Clinical trial results of an associated vaccine against cattle clostridiosis and escherichiosis

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Abstract. In veterinary practice, gastrointestinal diseases of newborn calves, caused by the association of pathogenic bacteria, in particular Clostridium perfringens and Escherichia coli, are often observed. The results of our research have shown that in the Volga Federal District of the Russian Federation, anaerobic enterotoxemia in newborn calves is most often caused by Clostridium perfringens serotypes A, C, D. For the specific prevention of this form of pathology, an associated vaccine has been developed containing inactivated antigens of Clostridium perfringens serotypes A, C, D and Escherichia coli, which produce adhesion factors K99 and A20. Clinical trials of the effectiveness of the vaccine on cattle were carried out in 3 large agricultural enterprises of the Republic of Tatarstan for the production of milk, permanently dysfunctional for clostridiosis and escherichiosis of newborn calves. Disease prevention began with the immunization of pregnant cows and heifers 60 days before the expected calving. The animals were vaccinated twice with an interval of 14 days at a dose of 10 cm3. Calves obtained from immunized cows were vaccinated twice subcutaneously at a dose of 3 cm3 at the age of 18-20 days. A total of 3156 cows and heifers were vaccinated in three dairy complexes. It was found that the associated vaccine is harmless for deep-walled cows and heifers, does not cause post-vaccination complications. Immunization of animals in the last months of pregnancy contributes to the accumulation of specific antibodies in colostrum, the intake of which provides passive protection of the newborn calf from infection with Cl. perfringens and E. coli bacteria.

1 Introduction

Gastrointestinal diseases of newborn calves caused by the anaerobic bacteria *Clostridium perfringens*, as well as *Escherichia coli*, cause enormous economic damage to livestock farms. The occurrence of the disease is associated with violations of the conditions of keeping and feeding, uncontrolled movement of animals from one farm to another, as well

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as the importation of young livestock from Western Europe and America [1-4]. Epizootological monitoring of large dairy complexes showed that clostridiosis in the form of enzootics occurs in all seasons, but most often in spring. It affects calves at 4-6 days after birth or during the fattening period and is accompanied by the development of bloody diarrhea. The spread of the causative agent of the infection is facilitated by its intensive accumulation in the external environment, unsatisfactory sanitary condition, irregular disinfection. One of the most important moments in the occurrence of enterotoxemia is the contamination of colostrum and untimely feeding it to calves, which causes, on the one hand, an increase in bacterial contamination of colostrum, on the other hand, untimely provision of the calf with immune globulins contained in colostrum [5-9].

Anaerobic enterotoxemia in animals is caused by anaerobic bacteria *Cl. perfringens* of various types: A, B, C, D, E and F. The results of our research show that in the Volga Federal District of the Russian Federation, enterotoxemia is most often caused in newborn calves by *Cl. perfringens* types A, C, D, in older fattening calves by *Cl. perfringens* types B, C, D. The disease is accompanied by intensive reproduction of the pathogen in the intestine and the accumulation of toxin, followed by its absorption into the blood [10-14].

Clostridiosis in newborn calves often occurs in mixed form with Escherichiosis. This form of the disease is very difficult, it is difficult to diagnose it, it is difficult to treat. The main causative agents of Escherichiosis in newborn calves are enterotoxigenic isolates of *E. coli* bacteria that produce adhesion factors K99 and A20.

The main causative agents of Escherichiosis in newborn calves are enterotoxigenic isolates of *E. coli* that produce adhesion factors K99 and A20. In the Russian Federation for the specific prevention of Escherichiosis various monovalent, combined vaccines have been developed and are successfully used, and there are no such drugs for clostridiosis of newborn calves, what prompted us to develop an associated vaccine for the specific prevention of mixed infection caused by anaerobic bacteria *Cl. perfringens* and enteropathogenic strains of *E. coli* [15].

2 Materials and Methods

The research was carried out in the laboratory of bacterial pathologies of animals of the Kazan Federal center for toxicological, radiation and biological safety and in 3 agricultural enterprises of the Republic of Tatarstan for milk production, that are unfavorable for clostridiosis and escherichiosis in newborn calves.

