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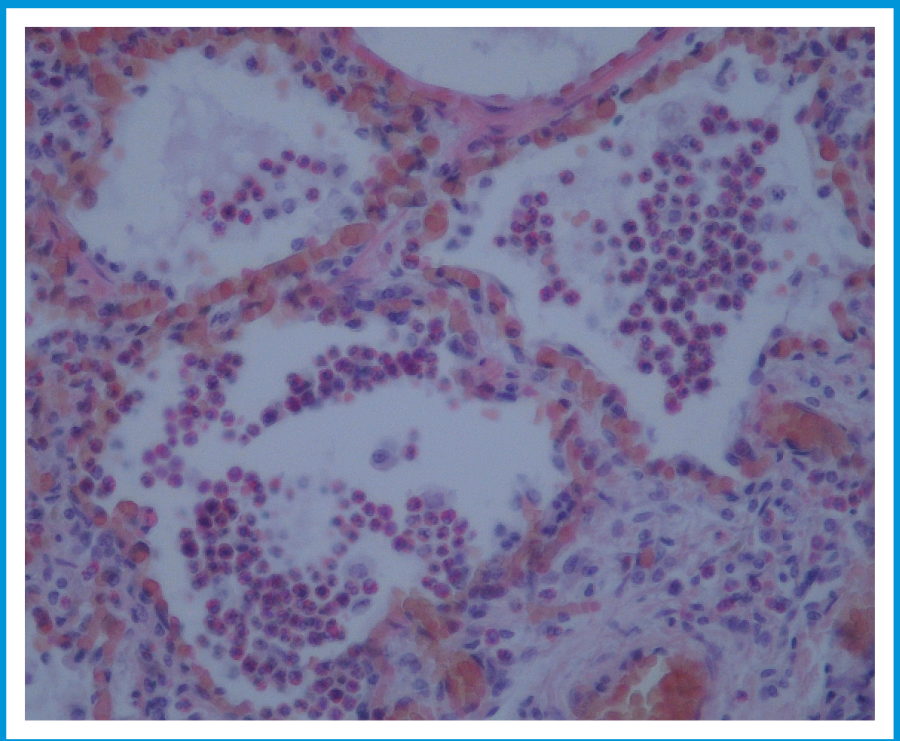

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Original Scientific Article

CAMPYLOBACTERS IN BROILER FLOCKS IN BOSNIA AND HERZEGOVINA: PREVALENCE AND GENETIC DIVERSITY

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Summary: Campylobacters are the most commonly reported bacterial gastrointestinal pathogens in humans. In the EU, the number of reported and confirmed human campylobacteriosis cases was 48.6 per 100,000 population in 2010. Poultry is considered to be the main reservoir of *Campylobacter* because they persist in the gastrointestinal tract of birds in industrial poultry flocks; *Campylobacter*-contaminated poultry meat and meat products are an important risk factor for campylobacteriosis in humans. The aim of this study was to establish the prevalence, genetic diversity and geographical relationships of *Campylobacter* isolates from an assortment of broiler flocks in Bosnia and Herzegovina. The calculated *Campylobacter* prevalence in faecal samples, based on isolation of *Campylobacter* spp. from selected broiler farms in the period from October 2009 to June 2010, was 62.0 %. At slaughter line, skin/carcass samples were positive in 18 out of 31 *Campylobacter*-positive farms (58.1 %). A total of 44 isolates (35 *Campylobacter jejuni* and nine *Campylobacter coli*) from caecal contents (n=31) and skin/carcasses (n=13) of chicken were genotyped by pulsed-field gel electrophoresis (PFGE) using *Sma* I. In general, the obtained *C. jejuni* and *C. coli* isolates exhibited limited genetic diversity. Only isolates with identical or very similar profiles were found on individual *Campylobacter*-positive farms. In addition, skin/carcass isolates showed the same or very similar profiles to campylobacters isolated from pooled caecal content originating from the same broiler batch. Accordingly, carcass cross contamination could not be observed in slaughter line samples.

Key words: *Campylobacter*; poultry; Bosnia and Herzegovina; PFGE; caecal contents; skin; carcass

Introduction

Bacteria from the genus *Campylobacter* have long been known as a causative agent of diarrhoea in cattle and septic abortion in cattle and sheep, but were only recognized as an important cause of human illness in the mid-1970s when *Campylobacter jejuni* was found to be responsible for infectious diarrhoea in man for the first time (1). Campylobacters preferentially inhabit the intestines of birds, including chickens, turkeys, quails, ducks, wild birds and even ostriches (2). Epidemiological studies have revealed a firm association between *Campylobacter* infections in humans and the handling and consumption of raw or undercooked poultry meat; this has been confirmed in many cases (3-8). It is commonly assumed that contamination of poultry meat with campylobacters occurs during slaughterhouse processing and that campylobacters survive throughout the food chain, posing a major risk to public health (4,9). In addition to poultry products, outbreaks of campylobacteriosis have been associated with the consumption of some other animal products, e.g., raw milk (10).

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In 2010, campylobacters continued to be the most commonly reported gastrointestinal pathogens in humans with a notification rate increasing from 45.6 per 100,000 population in 2009 to 48.6 per 100,000 population in 2010 (11). A typical seasonal pattern is often exhibited, especially in northern countries, with peaks during the warm summer months (11,12). The most commonly reported *Campylobacter* species in the EU is *C. jejuni*, accounting for 93.4 % of the confirmed human cases characterized at the species level in 2010 (11). Among the Member States, the prevalence of both the *Campylobacter* colonization in broiler batches (>72 %) and of the *Campylobacter* contamination of fresh poultry meat sampled at slaughter, processing or at retail (>70 %) can be extremely high; however, the prevalence greatly varies at the community level (11). Data demonstrate that the percentage of contaminated carcasses roughly reflects the *Campylobacter* prevalence in broiler batches and that the prevalence is much lower in northern than in central and southern European countries, probably due to different climatic conditions over the year (7,11,12). A geographical relationship of some *Campylobacter* genotypes has also been noticed (13,14).

In Bosnia and Herzegovina (BIH), detailed research on *Campylobacter* prevalence in primary poultry production had not been conducted until the present study. However, the prevalence in broiler flocks was partly studied, giving the main information on the extent of *Campylobacter* carcass contamination during the slaughtering process, since research was performed on poultry retail meat samples (6). Encouraged by the 2008 EU Baseline Study (12), the present research was performed as an initial investigation on the topic. Additionally, pulsed-field gel electrophoresis (PFGE) was employed to discover the genetic diversity of campylobacters on broiler farms and perhaps to demonstrate some geographical relationships of broiler farms, since PFGE has been proven to be appropriate for epidemiological studies (15,16) and a useful tool for identification of potential campylobacteriosis outbreaks (17). To date, PFGE has been used to evaluate the genetic diversity of *Campylobacter* isolates originating from poultry retail meat, human isolates and some isolates of live farm chickens (6) but not for *Campylobacter* isolates originating from different stages of broiler breeding.

The aim of our study was to determine the *Campylobacter* prevalence at different stages of the broiler production cycle, to analyze the genetic diversity of isolates from individual broiler flocks and to compare it among different broiler flocks in BIH.

Materials and methods

Samples

From October 2009 to June 2010, 50 broiler flocks originating from 29 municipalities were randomly selected for the isolation and identification of *Campylobacter* species within the scope of a pilot *Campylobacter* surveillance program conducted in BIH (Figure 1). With the highest density of poultry population, central and northern BIH were selected for sampling. Sampling (10 caeca per sample) of farms (Table 1) started with one-day-old chickens on their arrival at the farm (day 1) and was subsequently performed every seventh day until the end of breeding, when the animals were sent to the slaughterhouse (days 7, 14, 21, 28, 35 and 42). After collection, samples were transported to the laboratory within six hours in a cooling box (4-8 °C) and analysed according to recommended and standardized methods (12,18,19). In total, 3500 caeca (350 samples) were investigated. In addition, five skin/carcass samples were collected at the slaughter line from every *Campylobacter*-positive flock (155 samples in total). The first broiler flock that was confirmed as *Campylobacter*-positive was subjected to more intensive sampling, i.e., every

Table 1: Timetable of sampling for all 50 farms

Period of sampling	Farm numbers (1-50)
October 2009	1-4; 41-43
November	15, 16, 38, 45, 46
December	5-8, 17-19
January 2010	39, 40
February	9, 10, 20, 32-34
March	23, 24, 35-37, 48-50
April	11, 21, 22, 44
May	25, 26
June	12-14, 27-31, 47

Note: For farm numbers, see Table 2. For geographical distribution of farms, see Figure 1. Farms selected for PFGE typing are underlined (farms 1-14).

day after confirmation of *Campylobacter* infection until slaughtering (eight samples of 10 caeca each, in addition to the seven regular samples) and at 12 different positions of the slaughter line (seven skin/carcass samples, in addition to the five regular samples).

Campylobacter isolates

Isolation and identification of *Campylobacter* spp. from faecal material was performed according to the EU guidelines prepared for the 2008 Baseline Study on the prevalence of *Campylobacter* in broiler flocks and *Campylobacter*/*Salmonella* in broiler carcasses (18). Isolation and identification from broiler skin/carcasses was performed according to ISO 10272-1:2006 (19). Briefly, one inoculation loop of 10 pooled caecum contents was streaked onto the selective media mCCDA (modified Charcoal Cefoperazone Deoxycholate Agar) and Skirrow agar. Skin/carcass samples were enriched by the use of modified Bolton broth (1:9), incubated at 41.5 °C in a micro-aerobic atmosphere for 24-48 hrs, then streaked onto the mCCDA and Skirrow media and incubated at 41.5 °C in a micro-aerobic atmosphere for 24-48 hrs.

Bacteria from suspected *Campylobacter* colonies were examined for morphology and motility by dark-field microscopy. After sub-culturing on blood agar plates and antibiotic susceptibility disc-diffusion testing in nalidixic acid (30 µg) and cephalotin (30 µg), they were subjected to determination by selected biochemical tests (catalase, oxidase, indoxyl acetate and hippurate hydrolysis) and aerobic growth at 41.5 °C. Isolates identified as *C. jejuni* or *C. coli* were stored at -76 °C in a cryo-protective medium for PFGE genotyping.

PFGE typing

PFGE was conducted for selected *C. jejuni* and *C. coli* isolates, based on their geographical origin and its importance if occurring in major poultry production regions (Figure 1). From frozen beads, isolates were recovered on blood agar medium and subjected to PFGE genotyping employing *Sma*I restriction endonuclease according to the PulseNet standardised one-day protocol (20). The obtained fragments were electrophoretically separated under the following conditions: 18 h at 6 V/cm and 14 °C, with pulse-times from 6.7 s to 35.4 s employing the CHEF-DR II System (BioRad, USA). PFGE profiles (i.e., pulsotypes) were subjected to computer-assisted analysis with BioNumerics software (version 6.6; Applied Maths, Belgium). In brief, normalization was done according to molecular size standard (three lanes per gel), i.e., *Salmonella* serotype Braenderup H9812 (ATCC BAA-664). Similarity matrices were constructed using the band-based Dice coefficient with optimization and band-matching tolerance set to 1.5 %. Cluster analysis was based on the UPGMA algorithm and the cut-off value defining clusters of isolates was 90 % of similarity according to the dendrogram (21).

Nomenclature of isolates that were subjected to PFGE typing was based on the scheme CJ (for *C. jejuni*) or CC (for *C. coli*) followed by the farm name (abbreviation) and age of chicken at sampling of their caeca (in days; usually 35 or 42). Where chicken skin/carcass samples were *Campylobacter*-positive at the slaughter line, designation S was added to the isolate name (i.e., 42-S).

Results

Distribution of C. jejuni and C. coli

C. jejuni and/or *C. coli* were isolated from 31 (62.0 %) out of 50 investigated farms. From three of the *Campylobacter*-positive farms, both *C. jejuni* and *C. coli* were isolated (9.7 %), from 23 only *C. jejuni* (74.2 %) and from five only *C. coli* (16.1 %). Skin/carcass samples were *Campylobacter*-positive in 18 out of 31 positive farms (58.1 %). Skin/carcasses originating from 15 out of 26 *C. jejuni*-positive farms were positive for *C. jejuni* at slaughtering (57.7 %) and from three out of eight *C. coli*-positive farms positive for *C. coli* (37.5 %). Detailed results are shown in Table 2.

PFGE typing of C. jejuni

A total of 35 *C. jejuni* (CJ) isolates were subjected to PFGE typing: 22 faecal isolates originating from five municipalities (denoted 1-4, 5-8, 9-10, 11 and 13-14 in Figure 1, corresponding to locations Visoko, Gračanica, Srbac, Gradiška and Sarajevo, respectively) and 13 skin/carcass isolates from two farms (S2 and Sr1) (Table 2).

Table 2: *Campylobacter jejuni* and *Campylobacter coli* distribution and origin in *Campylobacter*-positive farms

Farm			Isolates			PFGE	
No.[1]	Name[2]	Location[3]	Ceaca[4] *Day 35*	*Day 42*	S[5]	Isolate name	
1	V1	Visoko	nd	CC	CC		CC V1-42
2	V2		nd	CJ and CC	nd	CJ V2-42	
3	V4		nd	CJ	nd	CJ V4-42	
4	V5		CC	CC	nd		CC V5-35 CC V5-42
5	G1	Gračanica	nd	CC	CC		CC G1-42
6	G2		CJ	CJ	nd	CJ G2-35 CJ G2-42	
7	G3		CJ	CJ	nd	CJ G3-35 CJ G3-42	
8	G4		CJ	CJ	nd	CJ G4-35 CJ G4-42	
9	Sr1	Srbac	CJ	CJ	CJ	CJ Sr1-35 CJ Sr1-42 CJ Sr1-42-S	
10	Sr2		nd	CJ	nd	CJ Sr2-42	
11	Gr1	Gradiška	CC	CJ and CC	nd	CJ Gr1-42	CC Gr1-35 CC Gr1-42
12	T1	Tarčin	CC	CC	nd		CC T1-35 CC T1-42
13	S1	Sarajevo	nd	CJ	nd	CJ S1-42	
14	S2		*Days 28, 33-42* CJ and CC[6]		CJ[7]	CJ S2-28 CJ S2-33…35 CJ S2-38…42 CJ S2-42-S1…S12	CC S2-37
15	BH1	Begov Han	CC	nd	CC		
16	O1	Orašje	CJ	nd	CJ		
17	Z1	Zenica	CJ	nd	CJ		
18	Te1	Tešanj	nd	CJ	nd		
19	Te2		CJ	nd	CJ		
20	P1	Pale	CJ	nd	CJ		
21	N1	Nemila	nd	CJ	CJ		
22	Kl1	Kladanj	nd	CJ	nd		
23	K1	Kakanj	CJ	nd	CJ		
24	Va1	Vareš	CJ	nd	CJ		
25	Gra1	Gradačac	nd	CJ	nd		
26	Tr1	Travnik	nd	CJ	CJ		
27	DG1	D. Golubinja	CJ	nd	CJ		
28	Ž1	Zepče	nd	CJ	CJ		
29	M1	Maglaj	nd	CJ	CJ		
30	Br1	Breza	CJ	nd	CJ		
31	Po1	Posušje	CJ	nd	CJ		

Note: S2 was the earliest *Campylobacter*-positive farm and was therefore subjected to more intensive sampling: in addition to days 1, 7, 14, 21, 28, 35 and 42 (*see text*), also at intermediate days 33, 34 and 36-41 and more intensively at slaughtering (12 skin/carcass samples from different positions on the slaughter line). From farm S2, 22 isolates were subjected to PFGE typing (21 *C. jejuni* and one *C. coli*). From all the *Campylobacter*-positive farms, 44 isolates (abbreviations CJ and CC that are underlined in Isolates column) were subjected to PFGE typing, namely 35 *C. jejuni* isolates from 10 farms and nine *C. coli* isolates from six farms.

