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Microbiological tests of fresh bull semen collected at breeding establishment

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SUMMARY

Bacterial and fungal contamination of the semen collected in production environment largely depends on the sanitary conditions of its collection as well as on the bacteria carrier state in breeding bulls. Since antimicrobials contained in the diluent used during semen product cryopreservation do not allow an objective assessment of semen contamination, a microbiological testing of fresh undiluted bull semen was carried out at the AO "Krasnoyarskagroplem" breeding establishment to identify the contamination source. The isolated opportunistic microorganism cultures were tested for their susceptibility to antibiotics for the purpose of effective treatment of bacteria carriers. The experiment was performed at the Department for Epizootiology, Microbiology, Parasitology and Veterinary and Sanitary Expertise of the Institute of Applied Biotechnology and Veterinary Medicine of the FSBEI HE "Krasnoyarsk State Agrarian University" and at the Veterinary Laboratory of the AO "Krasnoyarskagroplem" in 2017 and 2018. Semen was collected in accordance with GOST 32222-2013 and tested for veterinary and sanitary parameters according to GOST 32198-2013. Isolated microorganism cultures were tested for their susceptibility to antibiotics with disc-diffusion method according to the Methodical Guidelines 4.2.1890-04 "Testing of microorganisms for their susceptibility to antimicrobials" using discs containing eight antimicrobials. Analysis of microbiological test results showed that semen was rejected for sanitary reasons at the breeding establishment due to isolation of the following opportunistic microorganisms: *Pseudomonas aeruginosa* (6.4% samples) and *Proteus vulgaris* (8.5% sample) in 2017 and *Pseudomonas aeruginosa* (2.4% samples) in 2018. Other test parameters (total microbial count, coliform count) were within admissible limits. No anaerobes and pathogenic fungi were detected. Four *Pseudomonas aeruginosa* isolates and three *Proteus vulgaris* isolates recovered during the test have demonstrated susceptibility to ciprofloxacin that can be used for etiotropic treatment of bulls identified as bacteria carriers.

Key words: bulls, fresh semen, contamination, bacteria carrier state, opportunistic microorganisms, susceptibility to antibiotics

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Микробиологические исследования свежеполученной спермы быков-производителей на племпредприятии

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РЕЗЮМЕ

Контаминация бактериями и грибами спермы, полученной в производственных условиях, во многом зависит от санитарных условий при ее получении, а также от бактерионосительства быков-производителей. Так как наличие антибактериальных препаратов, входящих в состав разбавителя при криоконсервации спермопродукции, не позволяет объективно оценить степень ее обсеменения, для установления источника контаминации было проведено микробиологическое исследование свежеполученной неразбавленной спермы быков на племенном предприятии АО «Красноярскагроплем». Для эффективного лечения бактерионосителей определяли чувствительность выделенных культур условно-патогенных микроорганизмов к антибиотикам. Эксперимент выполнен в 2017 и 2018 гг. на базе кафедры эпизоотологии, микробиологии, паразитологии и ветеринарно-санитарной экспертизы института прикладной биотехнологии и ветеринарной медицины ФГБОУ ВО Красноярский ГАУ и ветеринарной лаборатории АО «Красноярскагроплем». Сперму отбирали в соответствии с ГОСТ 32222-2013, ветеринарно-санитарный контроль материала проводили по ГОСТ 32198-2013. Чувствительность

к антибиотикам выделенных культур микроорганизмов определяли диско-диффузионным методом согласно методическим указаниям МУК 4.2.1890-04 «Определение чувствительности микроорганизмов к антибактериальным препаратам» с использованием дисков, содержащих восемь препаратов. Анализ результатов микробиологических исследований показал, что выбраковка спермы по санитарным показателям на племенном предприятии происходила за счет выделения условно-патогенных микроорганизмов: в 2017 г. – синегнойной палочки (6,4% проб) и протея (8,5% проб); в 2018 г. – синегнойной палочки (2,4% образцов). Остальные показатели (общее микробное число и коли-титр) находились в пределах допустимой нормы. Анаэробы и патогенные грибы обнаружены не были. Четыре выделенных в 2017–2018 гг. изолята *Pseudomonas aeruginosa* и три изолята *Proteus vulgaris* проявили чувствительность к ципрофлоксацину, который можно использовать для этиотропной терапии быков в случае установления у них бактерионосительства.

