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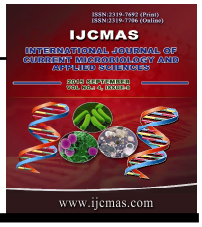

Original Research Article

Isolation and Identification of *Staphylococcal* Strains Producing Enterotoxin in Cow Milk and Feaces of Calves Suffering from Enteritis

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A B S T R A C T

Staphylococcus aureus are one of the main causes of mastitis (subclinical form) in dairy cows, excreting with milk enterotoxin, which are the main sources of food poisoning in humans (especially young people and the elderly) and young animals. The role of pathogenic staphylococci in the aetiology of calf enteritis and mastitis in cattle has been described by several authors. However, the role of staphylococcal producing enterotoxin in the aetiology of these diseases is poorly understood. The present study aims at investigating the presence of staphylococci, identifying their species and determining whether they are producers of enterotoxin. The study was conducted in the Department of Microbiology of the Veterinary Academy in Moscow, K.I. Skriyabine, and the Kolkhoz, Dzerzhinsky. In this study, 48 strains of staphylococci have been isolated from milk and 47 from feces, which at 80% belong to the genus *S. aureus*, 7.36% – *S. saprophyticus*, 5.26% – *S. intermedius*, 4.21% – *S. hyicus*, 2.10% – *S. sciuri* and 1.05% – *S. caprae*. The morphological studies, dye, biochemical and biological properties of the 95 isolated strains of *Staphylococci* from milk and feces showed that 31 (32.63%) belong to the pathogenic strains of staphylococcal groups. Of the 31 strains isolated pathogenic staphylococci, 14 (45.16%) produce enterotoxin-D, 43.75% of which are derived from the feces of sick calves and 46.66% isolated from cow milk.

Introduction

Chad is a vast Sahelian pastoral country; He largely based its economy on the valuation of livestock products. Current estimates of the workforce are livestock between 10 and 16 million UBT (Tropical Livestock Unit) allocated as follows: 7 million cattle, about 3 million camels and 8 million sheep / goats. This number is managed by 80% pastoral systems characterized by extensive production whose mobility is a production strategy and risk management (PNDE, 2009). In general and in Chad particular, the increase in animal production requires the future industrialization of this area and the creation of large complex. However, infectious diseases such as enteritis due to staphylococci significantly reduce the increase in the number of cattle on dairy farms in the world in general and Chad especially. According to the National Livestock Development Programme (PNDE,

2009), Chad, trafficking, processing and marketing of milk is mainly owned by women activity. In Chad, it is especially the milk of cows and camels, which is consumed and / or transformed in the form of fermented whole milk, skim milk and fermented liquid butter. These activities are carried out in unsanitary conditions and products are thus exposed to microbial contamination and providing a source of food poisoning due to bacterial infections, dangerously threatening human and animal health.

The bibliographic data (Tingting Zhang *et al.,* 2014; Vernula *et al.,* 2012; Mekonnen *et al.,* 2011; Elmoslemany *et al.,* 2009; Loir *et al.,* 2003; Debuyser *et al.,* 2001; Bidjeh 1984; Bozena, 1980; Rea *et al.,* 1980; Kato *et al.,* 1980, etc.) indicate the possible role of Staphylococcus in carriers of these pathogens cows, which excrete them with milk thus constituting potential sources of diseases (food poisoning, gastroenteritis, etc.). According to the same sources, the role of pathogenic staphylococci in the aetiology of calf enteritis and mastitis in cows is indisputable. However, the role of staphylococci producing enterotoxin in the aetiology of these diseases is poorly understood. The objective of this study is to investigate the presence of staphylococci, identify their species and determine whether they are producers of enterotoxin.

Materials and Methods

This study is a fragment of the complex theme of the Department of Microbiology of Veterinary Academy of Moscow, step 4.03.01 of diagnosis of gastroenteritis caused by exotoxins of *Escherichia* and Staphylococci, as well as the increase of method of the body resistance of calves by humoral factors vis-a-vis colostrum these microorganisms. The study was conducted in the Department of Microbiology of the Veterinary Academy in Moscow, K.I. Skriyabine, and the Kolkhoz, Dzerzhinsky (Moscow, USSR).

The *strain of S. aureus N°.* 494 (ATSS23235) and the anti-staphylococcal enterotoxin *type D* serum donated by Dr. FC Fluer from Institute of Experimental Medicine, N.F. *Gamaley,* Medical Academy (Moscow, USSR) was used for the production of D-type enterotoxin (as standard sample) and the identification of enterotoxin-D in pathological materials. In the experiment, 10 rabbits chinchilla kind each weighing 2.5–3 grams; 180 kittens 1–3 months of age and weighing 350–600 g each; 150 white mice (8–12 g) were also used; feces were collected from 30 sick calves (enteritis) and 10 clinically healthy calves at Dzerzhinsky Kolkhoz.

