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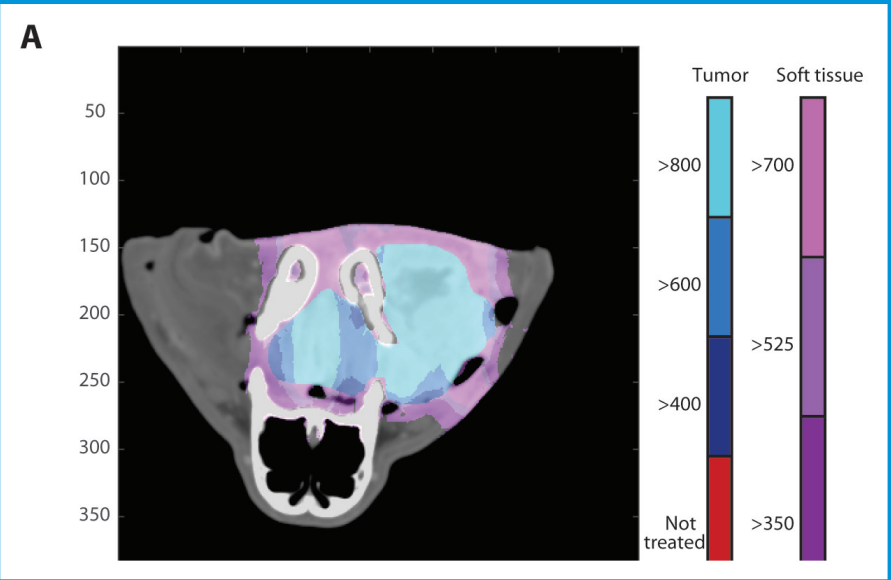

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Original Research Article

EVALUATION AND COMPARISON OF DNA EXTRACTION KITS FOR THE DETECTION OF *Clostridium difficile* IN SPIKED AND FIELD FAECES FROM PIGLETS BY USING REAL-TIME PCR

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Abstract: In complex samples (faeces, soil, food, etc.), *Clostridium difficile* is often present in the form of dormant spores that cause reduced effectiveness of DNA extraction. With the aim of determining an optimal DNA extraction procedure from spores, DNA extraction from faecal samples spiked with a known number of *C. difficile* spores and faecal samples from piglets was performed with three manual protocols, using two commercial kits and subsequent real-time PCR (rtPCR) DNA amplification. DNA extraction protocols, including mechanical disruption by bead beating, gave better results with rtPCR. The SmartHelix DNAid Complex Kit proved to be more efficient than the QIAamp DNA Stool Mini Kit, suggesting that an optimal combination of mechanical, enzymatic, and chemical lysis seems to be required for the best results.

Key words: *Clostridium difficile*; DNA extraction; real-time PCR; piglets; faeces; spores

Introduction

Clostridium difficile infection is one of the most common causes of nosocomial diarrhoea. Subclinical colonisation has been described in humans and in several animal species, which seem to be a significant reservoir of *C. difficile* and a potential source of bacteria for community-acquired *C. difficile*-associated disease (1, 2). The detection of low numbers of *C. difficile* in samples (e.g. food, environmental samples, and asymptomatic humans and animals) is extremely important, as an exact infectious dose is not completely known, but remains a challenge. Enrichment culture is the preferred 'gold standard' method for the isolation of *C. difficile*, but is time-consuming and labour intensive, although it provides an isolate for further studies (3). Real-time PCR (rtPCR) could potentially serve as a rapid screening test, but culture-positive/rtPCR negative samples represent a drawback to this method. It could be improved by an enrichment step prior rtPCR or with a better DNA extraction method (4, 5). A resistant spore coat is difficult to lyse; therefore, the release of nucleic acid is limited and consequently reduces the effectiveness of *C. difficile* DNA extraction methods. In this case, a mechanical disruption method is required. Bead-beating has been shown to be one of the most

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effective techniques for DNA extraction from soil samples and from *Bacillus* sp. spores (6, 7). Freifeld et al. (8) described a novel lysis microreactor with heat, and chemical and physical (shear flow) disruption, followed by PCR, for a rapid diagnosis of *C. difficile*. In our study, we attempted to develop an optimal DNA extraction method from the faeces of piglets without diarrhoea in order to improve the sensitivity of the rtPCR for the detection of *C. difficile*.

Materials and methods

For the spore preparation, *C. difficile* (strain 51377, A+B+CDT+) was plated onto 5% sheep blood agar (Columbia blood agar base; Oxoid, United Kingdom) (anaerobically, 37 ºC, five days) and the spores were purified using HistoDenz (Sigma-Aldrich, USA), based on a previously published protocol (9). The spores were stored in sterile distilled water at 4 ºC. Ten-fold dilutions (10^{-1} to 10^{-6} dilutions) of purified spores were prepared. The number of spores was estimated using dark-field microscopy and colony count on blood agar.

To evaluate the content of free *C. difficile* DNA in the experimental spore suspension, the 10^{-1} dilution was filtered (0.45 µm; Macherey-Nagel, Germany) to remove spores. The filtrate was used as a DNA source and amplified nine times with each rtPCR as described further in the text. Comparably, the unfiltered spore suspension (dilution 10^{-1}) was also amplified with rtPCR.

Dilutions of purified spores (100 µl) were used for spiking *C. difficile* – negative (rtPCR and enrichment culture negative) faecal specimens (1 g) to reach the spore concentrations presented in Table 1. Three sets of individually spiked faeces were prepared for DNA extraction in order to enable testing in triplicate. DNA was extracted using three extraction protocols (in triplicate with two negative controls). Protocol A was QIAamp DNA Stool Mini Kit (Qiagen, Germany) (QS) used according to the manufacturer's protocol. Meanwhile, for protocol B (QSB) the same kit was used, but mechanical disruption was performed before incubation at 95 ºC. Samples were mixed with 400 µL of ASL buffer (from the kit) and 370 mg ≤ 106 µm of glass beads (Sigma-Aldrich, USA), followed by bead beating (6400 rpm for 90 s) on a MagNA Lyser instrument (Roche Diagnostics, Germany). The samples were then centrifuged at 20000 × *g* for 3 min. Supernatants were transferred to new tubes and mixed with 1000 µL of ASL buffer. Samples were afterwards incubated at 95 ºC for 5 min and further processed according to the manufacturer's protocol. In protocol C, we used a SmartHelix DNAid Complex Kit (ExVivon, Slovenia) (SH), intended for extraction of bacterial DNA from complex samples or from bacteria with hard cell walls. The name and manufacturer of this commercial extraction kit were changed after the study, and it is now known as DNA Isolation from Complex Samples (Institute of Metagenomics and Microbial Technologies, Slovenia, info@immt.eu). DNA extraction was carried out according to the manufacturer's instructions. MagNA Lyser instrument (Roche Diagnostics, Germany) was used for bead beating, three times at 6400 rpm for 45 s.

The efficiency of DNA extraction from spores was tested with two real-time PCR assays: TaqMan real-time PCR (TMrtPCR) and LightCycler real-time PCR (LC rtPCR) (5, 10). Toxin gene *tcdB* was amplified with and without internal control three times.

All DNA extraction procedures were also tested on rectal swabs from apparently healthy piglets without diarrhoea (< 10 days old; n=40) collected from a large farm. Samples were taken in duplicate. One rectal swab per animal was washed in 1 mL sterile distilled water and 200 µL was used for each of the three tested DNA extraction protocols described above. Toxin gene *tcdB* was amplified with both rtPCR assays with and without internal control. The remaining rectal swab was used for bacteriological cultivation using a selective enrichment cultures (5).

Results

A comparison of spores counted under a microscope (10^6 spores mL^{-1} in stock solution) and the colony count approach ($4x10^5$ spores mL^{-1} in stock solution) gave comparable results.

The detected level of free *C. difficile* DNA in filtered suspension (dilution 10^{-1}) was close to the rtPCR detection limit: in nine reactions performed per each PCR type, only one LC rtPCR and one TMrtPCR were positive with threshold cycle (Ct) values ≥ 40. In comparison, the unfiltered spore suspension without DNA extraction was rtPCR positive with Ct values ≥ 40, while samples spiked

Table 1: Molecular detection of *Clostridium difficile tcdB* gene in faecal samples spiked with spores

DNA extraction protocol	number of *C. difficile* spores g^{-1} of faeces	10^4	10^3	10^2			10^1			1			0
	samples	E 1-3[b]	E 1-3	E 1-3			E 1-3			E 1-3			E 1-3
QIAamp DNA Stool Mini Kit	LC/TM[a] 1	pos[c]	pos	pos	neg[d]	pos	neg						
	LC/TM 2			neg	+[e]/–[f]	neg	neg	neg	neg	neg			neg
	LC/TM 3			–/+	–/+	pos	–/+						
QIAamp DNA Stool Mini Kit with bead beating	LC/TM 1	pos	pos	pos	–/+	neg	neg						
	LC/TM 2			–/+	neg	neg 1	–/+	neg	neg	neg			neg
	LC/TM 3			pos	neg	–/+	–/+						
SmartHelix DNAid Complex Kit	LC/TM 1	pos	pos	pos			pos	neg	+/–				
	LC/TM 2							neg	neg	neg	pos	neg	neg
	LC/TM 3							+/–	neg				

[a] LC/TM, LightCycler real-time PCR/TaqMan real-time PCR results (amplification repeated three times - 1, 2, 3)
[b] E 1-3, DNA extraction triplicates
[c] pos, positive LightCycler and TaqMan real-time PCR result
[d] neg, negative LightCycler and TaqMan real-time PCR result
[e] +, positive real-time PCR result (Ct value > 40)
[f] –, negative real-time PCR result

Table 2: Comparison of different DNA extraction protocols/kits used for molecular detection of *Clostridium difficile* tcdB gene in animal samples compared with culture results (n=40)

		LC[a]-QS[c]		LC-QSB[d]		LC-SH[e]		TM[b]-QS		TM-QSB		TM-SH	
		pos[f]	neg[g]	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg
culture pos	29	11	18	14	15	25	4	10	19	15	14	27	2
culture neg	11	6	5	8	3	11	0	7	4	7	4	11	0
total	40	17	23	22	18	36	4	17	23	22	18	38	2

[a] LC, LightCycler real-time PCR
[b] TM, TaqMan real-time PCR
[c] QS, QIAamp DNA Stool Mini Kit
[d] QSB, QIAamp DNA Stool Mini Kit with bead beating
[e] SH, SmartHelix DNAid Complex Kit
[f] pos, positive result (samples with sigmoid curve)
[g] neg, negative result

with the 10^{-1} spore dilution presented Ct values between 28.28 and 33.70 after DNA extraction with different DNA extraction protocols/kits.

When the SH extraction method was used, the rtPCR results for spiked samples showed better efficiency/sensitivity, since the sample with one *C. difficile* spore g^{-1} of faeces was rtPCR positive, while the QS and QSB samples were positive at 10-100 spores g^{-1} of faeces (Table 1). Similarly, a ten-fold difference was observed for 100% probability of detection (Table 1). Positive samples spiked with 100 and 10 spores g^{-1} of faeces that were positive only with one rtPCR assay (in Table 1 indicated as –/+ or +/–) had Ct value > 40, which suggested that the samples were near the limit of detection of the assays. Inhibition of rtPCR was not observed.

Comparison of three DNA extraction protocols on rectal swabs proves that SH yielded the highest number of rtPCR positive samples, followed by QSB and QS (Table 2). The Ct values for SH samples were on average 6-8 cycles lower in comparison to the QSB samples and 8-12 cycles lower than the QS samples (data not shown). Furthermore, 38

(95%) samples were rtPCR positive. Among these samples, 13 (34.2%) were positive when DNA was extracted with SH, but negative with other protocols. In 8 (21.1%) samples, the LC rtPCR and TMrtPCR results were not the same; two samples were positive with TMrtPCR only when SH was used, and in 6 samples (4 QS and 2 QSB samples) either LC rtPCR or TMrtPCR was positive. In all samples with different LC rtPCR/TMrtPCR results, the Ct values for positive rtPCR samples were higher than 40 and reproducibility was not 100%.

Discussion

The sensitivity of the rtPCR assays also depends upon high DNA yield and purity, which may be achieved with an optimal DNA extraction method. The efficient lysis of the clostridial spores and removal of PCR inhibitors are the major challenges to improve DNA extraction from complex samples (e.g. faeces, soil, food). Faecal samples with a known number of *C. difficile* spores were prepared and subjected to three DNA extraction protocols, followed by rtPCR. The possibility of false positive results due to free *C. difficile* DNA in samples was ruled out via a filtered spore suspension experiment, where the rtPCR results demonstrated very low amounts of free *C. difficile* DNA in spore suspensions, well under the limit of detection in the more diluted samples. Therefore, the rtPCR results obtained in experiments comparing different DNA extraction kits were most likely due to efficient DNA extraction from spores and not due to the presence of free DNA in samples.

The results of spore quantification differed slightly between the two applied methods. A lower number of *C. difficile* in stock solution was detected when the colony-forming unit (CFU) approach was used (5.6 log10 vs. 6 log10). In our study, blood agar was used, which is not supplemented with taurocholate to support the germination of spores; therefore, the number of CFU would probably be higher using a supplemented medium (11).

The evaluation of three DNA extraction protocols for spiked and field samples showed that SH provided the greatest DNA yield for the detection of *C. difficile* with rtPCR. However, the testing of spiked QS and QSB samples revealed no difference between these two extraction protocols, while QSB results were better when testing field samples (22 QSB positive in contrast to 17 QS positive samples). A possible explanation for this could be that the beads used in QSB extraction could also improve the lysis of a cell wall of vegetative bacteria in swabs.

A limitation of this study is the fact that only two commercial kits were compared. QS has been widely used in our laboratory for DNA extraction from faecal samples; in order to improve the sensitivity of *C. difficile* rtPCR assays, bead-beating was introduced prior to QS protocol. Furthermore, SH was found to be effective for *Mycobacterium avium* subsp. *paratuberculosis* (Map) DNA extraction from faecal samples from sub-clinically Map-infected shedders (12). As Map possesses a complex, resistant, lipid-rich cell wall, which is also difficult to lyse during DNA extraction, we surmised that SH could contribute to the efficiency of DNA extraction from *C. difficile* spores in complex samples, especially in samples with a low number of bacteria (e.g. animal shedders).

In this study, all the samples were amplified with LC rtPCR and TMrtPCR. Among 40 field samples, LC rtPCR and TMrtPCR results differed in 8 of them, but in the entire lot Ct values were higher than 40, indicating a small number of *C. difficile* organisms in the sample, probably close to the limit of detection of the rtPCR assays used.

To the best of our knowledge, only two studies dealing with the comparison of culture and in-house rtPCR assays for the detection of *C. difficile* in animal samples have been published (4, 5). Both studies reported 7–11% culture-positive/rtPCR-negative and 11% culture-negative/rtPCR positive-samples. The differences between culture and rtPCR results could be connected with samples (especially rectal swabs), because faeces are never a homogeneous medium, and the concentration of bacteria may vary. In our study, culture-positive/rtPCR-negative samples were reduced to 5% when the SH kit was used. Furthermore, the percentage of culture negative/rtPCR positive samples was in agreement with previously published studies for QS samples (15–17.5%), while improvement was observed using QSB (17.5–20%) and SH (27.5%) protocols, showing that the application of a better DNA extraction procedure, including bead-beating, increases the number of positive rtPCR samples.

Both SH and QS have similar processing times and are based on silica membrane spin columns, but QS is considerably more expensive than SH is. Superior SH results indicate that an optimal

extraction procedure should combine mechanical and enzymatic/chemical lysis. Moreover, QSB and SH comprise glass beads for mechanical disruption, while zirconia/silica beads could also be efficient for destroying spores and highly resistant cell walls (6, 13).

In conclusion, the selection of an optimal DNA extraction method implicating mechanical disruption could significantly improve the detection of *C. difficile*.

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References

1. Hensgens MPM, Keesen EC, Squire MM, et al. *Clostridium difficile* infection in community: a zoonotic disease? Clin Microbiol Infect 2012; 18: 635–45.

2. Banks A, Brown DJ, Mather H, Coia JE, Wiuff C. Sentinel community *Clostridium difficile* infection (CDI) surveillance in Scotland, April 2013 to March 2014. Anaerobe 2016; 37: 49–53.

3. Crobach MJT, Dekkers OM, Wilcox MH, Kuijper EJ. European society of clinical microbiology and infectious diseases (ESCMID): data review and recommendations for diagnosing *Clostridium difficile*-infection (CDI). Clin Microbiol Infect 2009; 15: 1053–66.

4. Houser BA, Hattel AL, Jayarao BM. Real-time multiplex polymerase chain reaction assay for rapid detection of *Clostridium difficile* toxin-encoding strains. Foodborne Pathog Dis 2010; 7: 719–26.

5. Avbersek J, Cotman M, Ocepek M. Detection of *Clostridium difficile* in animals: comparison of real-time PCR assays with the culture method. J Med Microbiol 2011; 60: 1119–25.

6. Miller DN, Bryant JE, Madsen EL, Ghiorse WC. Evaluation and optimization of DNA extraction and purification procedures for soil and sediment samples. Appl Environ Microbiol 1999; 65: 4715–24.

7. Dauphin LA, Moser BD, Bowen MD. Evaluation of five commercial nucleic acid extraction kits for their ability to inactivate *Bacillus anthracis* spores and comparison of DNA yields from spores and spiked environmental samples. J Microbiol Methods 2009; 76: 30–7.

8. Freifeld AG, Simonsen KA, Booth CS, et al. A new rapid method for *Clostridium difficile* DNA extraction and detection in stool. J Mol Diagn 2012; 14: 274–9.

9. Sorg JA, Dineen SS. Laboratory maintenance of *Clostridium difficile*. Curr Protoc Microbiol 2009; 12: 9A.1.1–10.

10. Avbersek J, Zajc U, Micunovic J, Ocepek M. Improved detection of *Clostridium difficile* in animals by using enrichment culture followed by LightCycler real-time PCR. Vet Microbiol 2013; 64: 93–100.

11. Burns DA, Minton NP. Sporulation studies in *Clostridium difficile*. J Microbiol Methods 2011; 87: 133–8.

12. Logar K, Kopinc R, Bandelj P, Lapanje A, Ocepek M. Evaluation of combined high-efficiency DNA extraction and real-time PCR for detection of *Mycobacterium avium* subsp. *paratuberculosis* in subclinically infected dairy cattle: comparison with faecal culture, milk real-time PCR and milk ELISA. BMC Vet Res 2012; 8: e49 (10 pp.) https://bmcvetres.biomedcentral.com/articles/10.1186/1746-6148-8-49 (Dec. 2016)

13. Kim BH, Ramanan R, Cho DH, et al. Simple, rapid and cost-effective method for high quality nucleic acids extraction from different strains of *Botryococcus braunii*. PLoS ONE 2012; 7: e37770 (90 pp.) http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0037770 (Dec. 2016)

OVREDNOTENJE IN PRIMERJAVA KOMERCIALNIH KOMPLETOV ZA IZOLACIJO DNK ZA DOKAZOVANJE BAKTERIJE *Clostridium difficile* V BLATU PRAŠIČKOV Z METODO PCR V REALNEM ČASU

J. Avberšek, U. Zajc, I. Gruntar, B. Krt, M. Ocepek

Povzetek: V kompleksnih vzorcih (blato živali, zemlja, hrana, itd.) je bakterija *Clostridium difficile* pogosto prisotna v obliki spor, ki so vzrok za slabšo učinkovitost izolacije DNK. Da bi določili optimalni postopek izolacije DNK iz spor, smo s tremi različnimi protokoli in uporabo dveh komercialnih kompletov za izolacijo DNK izolirali DNK iz vzorcev blata z znanim številom *C. difficile* spor in vzorcev blata prašičkov. DNK smo pomnoževali z metodo PCR v realnem času (rtPCR). Boljše rtPCR rezultate smo dobili pri vzorcih, kjer smo pri izolaciji DNK uporabili tudi mehansko razbitje celic s kroglicami. SmartHelix DNAid Complex Kit je bil učinkovitejši komercialni komplet kot QIAamp DNA Stool Mini Kit, kar nakazuje na optimalno kombinacijo mehanske, encimske in kemične lize za najboljši izplen DNK.