Legend: CJ, *C. jejuni*; CC, *C. coli*; nd, not detected

[1], Farm numbers (1-31, *Campylobacter*-positive farms shown in Table 2; 32-50, *Campylobacter*-negative farms not shown in Table 2); [2], Abbreviated farm names; [3], Location of farms (for their geographical distribution according to municipalities, see Figure 1); [4], Caecal samples (age of chicken in days); [5], Skin/carcass samples from the slaughter line; [6], From farm S2, *C. jejuni* was isolated at days 28, 33-36 and 38-42, and *C. coli* at day 37; [7], From farm S2, 12 skin/carcass isolates of *C. jejuni* were obtained from 12 positions on the slaughter line

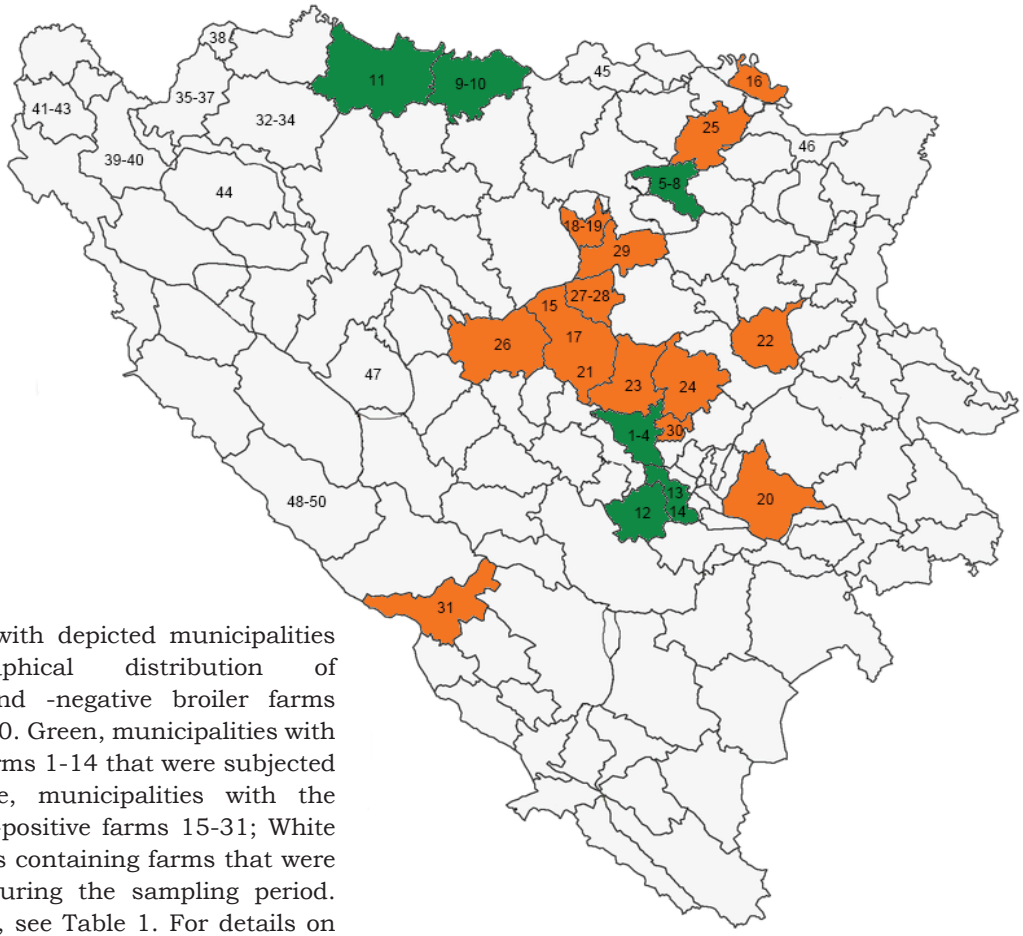

Figure 1: A map of BIH with depicted municipalities showing the geographical distribution of *Campylobacter*-positive and -negative broiler farms denoted with numbers 1-50. Green, municipalities with *Campylobacter*-positive farms 1-14 that were subjected to PFGE typing; Orange, municipalities with the remaining *Campylobacter*-positive farms 15-31; White (numbered), municipalities containing farms that were *Campylobacter*-negative during the sampling period. For timetable of sampling, see Table 1. For details on *Campylobacter*-positive farms 1-31, see Table 2

Pulsotypes revealed five clusters (A1, A2, B, C and D^+) containing 3-4 isolates (farm S2 was subjected to different sampling because of having the earliest *Campylobacter*-positive samples) (Figure 2). Isolates from the farm S2 were assigned to clusters A (A1 and A2), since they showed an 88.9 % similarity due to the difference in position of only one fragment. According to the 90 % cut-off value, cluster D^+ contained three isolates from farms G3 and G4; however, the second isolate from G4 was assigned to the same cluster as it showed a marked similarity of 80 % and did not have other neighbours by similarity in the dendrogram. Four CJ isolates (V4-42, Sr2-42, Gr1-42 and V2-42) with more distinct profiles were not assigned to any cluster.

In clusters A1 and A2 (isolates from S2) and C (isolates from Sr1), containing all CJ isolates from the slaughter line (noted as S), identical *C. jejuni* pulsotypes were revealed when caecal and S isolates were compared. Clusters A1 and A2 comprised 21 isolates with identical (cluster A1 or A2) or very similar (cluster A1 vs. A2) profiles, belonging to *C. jejuni* isolates obtained from animals of different age or from different positions on the slaughter line. Cluster D^+ contained four CJ isolates (G4-42, G3-35, G3-42 and G4-35) showing high genetic similarity, from two farms (G3 and G4) located in the same municipality (denoted 5-8 in Figure 1). Similarly, cluster B also contained isolates, namely three CJ isolates (G2-35, S1-42 and G2-42), from two different *Campylobacter*-positive farms (G2 and S1). However, these originated from geographically distant municipalities (denoted 5-8 and 13-14 in Figure 1), were sampled in two different time periods (G2 in December 2009 and S1 in June 2010; Table 1) and the pulsotypes in cluster B differed in one band in terms of number or position. In general, pulsotypes of *C. jejuni* isolates originating from different farms were heterogeneous in comparison with homogeneous pulsotypes of isolates belonging to the same broiler flock, with the exception of farms G2/S1 and G3/G4; however, the latter two shared the geographical area.

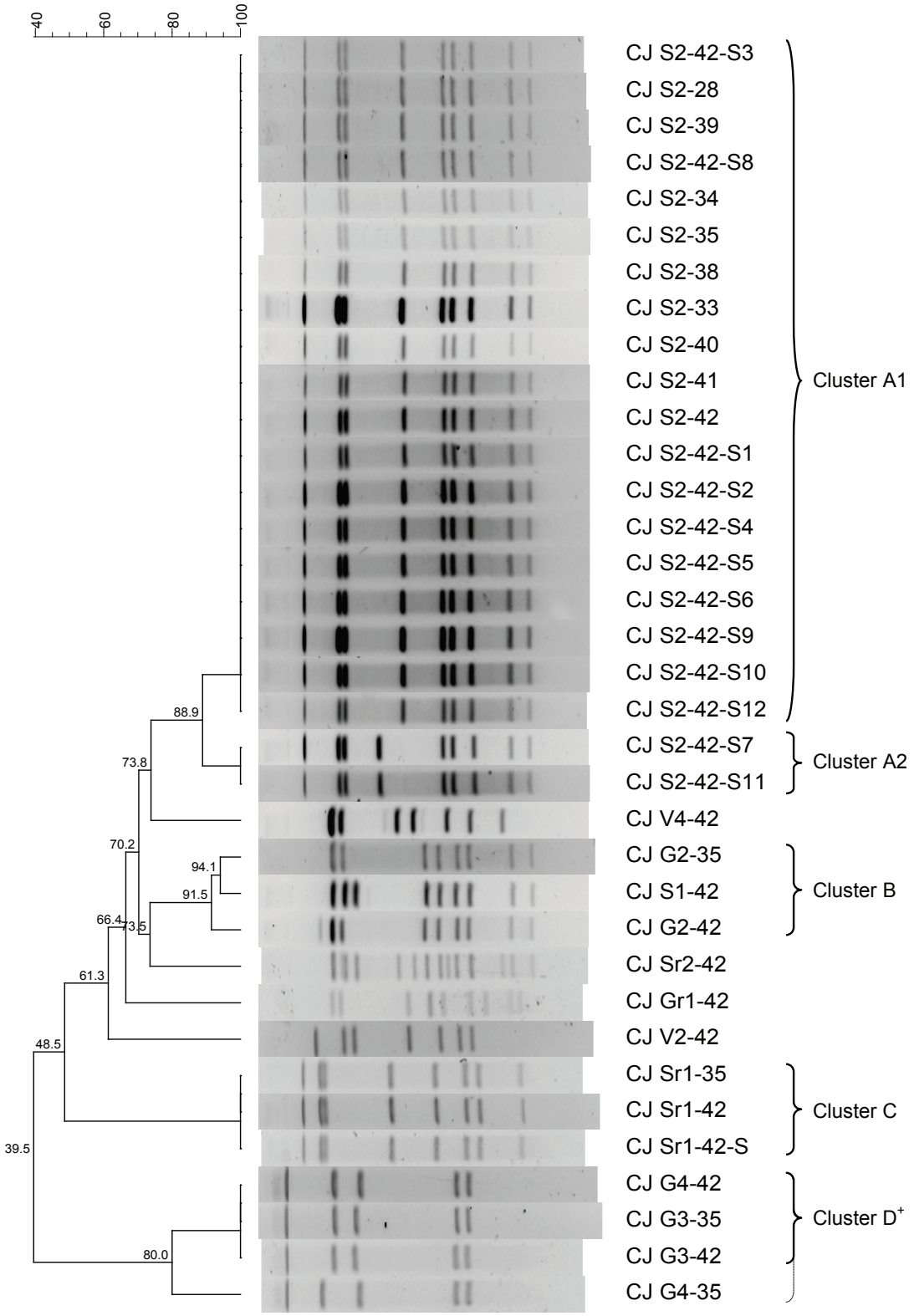

Figure 2: Dendrogram of 35 *Campylobacter jejuni* pulsotypes showing the genetic relatedness of isolates obtained in October 2009 - June 2010 from 10 out of 26 *C. jejuni*-positive broiler farms in BIH. Isolate name consisted of CJ (for *C. jejuni*) followed by the abbreviated farm name (G2, G3, G4, Gr1, S1, S2, Sr1, Sr2, V2 and V4), age of chicken at sampling (35 and 42; for farm S2, also 28, 33, 34 and 38-41) and, when needed for the skin/carcass samples, designation S (42-S; for farm S2, S1-S12 note different positions on the slaughter line). For details, see Table 2

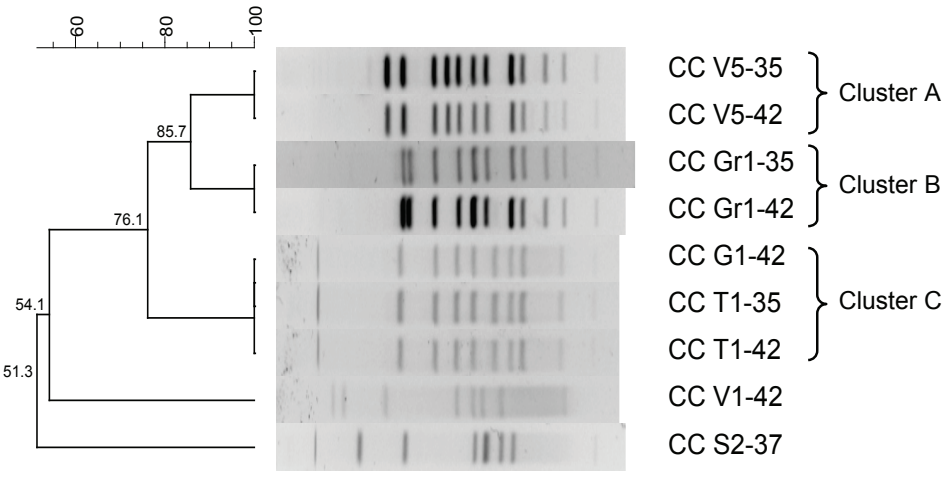

Figure 3: Dendrogram of nine *Campylobacter coli* pulsotypes showing genetic relatedness of isolates obtained in October 2009 - June 2010 from six out of eight *C. coli*-positive broiler farms in BIH. Isolate name consisted of CC (for *C. coli*) followed by the abbreviated farm name (G1, Gr1, S2, T1, V1 and V5) and age of chicken at sampling (35, 37 and 42). For details, see Table 2

PFGE typing of C. coli

A total of nine *C. coli* isolates obtained from the caecal contents of chickens were PFGE typed. Pulsotypes revealed three clusters (A-C; Figure 3) with 2-3 isolates exhibiting identical profiles and belonging to the same farm (V5 or Gr1; cluster A or B) or two separate farms (G1 and T1; cluster C) from two geographically distant municipalities (denoted 5-8 and 12, respectively, in Figure 1, that were, as shown in Table 1, sampled in two different time periods). In addition, two isolates from two different farms (V1 and S2) originating from two neighbouring municipalities (denoted 1-4 and 13-14, respectively, in Figure 1) exhibited distinct profiles.

In general, five pulsotypes (representing clusters A-C and two separate isolates) were observed, belonging to six locations from five municipalities (denoted 1-4, 5-8, 11, 12 and 13-14 in Figure 1) from three different geographical areas. However, clusters A and B (farm V5 and farm Gr1) contained isolates with similar profiles and cluster C (farms G1 and T1) isolates with identical profiles, although obtained over an extended time period and originating from poultry flocks in markedly different geographical areas.

Discussion

Bacteria of the genus *Campylobacter* remain the most frequently reported cause of human gastrointestinal disease in the EU (11,22). Poultry has often been associated with campylobacteriosis (23-28). To date, there have not been sufficient studies estimating the prevalence of *Campylobacter* spp. in primary poultry production in BIH. Bearing in mind the high prevalence of campylobacters in most European countries (11,22), the aim of our study was to carry out a more detailed research on *Campylobacter* prevalence at farm level. The obtained results can confirm the presence of *Campylobacter* spp. in BIH and also reveal their genetic diversity.

Our research showed that broilers in BIH are frequently colonised with *Campylobacter* spp. at farms and at slaughtering; contamination of carcasses, poultry meat and meat products consequently occurs, as has been confirmed by previous studies (6-8). During October 2009 and June 2010, the prevalence of campylobacters in the investigated farms was 62.0 %, which is in accordance with data from other countries, e.g., Germany 48.9 %, UK 75.0 %, France 76.0 %, Slovenia 78.2 % (12,22) and in some previously released publications (23,24,29,30). Given that the sampling period was predominantly during the colder period of the year and that campylobacters show a seasonal pattern (11,12,31-33), the actual prevalence could probably be expected to be even higher. Our results suggest that colonisation of caecum with campylobacters begins around the 28th day during poultry breeding, although it has been suggested that colonisation could occur much earlier (2,34). In our study, *C. jejuni* was more frequently isolated than *C. coli*, namely *C. jejuni* from 74.2 % and *C. coli* from 16.1 % of the *Campylobacter*-positive farms, which is consistent with other publications (11,12,22,31,35). In three cases, both *C. jejuni* and *C. coli* were isolated from the same farm in our study (9.7 %), also consistent with some previously released publications on

the presence of both *Campylobacter* species in a broiler flock (36,37).

The obtained PFGE results indicate a limited variability of pulsotypes belonging to *Campylobacter* isolates at farm level. Other publications suggest a greater genetic diversity of *Campylobacter* isolates, both within a farm and within geographical areas (37,38). Despite difficulties in the epidemiological research of *Campylobacter* bacteria caused by their diversity, our results suggest that a persistent and dominant type of *Campylobacter* strain could occur within a flock and, consequently, at the slaughter line. On the other hand, identical or very similar *C. jejuni* genotypes were obtained from two neighbouring farms (G3 and G4), although that could be a result of many circumstances, such as the presence of house flies (39), rodents, wild birds, flies or humans (e.g., transmission by protective clothing) as vectors (40). It was also revealed that certain *C. jejuni* and *C. coli* isolates obtained from farms in different geographical areas, and over extended time periods, showed marked genetic similarity. Vertical transmission of campylobacters could be suspected, especially if it was proven that both farms obtain animals from the same parent flock. Since evidence of vertical transmission of *Campylobacter* strains in chickens is lacking from publications (41,42), a more detailed sampling program must be performed in parent flocks and hatcheries. In addition, it can be concluded that certain genotypes can persist over time, revealing *C. jejuni* or *C. coli* isolates obtained in different time periods but showing very similar or identical genetic fingerprints.

Pulsotypes of *C. coli* showed somewhat higher homogeneity than those of *C. jejuni*; when a strain of *C. coli* was isolated more than once from a broiler flock, it showed an identical genotype profile (e.g., farms Gr1, T1 and V5). In addition, PFGE results revealed that cross-contamination of carcasses at the slaughter line is probably not present; although *C. jejuni* pulsotypes belonging to farm S2 were not identical (cluster A1 vs. A2 in Figure 2), the two pulsotypes that differed in the position of only one band (cluster A2) were very similar to others belonging to skin/carcass isolates from the same farm (cluster A1) and no similar pulsotypes could be observed belonging to samples from other poultry flocks.

