MEDWIN PUBLISHERS
Committed to create value for Researchers

Open Access Journal of Veterinary Science & Research
ISSN: 2474-9222

Boar Quality Semen Testing and Presence of Mycoplasma Organism

Milovanović A, Petrović T, Apić J, Lupulović D, Barna T, Lazić G and Lazić S*

Scientific Veterinary Institute, Novi Sad, Novi Sad, Republic of Serbia

***Corresponding author**: Lazić S, Scientific Veterinary Institute, Novi Sad, Novi Sad, Republic of Serbia Email: lazic@niv.ns.ac.rs

Research Article

Volume 2 Issue 3

Received Date: April 15, 2017

Published Date: June 13, 2017

Abstract

This paper describes results of mycoplasma organism and *Mycoplasma hyopneumoniae* detection in semen of boars. During routine examination of semen quality in 2015 and 2016, different percent of specific changes in the spermatozoa of 23 boars were observed and these samples were subjected to mycoplasma detection. These changes were manifested as frequent distal midpiece reflex abnormalities with sporadic coiled principal piece; but booth loops were filed with fine, netlike/reticular forms ("entrapped pseudocytoplasmatic droplet"). Based on the observed morphological forms it is suspected on the presence and influence of microorganisms, primarily of Mycoplasma origin.

PCR and real time PCR molecular methods were examined in all suspected sperm samples. The presence of *Mycoplasma spp* was found in 15 samples, of which, *Mycoplasma hyopneumoniae* was found in 8 samples. In the remaining seven samples differentiation to other mycoplasma species were not carried out.

This article is indicating that genital form of mycoplasma could manifest its effect on semen quality and this may be more significant than current literature data are indicating and recognizing as problem in boars. In the same time, its high incidence in suspected semen samples could be more stressed as a source of sexually transmitted infection.

Further estimation of *Mycoplasma* influence on boar semen quality is needed.

Keywords: Sperm; Boars; *Mycoplasma spp; Mycoplasma Hyopneumoniae*; PCR; Real Time PCR

Introduction

Profitability in pig industry largely depends on male and female fertility because pig is a multi-birth species. Production of sizeable and even litters, with high number of raised piglets is of paramount importance [1].

The sperm quality of ejaculates (concentration, motility, viability and abnormal morphology) is one of the primary factors for selecting males for livestock production, especially for artificial insemination [2]. Besides general guides for semen quality traits, boar often manifest different breeding results regardless to standard and sophisticated laboratory tests applied in semen examination. Data from the literature [3-6] have shown that these parameters have different correlation with fertility, from 0.06 to 0.86, and that none of the tests is consistently correlated with fertility.

Morphologic abnormalities are categorized as primary (associated with sperm head and acrosome), secondary (presence of droplet) and tertiary referred to other tail defects [7, 8]. This can indicate on locus of

defects and approximate recovery period. Number of textbook or articles specialized for detailed sperm morphology; its incidence, pathogenesis and effect on fertility are limited for bulls [9] and for boars [8, 10-13].

Factors influencing the quantity and quality of semen harvested from domestic animals are numerous [14]. Besides clear direct and indirect effects of different microorganisms presence in boars semen [15-17], presence of mycoplasma in boar reproductive tract is neglected as important, according to current literature data. Thus, genital Mycoplasmosis is not indicated and recognized as problem in boars. However, infection of the genitourinary tract with mycoplasmas is common in many animal species [18]. For example, strains of *Mycoplasma canis* were reported to cause orchitis and epididymitis following ductus deferens inoculation [19] and purulent endometritis following intrauterine inoculation [20] in dogs. Jurmanova and Sterbova [21] comparing mycoplasma positive samples with those that were negative revealed a significant correlation between semen contamination and impaired spermatozoa motility.

In cattle, studies have demonstrated that there is a significant and unpredictable variation in the numbers of organisms present in semen collected at different times from the same bull. The preputial cavity appears to be the main source of semen contamination, however, it has been demonstrated that the urethra is also heavily colonized [22].

Frozen-thawed bovine semen contaminated with *M. bovis* or *M. bovigenitalium* and used for oocyte insemination has negative impact on subsequent embryo development to the blastocyst stage. Isolation of motile spermatozoa by swim-up procedure, supplementation of culture media with standard antibiotics and washing embryos as recommended by IETS were not effective in rendering IVF embryos free from *M. bovis* and *M. bovigenitalium*. These results indicate that mycoplasmas present in semen can be transmitted through the IVF system and infect embryos [23].