Ključne besede: *Clostridium difficile*; izolacija DNK; spore; PCR v realnem času; prašički; blato

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Original Research Article

SWINE BRUCELLOSIS CAUSED BY *Brucella suis* BIOVAR 2 IN CROATIA

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Abstract: Brucellosis in swine was surveyed from 2011 to 2015 in 13 counties in Croatia. A total of 3230 breeding males were tested serologically, and positive reactions were confirmed in 42 (1.3%) males from 17 farms. A total of 641 sows with abortion or reproductive problems were tested, and positive reactions were confirmed in 34 (5.3%). Organs from 68 swine were tested for bacteria, and *Brucella spp.* was isolated from 47 (69.1%). *B. suis* was identified in 45 isolates from domestic swine and 2 isolates from wild boar in six counties in Croatia, and all isolates were found to be *B. suis* biovar 2 based on Bru-up/Bru-low, Bruce-ladder, Suis-ladder and RFLP-based PCR assays. These results indicate that brucellosis is difficult to eradicate in free-range and semi-free-range swine farming, particularly in areas where contact with wild boar is possible. Further disease control measures are required.

Key words: *Brucella suis* biovar 2; swine; abortion; prevalence; Croatia

Introduction

Brucella suis appears in most countries containing domestic swine and wild boar. *B. suis* infections spread easily on swine farms and are difficult to control; the most common clinical signs are abortions and infertility in sows, mortality of offspring and orchitis in breeding males (1). *B. suis* biovars (bv.) 1, 2 and 3 cause brucellosis in swine. *B. suis* bv. 1 has been reported in Latin America (2, 3) as well as in USA and China (4, 5). *B. suis* bv.3 has also been detected in the USA and China (4, 5). *B.suis* bv. 2 is the most frequent cause of infection in domestic swine in Central and Western Europe, where wild boar and hares serve as natural carriers (6-12).

In the Central European country of Croatia, infection of horses and swine with *B. suis* bv. 3 has been reported based on classical microbiological assays (13, 14). Genotyping of *B. suis* in Croatia based on multi-locus, variable-number tandem repeat analysis revealed the existence of various *B. suis* strains with more or less different geographic distributions (22); some of the strains were identical to ones identified in Hungary, Germany

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and France. One of the drivers of *B. suis* infection in Croatia appears to be extensive domestic swine-holding under conditions in which contact or even natural mating with infected wild boar is possible, which has already been reported in other European countries (14-21).

To gain additional insights into the epidemiology of brucellosis, the *B. suis* biovar(s) responsible and the factors that may drive *B. suis* infection in Croatia, we surveyed large number of breeding swine males and sows from herds with abortions and reproductive problems from 13 counties in the country. Surveyed swine were free-range or maintained under semi-intensive conditions.

Material and methods

Description of the sample

In Croatia, any abortion or appearance of clinical signs in breeding swine that raises suspicions of brucellosis must be reported to a veterinarian in order to facilitate early detection. In these cases, an authorised veterinarian must take appropriate samples and submit them to an authorised brucellosis testing laboratory. Young boars must also be serologically tested for brucellosis prior to their use in breeding programs, artificial insemination, or natural mating.

Between 2011 and 2015, swine were surveyed in free or extensive rearing systems in which abortion had appeared in gravid sows, or reproductive problems such as infertility, stillbirths or failure to fertilise. Most of these systems were small farms with a few sows and young breeding males, which were held extensively, left to roam freely in the forest or kept free-range under natural conditions. At each farm where brucellosis was confirmed in sows, all breeding males were tested serologically. When young boar tested positive, testing was also performed on the swine farms where the boar were used to fertilise sows. All serologically positive swine were removed from breeding and sampled for bacteriology at the time of slaughter. These samples were kept at 4°C andtested within 24 h.

Serological examination

Serum samples. Blood samples were collected from 641 sows from 62 herds as well as from3230 boars from the following 13 counties in Croatia:Bjelovar-Bilogora, Brod-Posavina, Karlovac, Koprivnica-Križevci, Krapina-Zagorje, Međimurje, Osijek-Baranja, Požega-Slavonia, Sisak-Moslavina, Varaždin, Virovitica-Podravina, Vukovar-Srijem, and Zagreb County.

Serological tests. Serum was assayed using the Rose Bengal test (RBT), complement fixation test (CFT), and the INgezim Brucella Porcina kit (Ingenasa, Madrid, Spain), which is an indirect enzyme-linked immunosorbent assay. Antigens for RBT and CFT were produced by the Institut Pourquier (Montpellier, France) and the Croatian Veterinary Institute (Zagreb, Croatia).Tests were conducted according to OIE guidelines(23, 24), while the INgezim kit was used according to the manufacturer's recommendations.

Bacteriological examination

Tissue samples. A total of 150 samples were collected at slaughter from 68 domestic sows, hogs and other swine from six counties. Samples comprised lymph nodes (parotid, submandibular, retropharyngeal, portal, subiliac, mesothelial, supramammary) (n = 62), spleen (8), testicles (18), foetuses (12) and uterus (50).

Bacteriological tests. Tissue samples were homogenised in a stomacher, and the suspension was directly cultured in duplicate on blood agar, *Brucella* agar and Farrell medium. One set of three plates was incubated at 37°C in a normal atmosphere, while the other set was incubated in a 10% CO_2 atmosphere. Colony growth and morphology was monitored daily, and colonies were subcultured and examined by microscopy. Isolates were confirmed as *Brucella* using classical microbiological biotyping based on microscopy, culture and biochemistry (23-25).

Polymerase chain reaction (PCR)-based biotyping

Isolates were confirmed to be *Brucella* using *Brucella* genus-specific PCR (26). The reference method to confirm *Brucella* species was Bruce-ladder multiplex PCR (27), while Suis-ladder multiplex PCR was used to determine *B. suis* biovars (28).

Table 1: Number of blood samples tested from pigs and wild boars

Year	Sows tested (n)	Breedings (n)	Positive sows (n/%)	Tested swine (n) -positive farms* (n/%)	Positive farms (n/%)	Wild boars tested (n)	Positive wild boars (n / %)	Positive hunting areas (n)
2011	211	32	10 / 4.7	511 – 27 / 5.3	4 / 12.5	1129	11 / 0.97	5
2012	170	19	7 / 4.1	314 – 12 / 3.8	2 / 10.5	896	4 / 0.44	2
2013	97	6	5 / 5.2	272 – 15 / 5.5	2 / 33.3	425	8 / 1.88	5
2014	116	3	9 / 7.8	392 – 10 / 2.6	1 / 33.3	445	3 / 0.67	1
2015	47	2	3 / 6.4	257 – 3 / 1.2	1 / 50.0	335	8 / 2.39	4
TOTAL	**641**	**62**	**34 / 5.3**	**1746 – 67 / 3.8**	**10 / 16.1**	**3230**	**42 / 1.3**	**17**

Results

Serological examination

Between 2011 and 2015, 3230 breeding males from 13 Croatian counties were serologically analysed, and positive reactions were confirmed in 42 (1.3%) boars from five counties (Table 1). Over the same period, 641 sows that aborted or displayed reproductive problems were serologically tested, and positive reactions were confirmed in 34 (5.3%). On farms containing sows positive for brucellosis, all other swine were serologically analysed, and positive reactions were found in 67 (3.8%) of swine on 10 farms in the same five counties (Table 1).

Bacteriological examination

Organs of 68 swine were tested bacteriologically, and *B. suis* bv. 2 was identified in 45 domestic swine (66.2%) from five counties (Bjelovar-Bilogora, Virovitica-Podravina, Sisak-Moslavina, Brod-Posavina and Zagreb) and 2 wild boars (2.9%) from Zagreb and Vukovar-Srijem counties.

PCR assay

A total of 47 *B. suis* isolates were typed using the Bru-up/Bru-low and Bruce-ladder PCR assays to identify genus and species, respectively, as well as the Suis-ladder PCR assay to assign biovar. Based on reference samples, all isolates were identified as *B.suis* bv. 2.

Discussion

This survey of a relatively large swine population from around Croatia confirms and extends previous findings that brucellosis poses a threat on small farms that share breeding males and in systems where swine are kept extensively or free-range at pasture and where contact with wild boar is possible. Several studies indicate that swine on farms typically become infected following the introduction of infected sows or breeding males, or through contact with infected wild boar(1, 14, 19, 21). Our findings of *B. suis* bv. 2 in 45 domestic swine and 2 wild boar from six Croatian counties highlights the difficulty of eradicating brucellosis from swine populations held semi-intensively or allowed to roam freely at pasture.

This survey is consistent with several earlier studies of Croatian countries bordering the Sava River, which identified *B. suis* bv. 2 as the cause of brucellosis in domestic swine and wild boar. In these counties, breeding swine are often held extensively at pasture or in forests, where contact is possible with many other swine as well as wild boar (8, 9, 14, 18, 19). Our findings are also consistent with studies in several Western European countries. Swine brucellosis caused primarily by *B. suis* bv. 2 has been reported in Austria, Germany, Portugal and Spain. A study of 36 swine herds in Sardinia found 33% to be positive, with the infecting strain in all cases being *B. suis* bv. 2 (1), and a study of 28 sows with reproductive problems in the Rome area found 89% to have brucellosis, with the infecting strain

being *B. suis* bv. 2 (21). However, *B. suis* infections have yet to be reported in Finland, Sweden, UK or Norway, and they have not been reported in Belgium since 1969 or in the Netherlands since 1973 (6).

The present survey detected *B. suis* bv. 2 in two wild boar, consistent with earlier reports of persistent *B. suis* bv.2 infection of wild boar in multiple regions of Croatia (8, 9, 14, 19). *B. suis* bv. 2 has been isolated from wild boar in many Central and Western European countries, including France (7, 15), Switzerland (16, 29), Germany (30), Belgium (11), Spain (17, 31) and Italy (12). Though direct evidence is lacking, it seems extremely likely that wild boars are a reservoir and source of infection for domestic swine. The two animal populations inhabit the same areas in nature and therefore indirect and direct (sexual) contact is possible.

Understanding *B. suis* epidemiology is important not only for the swine industry but also for other types of animal production, since the bacterium can spread easily from swine to other species. *B. suis* bv. 2 infection of dairy cows has been reported in Poland and Belgium (32, 33), and *B. suis* bv. 2 infection of roe deer (*Capreolus capreolus*) has been reported in Germany (34). *B. suis* infection of dogs used to hunt wild boar has been reported in the USA (35), and *B. suis* bv. 1 infection of armadillos (*Chaetophractus villosus*) has been described in Argentina (36). One report described *B. suis* infection of horses in Croatia (13), and while those authors identified the strain as bv. 3 based on biochemical assays, subsequent analysis of single-nucleotide polymorphisms suggest it maybe bv. 1 (37), while the observed zoonotic potency suggests it may be bv. 2 or perhaps a novel strain (18).

Previous work suggests that brucellosis is not a widespread problem among swine populations raised in intensive rearing conditions or on large farms with semi-intensive rearing (18, 19), which were covered in the present survey also.

The Croatian counties where the present survey detected swine brucellosis share long borders with several European countries: Slovenia, Hungary, Serbia, and Bosnia and Herzegovina. In this way, *B. suis* bv. 2 poses a regional threat for brucellosis control, which should be addressed through strong early-detection programs and rapid response in the event of confirmed cases.

References

1. Pilo C, Tedde MT, Orru G, Addis G, Liciardi M. *Brucella suis* infection in domestic pigs in Sardinia (Italy). Epidemiol Infect 2015; 143: 2170–7.

2. Meirelles-Bartoli RB, Mathias LA, Samartino LE. Brucellosis due to *Brucella suis* in a swine herd associated with a human clinical case in the State of Sao Paulo, Brazil. Trop Anim Health Prod 2012; 44(7): 1575–9.

3. Escobar GL, Jacob NR, Lopez G, Ayala SM, Whatmore AM, Lucero NE. Human brucellosis at a pig slughterhouse. Comp Immunol Microbiol Infect Dis 2013; 36(6): 575–80.

4. Pedersen K, Quance CR, Robbe-Austerman S, et al. Identification of *Brucella suis* from feral swine in selected states in the USA. J Wildl Dis 2014; 50(2): 171–9.

5. Dequi S, Donglou X, Jiming Y. Epidemiology and control of brucellosis in China. Vet Microbiol 2002; 90: 165–82.

6. Godfroid J, Käsbohrer A. Brucellosis in the European Union and Norway at the turn of the twenty-first century. Vet Microbiol 2002; 90: 135–45.

7. Garin-Bastuji B, Hars J, Calvez D, Thiebaud M, Artois M. Brucellose du porc domestique et du sanglier sauvage a *Brucella* suis biovar 2 en France. Epidemiol Sante Anim 2000; 38: 1–5.

8. Cvetnic Z, Mitak M, Ocepek M, et al. Wild boar (*Sus scrofa*) as reservoirs of *Brucella suis* biovar 2 in Croatia. Acta Vet Hung 2003; 51: 465–73.

9. Cvetnic Z, Toncic J, Spicic S, et al. Brucellosis in wild boars (*Sus Scrofa*) in the Republic of Croatia. Vet Med Czech 2004; 49: 115–22.

10. Abril C, Thoman A, Brodart I, Wu N, Ryser-Degiorgis MP, Overesh G. A novel isolation method of *Brucella* species and molecular tracking of *Brucella suis* biovar 2 in domestic and wild animals. Vet Microbiol 2011; 150(3/4): 405–10.

11. Gregoire F, Mousset B, Hanrez D, Michax C, Walravens K, Linden A. A serological and bacteriological survey of brucellosis in wild boar (*Sus scrofa*) in Belgium. BMC Vet Res2012; 8: e80 (8 pp.) https://bmcvetres.biomedcentral.com/articles/10.1186/1746-6148-8-80 (Dec. 2016)

12. De Massis F, Di Provvido A, Di Sabatino D, et el. Isolation of *Brucella suis* biovar 2 from a wild boar in the Abruzzo Region of Italy. Vet Ital 2012; 48(4): 397–404.

13. Cvetnić Ž, Špičić S, Čurić S, et al. Isolation of *Brucella suis* biovar 3 from horses in Croatia. Vet Rec 2005; 156(18): 584–5.

14. Cvetnić Ž, Špičić S, Tončić J, et al. *Brucella suis* infection in domestic pigs and wild boar in Croatia. Rev Sci Tech2009; 28(3): 1057–67 .

15. Hars J, Thiebaud M, Cau C, et al. La brucellose du sanglier et du lievre due a *Brucella suis* 2 en France. Faune Sauvage 2004; 261: 18–23.

16. Leuenberger R, Boujon P, Thur B, et al. Prevalence of classical swine fever, Aujeszky disease and brucellosis in a population of wild boar in Switzerland. Vet Rec 2007; 160(1): 362–8.

17. Muñoz PM, Boadella M, Arnal M, et al. Spatial distribution and risk factor of brucellosis in Iberian wild ungulates. BMC Infect Dis 2010; 10: e46 (14 pp.)

https://bmcinfectdis.biomedcentral.com/articles/10.1186/1471-2334-10-46 (Dec. 2016)

18. Špičić S, Zdelar-Tuk M, Račić I, Duvnjak S, Cvetnić Ž. Serological, bacteriological and molecular diagnosis of brucellosis in domestic animals in Croatia. Cro Med J 2010; 51(4): 320–6.

19. Špičić S, Zdelar-Tuk M, Račić I, et al. Sensitivity of actual laboratory diagnostic methods used for surveillance of swine brucellosis in Croatia. Int J Appl Res Vet Med 2013; 11(3): 167–73.

20. Špičić S, Benić M, Zdelar-Tuk M, et al. Brucellosis in Turopolje pig breeding in the 2008–2011 period: an overview of laboratory diagnostic and eradication system. Vet Arhiv 2016; 86(2): 243–52.

21. Barlozzari G, Franco A, Macri G, et al. First report of *Brucella suis* biovar 2 in a semi free–range pig farm, Italy. Vet Ital 2015; 51(2): 151–4.

22. Duvnjak S, Račić I, Špičić S, Zdelar-Tuk M, Reil I, Cvetnić Ž. Characterisation of *Brucella suis* isolates from Southeast Europe by multi-locus variable-number tandem repeat analysis. Vet Microbiol 2015; 180(1/2): 146–50.

23. OIE World Organization for Animal Health. OIE Manual of diagnosics tests and vaccines for terrestrial animals. Porcine brucellosis: chapter 2.8.5. Paris : OIE, 2009: 7 pp.

24. Alton GG, Jones LM, Angus RD, Verger JM. Techniques for the brucellosis laboratory. Paris : Institut Nacional de la Recherche Agronomique, 1988.

25. Corbel MJ, Gill KPW, Thomas EL. Methods for the identification of *Brucella*. Weybridge : Ministry of Agriculture Fisheries and Food, Central Veterinary Laboratory, 1983.

26. Serpe L, Gallo P, Finandza N, Scaramuzzo A, Fenizia D. Single-step method for rapid detection of *Brucella* spp. in soft cheese by gene-specific polymerase chain reaction. J Dairy Res 1999; 66: 313–7.

27. Lopez-Goni I, Garcia-Yoldi D, Marin CM, et al. Evaluation of a multiplex PCR assay (Bruce-ladder) for molecular typing of all *Brucella* species, including the vaccine strains. J Clin Microbiol 2008; 46: 3484–7.

28. López-Goñi I, García-Yoldi D, Marín CM. New Bruce-ladder multiplex PCR assay for the biovar typing of *Brucella suis* and the discrimination of *Brucella suis* i *Brucella canis*. Vet Microbiol 2011; 154: 152–5.

29. Wu N, Abril C, Hinić V, et al. Free-ranging wild boar: a disease threat to domestic pigs in Switzerland? J Wildl Dis 2011; 47: 868–79.

30. Melzer F, Lohse R, Nieper H, Liebert M, Sache K. A serological study on brucellosis in wild boars in Germany. Eur J Wildl Res 2007; 53: 153–7.

31. Closa-Sebastia F, Casas-Diaz E, Cuenca R, Lavin S, Mentaberre G, Marco I. *Brucella* species antibodies and isolation in wild boar in north-east Spain. Vet Rec 2010; 167(21): 826–8.

32. Szulowski K, Iwaniak W, Weiner M, Zlotnicka J. *Brucella suis* biovar 2 isolations from cattle in Poland. Ann Agric Environ Med 2013; 20(4): 672–5.

