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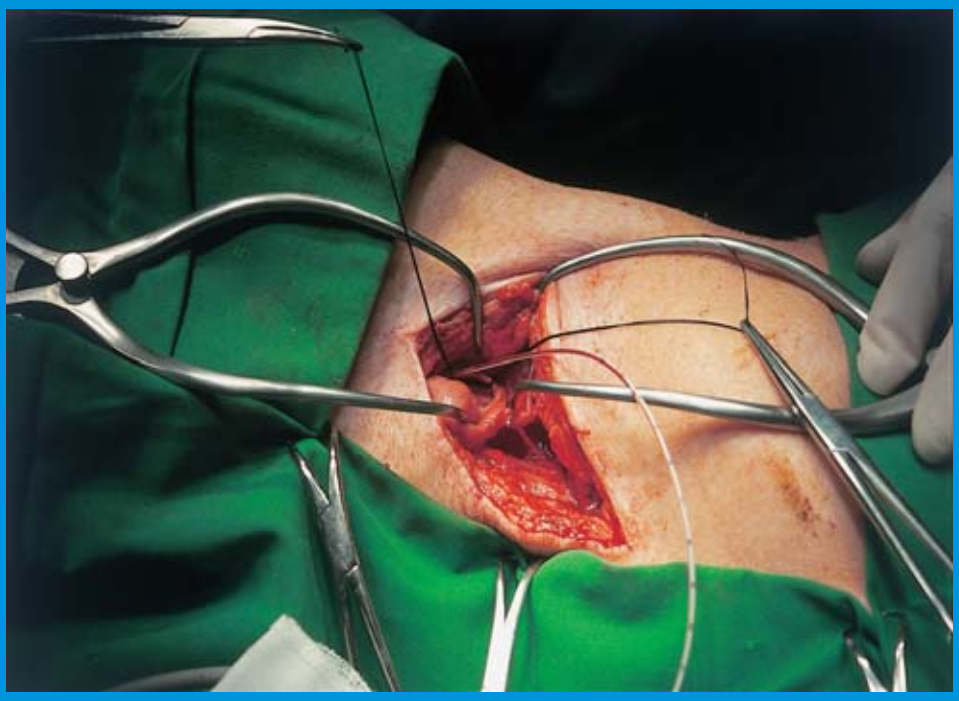
SLOVENIAN VETERINARY RESEARCH

SLOVENSKI VETERINARSKI ZBORNIK



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SLOVENIAN VETERINARY RESEARCH

SLOVENSKI VETERINARSKI ZBORNIK

Slov Vet Res 2005; 42 (1/2)

Slov Vet Res 2005; 42 (1/2) : 5-6

EDITORIAL

In June 2005, I was appointed to the position of Editor-in chief of Slovenian Veterinary Research. The new position presents at the same time a great challenge, a great responsibility and also a great burden. However, I believe with you, the readers and the contributors of Slovenian Veterinary Research, and with the members of newly appointed Editorial board (to whom I would like to thank for accepting their new responsibility), we can successfully run the journal.

To begin with, I must express gratitude and appreciation to my predecessor, emer. prof. dr. Srdan Bavdek for all the work he did in the past several years to improve the Slovenian Veterinary Research. To briefly mention his achievements during his tenure: Slovenian Veterinary Research started to be published quarterly instead of biannually, changed its appearance and started to be published only in English with summaries in Slovenian language. At the beginning, almost 50 years ago, the journal was a collection of doctoral theses presentations and reports on finished or running projects at the Veterinary faculty in Ljubljana and it was therefore appropriately called "Research Reports of the Veterinary Faculty at the University of Ljubljana". However, over the years, it became a proper professional and scientific journal. Prof. Bavdek therefore saw a need to rename it to Slovenian Veterinary Research. To reach international scientific community, the journal was published in two versions - English and Slovenian - since 1996. But to reduce both the publishing costs and burden for authors, last year Slovenian Veterinary Research began to be published only in English. However, summaries are kept in Slovenian language, with the aim to take basic care of professional Slovenian veterinary language, what was a very important role of Slovenian Veterinary Research in the past. Slovenian Veterinary Research moved with times and today, its readers can get all full text articles on the internet and thanks to the generous financial support from both Veterinary Faculty at University of Ljubljana, and Ministry of Science, Higher education and Technology of Slovenia, all content is accessible to anybody free of charge. Another important change was a new appearance. Although the old design served us well for more than 40 years, the change of design was well overdue. All this improvements were achieved mostly by enthusiasm of prof. Bavdek, but of course, he could not do all by himself. Therefore, I would also like to thank at this opportunity to all the members of the Editorial board that served with prof. Bavdek, and I believe special thanks should go to Ms. Zdenka Karlin, who worked with the Slovenian Veterinary Research from its infancy.

So, with all the improvements achieved by prof. Bavdek, what remains for the new Editor and the new Editorial board to do? Plenty, one could answer very promptly. If we want to build on the work of prof. Bavdek, the only way is the way forward. We would like to further improve the quality of Slovenian Veterinary Research, with our ultimate goal to make a Slovenian Veterinary Research truly internationally recognized and well reputed journal in the fields of veterinary medicine and biomedical sciences. To accomplish this, we need to address two issues. We need to attract more international contributors to submit manuscripts, and to realize this goal, Slovenian Veterinary Research must become more recognizable in international scientific community. Therefore, one of our first priorities will be to include Slovenian Veterinary Research in those international databases, from where it is currently missing. The most important of these are undoubtedly Medline/Pubmed, and in the future, SCI. However, to achieve this, we need first to address more burning issue - regular publishing. Slovenian Veterinary Research is currently battling with a serious problem of low submission rate. This has been a problem for several years, but became critical in the last two years. At the moment, due to lack of submissions, we are behind our publishing plan, and although, with current situation, we should be able to publish all planed issues for year 2005, the future for 2006 does not look very bright at this moment. However, this is not just the story of success or failure on the market, but it is a very important issue for Slovenian veterinary medicine at large. The Slovenian Veterinary Reserch is the only scientific journal in Slovenia covering this field of science. It therefore offers a unique and much needed opportunity to our (small) number of vet-

erinary scientists to publish their work at home. How to solve this issue? Attract more authors by making journal more internationally recognizable is, of course, the obvious answer. But here we run into the vicious circle. One of the main requirements to be included in both Medline and SCI is publishing regularly and on time, but this can be achieved only with a regular flow of the manuscripts to the journal. And here, the editorial board and the editor can do very little. Ultimately, the scientific journal is not made by Editor or Editorial board, but by its readers and by its contributing authors. The Editors can assure that the quality of the journal is kept at a certain level, the Editor and the Editorial board can steer the journal into different directions, they can make future plans and make promises, but ultimately, the responsibility for the scientific journal lies in the hands of the authors. Only when sufficient amount of high quality manuscripts will be regularly submitted to the journal, we will be able to move Slovenian Veterinary Research to a higher level. Therefore, I would like to call upon all of you who are reading this editorial, to help us to make our Slovenian Veterinary Research an even better journal.

What to say in the end? I accepted the position of new Editor-in-chief with enthusiasm and optimism. I believe we can achieve our aims. But in the modern science, teamwork is the most important and the same is true for running the journal. Therefore, I know I cannot achieve these goals alone. We need to work together, not just the Editorial board, but everybody involved with the journal, all its readers and authors. And then, and only then, we will be able, sometime in the future, to look back on the path we walked on, sat down and said, both with relief and satisfaction, it was a good path we choose.

Gregor Majdič, Editor in Chief

Slov Vet Res 2005; 42 (1/2): 7-10
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Original Research Paper

COULD LEPTIN LEVELS PREDICT BODY WEIGHT IN BOARS?

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Summary: Leptin is a regulator of body weight homeostasis in mammals. Blood levels of leptin are in direct correlation with the amount of adipose tissue in the body. In the present study, leptin levels and body weight were monitored through postnatal development in young boars. Sixteen boars were followed from 6 to 22 weeks of age. There was no significant correlation between leptin levels and body weight at the same ages. However, strong positive correlation was found between leptin levels in 6 weeks old piglets and body weights during puberty between 16 and 22 weeks of age. Correlation was especially strong ($p<0.001$) between leptin levels at 6 weeks and body weight of boars at 22 weeks of age. The results of this study therefore suggest that leptin levels in young piglets might be used as a prediction for body weight and growth during first 6 months of age.

Key words: physiology; leptin-blood; body weight; swine

Introduction

Leptin is a peptide hormone, produced by white adipose tissue (1, 2). Leptin gene was first identified in ob/ob strain of mice, that carry mutations in leptin gene and are consequently, severely obese (3). A similar obese strain of mice, db/db mice, carries a mutation in leptin receptor gene, a member of cytokine receptors (4). Leptin levels in blood are in direct positive correlation with the amount of white adipose tissue in the mammalian body (1, 2). Leptin enters the hypothalamus, the main site of leptin action, through median eminence. Increased levels of leptin cause decreased neuropeptide Y and agouti related protein expression and increased levels of melanocortin in hypothalamus. This pattern of neuropeptides expression results in decreased feeding and increased energy expenditure, while reduced levels of leptin have opposite effects, resulting in increased feeding. This mechanisms thus ensure long term body weight homeostasis (reviewed in (5, 6). However, many other molecules such as cocaine and amphetamine related transcript (CART), orexins, corticotropine realizing factor (CRF), endorphins and others are also involved in either regulation of appetite or energy consumption, although their connection to leptin is less established (reviewed in (5, 6). Mechanisms regulating leptin expression in adipose tissue are not clear yet. Leptin is constitutively expressed in white adipocytes. However, leptin levels are also dependent on the levels of glucocorticoid hormones and sex steroids (7) and estrogens can directly stimulate leptin expression in adipose tissue both in vitro and in vivo (8, 9). Through postnatal development, leptin levels steadily rise before and during puberty (10, 11) and leptin is also thought to be a metabolic signal, permitting entry into puberty (12) and some studies in humans suggested correlation between postnatal energy stores and leptin levels and obesity later in life. In the present study, we report that leptin levels in piglets are also predictable for total body weight in pubertal boars, independent on the amount of back fat.

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Material and methods

Animals

Sixteen boars were kept in standard condition and fed regular chow at commercial pig farm Ihan. Every 2 weeks from 6 weeks of age to 22 weeks of age, all boars were weighed and had blood samples taken. Blood was collected from vena cava. Evacuated tubes with separation gel (Vacutainer®, Becton Dickinson, Heidelberg,

Table 1: Leptin levels at different ages from 6 weeks until puberty (mean ± S.E.)

Age (weeks)	Leptin levels (ng/ml)
6 weeks	1.51 ± 0.12
8 weeks	2.44 ± 0.12
10 weeks	1.04 ± 0.03
12 weeks	1.95 ± 0.05
14 weeks	1.91 ± 0.07
16 weeks	2.49 ± 0.12
18 weeks	2.22 ± 0.11
20 weeks	2.20 ± 0.21
22 weeks	3.26 ± 0.15

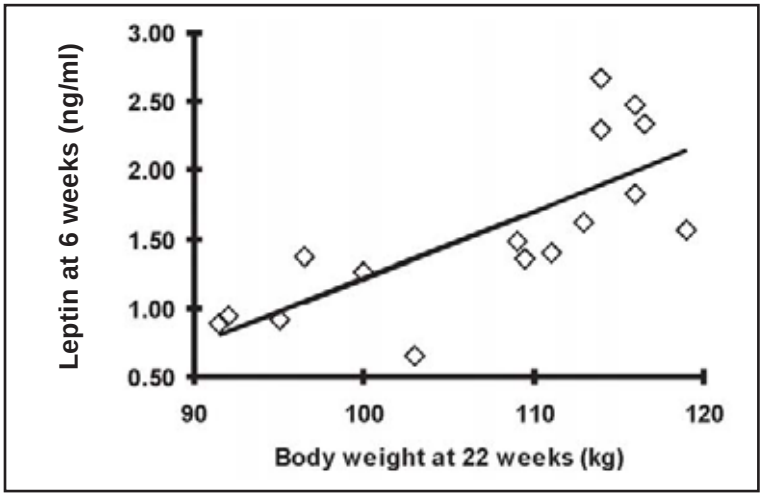

Diagram 1: Line fit plot for linear regression analysis to compare leptin levels at 6 weeks and body weight at 22 weeks

Germany) and anticoagulant were used for sample collection. Blood was centrufuged at 1000 rpm for 5 minutes and plasma was removed and stored at -20oC until used. All animal work was approved by Veterinary commission of Slovenia. At 16, 20 and 22 weeks, back fat thickness was measured using ultrasound device Krautkramer FGM2 (Agfa gevaert N.V., Mortsel, Belgium) at two points in lumbal region about 3 cm laterally from the spine. Average of two measurements was used for statistical analyses.

Hormone measurement

Serum levels of leptin were measured by Multi-species leptin RIA kit (LINCO Research, Inc., St. Charles, MO, USA), validated for porcine leptin, following original instructions of manufacturer (http://www.lincoresearch.com/products/xl-85k.html). For low values (mean 3.5 ng/ml) intra- and inter-assay coefficients were 7.71% and 8.72% respectively, for high values (mean 28.47 ng/ml) they were 8.73% and 5.71%, respectively.

Statistical analyses

Data (hormone levels, body weights and back fat thickness) were subjected to statistical analyses using SigmaStat software (Jandel scientific, Erkrath, Germany). Pearson correlation coefficient and linear regression was calculated to predict correlations between leptin levels, body weights and back fat thickness.

Results

Boars' body weight increased linearly from 6 weeks of age as expected. The mean body weight at the beginning of experiment was 11.59 ± 0.37 kg (mean ± S.E.) and at the conclusion of experiment 107.32 ± 2.16 kg (mean ± S.E.).
Leptin levels at 6 weeks of age were 1.51 ± 0.12 ng/ml (mean ± S.E.). Thereafter, the leptin levels steadily increased to reach the levels 3.29 ± 0.15 ng/ml (mean ± S.E.) at 22 weeks of age (Table 1). Thickness of back fat was measured at 16, 20 and 22 weeks of age. Thickness increased from 7.70 ± 1.25 mm at 16 weeks to 13.73 ± 2.81 mm at 22

Table 2: Pearson correlation coefficients between leptin levels at 6 weeks of age and body weights (BW) from 12 to 22weeks (* p<0.05, ** p<0.01, *** p<0.001)

Body weight	Leptin(6 weeks; ng/ml)
6 weeks	R = 0.1466
8 weeks	R = 0.4985
10 weeks	R = 0.4919
12 weeks	R = 0.5379*
14 weeks	R = 0.6116*
16 weeks	R = 0.7059**
18 weeks	R = 0.7551***

weeks (mean ± S.E.). Interestingly, statistical analyses did not show statistically significant correlation between leptin levels and measured back fat thickness at 16, 20 and 22 weeks. Similarly, there was no significant correlation between body weight and leptin levels at particular ages.

However, when all the data was subjected to correlation analyses, strong correlation was found between leptin levels at 6 weeks of age and body weights from 16 weeks until the end of experiment at 22 weeks of age (Table 1). Linear regression analyses confirmed these results and showed very strong correlation between leptin levels at 6 weeks of age and body weight at 22 weeks (Diagram 1). However, correlation between leptin levels at 8 weeks and body weight at 15 to 22 weeks disappeared and there was no correlation between body weight and leptin levels at any other age.

Discussion

Leptin is a central regulator of body weight in mammals. Secreted by adipose tissue into the circulation, it transmits signals about body fat deposits to the hypothalamus. Leptin levels are in many species strongly correlated with the amount of adipose tissue in the body (1, 2). In the hypothalamus, leptin is thought to regulate feeding and energy expenditure by regulating expression of several neuropeptides. The most important appears to be the neuropeptide Y/melanocortin systems (5, 6, 13)}. Besides regulating body weight homeostasis, leptin is thought to have several other functions. During postnatal period, leptin levels steadily rise (10, 11) and leptin appears to be one of the permissive factors allowing entry into puberty (12). Intriguing is also the difference between males and females in the levels of leptin that persist even after adjustment of data for adiposity (14, 15). The regulation of leptin expression in the adipocytes is not yet clear, although insulin, growth hormone, glucocorticoids and estradiol have all been implicated in the regulation of leptin gene expression (1). It is well documented that leptin levels are in direct positive correlation with the amount of white adipose tissue in many species. However, in our study, we failed to find such correlation at any studied age. One possible explanation is that boars differ from humans and rodents and there are possibly some other regulatory mechanisms that modulate levels of leptin secreted from white adipocytes. Perhaps more feasible explanation is that leptin levels and amount of adipose tissue are not directly correlated in fast growing animals, but would become correlated with the amount of adipose tissue in fully grown animals. However, in our study, we found that leptin levels in young boars are also strongly correlated with the body weight at around 6 months of age. This is intriguing, as it is difficult to explain, how leptin could act as a predictor for body weight several months later. One possible explanation is that animals with greater number of adipocytes would have higher levels of leptin (due to bigger number of adipocytes) already at early age, and higher number of adipocytes will ultimately result in higher body weight due to larger proportion of adipose tissue. However, the measurements of back fat thickness in studied boars did not show correlation between back fat and leptin levels at 22 weeks of age or correlation between leptin levels at 6 weeks and back fat thickness at either 16, 20 or 22 weeks. Another possible explanation could be connected to growth hormone. Houseknecht et al. (16) have reported that growth hormone in cattle upregulate leptin expression both in vivo and in vitro in the presence of insulin and dexamethasone. Therefore, higher levels of

leptin in young piglets could be at least partially due to higher intrinsic levels of growth hormone, and higher levels of growth hormone at early age could be responsible for bigger body weights in boars at 22 weeks of age. In conclusion, our study shows that leptin levels in 6 weeks old piglets are in strong correlation with body weights from 12 to 22 weeks and could be therefore potentially used in young animals as a marker for predicting pubertal body weight in boars.

Acknowledgement

The authors would like to thank the personnel at pig farm Ihan for their valuable help and Bostjan Drolc for his technical assistance. The study was supported by Ministry of Education, Science and Sport of Slovenia (P0-0501-0406).

References

1. Kalra SP, Dube MG, Pu S, Xu B, Horvath TL, Kalra PS. Interacting appetite-regulating pathways in the hypothalamic regulation of body weight. Endocr Rev 1999; 20(1): 68-100.

2. Elmquist JK, Elias CF, Saper CB. From lesions to leptin: hypothalamic control of food intake and body weight. Neuron 1999; 22(2): 221-32.

3. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. Nature 1994; 372(6505): 425-32.

4. Tartaglia LA, Dembski M, Weng X, et al. Identification and expression cloning of a leptin receptor, OB-R. Cell 1995; 83(7): 1263-71.

5. Harrold JA. Hypothalamic control of energy balance. Curr Drug Targets 2004; 5(3): 207-19.

6. Leibowitz SF, Wortley KE. Hypothalamic control of energy balance: different peptides, different functions. Peptides 2004; 25(3): 473-504.

7. Kristensen K, Pedersen SB, Richelsen B. Regulation of leptin by steroid hormones in rat adipose tissue. Biochem Biophys Res Commun 1999; 259(3): 624-30.

8. O'Neil JS, Burow ME, Green AE, McLachlan JA, Henson MC. Effects of estrogen on leptin gene promoter activation in MCF-7 breast cancer and JEG-3 choriocarcinoma cells: selective regulation via estrogen receptors alpha and beta. Mol Cell Endocrinol 2001; 176(1-2): 67-75.

9. Shimizu H, Shimomura Y, Nakanishi Y, et al. Estrogen increases in vivo leptin production in rats and human subjects. J Endocrinol 1997; 154(2): 285-92.

10. Garcia-Mayor RV, Andrade MA, Rios M, Lage M, Dieguez C, Casanueva FF. Serum leptin levels in normal children: relationship to age, gender, body mass index, pituitary-gonadal hormones, and pubertal stage. J Clin Endocrinol Metab 1997; 82(9): 2849-55.

11. Horlick MB, Rosenbaum M, Nicolson M, et al. Effect of puberty on the relationship between circulating leptin and body composition. J Clin Endocrinol Metab 2000; 85(7): 2509-18.

12. Clarke IJ, Henry BA. Leptin and reproduction. Rev Reprod 1999; 4(1): 48-55.

13. Elias CF, Aschkenasi C, Lee C, et al. Leptin differentially regulates NPY and POMC neurons projecting to the lateral hypothalamic area. Neuron 1999; 23(4): 775-86.

14. Rosenbaum M, Pietrobelli A, Vasselli JR, Heymsfield SB, Leibel RL. Sexual dimorphism in circulating leptin concentrations is not accounted for by differences in adipose tissue distribution. Int J Obes Relat Metab Disord 2001; 25(9): 1365-71.

15. Saad MF, Damani S, Gingerich RL, et al. Sexual dimorphism in plasma leptin concentration. J J Clin Endocrinol Metab 1997; 82(2): 579-84.

16. Houseknecht KL, Portocarrero CP, Ji S, Lemenager R, Spurlock ME. Growth hormone regulates leptin gene expression in bovine adipose tissue: correlation with adipose IGF-1 expression. J Endocrinol 2000; 164(1): 51-7.

ALI RAVEN LEPTINA LAHKO NAPOVE TELESNO TEŽO MERJASCEV?

N. Čebulj-Kadunc, V. Cestnik, G. Majdič

Povzetek: Leptin je peptidni hormone, ki v sesalskem organizmu ureja telesno težo. Proizvajajo ga bele maščobne celice, njegova koncentracija v krvi pa je v neposrednem razmerju s količino bele tolšče. V pričujoči raziskavi smo ugotavljali povezanost med telesno težo in vrednostmi leptina v krvi mladih merjascev. Šestnajst merjascev smo spremljali od 6. do 22. tedna starosti. Meritve niso pokazale statistično značilnih povezav med vrednostmi leptina ter telesno težo v isti starosti. Ugotovili pa smo statistično zelo značilno povezavo med vrednostmi leptina pri mladih merjaščkih (6 tednov) in telesno težo pri merjascih v puberteti (od 16 do 22 tednov). Povezava je bila zelo močna med vrednostjo leptina pri 6 tednih in telesno težo pri 22 tednih ($p < 0.001$). Rezultati te raziskave tako kažejo na možno vlogo vrednosti leptina pri mladih merjaščkih kot pokazatelja rasti in telesne teže pri živalih v puberteti.