Our results revealed and confirmed that different strains of *C. jejuni* and *C. coli* are present in different farms and geographical areas. In view of the considerable number of isolates, the results also indicated that a dominant *Campylobacter* strain may be present in a broiler flock and, consequently, at the slaughter line, consistent with other studies (43). If this hypothesis proves to be correct, it would enable epidemiological research and prevention of campylobacteriosis by linking a particular strain to its source and checking sources and transmission routs in a flock and poultry retail products. Prevention of *Campylobacter* contamination at the farm level would therefore be much more efficient if the critical points were highlighted and strict bio-security measures taken. For better understanding of the epidemiology of *Campylobacter* bacteria in a flock, it is necessary to design successful prevention programs at the farm level. With this in mind, an extensive surveillance program in BIH will be conducted during 2012 in order to gain more knowledge on the genetic diversity of campylobacters.

We believe that the obtained results have scientific value, especially since previous research of this kind in primary poultry production has not given enough data on the prevalence and diversity of specific *Campylobacter* strains. The obtained knowledge brings new possibilities to the epidemiological research of campylobacters and indicates the importance of cooperation between veterinary and public health laboratories.

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KAMPILOBAKTRI V REJAH PITOVNIH PIŠČANCEV V BOSNI IN HERCEGOVINI: PREVALENCA IN GENETSKA RAZNOLIKOST

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Povzetek: Bakterije iz rodu *Campylobacter* so najpogosteje prijavljeni bakterijski povzročitelji prebavnih obolenjih ljudi. V letu 2010 je bilo v Evropski uniji na 100.000 ljudi prijavljenih in potrjenih 48,6 primerov kampilobakterioz. Ker kampilobaktri naseljujejo prebavni trakt živali v industrijskih perutninskih rejah, je perutnina njihov glavni rezervoar; s kampilobaktri okuženo perutninsko meso in mesni izdelki predstavljajo pomemben dejavnik tveganja za kampilobakteriozo pri ljudeh. Namen našega dela je bil ugotoviti prevalenco, genetsko raznolikost in geografsko povezanost izolatov *Campylobacter* iz nabora rej pitovnih piščancev v Bosni in Hercegovini. Na podlagi izolacije bakterij iz rodu *Campylobacter* iz izbranih rej pitovnih piščancev v obdobju od oktobra 2009 do junija 2010 je bila izračunana prevalenca v vzorcih fecesa 62,0 %. Na klavni liniji so bili vzorci kože ali trupov pozitivni v 18 od 31 primerov rej, ki so bile pozitivne na kampilobaktre (58,1 %). Z metodo pulzne gelske elektroforeze (PFGE) smo z encimom SmaI genotipizirali 44 izolatov (35 *Campylobacter jejuni* in 9 *Campylobacter coli*) iz vsebine slepega črevesa (n=31) in kože ali trupov (n=13) piščancev. Pridobljeni sevi *C. jejuni* in *C. coli* so v splošnem izražali omejeno genetsko pestrost. V posameznih rejah, ki so bile pozitivne na kampilobaktre, smo našli samo seve z enakimi ali zelo podobnimi profili. Izolati iz kože ali trupov so imeli enake ali zelo podobne profile kot kampilobaktri, ki smo jih izolirali iz združene vsebine cekuma iz iste reje pitovnih piščancev, torej navzkrižnega okuževanja med vzorci na klavni liniji nismo opazili.

Ključne besede: *Campylobacter*; perutnina; Bosna in Hercegovina; PFGE; vsebina slepega črevesa; koža; trup

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Original Scientific Article

ASSOCIATIONS BETWEEN THE FAT TO PROTEIN RATION IN MILK, HEALTH STATUS AND REPRODUCTIVE PERFORMANCE IN DAIRY CATTLE

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Summary: The aim of the present study was to evaluate pregnancy rates of 232 dairy cows in relation to fat to protein ratio (FPR) in milk, using survival analysis. Pregnancy rates of cows inseminated within 90 and 120 days postpartum in a group of clinically healthy cows were 38 and 68 %, respectively. Lower pregnancy rates are observed in groups of cows with ketosis and reproductive disorders, 44 and 28 % for pregnancy rate within 120 days. The highest correlation between FPR and calving to conception interval (CC) was observed between 30 and 60 days postpartum ($r = 0.411$, $P < 0.001$). Diagnostic evaluation of FPR using ROC (receiver operating characteristics) analysis showed that FPR at 1.37 discriminates cows with CC below and above 120 days with an accuracy of 71 %. Survival curves for the subgroups of animals with FPR below or above 1.37 differed significantly in the case of clinically healthy cows, where CC in subgroups were 87 ± 28 and 122 ± 42 days, respectively. Although survival curves for subgroups for cows with diseases did not differ significantly we observed longer CC in all subgroups with FPR > 1.37 than in subgroups with FPR < 1.37. In all groups pregnancy rates within 90 and 120 days were lower in subgroups with FPR > 1.37 than in subgroups with FPR < 1.37. Therefore, FPR can be used by bovine practitioners to predict fertility problems in dairy herds.

Key words: dairy cows; fat to protein ratio in milk; calving to conception interval; ROC analysis; survival analysis

Introduction

Increased fat mobilization in a period of negative energy balance (NEB) coupled with decrease in dry matter and energy intake is shown in higher milk fat concentration and lower milk protein concentration in the postpartum period of dairy cows (1). In the last fifteen years a correlation between energy balance in the early postpartum period and milk composition has been reported, using different parameters such as fat to protein ratio (FPR), protein to fat ratio, fat/lactose quotient, milk yield and milk protein concentration (2, 3, 4, 5). Buttchereit *et al.* (6) showed FPR to be a suitable indicator of the energy status in postpartum dairy cows. FPR indicates low energy balance more reliably than body condition score (BCS) (7). A FPR threshold of 1.4 during the first month of lactation is commonly used by veterinary practitioners as a marker of NEB (8). FPR is also used as a diagnostic tool to estimate

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some metabolic disorders such as subclinical and clinical ketosis (1, 7).

Geishauser *et al.* (9) found that FPR in the first test milk could be useful as a predictor for subsequent abomasal displacement. Clinical mastitis appears most commonly in the first 30 days postpartum (10). Increased incidence of mastitis during early lactation or at peak production could result from a severe NEB (11, 12) severity of induced *E coli* mastitis has been related to some blood metabolite concentrations characteristic of NEB (13). Animals exposed to severe NEB suffer from impaired reproductive performance (14, 15, 16) which can be shown as absence of oestrus signs, delayed onset of cyclicity, failure to conceive at 1st artificial insemination (AI) and finally in prolonged calving to conception interval. Moreover, FPR was recently assessed as a predictor of calving to conception interval (CC) of individual cows, using fixed thresholds (17).

The aim of the present study was to evaluate practical use of milk and medical data records in association with reproductive performance in high yielding dairy cows. We evaluated pregnancy rates of dairy cows in relation to FPR in milk, using survival analysis. First, a complete receiver operating characteristics (ROC) analysis, was performed to provide an index of accuracy by demonstrating the limits of the test's ability to discriminate between healthy cows pregnant at day 90 (or day 120) after calving or not (18). Groups of clinically healthy cows and cows with ketosis, clinical mastitis or fertility problems were compared using Kaplan-Meier survival curves.

Material and methods

Animals and data

Records of 232 high yielding dairy cows (Holstein-Friesian), BVD and IBR-IPV free, in a period from April 2009 to June 2010 formed a basis for the study. Cows were kept in a free-stall barn system. The basic ration was composed of hay, grass and maize silage. According to the milk yield, protein concentrate (19 % digestible raw protein), roughly crushed maize grains and vitamin-mineral mixture were supplemented by a computerised feeding system. All cows could access basic ration and water *ad libitum* during the whole year in the stall. The voluntary waiting period of the herd was 80 days. They were inseminated by a well trained inseminator. Reproduction parameters, calving to 1st service (CFS), 1st service to conception (FSC) and calving to conception (CC) intervals were derived from farm records. All animals showing clinical signs of disease were examined and treated by bovine practitioners using standardized protocols. Treatment data were recorded for every animal individually. Animals were divided into 5 groups according to occurrence of different diseases in the first 90 days post partum (p.p.) (Table 1): Group 1: clinically healthy cows; Group 2: cows with clinical ketosis (diagnose based on clinical signs and milk tests with sodium nitoprosside); Group 3: cows with clinical mastitis; Group 4: cows with fertility problems (included gynaecological disorders such as retained placenta, puerperal metritis, cystic ovarian disease and endometritis); Group 5: other cows.

Milk sampling and analysis

Daily milk yield was measured and milk samples were collected at regular test days performed in a 30 day intervals in the post partum period at three stages: stage 1, 0 – 30 days post partum; stage 2, 30 – 60 days post partum; stage 3, 60 – 90 days post partum. Samples were conserved with sodium azide and sent, at outdoor temperature, to a dairy research laboratory. Protein and fat were analysed in milk samples using Fourier Transform Infrared Spectroscopy (CombiFoss 6000).

Statistical analysis

Groups of cows were compared with respect to CFS, FSC, CC, milk yield and FPR at stages 1, 2 and 3, using One way analysis of variance in the case of normal distribution of the data or Kruskal-Wallis one way analysis of variance on ranks in the case of non-normal distribution of data. When a significant difference among the groups was found, further pair-wise multiple comparisons were performed using the Holm-Sidak method (normal data distribution) or Dunn's method (non-normal data distribution).

FPRs in stages 1, 2 and 3 were compared within each group using One way repeated measures analysis of variance or Friedman repeated measures analysis of variance on ranks, according

to the normal or non-normal distribution of the data, followed by pair-wise multiple comparison using the Holm-Sidak method and Tukey's test, respectively.

ROC analysis

First, a correlation between milk data records and reproductive parameters of clinically healthy cows was determined using Spearman rank correlation coefficient. 115 healthy cows were included in diagnostic evaluation of FPR. Receiver operating characteristics (ROC) analysis was used to evaluate FPR in stage 2 (30-60 days postpartum) to discriminate between cows with CCs above and below 120 days. Stage 2 was selected on the basis of the highest correlation between FPR and CC in stage 2, and the criterion value of 120 days was based on reproductive characteristics found in Slovenian dairy herds.

Sensitivity (proportion of cows with FPR below the cut-off value in cows with CC below 120 days) and specificity (proportion of cows with FPR above the cut-off value in cows with CC above 120 days) were calculated for all possible cut-off values (Analyse-it, General + Clinical Laboratory statistics, version 1.71). ROC curves, displaying true positive rate (sensitivity) against false positive rate (1-specificity) for the complete range of cut-off points were used to determine the cut off value that minimizes the sum of false negative and false positive results (19). This optimal cut off value is found closest to the upper left hand corner of the ROC curve. The selection was supported with the plot of sensitivity and specificity as a function of cut-off value, which provides a useful visualisation in selecting optimal cut-off values on the basis of the best balance of sensitivity and specificity (20). Area under the curve (AUC) provides an index of accuracy by demonstrating the limits of an FPR's ability to discriminate between cows with different CC (18).

Survival analysis

Differences in proportion of non-pregnant cows among groups of healthy cows, cows with ketosis, cows with reproductive disorders and cows with clinical mastitis were measured by Kaplan-Meier survival analysis (21). Kaplan-Meier survival curves were constructed and compared using Log Rank test following pair-wise multiple comparison using the Holm-Sidak method in the case of significant difference among the curves. Criteria of censored animals included cows that did not conceive until day 300 post partum and animals culled during the study. For each cow's group, survival curves for subgroups, divided by FPR at 1.37, were constructed and compared using Log rank test. Pregnancy rates were calculated as numbers of cows conceived within 90 and 120 days, divided by the total number of cows in a group (22).

Results

Milk data in lactating cows

Distribution of diseases and groups of cows are presented in Table 1. Reproductive performance and milk data in lactating cows are summarized in Table 2.

Cows that were culled for various reasons during the study are recorded in Table 3. Mean milk yield over all cows in the first 100 days was 3069 ± 716 kg (average ± SD) and did not differ between the groups of clinically healthy cows and cows with diseases ($P>0.05$). No significant difference in FPR between the groups is observed in stage 1 ($P>0.05$), whereas FPR in stages 2 and 3 differed among the groups ($P < 0.001$ and $P = 0.003$, respectively). FPR within the group of cows suffering from ketosis did not differ significantly between stage 1 and stage 3 FPR ($P > 0.05$), whereas in clinically healthy cows and cows with reproductive disorders or clinical mastitis FPR decreased from stage 1 to stage 3 with a difference close to statistical significance ($P=0.067$, $P=0.046$ and $P=0.048$ respectively).

Reproductive performance and comparison of CC among the groups

The CFS interval was calculated as 87 ± 32 days; a significant difference was observed among the two groups ($P=0.021$), corresponding only to the difference between cows with clinical mastitis and cows with fertility problems ($P<0.05$) (Table 2). The average CC in the lactating cows was 113 ± 49. A significant difference was observed among the groups according to the calving to conception (CC) interval ($P < 0.001$).

Table 1: Distribution of diseases and structure of groups of cows

Groups (N)	Subgroups (N)	disease				
		ketosis	EM	RP	Clinical mastitis	DA
Group 1: Clinically healthy cows (n=130)	/	–	–	–	–	–
Group 2: Cows with clinical ketosis (n=32)	15	+	–	–	–	–
	7	+	–	–	+	
	8	+	–	–	–	+
	1	+	+	+		–
	1	+	+	+	+	–
Group 3: Cows with clinical mastitis (n=32)	/	–	–	–	+	–
Group 4: Cows with fertility problems (n=36)	15	–	+	–	–	–
	11	–	–	+	–	–
	5	–	+	+	–	–
	2	–	+	_	+	–
	1	–	–	+	+	–
	1	–	+	+	+	–
	1	–		+		+
Group 5: Other cows (n=2)	/	–	–	–	–	+

Legend: EM: endometritis; RP: retained placenta; DA: abomasal displacement

Table 2: Reproductive performance and milk data records of dairy cows in different groups according to disease

Group	1	2	3	4	Total
	Healthy cows	Cows with clinical ketosis	Cows with clinical mastitis	Cows with fertility problems	
N	130	32	32	36	230
CC (days)	103 ± 39	132 ± 57	98 ± 46	153 ± 51	113 ± 49
FSC (days)	18 ± 29	50 ± 55	17 ± 33	53 ± 46	28 ± 40
CFS (days)	85 ± 31	85 ± 38	80 ± 27	100 ± 32	87 ± 32
Milk yield (kg)	3069 ± 716	3267 ± 693	3211 ± 797	2948 ± 714	3096 ± 726
FPR – stage 1	1.39 ± 0.31	1.48 ± 0.34	1.41 ± 0.27	1.45 ± 0.27	1.42 ±0.30
FPR – stage 2	1.35 ± 0.25	1.59 ± 0.30	1.37 ± 0.23	1.37 ± 0.25	1.39 ± 0.27
FPR – stage 3	1.30 ± 0.25	1.46 ± 0.19	1.29 ± 0.17	1.33 ± 0.18	1.32 ± 0.23

Legend:
CC: calving to conception interval;
FSC: 1st service to conception interval;
CFS: calving to 1st service interval;
FPR – stage 1: fat to protein ratio in milk between 0 and 30 days post partum;
FPR – stage 2: fat to protein ratio in milk between 30 and 60 days post partum;
FPR – stage 3: fat to protein ratio in milk between 60 and 90 days post partum.
Values are expressed as mean ± SD.

CC interval of clinically healthy cows and cows with clinical mastitis was comparable. Conversely, cows with ketosis and fertility problems required longer times to conceive (Table 2). Cows with clinical ketosis and fertility problems had a significant longer CC interval than those in the group of clinically healthy cows ($P < 0.05$). Significantly lower CC is observed in clinically healthy cows and cows with clinical mastitis than in cows with fertility problems ($P<0.05$) (Table 2).

The proportion of non-pregnant cows in different groups was evaluated using survival analysis (Fig. 1). Significant differences were observed among Kaplan-Meier survival curves for all groups ($P=0.003$). Pair-wise multiple comparisons showed significant differences between survival curves of clinically healthy cows and cows with fertility problems and between cows with clinical mastitis and cows with fertility problems ($P<0.05$). Survival curves for clinically healthy cows and cows with ketosis differ, but not significantly ($P=0.0794$). Pregnancy rates within 90 and 120 days postpartum in a group of clinically healthy cows were 38 and 68 %, respectively. Lower pregnancy rates within 120 days were observed in the group of cows suffering from ketosis and in the group of cows with reproductive disorders (44% and 28% respectively), whereas 69 % of cows with clinical mastitis conceived by day 120 postpartum.

