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The correlation between vaginal microflora and vaginal cytology in European bison

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Abstract: European bison increasingly face a number of reproduction disorders. It can result in an increased risk of their extinction. Thus, microbiological analysis of vaginal swabs seems to be an important diagnostic method in monitoring of European bison reproductive tract infections. However, the first research in this area was carried out only by Speck *et al.* in 2006, but its results are uncompleted. Thus, the aim of this study was the continuation of research on E. bison vaginal cytology extended by microbiological examination and correlation of obtained results.

The female E. bison included in our study came from either free ranging herd or enclosures. They were divided into three groups. The first group consisted of animals without any lesions in reproductive tract. Animals from the second group had pathological lesions in genital organs. The third group included pregnant females. Cytological and microbiological samples were collected from vaginal mucosa with a sterile swab from all individuals.

Microbiological analysis of vaginal swabs provided less specific test compared to vaginal cytology, as most of isolated bacteria species were found in all examined groups of E. bison females with equal frequency. However, in animals with gross pathological lesions, the characteristic changes in vaginal cytology confirming necropsy, were observed.

Key words: European bison, reproduction, vaginal cytology, microbiological swab

Introduction

Microbiological analysis of vaginal swabs is a routine laboratory test successfully used in diagnostics of reproduction disorders in cattle (Wang *et al.* 2013). Microbiological examination is very helpful in prevention of reproductive tract infections, and is of significant importance in cattle husbandry, as it helps to significantly decrease economic losses caused every year world-wide by bacterial infections of genital organs in cows. Thus, many studies have been conducted to determine the physiological microflora of bovine reproductive tract and etiological factors of genital organs infections in domestic cattle (Sens and Heuwieser 2013).

Despite that E. bison live in different environmental conditions, they also face a number of reproduction disorders (Olech 2009; Krasińska and Krasiński 2010). That may result in an increased risk of their extinction. Therefore, cytological evaluation of E. bison vaginal swabs has been carried out (Olbrych *et al.* 2013). The second important issue in prevention of reproductive tract disorders in this species, is microbiological analysis of vaginal swabs. However, the pioneer research in this area was carried out only in 2006 and its results are uncompleted (Speck *et al.* 2006). Thus, this study is the continuation of our previous research of E. bison vaginal cytology, extended by microbiological examination and correlation of obtained results.

Material and methods

All animals included in this study were culled at Białowieża Forest and in European Bison Breeding Centre at Smardzewice from 2011 to 2015. The animals came from either free ranging herd or enclosures. All examined animals were eliminated, because of clinically evident diseases. In most females gross examination revealed pathological lesions in various organs including: lung, liver, lymph nodes and urogenital tract. One individual was eliminated because of the severe lameness caused by gastrocnemius bursitis, as it was confirmed anatomopathologically. Another animal was eliminated because of left horn fracture and presence of abscess in left mandibular region. All individuals from Breeding Centre in Smardzewice were diagnosed with tuberculosis by laboratory tests. In all cases, macroscopic evaluation of reproductive organs for presence of any pathological lesions was also performed.

All females included in this study were divided into three groups. The first group consisted of animals without any lesions in reproductive tract, and included 8 individuals from 6 months to 20 years old. The second group consisted of 8 females from 6 to 22 years old, with pathological lesions in genital organs including: endometritis, ovarian cysts, purulent vaginitis and erosion of vulvar labia. The third group included pregnant females from 6 to 21 years old. All of them were in the first trimester of pregnancy. Estimation of the advance stage of pregnancy was performed during necropsy, based on the level of fetus and fetal membranes development.

Immediately after culling, the cytological material was collected from vaginal mucosa with a sterile swab. Then, the obtained samples were transferred by roller movement to microscopic slides and fixed with BD Cytofix™ Fixation Buffer. The material for microbiological culture was collected from vagina with sterile culture swab. Then, the material was transported to the laboratory.

Immediately after delivery, the cytological slides were fixed in methylene, rinsed in distilled water and stained about 35 minutes in Giemsa, diluted in distilled water. Then, the slides were rinsed with distilled water and air-dried. The microscopic

analysis and photographic documentation were drawn using an optical microscope Nikon Eclipse with magnification 10× – 40×.

Samples collected for bacteriological culture were plated onto Columbia blood agar and MacConkey agar. Agar plates were incubated under microaerophilic conditions at 37°C for 48 hours. Identification of bacterial isolates was carried out using conventional diagnostic microbiology methods, based on their phenotypic characteristic.