33. Fretin D, Mori M, Czaplicki G, et al. Unexpected *Brucella suis* biovar 2 infection in a dairy cow Belgium. Emerg Infect Dis 2013; 19(12): 2053–4.

34. Sting R, Schwabe I, Oehme R, Elschner MC, Melzer F. First report of a *Brucella suis* infection in roe deer (*Capreolus capreolus*). Berl Munch Tierartzl Wochenschr 2014; 127(3/4): 120–2.

35. Ramamoorthy S, Woldemeskel M, Ligett A, Snider R, Cobb R, Rajeev S. *Brucella suis* infection in dogs, Georgia, USA. Emerg Infect Dis 2011; 17(12): 2386–7.

36. Kin MS, Fort M, De Eschaide ST, Casanave EB. *Brucella suis* in armadillos (*Chaetophractus villosus*) from La Pampa, Argentina. Vet Microbiol 2014; 170(3/4): 442–5.

37. Fretin D, Whatmore AM, Al Dahouk S, et al. *Brucella suis* identification and biovar typing by real-time PCR. Vet Microbiol 2008; 131: 376–85.

BRUCELOZA PRAŠIČEV, POVZROČENA Z BAKTERIJO *Brucella suis* BIOVAR 2 NA HRVAŠKEM

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Povzetek: Brucelozo pri prašičih smo spremljali od leta 2011 do leta 2015 v 13 hrvaških občinah. S serološko analizo smo preverili prisotnost bruceloze pri 3230 samcih v razplodu. Pozitivne reakcije smo ugotovili pri skupno 42 merjascih s 17 farm, kar predstavlja 1,3 % živali. S serološkimi testi smo preverili prisotnost protiteles proti bruceli tudi pri 641 plemenskih svinjah, ki so zvrgle ali imele težave z zabrejitvijo. Pozitivna reakcija je bila ugotovljena pri 34 svinjah, kar predstavlja 5,3 % vseh testiranih živali. Notranje organe 68 svinj iz šestih občin smo uporabili za osamitev bakterij *Brucella spp*. Bakterije smo ugotovili pri 47 vzorcih (69,1 %). Bakterijo *Brucella suis* smo odkrili v vseh 47 vzorcih, izmed katerih jih je bilo 45 od domačih plemenskih svinj, dva vzorca pa sta bila od divjih svinj. Vse izolirane bakterije so pripadale sevu *B. suis* biovar 2, kot so pokazale dodatne analize z uporabo metod Bru-up/Bru-low, Bruce-ladder, Suis-ladder in RFLP. Ti rezultati kažejo, da je popolno izkoreninjenje bruceloze težavno, še posebej v prostih rejah prašičev, kjer obstajajo možnosti stika z divjimi prašiči. Zato bi bilo v prihodnje potrebno razmisliti o dodatnih načinih nadzora nad to nevarno boleznijo prašičev.

Ključne besede: *Brucella suis* biovar 2; prašiči; zvrg; pojavnost; Hrvaška

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Original Research Article

MOLECULAR DETECTION AND SEROPREVALENCE OF MYCOPLASMAS IN CLINICALY HEALTHY WORKING DOGS

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Abstract: In this study seroprevalence and prevalence of mycoplasmas in clinically healthy dogs were studied. Thirty-four working dogs of various breeds, gender and age were included in this research. Among them, 27 were working dogs from Slovene armed forces and 7 were working sheepdogs. We used dot-immunobinding assay (DIBA) as a serological test for the detection of specific antibodies to *Mycoplasma cynos*, *Mycoplasma canis* and *Mycoplasma molare* and consensus PCR for detection of genes for 16S rRNA or 16S/23S IGS region of mycoplasmas. Specific antibodies against at least one of the canine mycoplasmas were detected in 94.1% dogs. Of them 23.5% samples showed positive reaction only to *M. cynos*, 20.6% were positive only to *M. canis* and none of the samples were positive only to *M. molare.* Altogether 47.0% of samples were positive to *M. cynos* and *M. canis* whereas only one dog (2.9%) had specific antibodies to all three mycoplasmas tested. The presence of mycoplasmas detected by PCR was 57.14% in younger dogs (≤ 1 year) and 18.5% to 35.3% in older dogs, depending on year of the sampling. Genital swabs were PCR-positive in more cases (60%) in comparison with oral swabs (46.7%). *M. canis* was detected in 40% of positive cases, in the same percent of samples mixed not determined mycoplasma infections were confirmed. Mycoplasma species such as: *M. cynos, M. edwardii, M. maculosum, M. spumans* were determined each in single cases and in one case mixed ureaplasma infection was confirmed.

Key words: working dogs; canine mycoplasmas; *Mycoplasma canis*, *Mycoplasma cynos*; DIBA; PCR

Introduction

Mycoplasmas are the smallest bacteria with the smallest number of genes that are still capable of self-replication. Some of them are pathogenic for humans and animals (1, 2). Up to now, 16 described mycoplasma species and two not fully described species have been isolated from dogs (3, 4). Mycoplasmas can be found in the upper respiratory tract of healthy dogs (5). Clinical studies have shown that they can be detected in the throat of every single healthy animal tested (3, 5). Based on different studies most pathogenic is *M. cynos* which is associated with canine infectious respiratory disease, frequent disease, especially in dog shelters (6, 7).

Some mycoplasmas can cause infections in different parts of the urinary and reproductive system. Most commonly *M. canis* was found, but also *M. spumans*, *M. edwardii*, *M. cynos*, *M. molare* and *M. maculosum* were isolated (8, 9). Canine mycoplasmas can be detected directly by culture or molecular methods and indirectly by serological methods. Culture is still the golden standard for the detection of mycoplasmas in

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samples, despite a long turnaround time and complex species determination that bring about an additional need for serological, biochemical or molecular testing. Compared to culture, molecular methods based on nucleic acid amplification tests such as PCR (polymerase chain reaction) are faster and easier for pathogen identification (3, 10). For detection of specific mycoplasma antibodies, serological methods are required such as enzyme immunoassays, immunofluorescence, agglutination tests and immunoblotting (7, 11, 12, 13). DIBA where mycoplasma cells are directly applied on the membrane can be used to detect specific antibodies (14). The aim of this study was to establish the prevalence of canine mycoplasmas especially those which are usually linked with clinical disease *(M. cynos, M. canis)* in clinically healthy working dogs in Slovenia. DIBA was used for simultaneous detection of specific antibody to 3 different canine mycoplasma species and consensus PCR was used for detection of the mycoplasma genomic DNA.

Materials and methods

Animals and samples

Thirty-four dogs of various breeds, gender and age were included in this research (Table 1). Among them, 27 were working dogs (aged between 2 to 10 years) from Slovene armed forces, stationed in the south-east part of Slovenia and 7 working sheepdogs (younger than 1 year), stationed on pastures in the south-west of Slovenia. Blood samples and swabs (oral, vaginal or preputial) were examined. The military working dogs were sampled in the years 2008 and 2009. First sampling of 27 military working dogs was done in the year 2008, but only 17 dogs were resampled next year. The sheepdogs were sampled in 2013.

Blood samples for complete blood count (CBC) including white cell differential count determinations were collected into EDTA-containing tubes (Microtainer TM, Beckton and Dickinson, Franklin Lakes, USA) by venipuncture of *v. jugularis*. For serologic testing, the blood was stored into serum separator tubes (Vacuette, Greiner Bio-One, Kremsmunster, Austria) and left for 30 minutes at room temperature to clot, then was centrifuged at 1300×g for 10 minutes to separate the serum. Serum samples were analysed for antibodies to *M. cynos*, *M. canis* and *M. molare*.

Vaginal or prepuce and oral swabs for molecular detection of mycoplasma were collected with a sterile cotton swab (Sterile, Meus S.r.l., Piove di Sacco, Italy) and transported to the laboratory in a cold pack. The swabs were stored at –70 °C until processing.

Bacterial strains

For DIBA type strains of *M. cynos* (strain H831^T), *M. canis* (strain PG14^T) and *M. molare* (strain H542^T) were used as antigens. The mycoplasma strains were cultured on modified Frey`s medium described previously (15). For DIBA broth cultures were harvested by centrifugation (20.000×g for 10 min) before reaching the stationary growth phase. *Mycoplasma* cells were washed in phosphate buffered saline (PBS, pH 7.2) and diluted 1: 500 for DIBA.

Blood analyses

The haematological parameters were determined by an automated laser haematology analyser H*1 (Siemens/Bayer (former Technicon), Munich, Germany) with species specific software (H*1 Multi-Species V30 Software, Tarrytown, NY, USA).

Dot-Immunobinding assay (DIBA)

DIBA was used to determine antibodies against to *M. cynos, M. canis* and *M. molare* in dog serum samples as was previously described (14, 16, 17). Briefly, two microliters of bacterial antigens, internal positive control (1:1000 diluted dog sera) and negative control (modified Frey`s medium) were dotted as separate dots on strips cut from PVDF membrane (Immobilon–P, Merck Millipore, Billerica, MA, USA). The strips were first blocked for one hour in 0.5% Tween 20 in PBS and then incubated in diluted (1:100) dog serum samples for one hour at room temperature, and after that washed three times for 15 min in PBS containing 0.05% Tween 20. Then they were incubated in diluted (1:2000) rabbit anti-dog horseradish peroxidase conjugated (HRP) antibodies (catalogue number: A6792) (Sigma Aldrich, St. Louis, MO, USA) for 45 min at room temperature. After two 10-minute washes in PBS containing 0.05% Tween 20 and one in PBS, strips were treated with

chromogenic substrate TrueBlue™ (Kirkegaard and Perry Laboratories, Milford, MA, USA). Evaluation of DIBA results by personal estimation of the intensity of the blue color on the place of the reaction was done immediately after the test was finished.

DNA extraction, PCR and sequencing

Prior DNA extraction 2 ml of sterile PBS was added to genital and oral swabs and vortexed vigorously. Total DNA was extracted using the commercial reagents of QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

For samples obtained in 2008 the semi-nested PCR for amplification of 1500 bp long 16S rRNA gene described by Johansson (18) and colleagues was used. For samples obtained in 2009 and 2013 the PCR for amplification of approximately 620 bp long 16S/23S IGS region of different canine mycoplasma species were used (19). The extracted DNA of *M. canis* (strain PG14^T) was used as positive control in the PCR assays.

The PCR products were analyzed by electrophoresis on a 1.8% ethidium bromide stained agarose gel. DNA fragments were excised from the gel and purified with Wizard PCR Preps DNA Purification System (Promega, Madison, WI, USA) and sent for sequencing to Macrogen laboratory (Macrogen Inc, Amsterdam, the Netherlands).

The nucleotide sequences were downloaded using Chromas (Technelysium Pty Ltd., Queensland, Australia). Nucleotide sequence data were analyzed by BLAST (20) for finding similar nucleotide sequences from NCBI sequence database. Sequences with ≥ 99% nucleotide homology with available sequence in database were determined as mycoplasma species. In the cases of mixed sequencing chromatograms mixed mycoplasma infection (*Mycoplasma* spp.) was specified.

Results

Animals

Clinical examination prior sampling and haematological results for 34 dogs did not show any signs of diseases.

DIBA analysis of canine blood serum

DIBA test was used for simultaneous detection of specific antibodies against *M. canis*, *M. cynos* and *M. molare*. 34 dog serum samples were tested, 27 from military working dogs sampled in 2008 and 7 from sheepdogs taken in 2013 (Table 1). Specific antibodies against at least one of the mycoplasma species were detected in 32 (94.1%) samples. Among them 8/34 (23.5%) dogs were positive only to *M. cynos*, 7/34 (20.6%) dogs were positive only to *M. canis* and none of the samples were positive only to *M. molare.* On the other hand, 16/34 (47.0%) samples were positive to both *M. cynos* and *M. canis* and only one dog No 18 (2.9%), had specific antibodies to all three mycoplasmas tested.

PCR analysis and DNA sequencing results of canine swabs

In 2008 18.5% (5/27) swabs of different male military dogs were PCR positive, among these two oral (7.4%) and 3 preputial swabs (11.1%) (Table 1). As presented in Table 1 the analyses of partial sequences (680 to 1082 nt) of 16S RNA gene of positive samples from 2008 showed mixed mycoplasma infections in 2 preputial swabs (dogs No. 11, 30) and 2 oral swabs (No. 16, 31). In one preputial swab dog No. 18 mixed infection with ureaplasma was detected.

Seventeen military dogs were sampled again in 2009. Six dogs (35.3%) were positive, 5 genital swabs (29.4%) and 1 oral swab (5.9%). The prevalence of mycoplasmas in sheepdogs was 57.1% (4/7), 4 oral swabs (57.1%) and one genital swab (14.3%). In female sheepdog No. 1 both oral and vaginal swabs were positive. The results of nucleotide sequence analysis of positive samples are shown in Table 1. *M. cynos* was confirmed in 20% (1/5) of positive oral swabs. *M. canis* was confirmed in 80% (4/5) of positive oral swabs. In positive genital swabs (6/24), *M. canis* was determined in 50% (3/6) cases, in one case together with *M. spumans*. *M. edwardii* and *M. maculosum*, were present in 16.7% of positive genital samples, not at the same time. In one case, in preputial swab of male dog no. 11, mixed not determined mycoplasma infection was confirmed as in the first testing at 2008.

Table 1: Results of mycoplasmal DNA and specific antibodies screening in 34 working dogs using molecular PCR and serological method DIBA

Dog no.	Bread	Gender	Age*	PCR **2008	PCR **2009 / 2013	DIBA	
						M. canis	*M. cynos*
1	TOR	F	4m	/	*M. edwardii* (V), *M. cynos* (O)	**neg**	**pos**
2	TOR	M	6m	/	*M. canis* (O)	**pos**	**pos**
3	KSD	F	6m	/	neg	**pos**	**pos**
4	KSD	M	1	/	*M. canis* (O)	**pos**	**pos**
5	KSD	M	1	/	*M. canis* (O)	**pos**	**pos**
6	TOR	F	1	/	neg	**pos**	**pos**
7	TOR	F	1	/	neg	**pos**	**neg**
8	GSD	M	2	neg	neg	**pos**	**pos**
9	MN	M	3	neg	nd	**pos**	**pos**
10	MN	M	2	neg	neg	**pos**	**neg**
11	LR	M	2	*Mycoplasma* spp. (P)	*Mycoplasma* spp. (P)	**neg**	**pos**
12	MN	M	5	neg	*M. canis* (P)	**pos**	**pos**
13	MN	M	7	neg	nd	**pos**	**neg**
14	MN	M	2	neg	*M.canis.* (P)	**pos**	**neg**
15	MN	M	3	neg	nd	**pos**	**neg**
16	LR	M	2	*Mycoplasma* spp. (O)	nd	**neg**	**pos**
17	X	F	4	neg	*M. canis, M.spumans* (V)	**neg**	**pos**
18	MN	M	7	*Ureaplasma spp.* (P)	nd	**pos**	**pos**
19	MN	M	10	neg	neg	**pos**	**neg**
20	GSD	F	5	neg	*M. maculosum* (V)	**neg**	**neg**
21	MN	M	6	neg	nd	**neg**	**pos**
22	MN	M	5	neg	neg	**neg**	**pos**
23	GSD	M	4	neg	neg	**pos**	**neg**
24	GSD	F	5	neg	*M. canis* (O)	**pos**	**pos**
25	GSD	M	7	neg	neg	**pos**	**pos**
26	GSD	M	3	neg	neg	**neg**	**neg**
27	MN	M	2	neg	neg	**pos**	**pos**
28	GSD	M	9	neg	neg	**pos**	**pos**
29	MN	M	4	neg	nd	**pos**	**pos**
30	GSD	M	7	*Mycoplasma* spp. (P)	nd	**pos**	**pos**
31	MN	M	2	*Mycoplasma* spp. (O)	nd	**pos**	**pos**
32	GSD	M	4	neg	neg	**neg**	**pos**
33	MN	F	3	neg	neg	**neg**	**pos**
34	MN	M	2	neg	nd	**pos**	**pos**

Dog numbers 1-7 present samples of sheepdogs, dog numbers 8-34 present samples of military dogs. *age at first sampling, m month; ** year of the sampling; TOR Tornjak- Bosnian and Herzegovininan- Croatian shepherd dog; KSD Karst shepherd dog; GSD German shepherd dog; LR labrador retriever; X mixed breed; MN Malinois; O oropharyngeal swab; P preputial swab; V vaginal swab, nd not done.

Discussion

This is the first extended mycoplasma detection study in healthy working dogs where mycoplasma specific antibodies and molecular mycoplasma detection from oral and genital samples were included. Rare studies were done regarding the prevalence of mycoplasmas in dogs (3, 5, 8, 21) and there is no published data regarding seroprevalence of mycoplasma infections in healthy dogs. In a previous study, where diseased and healthy dogs were included, the haemagglutination-inhibition (HI) assay revealed ~ 47% of dogs with antibodies to *M. cynos* (13). The HI assay is very specific but may be influenced by *M. cynos* strain causing infection and its strain used as HA antigen. In this study for simultaneous detection of specific antibodies against dog mycoplasmas *M. cynos*, *M. canis* and *M. molare* DIBA was used as a serological test. The presence of specific antibodies was confirmed in 32/34 (94%) tested samples, at least against one species of mycoplasma in question. With regard to previous studies, where Doig and others (8) reported 33.3% to 76.19% (16/21) prevalence of *M. canis* in mycoplasma positive genital swabs and Chalker and others (6) found 21.8% prevalence in oral swabs of healthy dogs, we can speculate that such high number of positive reactions to *M. canis* and *M. cynos* antigens in our study is probably the result of cross reactivity between different mycoplasmas. Despite the simplicity of DIBA the main disadvantage is that the evaluation of the test is based on subjective decisions and that is why it is sometimes hard to define the intensity of the reaction with specific antigens. This could be avoided using densitometry that can provide a quantitative evaluation of the result (23, 24). Regarding high serological prevalence in tested dogs, antibody titer determination and demonstration of increased antibody titer two to three weeks apart should be performed to establish the criteria for confirmation of acute onset of infection.

With PCR method and sequencing, the presence of mycoplasmas was detected in 57.1% (4/7) of younger dogs (≤ 1 year) and in 33.3% (9/27) of older dogs. It has been reported that the infection with mycoplasmas is more common in young dogs than in older ones (5). In 2008 mycoplasma and ureaplasma were confirmed only in male dogs whereas the prevalence of mycoplasmas was higher 50% (4/8) in females compared with 37.5% (6/16) in males sampled in 2009 and 2013. Genital swabs were positive in 60% (9/15) cases and oral in 46.7 % (7/15) cases. In the study by Rosendal (22) on healthy dogs, mycoplasmas were detected in all naso-oro-pharyngeal cavity and in 70 to 75% cases of genital samples. Very high prevalence (86.7%) of mycoplasmas in upper respiratory tract of dogs without respiratory signs was reported by Schulz and others (25). These results coincide with our results, where 94% of the tested dogs were seropositive. On the contrary, Hong and Kim (21) detected mycoplasma only in diseased dog in a group of laboratory dogs. Similarly, to our study, the detection was done by molecular methods directly from clinical samples without previous isolation of the mycoplasma, while in studies mentioned above (22, 25) isolation of bacteria was performed. The difference between results in different studies is probably also due to the different health background of included dogs; from dogs that were euthanized because of other diseases (25) to laboratory dogs (21). Dogs included in this study were healthy, without any history of respiratory infections or reproductive problems. The good general health status of the dogs could influence the lower mycoplasma presence. In one case, *M. cynos,* and in three cases, *M. canis*, were simultaneously confirmed by PCR and by serology. Although positive serology results in this study indicate exposure to mycoplasma in the past, it is also possible than an undetectable quantity of mycoplasma is present in mucosa and affects serology results. Unfortunately, because of the small number of samples in our study, it is difficult to compare the results with other studies where a larger number of dogs was included.