Diagnostic evaluation of FPR

The diagnostic ability of FPR was evaluated in the group of clinically healthy cows.

First, Spearman rank correlation coefficients were calculated between reproduction parameters, milk yield and FPR in all stages. Milk yield did not correlate with any of reproductive parameters, but significant correlations were observed with FPR in stage 1 ($r=0.190$, $P=0.0325$), stage 2 ($r=0.279$, $P=0.0015$) and stage 3 ($r=0.200$, $P=0.0243$).

CFS correlated with FPR in stage 1 ($r=0.233$, $P=0.0108$) and stage 2 ($r=0.246$, $P=0.0072$). No correlation was observed between CFS and FPR in stage 3 or between SP and FPR in stage 1 ($P>0.05$). Low correlations between FSC and FPR in stage 2 and stage 3 were observed ($r=0.290$ $P=0.0017$; $r=0.254$, $P=0.0062$); similar correlations were found between CC and FPR in stages 1 and 3 ($r=0.274$, $P=0.0031$; $r=0.2565$, $P=0.0042$). The strongest correlation between CC and FPR was observed in stage 2 ($r=0.411$, $P<0.001$). FPR in stage 2 was therefore further evaluated diagnostically.

Selection of optimal cut-off values of FPR

Area under the ROC curve (AUC) for the criterion value of 120 days post partum (AUC = 0.726; $P<0.0001$) indicates that FPR is valuable in distinguishing cows with different CC (Fig. 2).

The best balance between sensitivity and specificity is observed, with 71 % accuracy, at an optimal cut-off point of FPR = 1.37, corresponding to a sensitivity of 74 % and specificity of 68 %. 90 % sensitivity was found for the cut-off at 1.22, whereas cut-off at 1.53 provides over 85 % specificity of FPR (Fig. 3).

Comparison of CC according to FPR in healthy cows and cows with ketosis, fertility problems or clinical mastitis

Survival curves for subgroups, divided by FPR at 1.37, differed only for clinically healthy cows ($P=0.007$; Fig. 4A), showing CC intervals in subgroups with FPR below and above 1.37 were 87 ± 28 and 122 ± 42 days, respectively. Pregnancy rates within 90 or 120 days were higher in a subgroup of FPR < 1.37 than in a subgroup with FPR > 1.37. Subgroups of FPR < 1.37 has a pregnancy rate of 52 % at 90 days and 79 % at 120 days, whereas a subgroup of FPR > 1.37 has a pregnancy rate of 19 % at 90 days and 53 % at 120 days.

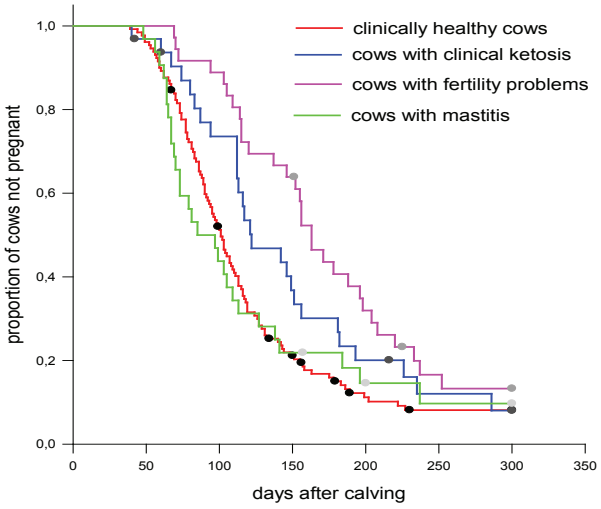

Figure 1: Kaplan-Meier survival analysis for the proportion of lactating dairy cows non pregnant, according to disease status

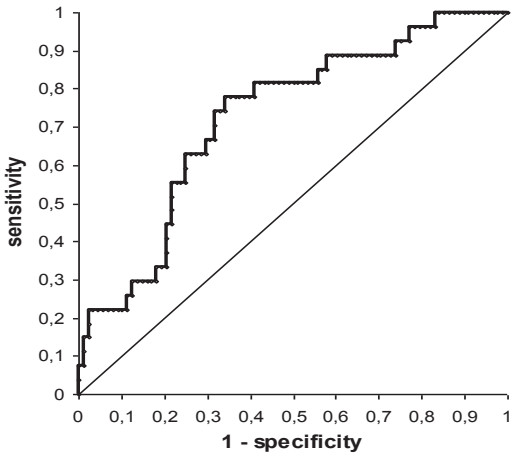

Figure 2: ROC curve of FPR for identifying healthy cows with pre-selected minimum of postpartum period at 120 days
Characteristics for the ROC curve are as follows: Area under the curve (AUC): 0.726, P < 0.0001

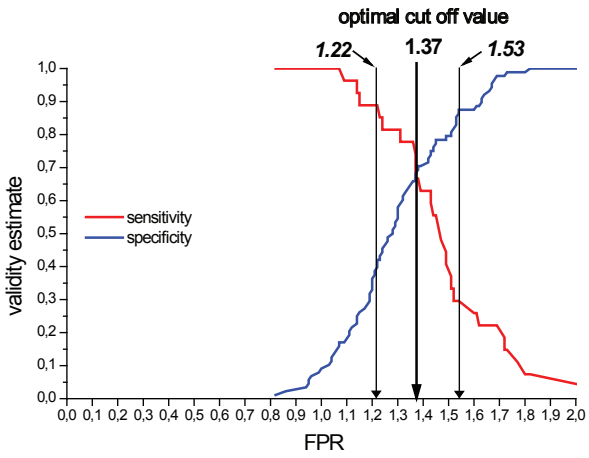

Figure 3: Plot of diagnostic parameters of FPR according to pregnancy status at 120 days post partum for the selection of optimal cut-off values

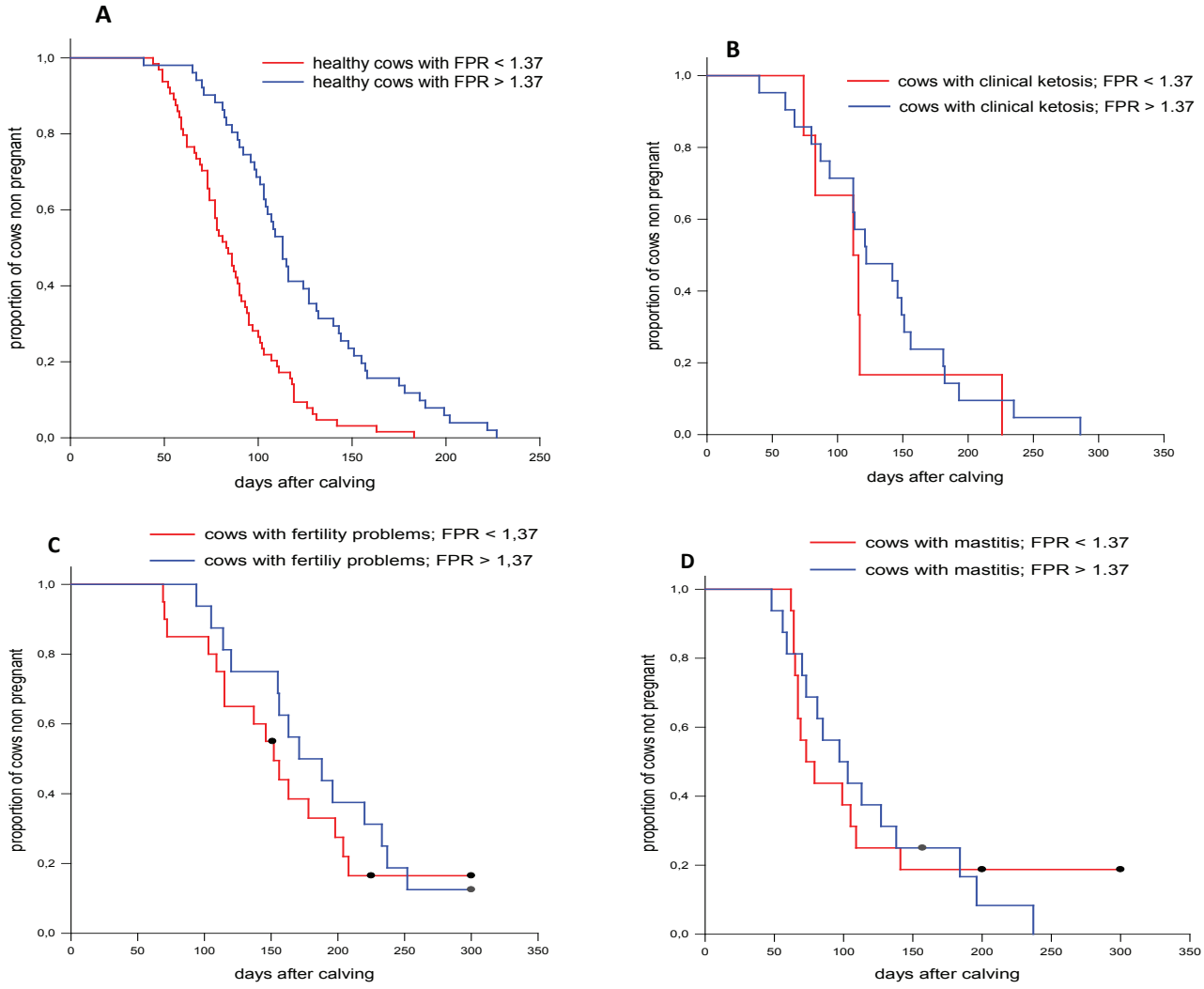

Figure 4: Kaplan-Meier survival analysis for the proportion of cows clinically healthy cows (A), cows with clinical ketosis (B), cows with fertility problems (C) and cows with clinical mastitis (D) non pregnant, according to the cut off value of FPR at 1.37

Table 3: Reproductive performance of dairy cows in different groups according to the diseases

	Groups			
	1	2	3	4
	Healthy cows	Cows with clinical ketosis	Cows with clinical mastitis	Cows with fertility problems
total group				
N	130	32	32	36
cows censored (%)	12	16	13	17
mean CC (days)	103 ± 39	132 ± 57	98 ± 46	153 ± 51
pregnancy rate at 90 days (%)	38	22	50	8
pregnancy rate at 120 days (%)	68	44	69	28
subgroup; FPR < 1.37				
N	73	6	16	20
cows censored (%)	12	0	19	20
mean CC (days)	87 ± 28	121 ± 54	82 ± 24	136 ± 48
pregnancy rate at 90 days (%)	52	33	56	15
pregnancy rate at 120 days (%)	79	83	75	35
subgroup; FPR > 1.37				
N	57	26	16	16
cows censored (%)	11	19	6	13
mean CC (days)	122 ± 42	135 ± 59	111 ± 56	171 ± 50
pregnancy rate at 90 days (%)	19	19	44	0
pregnancy rate at 120 days (%)	53	35	63	19

Legend: FPR: fat to protein ratio; CC: calving to conception interval

Survival curves for subgroups for cows with diseases did not differ significantly (P>0.05) (Fig. 4: B, C, D), although longer CC in all subgroups with FPR > 1.37 than in subgroups with FPR < 1.37 were observed (Table 3).

Discussion

The aim of this study was to evaluate fat to protein ratio (FPR) in milk for the first three months of lactation and find a threshold which is most appropriate to predict reproductive performance of dairy cows. Results of many studies in the past decade have shown significant correlations between body condition score, energy balance, mobilization of body reserves, blood metabolite concentrations, milk traits and different production and reproductive disorders (3, 4, 6, 15, 23).

Our results demonstrated poor reproductive performance in cows with ketosis and reproductive disorders. They had significantly longer CFS and CC intervals than healthy cows and cows with clinical mastitis. Pregnancy rates were calculated as the proportion of cows conceived within 90 or 120 days (22). A lower proportion of pregnant

cows were observed in groups with ketosis and fertility problems, while the proportion of cows with clinical mastitis conceiving up to day 120 postpartum is similar to that for healthy ones. Prolonged CC intervals in cows with clinical ketosis were probably related to exposure to NEB, which can be caused by either high milk yield or displaced abomasum. Cows in NEB are subject to increased risk of clinical mastitis (12). The group of mastitic cows in the first three months post partum, contrary to our expectation, did not differ significantly from the clinically healthy one in FPR, CFS, FSC or CC intervals. Reasons may be found in rapid response to treatment, better management and care of treated animals, but also in the statistically smaller number of mastitic cows included in the study compared to healthy ones.

Buttchereit *et al.* (6) showed FPR as a suitable indicator of the energy status of dairy cows during the most critical period for their metabolic constitution. FPR is also used as a diagnostic tool for estimating nutritional imbalance and some metabolic disorders such as subclinical or clinical ketosis (1, 7). Therefore in our study FPR was evaluated by correlation with reproduction parameters and its association with certain diseases.

The strongest correlations were observed between FPR and milk yield, CFS, FSC and CC intervals in stage 2. In our previous study (17) the strongest correlations were observed in stage 3, but clinically healthy and diseased animals were not differentiated. From the diagnostic point of view, the strongest correlations in stage 2 enable us to predict animals at risk before the voluntary waiting period ends. Although FPRs were calculated for only three lactation stages, the tendency to decrease is evident in all groups and comparable to the results of Buttchereit *et al.* (6).

ROC analysis showed that the optimal cut-off value at 1.37 in our study allowed discrimination between cows with CC above 120 days and cows with CC below 120 days with an accuracy of 71 %. High sensitivity of FPR was found at a cut-off value of 1.22, which enabled around 90 % correct identification of cows with CC lower than 120 days. On the other hand, cows with FPR more than 1.53 were over 85 % correctly identified as cows with CC above 120 days.

It appears that the results of optimal cut-off values of FPR differ among studies due to the numbers of animals or herds included in the studies, their general nutrition status, lactation time frame and the statistical methods used (1, 7, 8, 17, 24). Nevertheless it is clear that cows with FPR values above 1.4 in early postpartum are at high risk of NEB-dependent disorders such as ketosis, displaced abomasum and fertility problems (1).

Survival curves calculated for subgroups of animals with FPR below and above 1.37 differed significantly only in the case of clinically healthy cows. According to survival curves, pregnancy rates within 90 and 120 days were higher in the subgroup of FPR < 1.37 than in the subgroup with FPR > 1.37 (19 and 53 %). This indicates that even some clinically healthy cows (FPR>1.37) are exposed to intensive NEB.

Although survival curves for subgroups for cows with diseases did not differ significantly (P>0.05), pregnancy rates within 90 and 120 days in all groups were lower in subgroups with FPR > 1.37 than in subgroups with FPR < 1.37. The reason that no significant difference is observed between subgroups of cows with ketosis could be the small number of cows with FPR < 1.37. However, longer CC intervals were observed in all subgroups with FPR > 1.37 than in subgroups with FPR < 1.37. It can thus be concluded that FPR is strongly associated with the pregnancy rate in NEB-related diseases such as clinical ketosis, whereas in cows suffering from other diseases the increased FPR contributes to prolonged CC, but the effect is not as strong as in cows with clinical ketosis.

In our previous study it was shown that FPR in milk could be an indicator of the ability of a cow to adapt to the demands of milk production and reproduction in the post partum period, resulting in prolongation of the latter (17). It is also in accordance with the fact that the rate of mobilization of body reserves is directly related to the postpartum interval to first ovulation and to lower conception rate (25).

The present study clearly demonstrates that milk data records (e.g. FPR) and medical data can be used by bovine practitioners to analyse and to predict some fertility problems, mainly failure to conception and address metabolic disorders such as ketosis more quickly. The results presented here offer a simple and useful tool for assessing energy balance in a dairy herd in order to predict reproductive performance.