Ключевые слова: быки-производители, свежеполученная сперма, контаминация, бактерионосительство, условно-патогенные микроорганизмы, чувствительность к антибиотикам

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INTRODUCTION

Dairy cattle farming is a priority sector in the livestock industry of the Krasnoyarsk Krai and defines its successful development. The best cattle gene pool of the world, outstanding stud bull genotypes are currently used for animal reproduction in the Krai livestock industry owing to development of semen collection, cryoconservation and artificial insemination technology [1, 2].

At the breeding establishment, the high priority is given to control of donor bull semen for quality [3].

Bacterial and fungal etiological agents transmitting with semen and responsible for infectious diseases in parent population pose a danger during artificial insemination [4–12].

Bacterial and fungal contamination of the semen collected in production environment largely depends on sanitary conditions during the semen collection as well as bacteria carrier state of breeding bulls [3, 13, 14].

Special media containing antimicrobials are added to the semen to maintain its fertilizing ability for a long time [15–17]. Previous studies showed that antimicrobial components contained in the diluent used during cryoconservation not always inactivate all contaminants that resulted in rejection of already cryoconserved semen [18].

Since presence of antimicrobials does not allow an objective assessment of semen contamination, a microbiological testing of fresh undiluted bull semen was carried out at the AO "Krasnoyarskagroplem" breeding establishment to identify the contamination source.

The test objectives were as follows:

1. Analysis of results of microbiological tests of semen for the purpose of the semen veterinary and sanitary assessment.

2. Tests of the isolated microorganism cultures for their susceptibility to antimicrobials.

Recently, rise in antimicrobial resistance of infectious agents has been reported in the Russian Federation, as well as throughout the world, that results in microorganism survival during etiotropic therapy.

Currently, it is one of the serious threats to health. Improper use of antibiotics accelerates this process making more and more infectious diseases very difficult to treat, that is associated with a long course of the disease and an increased economic costs.

The main factors contributing to the development of antibiotic resistance of microorganisms are as follows: unjustified prescribing of antibiotics for the treatment of mild diseases; use of broad-spectrum drugs instead of available drugs with a narrow spectrum of action; prescribing drugs without taking into account the etiological spectrum of pathogens and their susceptibility.

Surveillance of antimicrobial resistance spread is a strategic task in veterinary field. Phenotypic (conventional) and molecular genetic methods are used for assessment of microorganism resistance to antimicrobials [19].

There is *AMRmap* database in Russia that contains data on monitoring of microorganisms for their antimicrobial resistance.

MATERIALS AND METHODS

The experiment was performed at the Department for Epizootiology, Microbiology, Parasitology and Veterinary and Sanitary Examination of the Institute of Applied Biotechnology and Veterinary Medicine of the FSBEI HE "Krasnoyarsk State Agrarian University" and at the Veterinary Laboratory of the AO "Krasnoyarskagroplem" in 2017 and 2018. Microbiological tests were carried out at the Veterinary Laboratory of the AO "Krasnoyarskagroplem".

Holstein, Red-and-White, Black-and-White, Simmenthal, Hereford and Aberdeen-Angus breeding bulls were tested.

Fresh semen (141 samples) collected from the breeding bulls was tested. The semen was collected aseptically, one ejaculate from each bull in a sterile tube in accordance with GOST 32222-2013 "Products for Reproduction. Semen. Methods for Semen Collection" [20].

The semen quality was tested for the following veterinary and sanitary parameters: total viable count –

microbial count in 1 cm^3 (CFU/cm^3), coliform count (coli-titre) in 1 cm^3; presence of pathogenic, opportunistic microorganisms and anaerobes.

