

BACTERIA MAY DETERIORATE PROGRESSIVE MOTILITY OF BOVINE SPERMATOZOA AND BIOCHEMICAL PARAMETERS OF SEMINAL PLASMA

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doi: 10.15414/jmbfs.2020.9.4.844-847

ARTICLE INFO

Received 15. 8. 2019
Revised 8. 10. 2019
Accepted 23. 10. 2019
Published 3. 2. 2020

Regular article



ABSTRACT

Bacterial contamination of ejaculates has captured the interest of the wide andrological community. Interactions between spermatozoa and bacteria are partially known. The aim of our study was to evaluate qualitative bacterial composition of fresh bovine semen and to reveal associations amongst the quantity of bacteria (Colony Forming Unit – CFU/mL) present in bovine ejaculates with progressive motility of spermatozoa and biochemical parameters of seminal plasma. Fresh semen (n=20) was collected from Holstein-Friesian bulls. Progressive motility (PROG \geq 25 μ m/s) was analysed using Computer-assisted sperm analysis (CASA). Seminal plasma was separated and subjected to assessment (Randox RX Monza analyzer) of following biochemical parameters: Magnesium (Mg), Calcium (Ca), Phosphor (P), Urea, Aspartate transaminase (AST), Alanine transaminase (ALT), Alkaline phosphatase (ALP), Cholesterol, Bilirubin, Triglycerides, Albumin and Uric acid. The identification of bacteria showed the prevalence of the genus *Staphylococcus*. The Pearson correlation analysis showed a negative significant (P<0.001) correlation between CFU and PROG. Further, positive significant correlations were observed amongst PROG and biochemical parameters (P<0.01 in case of Mg, Urea, ALP, ALB; P<0.05 in case of Ca, ALT, Bilirubin and Uric acid). The results show negative significant correlations amongst CFU and biochemical parameters (P<0.001 in case of Mg; P<0.01 in case of ALT, ALP and P<0.05 in case of Uric acid). In conclusion, our results reveal that bacteria may affect not only on the sperm motility, but also on the composition of the seminal plasma. More detailed studies are needed to reveal the mechanisms of interaction amongst semen and bacteria.

Keywords: spermatozoa, bacteria, biochemical parameters, progressive motility, seminal plasma

INTRODUCTION

Artificial insemination (AI) represents the result of high economic pressure and simultaneously the highest possible milk production. The cryopreservation of semen samples is one of the attractive advances in current AI programs. Unfortunately, cryopreservation possesses a high risk related to bacterial contamination of ejaculates (Sannat *et al.*, 2015). Ubiquitous microbes have ample chances to contaminate samples during collection, processing and storage. Despite sterilization, artificial vagina, glassware and accessories, extender or laboratory environment are the most common sources of bacterial contamination of samples during processing (Rana *et al.*, 2012). Obviously, bacteria are present under physiological or pathological conditions in the ejaculate. Bacteria may contaminate the ejaculate all way from the testes, through epididymis, vas deferens, accessory glands to urethral opening and foreskin (Marcus *et al.*, 1994). The use of a microbiologically active insemination dose may not only reduce birth rate, but also transmit diseases amongst healthy populations (Thibier and Guerin, 2000). Based on previous studies, bacteria can induce oxidative stress in spermatozoa and damage sperm membranes, which may lead to subfertility. Some bacterial strains may cause the agglutination of motile sperm, leading to the premature acrosomal reaction and morphological alterations of the sperm head. Moreover, the inflammatory response inside the genitourinary tract leads to increased level of leukocytes and this is reflected in the increased reactive oxygen species (ROS) generation (Moretti *et al.* 2009; Sabeti *et al.*, 2016). Although, the studies above reported that bacteria may damage the sperm cells, there is no available information about the effect of the presence of bacteria on the seminal plasma. Therefore, the aim of this study was to determine composition of bacterial load in fresh bovine ejaculates and to correlate bacterial load with progressive motility of spermatozoa and with levels of selected biochemical parameters in seminal plasma.

