
Purification of Staphylococcal Enterotoxin Type D and Obtaining Its Antiserum

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Abstract: Uncontrolled use of antibiotics in the treatment of calves suffering from gastroenteritis and dairy cows suffering from mastitis, created in pathogenic staphylococci enterotoxin producers some resistance to these drugs. These pathogens enterotoxin producers are often excreted with milk from cows with mastitis. That is why the specific and topical issue remains the isolation and identification of strains producing staphylococcal enterotoxin from cow milk and faeces of calves suffering from enteritis. Given all this, we have set a goal to get staphylococcal enterotoxin type D for use as mastitis diagnostic kit and enteritis and antigen for obtaining the antiserum. To obtain the staphylococcal enterotoxin type D we used *strain S. aureus No. 494 (ATSS23235)* courtesy offered by Dr. FC Fluer from Institute of Experimental Medicine, NF Gamaley, Medical Academy (USSR). Verification of the specificity of the enterotoxin was performed using 5 *strains of S.aureus* producing different enterotoxins (enterotoxin - A; enterotoxin-B; enterotoxin C₁; C₂ and enterotoxin - E) as well as non-enterotoxigenic staphylococcal strain (*S. aureus* Wood 46). In addition to the staphylococcal strains, five *strains of Escherichia coli*, producing thermostable and thermolabile enterotoxins were also used in the experiment. The method of purification of enterotoxin-D by precipitation twice chromatography on *km - cellulose* and gel filtration on *Sephadex G-75* allows to obtain pure enterotoxin-D having the biological properties, immunological and antigenic characteristics. Immunization of rabbits with purified enterotoxin-D associated with incomplete Freund's adjuvant provides antiserum very active and specific.

Keywords: *Staphylococcus aureus*, Milk, *faeces*, Enterotoxin, Antiserum, Enteritis

1. Introduction

Some researchers VA Alikhaev *et al.*; PB Barto *et al.*; MA Volovenko and BV Venskiyavitchen [1; 32; 40; 41] reported that a high percentage of recorded gastro enteritis in calves is mainly due to the ingested milk from cows suffering from mastitis. In the aetiology of gastro-enteritis of calves, it is important to also consider non-compliance with hygienic rearing conditions of these young animals. Many authors believe that young animals become contaminated with microorganisms such as enterotoxigenic *Staphylococcus aureus* and *Escherichia coli*, through the ingestion of colostrum or milk from cows suffering from mastitis [3]. The role of enterotoxigenic staphylococci in the aetiology of gastro-enteritis in calves remains to be elucidated. There are in the literature data on the purification methods of staphylococcal enterotoxin type D and its identification in the

milk or colostrum and other food. However, these authors have used more complex methods for purification and for obtaining the antiserum. Also, the methods used by these authors are difficult to apply in laboratories in developing countries [4]. Milk from cows with mastitis represents a great danger to human health, particularly for infants and the elderly [18; 22].

Until the 20th century, most of authors [5; 12; 19; 20; 33; 34], used kittens for biological test to identify the staphylococcal enterotoxin, but rarely mice and pups, as they are insensitive. By cons, in the United States of America, the monkey genus *rhesus Macacca* was used for this test. Also note that the identification of staphylococcal enterotoxin by the biological test is laborious and time consuming and the results are still not satisfactory. Also, for the biological testing a significant number of animals require; In addition, this method only allows detecting the presence of enterotoxin, but does not give rise to know the serotype of the offending enterotoxin

inducing Intoxication. The aim of this work is to develop a simple method of purification of staphylococcal enterotoxin type D for use as an antigen in immunization of rabbits in order to obtain the antiserum for diagnosis of enteritis due to staphylococci enterotoxin-D producing.