Results

The first group consisted of females without any gross pathological lesions in reproductive system (Tabl. 1). It included individuals from Białowieża Forest and one animal from Smardzewice. Predomination of parabasal and intermediate cells was noted in the vaginal smears. They were scattered as a single cells or in small sheets throughout all the cytological smears. The majority of cells had decreased nucleus to cytoplasm ratio. The cytoplasm of most cells was basophilic (Fig. 1). Moreover, single granulocytes and some mucus strands were also observed.

Table 1. Results of microbiological analysis of vaginal swabs collected from the females without any gross pathological lesions in reproductive system (group 1).

Site of material collection	Date of material collection	Age (years)	Results of microbiological analysis
Białowieża	15.01.2013	0,5	*Corynebacterium pilosum* (single) *Trueperella pyogenes* (single)
Białowieża	16.01.2013	0,5	*Corynebacterium pilosum* (abundant) *Corynebacterium* spp. (moderate)
Białowieża	15.02.2012	0,5	No growth
Białowieża	29.01.2013	0,5	*Corynebacterium* spp. (single) Alfa-haemolytic *Streptococcus* spp. (single)
Smardzewice	21.01.2015	1	No growth
Białowieża	13.02.2012	4	No growth
Białowieża	21.10.2014	16	Saprophytic microflora
Białowieża	15.03.2011	20	Coagulase-negative *Staphylococcus* spp. (moderate)

The following bacteria were isolated from the first group: *Corynebacterium pilosum* and *Corynebacterium* species as well as alpha-haemolytic *Streptococcus* species and coagulase-negative *Staphylococcus* species. In three cases none bacteria have been isolated.

The second group included females with pathological lesions in genital organs (Table 2). All animals came from Białowieża Forest and reached sexual maturity.

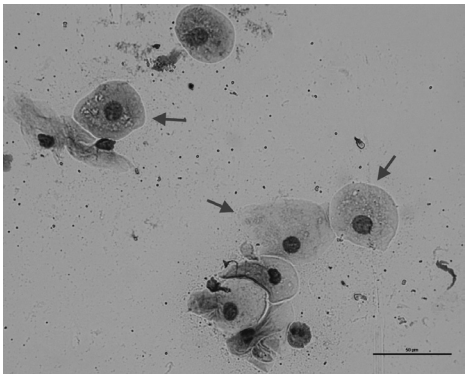

Figure. 1. Vaginal smear of 16-years old bison female. Note the cells with abundant cytoplasm and small nuclei (black arrows).

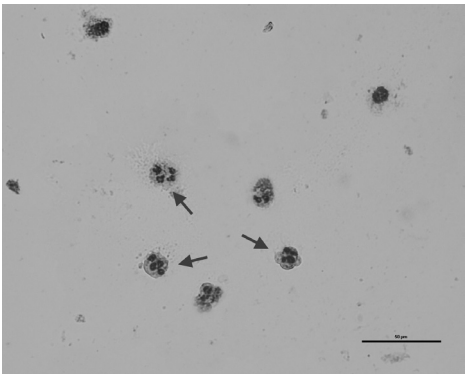

Figure. 2. Vaginal smear of 6-years old bison female. The neutrophils are present (black arrows).

Vaginal swabs from these females were obtained during the period from February 2012 to September 2014. The results of vaginal cytology examination were comparable with previous group. The parabasal and intermediate cells were predominant. However, neutrophils were abundant, especially in case of 20-years old cow culled in February 2012 (Fig. 2). The number of bare nuclei was also increased in this group. Bare nuclei were significantly more abundant than cells without evidence of cytolysis. Individually scattered cells were predominant in vaginal smears from this

Table 2. Results of microbiological analysis of vaginal swabs collected from the females with pathological lesions in genital organs (group 2).

Site of material collection	Date of material collection	Age (years)	Results of microbiological analysis
Białowieża	29.01.2012	6	Alfa-haemolytic *Streptococcus* spp. (single) *Corynebacterium pilosum* (moderate)
Białowieża	19.12.2012	8	*Corynebacterium* spp. (moderate) *Trueperella pyogenes* (single)
Białowieża	21.10.2014	9	*Corynebacterium* spp. (moderate)
Białowieża	07.11.2013	18	*Pseudomonas* spp. (moderate)
Białowieża	29.02.2012	10	*Corynebacterium renale* group (moderate)
Białowieża	07.11.2013	20	Coagulase-negative *Staphylococcus* spp. (single) Alfa-haemolytic *Streptococcus* spp. (moderate)
Białowieża	14.02.2012	20	No growth
Białowieża	18.12.2012	22	*Enterococcus* spp. (moderate) Non-haemolytic *Escherichia coli* (single) Alfa-haemolytic *Streptococcus* spp. (moderate)

Table 3. Results of microbiological analysis of vaginal swabs collected from pregnant females (group 3).