Ključne besede: fiziologija; leptin-kri; telesna teža; prašiči

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Original Research Paper

CIRCULATING LEPTIN CONCENTRATIONS IN LIPIZZAN HORSES AND JEZERSKO-SOLCHAVA SHEEP

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Summary: Leptin is a protein hormone synthesized in adipocytes of the white adipose tissue, whose level of expression and secretion parallels the quantity of body fat. Investigations in different species have identified leptin as an indicator of nutritional status and an important regulator of appetite, energy metabolism and body composition. The aim of our work was to measure circulating leptin levels in two Slovenian autochthonous breeds, Lipizzan horses and Jezersko-Solchava sheep, in order to study the dependence of its concentration on age, body weight and gender.
Serum leptin concentrations in 89 Lipizzan stallions and mares, 47 Jezersko-Solchava lambs of both sexes and 48 Jezersko-Solchava rams were measured with a commercial radioimmunoassay (RIA) kit. The significant elevations paralleled age and weight gain, as reported in other animal species and breeds. In young Lipizzan horses, the lowest leptin concentrations (1.064 ng/ml ± 0.19 ng/ml) were observed in yearling colts and the highest (1.94 ng/ml ± 0.33 ng/ml) in 4-year old fillies. In adult Lipizzan stallions and pregnant mares, the values were 1.99 ng/ml ± 0.20 ng/ml and 1.19 ng/ml ± 0.15 ng/ml, respectively. In Jezersko-Solchava sheep, the lowest concentrations (0.10 ng/ml ± 0.09 ng/ml) were found in lambs weighing 1 - 5 kg and the highest (0.81 ng/ml ± 0.12 ng/ml) in 12-month old rams.
Agreement of these leptin concentrations with values reported in the literature indicates the suitability of the multispecies RIA kit for measuring circulating leptin concentrations in equine and ovine species.

Key words: leptin-blood-physiology; age factors; body weight; sex; radioimmunoassay; horses; sheep

Introduction

The protein hormone leptin is synthesized in white adipose tissue, the levels of its expression and secretion paralleling the amount of body fat. Blood peptide carriers, which bind to it, regulate its biological half-life and activity (1, 2). Investigations in humans, rodents and domestic animals have shown the role of leptin to be an indicator of nutritional status, informing the central nervous system about the status of energy reserves and their balance, and regulating the appetite, energy metabolism and body composition (1, 2, 3). The primary role of leptin is not prevention of obesity, but regulation of physiological adaptation to starvation (4). Leptin receptors have been demonstrated in hypothalamic regions, regulating appetite, growth and reproduction, indicating the influence of leptin on secretion of various neurotransmitters, neuropeptides and hormones (1, 2, 4). Leptin can also act directly on some peripheral tissues like liver, skeletal muscle, pancreas and suprarenal cortex (1, 2, 4, 5, 6) and has an important influence on immunity, angiogenesis and haematopoiesis (1, 2, 7).

Leptin plays a crucial role in controlling reproduction, as demonstrated (2) in animals carrying mutation in Ob gene encoding leptin (ob/ob mice). It acts directly on gonadotropin secretion, but receptors in ovaries, testes and uterus indicate the additional possibility of direct leptin action on reproduction (1, 2, 8). Insufficient nutrition, or starvation, leads to decreased secretion of gonadotropin in most animals, delaying, or even hindering, the onset of puberty or cyclic ovarian activity in females and causing hypogonadotropism and infertility in males (2, 7, 8, 9, 10). Irrespective of energy status, circulating leptin levels are increased during pregnancy in humans, rodents and ewes, decreasing just before parturition (1, 2, 8).

Circulating leptin concentrations can be measured by enzyme (EIA) or radio immunoassays (RIA) 1). Only one commercial multispecies RIA kit is currently available, based on antibodies raised against human leptin that display broad crossreactivity to leptin molecules of several species. It is recommended by the manufacturer that investigators test the suitability of the assay for the

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Table 1: Serum leptin concentrations in Lipizzan horses of various age groups

	Stallions		Mares	
Age group	**Number (n)**	**Leptin (ng/ml)**	**Number (n)**	**Leptin (ng/ml)**
foals*	7	1.064 ± 0.19	18	1.18 ± 0.08
1 year	6	0.90 0.16	6	1.09 ± 0.06
2 years	6	1.12 0.19	6	1.41 ± 0.14
3 years	6	0.92 0.13	6	1.19 ± 0.12
4 years	6	1.69 0.32	6	1.94 ± 0.33
adult**	8	1.99 0.20	8***	1.19 ± 0.15

** 1 - 4 months; **7-19 years; ***pregnant*

Table 2: Serum leptin concentrations in lambs of various body weights

Body weight	**Number (n)**	**Leptin (ng/mL)**
1 - 5 kg	8	0.10 ± 0.09
6 - 10 kg	14	0.924 ± 0.06
11 - 15 kg	10	0.76 ± 0.09
16 - 20 kg	11	0.69 ± 0.06
21 kg and more	10	0.855 ± 0.16

Table 3: Serum leptin concentrations (ng/mL) in rams of various ages

Age (months)	**Number (n)**	**Leptin (ng/mL)**
1	22	0.97 ± 0.05
2	9	0.66 ± 0.026
3	17	0.78 ± 0.08
4 - 5	12	1.278 ± 0.06
6	12	1.42 ± 0.08
7	12	1.488 ± 0.10
12	12	1.808 ± 0.12

species of interest and establish their own reference values. The aim of this work was to determine variations in leptin levels in Lipizzan horses and Jezersko-Solchava sheep, having first established the assay for these species.

Materials and methods

89 Lipizzan horses of both sexes and various ages were selected at random in a stud farm, and 48 Jezersko-Solchava rams and 53 lambs of both sexes from a sheep farm. All animals were kept under the standard breeding conditions specific for each species. Categorisation of animals is shown in the Results section (see Tables 1, 2 and 3).

Blood was sampled, by jugular venipuncture, on the same day from all animals kept at the same location. Sampling was performed in accordance to animal welfare legislation and approved by the Veterinary administration of the Republic of Slovenia.

Blood serum was prepared using commercial evacuated tubes (Vacutainer®, Becton Dickinson, Heidelberg) and kept frozen (-20 °C ± 2 °C) until analysed. Serum leptin concentrations were measured by a commercial radioimmunoassay kit (Multi-Species Leptin RIA Kit, Linco, USA). Intra- and inter-assay coefficients of variability (CV) were for high values (X = 28.16 ng/ml) 11.3 % and 10.3 %, respectively, and for low values (X = 4.35 ng/ml) 13,2 % and 8.3 %, respectively. Statistical analysis was performed by SPSS for Windows, Release 12.01 (SPSS Inc.) with subprograms Paired Samples T-Test and Analysis of variance. Correlations are reported as Pearson correlation coefficients. Results were evaluated as statistically significant on the level of $P < 0.05$, and presented as mean standard error of mean (X ± s.e.m.).

Results

Leptin concentrations in Lipizzan horses are listed in Table 1. The mean value was 1.23 ± 0.52 ng/mL. The lowest leptin concentrations for both sexes were observed in yearlings, the highest in 4 year old mares and adult stallions ($P < 0.05$). A positive, statistically significant correlation was established between leptin concentration and age of horses ($r = 0.7878$, $P<0.01$). Serum leptin concentration in all age groups of young horses was insignificantly higher in fillies than in colts, but significantly lower in pregnant mares than in stallions ($P<0.01$).

Discussion

Although the serum leptin concentrations in Lipizzans were in the lower range of normal values reported for horses (11, 12), the variations and correlations depending on age and gender, determined in other breeds of horses (2, 11), were also confirmed in this breed. Lower serum leptin concentrations in younger, growing animals indicate their lower body fat mass and greater nutritional needs than those of adult horses. The latter have greater body fat mass and lower nutritional needs, depending on their level of activity (11). In foals and young Lipizzan horses (1 to 4 years), leptin concentrations in females were higher than in males, as reported for other breeds (11). On the other hand, leptin concentrations in adult Lipizzans were higher in stallions than in pregnant mares, indicating higher energy demands during late pregnancy (11).

Serum leptin concentrations in Jezersko-Solchava lambs and growing rams are in general accordance with the values in other breeds of sheep (3, 13), but in lambs the levels are lower than reported (14). The deviations in these lambs could be attributed to the breed differences, the variety of sampling protocols or the use of different assay systems (15). Sexual dimorphism, as determined for children, horses and sheep (12, 15), with females having higher leptin concentrations than males, was not evident in these lambs, as reported for twin lambs of different sexes (16). Our further calculations were performed without considering the sex of lambs. Serum leptin concentration in Jezersko-Solchava lambs decreased insignificantly from the group weighing 1 - 5 kg to that of 16 - 20 kg, followed by a slight, insignificant increase in the group of 21 kg and over. The decrease in circulating leptin concentration in lambs after the 5th day of age probably ensures elevated appetite and deposition of fat (14). With approaching puberty, circulating leptin levels gradually rise (16). Significant increases in circulating leptin levels were found in Jezersko-Solchava rams from the 1st to 12th month of age. Progressive elevation of leptin concentrations can be attributed to the elevation of body fat supplies, as leptin concentrations in rams parallel the amount of body fat (1, 15).

In conclusion, circulating levels of leptin in Lipizzan horses and in Jezersko-Solchava sheep were consistent with values reported by other authors (3, 11, 12, 13), as were the significant elevations of circulating leptin concentration, which correlated with age and weight (10, 11, 14, 16). The sexual dimorphism in leptin levels, confirmed in adult horses, is most probably the consequence of feedback action of sexual steroid hormones on leptin secretion (11).

Agreement of leptin concentrations determined in the presently examined species with values reported in the literature indicates the suitability of the multispecies RIA kit used for measurement of circulating leptin concentrations in equine and ovine species.

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References

1. Chilliard Y, Bonnet M, Delavaud C et al. Leptin in ruminants. Gene expression in adipose tissue and mammary gland, and regulation of plasma concentrations. Dom Anim Endocrinol 2001; 21: 271-95

2. Houseknecht KL, Baile CA, Matteri RL, Spurlock ME. The biology of leptin: a review. J Anim Sci 1998; 76: 1405-20.

3. Blache D, Tellam RL, Chagas LM, Blackberry MA, Vercoe PE, Martin GB. Level of nutrition affects leptin concentrations in plasma and cerebrospinal fluid in sheep. J Endocrinol 2000; 165: 625-37.

4. Barb CR, Hausman GJ, Houseknecht KL. Biology of leptin in the pig. Dom Anim Endocrinol 2001; 21: 297-317.

5. Ingvartsen KL, Boisclair YR. Leptin and the regulation of food intake, energy homeostasis and immunity with special focus on peripartum ruminants. Dom Anim Endocrinol 2001; 21: 215-50.

6. Kadokawa H, Briegel JR, Blackberry MA, Blache D, Martin GB, Adams NR. Relationships between plasma concentrations of leptin and other methabolic hormones in GH-transgenic sheep infused with glucose. Dom Anim Endocrinol. 2003; 24: 219-29.

7. Henry BA, Golding JW, Tilbrook AJ, Dunshea FR. Intracerebroventricular infusion of leptin elevates the secretion of luteinising hormone without affecting food intake in long-term food-restricted sheep, but increases growth hormone irrespective of body weight. J Endocrinol 2001; 168: 67-77.

8. Spicer LJ. Leptin: a possible metabolic signal affecting reproduction. Dom Anim Endocrinol 2001; 21: 251-70.

9. Adam CL, Archer ZA, Miller DW. Leptin actions on the reproductive neuroendocrine axis in sheep. Reproduction 2003; 61(Suppl): 283-97.

10. Barb CR, Kraeling RR. Role of leptin in the regulation of gonadotropin secretion in farm animals. Anim Reprod Sci 2004; 82/83: 155-67.

11. Buff PR et al. Leptin in horses: tissue localization and relationship between peripheral concentrations of leptin and body condition. J Anim Sci 2002; 80: 2942-8.

12. Cartmill JA, Thompson DL, Storer WA, Gentry LR, Huff NK. Endocrine responses in mares and geldings with high body condition scores grouped by high vs. low resting leptin concentrations. Dom Anim Endocrinol 2003; 24: 1-14.

13. Delavaud C, Bocquier F, Chilliard Y, Keisler DH, Gertler A, Kann G. Plasma leptin determination in ruminants: effect of nutritional status and body fatness on plasma leptin concentrations assessed by a specific RIA in sheep. J Endocrinol 2000; 165: 519-26.

14. Bispham J, Budge H, Mostyn A, Dandrea J, Clarke L, Keisler DH, Symonds ME, Stephenson T. Ambient temperature, maternal dexamethasone, and postnatal ontogeny of leptin in the neonatal lamb. Pediatric Res 2002; 52: 85 - 90.

15. Erhardt RA, Slepetis RM, Siegal-Willott J, Van Amburgh ME, Bell AW, Boisclar YR. Development of a specific radioimmunoassay to measure physiological changes of circulating leptin in cattle and sheep. J Endocrinol 2000; 166: 519-28.

16. McFadin EL, Morrison CD, Buff PR, Whitley NC, Keisler DH. Leptin concentrations in periparturient ewes and their subsequent offspring. J Anim Sci 2002; 80: 738-43.

KONCENTRACIJA LEPTINA V KRVI LIPICANSKIH KONJ IN OVC JEZERSKO-SOLČAVSKE PASME

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Povzetek: Leptin je beljakovinski hormon, ki nastaja v adipocitih belega maščobnega tkiva. Njegovi ekspresija in sekrecija sta premo sorazmerni količini telesnih maščob. Raziskave pri številnih vrstah živali kažejo, da je leptin indikator prehrambenega stanja in pomemben regulator apetita, energetskega metabolizma in sestave telesa. Namen našega dela je bil izmeriti koncentracijo leptina v krvi dveh slovenskih avtohtonih pasem živali, lipicanskih konj in jezersko-solčavskih ovc, da bi proučili razlike v koncentracijah, ki so odvisne od starosti, telesne mase in spola.

Koncentracije leptina v serumu 89 lipicanskih žrebcev in kobil, 47 jezersko-solčavskih jagnjet obeh spolov in 48 jezersko-solčavskih ovnov so bile izmerjene s komercialnim radioimunskim testom (RIA). Značilen dvig koncentracije leptina v krvnem serumu je bil premo sorazmeren z naraščanjem starosti ali telesne mase, kot je že bilo ugotovljeno pri drugih vrstah oziroma pasmah živali. Pri mladih, rastočih lipicanskih konjih je bila najnižja koncentracija leptina (1.064 ng/ml 0.19 ng/ml) izmerjena pri enoletnih žrebičkih in najvišja (1.94 ng/ml 0.33 ng/ml) pa pri žrebicah v 4. letu življenja. Tudi pri odraslih lipicancskih žrebcih in brejih kobilah sta bili vrednosti 1.99 ng/ml 0.20 ng/ml oziroma 1.19 ng/ml 0.15 ng/ml. Pri jezersko-solčavski pasmi je bila najnižja koncentracija leptina izmerjena pri jagnjetih s telesno maso 1 - 5 kg (0.10 ng/ml 0.09 ng/ml) in najvišja (0.81 ng/ml 0.12 ng/ml) pri 12 mesecev starih ovnih.

Izmerjene vrednosti pri obeh preiskovanih vrstah živali in njihova skladnost s podatki iz literature potrjujejo primernost uporabljenega RIA testa za merjenje koncentracije leptina pri konjih in ovcah.

Ključne besede: leptin-kri-fiziologija; starostni faktorji; telesna teža; spol; radioimunski test; konji; ovce

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Original Research Paper

THE CONTENTS OF Cu, Zn, Fe AND Mn IN SLOVENIAN FRESHWATER FISH

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Summary: From 1999 to 2003, 141 samples of freshwater fish from various Slovenian rivers and brooks were analysed for their content of Cu, Zn, Fe and Mn. The concentrations of elements were determined in the muscle/skin (in natural proportion), head, liver and kidney. Brown trout *(Salmo trutta m. fario)*, marble trout *(Salmo marmoratus)*, brook trout *(Salevelinus fontinalis)*, rainbow trout *(Oncorhynchus mykiss)*, grayling *(Thymallus thymallus)*, chub *(Leuciscus c. cephalus)*, nase *(Chondrostoma nasus)*, Danube roach *(Rutilus pigus virgo)* and barbel *(Barbus barbus)* were examined. Flame atomic absorption spectrometry was used. The distribution of trace elements in various tissues was studied. It was found that the distribution is specific for each element and also varied by fish species. The contents of elements were the lowest in fish muscle for all species. The target tissues for the elements examined are the metabolically active ones, such as the liver and kidney.

Key words: food contamination; food analysis; copper; zinc; iron; manganese; spectrometry; fishes

Introduction

Fish represent a high quality source of dietary protein, but could lose these properties due to environmental contamination. Contaminants can be introduced into the environment through natural sources, industry, urbanisation and intensive agriculture. In aquatic ecosystems, heavy metals have received considerable attention due to their toxicity and accumulation. Some metals are toxic for living organisms even at low concentrations. Others are essential and necessary in trace amounts for the functioning of biological systems but can also be toxic at higher concentrations (1, 2). Aquatic organisms, especially fish located at the end of the aquatic food chain, are widely used for biological monitoring variation of environmental levels of anthropogenic pollutants (1, 2, 3, 4, 5). Fish receive trace elements, either directly from the water through their grills, or indirectly from food through the alimentary tract (2). Together with biological factors such as feeding behaviour and interactions between elements, chemical factors such as acidity, buffer capacity, and the presence of calcium and organic compounds in water may influence the bioavailability and accumulation of heavy metals in fish (2, 6, 7).

The aim of this study was to determine the content of Cu, Zn, Fe and Mn in the muscle/skin in natural proportion, heads, livers and kidneys of freshwater fish caught in Slovenian rivers and brooks. The distribution of elements in the fish body and the differences in level of accumulation in various fish species were investigated.

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Material and methods

Samples

From 1999 to 2003, 141 freshwater fish samples caught in various Slovenian rivers and brooks were analysed. The fish were sampled by local fisherman and sent to the laboratory either fresh or frozen. Samples were sorted by date and location of catch, fish species, length and weight. 74 samples of various species of salmonids such as brown trout (*Salmo trutta m. fario*), marble trout (*Salmo marmoratus*), brook trout (*Salevelinus fontinalis*) and rainbow trout (*Oncorhynchus mykiss*), 23 samples of chub (*Leuciscus c. cephalus*), 12 samples of nase (*Chondrostoma nasus*), 9 samples of Danube roach (*Rutilus pigus virgo*), 12 samples of grayling

Table 1: Fish species, rivers and number of samples analysed

River \ Species		Salmonids	Chub	Nase	Danube roach	Grayling	Barbel	Total
Sava	I	13	3	1		3	3	**23**
Javornik		3						**3**
Tržiška Bistrica		4						**4**
Kokra		1						**1**
Sora		6	2	2		2	2	**14**
Kamniška Bistrica[a]		2	1					**3**
Ljubljanica		2	12	2	5	7		**28**
Obrh		1						**1**
Bober (brook)		7						**7**
Savinja		2	3	3			3	**11**
Sopota (brook)		2						**2**
Krka					2			**2**
Lahinja and Krupa		1	1	1	1			**4**
Drava	II		1	3	1		2	**7**
Bistra		1						**1**
Meža		6						**6**
Šentanelska reka		2						**2**
Soča[b]	III	11						**11**
Nadiža		2						**2**
Idrijca		6					1	**7**
Hubelj		1						**1**
Rižana		1						**1**
Total		**74**	**23**	**12**	**9**	**12**	**11**	***141***

[a] including the Debenski potok and Titanova mlinščica brooks
[b] including the Lepena and Doblarec brooks

(*Thymallus thymallus*) and 11 samples of barbel (*Barbus barbus*) were examined in the study. The number of samples, fish species and location of catch (name of open water) are listed in Table 1.

The rivers and brooks in Table 1 are sorted in three groups (I, II, III). Group I includes the Sava River with subsidiary streams and Group II includes the Drava River with subsidiary streams. The rivers and brooks in the first two groups flow to the Black Sea. In Group III are rivers and brooks that flow to the Adriatic Sea. From 1 to 3 fish were prepared per sample, according to the procedure described in the Association of Official Analytical Chemists (8). Before preparation the fish were washed, dried and scaled. For each fish, the head, liver and kidney were sampled and fillets in natural proportion were prepared from muscle and skin. The fillets were then minced with a Büchi-400 homogenizer. Different tissues were packed separately and kept below –18° C until the day of analysis. Some of the fish samples received were already dissected and without viscera and/or heads, and in those cases only the muscle/skin was prepared. In some cases only the liver and kidney were received, but for all of them the data regarding species, weight, length, date and location of catch were available.

Reagents

Standard solutions of Cu, Zn, Fe and Mn were prepared from commercial stock standard solutions (Merck) at concentrations of 1000 mg/L. Double deionised water was used throughout. Working standard solutions were prepared by dilution of stock standard solution with the addition of hydrochloric acid, so that the acid concentration in working standard solutions matched the acid concentration in digested solutions. All reagents used were an analytical-reagent grade.

Sample preparation

10 g of muscle/skin or whole head, liver or kidney were weighed in quartz-glass crucibles, dried

Table 2: Results of analyses of certified reference materials. Mean and standard deviation in mg/kg. Number of analysis in parentheses

Metal		BCR N°185R	BCR N°422	BCR N°186
Cu	Found	0.533 ± 0.021 (6)	0.84 ± 0.06 (4)	28.8 ± 1.2 (5)
	Certified	0.544 ± 0.017	1.05 ± 0.07	31.9 ± 0.4
Zn	Found	128.6 ± 2.6 (6)	19.2 ± 0.4 (6)	119 ± 7 (6)
	Certified	138.6 ± 2.1	19.6 ± 0.5	128 ± 6
Fe	Found	/	4.66 ± 0.59 (5)	278 ± 17 (6)
	Certified	/	5.46 ± 0.30	299 ± 10
Mn	Found	10.66 ± 0.24 (6)	0.512 ± 0.023 (6)	8.0 ± 0.5 (6)
	Certified	11.07 ± 0.29	0.543 ± 0.028	8.5 ± 0.3

in a drying oven at 105° C and ashed overnight at 450° C ± 25° C in a programmable furnace. The ash was dissolved with diluted hydrochloric acid (1/1, v/v). The solution was evaporated to dryness. The remains were redissolved with diluted hydrochloric acid (1/10, v/v), filtered into a 50 mL volumetric flask and diluted with water to the mark.

