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Short communication

ANTIMICROBIAL RESISTANCE AS A PROBLEM FOR THE QUALITY OF BOAR SEMEN

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The aim of the study was to determine whether the bacteria from the environment and from the mucous membrane of the boar prepuce have antimicrobial resistance and whether the result obtained is similar/same to the bacteria that can be found in native boar semen. The study addresses the problem of the presence of primarily resistant bacterial strains in the boar sperm, which, due to their reduced sensitivity, cannot be suppressed by antibiotics used in the semen dilution agent, as well as to emphasize the importance of microbiological monitoring of the boar mucous membranes and ambient surfaces before and during their exploitation. Such an examination could contribute to the interchangeable design of the dilution agent for the boar semen relative to the antibiotic content.Resistant strains of bacteria from prepuce swabs and swabs taken from the facility, as well as from native boar semen were isolated. The presence of these bacteria affected the quality of the semen. In conclusion, it should be pointed out that bacterial monitoring of the prepuce and surface of the facility can indicate possible problems related to the quality of semen, and that the design of the dilution agent for boar semen should be adjusted to the established resistance of isolated bacteria.

Key words: bacteria, boar semen, resistance, semen quality

INTRODUCTION

The number, mobility and morphological characteristics of spermatozoa, as well as plasma semen quality, can be changed under the influence of different agents [1,2]. According to available data [3,4], the usual number of bacteria in the sperm is 10^3 - 10^5 cfu/ml. The most commonly isolated bacteria are non-pathogenic microorganisms, which do not pose a threat to sperm quality individually, but if their number exceeds 10^6 cfu/ml and if, most often, there are several different types of microorganisms, then their impact on semen quality is significant. The unwanted impact of bacteria

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on sperm quality is the result of a change in the mobility of spermatozoa and in the structure of their membrane [5-8], damage to the head, body and tail of spermatozoids [9-12], as well as the occurrence of agglutination of spermatozoa [7,13]. The negative influence of the presence of bacteria on sperm quality is due to the presence of alpha and beta haemolysin, spermotoxins which alter their mobility cause agglutination or damage certain structures on the spermatozoid membrane [14].

Microorganisms colonize animal facilities and animals that occupy them. Phenotypic characteristics can change depending on the different agents used on the farms (antibiotics, disinfectants). Due to the risk of the occurrence and spread of antimicrobial resistance, the tendency is, in modern livestock production, to reduce the use of antibiotics. However, bacterial strains with reduced sensitivity to one or more classes of antibiotics can often be found on the farms. The finding of such bacteria may be the result of the introduction of resistant strains through equipment, utensils or new animals, or as a result of inadequate use of antimicrobial drugs on the farm itself. These types of bacteria can be transmitted to the reproductive tract of the boar and found in the sperm. In such circumstances, antibiotics that are found in the semen dilution agent will not be able to suppress the growth of bacteria and prevent their adverse effects on the quality of semen and spermatozoa.

In our study, we analysed microbiologically swabs of prepuce and swabs of ambient surfaces in the facilities, as well as native boar semen. In all isolated strains, we determined the sensitivity to antimicrobial resistance.

The aim of the study was to determine whether the bacteria from the environment and from the mucous membrane of the prepuce showed antimicrobial resistance and whether the result obtained was similar to the bacteria that could be found in native semen. The study should provide an answer to the problem of presence, primarily resistant bacterial strains in the boar sperm which, due to the reduced susceptibility/sensitivity, cannot be suppressed by antibiotics, contained in the semen dilution agent, as well as to emphasize the importance of microbiological monitoring of the mucous membrane of boars and ambient surfaces in facilities housing boars, before and during their exploitation. Such an examination could contribute to the interchangeable design of semen dilution agents in relation to the content of antibiotics.

MATERIALS AND METHODS

The study was conducted on three pig farms with their own reproduction centres. Boars were 10-22 months of age, they were fed twice daily 2.5 kg of feed containing 16% protein. They were raised in individual boxes. The farms differed in regard to the number of sows and the number of boars in production. Microbiological tests, as well as the control of the mobility and morphology of spermatozoa did not include all boars from the reproduction centres on farms, but only those who had poor or variable results during exploitation and/or sows which were inseminated with their

semen had no progeny. A total of 56 native semen samples were taken from three farms. In addition to semen samples at the same time, prepuce swabs and swabs from the floor of the animals' housing facilities were also taken. A total of 56 prepuce swabs and 25 (from grid plastic) floor swabs were examined in total.

