.

## 2 Materials and Methods

The work was carried out in the Federal Center for Toxicological, Radiation and Biological Safety, as well as in cattle farms of the Volga Federal District of the Russian Federation, unfavorable for anaerobic enterotoxemia. When developing the enzyme-linked immunosorbent assay, we used production strains of *Cl. perfringens* type A (no. 28), type B (LD-1), type C (no. 392) and type D (no. 213).

Research was conducted in accordance with the requirements of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS No. 123, Strasbourg, 1986). Research was approved by the bioethical commission of the Federal Center for Toxicological, Radiation and Biological Safety (protocol 2022–05/1, dated May 01, 2022).

When developing an immunoassay test system, studies were carried out to obtain the components of the test system and to determine their sensitivity and specificity.

To obtain the antigen necessary for sensitization of polystyrene plates, industrial strains of *Cl. perfringens* were grown on blood nutrient agar for 24 hours, then the bacterial mass was washed off with 0.85% sodium chloride solution. To free the cells from the remnants of the nutrient medium, the bacterial mass was washed 3 times with 0.85% sodium chloride solution. Then, suspensions of *Cl. perfringens* bacteria of each strain with a concentration of 20 billion microbial cells in 1 cm³ were separately prepared. Bacterial suspensions were mixed in equal proportions and thus a suspension containing 5 billion microbial cells in 1 cm³ of each *Cl. perfringens* serotype (A, B, C, D) was obtained. The resulting suspension, containing 20 billion microbial cells in 1 cm³, was sonicated on an ultrasonic disintegrator in the 20 MHz mode for 15 minutes at 4 °C. The suspension was centrifuged at 3000 rpm
for 30 minutes. The supernatant fluid containing endotoxins was used to obtain antigen. To precipitate endotoxin, ammonium sulfate was added to the suspension to 40% saturation and purified by differential dissolution of the precipitate in 0.02 M phosphate buffer, followed by dialysis against tap water.

The production of a control positive serum to the antigen of bacteria *Cl. perfringens* was carried out by hyperimmunization of 7-month-old cattle with inactivated antigens of bacteria *Cl. perfringens* of serotypes A, B, C and D. For this purpose, corpuscular antigens and toxoids of industrial bacterial strains were used. The corpuscular antigen was a suspension of formalin-inactivated bacteria *Cl perfringens*, containing 2.5 billion microbial cells of each serotype in 1 cm³. The corpuscular antigen was a suspension of formalin-inactivated bacteria *Cl. perfringens*, containing 2.5 billion microbial cells of each serotype in 1 cm³. To obtain toxoid, bacteria *Cl perfringens* were grown in a liquid nutrient medium for 7 hours, then the toxin was inactivated with formalin at 37 °C for 10 days. Then the resulting toxoid was purified by centrifugation and filtration of the culture fluid through bacterial filters.

The animals were hyperimmunized 4 times with an interval of 14 days. In this case, the animals were simultaneously injected with a corpuscular antigen subcutaneously and anatoxin - intramuscularly in increasing doses. 20 days after the last administration of the antigen, a blood sample was taken from the jugular vein of the animals to determine the titers of specific antibodies. Production blood sampling was performed in the presence of specific antibodies to *Cl. perfringens* bacteria in the blood serum of bulls-producers in titers of at least 1: 12800 in ELISA.

Control negative serum, free from specific antibodies to bacteria *Cl. perfringens*, was obtained from 7-month-old cattle from a farm free from infectious enterotoxemia.