The addition of *M. bovis* to unextended and extended fertile Holstein bull semen significantly reduced sperm penetration rates and the mean number of sperm per penetrated zona pellucida-free hamster oocytes. Similarly, the ability of spermatozoa to form pronuclei and the activation of penetrated oocytes were adversely affected by *M. bovis*. No apparent effect on sperm motility was detected [24].

Recent evidence suggests that *M. bovis* strains in Europe are becoming resistant to antibiotics traditionally used for treatment of mycoplasma infections in particular oxytetracyclines, tilmicosin, and spectinomycin [25].

All cited articles suggest that stress of *Mycoplasma* effects is evident in bovine reproduction. Opposite, up to now, *Mycoplasma spp.* is not detected as direct disturber of reproductive traits in pig production, at least not in boar.

Transmission of *M. suis* by semen is rare, since it only occurs in the case of blood contamination [26]. Shin et al. [27] demonstrated a pathogenic strain of *M. hyorhinis* thought to cause abortions in sows. However, the clinical relevance of *Mycoplasma spp.* in relation to reproductive performance remains doubtful. Its effect on semen quality is even less clarified according to actual literature review.

Mycoplasma organisms are common causes of various diseases in domestic animals and birds. In swine is usually proved by *Mycoplasma hyopneumoniae* and *Mycoplasma hyorhinis*, although they can also find other types of mycoplasma, such as *Mycoplasma flocculare, Mycoplasma hyopharyngis*, *Mycoplasma lipophilum*, which are non-pathogenic for pigs [28-30].

There is almost general agreement that mycoplasmas play a major etiological role in porcine pneumonia. *Mycoplasma hyopneumoniae* is a common causative agent of enzootic pneumonia, and chronic respiratory disease in pigs, which causes great economic losses in pig farming all over the world [31-33]. In affected herds with *M. hyopneumoniae* a large number of pigs with severe clinical pneumonia with a smaller number of dead pigs were diagnosed [34]. Chronic form of the disease is widespread in swine populations and the disease is characterized by high morbidity but a small percentage of mortality [35]. Frequently, *M. hyopneumoniae* with *Porcine reproductive and respiratory syndrome virus (PRRSV), Porcine circovirus type 2 (PCV2), Pseudorabies virus*, causing very severe forms of pneumonia, typically resulting in death [36, 37, 18].

The presence of bacteria in the boar's semen significantly affects the reproductive ability of sperm for artificial insemination. It has been proved the presence of Gram negative bacteria in the prepared dose of semen for artificial insemination of sows, which are most often of faecal origin [38,39]. However, there is little literature data that indicate that the presence of *Mycoplasma* have impact on sperm quality. Infections and secretion from urogenital tract with *Mycoplasma spp.* is possible in boars in the case of their penetration and cross into the bloodstream [18].

A better understanding of the possible anomalous forms in the ejaculate is of interest to obtain the best possible qualitative estimation of the doses in artificial insemination. Qualitative exploration of a sperm sample (ejaculate or dose) could allow the detection of the causal factor and then it would be possible to improve the results [12]. For this reason, some abnormal forms were often noted on sperm cells that can be referred as indicative to set up a doubt for presence of *Mycoplasma* strains.

Material and Methods

Semen quality control in boars is carried out at the Scientific Veterinary Institute "Novi Sad" based on continuous cooperation with the farm's centers for boars' semen production or semen was delivered to institute by occasion. Seven stud boar facilities ranging from 10 to 90 boars (Jorkshire, Durock and Landrace breed) that served for semen production were continuously analyzed trough a year for semen quality control and 5 studs were randomly delivering semen of suspected quality and with obvious fertility problems. Number of sows per farm were different and ranged from 150 - 1,200.

Boars and farm technology were very different between farms. Strict hygiene measures for disease control and prevention were not implemented on all studs. The boars were kept in the individual boxes, with air-condition and fed according to recommendations. Dose samples from regular control were collected at 2 month intervals and randomly subjected to bacterial analyses. Each case of sudden drop in semen quality and in cases of constant low semen quality in some boars was obligatorily subjected to bacterial count (CFU/mL), bacterial determination and for antibiotic sensitivity test. Semen was taken by manual fixation with gloved-hand technique. Pre-sperm fraction was discarded and 20 ml of semen was taken directly in sterile plastic pots for bacterial survey and *Mycoplasma* detection. Rest of semen was regularly processed for dose production. Three hours after collection, the semen was transferred to the Laboratory of reproduction at the Veterinary Institute "Novi Sad" in clima boxes and processed for bacterial control and PCR analyses. Semen quality was analyzed 24 hours after collection (we noted that sperm motility was better-stabilized, 24 hours after dilution in semen extender).