As is the case in other studies (6, 8), we confirmed that *M. canis* is one of the most common mycoplasma species present in dogs. In our study, altogether, *M. canis* was detected in 46.7% (7/15) mycoplasma-positive dogs. We obtained 33.3% (1/3) prevalence of *M. canis* in positive vaginal swab and 40% (2/5) prevalence of *M. canis* in mycoplasma positive preputial swabs. In positive oral swabs *M. canis* was confirmed in 57.1% (4/7). As was mentioned earlier, for true estimation of prevalence a larger number of dogs should be tested. Second most common mycoplasma infection was mixed infection. Infections with at least 2 *Mycoplasma* species (usually *M. cynos* and *M. canis*) in 42.9% of bitches with no clinical signs

or any other disturbances in reproduction cycle and in 13.3% prepuce swabs of healthy male dogs with at least two mycoplasmas were confirmed by Doig and others (8) in previous study. We found mixed infection in 33.3% (1/3) positive vaginal swabs and in 83.3% (5/6) mycoplasma or ureaplasma positive preputial swabs. The mixed infection was detected also in 28.6% (2/7) positive oral swabs. In one case (dog no. 17) mycoplasma species were determined successfully, in all other cases with mixed infection PCR cloning should be done.

Despite the differences between the seropositive results and positive PCR samples, PCR is a suitable method for fast diagnostics, but application of consensus primers for clinical samples should not be the method of choice. The main disadvantages are nonspecific positive reactions and in case of mixed infections the pathogen species can be masked by others. Based on known data obtained in previous studies the use of species specific primers would make this diagnostic method more suitable for routine use (6, 21). In cases when direct detection of pathogen fails, serology can help with diagnostics. After optimization of the method, DIBA could be included in the routine clinical practice as a screening serological test.

Despite several studies, comprehensive data about mycoplasma presence in dogs is missing. Since data about prevalence of mycoplasmas in different studies is controversial, regardless of the method of detection (PCR or culture) or chosen group of dogs, further studies should be done to accurately evaluate positive results, also in the context of pathogenicity of canine mycoplasmas.

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References

1. Razin S, Yogev D, Naot Y. Molecular biology and pathogenicity of mycoplasmas. Microbiol Mol Biol Rev 1998; 62: 1094–156.

2. Rosengarten R, Citti C, Glew M, et al. Host-pathogen interactions in mycoplasma pathogenesis:virulence and survival strategies of minimalist prokaryotes. Int J Med Microbiol 2000; 290(1): 15-25.

3. Chalker VJ. Canine mycoplasmas. Res Vet Sci 2005; 79: 1–8.

4. Spergser J, Langer S, Muck S, et al. *Mycoplasma mucosicanis sp. nov.*, isolated from the mucosa of dogs. Int J Syst Evol Microbiol. 2011; 61(4): 716–21.

5. Randolph JF, Moise NS, Scarlett JM, et al. Prevalence of mycoplasmal and ureaplasmal recovery from tracheobronchial lavages and prevalence of mycoplasmal recovery from pharyngeal swab specimens in dogs with or without pulmonary disease. Am J Vet Res 1993; 54: 387–91.

6. Chalker VJ, Owen WMA, Paterson C, et al. Mycoplasma associated with canine infectious respiratory disease. Microbiology 2004; 150: 3491–7.

7. Rycroft AN, Tsounakou E, Chalker V. Serological evidence of *Mycoplasma cynos* infection in canine infectious respiratory disease. Vet Microbiol 2007; 120: 358–62.

8. Doig PA, Ruhnke HL, Bosu WT. The genital mycoplasma and ureaplasma flora of healthy and diseased dogs. Can J Comp Med 1981; 45: 233–8.

9. Ülgen M, Cetin C, Sentürk S, et al. Urinary tract infections due to *Mycoplasma canis* in dogs. J Vet Med A Physiol Pathol Clin Med 2006; 53: 379–82.

10. Waites KB, Xiao L, Paralanov V, Viscardi RM, Glass JI. Molecular methods for the detection of Mycoplasma and ureaplasma infections in humans: a paper from the 2011 William Beaumont Hospital Symposium on molecular pathology. J Mol Diagn. 2012; 14(5): 437–50.

11. Busolo F, Tonin E, Conventi L. Enzyme-linked immunosorbent assay for detection of *Mycoplasma pneumoniae* antibodies. J Clin Microbiol 1980; 12: 69–73.

12. Kumar A, Srivastava NC, Singh VP, et al. Electrophoretic analysis of indian isolates of *Mycoplasma agalactiae* and *Mycoplasma bovis* by SDS – PAGE and immunoblotting. Vet Med Int 2014; 2014: e892421 http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3998006/ (5. 7. 2014)

13. Kastelic S, Cizelj I, Narat M, et al. Molecular characterisation of the *Mycoplasma cynos* haemagglutinin HapA. Vet Microbiol 2015; 30; 175(1): 35–43.

14. Benčina M, Cizelj I, Berčič RL, Narat M, Benčina D, Dovč P. Shared epitopes of avian im-

munoglobulin light chains. Vet Immunol Immunopathol. 2014; 158(34): 175–81.

15. Berčič RL, Cizelj I, Benčina M, Narat M, Bradbury JM, Dovč P, Benčina D. Demonstration of neuraminidase activity in *Mycoplasma neurolyticum* and of neuraminidase proteins in three canine Mycoplasma species. Vet Microbiol 2012; 155(2/4): 425–9.

16. Narat M, Biček A, Vadnjal R, Benčina D. Production, characterization and use of monoclonal antibodies recognizing IgY epitopes shared by chicken, turkey, pheasant, peafowl and sparrow. Food Technol Biotechnol 2004; 42(3): 175–82.

17. Benčina D, Narat M, Bidovec A, Zorman-Rojs O. Transfer of maternal immunoglobulins and antibodies to *Mycoplasma gallisepticum* and *Mycoplasma synoviae* to the allantoic and amniotic fluid of chicken embryos. Avian Pathol 2005; 34(6): 463–72.

18. Johansson KE, Heldtander MU, Pettersson B. Characterization of mycoplasmas by PCR and sequence analysis with universal 16S rDNA primers. Methods Mol Biol. 1998; 104: 45–65.

19. Chalker VJ, Brownlie J. Taxonomy of the canine Mollicutes by 16S rRNA gene and 16S/23S rRNA intergenic spacer region sequence comparison. Int J Syst Evol Microbiol 2004; 54: 537–42.

20. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignement search tool. J Mol Biol 1990; 215: 403-10.

21. Hong S, Kim O. Molecular identification of *Mycoplasma cynos* from laboratory beagle dogs with respiratory disease. Lab Anim Res 2012; 28: 61–6.

22. Hawkes R, Niday E, Gordon J. A dot-immunobinding assay for monoclonal and other antibodies. Anal Biochem 1982; 119: 142–7.

23. Furuya K, Noro S, Yamagishi T, et al. Adsorption of influenza viruses to nitrocellulose membrane filters by filtration and their quantitative densitometric determination. J Virol Methods 1984; 9: 193–9.

24. Rosendal S. Canine mycoplasmas: their ecologic niche and role in disease. J Am Vet Med Assoc 1982; 180: 1212–4.

25. Schulz BS, Raufeisen K, Weber K, Laberke S, Hartmann K. Comparison of the prevalence of Mycoplasma species in dogs with and without respiratory disease. Berl Munch Tierarztl Wochenschr. 2015; 128(7/8): 304–9.

MOLEKULARNA DETEKCIJA IN SEROPREVALENCA MIKOPLAZEM PRI KLINIČNO ZDRAVIH DELOVNIH PSIH

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Povzetek: Namen raziskave je bil določiti seroprevalenco in prevalenco mikoplazem pri klinično zdravih delovnih psih. V raziskavo je bilo vključenih 34 delovnih psov različnih pasem in starosti, od tega 27 psov iz Slovenske vojske in 7 ovčarskih psov. Za dokazovanje specifičnih protiteles proti bakterijam *Mycoplasma cynos, Mycoplasma canis in Mycoplasma molare* smo uporabili metodo točkastega imunskega odtisa (ang. Dot Immuno Binding Assay- DIBA) in konvencionalni PCR, ki temelji na pomnoževanju odseka gena za ribosomalno RNK 16s ali intergenskega odseka genoma med genoma ribosomalnih RNK 16s in 23s. Specifična protitelesa proti vsaj eni od izbranih vrst mikoplazem so bila ugotovljena pri 94.1 % psov. Med njimi je 23.5 % vzorcev reagiralo pozitivno samo na *M. canis*, 20.6 % samo na *M. canis* in noben od vzorcev ni reagiral pozitivno samo na *M. molare*. Skupno je 47.0 % vzorcev reagiralo pozitivno na *M. canis* in *M. canis* hkrati, en pes (2.9 %) je imel specifična protitelesa proti vsem trem testiranim mikoplazmam. Z metodo PCR smo mikoplazme dokazali v vzorcih 57.1 % psov mlajših od enega leta, in pri 18.5 % do 35.3 % starejših od enega leta, odvisno od leta vzorčenja. Genitalni brisi so bili pozitivni v 60 % primerov v primerjavi z oralnimi kjer je bil delež 46.7 %. *M. canis* je bila ugotovljena v 40 % pozitivnih primerov, v enakem deležu so bile ugotovljene tudi mešane nedeterminirane mikoplazemske okužbe. Mikoplazme, kot so *M. cynos*, *M. edwardii*. *M. maculosum* in *M. spumans* so bile ugotovljene posamično. V enem primeru je bila ugotovljena mešana okužba z ureaplazmami.

Ključne besede: delovni psi; pasje mikoplazme; *Mycoplasma canis*; *Mycoplasma cynos*; DIBA; PCR

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Original Research Article

CORTISOL CONCENTRATIONS IN HAIR, BLOOD AND MILK OF HOLSTEIN AND BUSHA CATTLE

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Abstract: Cortisol levels were measured in hair, blood and milk in two different cattle breeds, kept under different breeding conditions and with different genetic merit for milk production. Cows and heifers of Holstein and Busha breeds were selected for the study. Cortisol concentration was determined by immunoassays. Cortisol accumulation was determined in proximal (close to the skin) and distal (far from the skin) segments of the hair shaft. The influence of hair colour and washing prior to extraction and analysis was also examined in order to establish additional factors that may have an impact on hair cortisol concentrations. Concentrations of cortisol determined in the proximal and distal segments of the shaft were significantly higher in Holstein than Busha cows and heifers ($P<0.05$ and $P<0.01$, respectively). In Holstein cows, no significant difference was found between concentrations in black and white hair. In hair washed with isopropanol, cortisol concentration was significantly lower compared to unwashed hair ($P<0.01$). Thus, cortisol concentration in hair varies with the technique of hair processing (washing), but not with colour in Holstein cows. Blood serum cortisol concentrations in Holstein cows and heifers were significantly higher than in Busha cows and heifers, ($P<0.01$ and $P<0.05$, respectively). Milk cortisol in Holstein cows was significantly higher than in Busha cows ($P<0.05$). The higher cortisol concentrations in Holstein cows are assumed to be the result of intensive breeding and physiological adaptation to high milk production.

Key words: cattle; cortisol; hair; blood; milk

Introduction

Determination of blood cortisol concentration is used as a standard procedure for evaluating stress in farm animals, since excess cortisol is synthesized and released into the systemic circulation under stressful conditions (1). Environmental and management changes are main stressors influencing cattle physiology (2). In general, cortisol helps to maintain homeostasis in the body by aiding energy metabolism, reproduction, immune response, inflammatory processes, growth and brain function. Nevertheless, prolonged elevation of glucocorticoid levels negatively influences reproductive activity or immune response (3, 4). Due to its involvement in lactation, it may be assumed that breeds with different genetic aptitudes for milk production have different hypothalamic-pituitary-adrenal (HPA) axis activities since, in high-producing cows, the HPA axis must be recruited in order to re-establish homeostasis (5).

Cortisol can be measured in blood, saliva, milk and hair and its metabolites in urine and faeces (2, 6, 7). Since blood sampling is a stressful factor, cortisol is usually determined in body excreta and integuments, like hair, wool, and coat. The

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latter are milk, faeces, saliva, urine and hair and concentrations of cortisol and its metabolites are well established in these sources (2, 5, 7, 8, 9).

Cortisol concentration in the blood of cattle can vary due to circadian rhythmicity (10) and several extrinsic factors, such as cold, heat, humidity and wind (11). Cortisol concentrations in blood, saliva and urine reflect the HPA axis function shortly after its activation, faecal cortisol (due to the passage of gut contents) shows HPA activity from two days prior to measurement, while cortisol in hair reflects the concentration of cortisol that has been produced over longer periods (weeks or even months) (2). For this reason, the determination of cortisol in hair has been used for estimating chronic stress in animals (12, 13).

Based on such measurements in hair, most authors have emphasized that the transfer of cortisol from blood to hair is a reflection of the HPA axis activity (7, 14). It may thus be considered that the changes in the environment and management system, perceived as stressors for cattle, can have an impact on levels of cortisol in hair (2).

It is assumed that high-yielding cows, such as Holstein, are commonly exposed to various stress factors (heat, cold, high humidity, being handled) which, combined with high milk production, affect negatively the maintenance of the homeostatic mechanism, leading to a variety of metabolic and reproductive disorders (15). In contrast, natural grazing, as the unique feeding programme for extensive breeds such as Busha cattle, has a very favourable impact on the behaviour of animals and reduces their exposure to stress (16). Holstein and Busha are breeds with different breeding management and milk production. High-yielding Holstein cows are distributed all over the world, while the autochthonous low-yielding Busha breed is characteristic for the Balkan area. Unlike the Holstein breed, that is genetically adapted to high milk production and is kept under controlled feeding and breeding conditions, Busha is characterized by modest needs for food, low milk production and free grazing on pastures (17). High milk production in the Holstein breed is combined with a modified endocrine status (as increased somatotropin and decreased insulin concentrations) that allows increased milk production without metabolic disorders (18).

The aim of this study has been to establish baseline cortisol concentrations in cattle breeds that differ in both breeding conditions and their genetic merit for milk production. Cortisol accumulation in hair has been determined by measuring its concentration in the proximal and distal parts of the hair shaft and in the hair of different colours (black and white). Additionally, to distinguish the amounts of cortisol within or on the surface of the hair shaft, its concentration was determined in unwashed and washed hair. The influence of age on cortisol accumulation in hair was estimated by determining its concentration in the hair of heifers and cows. Moreover, blood and milk cortisol concentrations were compared in different breeds.

Materials and methods

Animals

Cows of Holstein (n=25) and Busha breeds (n=13) and heifers of Holstein (n=12) and Busha (n=11) breeds were selected. Heifers were 12 to 20 months of age, and cows between 4 and 8 years of age. Hair, blood serum, and milk cortisol were determined in 13 Holstein and 13 Busha cows. An additional twelve Holstein cows were selected solely for comparison of the cortisol contents of white and black hair. Holstein cattle were kept under a loose housing system in a deep litter system. They were kept in different groups formed according to their productive phase; i.e. heifers, milking cows and dry cows were kept separately. Holstein cattle were fed with total mix ration twice a day, with water consumed *ad libitum*. Busha cattle were kept free in an extensive production system, fed on mountain pastures for 24 hours a day, consuming water *ad libitum,* without the addition of concentrated feed. Covered areas on the pasture were used for shelter during poor weather conditions, like rainy and/or sunny days. All cows were in the stage of mid to late lactation. Holstein cows were milked twice daily, while Busha cows were not milked, but suckled by their calves.

The animal-related component of the study was approved by the Ethical Committee of the Faculty of Veterinary Medicine, University of Belgrade in accordance with the National Regulations on Animal Welfare.

Sampling

Hair samples (0.5 g) from cows and heifers of the two breeds were taken from the middle area of the tail, directly next to the skin surface, using an electric hair clipper. The hair length was 2 cm on average. To estimate any relation between hair colour and cortisol concentration, both black and white hair samples were taken from 12 Holstein cows. This was done with Holstein cows only, since Busha cows have single-coloured wild-type hair. To determine whether cortisol concentrations varied along the length of the hair shaft, hair samples were cut approximately in half into proximal and distal sections in both cows and heifers. Furthermore, any differences in cortisol level between unwashed and washed hair were determined using hair samples only from Holstein and Busha heifers. The hair samples obtained were packaged in plastic zip pouches and stored at -20 °C until analysed.

Blood samples (9 mL) were taken at the same time in the morning from Holstein cattle before feeding and from Busha cattle before pasture. Blood samples were taken from the jugular vein using vacutainers without anticoagulant and allowed to clot at room temperature for not more than 30 minutes. The tubes were then centrifuged at 2500 g for 10 minutes. Serum was decanted, portioned into aliquots of 1.5 mL, and stored in polypropylene microtubes at -20 °C until analysed.

Milk samples (30 mL) were taken from Holstein cows during regular morning milking, and from Busha cows at the same time as the blood samples. Milk samples were frozen at -20 °C and stored until analysed.

Procedures

Extraction and determination of hair cortisol

Hair was ground using a ball mill Millmix 20 (Tehtnica, Železniki, Slovenia). Approximately 0.2 g of hair sample was placed in liquid nitrogen for 10 seconds, then pulverized by grinding for 5 minutes at 1500 RPM in a cooled ball mill chamber. Cortisol was extracted from the 0.1 g of hair powder with 1.1 mL of 55% methanol. The mixtures were shaken at 500 RPM for 30 minutes, then centrifuged at 2500 g for 20 minutes at 4 °C (19). The supernatant was collected and stored in plastic tubes at -20 °C until analysis. The washed hair samples were processed in the same manner.

For the determination of cortisol concentration in washed hair, 0.2 g of hair sample was placed in a plastic tube and 3 mL of isopropanol (Carlo Erba, Milano, Italy) added. The tubes were shaken gently at 300 RPM for 3 minutes, the isopropanol then decanted and the washing procedure repeated (7). Hair samples were transferred from tubes to glass beakers and dried at 40 °C for 2 hours.

Cortisol concentrations in hair extracts were determined using a commercial, Cortisol Enzyme-linked immunosorbent assay (ELISA) kit (Demeditec, Kiel, Germany), following the instruction manual. The absorbance was measured with a microtiter plate photometer Multiskan FC (Thermo Fisher Scientific, Waltham, USA) at 450 nm. Concentrations of cortisol in hair extracts were expressed as ng per g of hair.

Partial validation of the ELISA kit, which included determination of the intra-assay and inter-assay coefficients of variation (CV) and recovery for hair cortisol was assessed. Samples of hair extracts with low and high cortisol concentrations were run 20 times in one ELISA test and repeated in triplicate in the next ELISA test. Recovery was tested by adding known amounts of hydrocortisone (Sigma-Aldrich, St. Louis, USA) to hair samples with previously determined cortisol concentrations.