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POVEZAVA MED RAZMERJEM MAŠČOB IN BELJAKOVIN V MLEKU, ZDRAVSTVENIM STATUSOM IN REPRODUKCIJSKO SPOSOBNOSTJO KRAV MOLZNIC

O. Podpečan, J. Mrkun, P. Zrimšek

Povzetek: V raziskavi smo z analizo preživetja ovrednotili deleže brejosti 232 krav mlečne pasme v povezavi z razmerjem med maščobami in proteini (koeficient M/B) v mleku. Delež brejih krav v obdobju 90 oziroma 120 dni po porodu je bil 38 oziroma 68 %. Pri kravah s ketozo in reprodukcijskimi problemi smo po 120 dneh po porodu ugotovili nižji delež brejih krav in sicer 44 oziroma 28 %. Najvišjo korelacijo med poporodnim premorom (PP) in razmerjem M/B smo ugotovili v obdobju med 30 in 60 dnevi po porodu ($r = 0,411$; $P < 0,001$). Na podlagi diagnostičnega vrednotenja razmerja M/B z uporabo krivulj ROC (receiver operating characteristics) smo ugotovili, da razmerje M/B pri 1,37 z 71 % zanesljivostjo loči krave s poporodnim premorom pod 120 dnevi in nad tem obdobjem. Krivulje preživetja za podskupine krav z razmerjem M/B nad 1,37 in pod 1,37 so se statistično značilno razlikovale pri zdravih kravah, kjer je povprečni poporodni premor znašal 87 ± 28 dni za krave z razmerjem M/B pod 1,37 in 122 ± 42 dni za krave z razmerjem nad 1,37. Krivulje preživetja za omenjene podskupine se pri kravah z različnimi boleznimi niso statistično značilno razlikovale, čeprav smo pri vseh skupinah opazili daljši poporodni premor pri kravah, ki so imele razmerje M/B višje od 1,37. Delež brejih krav v obdobju med 90 in 120 dnevi po porodu je bil pri vseh skupinah višji v podskupini z razmerjem M/B pod 1,37 v primerjavi s kravami, kjer je bilo razmerje M/B višje od 1,37. Rezultati raziskave nam dokazujejo, da je razmerje M/B lahko v pomoč veterinarjem praktikom pri predvidevanju težav s plodnostjo v čredah krav mlečnih pasem.

Ključne besede: krave mlečnih pasem; razmerje med maščobami in proteini v mleku; poporodni premor; analiza ROC; analiza preživetja

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Original Scientific Article

EXPRESSION OF MYOSIN HEAVY CHAIN ISOFORMS IN LONGISSIMUS MUSCLE OF DOMESTIC AND WILD PIG

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Summary: The expression of myosin heavy chain (MyHC) isoforms in the myofibers of domestic and wild pig was studied to characterize muscle tissue differences related to species domestication and selection. Muscle samples were obtained from longissimus muscle of five two years old wild and domestic Large White pigs. Four different MyHC isoforms (MyHC-I, MyHC-IIa, MyHC-IIb, MyHC-IIx) were determined in the myofibers of both, domestic and wild pig, and allowed the distinction of I, IIa, IIx/b and IIb myofiber types. Oxidative types I and IIa and type IIx/b myofibers were notably more abundant in longissimus muscle of wild than domestic pig. On the contrary, the number of glycolytic IIb myofibers prevailed in domestic pig. The cross sectional areas (CSA) of different MyHC myofiber types were 2 to 3 times smaller in wild than in domestic pig. In wild pig, CSA was more homogeneous between myofiber types with no difference between CSA of types I, IIx/b and IIb myofibers, whereas IIx/b and IIb myofibers exhibited greater CSA in domestic pigs. Type IIa myofibers were the smallest ones in both, domestic and wild pig. The presence of MyHC-IIb isoform was clearly established in the myfibers of wild pigs denoting that its expression is not just the result of the intensive selection for growth efficiency and muscularity. On the other hand, it is evident that domestication and breeding goals in pigs resulted in the hypertrophy of all myofiber types, in particular of those in which MyHC-IIb isoform is expressed.

Key words: myosin heavy chains; myofiber; immunohistochemistry; domestic pig; wild pig

Introduction

Skeletal muscles of mammals are composed of heterogeneous myofibers, in which distinct sets of structural proteins and metabolic enzymes are expressed. Such heterogeneity of skeletal muscles is related to the diversity of myofibrillar proteins, in particular myosin heavy chains (MyHC). In mammalian skeletal muscles up to 9 MyHC isoforms have been identified, each encoded by a distinct MyHC gene (1). Some of them are expressed transitorily during development or only in some functionally specialized muscles (2).

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In the adult mammalian locomotor skeletal muscles four MyHC isoforms have been described: one slow (MyHC-I) and three fast isoforms (MyHC-IIa, MyHC-IIx and MyHC-IIb). Studies on isolated myofibers in rodents showed that the maximal shortening velocity increased in the following pattern: I<IIa<IIx<IIb (3). Slow twitch type I myofibers consisting of predominantly of MyHC-I isoform exhibit a sustained, tonic contractile motor activity, whereas type II myofibers consisting of fast MyHC isoforms are recruited by acute motor neuron activity (4). The activities of metabolic enzymes generally match the energetic demands of each MyHC. Type I myofibers have greater oxidative capacity to support sustained contraction, type IIb myofibers are predominantly glycolytic and use

glycogen for short bursts of activity, whereas IIa and IIx myofibers are intermediate to I and IIb myofibers (1). In agreement with a predominant oxidative metabolism, type I and IIa myofibers contain also a lot of myoglobin, mitochondria, and phospholipids, contrary to gylcolytic IIb myofibers (5).

The expression of the fast MyHC isoforms seems to be species specific. In fast-twitch myofibres of small mammals (rodents and lagomorphs) all three fast MyHC isoforms (-IIa, -IIb, -IIx) are expressed (6). On the contrary, only two isoforms (-IIa and -IIx) were demonstrated in the locomotor skeletal muscles of humans (7, 8), some large animals like cats (9), dogs (10), cattle (11), goats (12), horses (13) and bear (14).

With the exeption of lamma, the domestic pig is the only large mammal in which all three fast MyHC isoforms (-IIa, -IIx, -IIb) have been demonstrated up to now. The MyHC-IIb positive myofibers are numerous especially in the so called white muscles in the peripheral region of the muscle fasciculi (15, 16). The reasons for the high expression of MyHC-IIb isoform in domestic pig muscles are not known. Domestic pig is a meat producing species, which has been intensively selected for growth rate and muscularity. Thus, it has been suggested that high MyHC-IIb isoform expression is related to genetic improvement and breeding conditions (17). Several studies compared muscle characteristics of wild and domestic pig, which demonstrated a large increase in the size of the myofibers and a switch in myofiber type composition towards the fast-twitch glycolytic character in the modern breeds of domestic pig (18, 19). Most of these studies were based on the histochemical classification of myofibers according to the myosin ATPase reaction and metabolic enzyme activities such as succinate dehydrogenase (SDH) and α-glycerophosphate dehydrogenase (α-GPDH) activities. However, the staining pattern of the myosin ATPase is ambiguous due to its pH sensibility and can lead to the misclassification of the myofiber types (20). Furthermore, hardly any evidence is available about the immunohistochemical expression of MyHC isoforms in muscles of wild pig. Therefore, the main objective of this study was to examine the myofiber profiles based on the expression of MyHC isoforms in the muscle of adult sows of the Large White breed as compared to wild pig in order to improve the understanding of the effect of genetic progress in pig breeding on myofiber type composition. We focused on longissimus muscle, which is a typical fast-twitch glycolytic muscle. This muscle is of particular interest, because it is used as an indicator of muscular development in breeding programs for pigs. Moreover, a condition related to low meat quality also known as pale, soft and exudative (PSE) meat occurs mainly in muscles with predominantly glycolytic metabolism.

Materials and methods

Muscle samples

Muscle samples were obtained from five two years old sows of Large White breed (carcass weight between 185 and 200 kg) and approximately two years old sows of wild pig (carcass weight between 46 and 60 kg). All five wild pigs were shot in the same season on the basis of the regular annual bag. Approximately one cubic centimetre of muscle samples were taken from the central part of the longissimus muscle (at the level of the last rib) within 24 hour *post-mortem* in both wild and domestic pigs. Samples were frozen in liquid nitrogen and stored at -80°C. Transverse serial cryosections (10 µm) of muscle tissue were cut with Leica CM 1800 cryostat at -17°C, mounted on APES-covered slides and air-dried.

Enzyme-immunohistochemistry

To show MyHC isoforms expression, four monoclonal antibodies specific for MyHC isoforms were used: NLC-MHCs (Novocastra laboratories, Newcastle on Tyne, UK) reactive with slow-twitch MyHC-I, antibody SC-71 specific for MyHC-IIa of rat, BF-F3 specific MyHC-IIb of rat and BF-35 specific for all MyHC isoforms except -IIx of rat. SC-71, BF-F3 and BF-35 antibodies were purchased from The Developmental Studies Hybridoma Bank, University of Iowa. Serial muscle cryosections were incubated with primary antibody in a humidified box overnight at 4˚C. The binding of the primary antibody was detected with the peroxidase-conjugated secondary antibody and visualised by Dako REAL™DAB+Chromogen (Köpenhagen, Denmark). To determine the metabolic profile of the myofibers, activity of the mitochondrial oxidative enzyme succinate dehydrogenase (SDH) was histochemically

demonstrated according to Nachlas et al. (21). The sections were counterstained with hematoxylin, dehydrated and mounted with Synthetic Mountant (Shandon, USA).

Analysis of serial cryosections

Nikon Microphot FXA microscope (Nikon Instruments Europe B.V., Badhoevedorp, The Netherlands) and Lucia-G image analysing system (Laboratory Imaging Ltd., Prague, Czech Republic) were used to analyse the serial sections processed for immuno- and enzyme-histochemical staining. Approximately 400-500 myofibers in five randomly selected fascicles were analysed per each muscle in order to determine the pattern of MyHC expression. The selected fascicles were the same for all antibodies on serial sections. The average proportions of myofiber types were determined according to the MyHC isoform expression and cross-sectional areas (CSA) of myofiber types were established. When measuring the CSA, the attention was paid to the delimitation of myofibers on the borders of endomysium.

Data analysis

Data were submitted to one-way analysis of variance (MIXED procedure by SAS Inc., Cary, NC, USA) including fixed effect of pig genotype (wild, domestic) or myofiber type. Means were compared using Tukey's test.

Results

Using monoclonal antibodies specific to MyHC isoforms, we could demonstrate the presence of four different MyHC isoforms (Figure 1). Thus slow myofibers (type I) were reactive with NLC-MHCs antibody, and all myofibres that remained unstained were presumed to be fast type II myofibers. A certain number of type II myofibers reacted with the SC-71 antibody specific to MyHC-IIa isoform of rat. In both, domestic and wild pigs, this antibody labelled the myofibers with different staining intensities. According to Lefaucheur et al. (17), the SC-71 antibody recognizes both MyHC-IIa and MyHC-IIx in pig; however the affinity of SC-71 is higher for MyHC-IIa. Consequently strongly stained fibres were classified as type IIa myofibers, and moderately stained ones as myofibers with prevailing MyHC-IIx isoform. Myofibers which reacted with both NLC-MHCs and SC-71 antibodies were hybrid type I/IIa myofibers, however their number was inferior to 0.5% and were thus excluded from the morphometric analysis. Antibody BF-35, which recognises all MyHC isoforms of rat, except MyHC-IIx, reacted strongly only with type I and type IIa myofibers. Indeed, it seems that BF-35 recognizes all MyHC isoforms, except MyHC-IIx and -IIb in pig skeletal muscle, likely with a higher background in domestic pig (Figure 1). The same myofibers reacted moderately or strongly with the antibody BF-F3, which is specific to MyHC-IIb isoform in pig muscles. Therefore, BF-35 negative myofibers can be divided into two subgroups: pure IIb myofibers (strongly stained with BF-F3) and hybrid IIx/b myofibers (weakly stained with BF-F3). According to the SDH staining the myofibers of wild and domestic pigs also showed different enzyme activity. Type I and IIa myofibers were intensively stained (highly oxidative) in both wild and domestic pig, whereas IIx/b myfibers were weakly stained and IIb myofibers totally negative.

The percentage (%) of oxidative type I and type IIa myofibers was significantly higher in longissimus muscle of wild than domestic pig (Table 1), whereas the proportion of glycolytic IIb fibers was higher in domestic pigs. In regard to the size of myofibers (Table 1), CSA of all myofiber types was 2 to 3 times smaller in wild than domestic pig. The CSA of myofiber types was more homogeneous in wild than domestic pigs. The myofiber hypertrophy of domestic pigs affected more type II than type I myofibers, above all the type IIb myofibers, which had the biggest CSA. Myofibers IIa were the smallest in both, domestic and wild pig.

Discussion

Distinctive differences in myofiber type composition of longissimus muscle between wild and domestic pig of modern breed have been mentioned by many authors. In a study performed by Rede et al. (22) wild pig had higher proportion of type I myofibers than domestic pig. Similar results were presented by Essén-Gustavsson and Lindholm (23), showing that muscles of wild boar had much higher oxidative metabolism than those of Landrace pigs. Szenkuti and Schlegel (24)

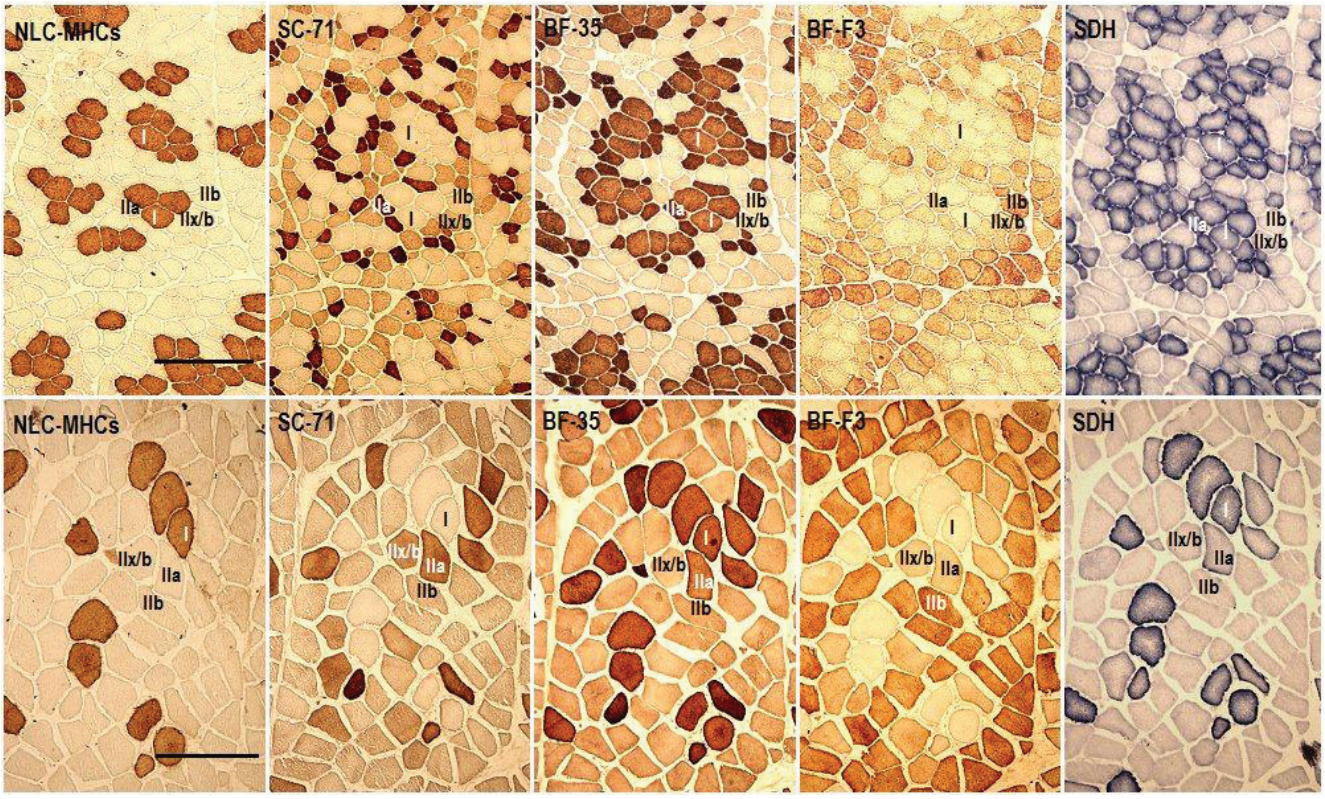

Figure 1: Antibodies reactivity and SDH activity in wild (upper row) and domestic pig (lower row). Bar = 250µm

Table 1: The proportion and cross sectional area (CSA) of myofiber types (based on MyHC isoforms) in the longissimus muscle of domestic and wild pigs

		Myofiber type			
Item		I	IIa	IIx/b	IIb
Proportion, %	Wild	17.0 ^{a,y} ±1.4	26.3 ^{b,y} ±1.3	31.0 ^{b,y} ±2.1	25.7 ^{b,x} ±0.8
	Domestic	10.8 ^{a,x} ±0.5	11.5 ^{a,x} ±0.5	15.7 ^{a,x} ±1.1	62.0 ^{b,y} ±1.8
		I	IIa	IIx/b	IIb
CSA, µm^2	Wild	5140 ^{b,x} ±272	3183 ^{a,x} ±191	4854 ^{b,x} ±218	5238 ^{b,x} ±1048
	Domestic	11638 ^{b,y} ±471	9231 ^{a,y} ±557	14244 ^{c,y} ±570	14827 ^{c,y} ±521

Data (mean±s.e.) measured in 2 years old wild (n=5) and domestic (n=5) pigs/sows.
Different letters (a-c) denote significant (P<0.05) differences between myofiber types.
Different letters (x-y) denote significant (P<0.05) differences between wild and domestic pigs.

suggested that myofiber composition is genetically determined by showing that despite intensive physical exercise domestic pigs exhibit higher proportion of glycolytic myofibers compared to wild pigs kept in a pen with restricted physical activity. The majority of these studies were based on myosin ATP-ase and metabolic enzyme histochemistry. Recent studies showed that the resolution of commonly used histochemical techniques is not sufficient to characterize adequately myofiber diversity. A better approach is offered by immunohistochemical techniques, although they are also limited by the antibody specificity (25).