On some farms, security level for boar was not strong enough. Facilities were directly connected with sows' objects and boars were even used for estrus detection and same person was working with different pig categories and then continued to produce semen.

Boars' semen quality control consisted of:

1. CASA (Computer Assisted Sperm Analysis, ISAS, Proiser, Spain) for assessing concentration, total and progressive motility and spermatozoa speed values;
2. flow cytometry analyses (Guava Milipore-IMV, USA) for sperm chromatin structure assay - SCSA test (acridine orange, Invitrogen), and test of membrane and acrosome integrity (PNA-FITC/PI, Invitrogen);
3. direct and detailed cyto-morphological examination of the stained eosine-nigrosine sperm sample were carried out under oil immersion with phase contrast objective, 1000x magnification (Olympus BX-40, Japan). Photos were taken with addition magnification by Olympus SP-500UZ digital camera. The spermatozoa morphology was assessed according to Barth and Oko [9] and semen was classified according to quality criteria in four class (I, II, III and out of class).

The data obtained by CASA, flow cytometry and cyto-morphologic examination were used for final semen quality evaluation. The doses were discarded (scored as "out of class") in the following situation: CASA parameters-total motility spermatozoa <60%, progressive motile under 30%, agglutinations more than 40%; flow citometry-membrane integrity <30%, acrosome defect ≥30%); and cytology sperm quality parameters - total live spermatozoa <70%, live with intact acrosome <60%, damaged acrosome and protoplasmatic droplet ≥30% or total abnormal spermatozoa ≥40%). In some cases, compensation of semen quality with higher number of spermatozoa was advocated.

During the routine laboratory testing of boar sperm samples, microscopic changes were observed on the spermatozoa similar to those that we noticed in dogs positive on *Mycoplasma* organisms, related to low/absence of conception, gross sperm damages and no specific bacterial contamination (or low level of CFU/ml). These observed changes were manifested as distal midpiece reflex abnormalities with sporadic coiled principal piece; but booth loops were reconnected with fine nets (so called "entrapped pseudocytoplasmatic droplet"). These forms of spermatozoa with gross lesions and low cyto-morpology rate for live normal spermatozoa with intact acrosome we have first detected in dogs that were positive on PCR reaction [26]. Low bacterial load or its absence encouraged us to start collecting and analyzing boars semen on the presence of mycoplasma as reflection of similar morphologic changes, knowing that in sows/boars there are no clear evidence of its reproductive effects, above all, its effect on sperm production and maturation in boars, opposite to dogs.

In 2015 and 2016, 23 semen samples of suspected boars were selected and subjected to mycoplasma detection from 8 different farms.

Detection of *Mycoplasma Hyopeumoniae* by Real Time PCR

In order to determine the extent of *M. hyopneumoniae* infections in diseased pigs a real time PCR assay was carry out. Extraction of total DNA from samples of boars semen was done with "QIAamp® DNA Mini Kit", (Qiagen, Germany) following manufacturer's instructions.

M. hyopneumoniae real-time PCR was performed by using TaqMan Universal PCR Master kit (Applied Biosystems) and previously described primers designed to amplify the P97 cilium adhesion gene (mhp183) [40]. The product of this well-characterized gene is important for the adherence of *M. hyopneumoniae* to ciliated epithelium within the respiratory tract, and because it is thought to be necessary for virulence, this gene is likely to be present in all pathogenic isolates of *M. hyopneumoniae*. The forward and reverse primers and probe sequences were as follows: forward Mhp183 F (5`-CCAGAACCAAATTCCTTCGCTG-3`), reverse Mhp183 R (5`-ACTGGCTGAACTTCATCTGGGCTA-3`) and probe Mhp183 P (5`-FAM-AGCAGATCTTAGTCAAAGTGCCCGTG-BHQ_1-3`) [40]. The 25 µl PCR reaction mix included 1 µl (800 nM) of each primer, 1 µl (200 nM) of dual labeled fluorogenic probe, 12.5 µl of TaqMan Universal PCR Master Mix, 4.4 µl of nuclease-free water, and 5 µl of DNA template. The parameters for the real-time assay performed on 7500 ABI PCR instrument was set as follows: 1 cycle of 50°C for 2 min, initial denaturation at 95°C for 15 min, and 40 cycles of 95°C for 15 sec, and annealing and extension at 60°C for 1 min.

