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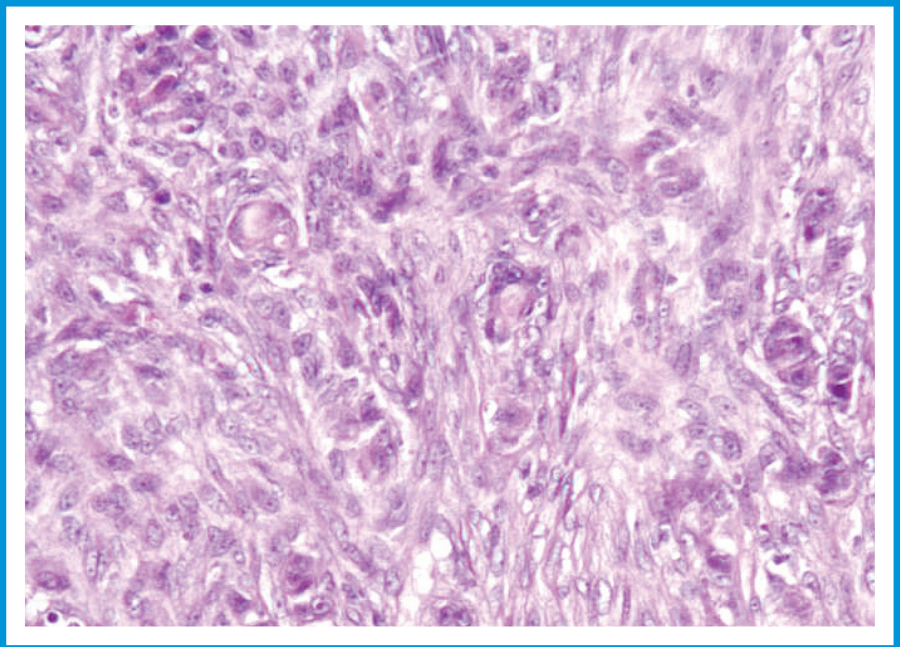

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Original Scientific Articles

Case Report

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Original Scientific Article

OCCURRENCE OF ANTI *Toxoplasma* ANTIBODIES IN OWNED DOGS FROM ITALY: A RETROSPECTIVE STUDY

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Summary: Toxoplasma infection in human patients is still an important problem in Italy. Dogs seem to have a role in the epidemiology of human toxoplasmosis, being their presence associated with increased seroprevalence to *Toxoplasma gondii* in humans. Dogs can act as intermediate hosts of this parasite being able to harbor tissue cysts, but this way their reservoir importance for human infection is negligible. Their impact on human health could be due to their role in contaminating the household environment, so permitting the exposure to *T. gondii* the inhabitants. Serum samples of N. 1811 owned dogs randomly collected were examined by IFAT for antibodies against *T. gondii*. One hundred ninety two sera out of 1811 (10.6%) scored positive, with titers ranging from 1/20 to 1/640. Seroprevalence was significantly ($P< 0.01$) higher in adult than in juvenile dogs. On the contrary, it not significantly differed with regards to gender and feeding habits. This is the first report of occurrence of antibodies in an extensive specimen of dogs in Italy. The results of the present survey would indicate a relatively high occurrence of antibodies against *T. gondii* among owned dogs in the investigated area, confirming that attention should be paid in the management of this domestic species.

Key words: dog; *Toxoplasma gondii*; IFAT; seroprevalence

Introduction

Toxoplasma gondii is a zoonotic intracellular protozoan parasite with a worldwide distribution, infecting a large range of vertebrates. Humans become infected postnatally by ingesting tissue cysts from undercooked meat, consuming food or drink contaminated with oocysts, or by accidentally ingesting oocysts. Up to one-third of the world's population is chronically infected (1) and toxoplasmosis has been targeted by Center for Disease Control and Prevention as one of the five top priority parasitic diseases for public health action. However, only a small percentage of exposed adult humans or other animals develop clinical signs of disease (2). Toxoplasmosis is usually a self-limiting disease in immunocompetent individuals, but it is an important cause of morbidity and mortality in immunosuppressed individuals and can cause inflammation of the retinae in healthy adults (3). In human infants congenitally infected *T. gondii* can causes mental retardation, loss of vision and other health problems.

Dogs can act as intermediate hosts of this parasite being able to harbor tissue cysts, but

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this way their reservoir importance for human infection is negligible. Their impact on human health could be due to their role in contaminating the household environment so permitting the exposure to the inhabitants (4) by ingesting or rolling in cat feces that contain sporulated *T. gondii* oocysts. This result shows that canine feces could pose a risk of *T. gondii* infection to other species including humans because dogs may serve as mechanical vectors for parasite (5). There are few reports of primary toxoplasmosis in dogs, even if canine toxoplasmosis has been reported in several European and Asian countries as well as the United States (6) with common clinical signs including encephalitis, hepatitis and pneumonia (7). However seroprevalence rates are reported in different areas to vary from 85% in Turkey to 5% in China as reported by Tenter, (2000) (8). To the best of our knowledge there is a lack of data concerning the seroepidemiology of *Toxoplasma* infection in dogs from Italy, except for a report carried on 104 animals in a restricted area from South Italy (9).

The aim of the present paper was to investigate retrospectively about the occurrence of anti *Toxoplasma* antibodies in a sample of owned dogs from Central Italy, submitted to the lab of serology of Department of Veterinary Sciences of the University of Pisa for the annual control for prophylaxis of leishmaniosis. This information could be useful to evaluate the possible circulation of the parasite among dog population living in strict contact with humans.

Materials and methods

Serum samples of N. 1811 owned dogs were randomly collected from those submitted to the lab of serology of Department of Veterinary Sciences of the University of Pisa, for the annual control for prophylaxis of leishmaniosis. The animals were 1052 males and 759 females, 269 of them were young (age ≤ 1 year), the other 1542 adult with ages ranging to 13 months to 15 years. All the animals had an indoor/outdoor lifestyle, spending part of their life outdoor every day and belonged to many different breeds, except toy dogs. Six hundred forty nine (35.8%) dogs were fed on commercial pet food, 531 (29.4%) on homemade dog food and 631 (34.8%) on both.

Anti *Leishmania* specific antibodies have been detected by immunofluorescent antibody test (IFAT) performed as described elsewhere (10) and positive and negative results for each dog have been recorded.

To evaluate the presence of anti *Toxoplasma* antibodies an IFAT was performed on sera, using Toxospot® (BioMérieux, Marcy l'Etoile, France) as antigen and an anti dog-IgG FITC antibody produced in rabbit (Sigma-Aldrich; PBS dilution 1:32). All serum samples were screened with a threshold dilution 1:20, and positive ones were end-titrated using 2-fold dilutions. Cut off dilution was chosen following Macrì et al (2009) (11).

The differences between the seroprevalence values obtained from animals of different gender and age, and fed on different food were evaluated by means of x^2 test.

Table 1: Distribution of antibody titers and coinfection with *Leishmania* of *Toxoplasma* seropositive dogs.

anti *Toxoplasma* antibody titres	number of animals	(%)	animals coinfected with *Leishmania*	(%)
20	26	13.5	1	3.8
40	132	68.8	9	6.8
80	27	14.1	5	18.5
160	4	2.1	2	50
320	2	1	0	0
640	1	0.5	0	0
total number	192		17	

Results

One hundred ninety two sera out of 1811 (10.6%) revealed anti *Toxoplasma* antibodies, with titers ranging from 1/20 to 1/640. Among the whole sample 92 (5.1%) sera scored positive for *Leishmania* antibodies and 17 of them (8.8%) resulted coinfected by *Toxoplasma*. More detailed data are reported in Table 1.

One hundred four out of 1052 (9.9%) males and 88 out of 759 (11.6%) females scored positive for antibodies against *Toxoplasma*, respectively. Nine young animals out of 269 (3.3%) and 183 out of 1542 adult (11.8%) were seropositive, also.

Among seropositive animals 72 (37.5%) were fed on both commercial and homemade food, 53 (27.6%) on homemade food and 67 (34.9%) on commercial pet food.

Seroprevalence was significantly ($P<0.01$) higher in adult than in juvenile dogs. On the contrary, the comparison of antibody presence was not significantly different with regards to gender and feeding habits.

Discussion

This retrospective study showed that 10.6% of examined dogs had antibodies against *T. gondii*. This remark agrees with data from literature even if there is a wide range of prevalences, due both to different serological techniques employed and to canine population selected. Recent surveys on domestic dogs have been performed in Portugal (12) with a global prevalence of 38%, in China (13) with 21.5% of positives and in Korea with 5.1% of domestic dogs positive versus 18.5% of stray dogs (14). Our data fully agree with Yang et al (2013) (15) who reported 10% of infected pet dogs from the Northeast of China. On the basis of this value the Authors stated that this relatively high prevalence of *T. gondii* infection in pet dogs, may pose a risk for human health.

There was not observed any correlation between gender and seropositivity, and this finding is in agreement with other Authors (14-18), while a positive correlation between age and seroprevalence, was reported by others (13,16). Feeding habits seem do not impact on presence of antibodies as observed by Ali et al. (2003) (16).

Occurrence of anti *Toxoplasma* antibodies was reported by IFAT in 17% of 104 dogs from the province of Benevento, Italy (9). To the best of our knowledge this is the only report by this country. *Toxoplasma* infection in human patients is still an important problem in Italy. Despite a substantial decrease in *T. gondii* seroprevalence in humans (from 40 to 20-30% in the adult population in the last 20 years) (19) in fact, 1-2 congenital *Toxoplasma* cases per 10,000 births are currently estimated (20) and 1-4% of them are at risk of death or serious neurological sequelae (21). Dogs seem to have a role in the epidemiology of human toxoplasmosis. Previous studies indicate that the presence of dogs is associated with increased seroprevalence to *T. gondii* in humans (22, 23). Free ranging stray dogs can act as sentinel, furthermore being the most common pets in the world, dogs also reflect the extent of *T. gondii* infection in the domestic environment (24) and can act as reservoir, living in strict contact with people and cats.

The results of the present survey would indicate a moderate occurrence of antibodies against *T. gondii* among owned dogs in the investigated area, confirming that attention should be paid in the management of this domestic species.

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PRISOTNOST PROTITELES PROTI TOKSOPLAZMI PRI DOMAČIH PSIH V ITALIJI: RETROSPEKTIVNA ŠTUDIJA

G. Rocchigiani, S. Nardoni, E. Amato, C. Tempori, F. Mancianti

Povzetek: Okužba s toksoplazmo je v Italiji še vedno pomemben zdravstveni problem. Predvideva se, da imajo psi pomembno vlogo pri epidemiologiji človeške toksoplazmoze, saj je njihova prisotnost v okolju ljudi povezana s povečano koncentracijo protiteles proti *Toxoplasmi gondii* pri ljudeh. Psi so lahko vmesni gostitelji tega parazita, ki lahko preživi v tkivnih cistah, vendar je pomen tega rezervoarja za prenos okužbe na ljudi zanemarljiv. Bolj pomembna je vloga psov pri onesnaževanju okolja s *T. gondii* in s tem na povečano stopnjo izpostavljenosti ljudi. V raziskavi smo pri 1811 naključno izbranih psih analizirali prisotnost protiteles proti *T. gondii* v serumu z analizo IFAT. Ugotovili smo, da je bilo 192 psov (10,6 %) pozitivnih, s titri od 1 : 20 do 1 : 640. Prisotnost protiteles je bila statistično značilno ($p < 0,01$) višja pri odraslih psih v primerjavi z mladimi, medtem ko ni bilo razlik med spoloma in tudi ne glede na različne prehranjevalne navade. To je prvo poročilo o pojavljanju protiteles proti *T. gondii* v obsežnem vzorcu psov v Italiji, ki nakazuje, da je seroprevalenca protiteles proti *T. gondii* pri psih relativno visoka. Zato je potrebno pozornost nameniti tudi kontroli okužb pri psih.

Ključne besede: pes; *Toxoplasma gondii*; IFAT; seroprevalenca

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Original Scientific Article

THE HEAVY METAL CONTENTS (Cd, Pb, Cu, Zn, Fe AND Mn) AND ITS RELATIONSHIPS WITH THE SIZE OF THE RUDD (*Scardinius erythrophthalmus*) FROM LAKE CERKNICA, SLOVENIA

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Summary: The concentrations of Pb, Cd, Cu, Zn, Fe and Mn were measured in different tissues of rudd (*Scardinius erythrophthalmus*) from Lake Cerknica, Slovenia. The content of heavy metals in rudd muscle/skin was low; therefore, rudd from Lake Cerknica are suitable for human consumption. Among the heavy metals studied, Pb was not detected in rudd tissues and Cd was undetectable in meat samples. The content of other elements was also the lowest in the meat samples. The highest concentrations of Cd and Zn were found in the kidneys, and those of Cu, Mn and Fe in the livers. The relationships between fish weight or length and metal concentrations were investigated. In the meat and liver of the rudd, the Pearson correlation analysis for Zn and Mn revealed a negative association related to size (length, weight). However, the concentrations of Fe in kidney and Cd in liver and kidney increased with the size of rudd.

Key words: lead; cadmium; copper; zinc; iron; manganese; rudd; size

Introduction

In aquatic ecosystems, heavy metals merit considerable attention due to their toxicity and accumulation in biota. High levels of heavy metals in freshwater environments may occur due to the natural weathering of minerals in sediment and bedrock, or as a result of anthropogenic activities, such as mining. Some of these elements are toxic to living organisms even at low concentrations; others are biologically essential and only become toxic at high concentrations. Fish are at the top of the aquatic food chain and, therefore, are often used as bio-indicators of water pollution with heavy metals (1-6).