During work on the development of an associated vaccine against clostridiosis and escherichiosis in calves, the following materials were used:

– nutrient media: nutrient agar, blood nutrient agar, beef-extract broth, meat-liver-casein medium, Hottinger broth, thioglycolic medium, Sabouraud agar, Kitt-Tarozzi, Endo, Minka media;

– industrial strains of microorganisms: *Cl. perfringens* No. 28 (type A), No. 392 (type C) and No. 213 (type D); *E. coli* "KV-1" and "PZ-3", producing adhesion factors K 99 and A 20, respectively;

– aluminum hydroxide gel 6%;

– enzyme immunoassay for the determination of specific antibodies to bacteria *Cl. perfringens*;

– diagnostic sera: antitoxic *Cl. perfringens* types A, C, D and "O"-coli agglutinating.

Equipment: Masterclave 60 Media Preparator, electric dry-air thermostat TS-1/80 SPU, Air sterilizer GPD-320, automatic vertical steam sterilizer SPVA-75-1-NN, microbiological safety cabinet BMB-II "Laminar-S" -1.2 (231-120), medium-temperature refrigeration cabinet SHH-0.80M, biological microscope Micromed-2 (2-20), electronic scales A&D

EK-600i and EK-6000i, table pH meter STARTER 3100, pharmaceutical refrigerator "POZIS" HF- 250-3, single and mono-channel pipette "Black".

Experimental animals:

– white mice weighing 14-16 g;
– rabbits weighing 2.5-3.0 kg;
– cattle of different sex and age groups.

The pathogenesis of anaerobic enterotoxemia is based on a toxic factor, and, therefore, the vaccine should develop antitoxic immunity in immunized animals [2, 5, 7]. For the production of toxoids, vaccine strains *Cl. perfringens* were grown on meat-liver-casein medium under vaseline oil in 20-liter ¾-filled glass bottles for 6-9 hours at a temperature of 37 -38 °C. At the same time, the concentration of *Cl. perfringens* bacteria in growth nutrient media reached over 4 billion / cm^3 of microbial cells. The resulting culture was tested for toxicity in 10 white mice, to which the samples were injected intraperitoneally at a dose of 0.5 cm^3. Animals died within 12 hours after administration of the culture suspension.

Inactivation of culture suspensions of *Cl. perfringens* was carried out in a thermostat for 10 days by adding 37-38% formalin at the rate of 7 cm^3 per 1 liter of suspension. Formalin was injected fractionally: completion of the incubation of bacteria, and the remaining 2 cm^3 on the 4th day of inactivation.

The biomass of *E. coli* was obtained in 1.5 liter matt flasks with nutrient agar (PZ-3 strain, producing A20 adhesion factor) and Mink's medium (KV-1 strain, producing K99 adhesion factor). Thermostable and thermolabile toxins of *E. coli* were obtained by growing industrial strains in Hottinger broth at 37-38 °C for 5-7 days. Inactivation of *E. coli* biomass and their toxins was carried out with formalin up to 0.5% of the final concentration.

An associated vaccine was made with the following ratio of components in 1 liter of vaccine:

– cell suspensions of *Cl. perfringens* bacterial strains No. 28 (type A), No. 392 (type C), No. 213 (type D) in a culture medium with a concentration of $3.5 \cdot 10^{12}$ - $4.0 \cdot 10^{12}$ m.c. in 1 cm^3 - 150 cm^3 each;
– cell suspensions of *E. coli* strains KB-1 and PZ-3, producing adhesion factors K99 and A20, respectively, in physiological solution with a concentration of $100 \cdot 10^{12}$ - $120 \cdot 10^{12}$ m.c. in 1 cm^3 - 30 cm^3 each;
– 6% aluminum hydroxide gel - 100 cm^3;
– formalin - 4.0;
– thermostable and thermolabile toxoids of *E. coli* KV-1 and PZ-3 strains in a ratio of 1:1 in a culture medium with DSA titers of 1:8-1:16 - up to 1 liter.

Each batch of the vaccine was tested for appearance, sterility, harmlessness, and antigenic activity.