MATERIAL AND METHODS

Semen collection and processing

Ejaculates (n=20) were obtained from sexually mature healthy Holstein-Friesian breeding bulls using an artificial vagina (Slovak Biological Services, a. s., Lužianky, Slovakia). The samples were transported to the laboratory within 15 minutes in thermos due to keeping a constant temperature. Immediately upon arrival, 100 μ L of each sample were aliquoted in test-tubes and stored at -20°C for the microbiological assessment. Only samples with a minimum of 70% motility and 1×10^9 sperm/mL were used for further experiments to meet the quality criteria given for the corresponding breed.

Progressive motility assessment

The computer-assisted sperm analysis (CASA; Version 14.0 TOX IVOS II; Hamilton-Thorne Biosciences, Beverly, MA, USA) was used to determine progressive motility. Samples were diluted in physiological saline solution (PS; sodium chloride 0.9% w/v, Bieffe Medital Grosotto, Italia) in a dilution ratio of 1:40. Subsequently, 10 μ L of each sample were placed into the Makler counting chamber (depth 10 μ m, 37°C; Sefi Medical Instruments, Haifa, Israel) and progressive motility of spermatozoa was assessed in 10 fields at a minimum 30 cells per field. Progressive motility of spermatozoa is determined by a velocity of \geq 25 μ m/s.

Cultivation and identification of bacteria

One hundred μ L of each sample were cultured in the blood agar, Gassner agar and Tryptic soy agar. The cultures were maintained at 37 °C during 24-48 h under aerobic conditions. The quantity of bacteria was evaluated by counting

Colony Forming Units (CFU/mL). Purification of all microorganisms was done by four ways streak plate method after the first cultivation.

Qualitative bacterial analysis of semen samples was performed by Matrix-assisted laser desorption ionization – time of flight (MALDI-TOF MS; Bruker Daltonics, Germany). Fresh overnight isolates were prepared in accordance to the manufacturer’s recommendations using the ethanol-formic acid extraction method. Sample spots were overlaid with 2 µL of matrix solution (saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid) and left to dry for 15 min in room temperature. Raw spectra of each isolate were compared to a spectra database in Biotyper software (version 2.0; Bruker Daltonics, Germany) without any user intervention (Hleba et al., 2017).

Biochemical analysis

Magnesium (Mg), Calcium (Ca), Phosphor (P), Urea (U), Aspartate transaminase (AST), Alanine transaminase (ALT), Alkaline phosphatase (ALP), Cholesterol (CHOL), Bilirubin (BILI), Triglycerides (TG), Albumin (ALB) and Uric acid (UA) were measured using the commercial kits DiaSys (Diagnostic Systems GmbH, Holzheim, Germany) on the Randox RX Monza analyzer (Crumlin, United Kingdom) (Kováčik et al., 2017).

Statistical analysis

All data were subjected to statistical analysis using the GraphPad Prism program (version 6.0 for Windows, GraphPad Software incorporated, San Diego, California, USA, <http://www.graphpad.com/>). Results of the mean quality parameters of ejaculate are quoted as arithmetic mean (AM) ± standard deviation (SD). Pearson correlation coefficient analysis for paired samples was used to evaluation of correlations between the quantity of bacterial CFU and quality parameters of semen. The level of significance for the correlation analysis was set at *** (P<0.001); ** (P<0.01); * (P<0.05).

RESULTS AND DISCUSSION

Mean values of sperm quality parameters

In this study we assessed the relationship amongst bacterial load and their interactions with progressive motility and biochemical parameters in seminal plasma of bovine ejaculates. Mean values of progressive motility, CFU and biochemical parameters are displayed in Table 1.