2. Materials and Methods

To obtain the staphylococcal enterotoxin type D we used strain *S. aureus* No. 494 (ATSS23235) courtesy offered by Dr. FC Fluer from Institute of Experimental Medicine, NF Gamaley, Medical Academy (USSR). To verify the specificity of the enterotoxin we used 5 strains of *S. aureus* enterotoxins producing such as: FRI 722- enterotoxin- A; FRI-243-enterotoxin- B; FRI-137-enterotoxin C₁; FRI-493-enterotoxin C₂ and FRI-326 enterotoxin-E), and non-enterotoxigenic staphylococcal strain (*S. aureus* Wood 46). In addition to the staphylococcal strains, five strains of *Escherichia coli* (*E. coli* P16, *E. coli* 1012, and *E. coli* 015 - H10407, thermostable enterotoxin-producing; *E. coli*-P99, producing heat-labile enterotoxin, have also been used in the experiment. The morphological, dyeing, biological and enzymatic properties of 7 strains of *S. aureus* indicated above as well as strains isolated from faeces of calves with diarrhea and cow milk-producing staphylococcal enterotoxin-D were studied by the method of KI Mateveev; BA Nourmatov *et al.* and AK Akatov *et al.* [24; 23; 25]. In the experiment, 10 rabbits, genus *chinchilla* weighing 2.5-3 grams each; 180 kittens 1-3 months of age and weighing 350-700 g each, were used. Material containing the enterotoxin is obtained according to the method of CE Donnelly *et al.* [31] by cultivating the *S. aureus* strain No. 494 in the plastic bag under continuous stirring at a rate of 280-300 rounds / min for 24-48 hours and the ventilation method, which involves passing air through medium through the two filters using a micro compressor mark VK-1 (220V, 50Hz, 10watt). To obtain the maximum concentration of staphylococcal enterotoxin type D, 5 different culture media were tested. These are: Medium n° 1. [26], modified by VI Bougrova *et al.* [6]; Medium n°2. [29]; Medium n°3. [30], Medium n°4 [42] and Medium n°5 [46]. All of these media are prepared on the basis of casein hydrolyzat and supplemented amino acids and vitamins in different concentrations. The activity of the enterotoxin is set to kittens by the method of [33]. Purification of enterotoxin is performed according to the method of K. Bidjeh, [4], which comprises of passing (2 times) the solution containing enterotoxin on km- cellulose and on sephadex G-75 gel. The homogeneity of the enterotoxin has been defined by the disc electrophoresis method as in acid system [39] that in the basic system [28] in a gel (7.5 %) of polyacrylamyl using the reagents and the electrophoresis apparatus (*Reanal*, Hungary). Some physicochemical properties of enterotoxin-D were studied as follows: heating at 80, 90 and 100°C. for 15, 30 and 60 minutes, and by studying the action of certain enzymes such as trypsin, pepsin, renin and hemotrypsin (all at a concentration of 0.3%).

For antisera, 8 rabbits were immunized according to the

method of EP Casman *et al.* [46]. Purified enterotoxin mixed with Freund's incomplete adjuvant in equal parts, injected into rabbits intramuscularly at increasing dosis (mg protein / ml) - 0.10; 0.10; 0.15; 0.2; 0.3; 0.4; 0.6; 0.8; 0.9; 1.2; 1.6; 1.6; 1.6 at 6- 7 days interval. In the control group, two non-immunized rabbits were used. The action and specificity of the antiserum have been defined by the agar immunodiffusion reaction (AGID) in petri dishes by the method of Ouchterlony O. [38] and in tubes. For the reaction of immunoelectrophoresis (IEP) is the method of P. Graber *et al.* [48] was used. 10 days after the 13th injection, rabbits were bled. After clotting, the clot is removed and the serum is obtained by centrifuging the contents of the tubes at 3000 rpm / min for 30 minutes and stored at -20 ° C. The resulting serum of each animal is considered as a serial. A total of 4 series of serum was obtained. The sera are then distributed in tubes and kept at -20°C for further use.