Detection of *Mycoplasma spp* by PCR

Detection of the genome of *Mycoplasma spp.* was performed using molecular PCR method. This test, as well as highly sensitive and specific, was used to directly determine the presence of mycoplasma in the samples of boar sperm. Extraction of total DNA was done with "QIAamp® DNA Mini Kit", (Qiagen, Germany) following manufacturer's instructions. PCR method was carried out using the "HotStar Taq Master Mix Kit" (Qiagen, Germany), with small modification of manufacturer's instructions. Briefly, the amplification reaction was carried out in a volume of 25 µl containing 3 µl of DNA, 12.5 µl of "HotStarTaq Master Mix" and 25 pmol of each primer. The sample of DNA isolate of *Mycoplasma hyopneumoniae* was used as a positive control and DNA of PCR clean water was used as a negative control in each of PCR reaction. In the PCR reaction were used specific primers to detect all species of the genus *Mycoplasma* as well as other species of the class *Mollicutes* (*Spiroplasma* and *Ureoplasma Acholeoplasma* types), and which are specific for 16S rRNA part of the genome: forward GPO3 5'-GGGAGCAAACAGGATTAGATACCCT-3' (position in the genome 774-798 in 16S rRNA gene) and reverse: MGSO 5'-TGCACCATCTGTCACTCTGTTAACCTC-3' (position in the genome 1055-1029 in 16S rRNA gene), which previously describe by Van Kuppeveld et al. [41] and Ossewaarde et al. [42]. Amplification conditions (Thermocycler Gradient, Eppendorf, Germany) were as follows: 95^{0}C 15 min, 40 cycles of 95^{0}C 30s, 55^{0}C 30s, 72^{0}C 30s, and a final extension at 72^{0}C for 10 minutes. PCR fragments were separated by electrophoresis (Hoefer HE 33 Mini Submarine, „Amersham Biosciences") on 1.5% agarose gel („Invitrogen Life Technologies", Great Britain) and were visualized on a transilluminator („TFX-35.M", Vilber Lourmat, France).

Results and Discussion

Sperm cells in some situation can gave characteristic types of anomalies [43], each one due to a specific causal agent, but in most cases these effects on sperm morphology is unknown. A better understanding of the possible anomalous forms in the ejaculate is of interest to obtain the best possible qualitative estimation of the doses and risk factors in semen production for artificial insemination.

Nets like forms (pseudodroplets) in midpeace bending or in tail loops were main morphology characteristic of sample selection of analyzed group and subjected to *Mycoplasmal* detection.

The presence of *Mycoplasma spp.* was found in 15/23 samples (65.21%), of which, *M. hyopneumoniae* was found in 8 samples. In the remaining seven samples differentiation to other mycoplasma species were not performed.

Most of samples were classified as *"Out of class"* according to above mentioned criteria (19/23; 82,60%). With higher presence of suspected morphology traits that we considered to be indicative for mycoplasmatic infection, the higher incidence of positive reaction was noted. Only 2 cases with distal mid piece reflex abnormalities with very high incidence (50% and more)-bent principal piece with entrapped cytoplasmic droplet were not positive, which can indicate on continuous shedding of *Mycoplasma* and/or divergence of possible causative agent presence in semen and its effects on semen morphology (slowly development/regression of sperm abnormalities in affected animals).

One positive semen sample had 82% of live sperm cells with intact acrosome on cyto-morphological smear, 86% total motile sperm cells on CASA and 83% in flow cytometry. Semen exhibited slightly progressive motility (31.5%), with a mild increase in the proportion of spermatozoa with protoplasmic droplets (19%, the lower limit is 15%), which indicates a problem in the maturation of sperm cells. Regardless, this kind of semen quality cannot be declared to be reason of increased repeat breeding on farm in sows and gilts, but it is a way of further spread of infection, due to the proven presence of the genome of *Mycoplasma spp.* in sperm of breeding boars.

Suspected boars were mainly with no gross bacterial contamination.

According to Barth and Joko [9] this form of sperm tail defects is uncommon in bulls and primarily occurs in association with distal midpiece reflex defect originating in the epididymis, with sporadic coiled principal piece.