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However, when fish are used as bio-indicators for water pollution, the obtained results must be correctly interpreted, taking into consideration several factors that may affect the accumulation of different pollutants in the body. For example, the bioaccumulation of heavy metals in fish is related to the physical and chemical properties of water, such as salinity, pH value, hardness, temperature and concentration of organic material in water. The eating behavioural patterns, age, sex, spawning of the fish, as well as the season of fish capture were also found to be essential for metal accumulation in fish tissues (1-3, 6).

The aim of our study was to determine the amount of lead (Pb), cadmium (Cd), copper (Cu), zinc (Zn), manganese (Mn) and iron (Fe) in different tissues of the rudd (*Scardinius erythrophthalmus*)

from Lake Cerknica. Concentrations of the above-mentioned elements were measured in livers, kidneys and muscle tissue with skin. In this research, the relationship between fish size and metal concentrations in different tissues were also studied.

Materials and methods

In this study, 30 rudd (*Scardinius erythrophthalmus*) caught in the Lake Cerknica were analysed. Rudd is a benthic cyprinid fish with omnivorous feeding habits. The rudd diet primarily contains various macrophytes, bryophytes, and filamentous algae along with some animal material and detritus. Rudd show a size-dependent diet shift from microcrustaceans while small, to macro-invertebrates at larger sizes. The lipid content in fish affects metal concentration in the tissues (7). In order to avoid this effect, the rudd were sampled in a one-month period in September 2004. Lake Cerknica is a large intermitted lake situated in the southwest of Slovenia. Its area usually reaches 28 km² but can also reach up to 38 km². According to the literature (8), water in the Lake Cerknica had in year 2003 pH between 7.9 and 9.1. The content of metals in water was mostly below the limit of quantification. The sediment in the lake contained measurable quantities of metals. However, the levels were low except for a slightly higher content of Cd. Nevertheless, the sediment of the tributary named Cerkniščica contained slightly higher levels of Cu, Zn and Pb.

The concentration of Pb, Cd, Zn, Cu, Mn and Fe content in fish tissues were determined with a Varian SpectrAA 220 flame atomic absorption spectrophotometer, using deuterium background correction. The collected fish were rinsed with water. The total body length (cm) and body weight (g) of each fish were recorded. The age of fish was determined from scale samples according to the annual ring structure (Table 1).

The scales, head with gills and abdominal contents were removed. From the abdominal contents, the liver and kidney were separated. Muscle with skin was homogenized without bones. Until the analysis, the samples were stored separately in tightly closed containers at -18°C. Each of studied tissues (whole liver, whole kidney and 10 g of homogenized muscle) was weighed in a quartz crucible. Samples were dried in a drying oven at 103°C and then put into a cold programmable furnace. The temperature in the furnace was increased slowly at a maximum rate 50°C/h to 450°C and left at this temperature overnight. Ash was wetted with 5 mL of water and 5 mL concentrated hydrochloric acid, and the solution was evaporated on a hot plate. The residue was dissolved in 50 mL of diluted hydrochloric acid (0.25 M). The contents of Zn, Cu, Mn and Fe were measured directly from the acidic solution of ash. An external calibration curve was used for the evaluation of the results. For Pb and Cd, a pre-concentration step was used. They were determined as diethylammonium diethyldithiocarbamate complexes extracted into methylisobutylketone (9). For the evaluation of the results, the method of standard addition calibration was used. The accuracy of the methods used was checked via analysing the standard reference materials (BCR 185R Bovine liver, BCR 422 Cod muscle, BCR 184 Bovine muscle and BCR 186 Pig kidney). The recovery rates ranged from 90% to 110% for all investigated elements. The limits of quantification (LOQ) expressed as mg of metal per liter of solution obtained after digestion were 0.01 (Pb), 0.0006 (Cd), 0.006 (Zn), 0.015 (Cu), 0.02 (Mn) and 0.03 (Fe).

The Pearson correlation test was used to check for significant relationships between individual heavy metal concentration and length or weight. The level of significance was set at a probability lower than 0.05. Samples with a detectable amount of metal were used for calculations. All calculations were carried out using the Microsoft Excel program for Windows XP.

Results and discussion

The Cd, Zn, Cu, Mn and Fe contents in fish tissues are presented in Table 2. The level of Pb was below LOQ in all analysed samples. Cd was also undetectable in muscle/skin samples; therefore, all analysed samples of rudd were suitable for human consumption. According to the Commission Regulation no. 1881/2006 the maximum acceptance level of Pb and Cd in the meat of the fish is 0.30 and 0.050 mg/kg ww (wet weight), respectively (10).

The results of this study are in accordance with previous studies done in Slovenia, which showed that Pb contents in fish tissues from

Table 1: Age, weight, length and number of analysed rudd (*Scardinius erythrophthalmus)*

n	Age (years)	Weight - W (g)	Length – L (cm)
30	1–6	25.7–603.6	13.0–32.0

Table 2: Concentrations of Cd, Zn, Cu, Mn and Fe in rudd tissues

	Element	n_0	n	Concentration (mg/kg wet weight)			
				min	max	me	av
Muscle with skin	Cd	30	30	all < 0.003			
	Zn	30	30	6.8	26.0	16.0	16.4
	Cu	30	30	< 0.08	0.29	0.14	0.14
	Mn	30	30	0.11	0.32	0.20	0.20
	Fe	30	30	2.3	4.9	3.2	3.1
Liver	Cd	30	23	0.046	0.359	0.115	0.151
	Zn	29	29	34.7	366	62.8	94.0
	Cu	30	30	6.56	85.0	17.8	27.7
	Mn	30	28	0.66	5.10	1.17	1.61
	Fe	30	30	90.2	519	168	199
Kidney	Cd	29	24	0.138	1.850	0.573	0.706
	Zn	29	29	68.0	373	165	174
	Cu	29	20	0.33	1.08	0.70	0.70
	Mn	29	18	0.33	0.80	0.54	0.53
	Fe	29	29	32.2	179	104	108

n_0 – number of samples; n – number of samples were, based on the LOQ concentration of element could be determined; me – median; av – average

Slovenian rivers are generally low. The meat of rudd from the Šalek lakes, situated in the close vicinity of the Šoštanj Thermal Plant, a great source of anthropogenic emission of metals into the environment, also contained very low levels of Pb (in average 0.02 mg/kg ww) (11). In our previous study, higher levels of Pb were found only in the muscle/skin of some fish from the lower flows of the Drava (max. 1.21 mg/kg ww) and Sava rivers (max. 0.32 mg/kg ww) and in all analysed muscle/skin samples of fish from the Mežica valley (0.39–0.77 mg/kg ww), where a Pb smelter used to operate (12).

The highest concentrations of Cd were found in the kidneys, with a median level 0.573 mg/kg ww (Table 2), while the content in the livers was 0.115 mg/kg ww. The level of Cd in kidneys and livers is an important indicator of environmental pollution. In tissues, Cd binds to metalothionein, which is synthesized in the liver and then transferred to the kidneys. Higher Cd content in the kidneys than in the liver is considered to be a consequence of long-term exposure, but in the case of acute poisoning more Cd is found in the liver than in the kidneys (2). Taking into account this fact, we can conclude that Cd content is a consequence of long-term exposure. In the study on freshwater fish from Slovenian rivers, sampled in the period from 1999 to 2003 (12), only 28% of the kidneys contained more than 0.5 mg/kg ww of Cd. Higher amounts of Cd were found in fish kidneys from the lower flows of the Drava, Meža and Ljubljanica rivers. According to the data of the Agencije republike Slovenije za okolje (Slovenian

Table 3: Pearson correlation coefficient (r) and level of significance (p) of correlation between element content and mass, and element content and length of the rudd

	Muscle/skin					
	Weight	Length	Cu	Zn	Mn	Fe
Cu	0.29	0.33	1.00			
Zn	–0.79***	–0.76***	–0.39*	1.00		
Mn	–0.43*	–0.48**	–0.19	0.21	1.00	
Fe	–0.18	–0.09	0.14	0.25	–0.33	1.00
Kidney						
Cu	–0.27	–0.32	1.00			
Zn	0.38*	0.28	–0.05	1.00		
Mn	0.20	0.21	–0.18	0.17	1.00	
Fe	0.46*	0.47*	0.09	0.38*	0.52*	1.00
Cd	0.66***	0.67***	–0.16	0.07	0.68**	0.56**
Liver						
Cu	–0.18	–0.19	1.00			
Zn	–0.46*	–0.50**	0.18	1.00		
Mn	–0.54**	–0.63***	0.05	0.06	1.00	
Fe	0.30	0.30	0.10	0.04	–0.30	1.00
Cd	0.57**	0.59**	–0.01	–0.33	–0.25	0.23

*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; in cases without mark, r is not statistically different from 0.
Number of analysed samples is in Table 2.

Table 4: Regression equations relating the element content in different tissues to the weight and length of the rudd (equation is given only in cases in which the correlation is statistically significant)

	Muscle/skin	Kidney	Liver
Zn	X = –0.02 w + 22.61	X = 0.11 w + 137	X = –0.18 w + 153
	X = –0.65 l + 32.21		X = –6.65 l + 256.6
Mn	X = –0.0001 w + 0.23		X = –0.003 w + 2.6
	X = –0.004 l + 0.03		X = –0.12 l + 4.54
Fe		X = 0.06 w + 88.5	
		X = 2.08 l + 57.0	
Cd		X = 0.002 w + 0.057	X = $3x10^{-4}$ w + 0.02
		X = 0.068 l – 1.084	X = 0.014 l – 0.231

w – weight, l – length

Environment Agency), the Drava and Meža rivers in 2002 and 2003 contained high levels of Cd in the sediment (Drava at Ormož 1.8 mg/kg and at Maribor Island 9.7 mg/kg; Meža at Otiški Vrh 7.9 mg/kg and at Podklanc 34 mg/kg) (13, 14), so the high Cd contents can be attributed to the environmental pollution. The Cd content in the sediment of Lake Cerknica was found to be rather low (Gorenje Jezero 1.1 mg/kg, Dolenje Jezero 0.37 mg/kg and Cerkniščica 0.63 mg/kg) (8); therefore, the high content of Cd in the kidneys of the rudd in our study cannot be explained only by pollution of water environment but also by the feeding habits of the rudd. It is an omnivorous fish, feeding on zooplankton, aquatic insects, filamentous algae, higher aquatic plants, and occasionally on fish eggs or small fish. Amundsen et al. (1) found that fish feeding with invertebrates contained more Cd in the tissues than piscivorous fish. Fish that eat zooplankton, aquatic insects, fingerlings and shrimp also contained higher levels of Cd than herbivorous fish did (6). Guerrin et al. (15) analysed rudd caught in a fishpond in France, and found less Cd (0.134–0.190 mg/kg ww) in kidneys than we did, although the sediment of the French fishpond contained similar amount of Cd as Lake Cerknica did (< 1 mg/kg). However, in the study, only four samples of rudd were analysed, and they were also smaller (average weight 220 g) than the rudd in our study (average weight 381 g).

The lowest concentrations of Zn, Cu, Mn and Fe were found in muscle/skin. The highest amounts of Zn were found in the kidneys, while the highest amounts of Cu, Fe and Mn were found in the liver. Levels of Zn, Fe and Mn in rudd tissues were found to be similar to the contents found in our previous study (16) considering similar fish species, such as Danube roach (*Rutilus pigus virgo*) and chub (*Leuciscus c. cephalus*), which belongs to the carp family (Cyprinidae) and are also omnivorous the same as rudd. A somewhat different situation was found regarding Cu contents. In our study, we found higher concentrations of Cu in the rudd liver (average 27.8 mg/kg ww) than we did in the liver of the Danube roach (1.35 mg/kg ww) and chub (2.31 mg/kg ww) analysed in a previous study (16). The content of Cu in the sediment of Lake Cerknica in 2003 was low (11 mg/kg Dolenje jezero) (8) compared to the sediment of Slovenian rivers (75 mg/kg Drava – Ormož, 350 mg/kg Ljubljanica – Zalog, 45 mg/kg Soča – Solkan) (14). Therefore, the high content of Cu in the liver of rudd was probably due to the higher accumulation of this element in the rudd's liver than in the Danube roach and chub liver. However, rudd in livers accumulates lower levels of Cu than salmonids. According to literature data (1) and the findings in our study (16), salmonids accumulate very high amounts of Cu in their livers (average 75 mg/kg).

The correlation analysis between the element concentrations in the fish tissues and their size (length or weight) revealed significant relationships. In Table 3, the Pearson correlation coefficient (r) and the level of significance (p) of the relationships between the tissue metal concentrations of the rudd and their weight or length are shown. A regression equation for the tissue metal concentrations and weight or length was calculated only for the elements for which the correlation is statistically characteristic (at a 95% confidence level). Equations are represented in Table 4. Lipid content in fish decreases during winter and spring and reaches its peak at the end of main feeding period – autumn. The metal concentration in tissues is affected by lipid content (7). In order to avoid this effect, the rudd were sampled in a one-month period in September. The lipid content in fish with the same size was similar, which was confirmed by good length-weight relationship that could be described with the formula $W = 0.0046L^{3.42}$ where W is weight and L is length (r = 0.99).

The content of Zn and Mn in the muscle/skin and liver tissue decreased significantly with fish size (weight and length). However, in the kidneys, the content of Zn increased with the body weight of the fish. The content of Fe in the kidneys and the content of Cd in the kidneys and liver also significantly increased with fish size.

In the tissues of rudd, significant correlation between some elements, such as the correlation between Mn-Cd, Fe-Cd, Fe-Zn and Fe-Mn in the kidneys and between Zn-Cu in the meat of the rudd, were observed.