Sample analysis

The Cu, Zn, Fe and Mn concentrations in the samples were determined by flame atomic absorption spectrophotometry (FAAS), using a Varian SpectrAA 220 instrument with deuterium background correction. The determinations were preformed by aspirating the final solutions into an AA spectrophotometer using an air-acetylene flame. Absorption wavelengths were as follows: 324.8 nm for Cu, 213.9 nm for Zn, 248.3 nm for Fe and 279.5 nm for Mn. Concentrations of metals in samples were evaluated using an external standard method.

The accuracy of the analytical procedure was checked by analyzing three different standard reference materials: cod muscle BCR 422, bovine liver BCR 185R and pig kidney BCR 186. The agreement for all the elements investigated was 80–98%. The results of analysis of standard reference material are shown in Table 2.

A *t-test* was used to statistically evaluate the sample data. A *p* value lower than 0.05 was considered to be significant. If the concentration of a metal was below the detection limit, a value of half of the detection limits was used in order to facilitate statistical comparisons

Results

The contents of the metals examined in various tissues of various fish species are given in Table 3.

The data in Table 3 reveal low levels of Cu and Mn in tissues and higher levels of Zn and Fe. The lowest concentrations of all elements were found in muscle/skin. The average concentrations of Cu in tissues of various fish species are shown in Fig. 1.

The highest concentrations of Cu were always found in the liver, followed by the kidney, head and muscle/skin. The ratio between the level of Cu in the liver and muscle/skin was particularly high in salmonids. The concentration of Cu in the liver of other fish species was approximately ten times lover than in salmonids and we did not observe any significant differences between them. The kidney, head and muscle/skin of barbel contained significantly higher levels of Cu than chub, nase, Danube roach and grayling. The kidney and muscle/skin, but not the head, of salmonids also contained significantly higher concentrations of Cu than the species mentioned above.

The average concentrations of Zn in the tissues of various fish species are shown in Figure 2.

Chub and Danube roach contained the highest amount of Zn in the kidney, and nase, grayling and barbel in the head. Salmonids contained an equal amount, and the highest amount of Zn in the head and liver. The kidney of barbel, nase, salmonids and grayling contained on average less than 25 mg Zn/kg. Higher concentrations were detected in the kidney of chub and the highest in the kidney of Danube roach. In the liver, concentrations of Zn were significantly higher in Danuble roach and chub than in barbel, nase and grayling. In the muscle/skin, the highest concentrations were also detected in Danube roach and the lowest in barbel and grayling. In the head, higher concentrations of Zn were detected in chub, nase and Danube roach than in salmonids, grayling and barbel.

Figure 3 presents the average concentrations of Fe in the tissues of various fish species, from

Table 3: Cu, Zn, Fe and Mn contents in tissues of various fish species, expressed in mg/kg wet weight

		Muscle and skin			Head			Liver			Kidney		
		N	min. - max.	average ± SD	N	min. - max.	average ± SD	N	min. - max.	average ± SD	N	min. - max.	average ± SD
Cu	Salmonids	60	<0.05 - 1.38	**0.36** ± 0.19	48	<0.05 - 0.82	**0.32** ± 0.17	42	3.43 – 324	**75** ± 69	38	0.29 – 4.02	**1.67** ± 0.87
	Grayling	12	<0.05 - 0.26	**0.17** ± 0.07	12	0.14 – 0.35	**0.23** ± 0.08	10	0.48 – 9.77	**2.63** ± 2.62	9	0.30 – 0.80	**0.57** ± 0.16
	Chub	15	<0.05 - 0.43	**0.22** ± 0.12	16	0.10 – 0.55	**0.32** ± 0.15	16	<0.06 – 8.39	**2.31** ± 2.36	14	<0.10 – 1.17	**0.50** ± 0.34
	Nase	12	<0.05 - 0.62	**0.30** ± 0.17	10	0.28 – 0.58	**0.40** ± 0.11	3	2.67 – 6.28	**4.09** ± 1.93	4	0.19 – 0.63	**0.44** ± 0.21
	Danube roach	8	0.11 - 0.30	**0.21** ± 0.08	5	0.18 – 0.45	**0.30** ± 0.13	5	0.37 – 2.32	**1.35** ± 0.87	4	0.21 – 0.78	**0.43** ± 0.25
	Barbel	10	<0.05 - 0.54	**0.37** ± 0.18	10	0.22 – 1.01	**0.53** ± 0.22	7	0.40 – 7.03	**2.31** ± 2.20	6	0.44 – 1.63	**0.94** ± 0.50
Zn	Salmonids	60	5.2 - 17.6	**9.7** ± 2.5	48	19.5 - 62.1	**33.9** ± 10.9	42	10.6 – 87.2	**35.3** ± 13.5	38	14.8 – 46.9	**23.1** ± 5.8
	Grayling	12	5.3 - 12.2	**8.5** ± 2.5	12	22.6 – 44.1	**33.5** ± 7.0	10	15.7 – 27.7	**22.4** ± 3.9	9	12.6 – 20.0	**15.4** ± 2.2
	Chub	15	4.4 - 13.3	**9.3** ± 2.3	16	12.8 – 80.8	**50.0** ± 17.4	16	13.6 – 81.0	**43.1** ± 18.7	14	50.2 – 404	**112** ± 93
	Nase	12	6.2 - 12.0	**9.4** ± 1.7	10	26.8 – 58.4	**42.7** ± 9.2	3	19.1 – 23.9	**21. 5** ± 2.4	4	17.8 – 22.5	**20.3** ± 2.2
	Danube roach	8	6.2 - 18.5	**12.8** ± 4.5	5	26.8 – 49.1	**41.7** ± 9.5	5	25.2 – 78.3	**54.1** ± 2.3	4	115 – 387	**270** ± 118
	Barbel	10	5.8 - 10.2	**7.8** ± 1.5	10	22.4 – 34.9	**30.1** ± 3.8	7	16.7 – 30.9	**20.4** ± 4.9	6	10.6 – 25.5	**16.1** ± 5.2
Fe	Salmonids	39	2.6 - 7.7	**4.8** ± 1.5	48	5.0 - 70.2	**15.4** ± 9.6	42	54 – 501	**178** ± 104	38	61– 325	**144** ± 54
	Grayling	12	2.9 - 5.8	**4.0** ± 0.8	12	8.9 – 22.5	**15.4** ± 4.6	10	36 – 175	**95** ± 48	9	110 – 205	**165** ± 31
	Chub	14	1.9 - 6.8	**4.1** ± 1.3	16	4.5 – 96.4	**18.3** ± 21.2	16	12 – 378	**72** ± 91	14	24 – 115	**71** ± 30
	Nase	11	4.1 - 9.2	**6.2** ± 1.9	10	10.9 – 40.8	**18.2** ± 9.2	3	65 – 193	**113** ± 70	4	100 – 179	**132** ± 35
	Danube roach	8	2.9 - 9.2	**4.8** ± 2.0	5	7.4 – 36.1	**17.2** ± 11.1	5	20 – 378	**172** ± 153	4	35 – 195	**111** ± 70
	Barbel	10	3.9 - 14.4	**6.7** ± 3.4	10	6.1 – 49.2	**19.1** ± 13.7	7	67 – 235	**144** ± 65	6	35 – 422	**166** ± 135
Mn	Salmonids	42	<0.07 - 0.35	**0.17** ± 0.08	48	0.21 - 3.89	**1.11** ± 0.73	42	0.40 – 2.61	**1.01** ± 0.45	37	0.16 – 1.25	**0.42** ± 0.24
	Grayling	12	0.14 - 0.37	**0.26** ± 0.08	12	1.21 – 8.03	**4.17** ± 2.36	10	1.04 – 3.13	**1.56** ± 0.59	9	0.21 – 4.00	**1.15** ± 1.42
	Chub	15	0.12 - 0.47	**0.28** ± 0.11	16	2.02 – 6.86	**4.08** ± 1.38	15	0.40 – 5.74	**2.18** ± 1.85	14	<0.14 – 0.91	**0.60** ± 0.21
	Nase	12	0.24 - 0.86	**0.47** ± 0.18	10	5.37 – 16.30	**9.95** ± 3.44	3	1.51 –2.66	**2.19** ± 0.60	4	0.85 – 1.09	**0.95** ± 0.11
	Danube roach	8	<0.07 - 1.43	**0.34** ± 0.45	5	1.52 – 6.72	**3.84** ± 2.18	4	0.82 – 1.42	**1.15** ± 0.31	4	0.42 – 0.77	**0.61** ± 0.14
	Barbel	10	0.26 - 1.20	**0.62** ± 0.31	10	2.72 – 12.94	**6.34** ± 2.96	7	0.46 – 2.45	**1.12** ± 0.71	6	0.29 – 8.09	**1.95** ± 3.03

N - number of samples

which it is evident that Fe accumulates in the liver and kidney. Salmonids and Danube roach contained higher concentrations of Fe in the liver than in the kidney, but barbel, nase and grayling had higher concentrations in the kidney than in the liver. Chub contained on average an equal amount of Fe in the liver and kidney and significantly lower concentrations than in other fish species. In the muscle/skin, the concentrations of Fe were significantly higher in nase and barbel than in chub, Danube roach, salmonids and grayling. No significant differences were observed in Fe concentrations in the head.

The average concentration of Mn in tissues of various fish species is presented in Figure 4. The highest concentrations of Mn were observed in the head of all fish species, followed by the liver, kidney and muscle/skin. All tissues of salmonids contained significantly lower levels of Mn than other fish species examined. Between chub, Danube roach and grayling no differences were observed in the content of Mn in the head, but significantly higher concentrations were detected in the head of nase and barbel. Significant differences between fish species in Mn contents were also observed for the muscle/skin, liver and kidney.

Discussion

The distributions of trace elements in tissues were specific for each element. The content of Cu, Zn, Fe and Mn in all fish species were the lowest in the muscle/skin. Similar results were also reported for a number of other fish species. Muscle is not an active tissue regarding the accumulation of metals (2, 3, 9). The contents of Fe and Zn in tissues were higher than the contents of Cu and Mn. These results were expected because Zn is present in many enzymes in the fish's organism and Fe is used to transport oxygen throughout the fish's body. The elements accumulate mainly in metabolically active tissues such as the liver and kidney, with the exception of Mn, which accumulates in the head. In the liver and kidney, metals are bound to metallothioneins, low molecular weight proteins with a high cysteine content. Metallothioneins are synthesized in the liver and kidney, and also in the gills after waterborne and dietary exposure and are considered to be involved in regulation of essential metals as well as Cd and Hg (1, 4, 5, 9). For all the elements studied, significant differences in concentration levels were found between various fish species, demonstrating that the concentration levels attained are species-related. The highest differences between fish species were observed in Cu content in the liver. Salmonids had approx-

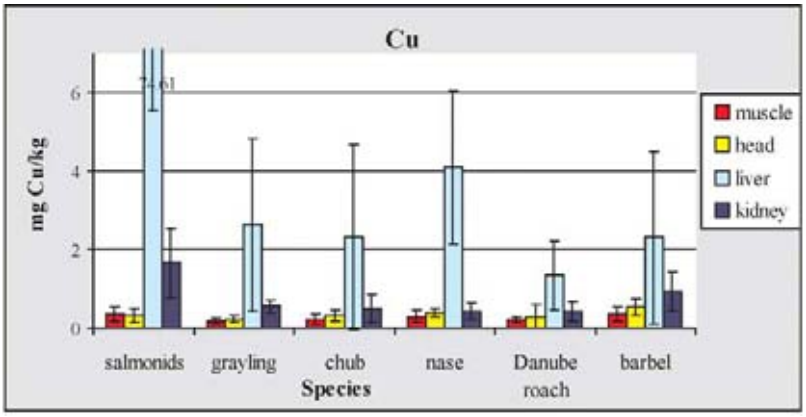

Figure 1: Average concentrations of Cu in tissues of various fish species (mg/kg wet weight)

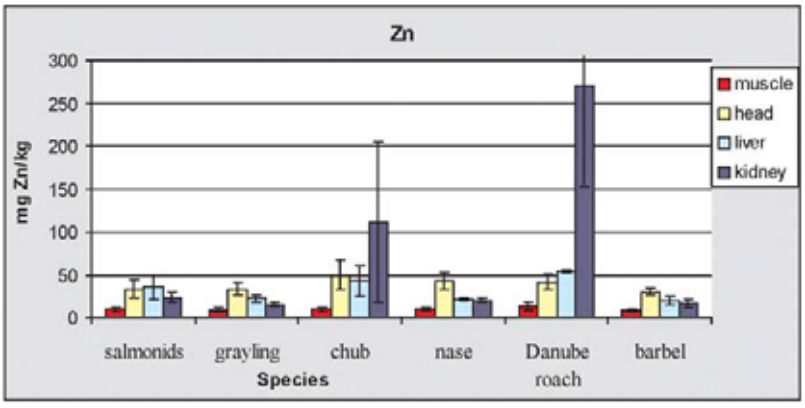

Figure 2: Average concentrations of Zn in tissues of various fish species (mg/kg wet weight)

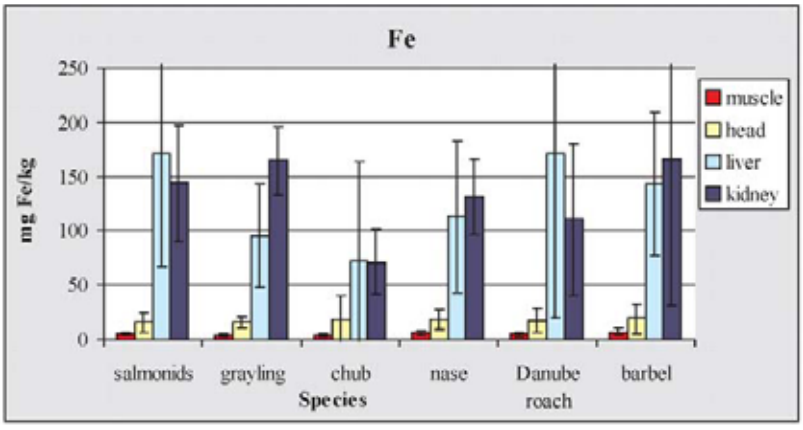

Figure 3: Average concentrations of Fe in tissues of various fish species (mg/kg wet weight)

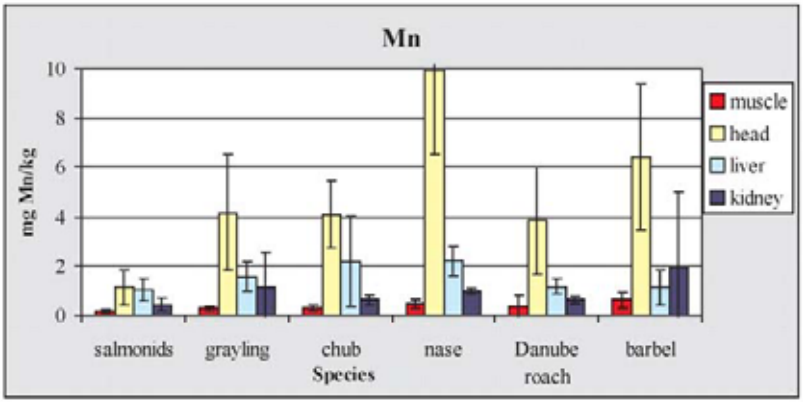

Figure 4: Average concentrations of Mn in tissues of various fish species (mg/kg wet weight)

imately an order of magnitude higher concentration than other fish species, which was also reported by Amundsen et al. (2). Various data in the literature indicate that the liver of brown trout from contaminated rivers contained around 200 to 300 mg Cu/kg wet weight (2, 6, 7). In our research only two samples of salmonids that were analysed contained more than 200 mg Cu/kg in the liver. Those samples were from a stream in the Soča Valley. However, Farkas et al. (11) reported that Cu concentration in the liver also increases with increasing length and weight of fish. The fish from the Soča Valley were much larger (1.2 kg and 3 kg) than the fish from contaminated water analysed by Olsvink et al. (6, 7), which weighed from 13-91 g. It could be concluded that the cause of the high amount of Cu in our samples is the weight of fish. Fish feeding on invertebrates have been found to have higher concentrations of Zn than piscivorous species (2). Our results largely support these observations, because Danube roach and chub had higher levels of Zn, especially in the kidney, than barbel and nase. All these fish species belong to family *Cyprinidae.* The Danube roach feeds on worms, molluscs, crustaceans and considerable amounts of plant matter, the chub feeds on algae and also on worms, molluscs, crustaceans as well as on small fish, but the nase feeds mostly on algal growth on stones (12). The highest concentrations of Zn in the kidney of Danube roach and chub were detected in fish from the Ljubljanica. These chubs were around 25 cm long, but larger chubs from the Ljubljanica contained lower values of Zn. Because the Zn concentrations in tissue decreased significantly with increasing length of the fish (2, 11), high levels of Zn in small chub and Danube roach are probably not a consequence of contamination. In all salmonid tissue, the levels of Mn were lower than in tissues of other fish species. Karadede and Ünü (9) also reported that Mn was not accumulated in salmonids. Cu, Zn, Fe and Mn are essential elements and are carefully regulated by physiological mechanisms in most organs. The differences observed between fish species can be explained by varying abilities for homeostatic control, detoxification and rejection, and varying fish feeding behaviour (9, 10).

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References

1. Kargin F. Metal concentrations in tissues of the freshwater fish Capoeta barroisi from the Seyhan river (Turkey). Bull Environ Contam Toxicol 1998; 60: 822-8.

2. Amundsen PA, Staldvik FJ, Lukin AA, Kashulin NA, Popova OA, Reshetnikov YS. Heavy metal contamination in freshwater fish from the border region between Norway and Russia. Sci Total Environ 1997; 201: 211-24.

3. Mansour SA, Sidky MM. Ecotoxicological studies. 3. Heavy metals contaminating water and fish from Fayoum Governorate, Egypt. Food Chem 2002; 78: 15-22.

4. Liang Y, Cheung RYH, Wong MH. Reclamation of wastewater from polyculture of freshwater fish: bioaccumulation of trace metals in fish. Water Res 1999; 33(11): 2690-700.

5. Carpené E, Gumiero B, Fedrizzi G, Serra R. Trace elements in fish from rearing poinds of Emilia-Romagna region (Italy). Sci Total Environ 1994; 141: 139-46.

6. Olsvik PA, Gundersen P, Andersen RA, Zachariassen KE. Metal accumulation and metallothionein in two populations of brown trout, Salmo trutta, exposed to different natural water environments during a run-off episode. Aquat Toxicol 2000; 50: 301-16.

7. Olsvik PA, Gundersen P, Andersen RA, Zachariassen KE. Metal accumulation and metallothionein in brown trout, Salmo trutta, from two Norwegian rivers differently contaminated with Cd, Cu and Zn. Comp Biochem Physiol C 2001; 128: 189-201.

8. AOAC. Official methods of analysis of the AOAC International. 15th ed. Arlington: AOAC International, 1990: 31.01.01.

9. Karadede H, Ünlü E. Concentrations of some heavy metals in the water, sediment and fish species from the Atatürk Dam Lake (Euphrates), Turkey. Chemosphere 2000; 41: 1371-6.

10. Allen-Gil SM, Martynov VG. Heavy metal burdens in nine species of freshwater and anadromous fish from the Pechora river, Northern Russia. Sci Total Environ 1995; 160/161: 653-9.

11. Farkas A, Salanki J, Specziar A. Age- and size-specific patterns of heavy metals in the organs of freshwater Abramis brama L. populating a low-contaminated size. Water Res 2003; 37: 959-64.

12. Povž M, Sket B. Naše sladkovodne ribe. Ljubljana: Mladinska knjiga, 1990: 86-178.

VSEBNOST BAKRA, CINKA, ŽELEZA IN MANGANA V SLADKOVODNIH RIBAH V SLOVENIJI

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Povzetek: V obdobju od leta 1999 do 2003 smo v 141 vzorcih sladkovodnih rib iz različnih slovenskih rek in potokov določili vsebnost Cu, Zn, Fe in Mn. Določili smo jo v mesu s kožo (v naravnem razmerju), v glavah, jetrih in ledvicah rib. Analizirali smo tkiva potočne postrvi *(Salmo trutta m. fario)*, soške postrvi *(Salmo marmoratus)*, potočne zlatovčice *(Salevelinus fontinalis)*, šarenke *(Oncorhynchus mykiss)*, lipana *(Thymallus thymallus)*, klena *(Leuciscus c. cephalus)*, podusti *(Chondrostoma nasus)*, platnice *(Rutilus pigus virgo)* in mrene *(Barbus barbus)*. Vsebnost elementov v tkivih smo določili z metodo plamenske atomske absorpcijske spektrometrije. Na podlagi rezultatov analiz smo proučili porazdelitev elementov po posameznih tkivih rib in ugotovili, da je le-ta specifična za vsak element in odvisna od vrste ribe. Največje razlike med vrstami rib smo opazili v vsebnosti Cu v jetrih in Zn v levicah. Koncentracija Cu v jetrih salmonidov je bila približno desetkrat višja (povprečje 74,61 mg/kg) kot v jetrih ostalih vrst rib. Ledvice salmonidov, lipanov, podusti in mren so vsebovale v povprečju manj kot 25 mg Zn/kg, ledvice klena povprečno 112 mg Zn/kg in platnice 271 mg Zn/kg. Razlike v vsebnosti Zn v ledvicah omenjenih vrst rib so najverjetneje posledica različnega načina prehrane. Ugotovili smo, da je bila vsebnost vseh preiskovanih elementov najnižja v mesu rib. Elementi se akumulirajo v metabolično aktivnih tkivih, kot so jetra in ledvice, z izjemo Mn, katerega najvišje koncentracije smo ugotovili v glavah rib (območje 0,21 - 16,30 mg/kg).