The enzyme-linked immunosorbent assay for the diagnosis of infectious enterotoxemia in animals consisted of the following components:

1. Polystyrene 96-well microplates for enzyme-linked immunosorbent assay adsorbed with *Cl perfringens* antigen - 2 pieces;
2. Control positive serum, lyophilized, ELISA titer 1: 6400 - 1: 12800, volume 1 cm³ - 1 ampoule;
3. Control negative serum, lyophilized, volume 1 cm³, - 1 ampoule;
4. Diagnostic antibodies against bovine immunoglobulins of the IgG class, labeled with horseradish peroxidase (conjugate), volume 0.2 cm³ - 1 ampoule;
5. Phosphate-buffered saline solution, 20-fold concentrate, 20 cm³ - 2 vials;
6. Detergent (Tween-80), 1.0 cm³ - 1 vial;
7. Citrate-phosphate buffer solution, volume 10 cm³ - 2 vials;
8. Chromogen (orthophenylenediamine), 5 mg - 2 vials;
9. Hydrogen peroxide (H₂O₂) 3 % solution, volume 10 cm³ - 1 vial;
10. 1 M sulfuric acid (stop reagent), volume 10 cm³ - 2 vials.

The enzyme immunoassay was performed in 96-well flat-bottomed plates for immunological reactions according to the generally accepted method. The results of the reaction were taken into account according to the readings of the optical density at a wavelength of 490 nm.

Preparation of working solutions and reagents. Before starting work, all reagents were kept for 30 minutes at room temperature.

The ELISA plate with *Cl perfringens* antigen adsorbed in the wells was ready to use.

Working solution for conjugate dilution - 0.01 M phosphate-buffered saline solution. The contents of the vial were brought to a volume of 400 cm³ with freshly prepared distilled water. It was allowed to be stored for no more than 1 month at a temperature of 4±2 °C.
Working solution for washing plates. To 500 cm$^3$ of a phosphate-buffered saline solution, 0.25 cm$^3$ of Tween-80 was added and stirred until it was completely dissolved.

Cattle sera, positive and negative, were dissolved with distilled water to 1 cm$^3$.

The contents of the ampoule with the conjugate were dissolved in phosphate buffered saline, which did not contain tween-80, until the working dilution.

The contents of the ampoule with hydrogen peroxide were dissolved in distilled water in the volume indicated on the ampoule label.

The substrate-indicator mixture was prepared immediately before use in the reaction as follows: the contents of the vial with orthophenylenediamine were dissolved in 10 cm$^3$ of citrate-phosphate buffer solution and 0.17 cm$^3$ of hydrogen peroxide was added. Stop-reagent is ready to use.

3 Results and Discussion

First, the main conditions of the ELISA reaction were standardized. When determining the optimal concentration of antigen for adsorption on a polystyrene 96-well plate, the intensity of the enzyme-linked immunosorbent reaction was assessed at antigen concentrations in solution: 2.0; 2.5; 5.0; 8.0; 10.0 μg/cm$^3$. As test samples used "positive" and "negative" control samples of blood serum of animals. Serum of cattle was used for the analysis. If the analysis was carried out within 24 hours after selection, the serum samples were stored at a temperature of 4 °C. For longer storage, the samples were frozen at minus 20 °C. Before the study, the frozen samples were quickly (within 5-10 min) heated in a water bath at a temperature of 37 °C. In case of precipitation, the samples were necessarily clarified by centrifugation for 10 minutes at 2000 g. Repeated freezing and thawing of samples was not allowed.

The selection of the concentration of sorption of the antigen was carried out in parallel testing of various dilutions of the conjugate. The most acceptable titer of the blood serum of animals was found at a dilution of the conjugate of 1:2500 and a concentration of the sorbed antigen of 5-8 μg/cm$^3$. Thus, this dilution of the conjugate, at an initial concentration of the sorbed antigen of 5-8 μg/cm$^3$, was considered optimal and was used in further work.

Determination of the optimal exposure time of the studied sera. The formation of stable immune complexes in the ELISA test system is a complex process and requires a certain time, for the establishment of which the intensity of the reaction was estimated in 3 replicates depending on the time of incubation of specific and negative sera in serial dilutions at 37 °C. In this case, the conjugate was used in a working dilution of 1: 2500. It was found that the optical density indices of the studied sera increased in the range from 15 to 60 min, and then stabilized. The observed effect was recorded for sera with different levels of specific activity. On this basis, a 60-minute exposure of the sera was considered sufficient to achieve a maximum and stable response.