Intra- and inter-assay CVs for hair cortisol were 7.68% and 8.93% for high (272 ng/g) and 7.49% and 7.90% for low (147 ng/g) cortisol values. On addition of hydrocortisone to hair samples in amounts of 250 ng/g and 125 ng/g recoveries were 124% and 101%, respectively.

Determination of blood and milk cortisol

Cortisol concentration in bovine blood serum was measured using the Radioimmunoassay (RIA) cortisol kit (INEP, Zemun, Serbia) according to the method described by Brkljačić (20). Milk samples were thawed in a water bath at 37 °C. The milk tubes were vortexed, then centrifuged at 2500 g for 20 minutes. Fat was removed from the surface using a vacuum pump and a few drops of rennet (SIRELA, Čačak, Serbia) added. The samples were incubated at 37 °C for 20 minutes, then centrifuged at 2500 g for 15 minutes to extract the milk serum. The separated milk serum was put into labelled tubes. 0.5 mL samples of the milk serum were placed in a test tube and dried under a nitrogen evaporator to

complete dryness. To the test tube with the dried milk serum, 125 µL of PBS was added and vortexed. The extract was used to perform the RIA test using the same Cortisol RIA kit used for determining cortisol concentrations in blood serum samples. Intra- and inter-assay coefficients of variation (CV) for cortisol concentrations in blood and milk were 5% and 10%.

Statistical Analysis

The data obtained were analysed statistically using STATISTICA v.8. (StatSoft, Inc., Tulsa, OK, USA) commercial software. The normality of data distribution was tested using Shapiro Wilk's W test. All the data were normally distributed, except data for Holstein blood cortisol concentrations. As average values, arithmetic means were determined for homogenous data and medians for heterogeneous values in the group. Cortisol concentrations in hair from cows and heifers of different breeds were compared using factorial ANOVA with the Fisher LSD *post hoc* test. Cortisol concentrations were compared statistically from white and black hair and from unwashed and washed hair and proximal and distal segments using dependent sample *t*-tests. Differences of average values of the blood cortisol between groups were computed using the Kruskall-Walis test and the Mann-Whitney U-test. Differences in milk cortisol concentrations between groups were analysed using independent sample *t*-test. *P* values <0.05 are considered significant.

Results

Hair cortisol concentrations were higher in Holstein than in Busha cows, in both compared proximal and distal hair segments ($P<0.05$ and $P<0.01$, respectively). Concentrations of cortisol in both the proximal and distal segments of hair were also higher in Holstein than in Busha heifers ($P<0.01$, respectively). There was no statistical difference between Holstein cows and heifers, either between the proximal segments of hair or between the distal segments of hair. In Busha cattle, cows had significantly higher cortisol concentrations than heifers, in both the proximal and distal segments of hair ($P<0.01$, respectively).

Cortisol concentrations from proximal and distal parts of hair from Holstein cows and heifers did not differ significantly. In contrast, in the Busha breed, in both cows and heifers, cortisol concentrations were significantly higher in proximal than in distal parts of the hair ($P<0.01$ and $P<0.05$, respectively). In general, average baseline hair cortisol concentration was higher in the proximal compared to the distal part, but significantly only in the Busha breed (Table 1).

Cortisol concentrations in the black hair of Holstein cows did not differ significantly from those in the white hair ($P=0.53$) (Table 2).

Cortisol concentrations in unwashed hair were significantly higher than those in washed hair in both Holstein and Busha cattle ($P<0.01$, respectively) (Figure 1). In the present study cortisol concentrations observed in washed hair of Holstein and Busha heifers were lower by 21% and 32%, respectively.

Blood serum cortisol concentrations did not differ significantly between cows and heifers, in either Holstein or Busha breeds. However, cortisol concentrations were significantly higher in Holstein than in Busha cattle, both for cows and heifers ($P<0.01$ and $P<0.05$, respectively) (Figure 2). Milk serum cortisol concentrations differed significantly ($P<0.05$) between examined breeds (Table 3).

Discussion

Determination of cortisol concentration in hair provides an estimate of long-term cortisol synthesis in an organism (14). Since the mechanisms and intensities of cortisol loading into and onto hair shafts are not completely clear, we sought information about cortisol levels in different parts of the hair. Thus, the hair shaft is considered as a three-dimensional object so cortisol levels were determined considering the activity of its accumulation on the surface and inside the hair and along the hair shaft. The colour of the hair was also considered. Finally, cortisol values in hair were compared with those in blood and milk. To the best of our knowledge, this is the first study that deals with hair cortisol in cattle in such an integrative and comparative manner, between two different breeds.

Reports of cortisol concentrations in various parts of the hair shaft differ. In humans and horses, the cortisol level decreases along the hair shaft (21, 22) while no differences were found in rhesus macaques (12) or dogs (13). As can be

Table 1: Comparison of hair cortisol concentrations (ng/g) in Holstein and Busha cattle

	Hair cortisol concentration (ng/g)	
Holstein cows	proximal part	distal part
Mean	233.92[A, a]	229.00[A, a]
SEM	16.78	18.59
range	103.00 – 293.00	80.00 – 313.00
Holstein heifers		
Mean	217.22[A, a]	190.00[A, a]
SEM	20.52	29.39
range	150.00 – 342.00	61.00 – 320.00
Busha cows		
Mean	188.85[B, a]	168.65[B, b]
SEM	13.52	13.27
range	109.00 – 300.00	95.50 – 275.50
Busha heifers		
Mean	120.54[C, a]	100.91[C, b]
SEM	9.54	37.81
range	67.00 – 166.00	24.00 – 161.00

[A, B, C] – Values in the same columns with different superscripts are significantly different
[a, b] – Values in the same rows with different superscripts are significantly different

Table 2: Comparison of cortisol concentrations (ng/g) between white and black hair in Holstein cows (N=12)

	Hair cortisol concentrations (ng/g)	
	white hair	black hair
Mean	172.58	189.42
SEM	24.47	25.76
range	64.00 – 332.00	87.00 – 358.00

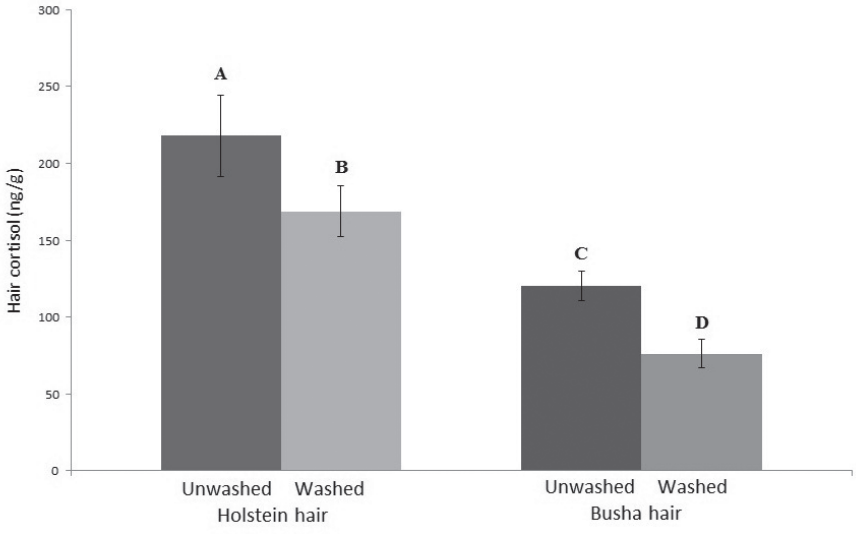

Figure 1: Cortisol concentration in unwashed and washed hair of Holstein and Busha heifers. Data plotted represent the mean ratio value ± SEM

[A,B,C,D] – Different superscripted letters denote mean values that are significantly different

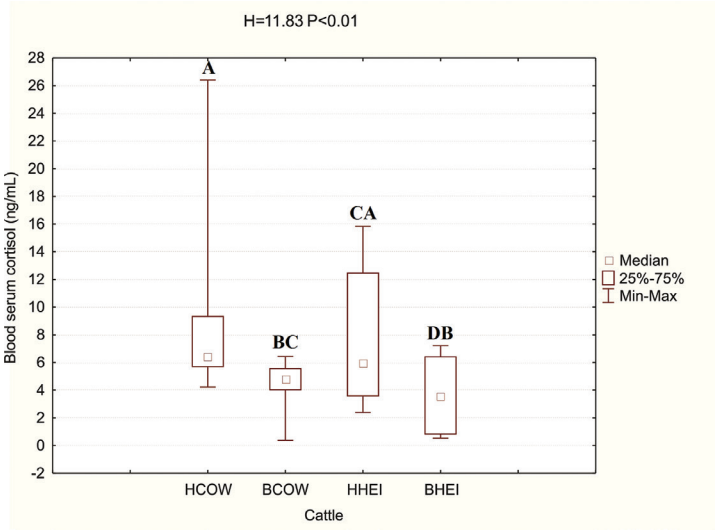

Figure 2: Blood serum cortisol in Holstein and Busha cattle

Data plotted represent the median, upper quartile, lower quartile and minimum and maximum.

^{A,B,C,D} – Different superscripted letters denote values which are significantly different
HCOW – Holstein cows, BCOW – Busha cows
HHEI – Holstein heifers, BHEI – Busha heifers

Table 3: Milk serum cortisol concentrations (ng/mL) in Holstein and Busha cows

	Hair cortisol concentrations (ng/g)	
Milk serum	Holstein	Busha
Mean	0.24a	0.17b
SEM	0.0[2]	0.0[2]
range	0.15 – 0.37	0.04 – 0.26

$^{a, b}$ – Values in the same rows with different superscripts are significantly different

seen from the results of this study, the cortisol concentration in Busha cows and heifers was significantly lower in the distal part of the hair, in comparison to proximal part. Since it was reported that solar (UV) radiation destroys cortisol (23, 24), we believe that this also happened with the cortisol in hair of Busha cattle. These were kept outside and exposed to varied weather conditions which might have resulted in gradual cortisol degradation in the hair. In contrast, the Holstein cattle were kept indoors and thus protected from outdoor environmental conditions.

Cortisol concentrations in hair of different colours were estimated only in Holstein cattle since the Busha breed is single-coloured. No significant difference was observed between cortisol concentrations in the black and white hair of Holstein cows. Previous studies on this topic are somewhat contradictory. In cattle, González-de-la-Vara et al. (25) and Burnett et al. (26) found a higher cortisol concentration in white than in black hair, while Tallo-Parra et al. (27) found higher concentrations in black hair. In contrast, in studies conducted on human hair, no association between cortisol concentration and hair colour was found (21). Other authors have suggested that accumulation of steroid substances in hair depends on interactions between different substances, as well as on the presence of both melanins (28). Thus, testosterone in bulls is present at a higher concentration in black than in white hair. Further, in the same study, it was shown that hormones such as oestradiol and testosterone in cows are present in similar proportions in black and white hair (29). It could be assumed that cortisol has the same affinity for black as for white hair, as described for oestradiol and testosterone (29). All these compounds are small lipophilic molecules that enter cells by passive diffusion and could thus accumulate equally in white and black hair. Similarly, no significant differences in hair cortisol concentrations were found in dogs of different colours (13).

To assess the relation between the amount of cortisol incorporated into the hair shaft and that located on the hair surface, cortisol levels in unwashed and washed hair samples taken from heifers of both breeds were determined. Lower

cortisol concentration in washed hair samples can be explained by the fact that hair is covered with sebum, sweat (30), and even saliva (4), all of which contain cortisol, and which are removed during washing.

The cortisol concentrations in the hair of cows measured in our study were higher than those described by Comin et al. (2), Burnett et al. (26), Tallo-Parra et al. (27), and Peric et al. (31), but were in the range of those reported for rhesus macaques (12) and for 15-day-old female calves (25). It is probable that the reason that higher concentrations were found is since cortisol was detected in the hair taken from the area of the tail, where its concentration is higher than in the hair from other parts of the body in dairy cattle (26). Higher concentrations of cortisol in the hair samples examined in our study could also be explained by the different methods of hair sample preparation. The samples used for comparison in this study were not washed with isopropanol as was done by other authors (2, 12, 26, 27), because we were interested in the entire concentration of cortisol located within the hair and on the hair shaft surface. Although washed hair samples are most commonly used for the determination of cortisol levels in hair, measurement of cortisol levels in unwashed hair may contribute to understanding the level of extra-potential sources of cortisol, such as sebum, sweat, and saliva.

The absence of significant differences of hair and blood cortisol levels between Holstein cows and heifers as observed in our study indicates that age has no significant impact on cortisol levels. The cortisol level in blood was the same in Busha heifers and cows, while it was significantly higher in the hair of Busha cows than that of heifers. It may be speculated that the significantly higher level of hair cortisol in Busha cows than in heifers may be a consequence of the longer period of cortisol accumulation in hair and having calf on foot. In our study, Busha cattle, as opposed to Holstein cattle, are kept outside and are thus exposed to more challenging environmental factors. As indicated by Dowling (32), inadequate environmental and feeding conditions may inhibit the shedding of hair in cows. Busha cattle, kept on pasture, are usually inadequately supplied with feed (33).

Furthermore, a goal of our study was to compare cortisol levels between breeds. As described by Sgorlon et al. (5) and Higashiyama et al. (34), Holstein cows have higher blood, milk and urine cortisol levels than other breeds do. The results obtained in this paper confirm the assumption that highly productive Holstein cows raised in intensive production systems have higher cortisol levels in biological fluids than those in low-yielding cattle breeds that are raised extensively on pasture, probably due to the greater exposure to stress combined with high milk production (16).

In the present study, hair cortisol concentrations in Holstein cattle were significantly higher than those in Busha cattle in both measured segments (proximal and distal). Peric et al. (31) compared hair cortisol concentrations in the Holstein breed and Swedish red x Montbeliarde cross-breed, and showed that the hair cortisol concentration was significantly higher in Holsteins. The lower hair cortisol concentration observed in Busha may be explained by the lower activity of the HPA axis in Busha cattle, since Bennett and Hayssen (13) confirmed the influence of HPA axis activity on hair cortisol concentrations in dogs. Lower cortisol concentrations in both segments of Busha hair than in segments in Holstein hair may be linked to the fact that the Holstein breed, in comparison to other cattle breeds, has the highest cortisol levels in all body fluids (5, 34). Many environmental and genetic factors may have an impact on cortisol concentrations (35). It may be speculated that the selection for high milk production in Holstein has an impact on the increased activity of the HPA axis and thus cortisol concentration. Cortisol, in synergy with other hormones, promotes mammary gland development (36). Since the Holstein breed is genetically selected for high milk production and milk production in Busha is in accordance with nutritional need of their calves, it may be supposed that increased rate of mammary tissue development is combined with increased cortisol levels in Holstein cattle.

The biological materials in which the concentration of cortisol may be correlated with cortisol from the hair are blood and milk. Blood and milk cortisol may provide information related to the HPA axis function shortly after the activation of short-term state of HPA axis activity (2); it fluctuates on a daily basis (37). The concentration of blood serum cortisol found here in the Holstein breed was significantly higher than in Busha. The blood cortisol concentration in Holstein cows is consistent with published values (38, 39). The concentration of blood serum cortisol in Holstein

heifers is, however, at variance with the results obtained by Bustamante et al. (40), who found higher blood cortisol levels in Holstein heifers. The obtained differences may be a consequence of different time of blood collection because the secretion of cortisol in cattle fluctuates daily (10). However, the specificity of the RIA kit used is also a factor that might influences the results. The blood concentration of cortisol in Holstein was significantly higher than that in Busha cattle in both cows and heifers. The lower cortisol concentrations in Busha cattle, in comparison to Holsteins, indicate differences between observed animals in the activity of the HPA axis.

Cortisol concentrations in milk serum were significantly higher in the milk of Holsteins than of Busha cows. These results are in line with those of Sgorlon et al. (5), who observed higher cortisol concentrations in milk of Holsteins than of Simmental cows. Since milk cortisol reflects the transition of the free fraction of blood cortisol, the results are expected and in accordance with higher blood serum cortisol levels. As described in Materials and Methods, we determined cortisol in milk serum rather than in skimmed milk, as done in many reports. Nevertheless, our results for cortisol concentration in milk serum are comparable with published values (41), since milk cortisol in the skimmed fraction is equally bound to whey proteins and casein (42).

Conclusion

As shown in our study, hair cortisol concentration is significantly higher in Holstein cows in heifers than in Busha cows and heifers. In addition, cortisol concentrations do not vary along the hair shaft in Holstein cattle; however, due probably to exposure to environmental conditions, in Busha cattle it was lower in the distal part of the hair. No significant differences were found in cortisol concentrations in hair of different colours. Additionally, it was estimated that 21% (Holstein) or 32% (Busha) of the entire hair cortisol is located on the surface of the hair shaft. Comparison of the concentrations of cortisol in the hair, blood serum, and milk from the two breeds of cows with different phenotypic characteristics, productive capacities, and breeding systems showed that cortisol concentrations in all three samples were higher in Holstein than in Busha cattle. Increased HPA axis activity in Holstein cattle probably contributes to their physiological adaptation to increased milk production.

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References

1. Mormède P, Andanson P, Aupérin B, et al. Exploration of the hypothalamic–pituitary–adrenal function as a tool to evaluate animal welfare. Physiol Behav 2007; 92: 317–39.

2. Comin A, Prandi A, Peric T, et al. Hair cortisol levels in dairy cows from winter housing to summer highland grazing. Livestock Sci 2011; 138: 69–73.

3. Minton JE. Function of the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system in models of acute stress in domestic farm animals. J Anim Sci 1994; 72: 1891–8.

4. Möstl E, Palme R. Hormones as indicators of stress. Domest Anim Endocrinol 2002; 23: 67–74

5. Sgorlon S, Fanyago M, Guiatti D, et al. Factors affecting milk cortisol in mid lactating dairy cows. BMC Vet Res 2015; 11: e259 (8pp.). https://bmcvetres.biomedcentral.com/articles/10.1186/s12917-015-0572-9 (30. 11. 2017)

6. Moberg GP, Mench JA. The biology of animal stress. Wallingford : CAB International, 2000.

7. Moya D, Schwartzkopf-Genswein KS, Veira DM. Standardization of a non-invasive methodology to measure cortisol in hair of beef cattle. Livest Sci 2013; 158: 138–44.

8. Morrow CJ, Kolver ES, Verkerk GA, et al. Fecal glucocorticoid metabolites as a measure of adrenal activity in dairy cattle. Gen Comp Endocrinol 2002; 126: 229–41.

9. Perez GC, Laita SGB, Portal JCI, et al. Validation of an EIA technique for the determination of salivary cortisol in cattle. Spanish J Agricult Res 2004; 2: 45–51.

10. Lefcourt AM, Bitman J, Kahl S, et al. Circadian and ultradian rhythms of peripheral cortisol concentrations in lactating dairy cows. J Dairy Sci 1993; 76: 2607–12.

11. Chen Y, Arsenault R, Napper S, et al. Models and methods to investigate acute stress responses in cattle. Animal 2005; 5: 1268–95.

12. Davenport MD, Tiefenbacher S, Lutz CK, et al. Analysis of endogenous cortisol concentrations in the hair of rhesus macaques. Gene Comp Endocrinol 2006; 147: 255–61.