The comparison of myosin heavy chain composition of different skeletal muscles in Large White and Meishan pigs clearly indicates that proportion of the different MyHC isoforms differ drastically between pig breeds and that breed affects mostly the MyHC IIx:IIb ratio in white muscles (26). According to muscle tissue properties wild pigs resemble Meishan pigs, which exhibit lower growth rate, poorer feed efficiency and lower meat content than conventional pig breeds (27). Wild pigs are physically highly active animals. Comparisons between wild and domestic pigs showed that they exhibit more oxidative and less glycolytic muscle metabolism and therefore a higher capacity to use lipids as an energetic substrate. Because oxidative metabolism decreases in the rank order I, IIa, IIx, IIb (17) these metabolic changes are consistent with the shift of MyHC profile toward a faster type in the domestic pig muscle. It may be noted, that domestic pigs included in the present study had somewhat higher percentage of slow-twitch oxidative type I and fast-twitch oxidative type IIa myofibers than reported in some other studies (19). This could be due to the breed used or because older pigs (approx. 2 years old) were used in our study. As demonstrated for some species, with aging the muscles become slower and more fatigue resistant as an adaptation to increasing body mass (14). The results of our immunohistochemical analysis are also in accordance with the studies in which the expression of MyHC isoforms in the longissimus muscle of different modern pig breeds or crossbreeds was quantified using a real time PCR at the mRNA level (26, 28, 29), or enzyme-linked immunosorbent assay on the protein level (28). A co-expression of IIx and IIb MyHC isoforms in pig muscles was also described by some other authors and was ascribed to the fine tuning of the expression of the IIx and IIb genes and its effect on myofiber plasticity (17).

Transition of MyHC isoforms is sequential, yet with a reversal pathway: I ↔ IIa ↔ II x ↔ IIb (1). It thus seems that breeding programs selecting for rapid, massive muscular growth pushed myofiber type to the right of this equation. Indeed, lower postnatal muscle growth was ascribed to decreased expression of glycolytic fibers in Chinese Meishan as compared to modern Yorkshire breed (31). Moreover, as shown here and in other studies, the MyHC-IIb isoform is the predominant isoform in the longissimus muscle. It contributes to the increased muscle mass which is the main goal of animal breeders (28). On the other hand, better water holding capacity, colour characteristics and tenderness are positively related to the presence of oxidative myofibers and hence the favourable MyHC isoforms would be -I, -IIa and -IIx (5). Overall, the available literature studies show that the prevalence of MyHC-IIb is probably responsible for the increased glycolytic potential of longissimus muscle. Moreover, when the CSA of myofibers increases, the density of capillary network in the endomysium of the same myofibers decreases particularly that of the largest myofibers (in our case type IIb), which makes it difficult to extract the lactate from them. Muscles composed predominantly of IIb myofibers are thus more susceptible to rapid post-mortem glycolysis which is negatively correlated with meat quality, in particular water holding capacity and may contribute to generating PSE meat condition (18, 32).

In conclusion, the present study showed that the expression of MyHC-IIb isoform in the muscles of domestic pig should not be considered as the sole consequence of intensive selection for muscularity and rapid growth. The fastest isoform i.e. MyHC-IIb is evidently expressed in the muscles of wild pigs, although less strongly than in domestic pig. It can also be deduced that due to the domestication and breeding goals, CSA of all myofiber types was considerably increased, in particular of those in which MyHC-IIb is expressed. The manifestation of MyHC isoforms is important for meat characteristics. For this reason the quantification of the expression of MyHC isoforms may represent a novel approach for future breeding programs, in particular in view of the balance between growth performance, muscularity and meat quality.

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IZRAŽENOST ISOFORM TEŽKIH MIOZINSKIH VERIG V NAJDALJŠI MIŠICI (*M. LONGISSIMUS*) PRI DOMAČEM IN DIVJEM PRAŠIČU

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Povzetek: Ugotavljali smo razlike v izraženosti izoform težkih miozinskih verig (MyHC) pri domačem in divjem prašiču kot posledico udomačitve in selekcije prašičev. Vzorce mišičnega tkiva smo odvzeli iz najdaljše hrbtne mišice (LM) petih divjih in petih domačih prašičev (pasma velika bela) starih dve leti. V mišičnih vlaknih obeh, domačega in divjega prašiča, smo imunohistokemično dokazali štiri različne izoforme MyHC (MyHC-I, MyHC-IIa, MyHC-IIb, MyHC-IIx), ki so omogočile razlikovanje tipov I, IIA, IIx/b in IIb. Vlakna tipa I, II in IIx/b so bila oksidativna in številnejša v LM pri divjem prašiču, pri domačem pa so prevladovala mišična vlakna IIb, ki so glikolitična. Prečni preseki vseh tipov mišičnih vlaken so bili dva- do trikrat manjši pri divjem prašiču kot pri domačem. Pri divjem prašiču je bila velikost vlaken različnih tipov tudi bolj izenačena kot pri domačem. Razlik v velikosti med vlakni I, IIx/b in IIb nismo ugotovili, medtem ko so pri domačem prašiču bila vlakna tipa IIx/b in IIb najdebelejša. Vlakna tipa IIa so bila najmanjša pri obeh, domačem in divjem prašiču. Prisotnost MyHC-IIb izoforme pri divjem prašiču dokazuje, da njena prisotnost v mišicah domačega prašiča ni zgolj odraz selekcije na boljši prirast in mesnatost. Razlika med divjim in selekcioniranim domačim prašičem se kaže predvsem v hipertrofiji vseh tipov mišičnih vlaken, posebej tistih z najbolj izraženo izoformo MyHC-IIb.

Ključne besede: težke miozinske verige; mišično vlakno; imunohistokemija; domači prašič; divji prašič

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Original Scientific Article

POST-MORTEM FINDINGS IN BOTTLENOSE DOLPHINS (*TURSIOPS TRUNCATUS*) IN THE SLOVENE SEA

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Summary: Between 1996 and 2012, a total of eleven bottlenose dolphins *(Tursiops truncatus)* were found dead in the Slovene sea. Only nine animals were dissected, since two were unsuitable for further examination due to severe post-mortal decomposition. Of the dissected dolphins, six were female and three were male; seven animals were adults, one subadult and one was a calf. At the dissection, pathological lesions in the lung prevailed; we established verminous pneumonia (N=5), lung worms in the bronchi (N=3), and multifocal nodular calcifications, probably calcified parasitic nodules (N=2). Among other pathological lesions, erosive and ulcerative oesophagitis (N=3); ulcerative gastritis (N=2); cutting injuries, excoriations and cicatrisations of the skin (N=2) were the most numerous. The most common histopathological lesions were also associated with parasites; we diagnosed parasitic nodules (N=3), verminous pneumonia (N =3) and bronchitis (N=1), and parasites in the lumina of bronchi (N=1). In six cases, parasitological examination revealed a presence of lung worms of the *Metastrongyloidea* superfamily.

We concluded that five bottlenose dolphins died of verminous pneumonia caused by nematodes of the order *Strongylida*, superfamily *Metastrongyloidea*; one animal died of chronic ulcerative oesophagitis and gastritis; one died after having been caught in a fishing net; one died due to endoparasitosis and emaciation, whereas the exact cause of death of one bottlenose dolphin could not be established due to severe post-mortal decomposition.

Key words: bottlenose dolphin; *Tursiops truncatus*; pathology; verminous pneumonia; Slovene sea

Introduction

The Slovene sea is the northernmost part of the Adriatic Sea with a surface area of 180 km^2, the average depth of 17 metres (1) and the deepest point – the Piran Punta – of 37.25 metres (3). The entire Slovene coast is 46.6 km long, and is composed of Eocene sedimentary flysch (1,4). The Slovene sea is shallow, it has a relatively high average sea temperature (15.8°C) (1), average salinity between 37 and 38‰ (2), average oxygen concentration between 6 mg/l and 9 mg/l (3) and a great amount of nutritive substances introduced by several large and small rivers, making it a rich habitat for numerous marine organisms (2).

Dolphins (*Delphinidae*) are the most numerous and diverse family of marine mammals, belonging to the order *Cetacea* and suborder *Odontoceti*. There are 36 genera in the *Delphinidae* family, of which several genera live in the Mediterranean Sea (5). Only one dolphin species, the bottlenose dolphin (*Tursiops truncatus*, Montagu 1821), lives in the Slovene sea (6-8), where one hundred

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and one dolphins living in groups of up to 43 individuals, have recently been identified. Annual mark-recapture density estimates 0.069 dolphins/km^2 (8). Other species of cetaceans, which are considered occasional in the region, are fin whale (*Balaenoptera physalus*), Risso's dolphin (*Grampus girseus*) and the striped dolphin (*Stenella coeruleoalba*) (9).

Bottlenose dolphins are prone to numerous infectious diseases. The most common are parasitic invasions, mostly in gastrointestinal and respiratory tracts, followed by bacterial, viral and fungal infections (10-16). Non-infectious diseases rarely occur: poisoning with biotoxins, sporadic neoplasia and congenital defects, organic disorders, mechanical injuries and suffocations have been reported (11, 16-17).

Between 1996 and 2012, eleven bottlenose dolphins were found dead in the Slovene sea. This study is the first to present the pathomorphological, bacteriological and parasitological findings discovered at the dissection of bottlenose dolphins found in the Slovene sea.

Materials and methods

Anamnestic data

Between October 1996 and August 2012, eleven bottlenose dolphins (*Tursiops truncatus*) were submitted for necropsy to the Institute of Pathology, Forensic and Administrative Veterinary Medicine at the Veterinary Faculty, University of Ljubljana. Five bottlenose dolphins had stranded on the coast of the Slovene sea, five were found dead in the open sea, one had been caught in a fishing net and managed to escape, but afterwards died in shallow water. Only nine animals were dissected - two carcasses were unsuitable for further examination due to severe post-mortal decomposition.

Of the nine dissected bottlenose dolphins, six were female and three were male. Seven of them were adults, one was subadult and one was a calf. One dolphin (case 6) was estimated to be 15 years old (18, 19). There are no exact data about the age of other dolphins. Four animals died in autumn, two in winter, two in summer and one in spring.

Data on sex, age and body weight of dissected animals, as well as the month and year of death and the site of their finding, are listed in Table 1.

Histopathological, bacteriological and parasitological examination

During the dissection, samples were taken for histopathological, bacteriological and parasitological examinations.

Samples for histopathological examination were fixed in 10% buffered formalin and embedded in paraffin. Four-µm-thick tissue sections were deparaffinised, stained with hematoxylin and eosin (HE) and examined under a light microscope.

Samples of the lung and intestine were taken for parasitological examination, and samples from several parenchymatous organs were taken for bacteriological examination. The parasitological and bacteriological examinations were performed at the Institute for Microbiology and Parasitology at the Veterinary Faculty in Ljubljana.

Results

Gross pathology

In all the examined bottlenose dolphins, pathological lesions in the lung prevailed: in five animals (cases 1- 5), multifocal verminous

Table 1: Characteristics of dissected bottlenose dolphins

Case	Dolphin sex, body length and age	Month and year of death	Site of finding
1	♂, approximately 300 kg	October 1996	Izola, Slovenia
2	♀, 226 cm	May 2001	Izola (Cape Ronek), Slovenia
3	♀, 297 cm	September 2002	Izola, Slovenia
4	♂, calf, 191 cm	January 2003	Strunjan, Slovenia
5	♀, 255 cm	October 2004	Piran, Slovenia
6	♀, 265 cm, 15 years (18, 19)	January 2005	Izola (White Rocks), Slovenia
7	♀, 265 cm	August 2007	Piran, Slovenia
8	♀, 272 cm	October 2008	Piran, Slovenia
9	♂, 297 cm	July 2011	Piran, Slovenia

pneumonia was detected (Fig. 1) and in two (cases 6 and 7), multifocal nodular calcifications, probably calcified parasitic nodules, were found. In the bronchi of three bottlenose dolphins (cases 2, 7 and 9), numerous or a few lung worms were found. In case number 7, numerous thread-like parasites were detected in the small intestine and in case number 3, parasitic nodules were also found in the stomach wall. In eight cases, a dilatation of the right or both ventricular chambers was determined. Transudate was found in body cavities of five bottlenose dolphins. In one animal, erosive and ulcerative stomatitis was established; in three cases erosive and ulcerative oesophagitis; in two, ulcerative gastritis; and in two cases cutting injuries, excoriations and cicatrisations of the skin, mostly in the region of fins, were detected. Lung adhesions were noted in one animal. The necropsy findings are presented in Table 2.

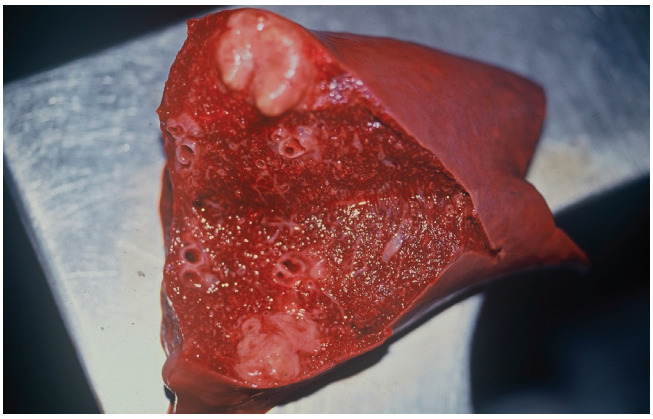

Figure 1: Verminous pneumonia in a bottlenose dolphin (case 4): greyish-white multifocal granulomas at the cut surface of the lung

Histopathological examination

The most common histopathological lesion was parasitic bronchopneumonia. Numerous adult nematodes were found in the bronchi of two bottlenose dolphins (cases 2 and 3) (Fig. 2A). In case number 3, a few desquamated epithelial cells and numerous degenerated neutrophil granulocytes were found in the bronchial lumina. There was significant thickening of the bronchial walls, with formation of granulation tissue, infiltrated with several eosinophil granulocytes, macrophages and lymphocytes. The infection spread through the interstitium into the walls of the surrounding alveoli. In the bronchial lumina, calcified adult parasites and amorphous calcifications were found; several small calcifications were observed also in the propria of these bronchi. In the lung parenchyma of two bottlenose dolphins (cases 1 and 5), there were multifocal parasitic nodules of various sizes, with adult parasites in the centre surrounded by a fibrous capsule; in two bottlenose dolphins (cases 6 and 7), round calcified nodules were found in the lungs and were most likely of the same aetiology. In case 4, nematodes in the lung parenchyma caused severe pyogranulomatous inflammation with abundant proliferation of the fibrous tissue, dense infiltration of eosinophil granulocytes, macrophages and lymphocytes in the alveolar walls and their lumina and extensive hyperplasia of the bronchus-associated lymphoid