To determine the appearance, color, presence of foreign matter, glass cracks, each vaccine vial was examined visually.

To test the vaccine for sterility, harmlessness, antigenic activity, a mixture of vaccine, combined under sterile conditions in a separate vial, from three vials of 15 cm^3 from each was used.

The sterility of the vaccine was tested by inoculating the vaccine on the following nutrient media: nutrient agar, Kitt-Tarozzi medium under vaseline oil, Sabouraud agar. The vaccine should not allow the growth of bacteria and fungi in any of the inoculated culture media.

To test the vaccine for harmlessness, a mixture of the vaccine from three vials was injected subcutaneously to 10 white mice at a dose of 0.5 cm^3 in the area of the shoulder blades, to 3 heads of cattle three immunizing doses in the middle third of the neck. The

vaccine is considered harmless if within 10 days after administration of the drug, white mice and cattle remain alive and healthy.

The immunogenic activity of the vaccine to *Cl. perfringens* was determined in rabbits and white mice in the reaction of neutralization. For this, blood from immunized rabbits was injected into white mice, and then they were infected intraperitoneally with a titrated lethal dose of *Cl. perfringens*. The immunogenic activity of the vaccine to. *E coli* was determined on white mice, which were first vaccinated and then infected with a lethal dose of E. coli.

A production test of the vaccine was carried out in dysfunctional farms, where the drug was administered to cows at a dose of 10 cm^3 twice 50-60 days before calving with an interval of 14 days. The effectiveness of the vaccine was judged by indicators of morbidity and safety of calves obtained from vaccinated and control unvaccinated cows.

The effectiveness of the associated vaccine was assessed by comparing the incidence and safety of calves in in permanently dysfunctional farms before and after the start of the use of the biological product.

3 Results and Discussion

3 series of the associated vaccine against Clostridiosis and Escherichizum of newborn calves were produced, laboratory tests of which showed that they are all sterile and harmless to animals. Study of the level of antitoxic antibodies in the neutralization reaction in white mice showed that the serum of vaccinated rabbits protects 80-90% of white mice after infection with titrated lethal doses of *Cl. perfringens* and *E. coli* bacteria. The titers of specific antibodies in twice vaccinated with the associated vaccine rabbits are presented in Table 1.

Table 1: Titers of specific antibodies in the blood serum of vaccinated rabbits (M ± m, n = 5)

Antigen name	Terms of the study	
	14 days after 1st vaccination	14 days after 2nd vaccination
Cl. perfringens type A	10,84±0,42	13,24±0,27
Cl. perfringens type C	11,24±0,22	13,84±0,22
Cl. perfringens type D	11,24±0,27	13,64±0,35
E. coli K 99	7,92±0,27	8,72±0,27
E. coli A20	8,52±0,22	8,92±0,27

The table shows that the associated vaccine has a sufficiently high antigenic activity, causes the formation of specific antibodies in blood serum in high titers.

A clinical trial of the effectiveness of the vaccine on cattle was carried out in 3 large agricultural enterprises of the Republic of Tatarstan for milk production, permanently dysfunctional with clostridiosis and escherichiosis of newborn calves. The study of the antigenic activity of the vaccine in cattle showed that the vaccine induces the formation of specific antibodies in animals after its double administration to antigens of *Cl. perfringens* bacteria in titers from 13.04 ± 0.27 to 13.84 ± 0.22 $\log_2$, to antigens of *E. coli* bacteria from 8.72 ± 0.27 to 9.12 ± 0.22 $\log_2$.

The protective properties of the blood serum of immunized cows were studied on 150 white mice weighing 14-16 g, divided into 3 groups of 50 animals each. Animals of the first group were injected with blood serum obtained from immunized cows, mice of the second group were immunized with blood serum obtained from control non-immunized cows, mice of the third group were not immunized; i.e. they were not injected with serum.

Immunization was carried out subcutaneously by injecting each white mouse with 0.5 cm^3 of freshly prepared serum taken in an equal volume from five cows. Mice were infected intraperitoneally 24 hours after immunization with titrated lethal doses of *E. coli* (K99 and A20) and *Cl. perfringens* (types A, C, and D). The results of these studies are presented in Table 2.