Site of material collection	Date of material collection	Age (years)	Results of microbiological analysis
Smardzewice	21.01.2015	6	No growth
Smardzewice	21.01.2015	14	Coagulase-negative *Staphylococcus* spp. (moderate) Alfa-haemolytic *Streptococcus* spp. (moderate) Beta-haemolytic *Streptococcus* spp. (single)
Smardzewice	21.01.2015	17	*Staphylococcus* spp. coagulose-negative
Białowieża	21.10.2014	18	No growth
Białowieża	20.12.2011	20	Non-haemolytic *Escherichia coli* (moderate)
Białowieża	26.02.2013	21	Alfa-haemolytic *Streptococcus* spp. (abundant) *Corynebacterium* spp. (moderate)

group, however some sheets of cells were also noted. Most of epithelial cells have basophilic cytoplasm, however some acidophilic cells were found. Abundant mucus strands were also observed.

Microbiological analysis showed predominance of alpha-haemolytic *Streptococcus* species as well as *Corynebacterium pilosum* and *Corynebacterium* species in material collected from this group. Moreover, bacteria from *Corynebacterium renale* group were isolated from 10-years old cow with ovarian cysts, and *Pseudomonas* species from 18-years old cow with endometritis and purulent vaginal discharge.

The third group consisted of pregnant females (Tabl. 3). It included 3 individuals from 18 to 22 years old from Białowieża Forest, and 3 slightly younger females from Smardzewice. In the vaginal smears obtained from these animal abundant mucus strands were observed (Fig. 3). Epithelial cells had abundant cytoplasm and they were arranged in numerous sheets. However, some individual cells were also

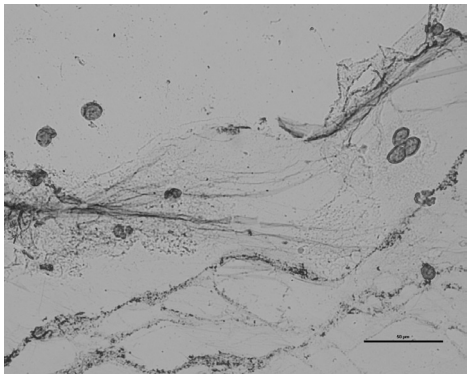

Figure. 3. Vaginal smear of 17-years old pregnant bison female. Abundant mucus strands are visible.

noted. In smears collected from two individuals (20-years old cow from Białowieża Forest and 17-years old cow from Smardzewice) the presence of moderate number of granulocytes and single erythrocytes was noted in microscopic visual fields.

The results of microbiological analysis are comparable with the first examined group.

Discussion

Cytological analysis is simple, quick, noninvasive and inexpensive procedure that can be used *ante mortem* under various field conditions. Because of that reasons, some authors have used this technique in wild animals species (Carlson and Gese 2008; Snoeck *et al.* 2011). Cytological analysis of vaginal swabs allows for the assessment of the phase of estrus cycle at the time of sample collection, and helps in determination of vaginal microflora (Olbrych *et al.* 2013). The results of vaginal swabs examination may indicate the necessity of performance other diagnostic tests including microbiological analysis. Therefore it becomes attractive and effective diagnostic tool in monitoring of reproductive capacity and overall health in wild animal populations, especially in case of threatened species.

Detailed analysis of vaginal cytology has been conducted on domestic cattle, a species closely related to European bison (Groppetti *et al.* 2012). Unfortunately, results of these studies cannot be compared with results of vaginal swabs analysis obtained from E. bison females. Domestic cattle are highly exploited for milk and meat production. Animals are kept in large farms under continuous veterinary surveillance. Differences in living conditions of domestic cattle and European bison as well as significant human interference in farm environment indicate differences in vaginal microflora composition in cattle and E. bison.

In year 2013 Olbrych *et al.* have published the results of cytological examination of E. bison vaginal swabs. Vaginal swabs were collected either post mortem from females culled during planned annual elimination or *ante mortem* from animals held in quarantine and underwent anesthesia. The results of vaginal cytology of individuals from the first group were confronted with pathological findings observed during necropsy. It allowed to fully verify results of vaginal smears analysis. Currently, this study has been continued and extended by microbiological analysis. However, the interpretation of bacterial culture results is difficult because of lack of sufficient data regarding physiological vaginal microflora in E. bison females. Some data in this field have been published by Speck *et al.* (2006). However, this study was conducted *ante mortem* on females under anesthesia. It cannot be excluded, that some of these animals had suffered from various diseases unrecognized clinically. Thus, bacteria isolated from examined females cannot be considered as physiological vaginal microflora. Moreover, Speck *et al.* (2006) divided examined animals into two group. One of them consisted of females 5 years old. The second one included animals older than 5 years. According