95 milk samples were collected from 24 cows positively reacted to mastitis test and 10 cows negatively reacted to the same test in the same Kolkhoz. The material containing D-enterotoxin obtained by cultivating the S. aureus strain in the dialysis plastic bag suspended in bottles stirring continuously at a rate of 280–300 rev / min for 24-18 hours, according to the method of Donnelly *et al.* (1967); 24–48 hours with the aeration method: passing air into the environment through two sterile filters using a micro compressor VK-1 brand, (220V, 50Hz, 10 wat). Five different culture media were tested for obtaining the staphylococcal D - enterotoxin at a maximum concentration. They are:

Medium 1 (Casman, 1958): Modified by Bougrova *et al.* (1974); This medium contains casein hydrolyzate (200 mg% a.az.), Plus (g / l) of iron sulfate 0.025 - 1; KH_2 PO_4, K_2 HPO_4, L-magnesium sulfate-0.2; 7-sodium chlorate; L-tryptophan -

0.075; L-cystein - 0.025; calcium pantotenat -0.0005; Thiamin hydrochloride - 0.0004; 0.0005 of niacin and distilled water - 1000ml. The pH is adjusted to 6.8.

Medium 2 (Jarvis *et al.,* 1970): This medium is prepared from: casein hydrolyzat (200mg am.az. %), yeast extract - 1% but this is supplemented by the following (mg / l): Thiamine-hydrochloride - 0.5; Niacin - 0.5; distilled water - 1000 ml. The pH is adjusted to 6.8.

Medium 3 (Noleto *et al.,* 1980): contains: Acid Casein - 30.0g; yeast extract-30.0g; distilled water - 1000ml; niacin- 10mg; thiamin hydrochloride- 0.5 mg. The pH is adjusted to 6.8.

Medium 4 (Favorite *et al.,* 1941): Containing – casein hydrolyzat - 30.0g; Acid Casein - 30.0g; distilled water-1000ml; Niacin - 10mg; thiamin hydrochloride - 0.5 mg. Adjust the pH to 6.8.

Medium No. 5 (Casman *et al.,* 1967) containing (g / l) Casein hydrolyzat 40.0; yeast extract - 10.0; K_2 HPO_4-1000ml of distilled water; thiamin hydrochloride-0.4 mg; calcium pantotenat - 0.5mg; Niacin - 1, 2mg. The pH is adjusted to 6.8. All ingredients were added aseptically into the medium through the filter Zeitz.

Prior the addition of ingredients, all media were sterilized by autoclaving at 120°C. For 30 minutes. The microbial mass obtained by these various methods was centrifuged at 6000 rev / min at 4°C for 30 minutes. Extraction of enterotoxin from the supernatant is made by adding 60% concentration of the solution of ammonium sulphate. Again centrifuged under the same regime as the previous step. The precipitate was diluted in distilled water (1/10 of the initial volume) and then dialyzed in running water and then in distilled water at + 4°C.

The sublimation of the substrate containing enterotoxin-D is made using a lyophilizer TP-15 brand made in German Democratic Republic (DRC). The protein concentration in the substrate is defined by the method of Lowry *et al.* (1951) using a CF-16 spectrophotometer (*Lomo)* at the wave length of 280 nm.

The effect of enterotoxin obtained is set to kittens by the method of Dolman *et al.* (1940). Statistical analysis of data was done according to methods of Lakin (1980).

Results and Discussion

The bacteriological study of 30 samples from faeces of calves suffering from enteritis showed that in 24 cases (80%), 47 staphylococcal strains have been isolated. The culture of isolated colonies of feces from clinically healthy calves (control group) on blood agar did not reveal the presence of staphylococci *(S. aureus).* By studying 95 milk samples from 24 cows, 48 staphylococcal strains were isolated from 47 (49.47%) samples. A total of 95 staphylococcal strains were isolated from faeces of calves and cow milk.

Isolation and identification of staphylococcal strains

The taxonomic identification of strains isolated strains showed that 76 (80%) belong to the genus *S.aureus;* 7 (7.36%) belong to the genus *S. saprophyticus;* 5(5.26%) - *S. intermedius, 4* (4.21%) - *S. Hyicus;* 2 (2.10%) - *S. sciuri* and 1 (1.05%) - belongs to the genus S. *caprae.* The study of the biochemical properties of staphylococcal strains *S.* 7 *aureus* (FRI-722, 243-FRI, FRI-137, Fri- 326, 493, 494 Wood and 46) selected in the library strain of microbiology Department of the Veterinary Academy in Moscow revealed that these strains produce the following enzymes: coagulase, DNA-

nase, phosphatase, lecitenase, hemolysin, lysozyme, plasmin and have a gold pigmentation.

They also, fermented mannitol in anaerobic conditions and have proved sensitive to novobiocin. They oxidized lactose, dextrose, sucrose, trehalose and galactose. From 8 isolated wild strains (chosen randomly), 6 (75%) had typical features of *S.aureus* and they have responded positively to the 3 tests characterizing pathogenic staphylococci (coagulase +, hemolysin, DNA-ase +).

