

Development of Antibacterial complex for Sanitation of Boar and Bull Semen

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Abstract. It is recognized that in case of full compliance with sanitary requirements, one can get from the bulls up to 72-74% of ejaculates, containing not more than 2,000 microbial cells per 1 ml, and from boars - 67-69% of ejaculates with microbial cells in amount of maximum 5,000 per 1 ml of semen. Most frequently ejaculates contain pathogens, opportunistic cells (blue pus bacillus), streptococci, micrococcaceae, saprophytes, olm, and fungi. The sensitivity of the semen microflora was tested to 24 antibiotics; among them 15 antibiotics were selected to be tested in terms of harmlessness to semen. Based on the research conducted we have developed a new antibacterial complex for sanitation of breeder semen that can be used for decontamination of ejaculates microflora providing at the same time increased holding period of spermatozoa, as well as birth of healthy offspring. A new complex is composed of the following ingredients (per 100 ml of the extender): cefotaxime - 10 mg, gentamicin - 8 mg, and oleandomycin phosphate - 60 mg. Cefotaxime and gentamicin have a different spectrum of antimicrobial action. An important property of both drugs is their chemical and antimicrobial compatibility. Application of the developed antibacterial complex reduces microbial contamination of semen by 6.4-8.3 times that indicates synergistic action of the ingredients against the semen microflora. Sanitation of breeder semen by means of novel antibacterial complex contributes to better seminal sowing. Breeding efficiency of pedigree stock, inseminated with semen, treated by new antibacterial complex, was 11.7-12.9% higher than that in control.

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1. Introduction

The presence of a variety of microorganisms in semen leads in reduction of survival rate and fertility of cells, and, as a consequence, results in non-viable offspring. Therefore the suppression of undesirable contaminant microbial activity in breeder semen is a mandatory condition for artificial insemination of the pedigree stock.

The microbial contamination of breeder semen has been studied by many researchers. Thus, the factors and the level of bacterial content in ejaculates are considered in works of V.K. Milovanov [1], J.O. Almquist et. al. [2, 3], V.M. Prokoptsev [4], and many other researchers. In these studies authors determined the total amount of bacteria and their species composition, as well as suggested a number of decontaminating substances.

Due to the large amount of antibiotics used in livestock industries, the question arose about antibiotic resistance of bacteria. According to P. Nooriander [5], in the Netherlands, several strains of penicillin resistant staphylococcus have increased over 15 years from 10-20 to 50-60%. Also, H. Trolldenier [6] noted in his work the increased

persistence of certain forms of bacteria to chloramphenicol (50-60%) and streptomycin (45%).

M. Sone [7] determined the effect of 9 antibiotics on the semen microflora and found that dibekasin, aminasin, and gentamicin are effective against all types of microflora. Polymyxin-B and holistin are effective against 6 species of semen microflora. Sulbenitsillin showed more effective results than penicillin against *Pseudomonas aeruginosa*; kanamycine gave different results, while streptomycin and penicillin showed low efficiency.

Analyzing the fertility of bovine semen, containing different antibiotics, A. Sevinc et. al. [8] found that a semen with chloramphenicol has the highest fertility (93.1%), followed by the semen containing penicillin and streptomycin (30-80%); the semen with ampicillin was the worst one (29%). Examining the semen of 22 bulls for the content of the bacterial flora, Saikia G.K. et. al. [9] noted the presence of various bacteria. All of the identified bacteria were studied in terms of their sensitivity to antibiotics, as well as the efficacy of these antibiotics. They obtained the following results: gentamicin (100%), kanamycin (94.5%), neomycin (89.2%),

ampicillin (83.7%), streptomycin (45.9%), penicillin (10.8%), polymyxin (2,7%), and bacitracin (0.0 %).