Ključne besede: hrana, kontaminacija; hrana, analize; baker; cink; železo; mangan; spektrometrija; ribe

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Original Research Paper

UV - SPECTROMETRIC METHOD FOR DETERMINATION OF BENZYLPENICILLIN SODIUM (PENICILLIN G) IN MODEL MIXTURES WITH DIFFERENT MILKS

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Summary: An investigation of 11 different milks from Bulgaria, Greece and France is carried out and parameters of milks are compared. The quantity of Benzylpenicillin Sodium (Penicillin G) in model mixtures with the mentioned milks is obtained. For the investigation of milks are used the followed methods: alkalimetric titration for determination of acidity and content of casein; lactodensitometry for measuring of density; bromthymol method for study of alkalescence; boiling tests for examination of freshness and stability. For determination of content of Penicillin G in model mixtures with milks is applied an existing UV - spectrometric method, after hydrolysis of antibiotic with solution of hydrochloric acid (pH = 4). The experimental results for the investigated parameters acidity, content of casein, density, alkalescence, freshness and stability correspond to the regulated standards for milk. Analytical parameters repeatability (precision) and accuracy are determined by the uncertainty of the result, in which are included: standard deviation (SD), relative standard deviation (RSD) and confidential interval ($\overline{X} \pm t.S\overline{X}$). The accuracy is determined by the degree of recovery (R).

Key words: milk-analysis; drug residues; penicillium G; spectrometry, ultraviolet

Introduction

The residues of β - lactam antibiotics in milk, obtained from animals with mastitis, may produce an allergic responces (1). The investigation of residues is conditioned by the requirements to certify the quality of milk. This problem is being realized by means of confirmation of correspondence of the eventual antibiotic residues in the milk with regulated from the European Community a highest degree of admissible antibiotic residues (2).

During the past few years Benzylpenicillin Sodium is the favorite agent for intramammary treatment of the mastitis in the veterinary practice (3). The most widespread method for analysis of Penicillin G in milk is HPLC (2, 4, 5).

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Materials and methods

I. Fresh cow's milk (not pasteurized):
Danone (Sofia - 17.05.2001; Batch. N:1222200); Elby (Sofia - 28.05.2001; Batch. N:0512025); Filipopolis (Sofia - 05.06.2001; Batch. N:4737261); Formalat (Dolni Lozen - 10.06.2001; Batch. N:002532); Home milk (Kasichene - 17.06.2001; --); Markelli (Kasanlak - 19.06.2001; Batch. N:924200); Zdravetz (Svishtov - 21.06.2001; Batch. N:0412002); Triki (Greece - 13.08.2001; Batch. N:5989); Family (Greece - 13.08.2001; Batch. N:979123); Family light (Greece - 14.08.2001; Batch. N:854769); Candia (France - 28.05.2001; Batch. N:002526).

II. Drug product: Benzylpenicillin Sodium.

III. Reagents:

1. Destilled water, 1 % solution of phenolphtalein in 95 % ethanol, 0.1 M solution of sodium hydroxide (used in methods IV. 1. and IV. 2.).
2. 2.5 % solution of cobalt sulphate in water, 35 % solution of formaldehyde (used in method

Table 1: Results for absorbance of hydrolyzed Benzylpenicillin Sodium and for Shovene ' s criterion for model mixtures (I - IV) with milk Danone

	Absorbance			
N	**A 150**	**A 200**	**A 250**	**A 300**
1.	0.10647	0.13911	0.18057	0.21040
2.	0.10432	0.14171	0.17651	0.21436
3.	0.10266	0.14060	0.17568	0.21946
4.	0.10683	0.13735	0.17602	0.21189
5.	0.10387	0.14464	0.17957	0.22024
6.	0.10581	0.14354	0.17387	0.21262
	Shovene' s criterion			
	Sh C 150	**Sh C 200**	**Sh C 250**	**Sh C 300**
1.	0.9056	0.7519	1.3939	1.0806
2.	0.4120	0.2016	0.2067	0.1138
3.	1.4249	0.2067	0.5335	1.1296
4.	1.1245	1.4005	0.3994	0.7163
5.	0.6867	1.2765	1.0000	1.3205
6.	0.5021	0.8734	1.2486	0.5394

A 150; A 200; A 250; A 300 - absorbance of hydrolyzed Benzylpenicillin Sodium (benzylpenicillenic acid) - respectively in model mixtures I (150 μg/ml); II (200 μg/ml), III (250 μg/ml); IV (300 μg/ml) with milk Danone.

Sh C 150; Sh C 200; Sh C 250; Sh C 300 - Shovene's criterion for the obtained quantity of hydrolyzed Penicillin G - respecrively in model mixtures I (150 μg/ml); II (200 μg/ml), III (250 μg/ml); IV (300 μg/ml) with milk Danone.

N - number of the individual measurements

IV. 2.).

3. 0.2 % solution of bromthymol blue in 95 % ethanol (used in method IV. 4.).

4. 6.81 % solution of potassium dihydrogen phosphate in water, 1 % solution of calcium chloride in water (used in method IV. 5.).

5. 95 % ethanol, solution of hydrochloric acid (pH = 4) (used in method V.).

All reagents are chemical pure and with analytical great quality.

IV. Methods for control of parameters of milk [6].

1. Alkalimetric titration for determination of acidity.

2. Method of Karunina - Stilovich - alkalimetric titration for determination of content of casein.

3. Lactodensitometry for measuring of density.

4. Bromthymol method for study of alkalescence.

5. Boiling tests for examination of stability.

6. Boiling test for examination of freshness.

V. Method for determination of Penicillin G in mixtures with milks by spectrometry.

Standard preparation: Accurately weighed quantity of Benzylpenicillin Sodium (0.015g, 0.020g, 0.025g, 0.030g) is disolved in solution of hydrochloric acid (pH = 4) and is diluted to 100.0 ml with the same solvent, to obtained respectively the standard solutions (1 - 4) with the followed concentrations of antibiotic: 150 μg/ml, 200 μg/ml, 250 μg/ml, 300 μg/ml.

Test preparation: The model mixtures (I - IV) are prepared by mixing of 1.0 ml milk with 0.015g, 0.020g, 0.025g and 0.030g Penicillin G respectively. The mixtures are treated with 95 % ethanol and are filtered. The filtrates are diluted to 100.0 ml with solution of hydrochloric acid (pH = 4), to obtained correspondingly model mixtures (I - IV) with the followed concentrations of antibiotic: 150 μg/ml, 200 μg/ml, 250 μg/ml, 300 μg/ml. After 60 min. of hydrolysis, for standards and for samples are measured at 322 nm the absorbances of hydrolyzed antibiotic (benzylpenicillenic acid) (7).

Results

I. Experimental data for parameters acidity, content of casein and density.

The obtained results for acidity of milks are: Danone (16.8°T), Elby (20.4°T), Filipopolis (18.5°T), Formalat (19.0°T), Home milk (16.2°T),

Table 2: Content of hydrolyzed Benzylpenicillin Sodium in model mixtures (I - II) with milk Danone

	Model mixture I		Model mixture I I	
N	**C 150**	**R 150**	**C 200**	**R 200**
1.	151.67	101.11	197.66	98.83
2.	148.60	99.07	201.35	100.68
3.	146.24	97.49	199.77	99.89
4.	152.18	101.45	195.15	97.58
5.	147.96	98.64	205.51	102.76
6.	150.73	100.49	203.95	101.98
$\overline{X}$ ± RSD	149.56 ± 1.56	99.71 ± 1.55	200.57 ± 1.93	100.29 ± 1.93
SD	2.33	1.55	3.87	1.94
RSD	1.56	1.55	1.93	1.93
S $\overline{X}$	0.95	0.63	1.58	0.79
P [%]	99.00	99.00	99.00	99.00
t	4.03	4.03	4.03	4.03
t. S $\overline{X}$	3.83	2.54	6.37	3.18
$\overline{X}$ ± t . S $\overline{X}$	145.73 ÷ 153.39	97.17 ÷ 102.25	194.20 ÷ 206.94	97.11 ÷ 103.47
E [%]	0.64	0.63	0.79	0.79

C 150; C 200 - concentration of hydrolyzed Benzylpenicillin Sodium at 60 min. after the beginning of hydrolysis [μg/ml] - respectively for model mixture I (150 μg/ml) and for model mixture II (200 μg/ml).

R 150; R 200 - degree of recovery (%) - respectively for model mixture I (150 μg/ml) and for model mixture II (200 μg/ml).
N - number of the individual measurements
SD - standard deviation
RSD - relative standard deviation

S $\overline{X}$ - mean quadratic error
P - confidential possibility (%)
t - coefficient of Student

$\overline{X}$ ± t . S $\overline{X}$ - confidential interval
E - relative error (%).

Markelli (18.0°T), Zdravetz (17.6°T), Triki (16.5°T), Family (17.0°T), Family light (18.0°T), Candia (16.0°T). The content of casein in milks is: Danone (3.20 %), Elby (4.00 %), Filipopolis (2.20 %), Formalat (2.23 %), Home milk (3.40 %), Markelli (2.75 %), Zdravetz (3.96 %), Triki (2.27 %), Family (2.64 %), Family light (2.60 %), Candia (3.02 %). The results for parameter density are: Danone (1.0302), Elby (1.0283), Filipopolis (1.0309), Formalat (1.0267), Home milk (1.0255), Markelli (1.0318), Zdravetz (1.0272), Triki (1.0274), Family (1.0296), Family light (1.0308), Candia (1.0296).

II. Quantity analysis of Penicillin G in model mixtures (I - IV) with milks.

In spectra of the placebo solution isn't exist peak at 322 nm and by this fact is confirmed the specificy of method. The linearity is studied and the concentration interval, where Beer's law is valid, is determined - 150 μg/ml ÷ 300 μg/ml. For standard solutions (1 - 4) of Penicillin G the measured absorbances of benzylpenicillenic acid at 60 min. after the beginning of hydrolysis are respectively : 0.10530, 0.14076, 0.17715, 0.21539.

The experimental results for the absorbances of benzylpenicillenic acid in model mixtures (I - IV) with milk Danone (A 150, A 200, A 250, A 300) and the data for the Shovene's criterion for the obtained quantity of hydrolyzed Penicillin G in every sample are presented in table 1. The results

Table 3: Content of hydrolyzed Benzylpenicillin Sodium in model mixtures (III - IV) with milk Danone

	Model mixture I I I		Model mixture I V	
N	**C 250**	**R 250**	**C 300**	**R 300**
1.	254.83	101.93	293.05	97.68
2.	249.10	99.64	298.57	99.52
3.	247.93	99.17	305.67	101.89
4.	248.41	99.36	295.13	98.38
5.	253.42	101.37	306.76	102.25
6.	245.37	98.15	296.14	98.71
$\overline{X}$ ± RSD	249.84 ± 1.43	99.94 ± 1.43	299.22 ± 1.91	99.74 ± 1.90
SD	3.58	1.43	5.71	1.90
RSD	1.43	1.43	1.91	1.90
$S_{\overline{X}}$	1.46	0.58	2.33	0.78
P [%]	99.00	99.00	98.00	98.00
t	4.03	4.03	3.37	3.37
t. $S_{\overline{X}}$	5.88	2.34	7.85	2.63
$\overline{X} \pm t \cdot S_{\overline{X}}$	243.96 ÷ 255.72	97.60 ÷ 102.28	291.37 ÷ 307.07	97.11 ÷ 102.37
E [%]	0.58	0.58	0.78	0.78

C 250; C 300 - concentration of hydrolyzed Benzylpenicillin Sodium at 60 min. after the beginning of hydrolysis [g/ml] - respectively for model mixture III (250 μg/ml) and for model mixture IV (300 μg/ml).

R 250; R 300 - degree of recovery (%) - respectively for model mixture III (250 μg/ml) and for model mixture IV (300 μg/ml).

N - number of the individual measurements
SD - standard deviation
RSD - relative standard deviation

$S_{\overline{X}}$ - mean quadratic error
P - confidential possibility (%)
t - coefficient of Student

$\overline{X} \pm t \cdot S_{\overline{X}}$ - confidential interval
E - relative error (%).

for the obtained quantity of hydrolyzed Benzylpenicillin Sodium in model mixtures (I - II) and (III - IV) with milk Danone, are pointed respectively in table 2 and table 3. The content of hydrolyzed antibiotic is calculated by using the data for the absorbances from table 1. For every kind of the examined mixtures are prepared 6 samples. In table 2 and table 3 are indicated for all mixtures: N - number of the individual measurements (1 ÷ 6); concentration of hydrolyzed Benzylpenicillin Sodium at 60 min. after the beginning of hydrolysis [μg/ml] - respectively: C 150, C 200 - for model mixtures (I - II) (table 2); C 250, C 300 - for model mixtures (III - IV) (table 3); degree of recovery (%) - respectively: R 150, R 200 - for model mixtures (I - II) (table 2); R 250, R 300 - for model mixtures (III - IV) (table 3); P - confidential possibility (%), t - coefficient; mean quadratic error ($S_{\overline{X}}$); relative error (E). The results for the quantity of Penicillin G in the investigated model mixtures (I - IV) with other milks and the data about the degree of recovery are summerized in table 4 and table 5. The content of antibiotic is calculated by using the data for the absorbances.

Discussion

The obtained results show that the acidity for milks Elby (20.4° T), Filipopolis (18.5° T) and Formalat (19.0° T) is higher than 18° T and for other milks corresponds to the regulated standards (16 ÷ 18° T). The data for the content of casein in milks suit standard requirements (2.2 % ÷ 4.0 %). Parameter density for milks Home milk (1.0255)

Table 4: Degree of recovery and content of hydrolyzed Benzylpenicillin Sodium in model mixtures (I - IV) with milks

N:	$\overline{X}$ ± RSD	$\overline{X}$ ± t . S $\overline{X}$	R $\overline{X}$ [%]	SD
Milk	**E L B Y**			
I	145.74 ± 1.85	142.91 ÷ 148.57	97.16	2.70
II	218.36 ± 0.44	217.36 ÷ 219.36	109.18	0.96
III	257.27 ± 0.38	256.24 ÷ 258.30	102.91	0.97
IV	307.22 ± 0.25	306.42 ÷ 308.02	102.41	0.76
Milk	**F I L I P O P O L I S**			
I	151.81 ± 0.92	150.35 ÷ 153.27	101.21	1.39
II	195.60 ± 0.35	194.88 ÷ 196.32	97.80	0.68
III	271.03 ± 0.26	270.28 ÷ 271.78	108.41	0.71
IV	294.96 ± 0.31	293.98 ÷ 295.94	98.32	0.92
Milk	**F O R M A L A T**			
I	142.02 ± 0.17	141.76 ÷ 142.28	94.68	0.24
II	197.50 ± 0.23	197.01 ÷ 197.99	98.75	0.46
III	245.14 ± 0.54	243.75 ÷ 246.53	98.06	1.33
IV	292.89 ± 0.80	290.42 ÷ 295.36	97.63	2.35
Milk	**H O M E M I L K**			
I	147.45 ± 0.33	146.94 ÷ 147.96	98.30	0.48
II	190.40 ± 0.38	189.63 ÷ 191.17	95.20	0.73
III	251.37 ± 0.47	250.11 ÷ 252.63	100.55	1.19
IV	286.38 ± 1.25	282.60 ÷ 290.16	95.46	3.59
Milk	**M A R K E L L I**			
I	143.75 ± 0.42	143.13 ÷ 144.37	95.83	0.60
II	187.33 ± 0.35	186.64 ÷ 188.02	93.67	0.65
III	260.23 ± 0.57	258.69 ÷ 261.77	104.09	1.48
IV	301.89 ± 0.30	300.91 ÷ 302.87	100.63	0.92

$\overline{X}$ - concentration of hydrolyzed Benzylpenicillin Sodium at 60 min. after the beginning of hydrolysis [μg/ml] - respectively for model mixtures: I (150 μg/ml); II (200 μg/ml), III (250 μg/ml); IV (300 μg/ml).

R $\overline{X}$ - degree of recovery (%) - correspondingly for model mixtures I (150 μg/ml)); II (200 μg/ml), III (250 μg/ml); IV (300 μg/ml).

N - number of model mixture
SD - standard deviation
RSD - relative standard deviation

S $\overline{X}$ - mean quadratic error
t - coefficient of Student

$\overline{X}$ ± t . S $\overline{X}$ - confidential interval

and Formalat (1.0267) is lower than 1.0270 and for other milks corresponds to the regulated standards (1.0270 ÷ 1.0320). The origin of all examined milks from healthy animals is confirmed by the obtained yellow colour by bromthymol sample. The freshness and the stability of milks are proved by the lack of coagulation, obrained from boiling tests. The Shovene's criterion: U_{Sh} C 150, U_{Sh} C 200, U_{Sh} C 250, U_{Sh} C 300 for samples is lower than standard Shovene's criterion: U_{Sh} St = 1.73 (N = 6). Analytical parameters repeatability (precision) and accuracy are determined by the uncertainty of the result, in which are included: standard deviation (SD), relative standard deviation (RSD) and confidential interval ($\overline{X}$ ± t.S $\overline{X}$). The accuracy is determined by the degree of recovery (R) [8].

From the experimental results is confirmed, that for milks Candia, Family light, Family, Triki and Formalat with 150 μg/ml antibiotic, the

Table 4: Degree of recovery and content of hydrolyzed Benzylpenicillin Sodium in model mixtures (I - IV) with milks

N:	$\overline{X}$ ± RSD	$\overline{X}$ ± t . S $\overline{X}$	R $\overline{X}$ [%]	SD
Milk	**ZDRAVETZ**			
I	153.97 ± 1.12	152.17 ÷ 155.77	102.65	1.72
II	191.98 ± 0.32	191.34 ÷ 192.62	95.99	0.62
III	248.24 ± 0.10	247.96 ÷ 248.52	99.30	0.26
IV	294.78 ± 0.36	293.67 ÷ 295.89	98.26	1.06
Milk	**TRIKI**			
I	141.74 ± 1.25	139.88 ÷ 143.60	94.49	1.78
II	199.38 ± 0.47	198.40 ÷ 200.35	99.69	0.93
III	245.89 ± 0.94	243.45 ÷ 248.32	98.35	2.32
IV	281.77 ± 0.80	279.41 ÷ 284.14	93.92	2.25
Milk	**FAMILY**			
I	141.08 ± 0.39	140.50 ÷ 141.66	94.06	0.55
II	185.45 ± 0.74	184.01 ÷ 186.88	92.72	1.37
III	232.13 ± 0.20	231.65 ÷ 232.61	92.85	0.46
IV	286.28 ± 0.45	284.93 ÷ 287.62	95.43	1.28
Milk	**FAMILY LIGHT**			
I	140.92 ± 1.33	138.97 ÷ 142.87	93.95	1.87
II	191.28 ± 0.31	190.66 ÷ 191.90	95.64	0.59
III	246.79 ± 0.38	245.81 ÷ 247.77	98.72	0.94
IV	280.12 ± 1.22	276.55 ÷ 283.69	93.37	3.41
Milk	**CANDIA**			
I	137.12 ± 0.82	135.94 ÷ 138.30	91.41	1.12
II	194.41 ± 1.36	191.63 ÷ 197.19	97.21	2.65
III	243.27 ± 1.37	239.77 ÷ 246.77	97.31	3.34
IV	298.23 ± 0.47	296.77 ÷ 299.69	99.41	1.39

$\overline{X}$ - concentration of hydrolyzed Benzylpenicillin Sodium at 60 min. after the beginning of hydrolysis [μg/ml] - respectively for model mixtures: I (150 μg/ml); II (200 μg/ml), III (250 μg/ml); IV (300 μg/ml).

R $\overline{X}$ - degree of recovery (%) - correspondingly for model mixtures I (150 μg/ml)); II (200 μg/ml), III (250 μg/ml); IV (300 μg/ml).

N - number of model mixture
SD - standard deviation
RSD - relative standard deviation

S $\overline{X}$ - mean quadratic error
t - coefficient of Student

$\overline{X}$ ± t . S $\overline{X}$ - confidential interval

recovery is lower than the recovery for standard (150 μg/ml) respectively with 8.59 %, 6.05 %, 5.94 %, 5.51 %, 5.32 %. For mixtures of 200 μg/ml Penicillin G with milk Elby, the enchancement in relation to standard (200 μg/ml) is 9.179 %. For milks Family and Markelli with 200 μg/ml Benzylpenicillin Sodium, the recovery is lower than the recovery for standard (200 μg/ml) with 7.28 % and 6.33 %. For mixtures of 250 μg/ml Penicillin G with milks Markelli and Filipopolis the enchancement in relation to standard (250 μg/ml) is 4.093 % and 8.414 %. For milk Family with 250 μg/ml Benzylpenicillin Sodium the recovery is lower with 7.15 % than the recovery for standard (250 μg/ml).

Conclusion

All parameters of the examined milks correspond to the regulated standards. The obtained results confirm the possibilities for using the

applied UV - spectrometric method for the determination of a high level of residue of Penicillin G in milk, namely, levels above 100 mg / ml. Method is not enough sensitive to detect Benzylpenicillin Sodium at the MRL level (4 μg /kg).

References

1. Zachariev Tz. Veterinary and sanitary expertize of milk and milk products. Sofia: Zemizdat, 1969: 101-2.

2. Sorensen LK, Rasmussen BM, Boison JO, Keng L. Simultaneous determination of six penicillins in cow's raw milk by a multiresidue high - performanse liquid chromatographic method. J Chromatogr B Biomed Sci Appl 1997; 694 (2): 383-91.

3. Anderson KL, Moats WA, Rushing JE, O ' Carrole JM. Detection of milk antibiotic residues by use of screening tests and liquid chromatography after intramammary administration of amoxicillin or penicillin G in cows with clinical mastitis. Am J Vet Res 1998; 59 (9): 1096-100.

4. Furusawa N. Rapid liquid chromatographic determination of residual Penicillin G in milk. Fresenius J Ana Chem 2000; 368 (6): 624-6.

5. Wang Ch, Li ShJ. Determination of 5 penicillins in milk by HPLC with pre - column derivatization. Fenxi Ceshi Xuebao 2000; 19 (6): 72-4.