Table 1 Mean values of progressive motility, bacterial load and biochemical parameters

PARAMETER	PROG [%]	CFU [log CFU/g]	Ca [mM/L]	P [mM/L]	Mg [mM/L]	U [mM/L]	UA [mg/dL]
VALUE	66.83	2.70	6.81	3.22	3.78	1.12	1.72
S. D.	7.59	3.10	0.85	0.58	0.60	0.27	0.30
PARAMETER	AST [µkat/L]	ALT [µkat/L]	ALP [µkat/L]	CHOL [mM/L]	BILI [µM/L]	TG [mM/L]	ALB [g/dL]
VALUE	0.13	1.11	70.86	1.70	14.35	0.43	1.42
S. D.	0.07	0.21	11.24	0.38	2.49	0.06	0.22

Legend: PROG – progressive motility, CFU – colony forming units, Ca – calcium, P – phosphorus, Mg – magnesium, U – urea, AST – aspartate transaminase, ALT – alanine transaminase, ALP – alkaline phosphatase, CHOL – cholesterol, BILI – bilirubin, TG – triglycerides, ALB – albumin, UA – uric acid.

Juyena and Stelletta (2013) analysed basic biochemical parameters in bovine seminal plasma. In comparison to their study, results of our measurements were similar with respect to Ca, P and Mg. In accordance to Navya’s (2012) results, the mean bacterial load in the neat semen from Holstein-Friesian bulls was 4.51±0.87 log CFU/mL which was in accordance to our measurements of bacterial load in semen. Cholesterol concentration in the seminal plasma was determined to be 1.70±0.38 mmol/L, which was several times higher in

comparison to other reports. Concentration of UA was determined to be 1.12±0.27 mg/dL, which was much lower when compared to our results. On the other hand, there were increased concentrations of TG (0.43±0.06 mmol/L) and U (1.12±0.27) in our samples when compared to Čevik’s et al. (2007) report. The mean bilirubin and albumin concentrations are consistent with the results obtained in a previous study (Tvrdá et al., 2013).

Table 2 Identification and quantification of bacterial colonies [log CFU/mL] in bovine ejaculates isolated on different culture media

Sample no.	Blood agar	Gassner agar	TSA	Identified bacteria
1	2.00	n	n	<i>B. cereus</i>
3	n	n	2.70	<i>S. cohnii, S. kloosii</i>
8	2.30	n	2.70	<i>S. cohnii, S. kloosii, B. cereus, M. luteus</i>
9	2.70	n	2.48	<i>B. cereus, B. licheniformis</i>
11	3.18	n	3.41	<i>S. xylosum, S. aureus, S. warneri, S. lentus, S. epidermidis, B. mycoides</i>
12	2.48	n	2.30	<i>B. cereus</i>
13	3.08	n	3.11	<i>S. cohnii, S. kloosii</i>
14	2.30	n	2.48	<i>S. kloosii</i>
17	n	2.00	2.00	<i>S. aureus, S. haemolyticus</i>
20	n	n	2.30	<i>S. aureus, S. haemolyticus</i>

Legend: TSA – tryptic soy agar, n – no isolates

In 10 semen samples out of 20 we identified bacteria on the selected growth media. Amongst the colonies isolated from bovine ejaculates, we have identified the following bacteria: *Bacillus cereus*, *Staphylococcus cohnii*, *Staphylococcus kloosii*, *Micrococcus luteus*, *Bacillus licheniformis*, *Staphylococcus xylosum*, *Staphylococcus aureus*, *Staphylococcus warneri*, *Staphylococcus lentus*, *Staphylococcus epidermidis*, *Bacillus mycoides* and *Staphylococcus haemolyticus*. Zampieri et al. (2013) reported that the MALDI-TOF method is as exact as quick. The accuracy of this technique was proven by the DNA sequencing of bacterial strains isolated from bovine ejaculates. Last available report targeting on the identification of bacteria in bovine ejaculates proven the prevalence of species from genus *Staphylococcus*, mainly *S. aureus* (16%), *S. intermedius* (11%), *S. coagulans-negative* (8%), *S. epidermidis* (10%). Moreover,