13. Bennett A, Hayssen V. Measuring cortisol in hair and saliva from dogs: coat color and pigment differences. Domest Anim Endocrinol 2010; 39: 171–80.

14. Comin A, Peric T, Corazzin M, et al. Hair cortisol as a marker of hypothalamic-pituitary-adrenal axis activation in Friesian dairy cows clinically or physiologically compromised. Livest Sci 2013; 152: 36–41.

15. Šamanc HA, Kirovski D. Adrenocorticotropic system of cattle. Belgrade : Veterinary Institute of Serbia, 2008.

16. Corazzin M, Piasentier E, Dovier S, et al. Effect of summer grazing on welfare of dairy cows reared in mountain tie-stall barns. Ital J Anim Sci 2010; 9: 304–12.

17. Simčić M, Čepon M, Horvat S, et al. Genetic characterization of autochthonous cattle breeds Cika and Busha, using microsatellites. Acta Agric Slov 2008; 2: 71–7.

18. Bonczek RR, Young CW, Wheaton JE, et al. Responses of somatotropin, insulin, prolactin and thyroxine to selection for milk yield in Holsteins. J Dairy Sci 1988: 71: 2470–9.

19. Snoj T, Cebulj-Kadunc N, Nemec Svete A, et al. Determination of sex hormones in rat hair after administration of testosterone propionate and estradiol valerate. Slov Vet Res 2012: 49: 27–34.

20. Brkljačić M. Inflammatory response in dogs naturally infected with *Babesia canis canis*: PhD thesis. Zagreb : Faculty of Veterinary medicine, University of Zagreb, 2012.

21. Kirschbaum C, Tietze A, Skoluda N, et al. Hair as a retrospective calendar of cortisol production-increased cortisol incorporation into hair in the third trimester of pregnancy. Psychoneuroendocrinology 2009; 34: 32–7.

22. Duran MC, Janz DM , Waldner CL, et al. Hair cortisol concentration as a stress biomarker in horses: associations with body location and surgical castration. J Equine Vet Sci 2017; 55: 27–33.

23. Wester LV, van der Wulp NRP, Koper JW, et al. Hair cortisol and cortisone are decreased by natural sunlight. Psychoneuroendocrinology 2016; 72: 94–6.

24. Roth LSV, Faresjö Å, Theodorsson E, et al. Hair cortisol varies with season and lifestyle and relates to human interactions in German shepherd dogs. Sci Rep 2016; 6: e19631. https://www.nature.com/articles/srep19631 (30. 11. 2017)

25. González-de-la-Vara MR, Valdez RA, Lemus-Ramirez V, et al. Effects of adrenocorticotropic hormone challenge and age on hair cortisol concentrations in dairy cattle. Can J Vet Res 2011; 75: 216–21.

26. Burnett TA, Augusto MLM, Bruna FS, et al. Factors affecting hair cortisol concentrations in lactating dairy cows. J Dairy Sci 2014; 97: 1–6.

27. Tallo-Parra O, Manteca X, Sabes-Alsina M, et al. Hair cortisol detection in dairy cattle by using EIA: protocol validation and correlation with faecal cortisol metabolites. Animal 2015; 9: 1059–64.

28. Stout PR, Ruth JA. 3H-Nicotine, 3-flunitazepam, and 3H-cocaine incorporation into melanin: A model for the examination of drug-melanin interactions. J Anal Toxicol 2001; 25: 607–11.

29. Gleixner A, Meyer HD, Heinrich H. Detection of estradion and testosterone in hair of cattle by HPLC/EIA. Fresenius' J Anal Chem 1997; 357: 1198–201.

30. Russell E, Koren G, Rieder M, et al. Hair cortisol as a biological marker of chronic stress: Current status, future directions and unanswered questions. Psychoneuroendocrinology 2012; 37: 589–601.

31. Peric T, Comin A, Corazzin M, et al. Hair cortisol concentrations in Holstein-Friesian and crossbreed F1 heifers. J Dairy Sci 2013; 96: 3023–7.

32. Dowling DF. Seasonal changes in coat characters in cattle. In: Proceedings of the Australian Society of Animal Production; 2nd Biennal Meeting. Melbourne, 1958: 69–80.

33. Prodanović R, Kirovski D, Vujanac I, et al. Insulin responses to acute glucose infusion in Busa and Holstein-Freisian cattle breed during the peripartum period: comparative study. Acta Vet Beograd 2013; 63: 373–84.

34. Higashiyama Y, Komatsu T, Fukasawas M, et al. Comparison of urinary cortisol levels in Holstein and Japanese shorthorn cows in response to breeding system and heat stress. J Anim Sci Adv 2014; 4: 1009–16.

35. Golden SH, Wand GS, Malhotra S, et al.

Reliability of hypothalamic-pituitary-adrenal axis assessment methods for use in population-based studies. Eur J Epidemiol 2011; 26: 511–25.

36. Brisken C, O'Molley B. Hormone action in mammary gland. Cold Spring Harbor Persp Biol 2010; 2: 1–15.

37. Verkerk GA, Phipps AM, Matthews LR. Milk cortisol concentrations as an indicator of stress in lactating dairy cows. Proc N Z Soc Anim Prod 1996; 56: 77–9.

38. Hudson S, Mullord M, Whittlestone WG, et al. Diurnal variations in blood cortisol in the dairy cow. J Dairy Sci 1975; 58: 30–3.

39. Šamanc H, Nikolić JA, Bugarski D, et al. Glycemia, glucocorticoids and adrenocortical reserve in postpartal dairy cows. Acta Vet Beograd 1999; 49: 281–8.

40. Bustamante HA, Rodríguez AR, Herzberg DE, et al. Stress and pain response after oligofructose induced-lameness in dairy heifers. J Vet Sci 2015; 16: 405–11.

41. Tucker HA, Schwalm JW. Glucocorticoids in mammary tissue and milk. J Anim Sci 1977; 45: 627–34.

42. Schwalm JW, Tucker HA. Glucocorticoids in mammary secretions and blood serum during reproduction and lactation and distributions of glucocorticoids, progesterone and estrogens in fractions of milk. J Dairy Sci 1978; 61: 550–60.

KONCENTRACIJA KORTIZOLA V DLAKI, KRVI IN MLEKU KRAV ČRNO-BELE PASME IN PASME BUŠA

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Povzetek: Ugotavljali smo koncentracijo kortizola v dlaki, krvi in mleku pri govedu dveh pasem, ki se razlikujeta po mlečnosti in pogojih reje. Raziskavo smo izvedli na kravah in telicah črno-bele pasme in pasme buša. Koncentracijo kortizola smo ugotavljali z imunoencimskimi metodami. Akumulacijo kortizola smo določili v proksimalnem (bliže koži) in distalnem (dlje od kože) delu dlake. Obenem smo ugotavljali vpliv barve in pranja dlake na koncentracijo kortizola. Tako v proksimalnem kot v distalnem delu dlake je bila koncentracija kortizola pri kravah črno-bele pasme v primerjavi s kravami pasme buša statistično značilno višja (P<0,01 in P<0,05). Pri primerjavi koncentracije kortizola med črno in belo dlako črno-belih krav nismo ugotovili statistično značilnih razlik. V dlaki, ki smo jo pred izvedbo določanja koncentracije kortizola oprali z izopropanolom, smo v primerjavi z neoprano dlako ugotovili statistično značilno nižjo vrednost kortizola (P<0,01). Rezultati torej kažejo, da je koncentracija kortizola v dlaki odvisna od načina priprave vzorca (pranje dlake), barva dlake pri črnobeli pasmi pa ne vpliva na koncentracijo kortizola.. V krvnem serumu krav in telic črno-bele pasme je bila koncentracija kortizola statistično značilno višja (P<0,01 in P<0,05) kot pri kravah in telicah pasme buša. Tudi v mleku krav črno-bele pasme je bila koncentracija kortizola statistično značilno višja (P<0,05) kot pri kravah pasme buša. Predvidevamo, da je višji nivo kortizola pri črno-beli pasmi rezultat intenzivne reje in fiziološke prilagoditve na visoko mlečnost.

Ključne besede: govedo; kortizol; dlaka; kri; mleko

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Case Report

EFFECT OF *Boswellia serrata* SUPPLEMENTATION IN ADDITION TO INSULIN ON GLYCEMIC CONTROL IN A DIABETIC DOG

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Abstract: Diabetes mellitus (DM) is a common disorder in middle-aged to older dogs. Treatment options, similar to those for humans, include insulin injection, dietary changes and exercise. Since some diabetic dogs may develop humoral immune responses to exogenous insulin resulting in treatment failure, the use of alternative medicine could represent an interesting new therapeutic strategy for DM in addition to the traditional insulin therapy. The main objective of this report was to evaluate a new therapeutic strategy for DM, based on the association of insulin injections with an orally administered extract of *Boswellia serrata* to improve glycemic control in a diabetic dog. A nine year old female neutered mixed breed dog diagnosed with DM was treated with increasing doses of a porcine insulin zinc suspension starting from 0.2 U/kg up to 0.6 U/kg *q* 12 hours. Twenty weeks after the start of insulin therapy the duration of effect and glucose nadir were ideal and clinical symptoms had disappeared but hyperglycemia, although less severe, persisted. Supplementation with a dry extract from the gum- resin of *Boswellia serrata* was initiated, at a dosage of 15 mg/kg *q* 12 hours, to improve insulin sensitivity or possibly increasing endogenous insulin secretion. *B. serrata* supplementation led to good glycemic control. No side-effect or adverse reaction were observed during the study.
The present case report provides the first evidence in veterinary medicine of a positive effect of dietary supplementation with boswellic acids associated with traditional insulin therapy on glycemic control in a diabetic dog. A major pitfall of the study is the lack of a control. More extensive clinical trials are required to provide definitive evidence of *B. serrata* efficacy.

Key words: diabetes mellitus; dog; boswellic acids; glycemic control

Introduction

Diabetes mellitus (DM) is a common disorder affecting middle-aged to older dogs and characterized by hyperglycemia and glycosuria and typical clinical symptoms such as polyuria, polydipsia and weight loss (1, 2). Treatment options, similar to those for humans, include insulin injection, dietary changes and exercise. The classification of DM in dogs follows the scheme used in human medicine: canine diabetes usually resembles type 1 diabetes in humans and is characterized by permanent hypoinsulinemia and an absolute necessity for exogenous insulin to maintain control of glycemia and avoid ketoacidosis (3). Since some diabetic dogs may develop humoral immune responses to exogenous insulin resulting in treatment failure, the use of alternative medicine, including herbal extracts, represents an interesting new therapeutic strategy for DM in association with the traditional insulin therapy as reported for diabetic patients in human medicine (4). Herbal drugs usually used for human

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therapies could also be administered to pets with chronic diseases mainly to avoid collateral effects, although few studies have evaluated the efficacy of medicinal plants in animals (5). Since ancient times, frankincense, the aromatic gum resin obtained from trees of the genus *Boswellia,* has been used in central African countries and in the Middle East for the prevention and treatment of various illnesses, especially chronic inflammatory diseases. *Boswellia serrata* is a species of the Burseraceae family from India, containing boswellic acids known to present anti-inflammatory, anti-hepatotoxic, antioxidant, anti-hyperlipidemic and hypoglycemic properties (6). In particular, boswellic acids seem possess antileukotrienic activity, showing efficacy in human chronic inflammatory disorders like bronchial asthma (7) and Crohn's disease (8). An experimental model of multiple low-dose streptozotocin induced type 1 diabetes in mice showed that hyperglycemia results from an autoimmune reaction against pancreatic beta cells. *B. serrata* gum resin extracts containing boswellic acids administered to the mice prevented hyperglycemia, islet inflammation, destruction of β-cells and increases in proinflammatory cytokines (9). According to a report by Schrott et al. (2014) (4), an alcoholic extract of the *B. serrata* resin in addition to insulin, also prevented insulitis in a human patient with type 1 diabetes, leading to a decrease of tyrosine-phosphatase antibodies. Therefore, the main objective of this report was to evaluate a new therapeutic strategy for naturally occurring DM in a dog based on the association of insulin injections with an orally administered extract of *B. serrata* to improve glycemic control.

Case presentation

A nine year old female mixed breed dog weighting 18 kg was referred to Bologna University Veterinary Teaching Hospital with a two-month history of weight loss and enuresis, worsened by polyuria and polydipsia in the last two weeks. The bitch had been neutered at the age of seven months and was housed outdoors, fed a homemade diet and regularly vaccinated and dewormed. On physical examination, the bitch was responsive, with normal pink mucous membranes, 39°C rectal temperature, normal corneal transparency and no abdominal abnormalities on palpation. Abdominal ultrasound showed only a mild diffuse liver impairment. All procedures were performed under informed consent of the owners for diagnostic and/or therapeutic purposes. Figure 1 shows a graphic timeline of the different therapeutic protocols and clinical analyses performed in this case study.

The hematological profile showed mild leucocytosis with mature neutrophilia, while the serum biochemistry profile reported the most important alterations, namely severe fasting hyperglycemia, hypertriglyceridemia, hypercholesterolemia, a moderate increase in alanine aminotransferase activity (ALT) and fructosamine and mild hypoalbuminemia. Urinalysis revealed marked glycosuria, proteinuria and albuminuria with increased urinary protein to creatinine ratio (UPC) and a mild ketonuria (Table 1).

Based on typical clinical signs and laboratory findings, DM was diagnosed and the bitch was treated with increasing subcutaneous doses of

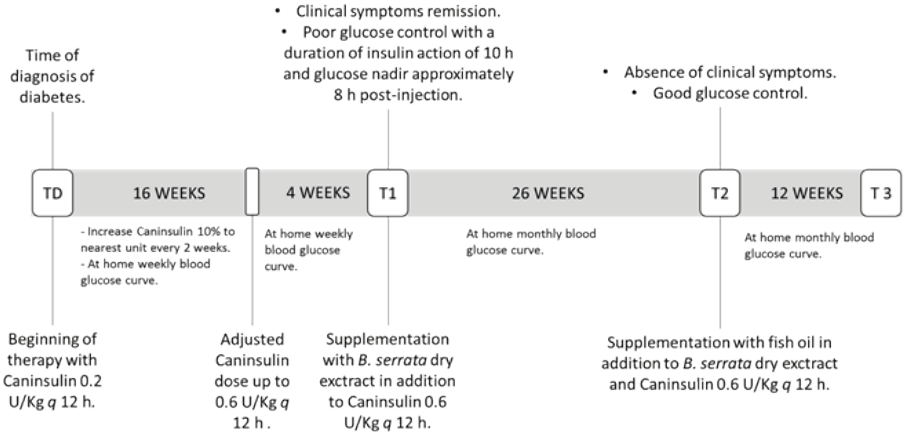

Figure 1: Graphic timeline of treatment plan and treatment goals; at time of diagnosis (TD), T1, T2 and T3 a full laboratory control, including haemato-biochemical and urine analyses, was done

Table 1: CBC, serum biochemistry and urinalysis results for a 9 years old mixed breed dog with polyuria and polydipsia at the time of DM diagnosis (TD) and after food supplementation with *B. serrata* dry extract in addition to insulin therapy (T3)

	TD	T3	Reference range
CBC			
RBC x 10^{12}/L	7.5	7.3	5.5-8.5
HCT fraction	0.48	0.52	0.37-0.55
HBG g/L	175	174	120-180
WBC x 10^9/L	**23.4**	8.4	6-17
PLT x 10^9/L	365	304	160-500
SERUM BIOCHEMISTRY			
Protein g/L	69	68	56-79
Albumin g/L	**26**	32	28-37
Creatinine µmol/L	84	66	57-119
ALT µKat/L	**0.97**	**2.17**	0.33-0.92
Triglycerides mmol/L	**15.65**	**2.09**	0.34-1.35
Cholesterol mmol/L	**10.02**	8.60	3.63-9.06
Glucose mmol/L	**29.69**	6.33	3.88-6.94
Fructosamine mmol/L	**0.52**	**0.55**	0.26-0.37
URINALYSIS			
Bilirubin µmol/L	Negative	**+**	Negative
Ketones mmol/L	2.58	Negative	Negative
Glucose mmol/L	**>16.65**	**>16.65**	Negative
Protein g/L	**0.3**	**0.3**	Negative
pH	**5.5**	6.5	6.5-7
SG	1040	1076	>1030
WBC	**+**	Negative	Negative
Blood	Negative	Negative	Negative
Epithelial cells	+	**+++**	Negative-+
UPC	**1.2**	0.3	0.0-0.4

Table 2: Average glucose concentrations obtained from glycemic curves (blood glucose concentrations were measured before and 2, 4, 6, 8 and 10 hours after insulin injection), fasting blood glucose, blood glucose nadirs (obtained 8 hours after insulin injection), serum fructosamine and triglycerides concentrations, measured as glycemic and lipemic control indicators. Urinary protein to creatinine ratio was also determined

	Average blood glucose (mmol/L)	Fasting blood glucose (mmol/L)	Blood glucose nadir (mmol/L)	Fructosamine (mmol/L)	Triglycerides (mmol/L)	UPC
TD	26.20	29.70	18.31	0.52	15.65	1.2
T1	23.70	28.36	17.48	0.52	nd	0.5
T2	8.21	14.21	2.77	0.68	10.37	0.5
T3	5.99	6.33	5.38	0.55	2.09	0.3

a porcine insulin zinc suspension (Caninsulin®, MSD Animal Health) starting from 0.2 U/kg, *q* 12 hours before feeding, up to 0.6 U/kg, with an increase equal to 10% to the nearest unit every two weeks. A specific commercial restricted-fat high-fiber diet as the sole source of food was also included in the therapy. Glycemia was monitored using a Glucocard G+ home glucometer (Menarini Diagnostic) in order to obtain a weekly blood glucose curve. The glycemic control was considered good when fasting blood glucose was below 16.65 mmol/L and the polyuria and polydipsia were absent, according to the owner's observation.

At T1 the bitch was re-examined. Although there was a complete remission of clinical symptoms of diabetes and the bitch gained 1.5 kg of weight, with a mild improvement of fasting blood glucose and blood glucose nadir, no change in serum fructosamine level was observed. Furthermore, the urinalysis revealed persistent glycosuria though with reduced proteinuria. The target results for a good glycemic control according to the AAHA diabetes management guidelines for diabetic dogs (1) were established as follows: nadir 4.44 to 8.32 mmol/L, time of nadir 8 hours after insulin injection, average blood glucose < 13.87 mmol/L, no single blood glucose > 16.65 mmol/L. To further reduce hyperglycemia, the patient's diet was supplemented every 12 hours with a dry extract obtained from *B. serrata* gum resin containing 300 mg of extract titrated to 65% in boswellic acids (15 mg/kg *q* 12 hours). Home monitoring was performed monthly and the owner reported a progressive improvement of glycemic control after four weeks of supplementation. A complete blood count, serum biochemistry profile and urinalysis were performed 26 weeks (T2) after the beginning of *B. serrata* supplementation to evaluate glycemic and lipemic control and the general health status of the bitch. At T2 blood glucose nadir showed a drastic drop to 2.77 mmol/L in the absence of clinical symptoms, while fasting blood glucose was 14.21 mmol/L and UPC stably remained slightly above the references range. Blood triglyceride concentration presented a remarkable decrease, although still widely above the reference range (Table 2). For this reason, additional supplementation was introduced with 9 ml fish oil, every 12 hours in the food. Twelve weeks (T3) after the beginning of fish oil supplementation, glycemic control showed a further decrease for both fasting blood glucose and average blood glucose and UPC also fell within the range. Blood triglyceride concentration was reduced fivefold and was very close to the reference range (Table 2). Serum ALT activity and fructosamine concentration were above the reference ranges throughout the study regardless of glycemic control (Table 1).