K. Ahmad with co-workers [10-12], and J. Masurova and P. Vinter [13] studied the effect of antibiotics on the quality of frozen-thawed bovine semen. Fertility of 1182 cows, inseminated with amikacin sulfate extended bovine semen in the dose of 500 mcg/ml, was 70.4% versus 70.5% in control. The investigation of certain antibiotics effect has shown that cefapirin and ceforanid are the most tolerant agents to spermatozoa (fertilization ability is 75.5%).

Thus, antibiotic resistance of bacteria requires the development of a new antibacterial complex for semen sanitation of animals, providing both increased holding period of semen, it's fertilizing ability, and ensuring the healthy offspring.

2. Materials and methods

The work was performed in the laboratories of the South-Kazakhstan State University named after M.Auezov, South Regional Laboratory, and "Dos-bi" and "Shubar" stock farms.

Meat-and-peptone agar (MPA) and meat-and-peptone broth (MPB), Kitt-Tarozzi medium, Saburo medium, Bulir's medium, and Endo agar were used for bacteriological studies. Morphological and tinctorial properties of isolated bacteria were studied by Gram staining smears. The study of bacteria motility was carried out by crushed drop method using a phase-contrast microscope. Hemolytic activity of cultures was determined while growing on MPA, supplemented with 5% of defibrinated sheep blood. Catalase reaction was carried out by applying 10 drops of 3.2% perhydrol solution on a culture lawn of MPA. The level of semen contamination and the extent and nature of the pseudo-monostorage of servicing bulls in livestock farms was determined by bacteriological studies.

Serological typification of isolated cultures was performed by carrying out the agglutination test on a glass using polyvalent O-serum pseudomonas, produced by Dnepropetrovsk plant on bacterial drugs production, according to the attached instructions.

During bacteria identification we guided by bacterium indicator of M.O. Bergey [14].

Susceptibility of isolated strains to antibiotics and chemical agents was determined by disc diffusion method on Mueller-Hinton Agar in accordance with methodological guidelines (MG) 4.2.1890-04 "Determination of the microorganisms sensitivity to antibiotics". Sensitivity of isolated cultures of blue pus bacillus pseudomonas, proteus, and E. coli to antibiotics was determined employing method of serial dilution in a nutrient broth.

3. Results

As a result of research we found that in case of full compliance with sanitary requirements, one can get from the bulls up to 72-74% of ejaculates, containing not more than 2,000 microbial cells per 1 ml, and from boars - 67-69% of ejaculates with microbial cells in amount of maximum 5,000 per 1 ml of semen. Most frequently ejaculates contain pathogens, opportunistic cells (blue pus bacillus), streptococci, micrococcaceae, saprophytes, olm, and fungi. In boars, microbial contamination of semen changes with advancing age by increasing the microbial count in the prepuce. As compared to young boars, microbial count in mature males at the age of 3 years was increased by 2.83-2.94 times, in boars over 4 years – by 4.12-4.57 times; coli titer decreased by 2.03 and 3.51 times, respectively.

The sensitivity of the semen microflora was tested to following 24 antibiotics: penicillin, streptocide, cefotaxime, gentamicin, ceftriaxone, enroxil, pharboxidin, angeil, tylosin, aminazin, nitox, olemorfoculine, carbenicillin, enroflon and polymyxin sulphate, streptomycin, brulamycin, erycyclinum, dicloxacillinum natrium, sulfatonum, neovetine, nystatin, and oleandomycin phosphate. To determine the minimum inhibitory concentration, formulations were tested at concentrations ranging from 6 to 100 mg/ml (U/ml).

All selected gram-positive semen microorganisms are sensitive to cefotaxime, gentamicin, and enroxil (88-92%). Most microorganisms are sensitive to enroflon and polymyxin sulphate, brulamycin, erycyclinum, and oleandomycin phosphate (64-79%).

Gram-negative semen microorganisms are sensitive to cefotaxime, gentamicin, enroxil, and nitox (80-87%). Most gram-negative bacteria strains have an average degree of sensitivity with regard to brulamycin, erycyclinum, and aminasin (33-46%).