For the essential elements, we assume that the content in tissues is homeostatically controlled, resulting in positive or negative correlation. Accumulation of the elements in the bodies is controlled by absorption, elimination and detoxification, which are highly dependent on the rate of metabolism. The rate of fish metabolism varies with age. The ratio between the surface area and the volume of fish, fish diet and the concentration of certain biologically important compounds

involved in the process of accumulation changes with the age of fish. All these factors affect the concentration of the metals in the fish tissues (18). Cu is an essential element, and most authors observed positive or negative correlation between its content in fish tissues and the size of fish (7, 19). However, in our study, the correlation was not confirmed. The negative correlation between Zn content in the muscle/skin or liver tissue and body size was also observed in other studies on other fish species (1, 7, 19). The negative correlation between the element content and the size of fish does not mean that the element is no longer absorbed by fish during growth. A negative correlation is linked with a different rate of absorption through the intestine and with the more efficient secretion of older fish. The fact that the main route of absorption is through the gills, and that gill size, relative to body size, diminishes with the size of the fish, could also be the reason for negative correlation between the element concentration and fish size.

The content of heavy metals in rudd muscle/skin was low; therefore, rudd from Lake Cerknica are suitable for human consumption. The element content in rudd tissues depends on the length and weight of fish. The concentration of elements in fish also varies among different fish species, and these two facts should be considered in comparative biomonitoring studies.

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VSEBNOST TEŽKIH KOVIN IN NJIHOVA POVEZAVA Z VELIKOSTJO RDEČEPERKE (*Scardinius erythrophthalmus*) IZ CERKNIŠKEGA JEZERA, SLOVENIJA

Z. Bajc, V. Jenčič, K. Šinigoj Gačnik

Povzetek: V tkivih rdečeperke (*Scardinius erythrophthalmus*) iz Cerkniškega jezera (Slovenija) smo proučevali vsebnosti Pb, Cd, Cu, Zn, Fe in Mn. Rdečeperke iz Cerkniškega jezera so primerne za prehrano ljudi glede vsebnosti težkih kovin, saj je bila njihova vsebnost v mišičnem tkivu nizka. Ugotovili smo, da je bila vsebnost Pb v vseh tkivih rdečeperke pod mejo zaznavnosti, Cd pa nismo zaznali v mišičnem tkivu rdečeperk (< 0,003 mg/kg). Tudi koncentracije Cu, Zn, Fe in Mn so bile najnižje v mišičnem tkivu. Najvišjo koncentracijo Cd in Zn smo ugotovili v ledvicah, najvišjo koncentracijo Cu, Mn in Fe pa v jetrih. Proučili smo tudi povezavo med maso oziroma dolžino rib z vsebnostjo kovin v tkivih. V ta namen smo izračunali Pearsonov korelacijski koeficient in ugotovili, da je ta za Zn in Mn v mišičnem tkivu in jetrih negativen, kar pomeni, da se koncentracija manjša z velikostjo rib. Za Fe v ledvicah ter za Cd v ledvicah in jetrih pa je Pearsonov koeficient pozitiven, kar pomeni, da koncentracija z velikostjo rdečeperke narašča.

Ključne besede: svinec; kadmij; baker; cink; železo; mangan; rdečeperka; velikost

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Original Scientific Article

POLYMORPHISMS OF THE *IGF1* GENE IN RUSSIAN SHEEP BREEDS AND THEIR INFLUENCE ON SOME MEAT PRODUCTION PARAMETERS

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Summary: Insulin-like growth factor 1 (IGF-1) plays an important role in the growth and development of muscle tissue in animals. Research into the structure of *IGF1* in sheep may provide important information for genomic marker assisted selection used to increase meat production. We investigated the structure of the *IGF1* gene and the effect of polymorphisms on lifetime meat productivity performance in the Russian Soviet Merino sheep breed. Alleles were detected in 15 rams using NimbleGen sequencing technology by Roche (USA). 18 single nucleotide polymorphisms (SNPs) were found in this breed. Only one SNP – c.-81T>C – was found in the coding region. All other SNPs were located in introns 5'UTR and 3'UTR. The c.-5363C>T, c.-5188G>C, c.-5186G>A and c.-4088G>A polymorphisms, presented together in two alleles of the gene, correlate with a high live weight in a heterozygous state. The synonymous substitution of c.81T>C in the exon was not found to have any influence on the analyzed meat production parameters. One of the detected SNPs – c.-91A>C – had a positive correlation with weight, height, croup parameters and other attributes in rams.

Key words: sheep; gene; *IGF1*; meat; SNP; selection

Introduction

Genomic marker-assisted selection is now a major global sheep breeding trend. Therefore, the search for new genes whose structural features affect the productive qualities of the animals has become an urgent aim for the present stage of research.

The greatest numbers of known genes that affect sheep meat production code for various regulatory peptides such as myostatin (1), calpastatin (2) and others. Another important regulatory protein, which controls growth and development in mammalian muscle structures, is insulin-like growth factor 1 (*IGF-1*). Along with IGF-2, growth hormone (GH) and growth hormone releasing hormone (GHRH), it is a member of the so-called somatotropic axis (GH / IGF-1 axis), which plays a key role in the growth of vertebrates (3, 4). IGF-1 mediates the stimulatory effect of growth hormone and testosterone on the growth and development of muscle fibers (5, 6).

A number of genetic polymorphisms of the IGF1 gene were found to relate to growth parameters in chickens (12), pigs (13) and goats (4). Single

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nucleotide polymorphisms (SNPs) describe the impact in the *IGF1* gene on a number of productive performances of sheep (14, 15, 16, 17, 18). Thus, a wide range of physiological functions of IGF-1 can be attributed to the candidate genes to identify genetic markers of meat production in farm animals (19, 20, 21).

Correlations have been detected between the concentration of *IGF-1* in the plasma of various species, the size of their fetus and the live weight of newborns (4, 7, 8). A positive correlation was found between the level of IGF-1 in the plasma, birth weight and muscle eye depth (9, 10, 11) of lambs at the age of 100 days.

The Soviet Merino breed of sheep is one of the most common in the Russian Federation. This fine-wool breed of meat-wool sheep, bred in the Soviet Union during the 1930s, is well adapted to the dry steppe climate and grazing. It is characterized by a good exterior, strong constitution, well-proportioned physique, strong skeleton and correct statement of limbs. Sheep of this breed have higher than average meat production for a meat-wool combination (22).

To date, there are no investigations pertaining to the structure of *IGF1* gene from Russian breeds of sheep. With this in mind, the aim of the work was the discovery of polymorphisms in the *IGF1* gene in Soviet Merino breed of sheep and an assessment of their effect on meat production.

Material and methods

All work was provided in the Genetic Laboratory of Science-Diagnostic and Veterinary Care Center (Stavropol State Agrarian University, Russian Federation). We have investigated rams (n = 15) at the age of one year of Soviet Merino breed, from livestock breeding farm of Stavropol Krai, Russian Federation. In order to obtain data about the maximum number of *IGF1* gene alleles we selected for the research 10 animals with maximum height and weight, and 5 animals of the same population with a minimum height and weight. All animals were healthy, were kept in optimal conditions and fed with a total mixed ration. To describe meat production analyzed parameters of body measurements.

DNA collection

Genomic DNA was extracted from blood samples obtained from the jugular vein under aseptic conditions. Blood samples were collected in Vacutainer® vials with stabilizer EDTA (Becton Dickinson and Company, Franklin Lakes, NJ, USA) and were transported to the laboratory at +4 C within 6 hours. DNA was extracted from 0.2 ml of blood using a kit PureLinkGenomic DNA MiniKit (Invitrogen Life Technologies, Grand Island, NY, USA).

Targeted enrichment and NextGeneration sequencing

In order to detect mutations in the genes there were performed target enrichment and sub-sequent sequencing of the investigated DNA fragments. For enrichment of target regions we used the NimbleGen technology (Roche NimbleGen, Inc., Madison, WI, USA). Probes for target regions were developed in cooperation with the firm Roche NimbleGen (USA). Libraries of DNA fragments of investigated animals, were prepared in accordance with the protocol Rapid Library Preparation Method Manual undergo the procedure of enrichment using NimbleGen SeqCap EZ Developer Libraries in accordance with the protocol (Roche NimbleGen, Inc., Madison, WI, USA).

Monoclonal amplification procedure of finished enriched target regions of DNA was carried out according to standard protocol emPCR Amplification Method Manual, Lib-L (Roche NimbleGen, Inc., Madison, WI, USA).

Sequencing was performed using a genomic sequencer GS Junior (Roche NimbleGen, Inc., Madison, WI, USA). The resulting sequencing fragments mapped to the reference genome assembly Ovis aries oviAri3 (The National Center for Biotechnology Information. Genome. (2012) Ovis aries (sheep), 2015) by software GS Reference Mapper v2.9 (Roche NimbleGen, Inc., Madison, WI, USA).

To describe a single nucleotide polymorphism (SNP) we use HGVS nomenclature (www.hgvs.org). We used this nomenclature based on transcript XM_012159668.1 (The National Center for Biotechnology Information. Genome. (2012) Ovis aries (sheep), 2015).

Statistical analysis

Phylogenetic analysis was performed using the software Unipro UGENE 1.15.1 (Unipro, Russia).

For statistical analysis used Student's t-test in Excel for Windows statistical plugin. Significant diference detected if $p<0.05$.

Results

As a result of *IGF1* gene sequencing in the Soviet Merino sheep, we found 18 single nucleotide substitutions (Table 1). The percentage of point mutations accounting for transitions was 67%. The coding region of the gene consists of only one of the identified SNPs – c.81T> C. This substitution is synonymous and does not change the encoded amino acid.

Phylogenetic analysis has shown 13 variants of *IGF1* gene according to 18 detected SNPs. The investigated animals were divided into six main genotype groups (A-F). Groups C and D consisted of three subgroups; group F – of four subgroups. One genotype (A) is identical to the reference *IGF1* gene (OAR_v3.1) and was found in 13% of cases.

In order to investigate the influence on meat production, we selected SNPs with higher frequencies. Most detected substitutions occurred in heterozygous form; some were detected simultaneously in the same animal. According to the transcriptional variant XM_012159668.1, the jointly identified substitutions c.-5363C>T, c.-5188G>C, c.-5186G>A and c.-4088G>A are located in locus 5' of the regulatory region, 5'UTR and the first intron. The research on the effect of SNP presence on lifetime productivity indicators showed that body size in animals with a heterozygous genotype is not significantly different from body size in wild homozygotes (Table 2). Meanwhile, live weights in these two groups differed significantly – by more than 4.5 kilograms.

The only substitution found in the exon (substitution c.-81T>C) had an insignificant effect on the lifetime productivity evaluation of any heterozygous or homozygous variant of Soviet Merino sheep.

The research on the impact of the c.-91A>C substitution on the body and live weight of the animals (half of which had the substitution) yielded interesting results (Table 2).

The carrier of homozygous mutant genotype of substitution c.-91A>C was only one of the investigated rams (shown in the last column of table 2). As shown in the table, the weight parameters are in the range of the group with the wild homozygous genotype. However, body size varies significantly in comparison with animals from the heterozygous group. A comparison between animals with wild homozygous and heterozygous genotypes revealed significant differences on a number of indicators: the average live weight of the heterozygotes was significantly greater than that of the homozygotes by 5.7%. The wither height was 4.2% greater in the heterozygotes, while the difference in croup height was 3.5%. The croup width of the heterozygous sheep was higher than the homozygotes by an average of 8.9%, while the croup length of the heterozygotes was a significant 6.6% greater. In addition, the back width was more than 4.4% the value of wild homozygotes. The dimension of back girth was greater in rams with the heterozygous genotype by 4.7%.

Discussion

Our studies of the individual link SNPs and their combinations on the lifetime productivity indices of the Soviet Merino sheep breed have shown that substitutions located in noncoding areas have an impact on a number of parameters. At the same time, the presence of the SNP c.-81 genotype located in the exon area, previously described as a g.271C>T (18) and recommended as a genetic marker (16), does not affect the animals' body size. This may be due to the fact that the substitution is synonymous and not accompanied by a change in the amino acid sequence of the protein produced. Due to the fact that, in a sample with different quantities of live weight (and only in the heterozygous form), the substitution is common in three-quarters of the animals, it cannot be used as a productivity marker.