Statistical analysis of these data showed the following bit combinations: (1) haemolytic activity with mannitol fermentation in anaerobic conditions; (2) anaerobic fermentation of mannitol with plasma coagulation; (3) production of DNase with plasma coagulation; (4) DNA-ase with mannitol fermentation; (5) hemolysin with DNA-ase; (6) with coagulase hemolysin. Between these parameters, it has been demonstrated that there is a measurable direct bond.

Obtaining enterotoxin

The comparative study of methods and culture media to obtain a maximum concentration of D-enterotoxin showed that the most suitable media are media 4 and 5. In media 1 and 3, yielded less enterotoxin concentration. The medium No. 2 has been found less suitable for the production of D-enterotoxin. The results of the comparative study of methods and culture media for production of enterotoxin are compiled in table 1, which we notice that these are the methods of cultivation by aeration and in plastic dialysis bags that are best for production of maximum staphylococcal enterotoxin type D. The analysis of the results of this study is statistically significant (P> 0.001).

Demonstration of enterotoxin presence in feces and in cow milk

In the study no positive reaction was found with feces from clinically healthy calves. Among 30 fecal samples from calves sick, 8 (26, 66%) reacted positively with antiserum enterotoxin by the immunodiffusion test agar (AGID); 2 (6.66%) yielded doubtful results by the same test. Thus the enterotoxin has been identified in 6 (75%) samples from sick calves aged from 5 to 8 days. In 12.25% of the cases, the results were also positive with the feces from the sick calves aged 1 to 7 days; 2 (25%) calves which the samples were positive with the anti-enterotoxin antiserum were aged between 4 and 7 days. 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 pasteurised milk with staphylococcal colonies producing enterotoxin-D at a concentration of 10^3 and 10^5 m / mL has revealed the presence of enterotoxin in any way whatever the weather incubation at 37°C for (3, 6, 18 and 24 hours). By cons, in the infected milk samples of *S. aureus* at a concentration of $10^6 – 10^9$ and incubated at 37°C for 18–24 h, there was secretion of enterotoxin. This means that the enterotoxin secretion rate depends on the quantity of microorganisms in the medium and incubation time for culture. The study of 95 samples of milk with mastitis test (5% dimastin solution) showed a positive reaction only in 36 cases. In contrast, with the immunochemical test (AGID), no sample was positive vis-a-vis the antiserum enterotoxin. However, among 17 samples of colostrum, 2 (11.76%) reacted positively with antiserum enterotoxin by AGID.

Table.1 Comparative study of methods and culture media for maximum production of *Staphylococcal* enterotoxin type D

Culture media	Method of culture					
	Agitation		***aeration***		***Plastic bags***	
	Incubation (hours)	Reaction Pets n = 4	Incubation (hours)	Reaction Pets N = 4	Incubation (hours)	Reaction Pets N = 4
1	24	1/4	24	3/4	24	3/4
	48	2/4	48	3/4	48	3/4
2	24	0/4	24	1/4	24	1/4
	48	1/4	48	2/4	48	2/4
3	24	1/4	24	3/4	24	3/4
	48	2/4	48	3/4	48	3/4
4	24	2/4	24	4/4	24	4/4
	48	3/4	*48*	4/4	48	4/4
5	24	1/4	24	4/4	24	4/4
	48	3/4	48	4/4	48	4/4

NB. - The culture media are defined in the "Materials and Methods"
- Incubation time in hours
- (N = 4) = Number of animals (kittens) used in each group of experience for the test of action of enterotoxin

This is explained by the fact that among dairy cows, the concentration of enterotoxin is so low that we cannot detect with IDG, while in cows that have given birth, the possibility of revealing the presence of enterotoxin is at the first milking.

Identification of strains of enterotoxigenic *staphylococci* in feces of calves suffering from enteritis and in cow milk

The morphological studies, dye, biochemical and biological properties of the 95 isolated strains of *staphylococci* from milk and feces (Hayek Marszalek) showed that 31 (32.63%) of the pathogenic strains belong to *staphylococci* groups, because they had been positive reaction characteristic of *S. aureus.* It should also be noted that of the 31 isolated staphylococci strains 14 (45.16%) produce D-enterotoxin of which 43.75% are derived from the feces of sick calves and 46.66% isolated from cow milk.

In conclusion, from pathological material, 48 staphylococcal strains have been isolated from milk and 47 from feces, which at 80% belong to the genus *S. aureus;* 7.36% - *S. saprophyticus;* 5.26% - *S. intermedius;* 4.21% *S. hyicus; 2.10%.S. sciuri* and 1.05% - *S. caprae.*

The morphological studies, dye, biochemical and biological properties of the 95 isolated strains of *Staphylococci* from milk and feces showed that 31 (32.63%) of the pathogenic strains belong to *Staphylococci* groups. Of the 31 strains isolated pathogenic *Staphylococci*, 14 (45.16%) produce D-enterotoxin, 43.75% of which are derived from the feces of sick calves and 46.66% isolated from cow milk.

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