To study the effect of antibiotics on the activity and vitality of spermatozoa, 15 samples were selected out of 24 antibiotics. The experimental study has shown that gentamicin, cefotaxime, and oleandomycin phosphate are the antibiotics that comply with the specified requirements.

In order to establish the optimal dose of each agent, bovine semen was extended by glucose-yolk citrate (GYC), while boars' semen was extended by glucose-chelating citrate-sulphate (GCCS-8); at that, the antibiotics were added to both extenders. Cefotaxime was added at the rate of 5, 10, 20, 30, 40, and 50 mg per 100 ml of extender. It is established that cefotaxime increases the absolute index of vitality of 9 bovine spermatozoa in a fairly wide range of concentrations from 5 to 40 mg per 100 ml of extender. As compared with the control ("Spermosan-3"), high vitality of spermatozoa was observed when

adding 10 (28.6%) and 20 mg (18.6%) of cefotaxime per 100 ml of extender. A similar increase of AIV in cryopreserved spermatozoa was found in extender, consisting of yolk, lactose, and glycerin (YLG); adding 10 mg of cefotaxime (per 100 ml of extender) led to an increase of AIV by 27.5%, while adding 20 mg resulted in AIV increase by 15.4%.

It is found that gentamicin is also harmless towards the semen within a wide range of concentrations (20-200 mg). Within the concentration range from 20 to 80 mg AIV increases from 12.1 to 20.1%. It was established that oleandomycin phosphate at a concentrations from 20 to 80 mg is harmless to spermatozoa as well.

To develop an antimicrobial complex, we have selected three antibiotics in appropriate concentrations. It is established that a positive effect of the agents is achieved within a broad range of concentrations, namely: 5-20 mg – for cefotaxime, 8-16 mg – for gentamicin, and 30-160 mg – for oleandomycin phosphate (Table 1). At these component concentrations AIV of spermatozoa increases within the range from 27.6 to 44.9%.

Table 1. The effect of different component ratios of a new antibacterial complex on the absolute index of vitality (AIV) of animal spermatozoa.

| Amount of antibiotic, mg/100ml of extender | | | Absolute index of vitality of spermatozoa in extended media (AIV, conventional units, %) | | | |
|--|------------|------------------------|--|-------|-------------------------|-------|
| Cefotaxime | Gentamicin | Oleandomycin phosphate | Bulls (GYC extender) | | Boars (GCCS-8 extender) | |
| | | | AIV | % | AIV | % |
| 5 | 8 | 30 | 1923.2 | 129.0 | 889.7 | 125.4 |
| 10 | 8 | 60 | 2160.4 | 144.9 | 1012.7 | 142.7 |
| 20 | 8 | 80 | 2004.0 | 134.4 | 943.2 | 133.1 |
| 10 | 16 | 100 | 2020.6 | 135.5 | 937.6 | 132.2 |
| 20 | 16 | 120 | 1984.5 | 133.1 | 926.1 | 130.5 |
| 10 | 16 | 140 | 1982.5 | 133.0 | 921.9 | 129.9 |
| 20 | 16 | 160 | 1902.1 | 127.6 | 879.3 | 123.9 |
| Control: Spermosan-3 | | | 1490.4 | 100.0 | 709.4 | 100.0 |

It is found out that the highest vitality of spermatozoa is provided by the following components (per 100 ml of extender): cefotaxime - 10 mg, gentamicin - 8 mg, and oleandomycin phosphate - 60 mg, which make up newly developed antibacterial complex for sanitation of animals' semen.

We have studied the efficiency of extended semen sanitation by adding a new antibacterial complex (Table 2). Microbiological studies have shown that the new antibacterial complex decontaminates the semen microflora at the level of 96.5-97.0%.

High decontamination level of semen microflora indicates that cefotaxime and gentamicin act synergistically against semen microflora, and, as chemicals, do not lead to inactivating interaction.