(Figures 1-4) Morphology of infertile dog semen, PCR positive for *Mycoplasma spp.* Degenerative forms with principal piece reflection around pseudodroplet and coiled tails with same effect are dominant forms. Also, presence of 4 lymphocytes can be noted on first Figure (eosine/nigrosine stain, magnification 1000×).

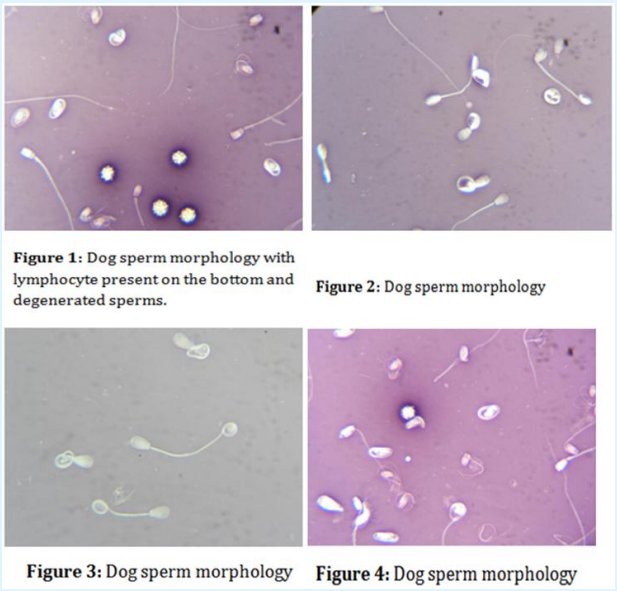

Figure 1: Dog sperm morphology with lymphocyte present on the bottom and degenerated sperms.

Figure 2: Dog sperm morphology

Figure 3: Dog sperm morphology

Figure 4: Dog sperm morphology

(Figures 5-8) Morphology of *Mycoplasma* PCR positive semen in boars. Midpiece and principal piece bending around netlike pseudodroplet are dominant forms. Coiled tails with same effect are very rare (eosine/nigrosine stain, magnification 1000×).

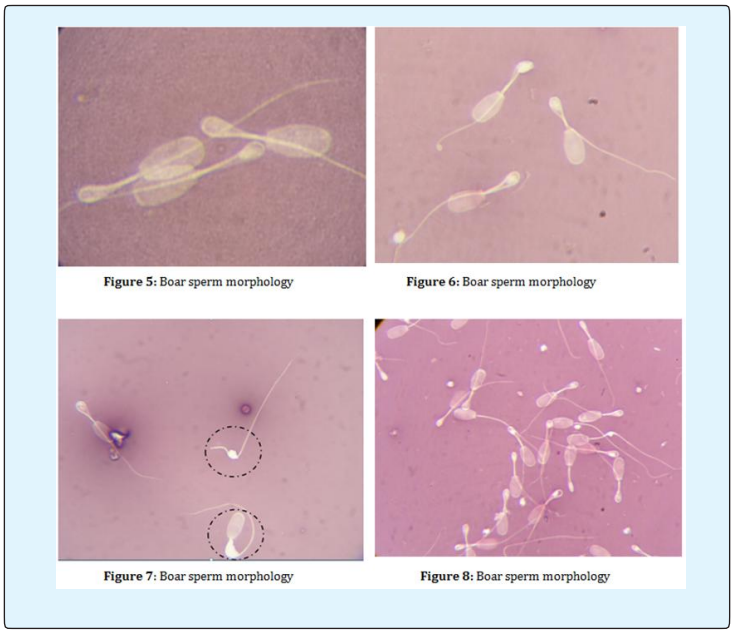

Figure 5: Boar sperm morphology

Figure 6: Boar sperm morphology

Figure 7: Boar sperm morphology

Figure 8: Boar sperm morphology

Mycoplasma spp. and *Mycoplasma hyopneumoniae* detection is given in Figures 9 and 10.