For microbiological examination, the semen and prepuce samples were collected according to the program of boar exploitation (three times in two weeks). The floor swabs from the facilities where the boars were housed were taken in several places by random sampling. All collected samples were stored at fridge temperature and brought to the laboratory in the next four hours. Isolation of bacteria was done by seeding boar sperm samples on nutrients -blood agar with 5% defibrated sheep blood, MacConeky and Sabouraud agar (Biokar, France) [15]. The total number of bacteria was determined by making dilutions (1-7 $\log_{10}$) according to ISO standard 8607 [16]. The isolated bacteria, after Gram staining and oxidative/fermentative (OF) test, were identified by determining the phenotypic characteristics by means of a biochemical series (Bacteriological Differentiation Discs, HiMedia).

The sensitivity test for isolated microorganisms was made using the diffusion method to 8 antibiotics (amoxicillin, amoxiclav, ceftriaxone, enrofloxacin, gentamicin, streptomycin, tetracycline, trimethoprim + sulfametaxazol (Bioanalysis, BioKar Diagnostics).

The boar semen was taken with the application of the necessary hygiene-technological procedures that limit the microbiological contamination of the sperm. The sperm was taken by a duble glove technique of fixing the penis by hand using a glove [3].

The cyto-morphological characteristics of spermatozoa were microscopically examined after eosin-nigrosin (AlfaPanon, Serbia) staining by means of immersion (x1000 magnification) on the microscope Olympus BX-40, Japan. The morphology of spermatozoa was assessed in accordance to Rozeboom [17].

Determination of the sperm count, total and progressive mobility of spermatozoa as well as linear examination were performed using CASA (Computer Assisted Sperm Analysis) (ISAS V.1.2., Proiser, Spain) [18]. The test of the membrane and spermatozoa acrosome integrity (Peanut Aglutinin-Flourescent Aglutination (PNA-FITC) / Propidium Iodide (PI), Invitrogen, USA) by flow cytometry (Guava Milipore-IMV, USA), was used to determine the percentage distribution of spermatozoa with intact or damaged acrosomes. Also, using the same apparatus, the SCSA test (DNA fragmentation test) with acridine orange (Acridine orange, Invitrogen) was used to examine the percentage distribution of spermatozoids with a damaged DNA structure. [19].

Statistical data processing was done at the level of 95% (ANOVA) with the help of the software package Statistica 7 (StatSoft Inc., 1984-2004).

Animal experimentation was conducted within standard ethical norms.

RESULTS AND DISCUSSION

Table 1 shows data on the number of examined boar semen samples and the number of samples with an increased bacteria count which were contaminated with more than three different bacterial species. The table provides data on the number of semen samples examined with a percentage presence of the total bacteria count. The total bacteria count represents all isolated strains that have been confirmed during the test.

Table 1. The number of examined boar semen samples, the presence and bacterial count

	Total number of boars in the facility	Number of examined boars	Number of materials with increased bacteria count	cfu/ml
Farm A	62	28	19 (67.86%)	$1 \times 10^3 - 4 \times 10^5$
Farm B	15	15	9 (60.00%)	$4 \times 10^4 - 6 \times 10^5$
Farm C	25	13	9 (69.23%)	$2 \times 10^3 - 3 \times 10^5$

During the exploitation of boars in reproduction centres, sterile conditions cannot be ensured. It is also not possible to expect that the boar semen obtained will be free from bacteria. The number of bacteria that can be expected in the native semen ranges from 10^3-10^5 cfu/ml [4] which corresponds to our findings. This number of microorganisms, depending on the presence of certain types of bacteria, usually does not lead to a change in sperm quality (motility, number of spermatozoa, acrosome damage) and are considered to be part of the normal flora (*Bacillus sp., Micrococcus sp., coagulase negative Staphylococcus*) of the urogenital tract [20]. Studies in Italy and Cuba confirmed the presence of bacteria in the 63% - 75% of native sperm samples, [21,22].