In our opinion, a complex of SNP c.-5363C>T, c.-5188G>C, c.-5186G>A and c.-4088G>A, could be considered as markers of increased meat production in the investigated sheep breeds. There are several reasons for this. Firstly, it is found in 2.5 times fewer cases than the wild homozygous genotypes; secondly, it revealed a significant correlation with the live weight of animals, which

Table 1: The frequency of *IGF1* gene polymorphic alleles in Soviet Merino sheep breed

	Name of SNP in HGVS nomenclature	RefSNP(rs) number	Genomic location	Biotype of SNP	Allele		Genotype		
1	c.-5412A>G	rs423903256	171328624	Upstream gene variant	T	C	TT	TC	CC
					0.83	0.17	0.8	0.07	0.13
2	c.-5363C>T	rs412597723	171328575	Upstream gene variant	G	A	GG	GA	AA
					0.9	0.1	0.8	0.2	0.00
3	c.-5188G>C	rs401028781	171328400	5'UTR variant	C	G	CC	CG	GG
					0.87	0.13	0.73	0.27	0.00
4	c.-5186G>A	rs422604851	171328398	5'UTR variant	C	T	CC	CT	TT
					0.87	0.13	0.73	0.27	0.00
5	c.-4088G>A	rs400113576	171327300	Intron variant	C	T	CC	CT	TT
					0.9	0.1	0.8	0.2	0.00
6	c.-4032G>A	rs421570650	171327244	Intron variant	C	T	CC	CT	TT
					0.97	0.03	0.93	0.07	0.00
7	c.-91A>C	rs430457475	171323303	Intron variant	T	G	TT	TG	GG
					0.7	0.30	0.47	0.47	0.06
8	c.81T>C	rs159876393	171323132	Synonymous variant	A	G	AA	AG	GG
					0.5	0.5	0.20	0.60	0.20
9	c.151+199G>A	rs402729264	171322863	Intron variant	C	T	CC	CT	TT
					0.7	0.3	0.40	0.60	0.00
10	c.151+463A>C	rs422974179	171322599	Intron variant	T	G	TT	TG	GG
					0.93	0.07	0.87	0.13	0.00
11	c.152-159G>A	rs424410885	171270326	Intron variant	C	T	CC	CT	TT
					0.80	0.20	0.67	0.27	0.06
12	c.152-47C>A	rs413216906	171270214	Intron variant	G	T	GG	GT	TT
					0.80	0.20	0.67	0.27	0.06
13	c.333+7C>T	rs419007446	171269979	Intron variant	G	A	GG	GA	AA
					0.83	0.17	0.73	0.20	0.07
14	c.333+88T>C	rs400681017	171269898	Intron variant	A	G	AA	AG	GG
					0.83	0.17	0.73	0.20	0.07
15	c.333+164C>T	rs406373781	171269822	Intron variant	G	A	GG	GA	AA
					0.87	0.13	0.80	0.13	0.07
16	c.333+259T>A	rs427977916	171269727	Intron variant	A	T	AA	AT	TT
					0.87	0.13	0.73	0.27	0.00
17	c.333+435A>G	rs420696231	171269551	Intron variant	T	C	TT	TC	CC
					0.83	0.17	0.73	0.20	0.07
18	c.*72C>G	rs159876382	171254333	UTR variant 3 prime	G	C	GG	GC	CC
					0.97	0.03	0.93	0.07	0.00

Table 2: Body measurements of rams with different *IGF1* genotypes (n represents number of animals; + represents wild type allele; M represents mutant allele; significantly different from wild type homozygotes if p<0.05)

Trait	Genotype						
	c.-5363, c.-5188, c.-5186, c.-4088			c.-91			
	+/+, (M±m, n=11)	+/M, (M±m, n=4)	p value	+/+, (M±m, n=7)	+/M, (M±m, n=7)	P value	M/M, (n=1)
Live weight (kg)	**51,18±0,96**	**55,83±0,27**	**0,001**	**49,70±0,98**	**52,51±0,23**	**0,04**	50,3
Height at wither (cm)	70,67±0,96	71,33±0,82	0,57	**69,50±1,07**	**72,43±0,51**	**0,02**	73
Height at croup (cm)	73,25±0,83	72,33±1,47	0,56	**71,88±0,87**	**74,41±0,54**	**0,02**	75
Width at croup (cm)	16,92±0,42	17,67±1,08	0,50	**16,13±0,32**	**17,57±0,47**	**0,03**	17
Length of croup (cm)	21,00±0,36	20,67±0,41	0,51	**20,12±0,30**	**21,45±0,52**	**0,04**	21
Carcass length (cm)	79,33±1,26	81,00±0,71	0,23	79,25±1,24	79,57±1,63	0,87	76
Chest width (cm)	23,83±0,46	24,33±1,47	0,73	24,13±0,71	23,71±0,51	0,62	24
Chest depth (cm)	30,42±0,58	31,00±1,22	0,64	30,88±0,79	30,14±0,64	0,46	31
Chest girth (cm)	99,08±1,12	99,00±2,83	0,98	98,63±1,56	99,57±0,74	0,57	99
Metacarpal girth (cm)	10,67±0,23	12,67±2,68	0,46	10,75±0,34	11,44±1,02	0,52	11
Metacarpal length (cm)	17,08±0,33	16,33±0,82	0,38	16,71±0,44	17,14±0,44	0,51	18
Metatarsus length (cm)	18,42±0,33	18,00±0,71	0,57	18,38±0,40	18,29±0,45	0,88	19
Loin width (cm)	13,42±0,24	14,00±0,71	0,42	13,36±0,35	13,71±0,31	0,45	13
Width of back (cm)	24,17±0,36	23,33±2,04	0,67	**23,71±0,39**	**24,75±0,27**	**0,03**	25
Half girth of back (cm)	78,33±1,25	73,33±7,12	0,48	**76,86±1,40**	**80,50±0,93**	**0,04**	83

represents an integrative measure of productive qualities; thirdly, the substitutions in positions c.-5188, c.-5186 (previously described in the article as g.855G>C), g.857G>A (16), C1511G, A1513G (17), g.179T>C and g.181G>C (23) have been proposed as genetic markers. In this regard, it is desirable that the directed mating of heterozygous animals with these substitutions be conducted in order to produce homozygous mutant allele carriers, which may have more pronounced upward deviations in weight. The absence of changes in the external measurement of the investigated animals with heterozygous genotypes does not form a valid basis for conclusions concerning the low markers of the investigated SNP complexes. There is a strong possibility that a more detailed investigation of such indicators in slaughtered animals could reveal a significant shift in the ratio of muscle mass, bone basis weight and internal organs.

The most important factor in the evaluation of the productive qualities of Soviet Merino breed of sheep is *IGF1* gene polymorphism at position c.-91. Heterozygous individuals that make up half of the samples are characterized not only by higher live weights, but also by an increase in the external body dimensions. Moreover, an increase was observed in parameters such as height, croup and back size, the last two of which are entirely dependent on the development of the muscles of the animals. From

this, it can be concluded that when a heterozygous variant of SNP c.-91A>C is detected in a lamb, it is possible to predict its parameters of superior meat production with a high degree of certainty. An examination of a single individual in a sample with homozygous mutant genotypes indicates that the use of this marker for breeding of animal lines with similar genotypes will eliminate wild homozygotes and increase productive performance across the whole breed.

Conclusion

The study indicates high variability of noncoding regions of the *IGF1* gene in sheep. A complex of four SNP c.-5363C>T, c.-5188G>C, c.-5186G>A and c.-4088G>A are presented together in two alleles, correlating with increasing live weight in heterozygotes. Substitution c.81T>C in exons is synonymous and did not affect any parameters of meat productivity. SNP c.-91A>C had a positive effect on weight, height, croup parameters and other variables in counted rams. New markers for marker-assisted selection in *IGF1* gene alleles were found.

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POLIMORFIZMI GENA *IGF1* PRI RUSKIH PASMAH OVC IN NJIHOV VPLIV NA NEKATERE PROIZVODNE PARAMETRE MESA

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Povzetek: Inzulinu podoben rastni faktor 1 (*IGF-1*) ima pomembno vlogo pri rasti in razvoju mišičnega tkiva pri živalih. Raziskave strukture gena IGF1 pri ovcah lahko zagotovijo pomembne nove podatke za selekcijo za povečanje prireje mesa na osnovi genetskih označevalcev. Proučevali smo strukturo gena *IGF1* in vpliv polimorfizmov na prirejo mesa pri ovcah rusko-sovjetske merino pasme. S tehnologijo NimbleGen za določanje zaporedja baznih parov smo analizirali alaele pri 15 ovnih. Našli smo 18 mononukleotidnih polimorfizmov (SNP), od katerih je bil samo en SNP - c-81T> C v kodirajočem področju. Vsi drugi SNP-ji so bili v intronih, 5'-UTR in 3'-UTR. Polimorfizmi c.-5363C> T, c-5188G> C, c-5186G> A in c.-4088G> A, ki so skupaj v dveh alelih, so bili v sorazmerju z višjo živo težo živali, če so bile te heterozigotne za ta alel. Sinonimna zamenjava c.81T> C v eksonu gena ni vplivala na analizirane parametre prireje mesa. Eden izmed odkritih SNP-jev – c.-91A> C – je bil pozitivno povezan s težo, višino, parametri v križu in drugimi latnostmi ovnov.

Ključne besede: ovce; gen *IGF1*; meso; SNP; izbor

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Original Scientific Article

POST-MORTEM DECREASE IN TEMPERATURE IN THE ORBIT OF DOGS FOR USE IN DETERMINING TIME OF DEATH

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Summary: Determination of time of death is a complex process taking into account numerous biological and environmental factors. These have to do with the changes taking place in the body immediately after death, mainly rigor mortis, lividity and the decrease in body temperature with the passage of time in specific ambient temperature and humidity conditions. Until recently body temperature was measured only in the rectum because the mechanisms of heat loss had been precisely established. Currently body temperature is measured in other tissues as well, including the soft tissues of the orbit.

The aim of this study was to evaluate the suitability of post-mortem measurement of the decrease in temperature in the orbit for determining the time of death of an animal (a dog) while taking into account the dynamics of changes in temperature measured in the rectum.

The carcasses of twenty dogs were examined. The temperature in the orbit and rectum was measured every half hour for 12 hours from the time of death. The body mass of the dog was found to affect the rate of the decrease in temperature in the orbital soft tissues. Because the dynamics of changes (decrease) in temperature in the orbit and rectum were uniform, temperature measurement at this site may be a valuable alternative method for determining time of death. Slight changes in ambient temperature and humidity did not affect the rate of cooling of the body.

Keywords: time of death; cooling of the body; temperature in the orbit; rectal temperature

Introduction

Precise determination of the time passed since the moment of death in humans or animals constitutes fundamental information allowing investigators to narrow a field of suspects and verify their alibis. Estimating the time of death as accurately as possible is the task of the expert physician examining the body. There are several more or less accurate methods for determining time of death, the most common of which is evaluation of the dynamics of post-mortem changes, particularly changes in rectal temperature. However, these analyses become less precise as time passes after the moment of death. For this reason attempts are made to develop a new, objective method enabling more precise determination of the time of death of an animal in the initial period after its death. This problem led the authors to attempt to use temperature measurement in forensic veterinary practice.

Due to the large number of cases in which animals are victims at the site of a crime, it is increasingly often necessary to determine the time of their death (1,2). When the time of death of an

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animal cannot be definitively determined on the basis of medical history, it is necessary to observe changes in the parameters of signs of death and to determine their dynamics. In forensic veterinary practice time of death is determined using methods based on evaluation of postmortem changes and measurements of the internal temperature of the animals. The measurements are most often made in the rectum, and this is currently one of the most objective methods for determining time of death (3,4,5).

Analysis of the available literature shows that only a few studies provide information concerning the practical use of the orbits as sites for measuring temperature with the purpose of determining time of death (6,7). For this reason we have chosen to attempt to develop a new method for use in forensic veterinary medicine enabling precise determination of time of death in the initial period after the death of an animal.

Attempts to determine time of death in humans on the basis of changes in body temperature date back to the mid-19th century, but the greatest progress was made at the end of the 1980s, when Henssge and colleagues developed nomograms making it possible to read off the time passed since the death of the individual. The proposed method takes into account the rectal temperature, the ambient temperature and the weight of the body (8, 9). Currently the achievements of Henssge and other researchers are exploited by computer programs, which has made it considerably easier to determine the moment of death (10,11).

Examinations were initially carried out by measuring the temperature in the rectum, but currently measurements made in other organs are used as well, such as the liver, the brain, and the vitreous humour of the eye (12).

The aim of the study was to evaluate the suitability of post-mortem testing of the decrease in temperature in the orbital soft tissues in comparison with rectal temperature in dogs in conditions of relatively constant air temperature and humidity.

Material and methods

The carcasses of twenty dogs aged 7 to 16 years were examined. The body mass of the dogs ranged from 4.5 to 48 kg. The animals were divided into two weight groups. The first group consisted of eleven small dogs (with body mass up to 12 kg) and the second consisted of nine large dogs (with body mass over 12 kg). The animals had been euthanized due to advanced age-related health problems or generalized cancer. All animals used in the study did not have damaged integument and were covered by short-hair coat. Only cases in which the time of death could be precisely and unquestionably determined on the basis of medical history were included in the study. The dogs' owners consented to the use of the carcasses as research material.

The carcasses were stored in a room in which the temperature, humidity and air flow were continuously measured. The results were recorded every 10 min using an anemometer (Airflow TA-440A). The physical parameters of the air, which were constant over the entire study period, were as follows: temperature 18°C, relative humidity 65% and mean air flow 0.1 m/s.

The temperature in the orbit and rectum was measured every half hour for 12 hours from the time of death. A needle probe was inserted into the orbital soft tissue in the vicinity of the medial canthus, moving along the medial rectus muscle towards the superior orbital fissure to a depth of 25 mm. A measuring probe was inserted into the rectum to a depth of 40 mm. The first measurement of internal temperature was made when the animal was euthanized. Temperature was measured with a TERMIO-25P electronic thermometer with accuracy of ± 0.01°C in conjunction with a 4 mm x 120 mm ST-02 temperature probe (Termoprodukt, Poland).

The data were analyzed with the use of statistical package SPSS 20.0PL (13). The t-test for independent variables and one-way ANOVA with Duncan's post-hoc test was carried out.

Results

The mean temperature measured in the orbit and the rectum in each time interval in the small and large dogs (Fig. 1) shows a gradual decrease over time. At the time of euthanasia the mean orbit temperature in the dogs was 38.34°C and the mean rectal temperature was 38.47°C. Statistical differences caused by body mass of dogs were not statistically confirmed.