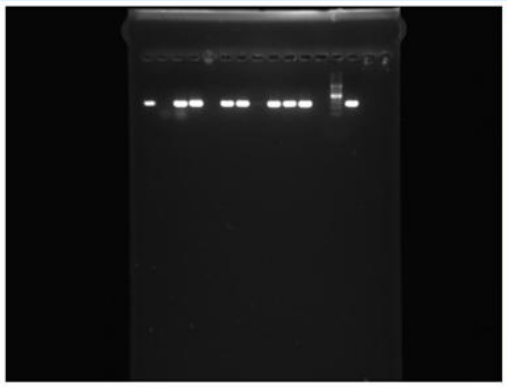

Figure 9: Detection of *Mycoplasma spp.* by molecular PCR method

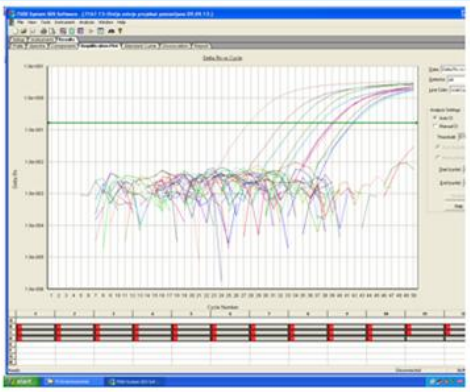

Figure 10: Detection of *M. hyopneumoniae* by molecular real time PCR method

Straw et al. [13] state that the problems in pigs caused by mycoplasmas (*M. hyopneimoniae, M. hyorhinis, M. hyosynoviae arthritis, M. haaemosius*) reflected disorders related to the respiratory tract, arthritis, polyserositis, otitits and anemia, depending on the pathogen. Furthermore, alleged that there is very little data about infection of the urogenital tract and disorders that could cause by infection with mycoplasmas. *M. hyopneumoniae* associated with enzootic pneumonia is playing a major role in the complex of respiratory diseases of pigs, which represents a continuous problem during production for swine producers. Transmission of *M. hyopneumoniae* in many herds begins with sow - piglet contact, and after the establishment of infection transmission occurs between individuals of the same box. Economic losses resulting from disorders provoked mycoplasmas are characterized by high morbidity in terms of lower progression of the diseased pigs on a daily basis as is due to poorer feed conversion and increased costs for medication.

Evidence of Mycoplasma organism in the boars' semen, as well as evidence of changes in sperm quality caused by *Mycoplasma*, according to the literature are not presented. However, infections of the urogenital tract with *Mycoplasma spp.*, in the case of their cross into the bloodstream can be excreted with sperm [18] is possible in boars. In this study, it has been demonstrated the presence *Mycoplasma spp*. in boars semen, which was probably caused by their contact with infected sows and other pigs as a consequence of low implementation of biosecurity measures. In Serbia, in commercial pig farms, *Mycoplasmal* infection is very widespread, and the *M. hyopneumoniae* is a common cause of respiratory syndrome pigs of all categories.

The observed changes of sperm cells in boars' semen were very similar to changes observed in dogs' spermatozoa infected with *Mycoplasma*.

Conclusion

High incidence of net forming pseudodroplet on bent parts of principal, midpiece or tail of boars' sperm cells was suspected to be provoked by *Mycoplasma spp.* presence in semen. Molecular PCR method and real time PCR confirmed *Mycoplasma spp.* in 15 sperm samples from 23; and *Mycoplasma hyopneumoniae* was detected from 8 positive samples.

This article is indicating that genital form of mycoplasma could manifest its effect on semen quality and this may be more significant than previously recognized. Its high incidence in suspected semen samples could be more stressed as a source of sexually transmitted infection.

Obviously, fertility of sperm positive for *Mycoplasma spp.* is reduced, which affect the conception of sows and far rowing rate, as well as the way of expansion and maintenance of Mycoplasma infection with all possible consequences in swine herds.

These findings have to stimulate additional research into the role of mycoplasmas in boar infertility. Our observations are just uncontrolled field reports confirmed by laboratory tests. Semen of reported animals was from apparently healthy animals. Experimental transmission studies on SPF and mycoplasma free boars are needed to demonstrated direct impact of some mycoplasma species, their pathogenicity and role in male and female porcine reproductive disease. Otherwise, the true role of *Mycoplasmal* isolates in reproductive disease still

remained in doubt. Typing is also needed to determine if specific strains are associated with disease.

As conclusion, further estimation of Mycoplasma influence on boar semen quality is needed.

Acknowledgements

This work was supported by the Ministry of Science and Technological Development of the Republic of Serbia, grants TR 31084 and TR 31071.

References

1. Gadea J (2005) Sperm factors related to in vitro and in vivo porcine fertility. Theriogenology 63(2): 431-444.

2. Bonet S, Briz M, Fradera A (1993) Ultrastructural abnormalities of boar spermatozoa, Theriogenology 40(2): 383-396.

3. Graham EF, Schmehl KL, Nelson DS (1980) Problems with laboratory assays, In: Proc 8th Tech Conf AI and Reprod, Milwaukee, Wis, NAAB, pp. 1-8.