Table 2 shows groups of ejaculates formed in relation to the number of isolates and bacteria in millilitre of semen.

Table 2. Number of bacteria (cfu/ml) and number of isolates per millilitre of ejaculate per group

Groups	No. of samples	No. of isolates	Av. No. of bacterial strains per sample	Average cfu/ml ±SD
G1 (≤5000 cfu/ml)	7	29	4,14	1.691 ± 1.298
G2 (≤10000 cfu/ml)	10	41	4,10	6.956 ± 3.012
G3 (≤100000 cfu/ml)	14	50	3,50	52.480 ± 27.726
G4 (>100000 cfu/ml)	7	33	3,11	345.555 ± 210.413
total (T) / Average (A)	38 (T)	153 (T)	3,71 (A)	101.670 ± 60.612 (A)

Table 3 gives data on the change of individual examined sperm parameters that indicate the change in semen quality in relation to the formed 4 groups of ejaculates (cfu/ml).

Table 3. The quality of the native boar sperm in relation to the bacteria count cfu/ml by groups*

Groups		G1	G2	G3	G4
Vol. of ejaculate (ml)		221.0	171.6	161.1	214.1
CASA	Total motility (%)	84.11	76.80	59.15[1,2]	60.42
	Progressive motility (%)	37.15	36.33	28.54	32.41
	VCL (μm/s)	61.8	67.4	46.7[3]	52.6
PNA-FITC	Σ L (%)	85.3	80.7	77.0	78.3
	LIA (%)	70.9	71.6	67.5	60.6
	Σ DA (%)	18.9	17.6	18.3	29.9[4]
Cito-morphology	Σ L (%)	91.8	78.3	75.5	68.9[5]
	LIA (%)	75.9	53.1[6]	49.4[7]	48.7[8]
	Σ DA (%)	6.7	7.3	9.7	21.3[9]

CASA (computer assisted sperm analysis); PNA-FITC (flow cytometry-membrane and spermatozoa acrosome integrity test); Cyto-morphology; * values displayed as mean value
VCL - curvilinear movement of sperm; Σ L - Total living sperm; LIA- living with intact acrosome; Σ DA – total with damaged acrosomes

Our study showed a statistically significant difference 95% ($p<0.05$) between groups (according to the number of bacteria in ml G1-G4) in overall motility in the group G1 and G2 in comparison with the G3 group, while the same statistical significance 95% ($p<0.05$) was established for linear movement in group G3 in comparison with other groups. A similar finding was obtained 95% ($p<0.05$) in the comparison of acrosome damage in G2 group compared to the G4 group.

Positive correlation with bacterial count was documented for acrosome defects (G2:G4) in flow cytometry and cytology test ($p\leq0.05$).

Sperm cytology indicated that the number of live and live spermatozoa with intact acrosome gradually decreased with bacterial contamination (G1-91.8% and 75.9%; G2-78.3 % and 53.1%; G3-75.5% and 49.4%; G4-68.9% and 48.7%, respectively) ($p\leq0.05$). There was no statistical significance between bacteria presence and the total number of abnormal forms.

The percentage of individual bacterial strains isolated from the test samples (native boar semen, prepuce and floor swabs) is shown in Table 4.

Table 4. Isolated types of bacteria from the boar semen, prepuce and floor swabs

	Boar semen (sample No. 56)	Prepuce swab (sample No. 56)	Floor (sample No. 25)
Micrococcus luteus	14.50%	11.00%	5.00%
Enterobacter spp.	54.00%	43.00%	68.00%
Staphylococus coagulase neg.	13.50%	22.00%	28.60%
Pseudomonas aeruginosa	75.00%	61.50%	82.50%
Escherichia coli	65.00%	53.33%	86.50%
Proteus vulgaris	15.00%	21.00%	33.00%
Str. α*-haemolyticus*	12.00%	28.50%	31.00%
Bacillus spp.	11.00%	14.00%	21.00%
Flavobacterium sp.	5.00%	00.00%	4.00%