6. Dimov N. Control of milk and milk products. Sofia: Zemizdat, 1974: 28 - 37, 42, 55-6.

7. Belikov VG. Pharmaceutical chemistry. Moscow: High school, 1985: 717.

8. Dimov NP. Industrial pharmacy - science and technology series. Vol 1: Chromatography in pharmaceutical analysis. Sofia: Chemical Pharmaceutical Research Institute (NIHFI), 1999: 205-29.

UV-SPEKTROMETRIČNA METODA ZA DOLOČANJE BENZILPENICILIN NATRIJA (PENICILINA G) V VZORČNIH MEŠANICAH RAZLIČNIH VRST MLEKA

D. D. Tzvetkova, D. P. Obreshkova, I. P. Pencheva

Povzetek: V raziskavi smo analizirali 11 različnih vrst mleka in Bolgarije, Grčije in Francije. Uporabili smo alkalimetrično titracijo za določanje kislinske stopnje in vsebnosti kazeina, laktodensitometrijo za merjenje gostote, bromtimolno metodo za proučevanje alkalescence in vrelne teste za preverjanje svežine in stabilnosti. V proučevanih vrstah mleka smo tudi določili količino benzilpenicilin natrjia (penicilina G). Pripravili smo vzorčne mešanice, antibiotik hidrolizirali s solno kislino (HCl, pH = 4) in uporabili obstoječo UV-spektrometrično metodo. Eksperimentalni rezultati kislinske stopnje, vsebnosti kazeina, gostote, alkalescence, svežine in stabilnosti so odgovarjali predpisanim standardom za mleko. Analitska parametra ponovljivosti (natančnosti) in točnosti smo določili z merilno negotovostjo rezultatov, v kar smo vključili standardni odklon (SD), relativni standardni odklon (RSD) in interval zaupanja ($\overline{X} \pm t.S_{\overline{X}}$). Točnost rezultatov smo določili s stopnjo izplena (R).

Ključne besede: mleko-analize; zdravila, ostanki; penicilin G; spektrometrija, ultravioletna

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Original Research Paper

SERUM ZINC AND COPPER CONCENTRATIONS IN RAMS EXPERIMENTALLY INFECTED BY *MYCOPLASMA AGALACTIAE*

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Summary: In this study, 15 Kivircik rams aged 1.5 years, were used. After determining the spermatologic characteristics of all individuals, five of the rams were vaccinated to constitute the control group. *Mycoplasma agalactiae* 99 M (AIK 2) strain was inoculated in all of the rams 34 days after the vaccination. Before the inoculation and 15 days after, semen and blood samples were collected. The agent was isolated in blood on the 7th day after inoculation. Before and after the inoculation, serum zinc and copper levels were measured by using an Atomic Absorbtion Spectrometry. When copper and zinc level results were evaluated it was observed that, before and after inoculation copper level showed a non-significant decrease whilst zinc level had a significant decrease ($p<0.05$). In post mortem examination, variable amounts of degeneration in the testes and an increase in the connective tissue of vesiculae seminales were observed.
In conclusion it was suggested that, zinc deficiency played an important role in the formation of disease symptoms.

Key words: Mycoplasma infections; *Mycoplasma agalactiae*; semen-analysis; zinc-blood; copper-blood; spectrophotometry, atomic absorption; sheep-male

Introduction

Contagious agalactia in sheep and goats is a very contagious, acute, subacute or chronic disease (1). Aytuğ and others (2) reported that the disease could be seen in most European, Mediterranean, middle East and African countries. Mycoplasma infection is characterised by mastitis, polyarthritis and keratoconjunctivitis lesions (2, 3, 4, 5). In Spain (6), in a herd of 200 sheep, 20 % had uni- or bilateral keratoconjunctivitis and *Mycoplasma agalactiae* was isolated from conjunctival fluids collected from 20 ewes and 3 lambs.

This infection occurs in animals at every age but advanced pregnant and lactating animals are more susceptible (2, 4, 7, 8). It is reported that the incubation period of the disease is 1-2 weeks and recovered animals continue to be carriers for seven months (2, 4, 9)

Zavaglı and others (10) reported that the disease causes no clinical finding in rams and bucks but Philpott (11) claimed that it makes balanoposthitis. Foggie and others, (12) isolated the agent from the testes of experimentally infected rams.

The principal way of diagnosis is agent isolation, however, complement fixation and indirect hemaglutination tests are commonly used (2, 9, 13, 14). Some researchers (15, 16, 17, 18, 19) suggested, ELISA was more successful in the diagnosis of subclinic mycoplasma infections. Recently PCR has been used to search *M. agalactiae* in sheep milk samples (20).

While Nelson and others (21) reported serum zinc levels of 4.86-6.08 µmol/l in a herd which suffered from cases of anorexia, wool eating, alopecia, hyperkeratosis and parakeratosis, Çamaş and others (22) have reported a level of 3.80-7.30 µmol/l for similar symptoms.

In ruminants, normal serum zinc levels are between 11 and 18 µmol/l, and animals with levels below 10.5 µmol/l are considered deficient (23). In Spain, values below 9.2 µmol/l are considered low for ovine, whereas levels above 12.2 µmol/l are

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considered normal (24).
Normal serum copper levels for sheep as reported by various researchers (25, 26) are 9.26-15.86 µmol/l and 12.56-18.84 µmol/l, respectively.

Deger and others (27) carried out a study to investigate changes in serum concentration of copper, zinc, and calcium in sheep naturally infested with lice. In their study, the concentrations of these elements in the infested animals were lower than in the healthy controls.

A study was carried out to determine the levels of trace elements (zinc, iron, copper), as well as of lead and cadmium, in both the soil and in organism in order to obtain more efficient economical results and healthier sheep breeds in Northwest Turkey by Or and others (28). In this study, while low zinc levels were determined in some districts, low copper levels were determined in full blood of 400 sheep from almost all districts of Northwest Turkey. The lowest values determined in copper in above study prove how serious the problem is in Northwest Turkey.

This study was performed to find out a possible relationship between zinc and copper trace element levels and clinical findings, specially genital defects (testis degeneration, spermatological differences, connective tissue increase in V.seminalis) in rams that experimentally infected by *M. agalactiae.*

Material and methods

The material of the study was 15 Kıvırcık rams aged 1.5 years, which were free from *M. agalactiae* and were maintained under the same conditions in the Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Istanbul University. The rams were treated for parasites for one month and were infected by a *M. agalactiae* 99 M (AIK 2) strain which was obtained from Pendik Veterinary Control and Research Institute. Every ram received $3.7x10^6$/ml colony microorganism subcutaneously.

After determining the spermatologic characteristics of all individuals, five of the rams were vaccinated to constitute the control group. *Mycoplasma agalactiae* 99 M (AIK 2) strain was inoculated in all of the rams 34 days after the vaccination.

Before vaccination and inoculation, six ejaculate samples were collected from each ram. Two ejaculates were discarded to minimise the effect of sexual rest and the remaining four ejaculates were evaluated to determine the spermatological characteristics. The rams were clinically observed for a 15 day incubation period. They were ejaculated by means of an electro ejaculator for nine times during this period. Seminal volume, mass activity, motility, concentration and morphological evaluations were carried out.

M. agalactiae isolation was carried out according to the literature (29) on semen, blood and organs after slaughter.

Blood samples were collected from each ram prior to inoculation and 15 days after inoculation. 10 cc blood was taken from V.jugularis into tubes without anticoagulant, kept at room temperature for 30 minutes and serum was separated by centrifuging at 5000 rpm for 3-5 minutes. These sera were stored at -20^0 C until analysis.

Serum copper and zinc measurements were carried out with a Shimadzu Atomic Absorbtion Spectrometry model AA-680 in accordance with the technique described in the references (30, 31). In order to prevent contamination from glass-ware, plastic materials were used during the measurements of trace elements.

For copper measurements, 0.5 µg/ml and 1 µg/ml copper standard solutions were prepared from 9987 Titrisol 1000+0.002 gram Copper ($CuCl_2 + H_2O$) Merck (E.Merck, D-6100 Darmstadt, Germany) standard stock solution. Bidistilled water was used as a blank solution. The device was operated to a 324.8 nm wave length, an air-acetilen gas mixture (8/1.8), a slit space 0.5 nm and B.G.C. mode were chosen. Blank and standard solutions were given to the device under these conditions. Then samples to be measured were given to the device and Copper concentrations were measured.

For zinc measurements, 0.5 µg/ml and 1 µg/ml zinc standard solutions were prepared from 9953 Titrisol 1000+0.002 gram zinc ($ZnCl_2$ + % 0.06 HCl) Merck (E.Merck, D-6100 Darmstadt, Germany) standard stock solution. Bidistilled water was used as a blank solution. The device was operated to a 213.9 nm wave length, an air-acetilen gas mixture (8/2.0), a slit space 0.5 nm and B.G.C. mode were chosen. Blank and standard solutions were given to the device under these conditions. The samples were given to the device like copper measurements and zinc concentrations were measured.

All rams were killed 79 days after inoculation and bacteriological and histopathological examination, of the samples taken from the testes, cauda epididymis, vesiculae seminalis and synovial fluids were carried out according to the literature (29).

Statistical evaluations of serum zinc and

Table 1: Mean values and standard deviations of serum Cu and Zn levels of control and treatment group rams

Parameter/Unit	Control (n=5)				Treatment (n=10)			
	Before Inoculation		After Inoculation		Before Inoculation		After Inoculation	
	X ± SD		X ± SD		X ± SD		X ± SD	
Copper (µmol/l)	13.22	0.43	13.14	0.39	13.46	0.48	12.85	0.45
Zinc (µmol/l)	9.91	0.24	9.90	0.27	9.44	0.25	8.39*	0.28

* *p<0.05*

copper element levels were done by student t test and of spermatological characteristics by Duncan test (32).

Results

Clinical results

No clinical effects except a slight temperature rise were observed in rams infected by *M. agalactiae*.

Blood serum results

Serum zinc and copper levels of infected rams before and after inoculation and their statistical evaluations are presented in Table 1.

When copper and zinc level results were evaluated it was observed that, before and after inoculation copper level showed a non-significant decrease whilst zinc level had a significant decrease ($p<0.05$).

Spermatological results

Characteristics of the control and treatment groups before and after inoculation are presented in Table 2.

At the comparison of average results, volume, mass activity, motility and concentration values of the treatment group decreased and the morphological defect ratio, specially head defects were observed to be increasing ($p<0.05$).

Microbiological results

The agent was not isolated from blood and semen samples collected before inoculation. The agent was isolated from heparin containing blood and semen samples collected from the 7th day post inoculation and cultured on PPLO media. No agent was isolated from the blood and semen of the control group. The agent was isolated from the testes, epididymis, V.seminalis and synovial fluids of the slaughtered rams. Tests for identification confirmed that the agent was *M. agalactiae*.

Histopathological results

At post-mortal macroscophic examinations, although the testes of the rams in treatment and control groups were normal in size, some of the treatment rams' testes were observed to be softer.

When the preparations from different testes regions of all rams were examined, distropfic changes of various degree were observed in all testes.

Discussion

M.agalactiae infection is an economic disease of sheep and goats characterised by mastitis, polyarthritis and ceratoconjunctivitis (2, 3, 4, 8, 9). Zavaglı and others (10) reported that the disease showed no symptoms in rams and bucks, also showed that rams are more resistant to experimental infections (33). In our study, we agreed with the above researches because no animal showed any symptom other than a slight temperature rise. In accordance with previous studies (2, 4, 9, 33, 34) we can isolate the agent from blood, testes, epididymis, V.seminalis and synovial fluids.

Burnet (35), in his early studies reported delay in growth, testicular atrophy, hyperkeratosis and

Table 2: Spermatological-morphological characteristics of control and treatment rams

Parameter/Unit	Control (n=5)				Treatment (n=10)			
	Before Inoculation		After Inoculation		Before Inoculation		After Inoculation	
	X	± SD	X	± SD	X	± SD	X	± SD
Volume (ml)	0.89[a]	0.61	0.97[a]	0.12	0.94[a]	0.34	0.74[b]	0.39
Mass activity (0-4)	3.45[a]	0.51	3.06[a]	0.62	3.64[a]	0.41	2.32[b]	0.91
Motility	87.25[b]	4.99	75.44[a]	10.96	90.54[a]	4.23	54.63[b]	21.12
Concentration ($x10^9$/m)	1.87[a]	0.56	2.01[a]	0.86	2.02[a]	0.33	1.39[b]	0.68
Head (%)	8.90[a]	3.51	8.67[b]	3.75	8.27[a]	2.99	12.34[a]	6.24
Mid Piece (%)	1.90[a]	1.92	2.18[a]	2.53	1.36[a]	1.42	1.68[a]	1.79
Tail (%)	5.75[a]	2.86	10.27[a]	5.20	4.21[b]	2.00	10.75[a]	5.88

a,b: The differences between the means of characters in each row that contain different letters are significant ($p< 0.05$)

anorexia with zinc deficiency, he also claimed that immune response is affected seriously by zinc deficiency. In our study, there was a considerable decrease (8.39 µmol/l) in the experimental incubation period in the treatment group and we supposed it was due to the degeneration in testicular tissue and defects in spermatogenesis.

Researchers (36) reported anemia, neutropenia, collagen and elastin formation defects in connective tissue, skeletal and cardiovascular lesions, neural demyelinisation degeneration and neonatal ataxia at a copper deficiency. In our study we investigated copper in relation to arthritis which is one of the main symptoms of the disease, but there was no arthritis in the treatment group and in accordance with this situation no significant decrease in copper levels were observed.

At the end of the study, it is concluded that Zn deficiency was important in the formation of symptoms.

References

1. Greco G, Corrente M, Martella V, Pratelli A, Buonavoglia D. A multiplex-PCR for the diagnosis of contagious agalactia of sheep and goats. Mol Cell Probes. 2001; 15:21-5.

2. Aytuğ, C.N., Alaçam, E., Özkoç, Ü., Yalçin, BC, Gökçen H. Sheep and goat diseases and breeding. Istanbul, Turkey. 1990: 171-4.

3. Ak S, Hasöksüz M, Uysal A, Ilgaz A, Minbay A. *M.agalactiae* ile enfekte edilen koçlarda serum antikor düzeyleri. J Pendik Vet Microbiol 1996; 27: 215-22.

4. Otlu S, Aydin F, Genç O, Taş C. Koyunlarda gözlenen kontagiyöz agalaksi hastaliği üzerinde klinik ve bakteriyolojik araştirmalar. J Pendik Vet Microbiol 1997; 28: 33-8.

5. Bergonier D, Berthelot X, Poumarat F. Contagious agalactia of small ruminants: current knowledge concerning epidemiology, diagnosis and control. Rev Sci Tech 1997; 16:848-73.

6. Rodriguez JL, Poveda JB, Rodriguez F et al. Ovine infectious keratoconjunctivitis caused by *M. agalactiae*. Small Rumin Res 1996; 22: 93-6.

7. Damassa AJ, Wakenel PS, Brooks DL. Mycoplasmas of goats and sheep. J Vet Diagn Invest 1992; 4:101-3.

8. Erdağ O. Türkiye'de koyun ve keçilerin salgin agalaksi hastaliğina karşi aşi hazirlama ve uygulama çalişmalari. Uluslararasi Mycoplasmosis ve Theileriosis Semp. 1989,11-13/10/1989, Ist.,16-19.

9. Arda M, Minbay A, Leloglu N, Aydin N, Akay Ö. Özel Mikrobiyoloji, Epidemiyoloji, Bakteriyel ve Mikotik Infeksiyonlar. Erzurum: Atatürk Üniv. Basimevi, 1992: 463-87.

10. Zavagli V. Bull. Off. Int. Epizoot. 1951; 36: 336 (In). Arisoy F, Erdag O, Cottew GS, Watson WA.

Investigations into contagious agalactiae of sheep and goats in Turkey. J Turkish Vet Med 1967; 37:11-7.

11. Philpott M. The dangers of disease transmission by artificial insemination. Br Vet J 1993; 149: 339-71.

12. Foggie A, Etheridge JR, Erdag O, Arisoy F. Contagious agalactia of sheep and goats preliminary studies on vaccines. J Comp Pathol 1970; 80: 345-58.

13. Petsaga-Tsimperi V, Sarris K. Mycoplasmosis of sheep and goats in northern Greece. Vet Glasn 1997; 51: 49-53.

14. Etheridge JR, Foggie A, Arisoy F, Erdağ O. Koyun ve keçilerin kontagiyöz agalaksisinde komplement fikzasyon ile teşhis. J Pendik Vet Res Inst 1969; 2:137-49.

15. Levisohn S, Davidson I, Carovergara MR. Use of an ELISA for differential diagnosis of M.agalactiae and M.mycoides subspecies mycoides (LC) in naturally infected goat herds. Res Vet Sci 1991; 51: 66-71.

16. Onoviran A, Taylor-Robinson D. Detection of antibody against *M. mycoides subs. mycoides* in cattle by enzyme-linked immunosorbent assay.Vet Rec 1979;105: 165-7.

17. Perez-Gomez S, San Martin Larraya M, Arriaga Oroquieta A. Epidemiology of contagious agalactia in Lacho sheep in Navarra province, Spain. Med Vet 1996; 13:55-60.

18. Romano R, Buonavoglia D, Montagna CO, Tempesta M, Buonavoglia C. Use of an ELISA to detect antibodies to *M.agalactiae* in ovine serum samples. Acta Med Vet 1995; 41:75-80.

19. Pepin M, Dufour P, Lambert M, et al. Comparison of three enzyme-linked immunosorbent assays serologic diagnosis of contagious agalactia in sheep. J Vet Diagn Invest 2003; 15: 281-5.

20. Tola S, Angioi A, Rocchigiani AM, et al. Detection of *M. agalactiae* in sheep milk samples by polymerase chain reaction. Vet Microbiol 1997; 54:17-22.

21. Nelson DR, Wolff WA, Blodgett DJ, Leucke B, Ely RW, Zachary JF. Zinc deficiency in sheep and goats: three field cases. J Am Vet Med Assoc 1987; 184: 1480-85.

22. Çamaş H, Bildik A, Gülser F. Toprak, Bitki ve Koyunlarin Kaninda Bazi Iz Elementlerle (Cu, Mo, Zn, Co, Mn) Sülfat (SO4) Miktarlarinin Araştirilmasi. Pro.no: VHAG-966. Van, 1994.

23. Lamand M. Place du laboratoire dans le diagnostic des carences en oligoéléments chez les ruminants = Importance of analytical laboratory in trace element deficiency diagnosis. Rec Méd Vét 1987; 163:1071-82.

24. Ramos JJ, Fernández A, Marca MC, et al. Niveles plasmáticos de zinc en ganado ovino de la provincia de Zaragoza (Ovine plasma zinc levels in Zaragoza province). Med Vet 1994; 11:610-4.

25. Martin DW, Mayes PA, Rodwell VW. Harper's review of biochemistry. 19th ed. Los Altos, California: Lance Medical Publications, 1983.

26. Ghosal AK, Mathur GN.: Zinc, copper and iron contents of blood serum of cattle, sheep in semiarid Tract of Rjasthan. Indian J Anim Sci 1992; 62(5): 441-2.

27. Deger Y, Dede S, Deger S. Short communication serum copper, zinc, and calcium concentrations in lice-infested sheep. Biol Trace Elem Res 2002; 88: 87-90.

28. Or ME, Kayar A, Kiziler AR, et al. Determination of the levels of some essential (Iron, Copper, Zinc) and toxic (Lead, Cadmium) metals in the blood of sheep and in samples water, plants and soil in Northwest Turkey. Vet Arh 2005; 75: 359-68.

29. Ak K, Ak S, Gürel A, et al. Koçlarda *M. agalactiae* spermatolojik özelliklere, genital organlara etkisi üzerine deneysel araştirmalar. J Pendik Vet Microbiol 1995; 26:139-55.

30. Brown A, Halls JD, Taylor A. Atomic spectrometry update-clinical materials, foods and beverages. J Anal Atom Spect 1986; 1: 21-35.

31. Brown AA, Taylor A. Applications of a slotted quartz tube and flame atomic absorption spectrophotometer to the analysis of biological samples. Analyst 1995; 110: 579-82.

32. Duncan DB. T-Tests and intervals for comparisions succested by data. Biometrics 1975; 31: 339-59.

33. Erdağ O, Arisoy F, Watson WA, Cottew GS. Türkiye'de koyun ve keçilerden izole edilen M. *agalactiae* suşlarinin tiplerinin ayrimi. J Pendik Vet Res Inst 1967; 1: 26-34.

34. Timoney JF, Gillespie JH, Scott FW, Barlough JE. The genera Mycoplasma and Ureaplasma. In: Hagan and Bruner's microbiology and infectious diseases of domestic animals. 8th ed. Ithaca, London: Comstock Publishing Associates, 1988: 304-5.

35. Burnet FM. New horizans in the role of zinc in cellular function. Clinical applications of recent advances in zinc metabolism. New York: Alan R. Liss Inc. 1982: 181-92.

36. Prasad AS. Trace elements and iron in human metabolism.New York: John Wiley and Sons, 1978: 17-54, 251-346.

KONCENTRACIJE CINKA IN BAKRA V SERUMU OVNOV, EKSPERIMENTALNO OKUŽENIH *Z MYCOPLASMA AGALACTIAE*

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Povzetek: V študiji smo uporabili 15 leto in pol starih ovnov pasme kivircik. Vsem smo določili spermatološke lastnosti, nato pa 5 ovnov zaščitno cepili, da so predstavljali kontrolno skupino. 34 dni po cepljenju smo vse živali okužili z mikoplazmo *Mycoplasma agalactiae* sev 99 M (AIK 2). Pred okužbo in 15 dni po njej smo živalim vsak dan jemali vzorce sperme in krvi. Mikoplazme smo izolirali iz krvi sedmi dan po okužbi. V krvnem serumu smo z atomsko absorpcijsko spektrometrijo določali raven bakra in cinka. Ko smo primerjali koncentracijo obeh elementov pred okužbo in po njej, smo ugotovili neznačilen padec vsebnosti bakra in velik padec vsebnosti cinka ($p<0.05$). Pri posmrtnem pregledu smo ugotovili različne stopnje degeneracije v modih in zvečanje količine vezivnega tkiva v mehurnici. Ugotavljamo, da ima pomanjkanje cinka pomembno vlogo pri oblikovanju simptomov bolezni.