Escherichia coli sp. (16%), *Pseudomonas aeruginosa* (13%), *Pseudomonas putida* (2%), *Pseudomonas testosteroni* (4%), *Micrococcus* sp. (11%), *Proteus mirabilis* (6%), and *Bacillus* sp. (4%) were identified in ejaculates of buffalo bulls (Andrabi et al., 2016). An older study reported the occurrence of the genus *Porphyromonas*, *Staphylococcus* ssp., *Lactobacillus* ssp., *Peptostreptococcus anaerobius*, *Streptococcus mitis*, *Alcaligenes faecalis*, *Neisseria weaveri*, *Haemophilus influenzae*, *Acinetobacter junii* and *Pseudomonas putida* in ejaculates obtained from Holstein bulls (González-Marín et al., 2011). Several previous studies have proven deleterious effects of bacterial load on quality of spermatozoa (Enwuru et al., 2016; Ďuračka and Tvrdá, 2018). However, there is no evidence about their effect on the composition of seminal plasma.

Table 3 Correlation analysis expressed through Pearson’s correlation coefficient (r) amongst progressive motility, CFU and biochemical parameters

	PROG	Ca	P	Mg	AST	ALT	ALP	CHOL	TG	BILI	ALB	U	UA
CFU	-0.736***	-0.264	-0.263	-0.921***	-0.206	-0.568**	-0.667**	-0.268	-0.122	-0.031	-0.305	-0.222	-0.397*
PROG	1	0.348*	0.206	0.592***	0.389	0.553*	0.837**	0.492	0.270	0.657*	0.626**	0.651**	0.572*

Legend: PROG – progressive motility, CFU – colony forming units, Ca – calcium, P – phosphorus, Mg – magnesium, U – urea, AST – aspartate transaminase, ALT – alanine transaminase, ALP – alkaline phosphatase, CHOL – cholesterol, BILI – bilirubin, TG – triglycerides, ALB – albumin, UA – uric acid; *** – P<0.001; ** – P<0.01; * – P<0.05.