The most common bacterial species that can be isolated from the semen are bacteria from the *Enterobacteriaceae* family, but *Pseudomonas aeruginosa* [23] can be isolated as a permanent finding. The most common bacteria of the *Enterobacteriaceae* family are: *Escherichia coli, Proteus* spp.*, Serratia* spp.*, Enterobacter* spp.*, Klebsiella* spp.*,*and the presence of *Staphylococcus* spp., *Streptococcus* spp. and *Pseudomonas* spp. has been confirmed [21]. However, the presence of *Pseudomonas aeruginosa* and some bacteria from the *Enterobacteriaceae* family (primarily *Escherichia coli*) can lead to diminished semen quality. Microorganisms in the sperm can adversely affect spermatozoa in different ways, direct contact of bacteria and spermatozoa or the action of various bacterial products such as toxins and various parts of the bacterial wall, pilli or fimbria [24].

The presence of *E. coli* is significant due to the toxins secreted by these bacteria and can lead to agglutination of spermatozoa in humans [25]. The impact of *Pseudomonas aeruginosa* on reproduction may be bilateral, by adverse effects on spermatozoa/sperm and on reproductive performances in sows [3]. Changes in semen quality can be manifested by binding bacteria to spermatozoa, auto agglutination of spermatozoa, reducing their motility, damaging acrosomes, reducing the response to the hypoosmotic test, activating leukocytes, creating antibodies to spermatozoids and cell lysis [21]. *Pseudomonas aeruginosa* produces haemolytic and non-haemolytic phospholipases. Studies show [26] that the haemolytic form of these enzymes is very significant because it leads to the degradation of phosphatidylcholine and sfingomyelins that are part of the membrane of eukaryotic cells. Also, *Pseudomonas aeruginosa* produces enzymes elastase and matoloprotease, which are important for tissue damage, or colonization [27]. Elastase is particularly interesting as a virulence factor because it provides protection against the immune system of the host by breaking down elastin and collagen. Some of the *Pseudomonas aeruginosa* pigments, such as *pyoverdin*, behave as a siderophore molecule that binds and transports iron molecules through the protein receptors on the external cell membrane and represents a virulence factor [28]. *Pyocyanin*, a blue green colour

pigment, in addition to its importance as a quick diagnostic method, is considered to be the main factor of virulence, as it affects numerous cell functions [29].

Table 5 shows the sensitivity/resistance of the most important bacterial species that were present in the tested materials.

Table 5. Resistance of individual bacterial isolates from semen, prepuce and floor swabs (%)

	AMX[1*]	AMC	CEFT	ENRO	GENT	STRE	TET	TSX
SPERM								
Pseud. aeruginosa	100%	100%	79%	32%	100%	100%	100%	100%
Escherichia coli	65%	62%	12%	45%	18%	31%	84%	74%
Enterobacter sp.	72%	51%	18%	39%	28%	39%	78%	69%
Proteus spp.	85%	80%	20%	47%	38%	41%	82%	71%
PREPUCE SWAB								
Pseud.aeruginosa	100%	100%	76%	31%	100%	100%	100%	100%
Escherichia coli	68%	69%	10%	47%	17%	29%	86%	77%
Enterobacter sp.	74%	53%	20%	39%	27%	36%	77%	71%
Proteus spp.	87%	83%	21%	49%	36%	38%	80%	73%
FLOOR SWAB								
Pseud. aeruginosa	100%	100%	78%	39%	100%	100%	100%	100%
Escherichia coli	69%	67%	14%	47%	15%	39%	87%	79%
Enterobacter sp.	70%	49%	18%	59%	33%	41%	80%	72%
Proteus spp.	88%	79%	18%	52%	42%	43%	84%	70%

[1]Antibiotic: AMX – Amoxicillin, AMC – Amoxiclav, CEFT – Ceftriaxone, ENRO – Enrofloxacin, GENT – Gentamycin, STRE – Streptomycin, TET – Tetracycline, TSX – Trimethoprim-sulphamethoxazole; * zones of inhibition were regarded according to manufacturer instruction

In our study, we analyzed the antimicrobial sensitivity/resistance of the most frequently isolated bacterias. Due to the ambient conditions, isolated bacteria from the prepuce swabs and boars semen, bacteria from the *Enterobacteriaceae* family and *Pseudomonas aeruginosa* were most common in the tested samples.