Ključne besede: mikoplazma infekcije; *Mycoplasma agalactiae*; sperma-analize; cink-kri; baker-kri; spektrofotometrija, atomska absorpcija; ovce; samci

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Original Research Paper

GENETIC MONITORING FOR SEVERE COMBINED IMMUNODEFICIENCY CARRIERS IN HORSES IN SLOVENIA

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Summary: Severe combined immunodeficiency disease (SCID) is an autosomal recessive trait affecting purebred and mixed breed Arabian horses. Similar diseases exist in humans, dogs and mice. First immunodeficiency with characteristics of SCID in Arabian horses was reported around 1960 in Australia. The disease was described as a syndrome in 1973. The disease prevents the production of functional lymphocytes in affected individuals, which leads to a complete loss of humoral and cellular immune response. The defect is a result of a genetic mutation (5 bp deletion) in a gene encoding DNA dependent protein kinase C (DNA-PKc). As SCID is recessive autosomal disease, clinical signs are present only in offspring from mating between two heterozygous carriers of the SCID mutation. Heterozygous carriers are asymptomatic, but can be detected by genetic testing.

To identify the normal and mutant versions of the DNA-PKc gene, blood samples were collected from all Arabian horses in Slovenia and DNA extracted from the samples. The specific sequence of DNA-PKc including 5 bp deletion causing SCID was amplified using polymerase chain reaction (PCR) and the size of amplified DNA band was determined on a 4% agarose gel.

After successful amplification and interpretation of the DNA sequences, only negative results were found. We can therefore conclude that at present time there are no carriers of the mutation in DNA - PKc in Slovenia.

Key words: horse diseases; severe combined immunodeficiency-diagnosis-genetics; genetics, population; polymerase chain reaction; horses; mutation

Introduction

Severe combined immunodeficiency disease (SCID) is an inherited defect affecting pure or mixed breed Arabian horses (reviewed in (1-3). Affected individuals have a deficiency in both the number and function of B and T lymphocytes, resulting in an incompetent immune system. Heterozygous carriers appear normal but could transfer mutations to their offspring. In homozygous offspring, disease has 100% mortality due to immunodeficiency (2-4). Genetic cause of a SCID is a 5-base pair deletion within the catalytic subunit of DNA dependent protein kinase (DNA-PKc). Mutation causes a frameshift at codon 3155 of the transcript, resulting in 967 amino acid deletion from the C-terminus that includes entire phosphatidylinositol 3-kinase domain, thus making DNA-PKC functionally inactive (5, 6). It has been established that DNA-PKc deficiency produces an incomplete block in V(D)J recombination - the lymphocyte-specific process that is necessary for the expression of antigen receptors on B and T cells. This defect in V(D)J recombination blocks differentiation of B and T lymphocytes, resulting in profound immunodeficiency (3, 6, 7). Due to immunodeficiency, affected foals are highly susceptible to secondary infections which usually, without any treatment, cause death during the first month of age (2). The origin of the mutation in DNA-PKc gene and subsequently of SCID is unknown. Although the origin of the gene mutation has not been found, it must have initially occurred in a popular stallion used extensively for breeding to enable the spread of the mutation through the population of Arab horses. The disease is normally occurring in Arab horses, but there is also one report of affected Appaloosa foal which

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had an Arab stallion in fifth generation in maternal line (8). Affected foals are clinically normal at birth, but develop signs of infection during the first two months of life (2, 9). They have a deficiency both in numbers and function of B and T lymphocytes. First clinical signs usually occur between two days and six weeks of age. They include elevated temperature and increase in heart and respiratory rates. Foals are unthrifty, lethargic, easily tire but still nurse and eat solid feed. Bilateral nasal discharge, coughing and dyspnea due to respiratory infection often occur. Chronic diarrhea is present in some foals as well as alopecia and dermatitis. Lymphopenia (<1000/μl) and failure of immunoglobulin (IgM) synthesis are constant findings together with the absence of skin hypersensitivity (8). Total white blood cells count may be low, normal or increased (9).

Percentage of affected foals and heterozygous carriers differ between countries. For example, it was reported that 2.3% of 257 foals from 9 USA states were affected and 25.7% of adult Arab horses were carriers of the mutation (10). In another study in Australia, the percentage of homozygous affected foals was higher (8.3 percent of 204 tested; (11) while in U.K., only 2.8 % heterozygous carriers out of 106 tested animals were identified (12). In Brazil, 1.5 % carriers out of 205 tested horses were found (13).

In Slovenia, 63 Arabian and 19 part-bred Arab horses are registered, but were not yet tested for the presence of mutation in DNA-PKc gene. Most of them were imported from Poland, Hungary and Tunisia.

An accurate diagnosis of SCID is not only important because of the grave prognosis but it also provides information that both parents must be mutation carriers and should not be used for further interbreeding. Horses heterozygous for the SCID trait appear healthy, and in the past, the only way to identify heterozygous horses was by use of progeny testing (14). However, identification of mutation enabled development of genetic testing that make identification of carriers easier.

Material and methods

Animals

All pure breed Arabian horses (128) in Slovenia were involved in the present study. None of the animals showed any clinical signs of SCID or any other disease. 3 ml of blood was collected from vena jugularis and stored in tubes containing EDTA.

DNA extraction

Blood was centrifuged for 5 minutes at 3000 rpm to separate blood cells from plasma. Plasma was removed and blood cells were used for DNA extraction using WIZARD genomic DNA isolation kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

PCR analyses

Primers amplifying 136 bp long fragment of normal (without 5 bp deletion in affected animals) horse DNA-PKc gene were chosen based on Genbank sequence of horse DNA-PKc. Sequence of the primers was:

5'-primer: 5'- GTTGGTCTTGTCATTGAGCTG-3'

3'-primer: 5'- GCATCCGGATATCTGTTTGTC-3'

PCR amplification was performed in 0.2 ml thin walled tubes with following conditions:

initial denaturation for 5 minutes at 95°C followed by 30 cycles of denaturation at 95°C for 30 seconds, primer annealing at 48°C for 30 seconds and extension at 72°C for 1 minute, followed by final extension at 72°C for 7 minutes.

Amplification products were electrophoresed on specific 4% agarose gel for separation of small fragments (Promega) containing Ethidum bromide together with the 100 bp DNA ladder (Promega). Electrophoresis was performed under constant voltage 120V for 30 to 45 minutes. Amplified bands were visualized on UV transilluminator and captured onto computer using Hybaid GelGrab system (Hybaid, Wallisellen, Switzerland).

Results

After successful amplification using polymerase chain reaction, only DNA bands of expected size were observed on the agarose gel (Figure 1, representative sample) in all samples.

Careful examination of amplified DNA bands did not reveal any difference between the lengths of the bands or especially double bands that would indicate the presence of 5 base pair deletion and thus heterozygosity. The results therefore show that there are currently no heterozygous carriers of SCID among Arabian horses in Slovenia.

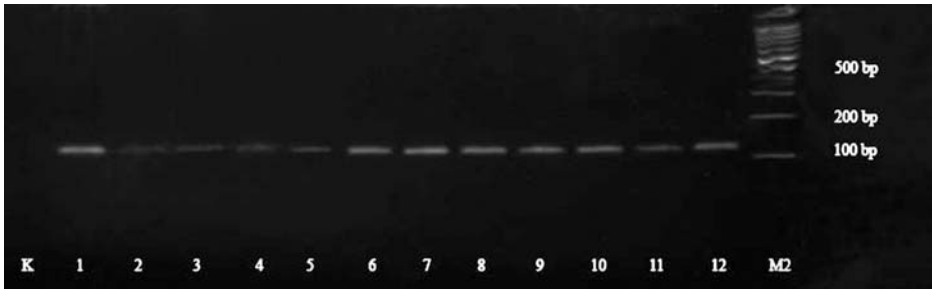

Figure 1: The results of DNA amplification with polymerase chain reaction. The picture shows a representative sample of 12 DNA samples from different horses.

Legend: M1 - DNA ladder (10 base pairs), K - control (no DNA), 1-12 - amplified DNK-PKc fragments; M2 - DNA ladder (100 base pairs).

Discussion

Even though hereditary diseases present only a small proportion of pathologies, they could present a significant problem with extensive economic losses. Inherited immunodeficiency diseases are important for the breeders, because animals without cellular and humoral immunity are unable to fight against foreign substances invading their bodies and consequently usually die due to secondary infections with opportunistic microorganisms (2, 3).

Studying genetic problems underlying certain hereditary diseases is important for several reasons. Firstly, knowing genetic causes of such conditions could help us understand basic physiological processes and how their malfunction leads to the development of the disease. Furthermore, understanding genetic causes of hereditary diseases usually also helps in development of diagnostic methods that could enable detection of healthy carriers of such diseases. Therefore, development of such diagnostic tests is extremely important for the breeders as detection of healthy carriers and their subsequent elimination from breeding programs could help with keeping populations free of certain hereditary diseases or even extermination diseases in certain populations and/or geographical areas. In last 20 years, development of molecular biology led to the development of ever simpler methods for mutation detection and unless the mutations that cause certain genetic disease are very heterogeneous, relatively simple and cost effective methods could be employed. PCR is extremely valuable tool that enables specific amplification of large quantities of DNA that can be used for subsequent analyses.

In the present study, all horses of Arab origin in Slovenia were tested for the presence of mutation in DNA-PKc gene that causes severe combined immunodeficiency syndrome. SCID is caused by a mutation in DNA-PKc which causes a defect in V(D)J recombination in lymphocytes (5, 6). Consequently, lymphocytes are unable to make antibodies against specific pathogens and therefore, affected animals do not have efficient immune system to defend against infections. In all known cases of SCID, disease was caused by a 5 bp deletion in DNA-PKc gene that causes a premature termination of the transcript (3, 10, 12). We employed a new simple method for detecting SCID mutations by using specific primers that amplified 136 bp long stretch of DNA-PKc gene that includes the deletion site. As there are no restriction sites within that 5 bp region to perform a restriction fragment length polymorphism (RFLP) analysis, we choose primers to amplify a fairly short fragment of DNA. Subsequently we used a high concentration (4%) of special agarose for detection of small fragments. According to manufacturer instructions as well as to our own experiences with this particular type of agarose gel, it is possible to detect differences of only 2 - 3 bp in short DNA fragments. Therefore, this method should be sufficient to detect carriers of the mutation in animals included in our study, as 5 bp difference between DNA fragments amplified from wild type and mutant allele should result in double band on the gel. In the present study, we did not find any mutation carriers, showing that epizootiological situation in Slovenia in regard to SCID is good. The origin of the disease is not known, however, the proportion of carriers differ between different countries. The highest percentage was reported in Arab horses in Australia and US (10, 11). Currently, most of Slovenian population of Arab horses can be traced to Hungary, Poland and Tunisia and although we do not have the data for the presence of SCID carriers in those countries, it is likely that occurrence of SCID in those countries is low, what would account for good situation in Slovenia.

SCID is a deadly disease and foals invariably die

within first months of age (2). Disease can be transmitted by natural mating, embriotransfer and artificial insemination. Because of simplicity and widespread use of new methods in animal breeding such as embriotransfer and artificial insemination, SCID as well as other inherited disease could spread easily between the countries. Therefore, it is even more important today than in the past to determine the presence of the carriers of genetic diseases. Classical methods such as selection against recessive genes are inefficient in complete elimination of disease as decrease in the frequency of the recessive gene causes an increasing proportion of recessive genes that are hidden from the effects of selection by occurring in heterozygous animals (12). Therefore, breeding programs for elimination of disease are much more efficient if carriers could be detected by testing their genome. With such methods, all the carriers could be detected and eliminated from breeding programs. Because SCID is not a sex-linked disease and the foal can inherit the defective gene from dam or sire, animals of both sexes must be tested. To prevent loss of some extremely important qualities of a horse, which is at the same time a carrier of the defective gene, some horses could still be used for the control breeding, but mating of two heterozygous animals should be avoided to prevent occurrence of disease with clinical signs. However, the mating of such animals should be carefully planned and recorded to prevent the spread of mutation in subsequent generations as 25% of offspring will inherit the mutation from their parent.

In some countries, SCID testing is already required for breeding Arab horses. In Slovenia, due to the absence of disease, small population of Arabian horses as well as little information about disease and cost the testing presents, the owners are generally not interested in testing their horses if tests would be commercial. However, due to favorable conditions at present, it would be desirable to introduce obligatory testing of all Arab horses imported to Slovenia for breeding purposes to prevent the introduction of SCID in Slovenian population of Arab horses.

References

1. Leber R, Wiler R, Perryman LE, Meek K. Equine SCID: mechanistic analysis and comparison with murine SCID. Vet Immunol Immunopathol 1998; 65(1): 1-9.

2. Perryman LE. Primary immunodeficiencies of horses. Vet Clin North Am Equine Pract 2000; 16: 105-16.

3. Perryman LE. Molecular pathology of severe combined immunodeficiency in mice, horses, and dogs. Vet Pathol 2004; 41: 95-100.

4. Lunn DP, McClure JT, Schobert CS, Holmes MA. Abnormal patterns of equine leucocyte differentiation antigen expression in severe combined immunodeficiency foals suggests the phenotype of normal equine natural killer cells. Immunology 1995; 84: 495-9.

5. Shin EK, Perryman LE, Meek K. A kinase-negative mutation of DNA-PK(CS) in equine SCID results in defective coding and signal joint formation. J Immunol 1997; 158: 3565-9.

6. Wiler R, Leber R, Moore BB, VanDyk LF, Perryman LE, Meek K. Equine severe combined immunodeficiency: a defect in V(D)J recombination and DNA-dependent protein kinase activity. Proc Natl Acad Sci U S A 1995; 92: 11485-9.

7. Priestley A, Beamish HJ, Gell D, et al. Molecular and biochemical characterisation of DNA-dependent protein kinase-defective rodent mutant irs-20. Nucl Acids Res 1998; 26: 1965-73.

8. Radostits OM, Gay CC, Blood DC, Hinchliff KW. Veterinary medicine: a textbook of diseases of cattle, sheep, pigs, goats and horses. Philadelphia: WB Saunders company 2000: 1727 - 8.

9. Horohov DW. The equine immune system. In: Reed SM, Bayly WM, eds. Equine internal medicine. Philadelphia: WB Saunders company, 1998.

10. Bernoco D, Bailey E. Frequency of the SCID gene among Arabian horses in the USA. Anim Genet 1998; 29: 41-2.

11. Nicholas FW. Introduction to veterinary genetics. Oxford: University Press, 1996.

12. Swinburne J, Lockhart L, Scott M, Binns MM. Estimation of the prevalence of severe combined immunodeficiency disease in UK Arab horses as determined by a DNA-based test. Vet Rec 1999; 145: 22-3.

13. Teixeira CS, Oliveira DAA, Kuabara MY. Prevalęncia da imunodeficięncia severa combinada em cavalos da raça Árabe em plantéis de Minas Gerais e São Paulo. Arq Bras Med Vet Zootec 2001; 53: 3.

14. Shin EK, Perryman LE, Meek K. Evaluation of a test for identification of Arabian horses heterozygous for the severe combined immunodeficiency trait. J Am Vet Med Assoc 1997; 211: 1268-70.

HUDO KOMBINIRANO POMANJKANJE IMUNOSTI PRI KONJIH V SLOVENIJI

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Povzetek: Hudo kombinirano pomanjkanje imunosti (severe combined immunodeficiency disease; SCID) je avtosomalna recesivna bolezen, ki se pojavlja pri arabskih konjih in križancih z njimi. Podobne bolezni obstajajo tudi pri ljudeh, psih in miših. Prvi primer bolezni z značilnostmi SCID je bil opisan leta 1960 v Avstraliji, leta 1973 pa je bila bolezen prvič natančno opisana in opredeljena kot genetski sindrom. Genetska napaka pri prizadetih živalih prepreči nastajanje delujočih limfocitov, zaradi česar so živali brez tkivne in celične imunske obrambe. Vzrok bolezni je mutacija (izbris petih baznih parov) v genu za od DNK odvisne proteinske kinaze C (DNK-PKc). Ker je SCID recesivna bolezen, se klinični znaki pojavijo samo pri homozigotnih potomcih heterozigotnih prenašalcev mutacije. Heterozigotni nosilci mutacije so popolnoma brez kliničnih znakov, zaradi česar jih lahko ugotovimo le z ugotavljanjem mutacije v genomu.
V predstavljeni raziskavi smo odvzeli kri vsem konjem arabske pasme v Sloveniji in iz nje osamili DNK. Z metodo verižne reakcije s polimerazo smo pomnožili del gena za DNK-PKc, ki je vključeval mutacijo (mesto izbrisa 5 baznih parov). Velikost pomnoženih verig DNK smo pregledali na 4% agaroznem gelu za ločevanje kratkih verig DNK. V vseh pregledanih vzorcih smo ugotovili le verige DNK pričakovane dolžine, iz česar lahko sklepamo, da trenutno v Sloveniji ni heterozigotnih prenašalcev mutacije v genu za DNA-PKc.

Ključne besede: konj, bolezni; genetika, populacijska; huda kombinirana imunska pomanjkljivost - diagnostika - genetika; verižna reakcija s polimerazo; konj

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Original Research Paper

SURGICAL INTRAVENOUS CATHETERISATION OF PIG

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Summary: For the single daily blood samples collection in swine blood venipuncture of the anterior caval vein is the most common method. If more frequent blood samples are needed then repeated venipuncture is less desirable, as it may cause damage to blood vessels and it could also influence the experimental results. Current methods utilized are therefore not suitable for frequent sampling due to the stress to which the pigs are exposed. To avoid the above-mentioned problems, several methods of catheterisation have been developed to allow for repeated sampling. In our experiment, an intravenous catheter was inserted into the jugular vein of a pig under general anaesthesia. No antibiotics were used perioperatively. Behaviour, appetite and body temperature were monitored twice daily. Blood samples were also taken twice daily for 5 days and were submitted to haematological and biochemical examinations. The values were compared to the results obtained from the samples, taken before the cannulation. Data were processed with a descriptive statistical method. According to the pig's behaviour, the animal did not display any clinical indications of pain or stress, provoked by the catheter. The catheter remained patent and on its primary position for the whole time of the experiment that was confirmed by an X-ray examination. The correct surgical intravenous cannnulation did not provoke any systemic inflammation process. Haematological and biochemical parameters of blood were not influenced by the catheterisation.

Key words: blood specimen collection; catheterization; *v. jugularis*; hematologic tests; swine

Introduction

Several techniques for the collection of swine blood are in current use. Venipuncture of the anterior caval vein is the most common method for obtaining blood samples, although other methods are used (1). If single daily blood samples are required, then jugular venipuncture is considered an acceptable collection protocol. If more frequent sampling is needed to asses various biomedical parameters (eg. pulsatile hormone secretion or hormonal responses to various secretagogues) in a conscious animal, then repeated venipuncture is less desirable as it increases stress and could potentially jeopardize the well-being of the animal (2). Beside it may also influence the experimental results. Moreover, it may cause damage to blood vessels, especially when frequent sampling is done (1). To avoid the above-mentioned problems, several methods of catheterisation have been developed for repeated blood sampling in pigs (3). They can involve surgical procedures under general anaesthesia (1, 3) or non-surgical cannulation of anesthetised pigs (2) or of pigs, restrained in the standing position with the use of a nylon or cotton snare placed around the snout (4, 5).

Sometimes the need to undertake biomedical research on conscious pigs also appears at our institute. The aim of our work was to insert a long-term intravascular catheter into the jugular vein of a pig without the use of antibiotics and to monitore its influence on some blood parameters.

Material and methods

A castrated male commercial slaughter pig cross of 35 kg was used in the experiment. It was stabled at the university´s pen 3 days prior to surgery to adapt to a new environment. The animal was allowed drinking water ad libitum and was fed 1 kg of a commercial feed twice daily.

Prior to the catheterisation procedure, blood was taken from vena cava cranialis to assess haematological and biochemical parameters. The investigated haematological parameters included red blood

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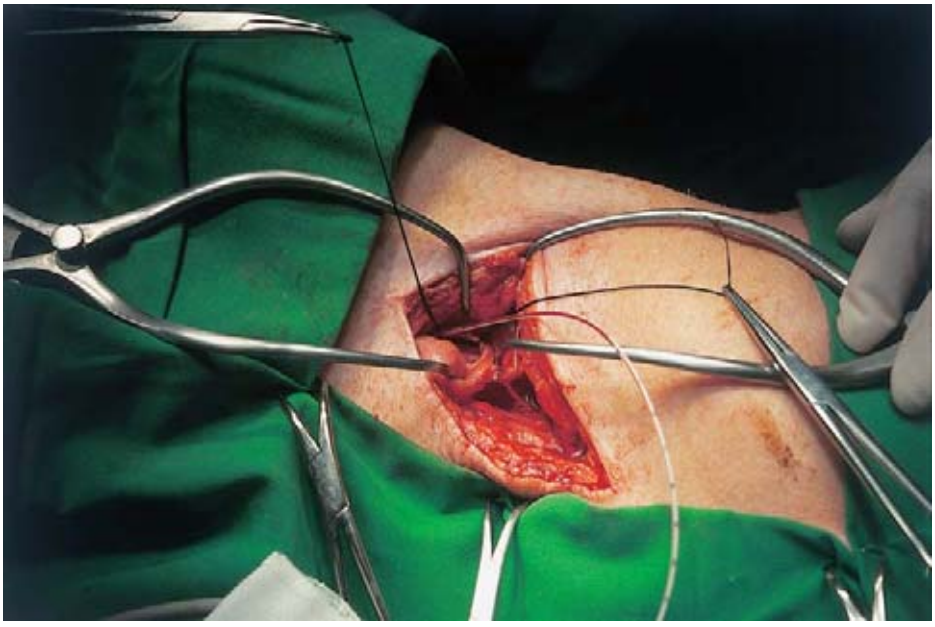

Figure 1: Cannulation of the jugular vein in pig

cells (RBC), haemoglobin (Hb), mean corpuscular volume (MCV), haematocrit (Ht), white blood cells (WBC), trombocytes (Trom.), neutrophils (Neu.), eosinophils (Eos.), basophils (Baso.), lymphocytes (Limp.), band neutrophils (BN) and monocytes (Mon.). The investigated biochemical paramethers were: bilirubin (Bil.), alanine aminotransferase (ALT), urea, iron (Fe), gama glutamyltransferase (GGT), proteins (Pro.), glutamate dehydrogenase (GLDH), potassium (K), magnesium (Mg), phosphorus (P) and creatine kinase (CK).