The results of the measurements in the rectum and orbit show that the mean temperature in the large dogs was higher than in the small dogs in

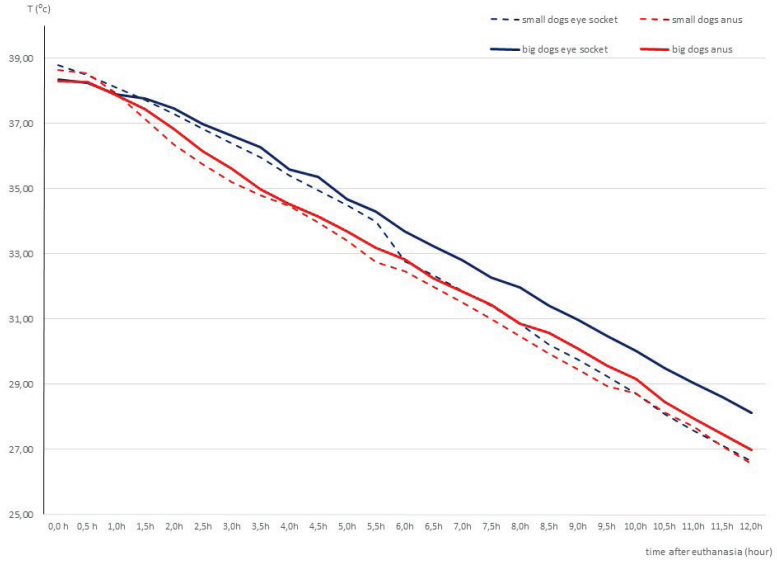

Figure 1: Mean rectal and eye socket temperature in the groups of small and large dogs

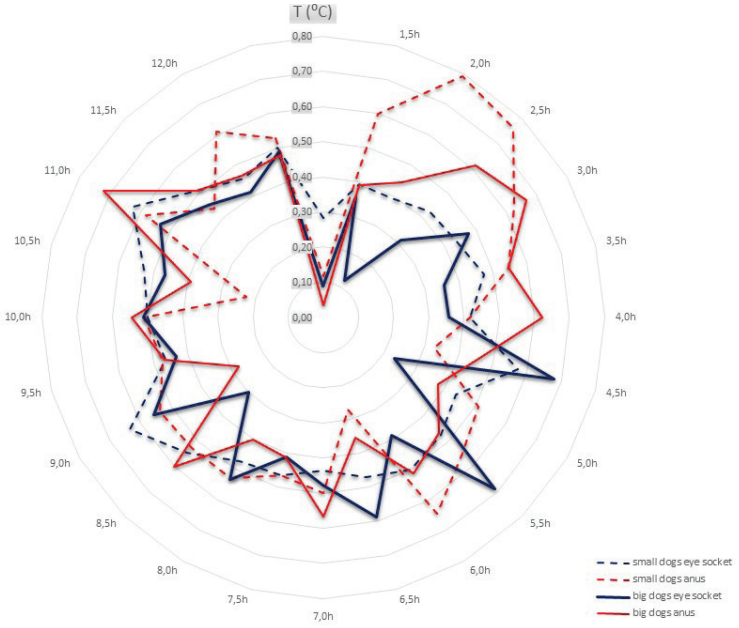

Figure 2: Difference in rectal and eye socket temperatures in the groups of small and large dogs at 30-minute intervals

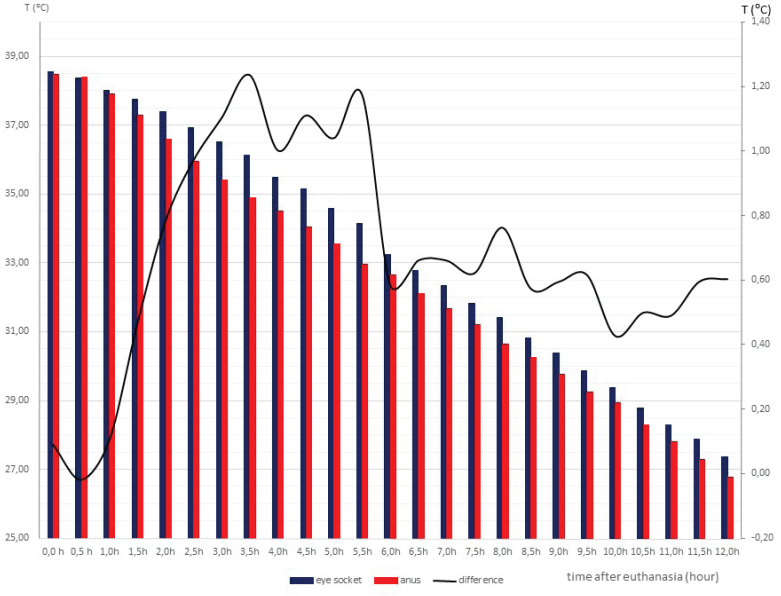

Figure 3: Mean temperature and differences in temperature between the eye socket and rectum

each time interval. At the same time the decrease in temperature in the orbit was slower than in the rectum, irrespective of the size of the dog (Fig. 1). The dynamics of temperature changes were more uniform in the orbit than in the rectum. Additionally, between 2nd and 4th hour after euthanasia in big dogs' group statistically sigificant difference was stated between mean value of temperature in eye and anus.

A comparative analysis was also made between the mean differences in temperature in the orbit Te and in the rectum Ta in the small and large dogs (Fig. 2). Greater temperature amplitude was noted for the rectum in the small and large dogs. The greatest difference in temperature, about 1.0°C, was noted in the rectum of small dogs between the first and third hour after death.

The dynamics of the decrease in temperature in the orbit and rectum were also analysed for both mass groups combined in the time intervals studied (Fig. 3). In the first two hours the difference in temperature between the orbit and the rectum did not exceed 1.0°C. Between 3 and 6 hours after death the difference in temperature between the two sites was highest, exceeding 1.2°C.

Discussion

The dynamics of the temperature changes in the orbit were more uniform than in the rectum, and the external atmospheric conditions had no effect on these changes. The results confirm the validity of using orbital soft tissues as a site for measuring temperature in the early period after death. A significant factor in support of this method is the lack of relationship between the rate of cooling in the orbit and body mass, as in the case of standard methods.

A similar conclusion was reached by Kaliszan & Hauser (6), who carried out research using the eyeball and orbital soft tissues of pig carcasses. They showed that the temperature of the eyeball decreases much faster than in the rectum, and observed no plateau effect (a stage in which the decrease in temperature is delayed in the initial post-mortem cooling period), which significantly distorted estimates based on measurements of rectal temperature. According to the authors, an additional argument in favour of using eyeballs and orbital soft tissues as sites for measuring the decrease in temperature in order to determine approximate time of death is the anatomical structure of these sites and the homogeneity of their localization between individuals on the cooling process. They also observed that low air movement (about 2 on the Beaufort scale) in the room where the measurements were made had no significant effect on the rate of cooling of the body.

In subsequent years Kaliszan (7) conducted a study enabling the use of a formula for estimation of the time of death based on temperature measurements in the human eyeball, developed on the basis of earlier comprehensive research on pigs. The possibility of using this method and its reliability can be explained by the similar anatomical structure and location of these organs in humans and pigs, and therefore presumably similar thermodynamic properties in these mammalian species. The author presented three cases in which measurements were made of the internal temperature of the eyeball shortly after death, at the site of the incident, and in this manner the time of death was precisely determined. The estimated time of death was confirmed during a police investigation.

The results obtained by Kaliszan (7) show that the method of determining time of death on the basis of post-mortem temperature measurements in the eyeball is sufficiently accurate in the early period after death, particularly when the body is situated in a relatively constant room temperature and in optimal atmospheric conditions (normal humidity and low air movement). According to the author, an additional argument in favour of the use of this means of temperature measurement is the way in which special touch probes are placed on the surface of the eyeball. This makes it possible to avoid the risk of damage to the rectum, particularly in cases of sexual assault. He also observed that the results of research carried out in pigs may enable more precise determination of the rate of cooling specific to the human eyeball, which could make the method even more precise.

Proctor et al. (14) also conducted a study using temperature measurements of dog carcasses to determine time of death. The analyses were carried out using the liver, brain, ear canal and rectum as sites for measuring the decrease in temperature. The study was conducted in a room in which the temperature was close to room temperature and the air movement was barely perceptible. They observed that sex and coat thickness had no effect on the rate of decrease in body temperature, but greater body weight and volume slowed down

the process. They were unable to definitively determine which of the measurement sites was most reliable in dead dogs on the basis of their study, but Al-Alousi et al. (15), after observing temperature changes in human organs, suggested that the most objective measurement site is the brain, followed by the liver, rectum and ear canal, in that order. According to Marcinkowski (16), rectal temperature decreases by 1°C per hour for 6 – 9 hours, but the plateau effect, when the decrease is very small, should be considered. This state continues until about 3 hours after death. In the following hours the decrease in temperature is slower and less regular than in the initial period (6, 7, 17). Moreover, in the last three decades veterinary forensic medicine has advanced and many studies have been conducted on dogs, pigs and deer (15, 18, 19, 20).

Introducing post-mortem measurement of the temperature of orbital soft tissues to forensic veterinary practice for the purpose of determining time of death in the initial period may lead to more precise estimates.

In conclusion, the decrease in temperature in the orbital soft tissues and rectum was uniform, so it enabling more precise determination of time of death. The body mass of the dogs affected the rate of cooling in the rectum and the orbital tissues. Measurements of the temperature of the orbital soft tissues may become a valuable method for determining time of death in dogs, and the use of this site is justified up to about 12 hours after death. The research should be continued in order to develop a mathematical model enabling determination of the time passed from the death of the animal to the discovery of the carcass. Because the dynamics of changes (decrease) in temperature in the orbit and rectum were uniform, temperature measurement at this site may be a valuable alternative method for determining time of death. Slight changes in ambient temperature and humidity did not affect the rate of cooling of the body.

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ZMANJŠANJE TEMPERATURE V OČNICI PSOV PO SMRTI KOT MOŽNOST ZA DOLOČANJE ČASA SMRTI

P. Listos, M. Gryzinska, J. Batkowska

Povzetek: Določanje časa smrti je kompleksen proces, pri katerem je potrebno upoštevati številne biološke in okoljske dejavnike. Ti vplivajo na spremembe, ki se dogajajo v telesu takoj po smrti, predvsem na mrtvaško otrplost, modrikavost in znižanje telesne temperature, ki so vse pogojene s časom ter pogoji okolja, kot so temperatura in vlažnost. Do nedavnega se je telesna temperatura merila le v danki, kjer je mehanizem toplotnih izgub natančno določen. Danes se telesna temperatura meri tudi v drugih tkivih, vključno z mehkimi tkivi očnice.

Cilj te raziskave je bil oceniti ustreznost merjenja znižanja temperature v očnici po smrti z namenom, da se določi čas smrti živali (pes), pri čemer se upošteva dinamika sprememb temperature, izmerjene v danki.

Pregledana so bila trupla dvajsetih psov. Temperatura v očnici in danki je bila izmerjena vsake pol ure do 12 ur po smrti. Ugotovljeno je bilo, da telesna masa psov vpliva na stopnjo znižanja temperature mehkih tkiv očnice. Ker so spremembe (znižanje) temperature v očnici in danki enake, je lahko merjenje temperature na tem mestu dragocena dodatna metoda za določanje časa smrti. Majhne spremembe v temperaturi okolja in vlažnosti niso vplivale na stopnjo hlajenja telesa.

Ključne besede: čas smrti; ohlajanje telesa; temperatura v očnici; rektalna temperatura

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Original Scientific Article

FREQUENCY OF YEASTS AND FILAMENTOUS FUNGI IN THE EXTERNAL EAR CANALS OF CATTLE IN IRAN

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Summary: Fungal microbiotas are saprophytic microorganisms that can act as opportunistic pathogens in animals. This study was carried out in order to isolate and identify the ear fungal biota from healthy cattle. The samples were taken using premoistened swabs from the right and/or left external ear canals of 32 healthy cattle and cultured onto Sabouraud glucose agar and modified Dixon's agar media. A total of eight different fungal genera were isolated from 29 (90.6%) of 32 healthy cattle. Both filamentous fungi and yeasts were isolated with the predominance of *Aspergillus* spp. (35.6%), *Candida* spp. (18.9%) and *Malassezia* spp. (16.8%). The most frequent *Aspergillus* spp. were A. *fumigatus* (16.8%), A. *glaucus* (14.9%) and A. *flavus* (4%). Among the fungal isolates, 46.5% and 17.8% colonies were associated with hyaline and dematiaceous fungi, respectively (p = 0.003). The recognized fungi, especially *Aspergillus* spp. and *Candida* spp., were colonized as saprophytic fungal contaminants in the external ear canals of healthy cattle.

Keywords: cattle; external ear canal; mycobiota; *Aspergillus*; *Candida*

Introduction

Fungi are part of the normal microbial biota in the external ear canals of animals (1,2). The normal microbiota could benefit the host by avoiding excessive growth of pathogenic microorganisms through a competitive process. Factors associated with the increase of fungal population in the ear canals of animals include increased humidity, long and pendant ears, the presence of hair in the ear, immune system deficiency, poor nutrition and abnormal hormonal status (3,4). The most common causes of hearing impairment in animals are otitis media, inflammatory processes or infections resulting in accumulation of fluid in the middle ear, which interferes with the tympanic vibrations (5).

Yeasts and filamentous fungi are frequently associated with otitis externa in human (6) and different animals such as horse (7), camel (8), sheep (9), dog and cat (10), rabbit (11), monkey (12) and elephant (13). The study of external otitis in the cattle is almost exclusively limited to parasitic otitis (14). However, large animals like cattle can

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be seen for non-parasitic external otitis (allergic, fungal, or bacterial). Without cytological data on external ear canals, it is almost impossible to study such conditions. Cytological examination of external ear canals is the key point of external otitis diagnosis and treatment. In different animals, the results of cytological examination of ear swab are used to choose the proper topical treatment, based on the presence and concentration of bacteria, fungi and inflammatory cells. It is a simple, practical and inexpensive diagnostic test, which gives immediate results. Interpretation of such a test is impossible without any data in healthy animals. There is little data regarding to the cytological examination of the external ear canals of healthy cattle. Many studies have used samples collected from only one ear per animal (15), others have used samples collected from one or two ears and considered them as different samples (16).