No side effects such as nausea, diarrhea or vomiting were observed during the study and no further serum and hematological parameters resulted altered.

Discussion

In late stage canine diabetes type 1, loss of β-cell function is irreversible and lifelong insulin therapy is mandatory to maintain glycemic control of the diabetic state (10, 11). Despite the pharmacotherapy approved for use in diabetic dogs, based on the administration of a porcine insulin zinc suspension (12) and the existence of other types of insulin tested in dogs (13, 14), it is still difficult to achieve adequate glycemic control in many diabetic canine patients, due to many causes such as insulin resistance or insulin-induced hyperglycemia. Most of the diabetic dogs are well controlled already at 0.5 U/Kg *q* 12 hours (1). Also in the case reported in this paper, therapy based on 0.6 U/kg *q* 12 hours of a porcine insulin zinc suspension resulted in the disappearance of clinical symptoms with an ideal duration of therapeutic effect (10 hours) and time of glucose nadir (8 hours post-injection). However, hyperglycemia, tough less severe, and persistent glycosuria were still present. Glycosuria in dogs typically develops when blood glucose concentration exceeds approximately 11.1 mmol/L and since average blood glucose in diabetic dogs should be less than 13.87 mmol/L, glycosuria may occur also in well controlled diabetic patients (1). In the present study, to achieve a better glycemic control, rather than increasing the dose of insulin, a food supplementation with a dry extract from *B. serrata* was introduced. This choice has the advantage to minimize the risk of hypoglycemia, which may often results in dogs with insulin excess or after excessive and strenuous exercise and to maintain the benefits obtained with the therapy based on 0.6 U/kg *q* 12 hours of insulin.

Different medicinal plants have been found to have a hypoglycemic effect both in normal

and diabetic human patients (15). Among herbal drugs, dry extract of the *B. serrata* gum resin have been used in traditional medicine for a variety of therapeutic purposes (16) without adverse effects or interferences with other drugs reported to date in experimental animals and humans (17, 18, 19). Boswellic acids, including O-acetyl-11-ketoβ-boswellic acid (AKBA) and 11-keto-β-boswellic acid (KBA), present in *B. serrata* gum resin, are novel, specific inhibitors of 5-lipoxygenase with a wide therapeutic potential (20). An alcoholic extract of *B. serrata* gum resin and two of its active compounds, AKBA and KBA, were recently reported to prevent hyperglycemia in multiple low-dose streptozotocin-induced diabetic mice as an animal model of type 1 DM, possibly by suppressing the production/action of pro-inflammatory cytokines and preventing insulitis in an autoimmune process (21, 9). Moreover, Schrott et al. (4) observed that treatment with *B. serrata* extract in a human patient with latent autoimmune type 1 diabetes led to a significant decrease of IA2 antibodies, a typical marker of autoimmune diabetes used in human medicine. There is also evidence that *B. serrata* supplementation with 900 mg/die for six weeks in human diabetes type 2 produces a remarkable decrease in fasting blood glucose (22). In the present study, clinical symptoms showed the typical features of canine diabetes and the diagnosis was achieved at a late stage. Therefore, it was not possible to clearly understand the etiopathogenesis and to classify the type of DM by assessing peptide C level to confirm an endogenous secretion of insulin, and/or by assessing the presence of autoantibodies to evaluate an autoimmune reaction. However, the good response to insulin therapy let us suppose that the dog was affected by type 1 DM. The hypoglycemic effect of *B. serrata* extract, administered in addition to insulin therapy, was evident and probably due to the improvement of a possible remaining endogenous insulin secretion through its antinflammatory action in an autoimmune process. Moreover, *B. serrata* supplementation in human patients affected by diabetes type 2 produces a significant decrease in blood cholesterol, LDL, fructosamine, ALT and AST activities and increased insulin levels (22). ALT activity in our case was over the reference range throughout the study, as already described for different hepatic enzyme activities in diabetic dogs probably due to hepatic lipidosis (23). However, the increased ALT activity was not accompanied by clinical symptoms and it may not be associated with progressive liver damage. A randomized clinical trial study in human type 2 diabetic patients, treated with 400 mg/die of *B. serrata* powder reported significantly lower glucose, HbA1c, insulin, total cholesterol, LDL and triglycerides levels with respect to a placebo group (24). In addition to its hypoglycemic effect, *B. serrata* could be used also as an anti-hyperlipidemic agent in diabetic patients (25), although in the present study an evident drop of serum triglycerides was obtained only after the administration of fish oil, as previously reported (26).

Despite the improvement in the glycemic control, the serum fructosamine concentration remained above the reference range throughout the study. This discrepancy between fructosamine and glucose serum concentration has already been reported in diabetic dogs (13, 27) and may be due to the delay between change in control of glycemia and change in blood glycated protein concentration. However, also specific canine genetic factors may influence variations in serum fructosamine concentration unrelated to glycemia as reported in Belgian Shepherds (28).

Conclusion

To the best of our knowledge, the only insulin preparation approved for use in dogs with DM is a porcine insulin zinc suspension that does not always provide optimal glycemic control. On the other hand, most DM dogs are type I insulin-dependent and oral hypoglycemic drugs are usually ineffective or accompanied by side-effects. The present case study provides the first evidence in veterinary medicine of a positive effect of dietary supplementation with dry *B. serrata* extract associated with traditional insulin therapy on glycemic and lipemic control in a dog with naturally occurring DM. The use of *B. serrata* extract in many inflammatory diseases did not produce any side-effects (6, 29) and could be a cheap and safe DM treatment option in addition to insulin therapy, when insulin alone cannot achieve good glycemic control.

The major pitfall of this study is the lack of a control case. A more extensive pharmacological study and interventional prospective clinical trials are needed to clarify the mechanism of

action of *B. serrata* extracts and to provide a definitive evidence of their efficacy and safety in the treatment of diabetes mellitus in dogs.

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References

1. Rucinsky R, Cook A, Haley S, Nelson R, Zoran DL, Poundstone M. AAHA Diabetes management guidelines for dogs and cats. J Am Anim Hosp Assoc 2010; 46: 215–24.

2. Nelson RW, Reush CE. Animal models of disease: classification and etiology of diabetes in dogs and cats. J Endocrinol 2014; 222(3): eT1-9. http://dx.doi.org/10.1530/JOE-14-0202

3. Catchpole B, Kennedy LJ, Davison LJ, Ollier WE. Canine diabetes mellitus: from phenotype to genotype. J Small Anim Pract 2008; 49: 4–10.

4. Schrott E, Laufer S, Lammerhofer M, Ammon HPT. Extract from gum resin of *Boswellia serrata* decrease IA2-antibody in a patient with "Late onset Autoimmune Diabetes of the Adult" (LADA). Phytomedicine 2014; 21(6): 786.

5. Russo R, Autore G, Severino L. Pharmaco-toxicological aspects of herbal drugs used in domestic animals. Nat Prod Commun 2009; 4(12): 1777–84.

6. Hartman RM, Morgan Martins MI, Tieppo J, Fillmann HS, Marroni NP. Effect of *Boswellia serrata* on antioxidant status in an experimental model of colitis rats induced by acetic acid. Dig Dis Sci 2012; 57: 2038–44.

7. Gupta I, Gupta V, Parihar A, et al. Effects of *Boswellia serrata* gum resin in patients with bronchial asthma: results of a double-blind, placebo-controlled, 6-week clinical study. Eur J Med Res 1998; 17: 511–4.

8. Gerhardt H, Seifert F, Buvari P, Vogelsang H, Repges R. Therapy of active Crohn disease with *Boswellia serrata* extract H 15. Z Gastroenterol. 2001; 39: 11–7.

9. Shehata AM, Quintanilla-Fend L, Bettio S, et al. 11-Keto-β-boswellic acids prevent development of autoimmune reactions, insulitis and reduce hyperglycemia during induction of multiple low dose streptozotocin (MLD-STZ) diabetes in mice. Horm Metab Res 2015; 47: 463–9.

10. Verkest KR, Rand JS, Flemann LM, Morton JM. Spontaneously obese dogs exibit greater post-prandial glucose, triglyceride and insulin concentration than lean dogs. Domest Anim Endocrinol 2012; 42(2): 103–12.

11. Gilor C, Niessen SJM, Furrow E, DiBartola SP. What's in a name? Classification of diabetes mellitus in veterinary medicine and why it matters. J Vet Intern Med 2016; 30: 927–40.

12. Monroe WE, Laxton D, Fallin EA, et al. Efficacy and safety of a purified porcine insulin zinc suspension for managing diabetes mellitus in dogs. J Vet Intern Med 2005; 19: 675–82.

13. Fracassi F, Boretti FS, Sieber-Ruckstuhl NS, Reusch, CE. Use of insulin glargine in dogs with diabetes mellitus. Vet Rec 2012; 170(2): 52.

14. Fracassi F, Corradini S, Hafner M, Boretti FS, Sieber-Ruckstuhl NS, Reusch, CE. Detemir insulin for the treatment of diabetes mellitus in dogs. J Am Vet Med Assoc 2015; 247: 73–7.

15. Ezuruike UF, Prieto JM. The use of plants in the traditional management of diabetes in Nigeria: pharmacological and toxicological considerations. J Ethnopharm 2014; 155: 857–924.

16. Poekel D, Werz O. Boswellic acids: biological actions and molecular targets. Curr Med Chem 2006; 13: 3359–69.

17. Singh GB, Atal CK. Pharmacology of an extract of salai guggal ex-*Boswellia serrata*, a new non-steroidal anti-inflammatory agent. Agent Actions 1986; 18: 407–12.

18. Ammon HPT. Modulation of the immune system by *Boswellia serrata* extracts and boswellic acids. Phytomedicine 2010; 17: 862–7.

19. Beghelli D, Isani G, Roncada P, et al. Antioxidant and ex vivo immune system regulatory properties of *Boswellia serrata* extracts. Oxid Med Cell Longev 2017; 2017: eID 7468064 (10 pp.) https://doi.org/10.1155/2017/7468064

20. Sharma A, Chhikara S, Ghodekar SN, et al. Phytochemical and pharmacological investigations on *Boswellia serrata*. Pharmacogn Rev 2009; 3: 206–15.

21. Shehata AM, Quintanilla-Fend L, Bettio S, Singh CB, Ammon HPT. Prevention of multiple low-dose streptozotocin (MLD-STZ) diabetes in mice by an extract from gum resin of *Boswellia serrata* (BE). Phytomedicine 2011; 18: 1037–44.

22. Ahangarpour A, Heidari H, Fatemeh RAA,

et al. Effect of *Boswellia serrata* supplementation on blood lipid, hepatic enzymes and fructosamine levels in type 2 diabetic patients. J Diabetes Metab Disord 2014; 13: 29–37.

23. Hess RS, Saunders HM, Van Winkle TJ, Shofer FS, Washabau RJ. Clinical, clinicopathologic, radio-graphic, and ultrasonographic abnormalities in dogs with fatal acute pancreatitis: 70 cases (1986–1995). J Am Vet Med Assoc 1998; 213: 665–70.

24. Azadmehr A, Ziaee A, Ghanei L, et al. A randomized clinical trial study: anti-oxidant, anti-hyperglycemic and anti-hyperlipidemic effects of Olibanum Gum in type 2 diabetic patients. Iran J Pharm Res 2014; 13: 1003–9.

25. Alam M, Khan H, Samiullah L, Siddique KM. A review on phytochemical and pharmacological studies of Kundur (*Boswellia serrata* Roxb ex Colebr.)-A Unani drug. J App Pharm Sci 2012; 2(3): 148–56.

26. Xenoulis PG, Steiner JM. Lipid metabolism and hyperlipidemia in dogs. Vet J 2010; 183: 12-21.

27. Briggs CE, Nelson RW, Feldman EC, Elliott DA, BVSc, Neal LA. Reliability of history and physical examination findings for assessing control of glycemia in dogs with diabetes mellitus: 53 cases (1995–1998). J Am Vet Med Ass 2000; 217: 48–53.

28. Forsberg SKG, Kierkzac M, Ljungvall I, et al. The Shepherds tale: a genome wide study across 9 dogs breeds implicates two loci in the regulation of fructosamine serum concentration in Belgian shepherds. Plos ONE 2015; 10(5): e0123173 (17 pp.) http://dx..doi.org/10.1371/journal.pone.0123173 (April 2016)

29. Reichling J, Schmökel HS, Bucher S, Saller R. Dietary support with *Boswellia resin* in canine inflammatory joint and spinal disease. Schweiz Arch Tierheilkd 2004; 146: 71–9.

VPLIV DODAJANJA BOSVELIJE (*Boswellia serrata*) OB ZDRAVLJENJU Z INZULINOM NA UREJANJE RAVNI GLUKOZE V KRVI PRI PSU S SLADKORNO BOLEZNIJO

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Povzetek: Sladkorna bolezen oz. *diabetes mellitus* (DM) je pogosta motnja pri psih v srednjih letih in starejših psih. Možnosti zdravljenja, ki so podobne kot pri ljudeh, vključujejo dodajanje inzulina, spremembe v prehrani in telesno aktivnost. Ker lahko nekateri psi s sladkorno boleznijo razvijejo imunski odziv na dodajanje inzulina, kar lahko vodi do neuspešnega zdravljenja, lahko uporaba alternativne medicine predstavlja zanimivo novo metodo zdravljenja DM poleg tradicionalnega zdravljenja z inzulinom. Glavni cilj raziskave je bil ovrednotiti novo metodo zdravljenja DM, ki temelji na hkratnem dodajanju inzulina in peroralnem dodajanju izvlečka bosvelije (*Boswellia serrata*) za izboljšanje urejanja ravni glukoze v krvi pri psu s sladkorno boleznijo. Devet let stara sterilizirana samica mešane pasme, ki je bila diagnosticirana s sladkorno boleznijo, je bila zdravljena z naraščajočimi odmerki suspenzije prašičjega inzulina in cinka (od 0,2 E/kg do 0,6 U/kg), ki jih je dobivala vsakih 12 ur. Dvajset tednov po začetku zdravljenja z inzulinom so bili učinki in najnižja vrednost izmerjene glukoze v normalnih mejah vrednosti, klinični simptomi so izginili, le hiperglikemija je bila še vedno prisotna, vendar pa je bila manj resna. Dodatek suhega izvlečka iz gumijeve smole bosvelije *Boswellia serrata* v odmerku 15 mg/kg vsakih 12 ur je povečal občutljivost na inzulin oz. morebitne preostanke endogenega izločanja inzulina. Dodatek *B. serrata* je pozitivno vplival na nadzorovanje sladkorne bolezni. Med zdravljenjem niso bili opaženi stranski ali nezaželeni učinki. Poročilo o opisanem primeru vsebuje prve dokaze o pozitivnem učinku prehranskega dopolnila z bosveličnimi kislinami v veterinarski medicini, povezanimi z običajnim zdravljenjem z inzulinom, pri nadzoru glukoze v krvi pri psu s sladkorno boleznijo. Najbolj neugoden del te študije je odsotnost kontrole. Za zagotovitev dokončnega dokaza o učinkovitosti *B. serrata* so potrebni obsežnejši klinični poskusi.

Ključne besede: *diabetes mellitus*; pes; bosvelična kislina; nadzor sladkorne bolezni

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Case Report

ELECTROCHEMOTHERAPY COMBINED WITH STANDARD AND CO_2 LASER SURGERIES IN CANINE ORAL MELANOMA

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Abstract: Oral melanomas commonly occur in elderly dogs resulting in darkly pigmented areas of the mouth, tongue and gums. The main object of this study was a severe melanoma diagnosed in the jaw of a 15-year-old dog and its palliative treatment with electrochemotherapy. The tumour spread throughout the jaw including bones. Electrochemotherapy (ECT) with bleomycin and calcium solution ($CaCl_2$) was combined with standard and CO_2 laser surgeries. The treatment resulted in good local control of the tumour mass after one ECT session with bleomycin and a second ECT session with calcium ions solution. ECT significantly reduced bleeding and enhanced success of the surgery. The combination of ECT with surgical debulking resulted in rapid recovery and regaining of physiological activities, including normal feeding by the dog.

This case demonstrates that the protocol combining ECT and surgery is promising in palliative melanoma treatment.

Key words: oral melanoma; CO_2 laser; electrochemotherapy; bleomycin; calcium ions

Introduction

Oral melanomas remain a therapeutic problem in veterinary medicine. Localization of the tumour in the oral cavity often results in late detection and advanced neoplastic process at the time of diagnosis. The average lifespan of a dog with oral melanoma depends on the biological behaviour of the tumour and its clinical stage at the moment of diagnosis. However, compared to dogs with melanomas localized in other regions, those with oral melanomas have the shortest survival times, ranging from 2 to 14 months (1-3). Typical treatment requires an aggressive local intervention for tumour control, usually accompanied by a wide surgical excision, sometimes involving partial mandibulectomy or maxillectomy (1). Early metastasis, in most cases before the time of diagnosis, and tumour recurrence are common in cases of oral malignant melanomas. Because the chances of cancer recurrence and rapid metastasis are very high, chemotherapy, immunotherapy, local and/or systemic adjuvant treatment, radiotherapy or electrochemotherapy (ECT) should be considered (3). Previous studies

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indicate that ECT with cytostatics is an effective treatment for various tumours in animals, and ECT is quite a simple method with short treatment sessions, low chemotherapeutic doses and insignificant side effects (4, 5). Here, we present a case of malignant oral melanoma treated with ECT during surgery in the Department of Surgery of Wrocław University of Environmental and Life Sciences in Wrocław, Poland.

Case presentation

A 15-year-old male crossbreed dog (weight 30 kg) was diagnosed with stage IV malignant melanoma of the oral cavity with involvement of the mandibular bone. The tumour mass infiltrated the entire left mandibular body tissue (Fig.1 A). Deformation of the facial area, difficulties in food intake, halitosis, drooling, and occasional bleeding had been observed by the owner for approximately 2 months. The patient could not be properly diagnosed by a veterinarian due to the dog's aggressiveness and concerns by the owner about sedation risk. RTG and CT indicated enlarged and distorted mandibular lymph nodes on the left side of the jaw. A large mass was located in the sublingual area and over the left mandibular body with local osteolysis of the bone. Histopathologic examination of a biopsy taken from the enlarged lymph node and oral tumour revealed malignant melanoma. The patient was diagnosed with stage IV of the disease with metastatic spread. A week after the first examination, the dog was unable to eat and the owner chose and approved the palliative treatment.