3. Results

Purification of enterotoxin - D and its physicochemical properties

Purification of enterotoxin-D is carried out as follows: after passing on km-cellulose (twice), washing is done on the gel *Sephadex* G-75 with distilled water until obtaining the pH neutral (7.0). After lyophilization, entering the second step on km-cellulose. After that, it was noted that only peak 3 had biological activity and the bioassay was positive on kittens. By repeating chromatography on km-cellulose, it has been found that a single peak eluting had a positive biological effect on kittens. The results of the disc -electrophoresis of eluent on polyacrylamil gel obtained after the 3rd purification step, showed that enterotoxin was not homogeneous because on the gel appeared two precipitation lines, indicating the presence of the second element in the solution (eluent), which is nothing other than α - hemolysin, which has been shown by the action hemolytic eluent on erythrocytes of rabbits. After 4th purification step on *Sephadex*, only the second peak of eluting possessed a biological action on kittens. The homogeneity of the enterotoxin is demonstrated by the appearance of a single precipitation line on the gel and the absence of hemolytic activity on erythrocytes of rabbits. It has been shown that both the purified enterotoxin and unpurified were resistant to the action of proteolytic effect of enzymes (0.3%) such as pepsin, trypsin, hemotrypsin and renin. Enterotoxin both purified and non-purified is destroyed at the temperature ranged between 85 and 100°C for 25 minutes as they lose their antigenic properties.

Obtaining antiserum enterotoxin- D and its specific action by immunodiffusion tests on agar (AGID), precipitation in tubes (IPT) and the immunoelectrophoresis test (IEP)

The activity of each serum is checked by the AGID and IPT tests. Both by the AGID as other immunochemical tests, the antiserum revealed the homogeneity of enterotoxin-D to the minimum concentration of protein of 10, 62 mg/ml, which is demonstrated by the appearance of one line of precipitation between the wells containing the enterotoxin-D and those

containing antiserum at different dilutions: from 1/2 to 1/32.

The specificity of the antiserum obtained was checked by various tests: Agar gel immuno diffusion (AGID), immuno-precipitation in tubes (IPT) and immunoelectrophoresis (IEP) vis-à-vis the heterogeneous enterotoxins (Table 1.). From this table, we can see that antiserum anti-enterotoxin- D has been very specific because it does not react with the filtrates of enterotoxigenic staphylococci producing enterotoxin types: A, B, C₁, C₂ and E and *S. aureus* strain producing α and β - hemolysin. As it can be seen from the said table 1, antiserum anti-enterotoxin-D does not react with any of these above mentioned antigens. Similar results were found with the supernatant of *E. Coli* N°1012, E; coli P16 and *E. coli* H10407 producers of thermostable enterotoxin (TS); *E. Coli* N°015-339, heat-labile enterotoxin Producer (TL). We recorded the same results with A-enterotoxin of *Cl. Perfringens*.

Table 1. Specificity of the antiserum anti-enterotoxin-D in AGID, PT and IEP tests.

Antigens	Protein concentration mg / ml	Results		
		IDG	IPT	IEP
S. enterotoxin-A	6000	-	-	-
S. enterotoxin-B	6000	-	-	-
S. enterotoxin C ₁	5000	-	-	-
Enterotoxin C ₂ S.	5000	-	-	-
S. -E enterotoxin	4000	-	-	-
α and β -S. hemolysins	6000	-	-	-
TS <i>E.coli</i> 1012	3900	-	-	-
TS P 16 <i>E. coli</i>	3900	-	-	-
TL <i>E. Coli</i> P99	3900	-	-	-
TS, TL <i>E. coli</i> H10407	3900	-	-	-
<i>E.coli</i> 015-339	3900	-	-	-
<i>CL. perfringens</i> -A	170	-	-	-
S. enterotoxin-D	4000	+	+	+

NB.: For staphylococcal enterotoxin types A, B, C₁, C₂, D and E, the supernatant that was used in the experiment. As for the enterotoxin-A of *Cl. Perfringens* is the purified product, which was used.

Demonstration of enterotoxin -D in the faeces of calves suffering from enteritis and in the milk or colostrum of cows suffering from mastitis.

In the study no positive reaction was found with faeces from healthy calves. By cons, among the 30 faecal samples from calves, 8 (26, 66%) reacted positively with antiserum anti-enterotoxin -D by AGID test; 2 (6.66%) yielded questionable results by the same test. The presence of enterotoxin - D has been demonstrated in 6 (75%) faecal samples from calves aged from 5 to 8 days. In 2 (25%) of cases, the results were also positive with faeces from calves aged 1 to 7 days. 2 (25%) calves, whose samples were positive with antiserum anti-enterotoxin-D, were aged between 4 and 7 days respectively. The age groups corresponding to the period when calves are often suffering from enteritis were divided as follows: 1, 3, 5 and 6 days. The study of infected pasteurilised milk with staphylococcal colonies producing enterotoxin- D at a concentration of 10³ and 10⁵/ ml did not reveal the presence of enterotoxin -D in any case regardless of the incubation time at 37 ° C for 3, 6, 18 and 24 hours. By cons, in the samples of