Table 2: Results of studying the protective properties of blood sera of immunized cows in white mice in the neutralization reaction in relation to *Cl. perfringens* and *E. coli*

Group of mice	Count, heads	Serum dose, cm^3	Infected with bacteria	Control results			
				Died		Survived	
				count	%	count	%
Experimental Introduced serum from immunized cows	10 10 10 10 10	0,5 0,5 0,5 0,5 0,5	*Cl. perfringens*, type A *Cl. perfringens*, type C *Cl. perfringens*, type D *E. coli* K99 *E. coli* A20	0 2 1 0 0	0 20 10 0 0	10 8 9 10 10	100 80 90 100 100
Control 1. Introduced serum from non- immunized cows	10 10 10 10 10	0,5 0,5 0,5 0,5 0,5	*Cl. perfringens*, type A *Cl. perfringens*, type C *Cl. perfringens*, type D *E. coli* K99 *E. coli* A20	8 10 9 8 10	80 100 90 80 90	2 0 1 2 1	20 0 10 20 10
Control 2. Serum not injected	10 10 10 10 10	- - - - -	*Cl. perfringens*, type A *Cl.* *perfringens*, type C *Cl. perfringens*, type D *E. coli* K99 *E. coli* A20	10 10 10 10 10	100 100 100 100 100	0 0 0 0 0	0 0 0 0 0

Table 3: Indicators of the effectiveness of the associated vaccine in cattle

Farm	Calves received	Got sick		Died		Preservation, %
		count	%	count	%	
before using the associated vaccine						
Dairy complex No. 1	1041	887	85,2	197	18,9	81,1
Dairy complex No. 2	1986	1901	95,7	473	23,8	76,2
Dairy complex No. 3	324	262	80,8	74	22,9	77,1
Total	3351	3050	91,0	744	22,2	77,8
after using the associated vaccine						
Dairy complex No. 1	1221	212	17,4	47	3,8	96,1
Dairy complex	1608	352	21,9	114	7,1	92,9

No. 2						
Dairy complex No. 3	268	48	17,9	13	4,8	95,2
Total	3097	612	19,7	174	5,6	94,4

From the presented table 3 it can be seen that the associated vaccine has a high prophylactic efficacy. The use of the vaccine made it possible to reduce the incidence of calves in permanently dysfunctional farms by 4.6 times, from 91.0% to 19.7%, and to increase safety by 16.6%, that is, from 77.8% to 94.4%.

Based on the results of research on the associated vaccine, regulatory documents that regulate its manufacture, control and use have been developed; a Russian patent for an invention has been obtained for the biological product. The results of studying the effectiveness of the use of the associated vaccine against clostridiosis and escherichiosis in calves in permanently dysfunctional farms are presented in Table 3.

4 Conclusion

It is known that in order to create colostral immunity in newborn calves, it is necessary to vaccinate pregnant cows and heifers 50-60 days before calving against major viral and bacterial infections. In this case, the calves will receive ready-made antibodies with colostrum. But vaccination should always be considered as a forced event, it should be carried out only in cases where there are epizootic indicators for this.

The development of immunity to perfringens toxins in sheep is well studied. However, in cattle it is not well understood, in this regard, difficulties arise in the prevention of anaerobic enterotoxemia in this species of animals. It is known that animals that have had anaerobic enterotoxemia acquire immunity, the intensity and duration of which depend on the strength and severity of the disease. We have made an experimental series of an associated vaccine against clostridiosis and escherichiosis in newborn calves. One of the main parameters for assessing the effectiveness of agents for the specific prevention of infectious diseases is their protective activity.

Therefore, the main stage of our research was the assessment of the effectiveness of the associated vaccine in a production environment on cattle. The research results have shown that the associated vaccine is harmless for deep-pregnant cows, the specific antibodies formed in their colostrum provide passive protection of newborn calves from infection with Cl. perfringens and E. coli bacteria. When it is used in permanently dysfunctional farms, the incidence of newborn calves decreases 4.6 times, their safety increases by 16.6%.

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