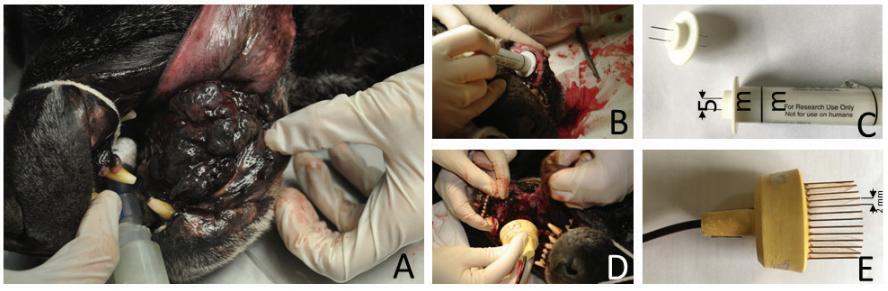

Figure 1: Oral melanoma in dog: A) The tumour in the mandible of the dog; B) two-needle array electrode during electrochemotherapy (ECT); C) disposable two-needle array tips; D) Petri Pulser during ECT; E) Petri Pulser electrode

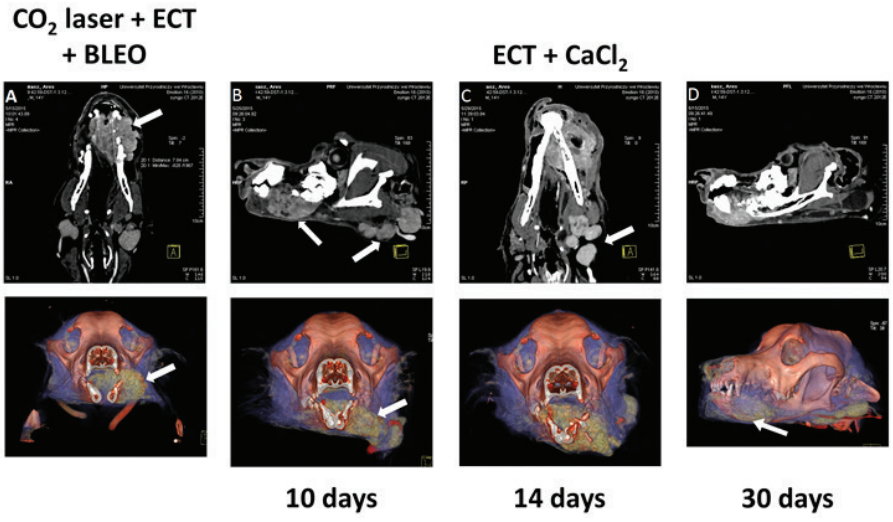

Figure 2: CT imaging before and after therapy. In the lower panel tumour mass tissue is represented in yellow-green colour and the tumour is indicated by white arrows: A) day of ECT; B) 10 days after ECT; C) 14 days after ECT; D) 30 days after last ECT session

Table 1: The detailed data of tumour mass before, during, and after treatment

Days after ECT	Width [cm]	Height [cm]	Length [cm]	Estimated tumour volume [cm^3]
day of ECT	7.07	4.83	7.84	140.11
10 days	6.05	2.95	6.71	62.67
14 days	7.95	3.40	8.68	122.78
30 days	6.55	2.70	6.40	59.23

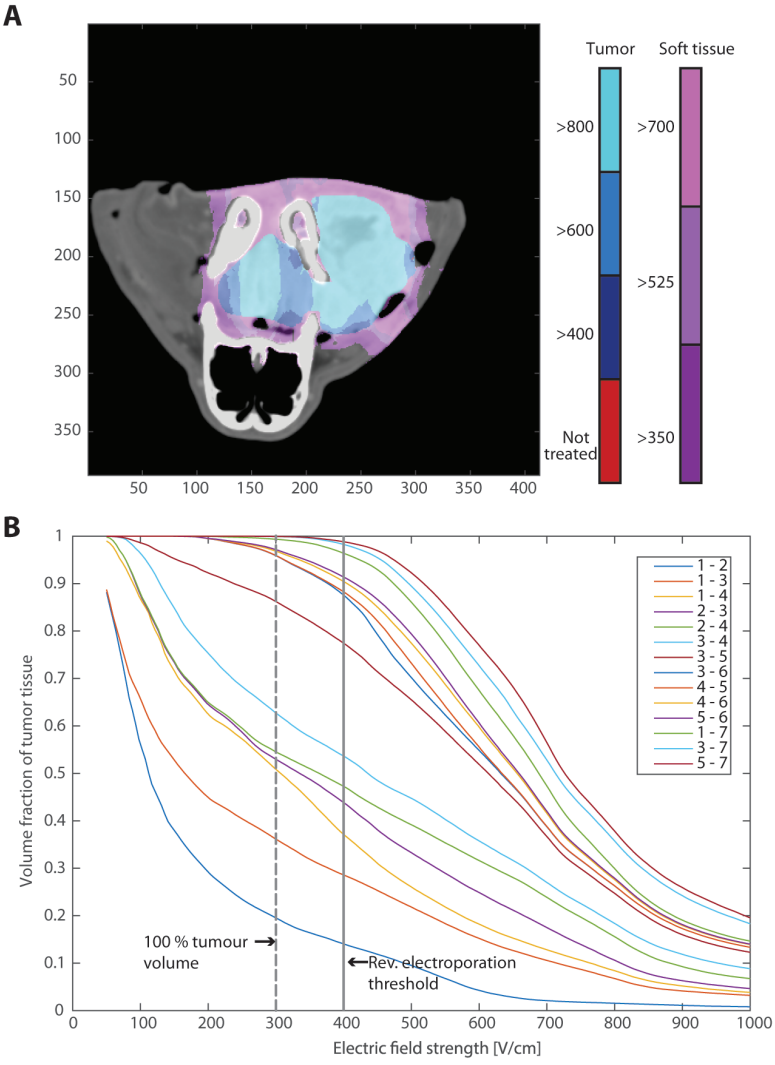

Figure 3: Electroporation field distribution modelling results from Visifield (8). A) The electroporation cross-section shows a representative slice and the local electric field coverage. B) Dose-volume histogram showing the cumulative coverage of the tumour with electric field. After the delivery of all pulses the whole tumour volume is covered with at least 300 V/cm electric field

Surgery-ECT treatment

The dog was examined from April to September of 2015. It was premedicated intramuscularly with medetomidine 0.3 ml (cepetor 1 mg/ml, ScanVet) and midazolam 0.6 ml (midanium 5mg/ml, Polfa S.A.). General anaesthesia was induced with 3 ml of propofol (scanofol 10 mg/ml, ScanVet) and after intubation with a 9 mm diameter cuffed tube, anaesthesia was maintained with isoflurane (aerrane, Baxter). Proper analgesia was assured during the surgery by infusion of fentanyl at a constant rate of 0.2 µg/kg/min (fentanyl WZF 50 µg/ml, Polfa Warsaw). Debulking of the tumour tissue was performed using a CO_2 laser with 0.25 mm spot diameter, 12 W power output, in a continuous wave mode. Coagulation with 1.4 mm spot diameter was insufficient and bleeding from the remaining tissue occurred. ECT included intravenous (i.v.) and intratumoural (i.t.) administrations of bleomycin (Bleomedac, medac Gesellschaft für klinische Spezialpräparate mbH) and exposure of the remaining tumour mass to the electric pulses. Bleomycin was dissolved in physiological saline and applied at a concentration of 0.3 mg/kg i.v. and at 3 mg/ml i.t. (total dose 4 ml). Bleomycin was applied by both i.v. and i.t., because of the very irregular shape of the tumour tissue and visible fragments of the remaining tissue that could not be surgically removed. The interval between i.v. and i.t. bleomycin administration and the application of electric pulses was 8 minutes. Electroporation was performed using an ECM 830 Square Wave Electroporation System (BTX Harvard Apparatus, purchased from Syngen Biotech, Poland). Two types of electrodes were used: 1) two-needle array (BTX model 532) (Fig.1 B and C) and 2) Petri Pulser Electrode (BTX model

45-0130) (Fig.1 D and E). In the two-needle array electrode, the needle spacing was 5 mm with a needle length of 20 mm. The needles were made of stainless steel and were attached to a handle 8 cm long. The Petri Pulser electrode consisted of 13 gold plated electrodes with needle diameter 0.5 mm and gap size 2 mm. In each application of electrodes, 8 square wave pulses of 100 μs each were delivered at 1 Hz, with the voltage-to-distance ratio set at 1300 V/cm (302 V for Petri Pulser electrode and 650 V for the needle array electrodes). After the treatment, the dog remained in the clinic for about 2 hours. It was examined daily for the first 3 days, then every week to evaluate the treatment effectiveness and possible local and systemic side effects. Standard and 3D CT imaging of the dog's mandibula before and after therapeutic procedures are presented in Fig. 2.

ECT enhanced the surgical effect, stopped bleeding during surgery and enabled rapid recovery of physiological activities. The day after the surgery the owner reported that the dog resumed eating. After 10 days the tumour mass decreased in visible areas of the local necrosis and no bleeding from the remaining tissue was observed. Enlargement of the mandibular lymph nodes and difficulty in swallowing were noted 14 days after the first ECT, and CT revealed enlargement of the metastatic spread in the lymph nodes along with swelling of the treated area (Fig. 2C and Tab.1). On that day ECT with calcium ions ($CaCl_2$ in low concentration at 5 mM, 10 ml delivered i.t.) was performed directly on the metastasis in the lymph nodes and on the remaining tumour mass. Only two-needle array electrodes were used and in each application the electric field was 8 square wave pulses of 100 μs each, delivered at 1 Hz and a voltage of 650 V. After 5 days strong inflammation occurred in the lymph nodes and during the next two days dexamethasone (0.1 mg/kg per day) was applied. The dog was examined on the 30th day after the second treatment and no metastases in the lymph nodes were observed (Fig.2 D). These observations may indicate that treatment with calcium ions, which induced strong inflammation, led to additional immune response. Unfortunately, the dog began to have seizures 2 months after the primary diagnosis, probably due to metastatic spread in the brain, prompting the owner to choose euthanasia.

Treatment planning

This case was treated by electrochemotherapy during surgery using fixed geometry electrodes. However, after conclusion of the case we investigated the possibility of using single needle electrodes of variable geometry in combination with computational treatment planning. This post-treatment approach was based on modelling the electric field distribution (5, 6), which could be especially effective for highly irregular, large mass tumours. The images from CT were uploaded to the web-based electric field visualization tool Visifield (www.visifield.com, University of Ljubljana, Slovenia) (7). Bone of the jaw, tumour tissue, and surrounding soft tissue were segmented (Fig.3A). The total reconstructed volume of the tumour tissue was 88 cm^3. Then, 7 individual needle electrodes with 4 cm exposed tips were inserted from the anterior side. Their number was chosen to provide sufficient electric field strength in the whole tumour. This provided coverage of the whole tumour mass with at least 300 V/cm electric field, and more than 98% of the tumour volume was covered with at least 400 V/cm electric field. This simulation showed that the whole tumour volume could be potentially treated in a single electrochemotherapy session and therefore eliminate the need for partial or complete surgical resection (Fig.3B). The only limitation is that no pulse generator is commercially available that allows the connection of more than 6 individual electrodes, so cables would have to be manually reconnected. The methods applied in electric field modelling and treatment plan optimization are presented in more detail in previous works (8-11).

Discussion

This case demonstrates that surgical methods can be effectively combined with ECT in palliative melanoma treatments. In the first treatment session, standard surgery and CO_2 laser surgery accompanied by ECT with bleomycin were applied. As previously reported, calcium electroporation can be highly efficient in eradicating tumours *in vivo* (13, 14) and, moreover, calcium solution is not toxic. Therefore, taking into consideration the condition of the dog, during the second treatment session we chose ECT with only calcium chloride. The treated metastatic nodules were not detectable

after one month. This may be due to a delayed response of the metastases following the first session with ECT with bleomycin, as was noted in other studies (15, 16). However, additional response of the immune system enhanced by electroporation with calcium ions could also contribute to the observed effects (13, 16, 17). We conclude that the final outcome was the result of the additive effects of laser surgery and ECTs with bleomycin and calcium. A combination approach using ECT and surgery seems to be promising in palliative melanoma treatment (18, 19), as was suggested previously for human patients.

Additionally, we show the possibility of performing pre-treatment planning using specialized software such as Visifield (www.visifield.com, University of Ljubljana, Slovenia). ECT is currently applied with standard operating procedures using predefined fixed electrode geometries (19, 20), or using individual patient treatment planning to predict the electroporation outcome related to the treatment procedure (7, 12). However, efficient ECT of large tumours with variable geometry electrodes could rely on realistic computer models to provide better results. In this way more details, including number of electrodes, electrodes positioning, and the resulting electric field distribution could be taken into consideration (21). Currently, this approach is applied only for human ECT, mainly for treatment of deep-seated tumours (7, 12, 20-22). Application of treatment planning in veterinary procedures could result in much more effective ECT.

Conclusions

We present a case of canine oral melanoma which was treated by ECT for the first time in Poland. Our observations indicate that ECT enhanced the surgical effect and stopped bleeding during the surgery. The treatment enabled normal feeding and faster recovery to physiological activities. The protocol combining ECT and surgery is promising in palliative melanoma treatment.

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References

1. Bergman PJ. Canine oral melanoma. Clin Tech Small Anim Pract 2007; 22(2): 55–60.

2. Desmas I. Canine oral melanoma. Vet Irel J 2013; 3(7): 398–401.

3. Bowlt K, Starkey M, Murphy S. Oral malignant melanoma in dogs. Vet Times 2013; 36: 27–9.

4. Cemazar M, Tamzali Y, Sersa G, et al. Electrochemotherapy in veterinary oncology. J Vet Intern Med 2008; 22(4): 826–31.

5. Tozon N, Kodre V, G. Sersa G, Cemazar M. Effective treatment of perianal tumors in dogs with electrochemotherapy. Anticancer Res 2005; 25(2A): 839–45.

6. Miklavcic D, Snoj M, Zupanic A, et al. Towards treatment planning and treatment of deep-seated solid tumors by electrochemotherapy. Biomed Eng Online 2010; 9: e10 (12 pp.) https://biomedical-engineering-online.biomedcentral.com/articles/10.1186/1475-925X-9-10

7. Edhemovic I, Brecelj E, Gasljevic G, et al. Intraoperative electrochemotherapy of colorectal liver metastases. J Surg Oncol 2014; 110(3): 320–7.

8. Marčan M, Pavliha D, Kos B, Forjanič T, Miklavčič D. Web-based tool for visualization of electric field distribution in deep-seated body structures and planning of electroporation-based treatments. Biomed Eng Online 2015: 14(Suppl 3): eS4 (13 pp.) https://biomedical-engineering-online.biomedcentral.com/articles/10.1186/1475-925X-14-S3-S4

9. Kos B, Zupanic A, Kotnik T, Snoj M, Serša G, Miklavčič D. Robustness of treatment planning for electrochemotherapy of deep-seated tumors. J Membr Biol 2010; 236(1): 147–53.

10. Kos B, Voigt P, Miklavcic D, Moche M. Careful treatment planning enables safe ablation of liver tumors adjacent to major blood vessels by percutaneous irreversible electroporation (IRE). Radiol Oncol 2015; 49(3): 234–41.

11. Zupanic A, Kos B, Miklavcic D. Treatment planning of electroporation-based interventions: electrochemotherapy, gene electrotransfer and irreversible electroporation. Phys Med Biol 2012;

57(17): 5425–40.

12. Groselj A, Kos B, Cemazar M, et al. Coupling treatment planning with navigation system: a new technological approach in treatment of head and neck tumors by electrochemotherapy. Biomed Eng Online 2015: 14(Suppl 3): eS2 (14 pp.) https://biomedical-engineering-online.biomedcentral.com/articles/10.1186/1475-925X-14-S3-S2

13. Frandsen SK, Gissel H, Hojman P, Tramm T, Eriksen J, Gehl J. Direct therapeutic applications of calcium electroporation to effectively induce tumor necrosis. Cancer Res 2012; 72(6):1336–41.

14. Gehl KJ, Frandsen SK, Eriksen JO, Giessel HM, Hojman PJ. Therapeutic applications of calcium electroporation to effectively induce tumor necrosis. Patent No: EP2797608. Aarhus: Herlev Hospital, Aarhus Universitet, 2014.

15. Caracò C, Mozzillo N, Marone U, et al. Long-lasting response to electrochemotherapy in melanoma patients with cutaneous metastasis. BMC Cancer 2013; 13: e564 (4 pp.) https://bmccancer.biomedcentral.com/articles/10.1186/1471-2407-13-564

16. Spugnini EP, Pizzuto M, Filipponi M, et al. Electroporation enhances bleomycin efficacy in cats with periocular carcinoma and advanced squamous cell carcinoma of the head. J Vet Intern Med 2015; 29(5): 1368–75.

17. Frandsen SK, Gissel H, Hojman P, Eriksen J, Gehl J. Calcium electroporation in three cell lines: a comparison of bleomycin and calcium, calcium compounds, and pulsing conditions. Biochim Biophys Acta 2014; 1840(3): 1204–8.

18. Calve CY, Famin D, André FM, Mir LMl. Electrochemotherapy with bleomycin induces hallmarks of immunogenic cell death in murine colon cancer cells. Oncoimmunol 2014; 3(4): e28131 (10 pp.) http://www.tandfonline.com/doi/abs/10.4161/onci.28131

19. Campana LG, Bianchi G, Mocellin S, et al. Electrochemotherapy treatment of locally advanced and metastatic soft tissue sarcomas: results of a non-comparative phase II study. World J Surg 2014; 38(4): 813–22.

20. Campana LG, Testori A, Mozzillo N, et al. Treatment of metastatic melanoma with electrochemotherapy. J Surg Oncol 2014; 109(4): 301–7.

21. Miklavčič D, Serša G, Brecelj E, et al. Electrochemotherapy: technological advancements for efficient electroporation-based treatment of internal tumors. Med Biol Eng Comput 2012; 50(12): 1213–25.

22. Pavliha D, Kos B, Zupanič A, Marčan M, Serša G, Miklavčič D. Patient-specific treatment planning of electrochemotherapy: procedure design and possible pitfalls. Bioelectrochemistry 2012; 87: 265–73.

ELEKTROKEMOTERAPIJA V KOMBINACIJI S STANDARDNO IN LASERSKO CO_2 KIRURGIJO PASJIH USTNIH MELANOMOV

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Povzetek: Oralni melanom se pogosteje pojavlja pri starejših psih, kot temno pigmentirano področje ustne sluznice, jezika in dlesni. V prispevku opisujemo primer paliativnega zdravljenja obsežnega melanoma na področju spodnje čeljusti 15-letnega psa z elektrokemoterapijo. Tumorska masa se je vraščala tudi v spodaj ležečo kost. Delno kirurško odstranitev tumorske mase s klasično in CO_2 lasersko kirurško metodo smo dopolnili z elektrokemoterapijo z bleomicinom in z raztopino kalcijevega klorida ($CaCl_2$). S kombinacijo terapij smo dosegli dobro lokalno kontrolo tumorja. Elektrokemoterapija neposredno po kirurškem posegu je tudi močno zmanjšala krvavitev. Pes je po posegu okreval hitro ob ohranitvi vseh fizioloških funkcij, vključno z normalnim hranjenjem.

Delna kirurška resekcija v kombinaciji z elektrokemoterapijo bi bila lahko alternativna možnost paliativnega zdravljenja oralnega melanoma.

Ključne besede: ustni melanom; laser CO_2; elektrokemoterapija; bleomicin; kalcijevi ioni

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