The most antibiotic resistance had *Pseudomonas aeruginosa*. This species was resistant to all antibiotics, at one hundred percentage, except ceftriaxone i enrofloxacin. We revealed the high deerge of antimicrobial resistance at the three examined bacterial species from the *Enterobacteriaceae* family too.

The antimicrobial resistance was the higest on antibiotics from a group of sintetic peniciline, tetracicline and trimetoprim+sulfometaxazolone.

Bacterial resistance can be natural, genetically determined or acquired by transferring the genetic material of other microorganisms, by plasmids or bacteriophages, and may be the result of a mutation under the pressure of antibiotics. The use of antibiotics on

farms can be one of the factors in the development of antimicrobial resistance. The spread of resistant strains within the farm as well as between animals can be achieved by direct contact or through utensils and equipment used on the farm. Bacteria that have developed antimicrobial resistance can colonize the areas in which the animals are located, as well as their skin and mucous membranes. Since the conditions in the facilities during sperm production are burdened by microorganisms [1], the finding of resistant strains in the environment where the animals are housed and on the mucous membranes of the prepuce can be a problem. One aspect of the problem is that such strains can be present in native sperm, especially if they are in an increased number, and if there are a number of different species present to affect semen quality, and the second aspect is that they, due to their resistance, will remain present in the semen after use of the dilution agent. The presence of bacteria in the sperm has been confirmed in the work of many authors [3]. The finding of bacteria in samples of diluted ejaculate ranges from 26 to 31.2% in studies [3,5] from America to Europe [30].

CONCLUSIONS

Microbiological analysis of samples of native boar semen, prepuce and floor swabs revealed types of bacteria which belong to conditionally pathogenic microorganisms.

Semen quality control revealed statistically significant differences that were related to groups with a higher total bacteria count (total motility, linear movement, total number of living and spermatozoa with damaged and intact acrosomes). This finding indicates that the presence of bacteria significantly affected the quality of the semen.

In the analysis of the sensitivity of the most important isolates from the samples of native sperm, prepuce and floor swabs, multiple resistance was found in the tested bacterial isolates (mainly Gram negative bacteria).

Studies have shown that the microbiological monitoring of the environment in which the boars are housed, in the production and of the mucous membranes of the urogenital tract, can indicate to us not only the expected bacterial flora in native semen, but also, due to the presence of resistant strains, the presence of an increased total bacteria count in the native but also in diluted semen. Such results could give a prediction to the variable design of the semen dilution agent in the part relating to antibacterial additives.

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Authors' contributions

JMZ carried out molecular tests and sequence analysis, designed phylogenetic trees and draft the manuscript. VM, BS, OS and BK designed and coordinated the study and drafted the manuscript. CC performed Next Generation Sequencing and sequence assembly and editing and drafted the manuscript. AP, NS, LJV and VR participated in the collection and preparation of samples, molecular tests and preparation of phylogenetic trees.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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ANTIMIKROBNA REZISTENCIJA KAO PROBLEM KVALITETA SEMENA NERASTA

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Cilj ispitivanja je da se utvrdi da li bakterije iz okoline i sa sluznici prepucijma poseduju antimikrobnu rezistenciju i da li je dobijeni nalaz sličan/isti sa bakterijama koje se mogu naći u nativnom semenu nerasta. Ispitivanje treba da odgovori na problem prisustva, pre svega rezistentnih bakterijskih sojeva u spermi nerasta koji zbog svoje smanjene osteljivosti ne mogu biti suprimirani antibioticima, koji se nalaze u razređivaču za seme, kao i da istakne značaj mikrobiološkog monitoringa sluznice nerasta i ambijentalnih površina pre i tokom njihove eksploatacije. Ovakvo ispitivanje bi moglo da doprinese izmenjivom dizajniranju razređivača za seme nerasta u odnosu na sadržaj antibiotika.

Izolovani su rezistentni sojevi bakterija iz briseva prepucijuma i briseva uzetih iz objekta kao i iz nativnog semena nerasta. Prisustvo ovih bakterija uticalo na kvalitet semena. Kao zaključak treba istaći da bakterijski monitoring prepucijuma i površina u objektu može ukazati na moguće probleme vezane za kvalitet semena nerasta kao i da se dizajniranje razređivača za seme nerasta prilagodi utvrđenoj rezistenciji izolovanih bakterija.