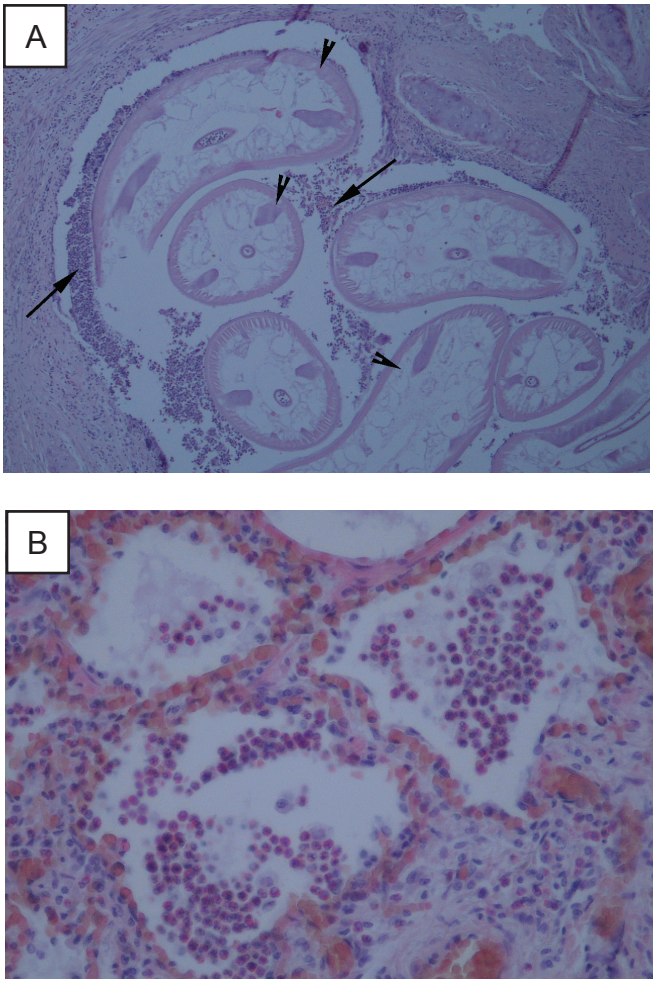

Figure 2: Verminous pneumonia in a bottlenose dolphin. A: Case 3. Numerous cross-sections of adult nematodes (arrowheads) and desquamated epithelial cells (arrows) in the bronchial lumina. HE staining, x 40; B: Case 4: Dense eosinophilic infiltrate in alveolar lumina and walls, thickened due to fibrous tissue proliferation. HE staining, x 200

Table 2: Results of the necropsy and additional examinations of dissected bottlenose dolphins

Case	Necropsy findings	Findings of histopathological examination	Findings of parasitological examination	Findings of bacteriological examination	Cause of death
1	chronic multifocal verminous pneumonia, focal adhesion of the left pulmonary lobe with pleura	encapsulated and incalcinated parasitic nodules in the lung; other organs were unsuitable for examination due to autolysis	lung: lung worms of the *Metastrongyloidea* superfamily	intestine: *E. coli*, *Pleisiomonas shigelloides*; spleen, liver, kidney and lung: negative	verminous pneumonia
2	chronic multifocal verminous pneumonia; obstruction of bronchi with numerous lung nematodes; hydrothorax; severe dilatation of ventricular chambers	parasites in the lumina of bronchi; other organs were unsuitable for examination due to autolysis	lung: lung worms of the *Metastrongyloidea* superfamily; intestine: negative	brain, lung, liver, spleen, kidney: unspecific microflora (contaminants); intestine: *Clostridium perfringens*	verminous pneumonia
3	severe pulmonary oedema and emphysema, severe dilatation of the right ventricle chamber, congestion of the lung and liver, multifocal verminous pneumonia, parasitic nodules in the stomach wall, erosive and ulcerative stomatitis, a cutting injury on the left pectoral fin and several smaller cuts and cicatrices of the skin	congestion of the liver, disseminated myocardial fibrosis, chronic verminous bronchitis, chronic hyperplastic gastritis and a parasitic nodule in the stomach wall; chronic plasmacytic enteritis	lung: lung worms of the *Metastrongyloidea* superfamily; intestine and liver: negative	intestine: undetermined pleomorphic gram-negative rod-shaped bacteria; liver, spleen and kidney: sterile; lung: numerous undetermined pleomorphic gram-negative rod-shaped bacteria	suffocation
4	chronic multifocal verminous pneumonia; dilatation of ventricular chambers; cutting injury on the right side of the lower jaw and bruises on the dorsal and caudal fin	pyogranulomatous verminous pneumonia, congestion of the parenchymatous organs	lung: negative	intestine, spleen, lung and kidney: negative	verminous pneumonia
5	chronic multifocal verminous pneumonia, pulmonary congestion and oedema, severe dilatation of ventricular chambers, hydrothorax, erosive and ulcerative oesophagitis, incapsulated abscess in the subcutis of the abdominal region	incapsulated and partially incalcined parasitic nodule in the lung, numerous calcifications in the lung, alveolar pneumonia, incapsulated abscess in the subcutis; other organs were unsuitable for examination due to autolysis	lung: lung worms of the *Metastrongyloidea* superfamily; intestine: negative	lung: negative; liver and spleen: *Pleisiomonas shigelloides* (single colonies), intestine: *Pleisiomonas shigelloides*	verminous pneumonia
6	small multifocal calcifications, congestion and pulmonary oedema, severe dilatation of the right ventricle chamber, ascites, hemorrhagic oedema in the subcutis of the right pectoral fin	multifocal calcifications in the lung; other organs were unsuitable for examination due to autolysis	lung and intestine: negative	intestine and peritoneal smear: negative; spleen: *Enterococcus faecalis*	exact cause of death could not be determined due to a severe autolysis of the body
7	emaciation, severe enteroparasitosis, multifocal nodular calcifications, congestion and pulmonary oedema, thread-like parasites in the bronchi, congestion of the tracheal mucosa, severe dilatation of ventricular chambers, hydrothorax, ascites, liver congestion, erosive and ulcerative oesophagitis numerous thread-like parasites in the intestine	focal verminous pneumonia and disseminated calcified nodules in the lung, nephrocalcinosis	lung: lung worms of the *Metastrongyloidea* superfamily; intestine: thread-like unidentified parasites	liver, kidney, lung and spleen: negative	verminous pneumonia
8	chronic multifocal ulcerative oesophagitis and gastritis, pulmonary oedema, hydrothorax, ascites, dilatation of ventricular chambers	two chronic gastric ulcers and gastritis, nephrocalcinosis, other organs were unsuitable for examination due to autolysis	lung and intestine: negative	liver, kidney, lung: *Enterococcus faecalis, E. coli, proteus* sp.; intestine: *E. coli, Enterococcus* sp.; spleen: negative	chronic ulcerative gastritis and oesophagitis
9	emaciation, erosive gastritis, thread-like parasites in the bronchi, lung adhesions, severe dilatation of ventricular chambers	organs were unsuitable for examination due to autolysis	lung: lung worms of the *Metastrongyloidea* superfamily; intestine: undetermined trematode eggs	organs were unsuitable for examination due to autolysis	endoparasitosis emaciation

tissue (BALT) (Fig. 2B). The pleura was diffusely thickened due to proliferation of the fibrous tissue and infiltration of inflammatory cells.

Uteri of many adult parasites from the bronchi and lung parenchyma were filled with embryonated eggs and larvae. According to their morphological characteristics, the parasites were classified into the superfamily *Metastrongyloidea* and order *Strongylida*.

In case 3, chronic hyperplastic gastritis and a parasitic knot in the stomach wall were established. In case 8, two chronic gastric ulcers and chronic nephrocalcinosis were diagnosed. In case 4, besides pneumonia, congestion of all other organs was established.

All other organs were severely autolytic and therefore unsuitable for histopathological examination.

The results of the histopathological examination are presented in Table 2.

Parasitological examination

In six cases (cases 1, 2, 3, 5, 7 and 9), parasitological examination established a presence of lung worms of the *Metastrongyloidea* superfamily. A more exact determination was not possible due to extensive post-mortal changes. In three cases (cases 4, 6 and 8), the parasitological examination of the lungs was negative. In cases 3, 5, 6 and 8, the parasitological examination of the intestine was negative. In case 7, determination of intestinal parasites was not possible due to severe post-mortal decomposition of the parasites. The results of the parasitological examination are presented in Table 2.

Bacteriological examination

Bacteriological examination was performed in eight cases. Organs of case 9 were unsuitable for examination due to autolysis. The results are presented in Table 2. Single bacterial colonies of *Plesiomonas shigelloides* were isolated in the intestines of cases 1 and 5 and in the liver and spleen of case 5. Numerous undetermined pleomorphic gram-negative rod-shaped bacteria were isolated in case 3. All other organs were negative or overgrown with putrefactive microflora.

On the basis of the dissection and the results of additional examinations we conclude that five bottlenose dolphins died of verminous pneumonia, one bottlenose dolphin, which had been caught in a fishing net, died of suffocation, one died of chronic ulcerative oesophagitis and gastritis, one died due to endoparasitosis and emaciation, whereas the exact cause of death of one bottlenose dolphin could not be established due to severe post-mortal decomposition.

Discussion

Only sporadic deaths of the bottlenose dolphins from the Slovene sea were recorded during our study: in 1996 and between 2001 and 2012 (except in 2006, 2009, 2010 and 2012) one dolphin per year was found dead. The annual average of stranded bottlenose dolphins in the Croatian Adriatic is 13.1 animals (20). Croatian scientists established an increased death rate among bottlenose dolphins in summer and autumn (20). Most deaths among dolphins in the Slovene sea also occurred in autumn, but the sample size was too small to draw any conclusions. Seven of nine bottlenose dolphins found in the Slovene sea were adult animals; however, we do not have the exact information about their age, except in case 6 (18, 19). The majority of bottlenose dolphins found on the Croatian coast were between six and seven years old (20).

The most common cause of death of the examined bottlenose dolphins was verminous pneumonia. Due to strong post-mortal decomposition, we could only partially determine the parasites, which were than classified into the superfamily *Metastrongyloidea*. The genera *Halocercus*, *Pharurus*, *Stenurus* and *Pseudalius* of the *Metastrongyloidea* superfamily often cause bronchitis and pneumonia in bottlenose dolphins (11). Italian researchers determined chronic bronchopneumonia caused by *Halocercus* sp. parasites in 85.7% of cases of death of bottlenose dolphins (13). *Halocercus lagenorhynchi* parasites were found in the lungs of numerous bottlenose dolphins with verminous pneumonia and bronchiolitis in Florida (10); Dailey *et al.* (21) found these parasites even in the lung abscess of dead new-born bottlenose dolphins.

One dolphin most probably died of chronic ulcerative oesophagitis and gastritis. Reidarson (11) reported that *Anisakis*, *Contracaecum* and *Pseudoterranova* nematodes, which inhabit the

gastrointestinal tract, predominantly cause gastritis and ulcerations, although in our case the parasitological examination yielded negative results.

Two of the examined animals had numerous skin injuries, which were most likely caused during their attempt to escape from fishing nets. Gomerčič *et al.* (16) established in seven out of 17 dissected bottlenose dolphins that they died as a consequence of entanglement in fishing nets.

In the intestines of two bottlenose dolphins and in the liver and spleen of one, single colonies of the bacterium *Plesiomonas shigelloides* were isolated. *Plesiomonas shigelloides* is widely spread in the aquatic environment (22) and causes several diseases in animals and humans with a weakened immune system (23, 24). We could find no reference in literature that the isolated bacterium could cause any diseases in bottlenose dolphins.

Establishing the cause of death of bottlenose dolphins is hindered or even prevented by strong post-mortal decomposition of their bodies, because there is usually a substantial time lapse between the actual death and the stranding of the body. The most common cause of death of bottlenose dolphins found in the Slovene sea was verminous pneumonia; however, skin lesions are also very common and are most likely the consequence of entanglement in fishing nets. We found no bacterial or fungal diseases or neoplasia in the examined bottlenose dolphins.

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PATOMORFOLOŠKE UGOTOVITVE PRI POGINULIH VELIKIH PLISKAVKAH (*TURSIOPS TRUNCATUS*) IZ SLOVENSKEGA MORJA

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Povzetek: Delfini (*Delphinidae*) so najštevilčnejša in najbolj raznolika družina morskih sesalcev. Spadajo v red Cetacea, podred Odontoceti. V družini je 36 rodov, ki naseljujejo vse oceane planeta. Številne rodove najdemo tudi v evropskih morjih, vendar le ena vrsta iz rodu Tursiops, velika pliskavka (*Tursiops truncatus*, Montagu 1821), živi v slovenskem morju, kjer so našteli sto eno žival. Zaradi svoje lege, plitkosti in stalnih dotokov celinskih voda predstavlja slovensko morje zelo specifično in bogato, vendar tudi zahtevno življenjsko okolje, zaznamovano z velikimi nihanji temperature, slanosti in vsebnosti kisika ter največjim plimovanjem v Jadranu.

Med letoma 1996 in 2012 smo v slovenskem morju našli enajst poginulih delfinov vrste velika pliskavka (*Tursiops truncatus*). Raztelesili smo jih devet, saj sta bili dve zaradi močne posmrtne razpadlosti neprimerni za preiskave. Šest pliskavk je bilo ženskega spola, tri pa moškega. Sedem pliskavk je bilo odraslih, ena je bila juvenilna, en pa je bil mladič. Pri vseh pliskavkah so prevladovale patološke spremembe na pljučih: diagnosticirali smo verminozno pljučnico (N=5), pljučne črve v bronhih (N=3) in multifokalne vozličaste poapnitve, najverjetneje poapnele parazitne vozliče (N=2). Pogosto smo ugotovili tudi erozivno in ulcerativno vnetje sluznice požiralnika (N=3) in ulcerativno vnetje sluznice želodca (N=2) ter vreznine, odrgnine in brazgotine po koži (N=2). Najpogostejše patohistološke spremembe so bile prav tako povezane s pljučnimi paraziti: ugotovili smo parazitne vozliče (N=3), verminozno vnetje pljuč (N=5) in bronhov (N=1) ter zajedavce v svetlini bronhov (N=1). S parazitološko preiskavo smo pri šestih pliskavkah našli pljučne črve iz naddružine *Metastrongyloidea*.

Na osnovi opravljenih preiskav smo ugotovili, da je pet pliskavk poginilo zaradi verminozne pljučnice, ki so jo povzročili nematodi iz reda *Strongylida*, naddružina Metastrongyloidea, po ena pliskavka pa je poginila zaradi kroničnega vnetja sluznice požiralnika in želodca, zaradi zapleta v ribiško mrežo ter zaradi endoparazitoze in posledične shujšanosti. Zaradi močne posmrtne razpadlosti trupla ene pliskavke nismo mogli ugotoviti vzroka njenega pogina.

Ključne besede: velika pliskavka; *Tursiops truncatus*; patologija; verminozna pljučnica; slovensko morje

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Case report

MALIGNANT MESENCHYMOMA OF THE AORTIC VALVE IN A DOG

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Summary: A malignant mesenchymoma of the aortic valve with an infiltrative growth into the myocardium in an 8-year-old intact male German shepherd is presented. Macroscopically, the aortic valve leaflets were markedly thickened, yellowish-grey and rough, with rounded edges, a rubbery consistency and greyish and smooth cut surface. Approximately 90% of the valve tissue was altered. Microscopically, alteration consisted predominantly of liposarcomatous, fibrosarcomatous and leiomyosarcomatous components and a focus of chondrosarcomatous cells. Focally, liposarcomatous cells infiltrated the myocardial muscle fibres. Mitoses were rare. Histopathologically, malignant mesenchymoma of the aortic valve was diagnosed. The diagnosis was confirmed by immunohistochemistry. A strong diffuse positive reaction for vimentin and a negative reaction for desmin were observed in all neoplastic components. In leiomyosarcomatous component a strong positive reaction for smooth muscle actin (SMA) was detected multifocally. Severe dilatation of all heart chambers, mild endocardiosis of the mitral and tricuspidal valves, severe congestion of the liver, spleen, kidneys and lungs with severe pulmonary oedema, moderate ascites and mild hydrothorax were also observed. We conclude that the dog died of chronic heart failure induced by malignant mesenchymoma of the aortic valve and endocardiosis of the mitral and tricuspidal valves.
Malignant mesenchymomas are uncommon tumours in animals and humans, rarely reported in dogs. To the best of our knowledge, this is the first report of a malignant mesenchymoma of the aortic valve with an infiltrative growth into the myocardium in animals.

Key words: malignant mesenchymoma; aortic valve; German shepherd; histopathology; immunohistochemistry

Introduction

Cardiac tumours are uncommon in dogs, with the prevalence of 0.19%. To date, haemangiosarcoma, rhabdomyo(sarco)ma, chondro(sarco)ma, leiomyo(sarco)ma, fibro(sarco)ma, myxoma, myxo-, lipo- and neurofibroma, haemangioma, lymphangioendothelioma, squamous cell carcinoma, mixed spindle cell and round cell sarcoma, pericardial mesothelioma and malignant myocardial mesenchymoma have been reported (1).

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Malignant mesenchymomas are soft tissue tumours of mesenchymal origin characterized by the presence of two or more unrelated, malignant cell lines, e.g. fibrosarcoma, liposarcoma, chondrosarcoma, leiomyosarcoma or rhabdomyosarcoma, in the same tumour. These tumours, rare in animals as well as in humans, have an unpredictable biological behaviour and can develop in different anatomic sites. Malignant mesenchymomas usually have a longer course than most malignant neoplasms and any one or all of the individual components may metastasize (2, 3). In animals, malignant mesenchymomas have to date been described in the liver (4), long

bones (5), spleen (6), heart (1, 7), soft tissue of the rear limb (8), intermandibular area (9) and dorsal area of the abdomen (10) in dogs, in the nasal cavity in a bull (11) and in the intra-abdominal soft tissue in a ferret (2).