Table 2. Sanitation efficiency of the breeder semen using standard "Spermosan-3" and new antimicrobial complex (number of tests in percentage terms). Notice: ^X cefotaxime - 10 mg; gentamicin - 8 mg; oleandomycin phosphate - 30 mg; ^{XX} cefotaxime - 15 mg; gentamicin - 15 mg; oleandomycin phosphate - 60 mg.

| Bacterial contamination degree (bacterial count) | «Spermosan-3» | | New complex ^X | | New complex ^{XX} | |
|--|-------------------------|----------------------------|--------------------------|----------------------------|---------------------------|----------------------------|
| | GYC extended bull semen | GCCS-8 extended boar semen | GYC extended bull semen | GCCS-8 extended boar semen | GYC extended bull semen | GCCS-8 extended boar semen |
| Germ-free | 5.8-13.8 | 4.2-10.9 | 42.6-89.6 | 36.4-81.6 | 43.7-89.1 | 34.2-87.6 |
| Up to 50 microbial cells per ml | 3.6-8.2 | 7.5-18.9 | 1.2-2.8 | 1.6-3.1 | 1.0-2.8 | 1.4-3.2 |
| Up to 100 microbial cells per ml | 6.4-17.2 | 6.8-17.4 | 1.4-2.84 | 2-5.2 | 1.2-2.82 | 1.3-3.4 |
| Up to 200 microbial cells per ml | 8.2-21.7 | 5.9-13.8 | 1.3-2.84 | 1-2.7 | - | - |
| Up to 300 microbial cells per ml | 3.1-8.2 | 5.7-14.5 | - | - | - | - |
| Up to 400 microbial cells per ml | 3.3-8.2 | 3-8.2 | - | - | - | - |
| Up to 500 microbial cells per ml | 4.5-10.9 | 3.6-8.2 | - | - | - | - |
| Over 500 microbial cells per ml | 3.6-8.2 | 2.8-5.3 | - | - | - | - |

To determine the effect of a new antibacterial complex on breeding efficiency of animals, the insemination of cows and sows was performed (Table 3). It was revealed that sanitation of breeder semen by means of a new antibacterial complex contributes to productive insemination. Fertility of cows using a new antibacterial complex was 11.7-12.9% higher than that in control. All the litters were healthy.

Table 3. Fertility of cows and sows, inseminated with the semen contained a new complex with harmonized components.

| Indicators | Bovine semen | | Boars semen | |
|----------------------------------|--------------|------------|-------------|------------|
| | Control | Experiment | Control | Experiment |
| Inseminated animals, beasts | 176 | 181 | 206 | 212 |
| Fecundated, beasts | 139 | 164 | 162 | 194 |
| Breeding efficiency, % | 78.9 | 90.6 | 78.6 | 91.5 |
| Result comparing with control, % | | +11.7 | | +12.9 |

New antibacterial complex for semen sanitation of servicing bulls and boars allows one to perform microflora decontamination at the level of 96.5-97.0%; increases the holding period of ejaculates by 27.6- 44.9%, enhances the fertility of cows and sows by 11.7-12.9%, and enables to obtain healthy litters.

4. Discussion

It is known that neither specific antibiotic, even having the most broad action spectrum, is able to fully decontaminate the semen of farm animals. Therefore modern antibiotic sanitation of animal semen should be based on combination of

pharmacokinetics data, drug toxicity information, and bacteriology. Thus, in particular, E.J. Ariens and A.M. Simonis [15] differ the cooperative interaction of antibiotics, when their combined use exceeds the contribution from individual effects, and antagonistic interaction, i.e. deteriorating the individual effect of each specific antibiotic.

R.B.Trustcott. and J.L. Ruhnke [16], when studying the sensitivity of antibiotics towards the bovine semen, found that the efficiency of lincomycin, spectinomycin, and tilosin combination is higher than that compared to rezoksatsin and gentamicin. Kozumplik J. [17], when studying the effects of apramicin on bacterial contamination of semen, found that the agent at concentrations of 200-250 mg in combination with 500 thousand IU of potassium penicillin muriate significantly improves the semen survival rate.