Despite many the published information on bacterial biota of ear canals, there is still an extraordinary lack of specific well-organized, comprehensive information on ear mycobiota in cattle. The aim of this study was to isolate and identify the fungal biota from the external ear canals of healthy cattle.

Materials and methods

Animals

A total of 32 healthy cattle were selected in this study from different locations in Tehran province, Iran. Animals of any age, breed or sex were eligible for enrollment. Cattle were included in the normal group if there was no previous history of skin or ear disease and no history of underlying metabolic disease. Animals in the normal group were not currently on medications other than preventive parasitic or flea and tick treatments. In addition, cattle were free of clinical signs of skin or ear disorders, with no evidence of inflammation or infection on cytological analysis of ear specimens. Cattle were not included if any ototopical medications or flushes were used in the previous two weeks or if the animal was administered any systemic antifungal medication in the previous four weeks. In this study, all conducted experiments on cattle were in accordance with the guidance of ethical committee for research on animals of University of Tehran, Iran.

Clinical examination and sample collection

Complete physical and dermatological examinations were performed prior to collection of ear samples in each cattle. Each animal had a fungal culture obtained from the right and/or left external ear canals at the furthest accessible level of the auditory canal. Collection of samples was performed by passing a sterile culture swab into the ear canal. Samples were transferred overnight to the Mycology Center, Amol University of Special Modern Technologies, Amol, Iran according to the submission protocol.

Laboratory methods

Direct microscopic examination was carried out on the samples mounted in 10% potassium hydroxide (KOH)/dimethyl sulfoxide (DMSO) (*Merck Co., Darmstadt*, *Germany*). In addition, each cotton swab was slowly rolled once onto a glass slide. The glass slides were air-dried for 10 min, fixed and stained with a modified Wright's stain kit (*RAL555, RAL Diagnotics France*) as prescribed by manufacturer. Using this staining technique, fungal elements were stained in blue (17).

For initial fungal cultures, the samples were inoculated onto Sabouraud glucose agar (*Merck Co., Darmstadt*, *Germany*) supplemented with chloramphenicol (0.005%), Mycosel agar (*Merck Co., Darmstadt*, *Germany*) and modified Dixon's agar for identification of saprophytes, dermatophytes and *Malassezia* spp., respectively. The cultures were incubated at both 25°C and 32°C and examined daily for two to four weeks. Homogenized mixtures were prepared from the hairs, which had been collected by pincetle and inoculated onto the media as well. Saprophytic colonies were inoculated onto Malt extract agar (*Merck Co., Darmstadt, Germany*), Czapek-dox agar (*Merck Co., Darmstadt, Germany*), Potato dextrose agar (*Merck Co., Darmstadt, Germany*) and Cornmeal agar containing Tween-80 (*Sigma Chemical Co., St Louis, MO, USA*) media for identification at the genus level (18). Subsequently, fungal genera were identified based on micro- and macromorphology, reverse and surface coloration and size of colonies grown on the above-mentioned media (19,20).

Candida spp. were also identified by germ tube production, micromorphology and chlamydospore production on Tween 80-corn meal agar and by Rap ID™ yeast identification system (*Remel, USA*) (21). The identification of *Malassezia* yeasts was based on the ability to use certain polyoxyethylene sorbitan esters (Tweens 20, 40, 60 and 80), catalase reaction, cremophor EL assimilation test, splitting of esculin and precipitate production on modified Dixon agar (22).

Real-time PCR assay for identification of Aspergillus species

Spore suspensions from 7-day cultures on Czapek-dox media were inoculated into 15 ml of yeast peptone dextrose broth (*Merck Co., Darmstadt, Germany*) media and incubated at 32°C for 48 to 72 h. Then, fungal DNA was extracted and purified by MagNA Pure LC DNA I isolation kit (*Roche, Mannheim, Germany*). The preparation and settings of the instrument were according to the manufacturer's instructions.

The LightCycler system (*Roche, Mannheim, Germany*) was used for amplification of *Aspergillus* DNA. LightCycler hot-start PCR was performed in glass capillaries with a LightCycler Fast Start DNA Master Hybridization Probes kit (*Roche, Mannheim, Germany*) as specified by the manufacturer. The primers and hybridization probes for *Aspergillus* species were those described by Loeffler *et al.* (23). The PCR master mix (10 µl) contained 1× Fast Start reaction mixture with Fast Start Taq DNA polymerase, reaction buffer, dNTPs, 1.6 µl of 25 mM $MgCl_2$, 1 µl of each primer (3 µM), and 1 µl (2 µM) of each hybridization probe. PCR was performed in a final volume of 20 µl (10 µl of master mix + 10 µl of DNA extract) with 10 min at 95°C, followed by 50 cycles of 15 s at 95°C, 10 s at 58°C and 20 s at 72°C, with a temperature transition rate (TTR) of 20°C / s. The PCR was followed by a melting temperature analysis cycle comprising 95°C for 10 s (TTR of 20°C/s), 50°C for 60 s (TTR of 20°C/s) and 75°C for 0 s (TTR of 0.1°C/s) to check the specificity of the PCR product. DNA extracts from the samples were analyzed in parallel with an extraction control and a PCR control containing fungal DNA.

Statistical analysis

Student's t-test was used to compare the differences among different fungi using SPSS software (Version 15). A *P*-value less than 0.05 was considered to be statistically significant.

Results

Of 32 examined cattle, different fungal genera were recovered from 29 healthy animals (90.6%), whereas three cattle (9.4%) did not have positive cultures. A total of 101 fungal colonies were isolated from animals, 53 colonies from right ears and 48 colonies from left ears. There was no statistically significant difference between right and left ears. The following fungal genera (no. 8) were recovered: *Aspergillus* spp. (35.6% of the total examined cattle), *Candida* spp. (18.9%), *Malassezia* spp. (16.8%), *Cladosporium* spp. (10.9%), *Mucor* spp. (9.9%), *Alternaria* spp. (5.9%), *Ulocladium* spp. (1%) and *Fusarium* spp. (1%) (Table 1). There were statistically significant differences between *Aspergillus* spp. and *Malassezia* spp. ($p = 0.007$), *Cladosporium* spp. ($p = 0.000$), *Mucor* spp. ($p = 0.000$), *Alternaria* spp. ($p = 0.000$), *Ulocladium* spp. ($p = 0.000$) and *Fusarium* spp. ($p = 0.000$). In addition, there were statistically significant differences between *Ulocladium* spp. and *Fusarium* spp. and *Candida* spp. ($p = 0.000$), *Cladosporium* spp. ($p = 0.003$), *Mucor* spp. ($p = 0.008$) and *Alternaria* spp. ($p = 0.025$).

The most frequent *Aspergillus* species was *A. fumigatus* (16.8%), followed by *A. glaucus* (14.9%) and *A. flavus* (4%). There were no statistically significant differences among various *Aspergillus* species.

Of 65 filamentous fungi detected, 47 (46.5%) and 18 (17.8%) colonies were associated with hyaline and dematiaceous fungi, respectively (Table 1). There was statistically significant difference between hyaline and dematiaceous fungi ($p = 0.003$). As shown in Table 1, the frequency of filamentous fungi (64.4%) was higher than yeasts (35.6%). There was no statistically significant difference between filamentous fungi and yeasts ($p = 0.125$). Of 29 positive samples from healthy cattle, 16.8% yielded positive yeast cultures. The frequency of *Malassezia* spp. (16.8%) was lower than *Candida* spp. (18.8%). There was no statistically significant difference between *Malassezia* spp. and *Candida* spp.

Table 1: Frequency of different fungi isolated from the external ear canals of healthy cattle

Cattle no.	Mycobiota of external ear canal								Total
	Filamentous fungi						yeasts		
	Hyaline (no., %)			Dematiaceous (no., %)			*Candida* (no., %)	*Malassezia* (no., %)	
	Aspergillus	*Mucor*	*Fusarium*	*Cladosporium*	*Alternaria*	*Ulocladium*			
1	2 (1.9)	0 (0)	0 (0)	0 (0)	1 (1)	0 (0)	0 (0)	1 (1)	4 (4)
2	1 (1)	1 (1)	0 (0)	1 (1)	0 (0)	0 (0)	1 (1)	1 (1)	5 (5)
3	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
4	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	1 (1)	2 (1.9)
5	2 (1.9)	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	4 (4)
6	1 (1)	0 (0)	0 (0)	1 (1)	0 (0)	0 (0)	0 (0)	1 (1)	3 (3)
7	0 (0)	2 (1.9)	0 (0)	0 (0)	1 (1)	0 (0)	1 (1)	1 (1)	5 (5)
8	3 (2.8)	0 (0)	0 (0)	2 (1.9)	0 (0)	0 (0)	0 (0)	1 (1)	6 (5.9)
9	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	0 (0)	2 (1.9)
10	2 (1.9)	0 (0)	0 (0)	0 (0)	1 (1)	0 (0)	1 (1)	0 (0)	4 (4)
11	1 (1)	0 (0)	1 (1)	1 (1)	0 (0)	0 (0)	0 (0)	1 (1)	4 (4)
12	1 (1)	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	0 (0)	3 (3)
13	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
14	3 (2.8)	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	5 (5)
15	1 (1)	0 (0)	0 (0)	0 (0)	1 (1)	0 (0)	1 (1)	1 (1)	4 (4)
16	2 (1.9)	0 (0)	0 (0)	1 (1)	0 (0)	0 (0)	1 (1)	0 (0)	4 (4)
17	0 (0)	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	2 (1.9)
18	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	0 (0)	1 (1)
19	1 (1)	0 (0)	0 (0)	1 (1)	0 (0)	0 (0)	1 (1)	0 (0)	3 (3)
20	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)
21	2 (1.9)	0 (0)	0 (0)	0 (0)	2 (1.9)	0 (0)	2 (1.9)	0 (0)	6 (5.9)
22	1 (1)	0 (0)	0 (0)	1 (1)	0 (0)	0 (0)	1 (1)	0 (0)	3 (3)
23	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	2 (1.9)
24	1 (1)	0 (0)	0 (0)	1 (1)	0 (0)	0 (0)	1 (1)	0 (0)	3 (3)
25	2 (1.9)	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	2 (1.9)	6 (5.9)
26	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	0 (0)	0 (0)	2 (1.9)
27	3 (2.8)	1 (1)	0 (0)	1 (1)	0 (0)	0 (0)	1 (1)	0 (0)	6 (5.9)
28	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	1 (1)
29	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	0 (0)	2 (1.9)
30	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
31	2 (1.9)	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	2 (1.9)	6 (5.9)
32	0 (0)	0 (0)	0 (0)	1 (1)	0 (0)	0 (0)	1 (1)	0 (0)	2 (1.9)
Total	**36 (35.6)**	**10 (9.9)**	**1 (1)**	**11 (10.9)**	**6 (5.9)**	**1 (1)**	**19 (18.9)**	**17 (16.8)**	**101 (100)**

Discussion

Domestic animals are often affected with external ear injuries followed by secondary infections. Little is known of the significance of resident microbiota in cattle ears and this knowledge would be very useful in assessing the accuracy of treatments. The mycological examination of the external ear canals of healthy cattle showed the presence of different fungi in 90.6% of the animals. The most common fungal isolate was *Aspergillus* spp. (35.6%), followed by *Candida* spp. (18.9%), *Malassezia* spp. (16.8%), *Cladosporium* spp. (10.9%), *Mucor* spp. (9.9%), *Alternaria* spp. (5.9%), *Ulocladium* spp. (1%) and *Fusarium* spp. (1%). In a study conducted by Duarte *et al.* (23), the most frequent fungus was *Malassezia* spp. (68.9%), followed by `Micelia sterilia' (17.8%), *Candida* spp. (15.5%), *Rhodotorula* spp. (11.1%) and *Aspergillus* spp. (4.4%). Saprophytic fungal organisms are ubiquitous in nature and are normal contaminants of body and mucosal surfaces; thus, it is not surprising that these organisms could be found transiently in the ear canals of cattle. Our results are also consistent with similar studies on saprophytic fungal contaminants from the external ear canals of other animals (8,12,17).

In this study, the most predominant *Aspergillus* species was *A. fumigatus* (16.8%), followed by *A. glaucus* (14.9%) and *A. flavus* (4%). In accordance with our results, previous studies indicated that different *Aspergillus* species were isolated from the external ear canals of various animals, especially cattle (7-9,24).

Of 65 filamentous fungi detected, 47 (46.5%) and 18 (17.8%) colonies were associated with hyaline and dematiaceous fungi, respectively (p = 0.003). There are no indications in literature for difference in the occurrence of hyaline and dematiaceous fungi from the ears of healthy cattle. From culture-positive samples, the occurrence of filamentous fungi (64.4%) was higher than yeasts (35.6%). According to our best knowledge, there are no previous reports concerning the saprophytic fungal contaminants, especially filamentous fungi, from the external ear canals of healthy cattle. Duarte *et al.* (25) showed that the positive cultures were 28% for filamentous fungi and 34.6% for yeasts in cattle. In this study, the frequency of filamentous fungi recovered from cattle ears were considerably greater than those reported in other studies (25,26). One possible reason for this difference may be related to the long incubation period of fungal cultures. In the present study, fungal cultures were monitored for four weeks, in contrast to other studies, in which fungal cultures were kept from two to 15 days.