Total microbial count (TMC) in 1 cm^3 was determined by inoculation of the semen at two dilutions (1:10 and 1:1,000) onto meat-peptone agar (MPA), one dilution per four dishes examined with two-layer agar plate method. Coliform count was determined by inoculation of the semen samples onto Bilur's medium for mannitol fermentation caused by coliforms. Results were assessed based on medium colour changes and gas production.

As for opportunistic microorganisms, meat peptone broth (MPB) supplemented with 10% glucose was used for detection of *Pseudomonas aeruginosa* and microscopy and chlor-formalin test were used for *Proteus vulgaris* identification.

For anaerobe isolation the semen was cultivated in Kitt-Tarozzi medium in accordance with GOST 32198-2013 "Product for reproduction. Semen. Microbiological analysis technique" [21] and Methodical guidelines for prevention of microbial contamination of breeding bull semen [3].

The mycological testing of the semen was carried out with the methods for assessment of the semen used for artificial insemination of livestock animals [22].

The isolated microorganisms were tested for their susceptibility to antibiotics with disc-diffusion method using discs containing ciprofloxacin (5 μg), streptomycin (30 mg), gentamycin (10 μg), tetracycline (30 μg), ampicillin (10 μg), ceftazidim (30 μg), imipenem (10 μg), polymyxin (300 U) in accordance with Methodical Guidelines 4.2.1890-04 "Tests of microorganisms for their susceptibility to antimicrobials" [23].

RESULTS AND DISCUSSION

Microbiological tests of 47 samples of fresh non-diluted semen carried out in 2017 showed absence of growth in Kitt-Tarozzi medium inoculated with the tested semen samples that was indicative of anaerobe absence in the tested samples. The highest TMC found in the samples was 3,290 CFU/cm^3 when the maximum level laid down in GOST 32198-2013 – 5,000 CFU/cm^3. No colonies were detected in six semen samples (12.8%) inoculated onto solid nutrient media. Mean TMC in tested samples was 275 ± 76 CFU/cm^3. Coli-titre in one sample was 0.1 cm^3.

Pseudomonas aeruginosa was detected three samples of fresh semen (isolates No. 3, 37, 42), that was equal to 6.4% of total number of the tested samples. Semen collected from such animals shall be rejected and bulls-semen donors shall be put under control for their bacteria carrier state.

Pseudomonas aeruginosa belongs to rod-shaped bacteria. It has the following pathogenic factors as motility and toxin production, it easily affects the organisms with compromised immune status [24].

Pseudomonads are capable of persisting in the body for a long time. Therefore, the said microorganisms have evolutionarily developed mechanisms of protection from the applied therapeutic measures and evasion of host immune response.

Biofilm formation is one of the most important factors in the microorganism colonization. In this case, the polysaccharide matrix of the cell becomes invisible to the immune system, and exopolysaccharides slow down antimicrobial diffusion [25].

Host cells are used for pseudomonads accumulation. Such an invasion is observed, as a rule, in epithelial cells during genitourinary tract infection, and pathogen contamination level is usually much higher at the port of the infection entry [24].

Walters M. C. et al. successfully combated *P. aeruginosa* biofilms using combination of tobramycin, ciprofloxacin and tetracycline having an effect on active cells in biofilm upper layer as well as colistin, antibiotic having an effect on non-active cells [26].

Results of tests of the recovered *P. aeruginosa* isolates for their antimicrobial resistance are given in Figure 1 and Table 1.

Obtained data showed that all three *P. aeruginosa* isolates demonstrated higher susceptibility to ciprofloxacin and lower susceptibility to streptomycin. Isolates No. 37 and 42 were also susceptible to gentamicin, tetracycline and ceftazidim. Isolate No. 42 was susceptible to ampicillin.

Pathogenic *Proteus* (*Proteus vulgaris*) (isolates No. 12, 15, 17, 19) was detected in 8.5% of total number of the tested samples. It is a gram-negative, spore-forming, facultative anaerobic small filamentous bacillus. *Proteus* is a representative of normal opportunistic microbiota of mammalian gastrointestinal tract.