The investigated haematological parameters were analysed with analyser ABC VET installed at the laboratory of the Clinic for ruminants of the Veterinary faculty.

The investigated biochemical parameters were analysed with analyser COBAS MIRA.

After a 24-hour period without feed and 12 hours without water, the animal was premedicated intramuscularly with midazolame (0.15 mg/kg, Dormicum, Roche), ketamine (10 mg/kg, Bioketan, Vetoquinol) and xylazine (0.4 mg/kg, Chanazine 2%, Chanelle Pharmaceuticals). Anaesthesia was induced by intravenous administration of propofol (1-2 mg/kg, Diprivan 10mg/ml, Zeneca Pharmaceuticals Ltd.) and then maintained also by propofol (10 mg/kg/h, Diprivan 10 mg/ml, Zeneca Pharmaceuticals Ltd.). Analgesia was included by morphine (0.2 mg/kg, Morphini Hydrochloridum, 20 mg/ml, Alkaloid Skopje) and butorphanol (0.3 mg/kg, Torbugesic, Fort Dodge Animal Health).

The surgical area of the neck was shaved, disinfected and draped. Under sterile conditions a left jugular sulcus was exposed between musculus brachiocephalicus and m. sternomastoideus, near the prescapular part of the pectoral muscle so that the left external jugular vein was reached. After the vein was lifted, two vascular tapes were placed around it 3 cm apart. The jugular catheter Intramedicut 2 (16G, Sherwood medical) was inserted 10 cm proximally. Two silk ligatures were placed around the vein. Distally from the place of the insertion, the vein was ligated (Softsilk 2/0, Autosuture), while proximally the vein was ligated over the catheter (Softsilk 4/0, Autosuture) so that the catheter was fixed in the desired position. The patency of the catheter was confirmed with the application of heparinised saline (2 I.U. of heparin (Heparin razt. za inj., 25000 I.U./5 ml, Krka) in 1 ml of saline (0.9% NaCl, Braun)). The subcutis on the left neck area was chanelled dorsally. The catheter was looped in the subcutaneous plane in order to provide additional lenght for neck movements. The rest of the catheter was led externally to the back of the neck where it was fixed with silk (Softsilk 4/0, Autosuture). The neck was taped with selfgripping plaster (Vivapore 15 x 10 cm, Tosama). The incision was closed routinely; subcutis with continuous sutures, using absorbable, synthetic multifilament material (Polysorb 2/0, Autosuture), and cutis with single interrupted sutures, using silk (Softsilk 2/0, Autosuture). No antibiotics were administered.

Blood samples were collected during a 5-day period after cannulation. They were taken twice

Table 1: Results of biochemical blood examination

Day	Bil. µmol /L	ALT U/L	Urea mmol/L	Fe µmol /L	GGT U/L	Pro. g/L	Albumin g/L	GLDH U/L	K mmol/L	Mg mmol/L	P mmol/L	CK U/L
0*	2.81	46	4.68	20.1	35	64.1	25.4	1.07	4.70	0.88	2.84	502
0(3pm)	3.33	43	2.46	6.1	20	60.8	24.7	1.16	4.41	0.78	3.09	725
1(8am)	1.97	51	4.70	20.3	27	68.6	28.8	1.51	4.47	0.91	3.08	1514
1(3pm)	2.17	50	4.27	14.1	22	68.1	28.5	1.03	4.49	0.85	2.84	1419
2(8am)	2.13	45	4.41	16.7	36	64.7	26.9	1.40	5.56	0.97	3.23	1089
2(3pm)	2.05	42	2.98	13.7	35	64.7	27.2	1.25	4.43	1.04	3.28	729
3(8am)	1.77	38	3.36	23.7	37	61.7	26.0	1.22	4.92	0.97	2.86	408
3(3pm)	1.93	37	3.94	13.7	38	63.2	27.1	1.42	4.95	0.97	3.06	453
4(8am)	1.28	38	4.09	23.6	24	68.0	28.6	1.68	4.87	0.87	2.92	281
4(3pm)	0.8	37	4.27	16.4	59	64.9	27.2	3.44	5.79	1.16	4.25	312
5(8am)	1.44	31	3.85	26.0	26	62.6	25.6	1.21	4.88	0.90	2.52	239
5(3pm)	0.84	33	3.59	26.0	26	66.0	27.3	2.70	4.61	0.88	2.48	246

daily, at 8 am and at 3 pm. Two syringes were used each time. The first one was used to remove the blood, mixed with heparine, from the catheter, while the second one was used to collect the blood sample. The mixture of blood and heparine from the first syringe was then returned back to the catheter and 1 ml of heparinised saline was added. The blood samples were subjected to the same examinations as the samples, taken before the cannulation. The pig´s behaviour, appetite and body temperature were also monitored twice daily.

Data were processed with a descriptive statistical method. Based on 11 results, the mean value ± and the standard deviation were calculated for each blood parameter and the data were compared with the values, gain from the samples that were taken before the catheterisation.

On day six after catheterisation, the animal was sedated with xylazine (0.4 mg/kg) and ketamine (10 mg/kg) and the position of the catheter was checked with X-ray examination. Then euthanasia was performed by a lethal dose of euthanasia solution (T-61, Hoechst Roussel) via the indwelling catheter.

The procedure was approved by the Veterinary Administration of the Republic of Slovenia, number 323-02-16/2003, date 10.2.2003.

Results

Results of biochemical blood examination before cannulation (0*), on the day of cannulation (day 0 at 3 pm), and twice daily on days 1, 2, 3, 4 and 5 after cannulation. The time of blood sampling is given in parenthesis.

Orientational normal biochemical values in pigs, enzyme activity measured at working temperature 37° C (6):

Bil.: up to 5.64 µmol /L
CK: 50 - 500 U/L
ALT: 31 - 58 U/L
Urea: 2,33 - 6.66 mmol/L
Fe: 18,0 - 35.0 µmol /L
P: 1,81 - 3.19 mmol/L
K: 4,5 - 6.2 mmol/L
GGT: 32 - 58 U/L
Pro.: 62.0 - 82.0 g/L
Albumin: 30.0 - 40.0 g/L
GLDH: up to 1.86 U/L
Mg: 0.83 - 1.42 mmol/L

Results of haematological blood examination before cannulation (0*), on the day of cannulation (day 0 at 3 pm), and twice daily on days 1, 2, 3, 4 and 5 after cannulation. The time of blood sampling is given in parenthesis.

Orientational normal haematological values in pigs (6):

RBC: 5,0 - 8.0 x1012/L
Hb: 10,0 - 15.5 g/dl
Neu.: 28 - 52%
Limp.: 40 - 64%

Table 2: Results of haematological blood examination

Day	RBC 10^{12}/L	Hb g/dl	MCV %	Ht %	WBC 10^9/L	Trom. 10^9/L	Neu. %	Eos. %	Baso. %	Limp. %	BN %	Mon. %
0*	6.12	10.1	57	34.7	24.4	329	44	2	0	54	0	0
0(3pm)	4.62	7.6	55	25.7	15.0	272	54	0	0	46	0	0
1(8am)	5.88	9.8	57	33.3	26.3	252	44	0	0	54	0	2
1(3pm)	5.78	9.6	56	32.3	24.5	260	43	2	0	53	0	2
2(8am)	5.45	8.8	59	32.0	22.8	198	40	12	0	48	0	0
2(3pm)	5.42	8.8	58	31.5	20.4	218	57	4	0	39	0	0
3(8am)	5.14	8.3	56	28.7	19.1	242	34	8	1	57	0	0
3(3pm)	5.15	8.4	56	28.8	20.0	262	36	2	0	62	0	0
4(8am)	5.41	8.7	56	30.4	20.6	284	49	2	1	47	0	1
4(3pm)	4.95	8.1	57	28.1	25.3	260	75	2	0	23	0	0
5(8am)	4.92	8.2	56	27.8	19.1	302	27	4	0	69	0	0
5(3pm)	4.69	7.8	56	26.3	18.4	282	45	2	0	53	0	0

WBC: 10.0 - 20.0 x109/L
Ht: 32 - 47%
MCV: 32 - 47%
Trom.: 250 - 600 x109/L
Mon.: 2 - 8%
Baso.: 0 - 2%
Eos.: 1 - 8%
BN: 0 - 4%

During the trial the pig behaved normally, having a good appetite, and had normal body temperature. No clinical manifestations of pain or stress due to the catheter or repeated blood collections were noticed. The catheter remained on its primary position and patent for the whole time of the experiment.

Discussion

Intravenous catheterisation of the pig was performed under general anaesthesia due to the fact that the cannulation of the jugular vein with the use of classical means of restraint can only be done in 15 to 20 minutes (5). This represents 15 to 20 minutes of stress for the animal and can lead to the alteration of some blood parameters. Conscious pigs, restrained by a snare, may interrupt cannulation efforts by making undesirable movements. It could also prove more hazardous for the investigators than working with anesthetized animals (4).

According to the pig's behaviour, the animal did not appear to be stressed by the catheter. Irritation or itching in the neck area were not evident. The catheter remained on its primary position for the whole time of the experiment, what was confirmed by the X-ray examination prior to the euthanasia of the animal. The fixation of the catheter did not have to be too tight and had to allow normal passage of the blood so that the blood collection was possible. The vein and the catheter remained patent for all 5 days of the experiment. The blockage of the vein or the obstruction of the catheter due to the thrombosis and phlebitis were reported (1).

The correct surgical intravenous cannnulation did not provoke any systemic inflammation process that was confirmed by monitoring blood parameters. The investigated blood parameters, with the exception of L and CK, were within the limits of the reference values. Initially elevated values of L and CK were at the end of the experiment within the limits of the reference values. Although the values of the investigated blood parameters were, according to the initial values, changing during the experiment (Tables 1 and 2), their mean values, considering standard deviation, did not deviate from the borders of the ref-

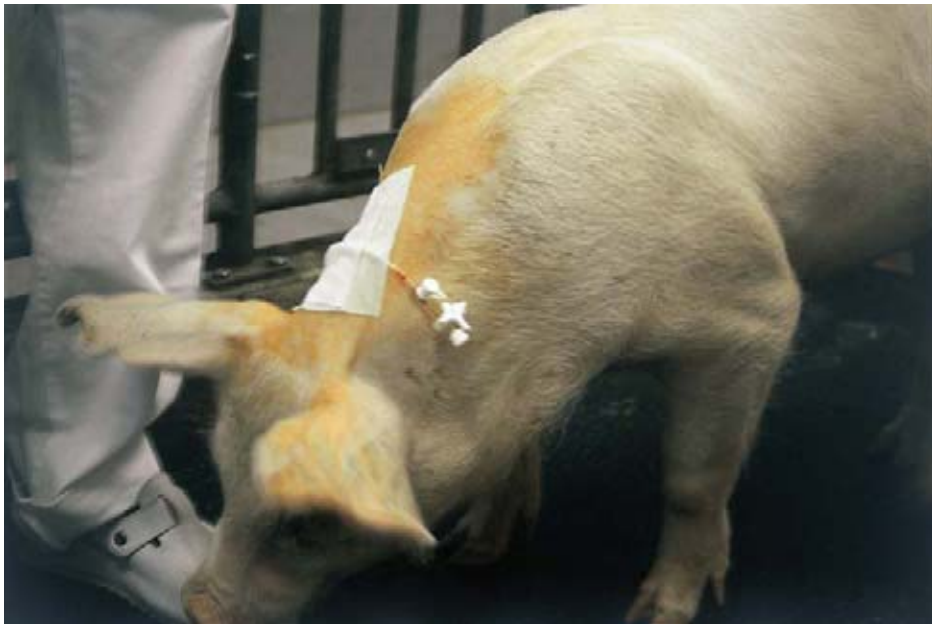

Figure 2: Pig with the intravenous catheter, two days after the cannulation.

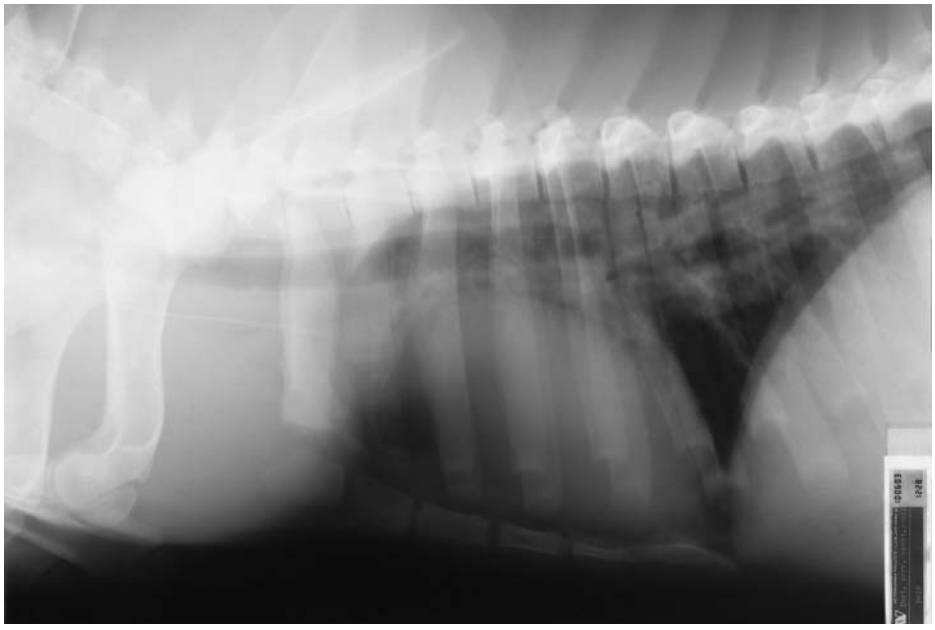

Figure 3: X-ray conformation of the intravenous catheter's position.

erence values. Pijpers et al.(1) reported the changes in rectal temperature, WBC counts, serum Zn and Fe levels, that may provide essential information about the reactions of defence mechanisms against infections. These effects of surgery and anaesthesia did not last longer then two days.

In our experiment, catheterisation did not have influence on the haematological and biochemical parameters of blood, however an adequate sample of animals should be tested in order to confirm these results.

References

1. Pijpers A, Noordhuizen-Stassen EN, Goedegebuure SA et al. Intravenous catheterisation of convential pigs without application of antimicrobial agents. Vet Q 1989; 11: 216-21.

2. Carroll JA, Daniel JA, Keisler DH, Matteri RL. Non-surgical catheterization of the jugular vein in young pigs. Lab Anim 1999; 33: 129-34.

3. Haberstroh J, Mand U, Lucke I, Breuer H, Ahrens M, von Specht BU. A technique of chronic intravascular catheterization for monitoring haemodynamic and laboratory values in conscious swine. Lab Anim 1997; 31: 70-3.

4. Smith CA, Ficken MD. Non-surgical cannulation

of the vena cava for chronic blood collection in mature swine. Lab Anim Sci 1991; 41: 274-8.

5. Matte JJ. A rapid and non-surgical procedure for jugular catheterization of pigs. Lab Anim 1999; 33: 258-64.

6. Jazbec I. Klinična laboratorijska diagnostika. Ljubljana: Veterinarska fakulteta, 1990.

KIRURŠKO VSTAVLJANJE VENSKEGA KATETRA PRAŠIČU

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Povzetek: Pri enkratnem odvzemu krvi prašičem se le-ta najpogosteje jemlje iz sprednje velike dovodnice (vena cava cranialis) z iglo. Pri pogostejšem jemanju pa ta način ni primeren, saj lahko povzroči poškodbe krvnih žil in vpliva na rezultate preiskav. Poleg omenjenega so metode, ki jih navadno uporabljamo za odvzem krvi prašičem, za večkratno jemanje v kratkem času neprimerne zaradi stresa, ki so mu živali pri tem izpostavljene. Da bi se izognili tem težavam, so razvili različne metode kateterizacije. V našem poskusu smo prašiču v splošni anesteziji vstavili intravenski kateter v jugularno veno. Antibiotikov nismo uporabljali. Po vstavitvi katetra smo dvakrat dnevno spremljali obnašanje, ješčnost in telesno temperaturo prašiča. Pet dni smo dvakrat dnevno prašiču jemali kri in opravili hematološke in biokemične preiskave. Dobljene vrednosti smo primerjali z vrednostmi hematoloških in biokemičnih preiskav krvi, odvzete pred vstavitvijo katetra. Podatke smo obdelali s pomočjo deskriptivne statistične metode. Po obnašanju prašiča smo sklepali, da kateter zanj ni bil moteč. Ves čas poskusa je kateter ostal prehoden in na mestu, kjer smo ga vstavili, kar smo potrdili z rentgenskim slikanjem prašiča. Kirurško vstavljanje katetra ni povzročilo sistemskega vnetnega procesa. Kateterizacija ni vplivala na vrednosti biokemičnih in hematoloških parametrov.

Ključne besede: krvni vzorec, zbiranje; kateterizacija; *vena jugularis*; hematološki testi; prašiči

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Case Report

LISTERIA MONOCYTOGENES SEPTICAEMIA IN A FOAL

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Summary: We describe *Listeria monocytogenes* septicaemia in a Slovenian warm-blood 3-day old filly. When admitted to the hospital, the foal was weak and severely dehydrated. Due to poor response to therapy and grave prognosis, the foal was euthanized. Postmortem examination was performed and significant findings included multifocal hepatitis, diffuse enteritis and pulmonary and spleen haemorrhages. The histopathological examination revealed pyogranulomatous hepatitis, mononuclear meningitis, acute septic embolic pneumonia, acute catarrhal enteritis as well as congestion and acute haemorrhages in most of examined tissues. *Listeria monocytogenes* was isolated on primary culture from the liver, spleen, kidney, lung, intestine and brain, and was therefore considered as the cause for the septicaemia in this foal. *L. monocytogenes* septicaemia in foals is rarely diagnosed, and the present report is, to our knowledge, the first to describe *L. monocytogenes* septicaemia in a foal in Slovenia. Despite being rare, a definitive diagnosis based on postmortem examination and bacteriological culture is important because it allows preventing transmission of the disease and/or development of healthy carriers. This may be of particular importance in herds where several foals are expected to be born.

Key words: Listeria infection-diagnosis; *Listeria monocytogenes*; sepsis; horses

Introduction

Listeriosis is an infectious disease caused by bacteria of the genus *Listeria*. These Gram-positive and facultative anaerobic rods are ubiquitous in the nature; however, especially its presence in poorly ensiled feed has always been the cause of great concern in the animal husbandry. The genus *Listeria* comprises six species, of which only two are pathogenic. *Listeria monocytogenes* is responsible for most infections in humans and animals. However, *L. ivanovii* may cause the significant numbers of cases of disease in domestic animals, especially in sheep (1). All domestic animals can be infected with *L. monocytogenes*, although sheep, cattle, goats and chickens are considered the most susceptible species (2). Clinical forms of listeriosis include encephalitis, abortion and, relatively uncommon, septicaemia in neonates (3, 4). In septicaemic listeriosis, small necrotic foci are often found in liver and other abdominal organs (5). Listeriosis in horses is rare (6, 7, 8, 9, 10, 11, 12, 13, 14). Even less reports are available for *L. monocytogenes* in foals. In 1943, Grini reported *L. monocytogenes* septicaemia in the equine neonate (6). Since then of L. monocytogenes septicaemia in equine neonates have been reported worldwide. In other reports, L. monocytogenes was isolated from a group of six ponies with fever, colic and jaundice (7), an aged mare and a two-year old foal with septicaemia (8), a three-week old foal with diarrhoea (9), two foals with necrotizing hepatitis and septicaemia (10), a six-day old foal with diarrhoea (11) and three foals with septicaemia and signs of diarrhoea and neurological disease (12). *Listeria monocytogenes* was also described as a cause of meningitis in a one-month old foal with combined immunodeficiency (13) and as a cause of abortion of a near-term equine foetus (14). Only two of the above-mentioned cases had a successful outcome (9, 12). All other diagnoses, including ours, were made post-mortem.

In Slovenia listeriosis most commonly occurs in ruminants (15). This is the first report of L. monocytogenes infection in a foal in Slovenia.

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Material and methods

Case history and clinical signs

A 3-day-old Slovenian thoroughbred filly was presented to the Equine hospital of the Clinic for reproduction and horses at the Veterinary Faculty Ljubljana for evaluation of generalised weakness. Parturition was normal. Initially, in the presence of owners, the mare allowed the foal to nurse. However, few hours later it completely rejected the foal. The foal was then bottle- fed.

On physical examination, the foal was weak and severely dehydrated. Rectal temperature of 37.2°C, pulse rate of 92 beats per minute and respiratory rate of 20 breaths per minute were within normal limits. The mucous membranes were pale and the capillary refill time prolonged on 3 seconds. Despite intermediate treatment with intravenous polyionic fluids (lactated Ringer's solution) and intravenous antibiotic (Ceftiofur Sodium), the foal's condition rapidly deteriorated and it had to be euthanised soon after admission. Complete blood count and serum biochemistry were available only after euthanasia. Significant abnormalities included sever leucopoenia, increased blood urea nitrogen (BUN) and creatinine, hypoalbuminaemia and hypoglobulinaemia.

Clinical examination of the mare did not show significant abnormalities. Mild nasal discharge was the only complaint reported by the owners regarding mare's health status during pregnancy. There was another pregnant mare present on the premises that did not show signs of any kind of disease for the past year. Animals did not travel from the premises and did not come in contact with other horses. Animals did not undergo preventive vaccinations and were dewormed every six months.