4. Amann PR (1989) Can the fertility Potential of a Seminal Sample Be Predicted Accurately? J Androl 10(2): 89-98.

5. Quintero-Moreno A, Rigau T, Rodriguez-Gilje (2004) Regression analyses and motile sperm subpopulation structure study as improving tools in boar semen quality analysis, Theriogenology 61(4): 673-690.

6. Didion BA (2008) Computer-assisted semen analysis and its utility for profiling boar semen samples, Theriogenology 70(8): 1374-1376.

7. Hafez B, Hafez ESE (2000) Reproduction in Farm Animals 7th (Edn.), Lippincott Williams & Wilkins, Philadelphia, pp. 98.

8. Donadeu M (2004) Advances in male swine artificial insemination (AI) techniques. The Pig Journal 54: 110-122.

9. Barth AD, Oko RJ (1989) Abnormal Morphology of Bovine Spermatozoa, Iowa State University Press, Ames, IA, US.

10. Martín RS (1982) Reproduccidn e Inseminacidn Artificial Porcina, Aedos, Barcelona, pp. 124.

11. Bonet S (1987) Estudio del eyaculado de un verraco estresado por la frecuencia de recogidas en inseminación artificial. Sci Gerundensis 13: 35-40.

12. Bonet S (1990) Immature and Aberrant Spermatozoa in the Ejaculate of Sus domesticus. Animal Reproduction Science 22: 67-80.

13. Straw BE, Zimmerman JJ, D'Allaire S, Taylor DJ (2006) Diseases of Swine 9th(Edn.), Blackwell Publishing Asia, Carlton, Australia, pp 701-717.

14. Foote RH (1978) Factors influencing the quantity and quality of semen harvested from bulls, rams, boars and stallions. J Anim Sci 47(S2): 1-11.

15. Maroto Martín LO, Muñoz EC, De Cupere F, Van Driessche E, Echemendia-Blanco D, et al. (2010) Bacterial contamination of boar semen affects the litter size. Anim Reprod Sci 120(1-4):95-104.

16. Sepúlveda L, Bussalleu E, Yeste M, Bonet S (2014) Effects of different concentrations of Pseudomonas aeruginosa on boar sperm quality. Animal Reproduction Science 150(3–4): 96-106.

17. Milovanović A, Barna T, Vasiljević T, Milanov D, Uzelac Z, et al. (2012) Quality of boars semen and number of bacteria in ejaculates. organizer Faculty of Agriculture, [editor in chief Slavča Hristov], Proceedings of the First International Symposium on Animal Science, November 8-10th 2012, Belgrade, Serbia, 1, Str. 71-79, Beograd, Faculty of Agriculture.

18. Maes D, Nauwynck H, Rijsselaere T, Mateusen a B, Vyt aP, et al. (2008) Diseases in swine transmitted by artificial insemination. Theriogenology 70(8): 1337-1345.

19. Laber G, Holzmann A (1977) Experimentally induced mycoplasmal infection in the genital tract of the male dog. II Andrological and microbiological investigations after exposure to mycoplasmas. Theriogenology 7(4): 177-188.

20. Holzmann A, Laber G, Walzl H (1979) Experimentally induced mycoplasmal infection in the genital tract of the female dog. Theriogenology 12: 355-370.

21. Jurmanova K, Sterbova J (1977) Correlation between impaired spermatozoan motility and mycoplasma findings in bull semen. Vet Rec 100(8): 157-158.

22. Doig PA (1981) Bovine Genital Mycoplasmosis. Can Vet J 22(11): 339-343.

23. Bielanski A, Devenish J, Phipps-Todd B (2000) Effect of Mycoplasma bovis and Mycoplasma bovigenitalium in semen on fertilization and association with in vitro produced morula and blastocyst stage embryos. Theriogenology 53(6): 1213-1223.

24. Eaglesome MD and Garcia MM (1990) The effect of Mycoplasma boris on fertilization processes in vitro with bull spermatozoa and zona-free hamster oocytes. Vet Microbiol 21: 329- 337.

25. Ayling, RD, Baker SE, Peek ML, Simon AJ, Nicholas RAJ (2000) Comparison of in vitro activity of danofloxacin, florfenicol, oxytetracycline, spectinomycin and tilmicosin against recent field isolates of Mycoplasma bovis. Veterinary Record 146(26): 745-747.