Bacteria of *Proteus* genus are mainly responsible for the disease in young livestock animals exposed to immunosuppression and to stress. *Proteus* infection

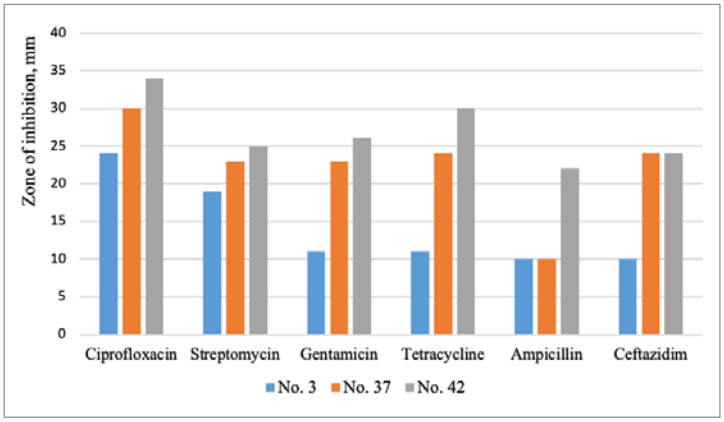

Fig. 1. Tests of P. aeruginosa isolates recovered in 2017 for their susceptibility

Table 1
Antimicrobial resistance of *P. aeruginosa* isolates recovered in 2017

Name of antibiotic	Susceptibility of isolates		
	No. 3	No. 37	No. 42
Ciprofloxacin	susceptible	susceptible	susceptible
Streptomycin	susceptible	susceptible	susceptible
Gentamicin	resistant	susceptible	susceptible
Tetracycline	resistant	susceptible	susceptible
Ampicillin	resistant	resistant	susceptible
Ceftazidim	resistant	susceptible	susceptible

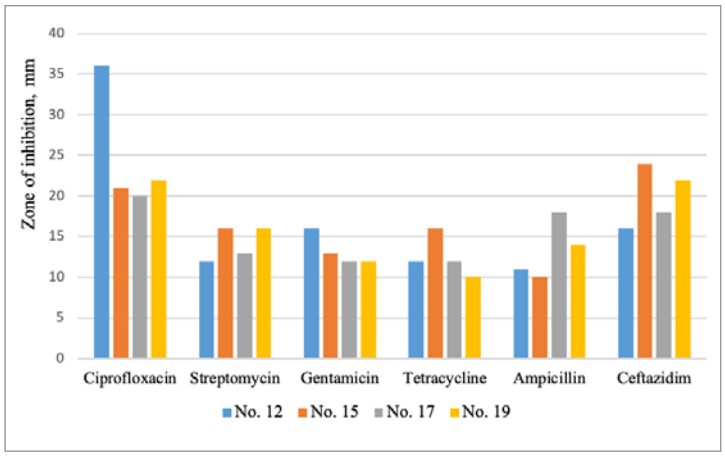

Fig. 2. Tests of P. vulgaris isolates recovered in 2017 for their antimicrobial resistance

Table 2
Antimicrobial resistance of *P. vulgaris* isolates recovered in 2017

Name of antibiotic	Susceptibility of isolates			
	No. 12	No. 15	No. 17	No. 19
Ciprofloxacin	susceptible	susceptible	intermediate	susceptible
Streptomycin	resistant	intermediate	intermediate	intermediate
Gentamicin	susceptible	intermediate	resistant	resistant
Tetracycline	resistant	intermediate	resistant	resistant
Ampicillin	resistant	resistant	susceptible	intermediate
Ceftazidim	intermediate	susceptible	susceptible	susceptible

outbreaks are reported sporadically, the pathogen is generally transmitted by alimentary route.

Proteus belongs to typical gram-negative bacteria containing beta-lactamases mediating resistance to antimicrobials such as penicillins, cefalosporins, carbapenems [27].

Semen collected from such bulls are rejected and the bulls are put under control for their bacteria carrier state. In this case, it is reasonable to test each freshly isolated microorganism culture for its susceptibility to antimicrobials for more effective treatment of bacteria carrier-bulls.

Results of tests of *P. vulgaris* isolates for their resistance to antimicrobials are given in Figure 2 and Table 2.