Gross pathology and histopathology

Blocks of liver, kidneys, lung, heart, stomach, intestines, mesenteric lymph nodes, adrenal gland and brain were fixed in 10 % buffered formalin and paraffin-embedded for histopathology. 4 µm thick tissue sections were then deparaffinised, stained with hematoxylin and eosin (HE) and examined under a light microscope.

Bacteriological examination

Samples of liver, spleen, lungs, intestines and brain for bacteriological examination were collected during necropsy. The samples were inoculated on nutrient agar (Oxoid, Hampshire, UK) supplemented with 5% of sheep blood and Drigalski agar (Oxoid) and incubated at 37°C for 24 hours. In addition, brain stem tissue was inoculated into selective enrichment media half Fraser and Fraser broth (Oxoid), and onto selective differential solid media Oxford and Palcam Agar (Oxoid). Bacteria were Gram stained and tested for catalase activity (Difco-BBL, Maryland, USA). The biochemical characteristics were evaluated using API *Listeria* (bioMerieux, Marcy l'Etoile, France) according to the manufacturer's instructions. CAMP reaction was determined on blood agar with *Staphylococcus aureus* (ATCC 25923) and *Rhodococcus equi* (CAPM 6312). Testing for motility was performed in tryptic soy broth (Biolife, Milan, Italy), which was incubated at 20°C and 37°C. Diagnosis was confirmed with AccuProbe *Listeria monocytogenes* hybridisation assay (Gen-Probe, San Diego, USA). The strain was serologically determined with commercial antisera (Bacto-Listeria O Antiserum, Difco-BBL). Susceptibility to the following antibiotics was tested by disc diffusion method on Mueller-Hinton agar (Merck, Darmstadt, Germany) with discs: amikacin, gentamicin, oxytetracycline, penicillin, trimethoprim-sulphamethoxazole, cefalexin, ceftriaxone, cefoperazone (all Difco-BBL) and ceftiofur (Rosco, Taastrup, Denmark). The mare's vaginal swab was inoculated onto blood agar and half Fraser and Fraser broth (Oxoid). The presence of specific antibodies in the mare's serum was tested by complement fixation assay (Institut Virion Serion GmbH, Wurzburg, Germany).

Results

Post-mortem findings

The foal was emaciated and dehydrated. The findings included acute diffuse gastroenteritis and typhlocolitis with enlarged and congested mesenteric lymph nodes, obstipation of large intestine with accumulated dry intestine content, congestion, disseminated haemorrhages and oedema of the lung and enlarged spleen due to severe congestion. Numerous petechial haemor-

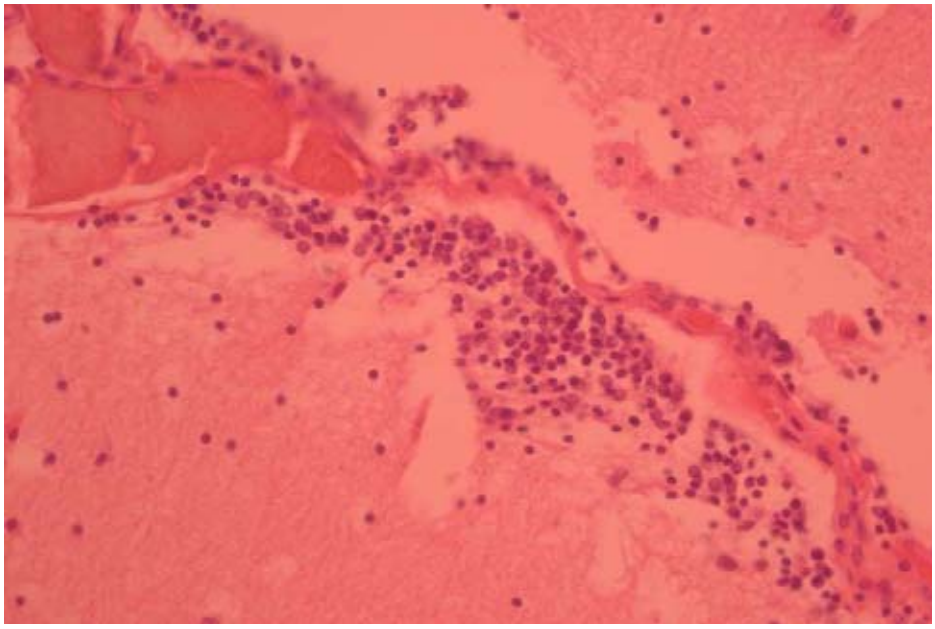

Figure 1: Cerebellar meninges, foal. Mononuclear cell infiltrate and congested blood vessels. HE×400

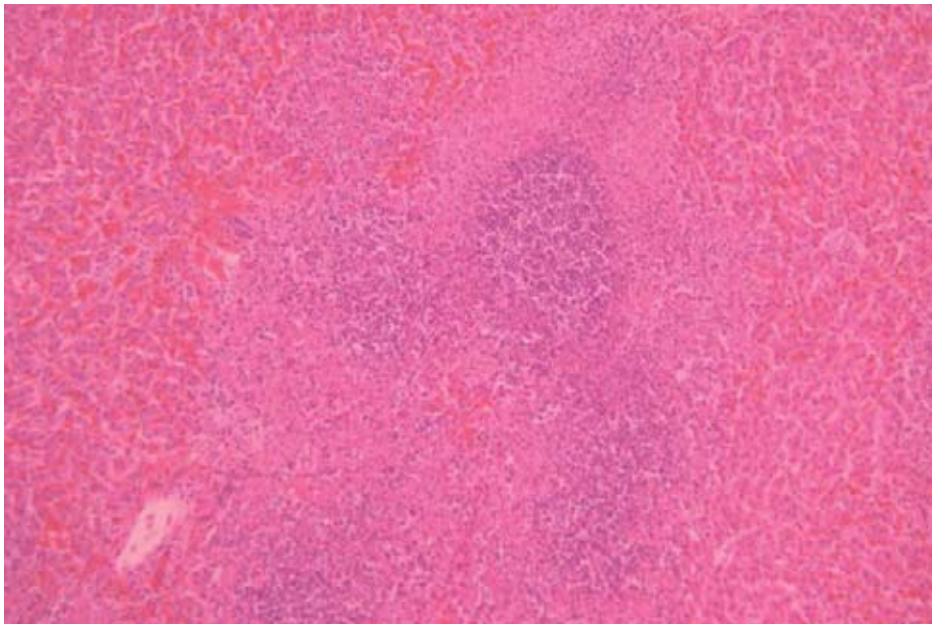

Figure 2: Liver, foal. Pyogranulomatous hepatitis. HE×400

rhages were found on serosal surfaces of the lung and spleen. Liver was congested and many randomly distributed white, clearly demarcated spots measuring up to 3 mm in diameter were visible on the surface and on the cross section. Right heart ventricle was dilated and congestion of the kidneys, adrenal gland, brain and meninges were also noted.

Histopathology

Brain was oedematous, blood vessels were congested and some were filled with large bacterial colonies and fibrinous clots. In cerebellar meninges, mononuclear cell infiltrate was found around congested blood vessels (Figure 1). Multiple pyogranulomatous inflammatory lesions, characteristic for listeriosis were distributed randomly in the parenchyma of hepatic lobules (Figure 2). Lungs were congested, atelectatic and oedematous with disseminated acute haemorrhages and accumulations of neutrophils in alveolar spaces, many pulmonary blood vessels were filled with rod- shaped bacteria. Lesions were diagnosed as an acute embolic pneumonia. Other lesions reflected gross pathology lesions:

catarrhal desquamative enteritis with hyperplastic Peyers patches, lymphopenic and hyperaemic mesenteric lymph nodes, reactive hyperplasia, extramedullar haematopoesis, congestion and multifocal haemorrhages of the spleen, multifocal cortical haemorrhages of adrenal gland and kidney cortex.

Bacteriological examination

Bacteriological cultures of the organs on blood agar yielded abundant growth of smooth transparent colonies, 1-2 mm in diameter, with narrow zone of beta-haemolysis. Gram stained cultures revealed short round-ended Gram-positive rods. The obtained numerical profile in API Listeria system was 6510, which is consistent with *L. monocytogenes*. *L. monocytogenes* produces haemolysin listeriolysin O, which enhanced the effect of S. aureus beta-toxin. Weak CAMP effect with equi factor of R. equi was also observed (16, 17, 18). The culture incubated in tryptic soy broth at 20°C showed the characteristic tumbling motility end-over-end. In 37°C broth culture the bacteria were not motile. After confirmation by standard cultural method, the strain was positive for *L. monocytogenes* in nucleic acid hybridisation assay. It was serologically identified as serotype 1. We also isolated Listeria from selective enrichment medium. The colonies had typical morphology on solid selective differential media, so further identification was not performed as *L. monocytogenes* had already been confirmed directly. The strain was sensitive to amikacin, gentamicin, oxytetracycline and trimethoprim-sulphamethoxazole, and intermediate sensitive to penicillin. However, the strain was resistant to cefalexin, ceftriaxone, cefoperazone, and ceftiofur.

The bacteriological examination of the mare' swab and the complement fixation assay were both negative.

Discussion

Gross and histopathology findings i.e. pyogranulomatous hepatitis, mononuclear meningitis, pneumonia, haemorrhages in the spleen and other organs and enlarged lymph nodes consistent with septicaemia and isolation of the *L. monocytogenes* in a pure culture from different tissues strongly suggest that *L. monocytogenes* was the etiologic agent for the disease in this foal. Severe suppurative bronchopneumonia with necrotizing vasculitis and microabscesses in liver caused by *L. monocytogenes* was described previously in a 6-day-old foal (11).

Listeria monocytogenes is ubiquitous and, although non-sporing, very resistant in the environment. Many healthy individuals are intestinal carriers. Carriage rate for animals depends on the species and is a little higher rate during indoor season, as compared to animals on pasture (19). Weber et al. found *L. monocytogenes* in 4.8% of 400 faecal samples of healthy horses (20). The route of infection is mainly by ingestion, through the nasal mucosa, conjunctiva or wound. Infection can also be transmitted by aerogenic route, transplacentally or to foals via the mare's milk (9, 11). In the case presented herein, we were not able to determine the source of infection. The mare's milk was not available any more, and the vaginal swab bacteriology and the complement fixation assay were negative. The foal did not have any contact with ruminants and there were no history of diseased horses or other animals on the farm. The animals had not been fed silage, the most frequent source of feed-borne listeriosis. In situation when hosts are immunosupressed due to stress, primary disease or infection, pregnancy, immunodeficiency disorder or high infective dose, clinical symptoms can develop. The most likely source of infection in this case may have been the contaminated environment and inadequate passive immunity. In fact, the passive transfer of immunity was probably not adequate in this foal with the serum globulin concentration of 19g/l (normal >20g/l). Serum IgG concentration, unfortunately, was not specifically determined.

In general, isolates of *L. monocytogenes*, as well as strains of other Listeria spp., are susceptible to a wide range of antibiotics, but show a high natural resistance to cephalosporins, especially to those that are broad spectrum (21). In our case, the antibiotic of choice was ceftiofur as the exact diagnosis could not be made by clinical observation alone. Anyway, the foal had been euthanised before possible effects of the therapy could be seen.

This report is the first to describe *L. monocytogenes* septicaemia in a foal in Slovenia. Although the disease is rare in horses, the possibility of *L. monocytogenes* infection should always be considered in cases expressing clinical signs such as those presented with this case. It is important to

define the diagnosis and etiology of the disease as, in the case of *L. monocytogenes*, interspecies disease transmission and/or development of healthy carriers can occur. This may be of particular importance in herds where we expect several foal to be born.

References

1. McLauchlin J, Jones D. Erysipelothrix and Listeria. In: Balows A, Duerden BI, eds. Topley and Wilson's microbiology and microbial infection, systematic bacteriology. Vol. 2. London: Arnold, 1999: 692.

2. Wesley IV. Listeriosis in animals. In: Ryser ET, Marth EH, eds. Listeria, listeriosis, and food safety. New York, Basel: Marcel Dekker, 1999: 39-40.

3. Low C, Linklater K. Listeriosis in sheep. In Pract 1985; 7: 66-7.

4. Low JC, Donachie W. A review of Listeria monocytogenes and listeriosis. Vet J 1997; 153: 9-29.

5. Quinn PJ, Carter ME, Markey B, Carter GR. Clinical veterinary microbiology. London: Mosby International, 2002: 170-4.

6. Grini O. Listerella monocytogenes som assak til septico-pyemi hos foll. Norsk Vet Tidskr 1943; 55: 57-104.

7. Emerson FG, Jarvis AA. Listeriosis in ponies. J Am Vet Med Assoc 1968; 152: 1645-6.

8. McCain CS, Robinson M. Listeria monocytogenes in the equine. In: Proceedings of the 18th Annual Meeting of the American Association of Veterinary Laboratory Diagnosticians. Portland, 1975: 257-61.

9. Wallace SS, Hathcock TL. Listeria monocytogenes septicaemia in a foal. J Am Vet Med Assoc 1995; 207: 1325-6.

10. Larochelle D, Mysore J, Walker R. Necrotizing hepatitis in foals with a Listeria monocytogenes septicaemia. In: 42nd Annual Meeting of American Association of Veterinary Laboratory Diagnosticians, 1999: 104.

11. Wilkins PA, Marsh PS, Acland H, Del Piero F. Listeria monocytogenes septicemia in a thoroughbred foal. J Vet Diagn Invest 2000; 12(2): 173-6.

12. Jose-Cunilleras E, Hinchcliff KW. Listeria monocytogenes septicaemia in foals. Equine Vet J 2001; 33 (5): 519-22.

13. Clark EG, Turner AS, Boysen DG, Rouse BT. Listeriosis in an Arabian foal with combined immunodeficiency. J Am vet med Ass 1978; 172: 363-6.

14. Welsh RD. Equine abortion caused by Listeria monocytogenes serotype 4. J Am Vet Med Assoc 1983; 182: 291.

15. Zdovc I, Mehle J. Current situation of animal listeriosis in Slovenia. In: Proceedings of ISOPOL XIV. Mannheim, 2001: 93.

16. Fraser G. The effect on animal erythrocytes of diffusible substances produced by bacteria. J Pathol Bacteriol 1964; 88: 43-53.

17. Skalka B, Smola J, Elischerova K. Routine test for in vitro differentiation of pathogenic and apathogenic Listeria monocytogenes strains. J Clin Microbiol 1982; 15: 503-7.

18. Prescott JF, Lastra M, Barksdale L. Equi factors in the identification of Corynebacterium equi Magnusson. J Clin Microbiol 1982; 16: 988-90.

19. Iida T, Kanzaki M, Maruyama T, Inoue S, Kaneuchi C. Prevalence of Listeria monocytogenes in intestinal contents of healthy animals in Japan. J Vet Med Sci 1991; 53: 873-5.

20. Weber A, Potel J, Schafer-Schmidt, R, Prell A, Datzmann C. Studies on the occurrence of Listeria monocytogenes in fecal samples of domestic and companion animals. Zentralbl Hyg Umweltmed 1995; 198(2): 117-23.

21. Hof H, Nichterlein T, Kretschmar M. Management of listeriosis. Clin Microbiol Rev 1997; 10: 345-57.

LISTERIOZNA SEPSA PRI ŽREBIČKI

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Povzetek: Opisali smo listeriozno sepso pri tridnevni žrebički slovenske toplokrvne pasme. Žrebička je bila ob sprejetju na kliniko slabotna in močno dehidrirana. Kljub terapiji se ji je zdravstveno stanje slabšalo, zato je bila potrebna evtanazija. Najočitnejše spremembe, ugotovljene pri raztelesbi, so bile multifokalni hepatitis, difuzno vnetje črevesja ter krvavitve po pljučih in vranici. S patohistološko preiskavo smo ugotovili piogranulomatozni hepatitis, mononuklearni meningitis, akutno septično embolično pljučnico, akutni kataralni enteritis ter polnokrvnost in akutne krvavitve v večini pregledanih tkiv. Iz jeter, vranice, ledvic, pljuč, črevesa in možganov smo izolirali bakterijo Listeria monocytogenes, ki smo jo določili kot povzročitelja sepse pri tej žrebički. Listeriozna sepsa je pri žrebetih le redko diagnosticirana in po nam dostopnih podatkih je to prvi opisan primer v Sloveniji. Čeprav je listeriozna sepsa redka, je povzročitelja pomembno ugotoviti, saj tako lahko preprečimo širjenje bolezni in/ali nastanek zdravih klicenoscev, kar je še posebej pomebno v čredah, kjer pričakujemo večje število novorojenih žrebet.

Ključne besede: Listerija infekcije-diagnostika; *Listeria monocytogenes*; sepsa; konji

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Article in a journal or newspaper: Fuji J, Otsu K, Zorzato F, et al. Identification of mutation in porcine ryanodine receptor asociated with malignant hyperthermia. Science 1991; 253: 448-51.

Article in proceedings of a meeting or symposium: Schnoebelen CS, Louveau I, Bonneau M. Developmental pattern of GH receptor in pig skeletal muscle. In: the 6th Zavrnik memorial meeting. Lipica: Veterinary Faculty 1995: 83-6.

NAVODILA AVTORJEM

Slovenski veterinarski zbornik (Slovenian Veterinary Research) objavlja izvirne prispevke, ki še niso bili objavljeni oz. poslani v objavo drugam. Za vse navedbe v prispevkih so odgovorni avtorji. Uredniška politika obsega publiciranje znanstvenih člankov, preglednih znanstvenih člankov, strokovnih člankov, povzetkov disertacij in drugih prispevkov, kot so kritične presoje o vsebini razprav, objavljenih v zborniku, kratke znanstvene prispevke, pisma uredniku in drugo. Avtorji pošljejo prispevke na naslov uredništva. Glavni urednik pregleda vse prispevke. Za vse članke je obvezna strokovna recenzija, za katero poskrbi uredništvo.

Prispevki naj bodo napisani v angleškem jeziku, z naslovom, povzetkom in ključnimi besedami tudi v slovenščini. Obsegajo naj največ 12 strani, kar pomeni 27 vrstic na stran s približno 75 znaki v vrstici. Prispevki naj bodo poslani v elektronski obliki v katerem koli urejevalniku besedil za okensko okolje. Zaželjena je uporaba elektronske pošte (slovetres@vf.uni-lj.si) in trije predlogi za možne recenzente. Besedilo naj ima dvojni razmik med vrsticami, pri čemer naj bodo vrstice na levi strani oštevilcene. Besedilo naj bo na levi strani od roba oddaljeno 4 cm.

Naslovna stran prispevkov se začne z naslovom, sledi ime in priimek avtorja. Kadar je avtorjev več, jih ločimo z vejicami. V naslednjih vrsticah je v rubriki Addresses of authors: za dvopičjem treba navesti polno ime in priimek ter naslov(e) avtorja(ev), tj. ustanovo, ulico s hišno številko, pošto in kraj. Vse navedene podatke ločujejo vejice. Sledi vrstica, kjer je treba navesti ime ter elektronski (E-mail:) in poštni naslov ter telefonsko številko (Phone:) odgovornega avtorja.

Sledi besedilo povzetka Summary v obsegu 16 do 20 vrstic (približno 1000 do 1500 znakov). V naslednji rubriki Key words: se za dvopičjem navedejo ključne besede. Posamezne besede ali sklopi besed morajo biti ločeni s podpičjem.

Znanstveni članki in tisti, ki so prikaz lastnih raziskav in dognanj, morajo vsebovati še naslednje obvezne rubrike, s katerimi avtor sam naslovi ustrezne dele besedila v prispevku: Introduction, Material and methods, Results, Discussion in References. Pregledni članki naj vsebujejo uvod, poglavja, ki so glede na vsebino smiselno naslovljena, in literaturo. Podatke o financerjih ali drugih zadevah, pomembnih za prispevek, npr. o tehnični pomoči, avtorji navedejo v rubriki Acknowledgements, ki se uvrsti pred rubriko References Za rubriko References sledijo spremna besedila k slikam.

Priloge, kot so tabele, grafikoni in diagrami naj bodo smiselno vključene v besedilo. Slikovni material naj bo poslan posebej v obliki bmp, jpg, ali tif.

Priloge in slike morajo biti poimenovane z besedami, ki jih opredeljujejo, in arabskimi številkami (npr. Table 1:, Figure 1: itn.). Za dvopičjem sledi besedilo oziroma naslov. Vsi navedki (reference), citirani v besedilu, se morajo nanašati na seznam literature. V besedilu jih je treba oštevilčiti po vrstnem redu, po katerem se pojavljajo, z arabskimi številkami v oklepaju. Prvi navedek v besedilu opredeli številko oziroma vrstni red ustreznega vira v seznamu literature. če se avtor v besedilu ponovno sklicuje na že uporabljeni vir, navede tisto številko, ki jo je vir dobil pri prvem navedku. Citirana so lahko le dela, ki so tiskana ali kako drugace razmnožena in dostopna javnosti. Neobjavljeni podatki, neobjavljena predavanja, osebna sporočila in podobno naj bodo omenjeni v navedkih ali opombah na koncu tiste strani, kjer so navedeni. V seznamu literature so viri urejeni po vrstnem redu. če je citirani vir napisalo šest ali manj avtorjev, je treba navesti vse; pri sedmih ali vec avtorjih se navedejo prvi trije in doda et al.

Da bi se morebitni popravki lahko objavili v naslednji številki, jih morajo avtorji pravočasno sporočiti glavnemu uredniku.

Načini citiranja

Knjiga: Hawkins JD. Gene structure and expression. Cambridge: University Press, 1991: 16.

Poglavje ali prispevek v knjigi: Baldessarini RJ. Dopamine receptors and clinical medicine. In: Neve KA, Neve RL, eds. The dopamine receptors. Totowa: Human Press, 1996: 475-98.

Članek iz revije ali časopisa: Fuji J, Otsu K, Zorzato F, et al. Identification of mutation in porcine ryanodine receptor asociated with malignant hyperthermia. Science 1991; 253: 448-51.

Članek iz zbornika referatov: Schnoebelen CS, Louveau I, Bonneau M. Developmental pattern of GH receptor in pig skeletal muscle. In: the 6th Zavrnik memorial meeting. Lipica: Veterinary Faculty 1995: 83-6.

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