In our study, the correlation analysis confirmed detrimental effect of bacterial load on the progressive motility of bovine spermatozoa, as there was strong negative (-0.736) and statistically significant ($P < 0.001$) correlation between PROG and CFU. **Moretti et al. (2009)** verified the prevalence of bacteria in human semen samples and the potential relationship with sperm quality. They pointed out that 86 men out of 230 men included in their study were considered fertile despite the fact that their semen samples were positive for various bacteria: *E. faecalis*, *E. coli*, *S. agalactiae*, *U. urealyticum*, *S. epidermidis*, *S. anginosus*, *M. morganii*. Except for the samples containing *S. agalactiae* and *S. anginosus*, the progressive motility was decreased when compared to WHO guidelines and significantly decreased when compared to controls. Apparently, an important role in the sperm quality may play hemolysin, a well-known virulence factor of enterococci and staphylococci (**Moretti et al., 2009; Pontieri, 2018**). The bacterial flagella and pili are considered to be an important determinant of pathogenicity. The mechanism of the bacterial damage of spermatozoa comes from the expression of the adhesive properties of the flagella and pili to mannose receptors at the surface of spermatozoa. Subsequently, the production of metabolic products and toxins originating from bacterial proliferation are in the direct contact with the sperm membrane (**Villegas et al., 2005; Moretti et al., 2009**). The correlation analysis revealed very strong (-0.921) and significant ($P < 0.001$) correlation between Mg concentration and CFU. Vice versa, significant positive correlation (0.592; $P < 0.001$) was observed between Mg concentration and PROG. As Mg is essential element during production of ATP, decreased concentration of Mg may be ultimately responsible for decreased sperm motility (**Valsa et al., 2013**). An important role in sperm metabolism play transaminases and phosphatases. Considering that Mg is essential element in spermiogenesis and as a cofactor in enzymatic reactions may Mg affect activity of these enzymes. In current study was observed negative correlation ($P < 0.01$) amongst activity of ALT (-0.568), ALP (-0.667) and CFU, which may be related to level of Mg in seminal plasma. **Yousef et al. (2007)** reported that the decrease in the activities of seminal plasma enzymes (AST, ALT) coincided with the decrease of semen quality. Therefore, the authors are in consensus that the presence of bacteria in semen may indirectly affect activity of transaminases through concentration of Mg in seminal plasma. A previous study (**Meena et al., 2015**) suggested that microbial contamination had an effect not only on the morphology, motility and quality parameters of spermatozoa, but also on the nutrient composition of seminal plasma. Considering that bacteria may compete for nutrients amongst each other (**Hibbing et al., 2010**), synergic effect of various opportunistic pathogenic bacteria may completely deprive nutrients in the seminal plasma. The role of ALP in semen is to catalyse the transport of phosphate groups into spermatozoa. Thereby, ALP contributes to processes associated with fertilization. A decreased ALP activity may be caused by infection of the epididymis. Moreover, a decreased ALP activity was correlated with a decreased spermatozoa concentration in various animal species (**Mollo et al., 1997; Pesch et al., 2006; Schäfer-Somi et al., 2013**). Despite that previous study did not consider albumin as a dominant protein (**Chard et al., 1991**), our study revealed its significant correlation (0.626; $P < 0.01$) with PROG. This correlation may be explained through its multiple-binding sites and free radical-trapping properties (**Tvrđá et al., 2013**). Although we expected a significant correlation between Ca and CFU, only a weak negative correlation (-0.264) was observed. On the other hand, significant correlation (0.348; $P < 0.05$) was observed between Ca and PROG. According to **Rahman et al. (2014)** Ca uptake is associated with the entire process of fertilization – from hyperactivation, chemotaxis, capacitation to the acrosome reaction and reaching an oocyte. Concentration of UA reflected in both, CFU and PROG. While the correlation with PROG was significant ($P < 0.05$) and positive (0.572), the correlation with CFU was significant ($P < 0.05$) and negative (-0.397). This phenomenon may be explained through potent antioxidative properties of UA. From the studies above it is apparent that various bacterial species may produce free radicals. On one hand, UA may scavenge hydroxyl radicals. On the other hand, UA converts to U which, according to several studies (**Allahkarami et al., 2017; Halo Jr. et al., 2018**), may be related to a decreased sperm motility, which is in conflict with our results. The correlation analysis revealed significant ($P < 0.01$) and positive (0.651) correlation between PROG and U.

CONCLUSION

Our study investigated the bacterial composition of fresh semen from healthy Holstein-Friesian breeding bulls. The results of bacterial identification showed the prevalence of species from *Staphylococcus* genus, mainly *S. aureus*, *S. haemolyticus*, *S. epidermidis*, *S. cohnii* and *S. kloosii*. Furthermore, *B. cereus*, *B. licheniformis*, *M. luteus*, *B. mycoides*, *S. xylosum* and *S. warneri* were identified in fresh semen. The second part of our study dealt with associations amongst bacterial load present in ejaculates, progressive motility of spermatozoa and biochemical composition of seminal plasma. The correlation analysis revealed strong negative association between bacterial load and concentration of Mg. The decreased amount of Mg may affect the activity of ALP and ALT. Furthermore, strong negative correlation was observed between progressive motility of spermatozoa and bacterial load. Therefore, we may conclude that the bacterial load may modulate the seminal plasma composition which may lead to an

impaired nutrition for spermatozoa. Further studies are needed to understand deeply the mechanisms behind the effects of bacteria on spermatozoa.

Acknowledgements: This study was supported by the European Community Project no. 26220220180: Building Research Centre „AgroBioTech“, by the Scientific Grant Agency of the Ministry of Education of the Slovak Republic and by the Slovak Research and Development Agency Project no. APVV-15-0544.

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