to literature, E. bison females may give birth for the first time at about 3 year of their life (Wróblewski 1927; Jaczewski 1958). Thus, choice of such study groups excludes analysis of correlations between vaginal microflora and reproductive activity. It also makes impossible to compare vaginal microflora between sexually immature and sexually mature cows. Moreover, in current study, significantly more obligatory anaerobic bacteria species were found in collected material. Despite vast range of culture media used in our study, including Columbia blood agar, chocolate agar, Schaedler agar, Gassner lactose agar and MacConkey II agar, and various conditions of incubation (microaerophilic *vs*. anaerobic environment), composition of physiological vaginal microflora has not been unequivocally established. Thus, diagnostic panel should be extended to cytological analysis of vaginal swabs. It is very important, especially if such tests will be used in monitoring of reproductive capacity of E. bison living in free ranging herds. Microbiological analysis should be made concomitantly with vaginal cytology or should be considered as an additional diagnostic test performed after cytodiagnostics. Obviously, such diagnostic approach is more expensive, but it provides more data for assessment of physiological vaginal microflora in European bison, and could be useful in monitoring of wild animal reproduction.

Vaginal cytology is a simple and quick procedure to perform without any specialist equipment under field conditions. Microscopic findings reflects state of reproductive system of examined animal. European bison are polyestral seasonal animals. Microscopic appearance of vaginal and uterine cervix epithelium changes cyclically depending on cyclical changes in ovarian endocrine activity, season and level of sexual maturity. In European bison, the mating season begins in August and may continue through October, so the offspring is born in May or June (Jaczewski 1958; Wróblewski 1927). Because all animals included in this study were culled during autumn-winter period, they were in *anaestrus* phase. Thus, further studies of vaginal smears collected in other seasons are needed to fully describe vaginal cytology during whole estrous cycle.

Conclusions

Microbiological analysis of vaginal swabs provided less specific test compared to vaginal cytology, as most of isolated bacteria species were found in all examined groups of E. bison females with equal frequency. It can be explained by small number of animals included in the study. However, in animals with gross pathological lesions, the characteristic changes in vaginal cytology confirming necropsy findings were observed. These results encourage for further microbiological studies of vaginal swabs. This is the only way to determine bacteria species belonging either to saprophytic or pathogenic vaginal microflora of European bison species.

Microscopic analysis of vaginal cytology smears is a good method in determination of phases of the estrus cycle in E. bison females. According to the time of the

samples collection all examined animals were in *anaestrus*. However, to fully describe changes in vaginal epithelial cells appearance during estrus cycle, analysis of cytological smears collected over the whole year is needed. It is of importance for appropriate monitoring of reproduction of wild living bison. Unfortunately, we have no possibilities to collect the material from E. bison in particular estrus phases so far. Thus, the study of microbiological and cytological vaginal swabs should be continued in future, as they may allow for more successful protection of this threatened species.

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Badania korelacji wymazów bakteriologicznych i cytologicznych z pochwy u żubra.

Streszczenie: U żubra coraz częściej pojawiają się problemy związane z rozrodem, co może prowadzić do zagrożenia istnienia tego dzikiego gatunku. Dlatego ważnym zagadnieniem wydają się badania bakteriologiczne wymazów pochwowych. Jednak w przypadku żubra pierwsze badania w tym zakresie zostały przeprowadzone dopiero w roku 2006 przez Speck z zespołem, a uzyskane wyniki nie są pełne. W związku z tym postanowiono przeprowadzić dalsze badania cytologiczne poszerzone o analizę bakteriologiczną oraz korelację uzyskanych wyników.

Część żubrów pochodziła ze stada wolno żyjącego, a część z zagród pokazowych. Żubrzyce zostały podzielone na trzy grupy. Grupa pierwsza to samice bez stwierdzonych podczas sekcji zmian anatomopatologicznych w obrębie narządów płciowych. Grupa druga to samice z stwierdzonymi zmianami w obrębie układu rozrodczego. Ostatnia grupa trzecia składała się z samic ciężarnych. Sterylną wymazówką pobrano wymazy z pochwy do badań cytologicznych i bakteriologicznych.

Wymazy bakteriologiczne pochodzące od badanych samic w porównaniu z wymazami cytologicznymi okazały się dużo mniej specyficzne. Większość bakterii występowała z taką samą częstotliwością we wszystkich trzech badanych grupach. Natomiast w wymazach cytologicznych, u samic z zaobserwowanymi podczas sekcji zmianami makroskopowymi, stwierdzono charakterystyczne zmiany obrazu mikroskopowego, potwierdzające wyniki badań anatomopatologicznych.