26. Heinritzi K (1999) Eperythrozoonosis. In: Straw B, et al. (Eds.), Diseases of swine 8th (Edn.), Iowa State University Press, Ames, IA, USA, pp: 413-418.

27. Shin JH, Joo HS, Lee H, Seok HB, Calsaming M, et al. (2003) Identification and Characterization of Cytopathogenic Mycoplasma hyorhinis from Swine Farms with a History of Abortions. J Vet Med Sci 65(4): 501-509.

28. Bereiter M, Young TF, Joo HS, Ross RF (1990) Evaluation of the ELISA and comparison to the complement fixation test and radial immunodiffusion enzyme assay for detection of antibodies against Mycoplasma hyopneumoniae in swine serum. Veterinary Microbiolgy 25(2-3): 177-192.

29. Kwon D, Choi C, Chae C (2002) Chronologic localization of Mycoplasma hyopneumoniae in experimentally infected pigs. Vet Pathol 39(5): 584-587.

30. Friis NF, Ahrens P, Hagedorn-Olsen T, Nielsen EO, Kokotovic B (2003) Mycoplasma hyopharyngis Isolation From Swine. Acta vet scand 44(1-2): 103-104.

31. Vicca J, Stakenborg T, Maesa D, Butaye P, Peeters J, et al. (2003) Evaluation of virulence of *Mycoplasma hyopneumoniae* field isolates. Veterinary Microbiology 97(3-4): 177-190.

32. Ruiz A, Galina L, Pijoan C (2002) Mycoplasma hyopneumoniae colonization of pigs sired by different boars. Can J Vet Res 66(2): 79-85.

33. Beilage E, Rohde N, Krieter J (2009) Seroprevalence and risk factors associated with seropositivity in sows from 67 herds in north-west Germany infected with Mycoplasma hyopneumoniae. Preventive Veterinary Medicine 88(4): 255-263.

34. Opriessnig T, Thacker EL, Fenaux YuM, Meng X-J, Halbur PG (2004) Experimental Reproduction of Postweaning Multisystemic Wasting Syndrome in Pigs by Dual Infection with Mycoplasma hyopneumoniae and Porcine Circovirus Type 2. Vet Pathol 41(6): 624-640.

35. Fano E, Pijoan C, Dee S (2005) Dynamics and persistence of Mycoplasma hyopneumoniae infection in pigs. Can J Vet Res 69(3): 223-228.

36. Opriessniga T, Madsona DM, Schalka S, Brockmeierb S, Shena HG, et al. (2011) Porcine circovirus type 2 (PCV2) vaccination is effective in reducing disease and PCV2 shedding in semen of boars concurrently infected with PCV2 and Mycoplasma hyopneumoniae. Theriogenology 76(2): 351-360.

37. Fablet C, Marois-Créhanb C, Graslandc B, Simond G, Rosea N (2016) Factors associated with herd-level PRRSV infection and age-time to seroconversion in farrow-to-finish herds. Vet Microbiol 192: 10-20.

38. Kuster CE, Althouse GC (2016) The impact of bacteriospermia on boar sperm storage and reproductive performance Theriogenology 85(1): 21-26.

39. Althouse GC, Kuster CE, Clark SG, Weisiger RM (2000) Field investigations of bacterial contaminants and their effects on extended porcine semen. Theriogenolgy 53(5): 1167-1176.

40. Strait EL, Madsen ML, Minion FCh, Christopher-Hennings J, Dammen M, et al. (2008) Real-Time PCR Assays To Address Genetic Diversity among Strains of *Mycoplasma hyopneumoniae.* Journal of Clinical Microbiology 46(8): 2491-2498.

41. Van Kuppeveld FJM, van der Logt JTM, Angulo AF, van Zoest MJ, Quint WGV, et al. (1992) Genus-and species-specific identification of mycoplasmas by 16S rRNA amplification. Appl Environ Microbiol 58(8): 2606-2615.

42. Ossewaarde JM, De Vries A, Bestebroer T, Angulo AF (1996) Application of a Mycoplasma Group-Specific PCR for Monitoring Decontamination of Mycoplasma-Infected Chlamydia sp. Strains. Appl Environ Microbiol 62(2): 328-331.

43. Sironen A, Uimari P, Nagy S, Paku S, Andersson M, Vilkki J (2010) Knobbed acrosome defect is associated with a region containing the genes STK17b and HECW2 on porcine chromosome 15. BMC Genomics 11: 699.