In this study, the results were positive for yeasts of *Candida* spp. and *Malassezia* spp. in 18.9% and 16.8% of the samples from the external ear canals of cattle, respectively. In a previous study by Duarte *et al.* (24), the presence of seven yeasts of the genus *Candida* (15.5%) was confirmed in the ears of cattle. In addition, the frequency of *Malassezia* spp. was value near to that found by Gustafson study with positivity of 16% from the external ear canals of 50 healthy cattle (27). Duarte *et al.* (25) exhibited that 34.6% of healthy cattle were positive for *Malassezia* spp. In another study by Duarte *et al.* (28), 39.6% of isolates from healthy cattle were positive for *Malassezia* spp. They showed a relatively high frequency of *Malassezia* spp. in Holstein cattle, especially in the summer months, indicating a correlation between the open and air-exposed ears of this breed and *Malassezia* number. Pendulous-eared zebu breeds and hybrids had higher levels of colonization, although this effect was more pronounced in humid regions. Among other reports on *Malassezia* occurrence in healthy cattle, Dufait (29) obtained two positive cultures of *Malassezia* spp. from the ear of six sampled cattle (33.3%). In a study based on direct microscopic examination, the frequency of *Malassezia* species was found to be 29% in samples collected from the external ear canals of 55 healthy cattle (30). The significant frequency of *Malassezia* spp. in healthy cattle may indicate that it is a member of the normal microbiota of the ears in these animals (25).

The ear microenvironment consists of resident organisms that are believed to live and multiply on the skin and transient organisms that are acquired from the environment. Currently, it is believed that saprophytic fungi are transient contaminants by airborne fungi or fungi in soil. Saprophytic fungal organisms transiently found on the skin can take advantage of changes in microenvironment or host defenses to establish infection (31). It is unlikely that these fungal saprophytes could be the primary cause of otitis externa; however, they could complicate cases of prolonged bacterial otitis treated with topical

antibiotics and corticosteroids (32). Since the introduction of antibiotic eardrops containing corticosteroids, there has been an increasing prevalence of otomycosis most commonly due to *Aspergillus* spp. and *Candida* spp., which were isolated in our study. The pathogenesis of ear infection is not clear, although in calves extension of infection from the pharynx via the Eustachian tube is the most common means of entry into the middle ear (33). In cattle, exudate fills the cavity, increases pressure, ruptures the tympanic membrane and discharges into the external acoustic meatus (34).

In conclusion, this study demonstrated that filamentous fungi were more common than yeasts from the external ear canals of healthy cattle. Future studies may contribute to clarify the importance of their possible participation in the aetiology of otitis externa.

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POGOSTOST KVASOVK IN NITASTIH GLIV V ZUNANJEM UŠESNEM KANALU PRI GOVEDU V IRANU

H. Shokri

Povzetek: Glivično mikrobioto predstavljajo saprofitski mikroorganizmi, ki lahko delujejo kot oportunistični patogeni pri živalih. Raziskava je bila izvedena z namenom izolacije in identifikacije glivične mikrobiote pri zdravem govedu. Vzorci so bili vzeti s pomočjo vlažnih tamponov iz desnega in/ali levega zunanjega ušesnega kanala pri 32 zdravih govedih in kultivirani na glukoznem agarju po Sabouraudu ter modificiranem agarskem mediju po Dixonu. Izolirali smo skupno osem različnih glivičnih rodov iz 29 (90,6 %) od 32 zdravih goved. Med nitastimi glivami in kvasovkami, ki smo jih izolirali, so prevladovali *Aspergillus* spp. (35,6 %), *Candida* spp. (18,9 %) in *Malassezia* spp. (16,8 %). Najpogostejši prestavniki rodu *Aspergillus* spp. so bili *A. fumigatus* (16,8 %), *A. glaucus* (14,9 %) in *A. flavus* (4 %). Med glivičnimi izolati je bilo 46,5 % in 17,8 % kolonij povezanih s hialinskimi oziroma pigmentiranimi glivami (p = 0,003). Prepoznane glive, zlasti *Aspergillus* spp. in *Candida* spp., so kolonizirale kot saprofitski glivični onesnaževalci zunanje ušesne kanale zdravih govedi.

Ključne besede: govedo; zunanji sluhovod; mikrobiota; *Aspergillus*; *Candida*

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Case Report

METASTASIZING OVARIAN CARCINOMA IN AN EURASIAN BROWN BEAR (*Ursus arctos arctos*): A CASE REPORT

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Summary: A case of ovarian carcinoma, never previously reported in bear is described. A 37-year-old, nulliparous, female Eurasian brown bear hosted at the Falconara Parco Zoo in Italy, showed neurological clinical signs including bilateral blindness and signs of hemiparesis involving both limbs of the left side.
A therapy based on fluid, dexamethasone sodium phosphate, ranitidine, ceftriaxone, propentofylline, and a vitamin B complex administration was started after the onset of symptoms. After about a week of therapy the bear was able to stand up and walk, partially recovered the vision and ate regularly. Despite this initial improvement, three weeks after the clinical onset the bear died. At necropsy a large tumourous mass involving the left ovary and spread of tumour metastases to the regional lymph node and brain has been found. Based on the typical histological and immunohistochemical features of neoplastic cells, this tumor was diagnosed as papillary to solid serous type ovarian carcinoma. Because of the scattered distribution pattern of neoplastic nodules, the involvement of the brain and lumbo-aortic lymph node was considered to be metastatic. Only few reports of neoplasms in Ursidae can be found in scientific literature and these include lymphosarcoma, osteoma, osteosarcomas, chondrosarcoma, squamous cell, biliary, thyroid, mammary, and hepatocellular carcinomas. According to these results, the presence of tumor should be considered in bears with neurological signs.

Keywords: brain; brown bear; metastases; ovary; tumor

Case description

Only few reports of neoplasms in *Ursidae* can be found in scientific literature (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14) and none of this refer about ovarian tumor.

A 37-year-old nulliparous female Eurasian brown bear (*Ursus arctos arctos*) 87 Kg in weight, hosted at the Falconara Parco Zoo, in Italy, was referred because found in lateral recumbency.

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The bear was borne in the Zoo of Verona, Italy, and moved in the Falconara Parco Zoo shortly after bird. The area that housed the bear was made of concrete, divided into different rooms, and equipped with windows of armored glass. The floor was in concrete and enriched with straw, leaves and dead branches. The animal was feed once a day with fruits and vegetables and once a week beef or chicken meat was administered. No other bears were hosted in the zoo and no previous health problems were recorded. Deworming were performed once a year with ivermectine.

The general conditions allowed to approach the bear without a dangerous situation occurs. In effect, the bear did not react to the approach of the people but she tried some slight reaction (feeble attempts to bite), when was touched. The bear resulted to be slightly dehydrated, appeared bilaterally blind at evaluation of the "response to the threat" and had signs of hemiparesis involving both limbs of the left side. Anyway, deep and superficial sensitivities were preserved. Widespread tremors and nystagmus were also present. The bear appeared conscious and was able to eat. A venous sample of blood was collected from the jugular vein to analyse the hematologic parameters reported in table 1.

Table 1: Results of the hemato-biochemical analysis. Reference ranges are from Ramsay 2003 (15), except for leukocytes, white subcellular population (16) and GGT (17)

Parameter	Result	Reference range
Erythrocytes (106/µl)	3.89	4.17–8.93
Hematocrit (%)	22	36.8–62.7
Hemoglobin (g/dl)	8.7	11.7–59.4
MCV (fl)	56.3	44.1-93.0
MCH (pg)	18.3	15.4-33.5
MCHC (g/dl)	31.5	14.0-63.8
Leukocytes (103/ µl)	38.6	3.9–37.6
Unsegm. neutrophils (103/ µl)	1.0	<0.01-3.07
Segm. neutrophils (103/ µl)	31.7	2.55-23.7
Lymphocytes (103/ µl)	5.1	<0.01-12.4
Monocytes (103/ µl)	0.3	<0.01-1.59
Eosinophils (103/ µl)	0.5	<0.01-2.58
Basophils (103/ µl)	0	<0.01-0.14
Platelets (106/ µl)	399	134–719
Total protein (g/dl)	9.1	5.7–8.8
Albumin (g/dl)	3.3	2.7–5.4
Calcium (mg/dl)	8.9	6.6–11.5
Phosphorus (mg/dl)	5.0	3.2–9.1
Sodium (mEq/l)	133	123–150
Potassium (mEq/l)	4.8	3.6–6.1
Chloride (mEq/l)	100	94–112
Creatinine (mg/dl)	1.7	0.5–3.9
Urea nitrogen (mg/dl)	42	4–43
Cholesterol (mg/dl)	211	172–1030
Total bilirubin (mg/dl)	0.5	0.0–1.4
Direct bilirubin (mg/dl)	0.1	0.0–0.1
ALT (IU/l)	58	10–101
AST (IU/l)	158	25–203
ALP (IU/l)	111	4–210
GGT (IU/l)	432	16–176

Number of erythrocytes, haematocrit and haemoglobin resulted to be decreased causing a moderate status of anemia. The number of leukocytes was increased with most of them represented by segmented neutrophils. Values for total protein were slightly increased while GGT (gamma-glutamyl transpeptidase) resulted to have a fourfold increased activity.

Based on the clinical suspicious of central nervous system damage, a therapy including fluid administration (ringer lactate only the first day), dexamethasone sodium phosphate (4 mg/Kg, IM, BID the first day and 2 mg/Kg IM, SID in the following days), ranitidine (6 mg/Kg PO BID; Ranitidina TEVA®, Teva Pharma Italy), ceftriaxone (1.5 g, IM, SID; ceftriaxone hexal®, Hexal Spa Italy), propentofylline (3 mg/Kg, PO, BID; Karsivan®, Intervet Italia Srl), and a vitamin B complex PO, SID (Stimulfos®, Teknopharma S.p.a.) has been started. Drugs to be given parenterally, were administered by dart using a blow pipe; PO therapy was administer with food. After about a week of therapy the bear was able to stand up and walk, partially recovered the vision and ate regularly. Nevertheless, three weeks after the clinical onset, she went back in decubitus and died after 12 hours.

Necropsy revealed an irregular aspect of left ovary, and old clots were noted throughout the pelvis. The right ovary appeared normal. On gross examination, a 20×15×8 centimeter and 435 g in weight left ovarian mass was evident. Grossly, the external surface was intact and smooth, and the cut surface was gray to yellow, predominantly solid and lobulated, with a few small areas of cystic change and hemorrhage.

A 2.5 cm-sized nodule occupying the lombo-aortic lymph nodes was also observed. Additionally brain metastases were noted, characterized by multiple lesions, typically at grey white junction, profuse perilesional edema, and relatively smooth margin.

Intimal calcification of the aortic arch and scattered petechiae on the mucous membrane of the urinary bladder were also observed.

Tissue samples from tumors and all organs were fixed in 10% neutrally-buffered formalin (pH 7.4), processed for paraffin embedding, sectioned at 4 μm and stained with haematoxylin and eosin (H&E) and used also for immunohistochemical characterization. On the histological examination, the ovarian tumor was composed of epithelioid to spindle-shaped neoplastic cells that were arranged in closely packed hollow or solid tubules with a fibrous stroma, resulting in a lobular pattern. Diffuse solid areas, microcystic pattern with eosinophilic secretion, and focal characteristic sieve-like structure were also noticed (Fig. 1). The tumor cells showed mild nuclear atypia and frequent mitotic figures. Nuclear pleomorphism and tumor necrosis was also found. Histology of brain metastases revealed a sharp interface between brain parenchyma and metastatic tumor. The neoplasm had areas of follicular architecture and areas of solid nests (Fig. 1), with tumor nuclei that were round to oval in shape with prominent nucleoli and finely granular chromatin. The neoplastic cells had abundant eosinophilic cytoplasm and mild pleomorphism. Some features of papillary carcinoma were occasionally observed. The tumor was intermixed with areas of fibrosis with hemosiderin deposition and calcification. Focal necrosis and perivascular macrophages were also noted. The adjacent brain parenchyma contained hemosiderin deposits, vascular sclerosis, and reactive astrocytes. Finally, microscopy of neoplastic nodules in the lymph nodes showed a similar histological pattern, with neoplastic cells being arranged in diffusely proliferating sheet-like cellular nests. Neoplastic cells were separated by variable amounts of fibrous stroma, but they were invasive to the stroma and lymphatic vessels. In all these three sites of neoplastic development, the neoplastic cells were largely polygonal with round to oval-shaped nuclei and abundant eosinophilic cytoplasm and prominent nucleoli with indistinct cellular borders sometimes forming rosettes, papillae, and duct-like structures; mitotic figures were observed frequently. In the lombo-aortic lymph nodes, the lymphoid tissue was mostly replaced by neoplastic cells forming a large tumor mass. In the brain, a scattered distribution of neoplastic cell aggregation of various sizes was evident, as well as larger discrete nodules.

Sections from neoplastic tissues of the ovary, brain, and lombo-aortic lymph node were immunohistochemically stained by the avidin-biotin-peroxidase complex (ABC) procedure (Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, CA, U.S.A.) and examined microscopically. Details of the specific primary antibodies used and the staining results for the neoplastic tissues are summarized in Table 2.