Test results showed that three (No. 12, 15, 19) out of four *P. vulgaris* isolates were highly susceptible to ciprofloxacin and isolate No. 17 was intermediately susceptible to ciprofloxacin. Isolates No. 15, 17, 19 were susceptible to ceftazidim, isolate No. 12 was susceptible to gentamycin and isolate No. 17 was susceptible to ampicillin.

In spring 2018, 42 samples of fresh semen from breeding bulls were collected and tested. Mean TMC in tested samples was 142 ± 70 CFU/cm^3 the highest TMC (2,020 CFU/cm^3) was detected in one sample (2.4% of total number of tested samples). Coli-titre in all samples was higher than 0.1 cm^3 whereas 14 samples (33.3%) were sterile. No anaerobes and pathogenic fundi were detected. *Pseudomonas aeruginosa* (pseudomonosis agent) was isolated from one sample (2.4%). Prepared pure *P. aeruginosa* culture was tested for its resistance to antimicrobials. Test results are given in Figure 3 and Table 3.

Obtained data showed that recovered *P. aeruginosa* isolate demonstrated the highest susceptibility to ciprofloxacin and imipenem.

In autumn 2018, 52 ejaculates were collected from breeding bulls and tested. No growth was observed in MPA and other nutrient media inoculated with the tested samples. No pathogenic fungi were detected in the bull semen subjected to microbiological testing. The semen was found to be suitable for artificial insemination based on the test results.

CONCLUSIONS

1. Analysis of results of microbiological tests of fresh semen collected from breeding bulls at the breeding establishment showed that the semen was rejected for sanitary reasons at the breeding establishment due to isolation of the following opportunistic microorganisms: *P. aeruginosa* (in 6.4% of samples) and *Proteus* (in 8.5% of samples) in 2017 and *P. aeruginosa* (in 2.4% of samples) in 2018.

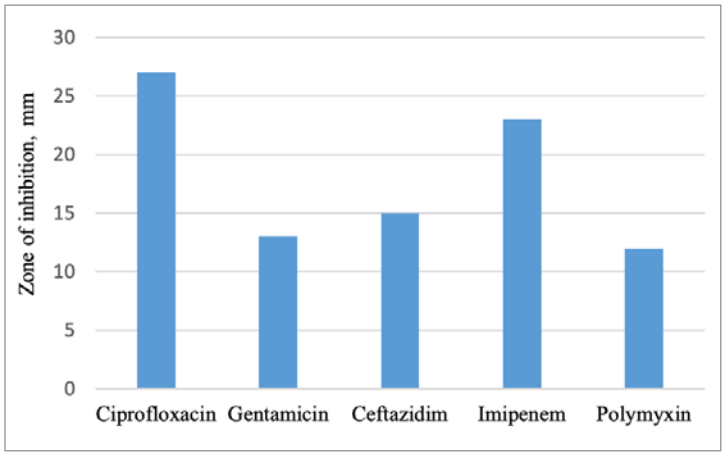

Fig. 3. Test of P. aeruginosa isolate recovered in 2018 for its antimicrobial resistance

Table 3
Antimicrobial resistance of *P. aeruginosa* isolate recovered in 2018

Name of antibiotic	Susceptibility of isolate
Ciprofloxacin	susceptible
Gentamicin	intermediate
Ceftazidim	intermediate
Imipenem	susceptible
Polymyxin	resistant

Other test parameters (total microbial count, coli-titre) were within admissible limits. No anaerobes and pathogenic fungi were detected.

2. Three *Pseudomonas aeruginosa* isolates and three *Proteus vulgaris* isolates recovered in 2017 and one *Pseudomonas aeruginosa* isolate recovered in 2018 and tested for their antimicrobial resistance demonstrated their susceptibility to ciprofloxacin that can be used for etiotropic treatment of bulls identified as bacteria carriers. Cultures of the same species but isolated from different animals demonstrated different susceptibility to other antimicrobials, therefore it is reasonable to test isolated cultures to susceptibility to antimicrobials for effective etiotropic treatment.

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