Pathomorphological and immunohistochemical features of a malignant mesenchymoma of the aortic valve in a German shepherd, accidentally encountered during necropsy, are reported. To the best of our knowledge, this is the first report of a malignant mesenchymoma of the aortic valve in animals.

Material and methods

Case history

Eight-year-old intact male German shepherd with no anamnestic and clinical data was necropsied at the Institute for Pathology, Forensic and Administrative Veterinary Medicine, Veterinary Faculty, University of Ljubljana for education purposes.

Necropsy, histopathological and immunohistochemical examination

Heavily altered aortic valve leaflets, myocardium, lungs, liver, kidneys and spleen were collected during the necropsy for histopathological and immunohistochemical examination. All samples were fixed in 10% buffered formalin, routinely embedded in paraffin, sectioned at 4 µm, stained with hematoxylin and eosin (HE) and Toluidine and examined under a light microscope.

Immunohistochemical assays were performed on 4 µm sections of formalin-fixed, paraffin-embedded tissue samples. The mouse monoclonal antibody anti-human smooth muscle actin (clone 1A4, DAKO), mouse monoclonal antibody anti-human desmin (clone D33, DAKO) and mouse monoclonal antibody anti-human vimentin (clone V9, DAKO), at 1:50 dilution were used for the immunolabelling. Antigen retrieval was performed by microwave treatment at medium power (550 W) for 15 minutes in 0.1M citrate buffer (pH 6.0) for desmin and vimentin, and in EDTA buffer (pH 9.0) for desmin. The sections were incubated with primary antibodies for one hour at room temperature in a humid chamber. Endogenous peroxidase activity was quenched in Peroxidase-Blocking Solution, Dako REAL™ (DAKO) for 30 minutes at room temperature. Subsequently, visualization kit DAKO REAL™ EnVision™ Detection System Peroxidase/DAB+, Rabbit/Mouse (DAKO) was applied according to the manufacturer's instructions. Sections were counterstained with Mayer's haematoxylin and mounted.

Sections of normal canine small intestine were used for positive control. Negative control included incubation with DAKO Cytomation Antibody Diluent (DAKO) without the primary antibody.

Results

The thoracic cavity contained 200 ml of red-tinged serous fluid and the abdominal cavity 500 ml. The liver, spleen and kidneys were severely congested, the other abdominal viscera were grossly normal. The lungs were severely congested and oedematous and partially displaced by the severely enlarged heart. The pericardial sac was without effusions. Atria and ventricles of the heart were markedly dilated. A mild endocardiosis of the mitral and tricuspidal valves was noticed. When the aorta was opened, yellowish-gray, rough, 0.5 cm thick aortic leaflets with rounded edges and rubbery consistency were observed. Approximately 90% of the valve tissue was altered. On the cut surface, the leaflets were pink-greyish and smooth.

Histologically, a large mass composed of neoplastic mesenchymal cells was observed within the aortic valves. The tumour consisted predominantly of liposarcomatous, fibrosarcomatous and leiomyosarcomatous components. An islet of chondrosarcomatous cells was incorporated in the fibrosarcomatous component. A liposarcomatous component, with dense infiltrates of eosinophils, was multifocally penetrated by wide, irregularly oriented fascicles of fibrosarcomatous cells. Between those fascicles narrow bundles of leiomyosarcoma were found. The islet of chondrosarcomatous tissue was surrounded by a thick strand of collagenous stroma. Vascular stroma was abundant. The mitotic rate ranged from 0 to 2 per high power field. Focally, the tumour infiltrated the myocardial muscle fibres. The tumour was diagnosed as a malignant mesenchymoma of the aortic valve. Immunohistochemical examination demonstrated

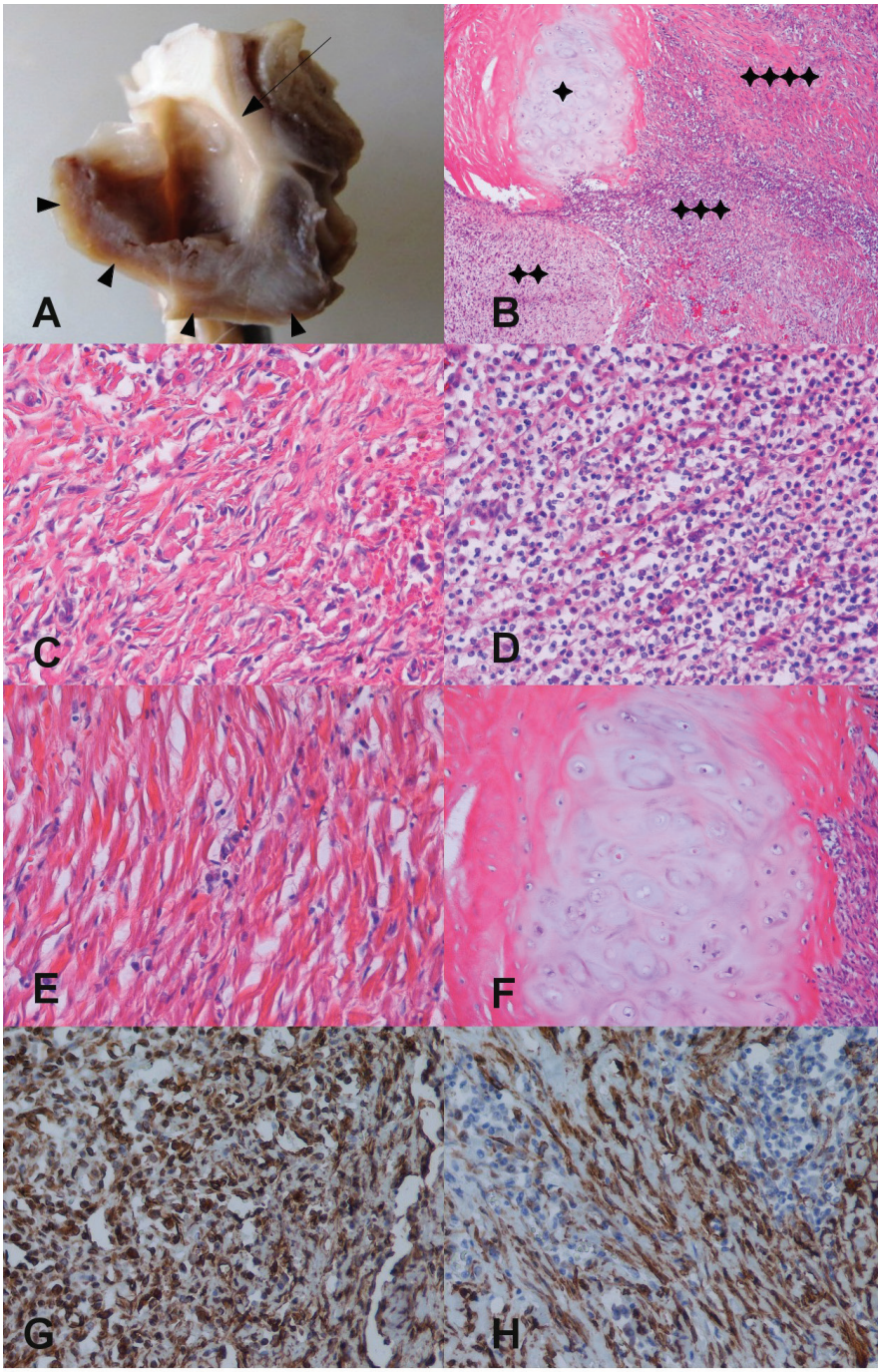

Figure 1: Macroscopic, histopathologic and immunohistochemical features of the malignant mesenchymoma of the aortic valve from a dog. **A**, cut surface of the aortic valve after formalin fixation. Note heavily altered, rough, pink-greyish valve leaflet (arrowheads) of the aorta (arrow). **B**, histological section from malignant mesenchymoma (MM) of the aortic valve showing chondrosarcomatous (**+**), liposarcomatous (**++**), leiomyosarcomatous (**+++**), and fibrosarcomatous (**++++**) components. Hematoxylin and eosin (HE), 40x. **C,** histological section of MM of the aortic valve showing fibrosarcomatous component. HE, 200x. **D**, histological section of MM of the aortic valve showing liposarcomatous component. HE, 200x. **E**, histological section of MM of the aortic valve showing leiomyosarcomatous component. HE, 200x. **F**, histological section of MM of the aortic valve showing an islet of chondrosarcomatous cells. HE, 100x. **G**, immunohistochemistry (IHC) of MM of the aortic valve showing strong diffuse positive labelling for vimentin. IHC, 200x. **H**, immunohistochemistry of MM of the aortic valve showing labelling for SMA. IHC, 200x

a strong diffuse positive reaction for vimentin. Furthermore, a strong SMA reactivity was noticed in areas with the leiomyosarcomatous component. The staining for desmin was negative. The results of immunohistochemical examination confirmed the previous histopathological diagnosis of malignant mesenchymoma.

The only pathological lesion diagnosed in other examined organs was a severe congestion.

Discussion

The described malignant mesenchymoma was diagnosed in the aortic valve. The tumour showed locally invasive growth into the myocardium, but no distant metastases were noted. Malignant mesenchymomas very rarely affect dogs. To date, they have been described in the liver (4), radius (5), spleen (6), soft tissue of the rear limb (8),

intermandibular area (9) and dorsal area of the abdomen (10) and in the heart (1, 7). The primary tumour may be locally invasive, but metastases to distant sites can occur (2). Metastases to the lung (7, 8, 9, 10), kidney, abdominal wall, mediastinum and parietal pleura (9), axillary lymph nodes, adrenal glands and liver (10), and mesentery (4) were observed in other cases of malignant mesenchymoma in animals.

Malignant mesenchymoma of the aortic valve had four malignant cell lines: liposarcomatous, fibrosarcomatous, leiomyosarcomatous, and chondrosarcomatous. This is in accordance with the diagnosis of malignant mesenchymoma. Malignant mesenchymoma is characterised by a combination of at least two unrelated neoplastic cell lines of mesenchymal origin within a single tumour (10). In dogs, other authors reported rhabdomyosarcomatous, myxosarcomatous and hemangiosarcomatous differentiation in the hepatic malignant mesenchymoma (4); fibrosarcomatous, rhabdomyosarcomatous, liposarcomatous and chondrosarcomatous elements in the malignant mixed mesenchymal tumour of the heart (1); leiomyosarcomatous, rhabdomyosarcomatous, chondrosarcomatous and liposarcomatous components in the malignant mesenchymoma of the heart base (7); liposarcomatous and osteosarcomatous (10), as well as osteosarcomatous and fibrosarcomatous cell lines (2) in soft tissue of the abdomen; areas of chondrosarcoma, leiomyosarcoma and osteoid in the soft tissue of the rear limb (8); and myxosarcomatous and osteosarcomatous tissue in soft tissue of the intermandibular area (9).

In the malignant mesenchymoma of the aortic valve, the reaction for vimentin was strongly positive and the reaction for desmin was negative. A strong multifocal reaction for SMA was detected only in the leiomyosarcomatous components. Results of immunohistochemical examination are in accordance with the previously described cases of malignant mesenchymoma in animals. In literature, immunohistochemistry has only been described in three cases of malignant mesenchymoma in animals: in a ferret a positive reaction for vimentin was found (2), in a dog a positive reaction for desmin and SMA was reported (1) and in another dog a positive reaction for vimentin and actin occurred (9). We believe that the German shepherd died of chronic heart failure caused predominantly by the malignant mesenchymoma of the aortic valve. Unfortunately, no data on the clinical signs and the course of the disease were available.

The described case is the first report of a malignant mesenchymoma of the aortic valve in animals. In humans to date, the only case of mesenchymoma involving heart valves was a chondrosarcomatous mesenchymoma, which was diagnosed in the mitral valve (12).

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MALIGNI MEZENHIMOM AORTNE ZAKLOPKE PRI PSU

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Povzetek: V prispevku predstavljamo primer malignega mezenhimoma v aortni zaklopki z infiltrativnim vraščanjem v srčno mišico pri osemletnem nemškem ovčarju. Na Inštitutu za patologijo, sodno in upravno veterinarstvo Veterinarske fakultete smo med raztelesbo psa, o katerem nismo imeli nobenih kliničnih podatkov, opazili zelo spremenjeno aortno zaklopko. Ta je bila zelo zadebeljena, rumenosiva, hrapava, gumaste konsistence, z močno zaobljenimi robovi, na prerezu gladka in sivkasta. Spremenjeno je bilo približno 90 % aortne zaklopke. S patohistološko preiskavo smo ugotovili, da je sprememba tumor, zgrajen iz štirih različnih komponent mezenhimskega izvora: iz prevladujočih liposarkomatozne, fibrosarkomatozne in leiomiosarkomatozne komponente ter majhnega otočka hondrosarkomatoznih celic. Liposarkomatozne celice so se na enem mestu infiltrativno vraščale med mišične celice srca. Mitoze so bile redke, in sicer od 0 do 2 mitozi na polje velike povečave. Žilna stroma je bila dobro razvita. Tumor smo diagnosticirali kot maligni mezenhimom aortne zaklopke ter diagnozo potrdili z imunohistokemično preiskavo, ki je pokazala močno difuzno pozitivno reakcijo na vimentin in negativno na desmin. Močno pozitivno reakcijo na aktin gladkih mišičnih celic (SMA) smo zgolj multifokalno ugotovili v leiomiosarkomatozni komponenti tumorja. Med raztelesbo psa smo ugotovili še: blago endokardiozo mitralne in trikuspidalne zaklopke, močno razširitev vseh srčnih votlin, močno polnokrvnost jeter, vranice, ledvic in pljuč, močen pljučen edem, zmeren ascites in blag hidrotoraks. Na osnovi vseh ugotovljenih sprememb menimo, da je pes poginil zaradi kronične odpovedi srca, ki je bila v največji meri posledica malignega mezenhimoma v aortni zaklopki in endokardioze mitralne in trikuspidalne zaklopke.
Maligni mezenhimom je redek tumor pri živalih in ljudeh, zelo redko je bil opisan pri psih. Po nam dostopnih podatkih iz literature je to prvi opis malignega mezenhimoma aortne zaklopke z infiltrativnim vraščanjem v srčno mišico pri živalih.

Ključne besede: maligni mezenhimom; aortna zaklopka; nemški ovčar; patohistologija; imunohistokemija

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Izterjava dolgov in upravljanje s terjatvami

Namen ustanovitve in delovanja podjetja MD svetovanje d.o.o. je pomagati podjetjem pri poslovanju z nudenjem produktov in storitev, ki ne spadajo v osnovno dejavnost podjetja. To dosegamo s celovito ponudbo predstavljenih produktov in storitev.

Zato smo naš moto Skupaj bomo uspešnejši! nadgradili še z motom in sloganom Vse za Vas na enem mestu!

Vizija

Postati vodilna neodvisna družba s celotno ponudbo za podjetja in posameznike na enem mestu in na ta način prihraniti podjetjem in posameznikom čas in denar.

Vse to nam bo uspelo s trdim delom in kakovostno izvedbo storitev in zaupanih nam nalog, predvsem če bomo sledili naslednjim načelom:

- zagotavljanje celovite ponudbe,
- vedno delo v dobro stranke,
- strokoven razvoj,
- organizacijsko izpopolnjevanje,
- zagotavljanje visoke stopnje kakovosti storitev z upoštevanjem predlogov naših strank,
- ustvarjanje novih delovnih mest,
- povečanje produktivnosti in dobičkonosnosti,
- visoko motiviran in usposobljen kader s primernim vodenjem, kar zagotavlja
- kakovost izvajanja storitev,
- postati vodilno podjetje, ki ponuja rešitve, ki podjetju omogočajo da si na enem
- mestu zagotovi vse dejavnosti, ki ne spadajo v njegovo osnovno dejavnost.

Prednosti poslovanja z nami:

- vse svoje potrebe in vizije uresničite s klicem na eno telefonsko številko,
- razbremenite se ukvarjanja z obrobnimi zadevami,
- posvetite se svojemu strokovnemu delu,
- informacijska tehnologija,
- prilagodljivost,
- zanesljivost,
- povečanje dobičkonosnosti,
- zmanjšanje stroškov dela,
- ...

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Zakaj MD Svetovanje d.o.o.

- visoka profesionalizacija,
- visoka strokovnost,
- visoka uspešnost,
- konkurenčne cene,
- vse na enem mestu.

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