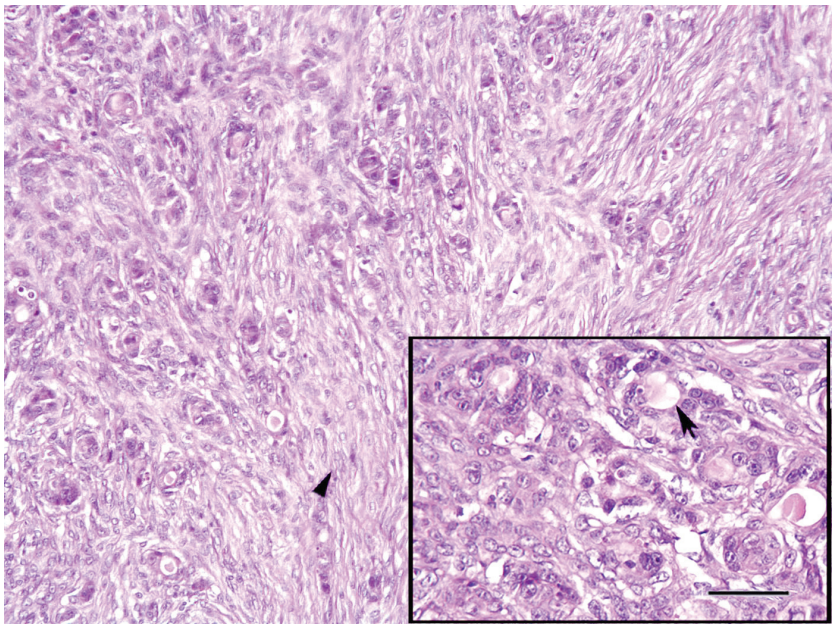

Figure 1: Low-power view of ovarian neoplastic tissue, characterized by epithelioid to spindle-shaped neoplastic cells that were arranged in closely packed hollow or solid tubules with a fibrous stroma (arrowhead); right lower insert: characteristic microcystic (sievelike) structure with eosinophilic secretion (arrow), interspersed in a diffuse solid area. (H&E, Bar = 250μm; insert = 100μm)

Table 2: Panel of antibodies utilized for tumour characterization

Primary antibody	Antibody clone	Dilution	Antigen retrieval	Results
Vimentin	V9	1:30	none	+/-
AE1/AE3	AE1 and AE3	1:50	HMAR	+++
EMA	E29	1:50	HMAR	+
CA 125	OC 125	1:25	HMAR	+
CEA	policlonal	1:400	HMAR	-
Calretinin [a)]	policlonal	1:100	HMAR	++
WT1 [b)]	policlonal	1:100	HMAR	+

[a)] made by ZYMED; [b)] made by LifeSpan Bioscience, Inc., [c)]HMAR = Heat Mediated Antigen Retrieval

Deparaffinized sections were blocked for endogenous peroxidase in 0.3% H_2O_2 with methanol for 30 min. Incubation of sections with the primary antibody was performed at 4°C for 16 h, followed by incubation with the biotinylated secondary antibody for 30 min, and with avidin peroxidase conjugate for 30 min at room temperature. Sections were developed in 0.05% 3,3'-diaminobenzidine/H_2O_2 solution. As positive control for each immunoreactivity, an ovary serous carcinoma, and a colonic carcinoma belonging to dog were employed. Additionally, a normal ovary and brain tissues from a brown bear (archive material) were also used. Samples from canine serous ovarian carcinoma were used for confirmation of the immunoreactivity for epithelial membrane antigen (EMA), Wilms Tumor 1 antigen (WT-1), and Cancer Antigen 125 (CA125). Neoplastic colonic tissue was used for confirmation of the immunoreactivity for Carcino-Embryonic Antigen (CEA), and EMA. Finally normal brown bear tissue from ovary and brain were used as positive control for cytokeratins and vimentin stain.

The utilized antibodies were concentrated antibodies made by *DAKOCytomation*, Denmark, ZYMED Laboratories Inc., San Francisco (for Calretinin polyclonal antibody), and LifeSpan Bioscience, Inc. (for WT-1 polyclonal antibody, LS-B4578). In order to confirm immunohistochemically the diagnosis of carcinoma of the ovary, all the specimens were initially marked with vimentin to control the primary processing of the analyzed case. First we chose a panel of antibodies to help

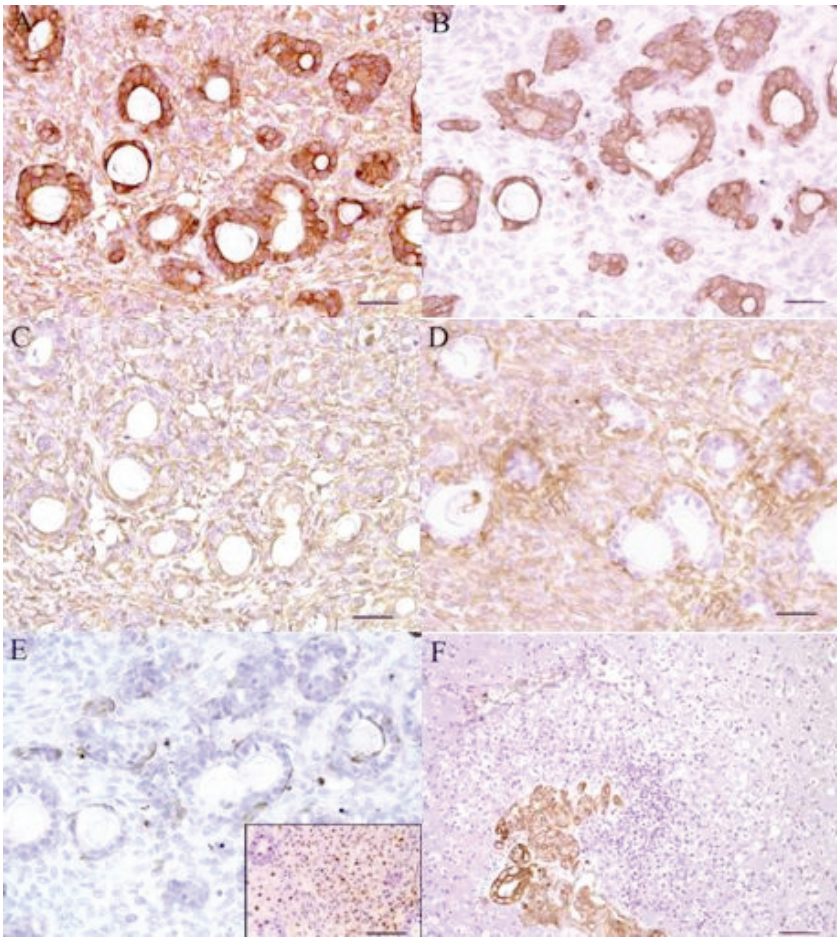

Figure 2: **A)** Diffuse and strong positivity of tumor cells, especially pseudo-cysts forming cells, for A1-A3 citockeratins. **B)** Diffuse positivity restricted to pseudo-cysts forming cells for EMA. **C)** Calretinin diffuse and light to moderate positivity of all tumoral cells. **D)** Similar pattern of diffuse and light positivity, with the exception of the pseudo-cysts forming cells, for CA125 antigen. **E)** Substantial negativity of all neoplastic cells for CEA antigen. Note in right lower insert the strong and diffuse nuclear positivity of the neoplastic cells for WT-1 antigen. **F)** Strong positivity of brain metastasis for citockeratins A1-A3. Note the intense inflammatory reaction and edema around the neoplastic tissue. (IHC stain. Bar = 100 μm; insert and panel F = 250 μm)

establish the epithelial origin of the tumour. Thus, we opted for cytokeratin AE1/AE3, and EMA antibodies. Since there is no specific marker to indicate the ovarian origin of the tumor, the differentiation of the studied tumor from possible ovarian metastasis was realized through several associated immunohistochemical reactions. Thus, the anti-calretinin antibody was used in attempt to help, together with the CA125, CEA, and WT-1 antibodies, to eliminate a mesothelioma belonging to the peritoneal cavity. The CEA staining allowed the separation of this tumor from ovarian metastasis originating in the gastrointestinal tract. Finally, WT-1 staining was used in attempt to speculate the serous, non mucinic origin of the ovarian neoplasia. The immunostaining intensity was evaluated using a four-degree-system in accordance with the model of Wauters, 1995. Immunohistochemistry stain was notable and intense for pan-cytokeratin (Fig. 2A, and F), moderate and diffuse for calretinin (Fig. 2C), light to moderate and "periacinar" for EMA (Fig. 2B), and focal and nuclear for WT-1 (Fig. 2E, insert). Not unexpected in such a high grade carcinoma, vimentin staining was positive in a minority of epithelial cells. Calretinin staining was focally strongly positive, also in brain and lymph node metastases. Additionally, neoplastic cells showed a moderate and focal stain for CA125 (Fig. 2D), resulting consistently negative for CEA antigen (Fig. 2E). In all the neoplastic samples (primary tumor and metastases) the inconspicuous and myxoid stroma resulted strongly positive for vimentin, as expected.. On the basis of the tumor architecture, and the immunohistochemical profile, the tumor was classified, according WHO guidelines (18), as ovarian carcinoma serous type, papillary to solid.

According to WHO classification, ovarian cancers are classified in three categories: epithelial tumors (arise from cells that line or cover the ovaries); germ cell tumors (originate from cells that are destined to form eggs within the ovaries); and sex cord-stromal cell tumors (begin in the connective cells that hold the ovaries together and produce female hormones) (18). Common epithelial tumors begin in the surface epithelium of

the ovaries are divided into serous, endometrioid, mucinous, and clear cell tumors subtypes. Unfortunately, extraovarian mesotheliomas, peritoneal carcinoma, or ovarian metastatic carcinomas belonging to gastrointestinal tract, represent some tumors that are adjacent to ovarian tissues and may be viewed as ovarian cancer, complicating the histological diagnosis.

In our case, the immunohistochemical analysis of primary tumor and metastases revealed a characteristic profile of the neoplasia. Anticalretinin antibody was used (presently considered to be the best marker for mesotheliomas) together with cytokeratins, EMA and CA125 antibodies (epithelial cells markers). The immunostaining for cytokeratin AE1/AE3 was of great importance in the determination of the epithelial origin of the tumour since, for this marker, primary neoplasia and metastases were positive at the level of tumour cells (Fig. 2A, and F). The positivity of the ovarian surface epithelium for calretinin, was very helpful since it represented the positive internal control. In our case, the light cytoplasmic and diffuse pattern of calretinin positivity in neoplastic cells, suggested an ovarian surface epithelial tumor (Fig. 2C). The immunostaining was frequently diffuse and the intensity of the reaction was strong positive. The EMA expression of neoplastic cells is tipical for poorly differentiated ovarian carcinomas (19), and this antibody emphasized acinar differentiation (Fig. 2B), often where it was not easily observed in haematoxylin and eosin preparations (20). Even if CA125 is not a specific marker of the ovarian origin of the tumor, some studies have shown a CA125 heterogeneous positivity in specimens of ovarian serous carcinomas (21). This type of heterogeneous staining, observed also in our case, was in favor of the ovarian origin (Fig. 2D). Since tumors with gastrointestinal origin are intense and diffusely positive for CEA (20), the negativity of this brown beer tumor supported the ovarian origin (Fig. 2E). Besides, when present, CEA expression is indicative of the mucinous type differentiation in ovarian tumors which are, at the same time, negative for CA125 (21).

Then, immunohistochemistry completed the present study of metastasizing ovarian tumor, allowing its separation from peritoneal mesotheliomas with ovarian extension and from ovarian metastasis of a primary gastrointestinal carcinoma.

The brain, along with the bone, liver, and lung, is one of the most common sites of metastasis of ovarian carcinomas. Weakness, depression, anorexia, unstable gait, alteration of the vision, slurred speech, dizziness or vertigo, head tilting are some of the neurological symptoms reported in human and animals with brain metastases (22, 23, 24) but, of course, they depend form the site of metastases implantation. Regarding clinical pathology, results were not indicative of specific diseases: many cancer patients have a mild normocytic, non-regenerative anemia associated with chronic disease. The increase in serum γ-glutamyltransferase was considered to be due to hepatic damage, but no metastases were found in the liver. Neutrophilia could be due to the reaction against the tumors and increasing in total protein could have been caused by slight dehydration.

In summary, we report here a case of malignant metastasizing ovarian tumor in a brown beer. Because of typical histological and immunohistochemical features of neoplastic cells, this tumor was diagnosed as papillary to solid serous type ovarian carcinoma. Because of the scattered distribution pattern of neoplastic nodules, the involvement of the brain and lombo-aortic lymph node was considered to be metastatic. According to these results, the presence of tumor should be considered in bears with neurological signs.

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METASTAZIRAJOČI KARCINOM JAJČNIKA PRI EVROAZIJSKEM RJAVEM MEDVEDU (*Ursus arctos arctos*): KLINIČNI PRIMER

Povzetek: V članku je opisan primer karcinoma jajčnika pri rjavemu medvedu. Evroazijska rjava medvedka, stara 37 let, brez mladičev, iz živalskega vrta Falconara v Italiji, je kazala klinične znake nevroloških motenj, in sicer obojestransko slepoto ter znake delne levostranske pareze obeh okončin.

Po opaženih kliničnih znakih je bila medvedka zdravljena z deksametazonom, ranitidinom, ceftriaksonom, propentofilinom, vitamini kompleksa B in dodajanjem tekočine. Približno po enem tednu so znaki pareze popustili, tako da je medvedka znova lahko hodila, delno se ji je povrnil vid in pričela je jesti. Kljub temu začetnemu kliničnemu izboljšanju je medvedka poginila tri tedne po pojavu bolezenskih znakov. Med raztelesbo smo našli močno povečan in tumorozno spremenjen levi jajčnik ter metastaze v regionalnih bezgavkah in možganih. Glede na značilno histološko sliko in imunohistokemične značilnosti tumorskih celic smo tumor diagnosticirali kot papilarni do solidni serozni tip karcinoma jajčnika. Zaradi razpršenega vzorca razporeditve žarišč tumorskih celic v možganih ter v ledveno-aortnih bezgavkah smo le-ta opredelili kot metastaze. V literaturi je opisanih le nekaj primerov tumorjev pri različnih vrstah medvedov (*Ursidae*), poročajo o primerih limfosarkoma, osteoma, osteosarkoma, hondrosarkoma, ploščatoceličnega karcinoma ter karcinoma žolčevodov, ščitnice, mlečne žleze in jetrnih celic. Glede na naše rezultate je potrebno pri medvedih z nevrološkimi kliničnimi znaki pri diferencialni diagnostiki pomisliti tudi na tumorje.

Ključne beside: možgani; rjavi medved; metastaze; jajčnik; tumor

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