To take into account and interpret the results obtained by the ELISA test system, the positive-negative threshold of the test system was determined, which was in the range <15% -> 22%. Subsequently, all samples, the value of the coefficient of specificity of which was less than the positive-negative threshold, were considered negative, and the samples with the value of the coefficient of specificity equal to or greater than this indicator - positive.

An interlaboratory commission test of the components of the test system for the specificity, sensitivity and reproducibility of the results of the enzyme-linked immunosorbent assay was carried out.

The experiments used a control positive serum against bacteria *Cl. perfringens*, control negative serum against bacteria *Cl. perfringens*, blood serum from calves vaccinated against infectious enterotoxemia and infectious enterotoxemia calves, as well as...
heterogeneous hyperimmune sera (salmonella, escherichiosis). At the same time, it was found that all components of the kit are active and specific in ELISA. The specific antigen does not react with heterogeneous hyperimmune sera (salmonella, escherichiosis), while with homologous sera (hyperimmune serum and blood sera obtained from vaccinated against infectious enterotoxemia and obviously sick animals) it gives a positive reaction in high titers - 1:3200 - 1:12800.

The production test of the enzyme-linked immunosorbent assay was carried out in cattle farms in 3 regions of the Volga Federal District of the Russian Federation, which were unsuccessful in terms of infectious enterotoxemia in calves.

A total of 1402 blood serum samples were examined, including 344 from animals with infectious enterotoxemia, 923 samples from those vaccinated against infectious enterotoxemia, and 135 from clinically healthy animals from permanently prosperous farms. The results of the serological study are presented in the table 1.

Table 1 - Results of the study of blood serum of cattle for the presence of specific antibodies to bacteria *Cl perfringens*

<table>
<thead>
<tr>
<th>Region name</th>
<th>Number of samples</th>
<th>The condition of the animals</th>
<th>React positively in ELISA for <em>Cl perfringens</em> number of samples</th>
<th>Total</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Republic of Tatarstan</td>
<td>534</td>
<td>sick, not vaccinated</td>
<td>521</td>
<td>97,5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>249</td>
<td>vaccinated</td>
<td>240</td>
<td>96,4</td>
<td></td>
</tr>
<tr>
<td>Samara Region</td>
<td>284</td>
<td>sick, not vaccinated</td>
<td>278</td>
<td>97,9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>56</td>
<td>vaccinated</td>
<td>53</td>
<td>94,6</td>
<td></td>
</tr>
<tr>
<td>Nizhny Novgorod Region</td>
<td>105</td>
<td>sick, not vaccinated</td>
<td>101</td>
<td>96,2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>39</td>
<td>vaccinated</td>
<td>37</td>
<td>94,8</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>344</td>
<td>sick, not vaccinated</td>
<td>330</td>
<td>95,9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>923</td>
<td>vaccinated</td>
<td>900</td>
<td>97,5</td>
<td></td>
</tr>
<tr>
<td>Republic of Tatarstan</td>
<td>135</td>
<td>clinically healthy, not vaccinated from a safe farm</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

The table shows that the ELISA method allows detecting specific antibodies to *Cl. perfringens* bacteria in 97.5 % of healthy vaccinated animals and 95.9 % of animals with infectious enterotoxemia. For the test system, regulatory documents have been developed for its manufacture, control and use.

4 Conclusion

An enzyme-linked immunosorbent assay for the determination of specific antibodies to *Cl. perfringens* bacteria has been developed and tested under production conditions. The proposed enzyme-linked immunosorbent assay is universal, has high specificity and sensitivity, does not require sophisticated equipment and is acceptable for conducting analyzes in large quantities. It can be used both for the diagnosis of infectious enterotoxemia in animals in permanently dysfunctional farms, and for determining the intensity of post-vaccination immunity. The research time is only 4 hours, while the laboratory tests currently available in the Russian Federation are up to 8 days.

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Conflict of interest. The authors declare that there is no known conflict of interest associated with this publication.
References