pasteurellised milk infected with *S. aureus* at a concentration of 10⁶ - 10⁹ and incubated at 37°C for 18-24 h, there was secretion of enterotoxin-D, Which allows deducing that the speed of secretion of enterotoxin-D depends on the quantity of microorganisms in the medium and the incubation time. The study of 95 samples of milk with 5% dimastin solution showed that the reaction was positive in only 36 (37.8%) cases. In contrast, with the immunochemical test (AGID), no sample was positive vis-a-vis the antiserum anti-enterotoxin-D. However, of 17 samples of colostrum tested, only 2 (11.76%) reacted positively with antiserum anti-enterotoxin-D by AGID test. This is explained by the fact that among the cow milk, the concentration of enterotoxin is so low that we cannot detect with AGID test, while in cows that have given birth, the possibility of revealing the presence of enterotoxin-D is the first milking.

4. Discussion

In general, increased animal production requires industrialization in this area and the creation of large livestock complexes. However, infectious diseases such as enteritis due to staphylococci significantly reduce growth in the number of cattle in dairy farms. The information collected in different scientific journals shows the possible role of enterotoxigenic staphylococci in enteritis of calves and mastitis in dairy cows excreting enterotoxigenic staphylococci with milk and constituting potential sources of diseases such as food poisoning, gastroenteritis, etc. [1; 2; 3; 4; 9; 10; 11; 13; 14; 16; 17; 27; 35; 39; 40; 41; 45; 50]. However, the role of staphylococci producing enterotoxin- D in the aetiology of these diseases is poorly understood and the simplified method of identifying the enterotoxin in faeces of calves suffering from enteritis has not been in focus. It is the same for the milk of cow carriers of pathogenic staphylococci. EH Kato *et al.*[35]; MS Berdoll [43]; KN Shinagawa Kunita [49]; EP Casman *et al.*[46] described the methods of purification and identification of the staphylococcal enterotoxin type D in milk and colostrum from cows, but the authors used for these purposes complex methods difficult to implement in the conditions of laboratories in developing countries. These shortcomings noted have led us to use the four-step purification of staphylococcal enterotoxin-D.

Today, the most effective diagnosis of enterotoxicoes caused by staphylococci can be done with the immunochemical test that gives accurate and satisfactory results. For use, it is necessary to have the purified enterotoxin and the antiserum [4; 21; 47; 15; 44]. However, in the veterinary field, the development of such reagents requires the development of special technology for extraction, purification of enterotoxin and obtaining the homologous antiserum.

By immunizing rabbits with enterotoxin-D, it was noted that it is only at the 9th injection that was able to detect antibodies directed against enterotoxin-D (1/2 of dilution). Then, antibody titers increased progressively up to 1/8 according to increase in the dose of the antigen. Also note that in the immunization process, 4 rabbits (50%) died,

demonstrating the toxicity of enterotoxin-D. The use of this immunization scheme of rabbits demonstrates once again that enterotoxin-D, like other staphylococcal enterotoxins have low antigenic property.

Many problems associated with the effect of colostrum from cows suffering from mastitis and carrier enterotoxigenic staphylococci. These problems have not previously found solutions, while the spread of enterotoxigenic calves are recorded every day and this number increases rapidly [40; 3]. The antiserum obtained is one of the possible solutions to these problems. As it will detect the presence of enterotoxin-D in the milk / colostrum from cows suffering from mastitis and in the faeces of calves suffering from gastroenteritis.

5. Conclusion

The method of purification of enterotoxin-D by precipitation twice chromatography on *km-cellulose* and gel filtration on *Sephadex G-75* allows to obtain pure enterotoxin-D having the biological properties, immunological and antigenic characteristics of staphylococcal enterotoxin.

Immunization of rabbits with purified enterotoxin-D associated with incomplete Freund's adjuvant provides antiserum very active and specific. Obtaining purified enterotoxin and the antiserum thus offering use the immunochemical method for the detection of enterotoxin-D in the supernatant of faeces of sick calves and dairy cows carrier pathogenic staphylococci.

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