K.Gangadhar et. al. [18] have experienced in India the effects of different antibiotics combinations on bacterial contamination of bovine semen. Investigated sanitation complexes consisted of the following components: 1- penicillin + streptomycin;

2- chloramphenicol + kanamycine + oxytetracycline; 3- chloramphenicol + kanamycine. The best decontamination was found when using chloramphenicol + kanamycine, which was offered for practical use.

Studies of K. Tamuli Madun and C.K. Rajkonwar [19] showed that the addition of 3.2 mg of gentamicin, 60 mg of penicillin, and 100 mg of streptomycin into 100 ml of GPSE extender increases the vitality of boar semen.

According to S.Roy and R.Choudhury [20], microorganisms of contaminated semen survive lyophilization. In an attempt to study the possibility of restoring life activity of bovine semen after lyophilization by means of adding dihydrostreptomycin sulphate and other agents did not bring much success.

J. Hovozka [21] offers 11 sanitizing complexes in combination with spiramycin, penicillin, streptomycin, colistin, lincomycin, and erythromycin. When using these complexes, fertility ranged from 58.7 to 82.7%. According to Kozumplik J. and Slehoferova L. [22], application of depotocin gives better effect than use of other antibiotics. It has a positive effect on the semen quality, increases fertility (19.89%), and sows farrow (1.08 beasts).

Balashov N.G. et al. [23, 24] reported that the addition of "Spermosan-PPK" reduces the microbial count in the semen by 7-23.5 times, and content of fungi by 3.2-13.0 times. "Spermosan-PPK" allows one to obtain boar ejaculates without E. coli in an amount 7-20.5 times greater than that when using Spermosan-3.

According to T. Stoyanov [25, 26], the introduction of penicillin with streptomycin into the extender reduces the activity of spermatozoa by 2.4 times. Introducing chloramphenicol and oxytetracycline reduces the activity of semen by 10 times, while gentamicin has no negative effect on the activity of sexual gametes, and increases fertility by 15.3%. In experiments conducted by K. Ganqadhar et.al. [27], the best decontamination was found when using chloramphenicol along with kanamycine.

Our results excel the above reported data in terms of the decontamination of ejaculates microflora and harmlessness towards the spermatozoa. We have developed a new antibacterial complex consisting of the following components (per 100 ml of exyender): cefotaxime -10 mg, gentamicin - 8 mg, and oleandomycin phosphate - 60 mg. Introduction of a new antibacterial complex into extender has increased the semen vitality of servicing animals by 27.6 - 44.9%, allowed one to reach decontamination efficiency of microflora up to 96.5-97.0%, and at the same, to increase the fertility of the animals by 11.7-12.9%.

Oleandomycin phosphate, as part of a new antibacterial complex for semen sanitation, plays important role as a stabilizing factor of semen viability. Cefotaxime and gentamicin have different spectrum of antimicrobial action. Their chemical and antimicrobial compatibility is an important property of these agents. Combined action may result in either a synergistic effect, additive antagonistic effect, or be just indifferent.

5. Conclusion

1. It is recognized that in case of full compliance with sanitary requirements, one can get from the bulls up to 72-74% of ejaculates, containing not more than 2,000 microbial cells per 1 ml, and from boars - 67-69% of ejaculates with microbial cells in amount of maximum 5,000 per 1 ml of semen. Most frequently ejaculates contain pathogens, opportunistic cells (blue pus bacillus), streptococci, micrococcaceae, saprophytes, olm, and fungi.

2. The studies on sensitivity of semen microflora and harmfulness of certain antibiotics resulted in development of a new antibacterial complex consisting of the following components: cefotaxime -10 mg, gentamicin - 8 mg, and oleandomycin phosphate - 60 mg.

3. Introduction of a new antibacterial complex into the semen extenders increases vitality of spermatozoa by 27.6-44.9%, decontamination of microflora – by 96.5-97.0%, and fertility of animals – by 11.7-12.9%.

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