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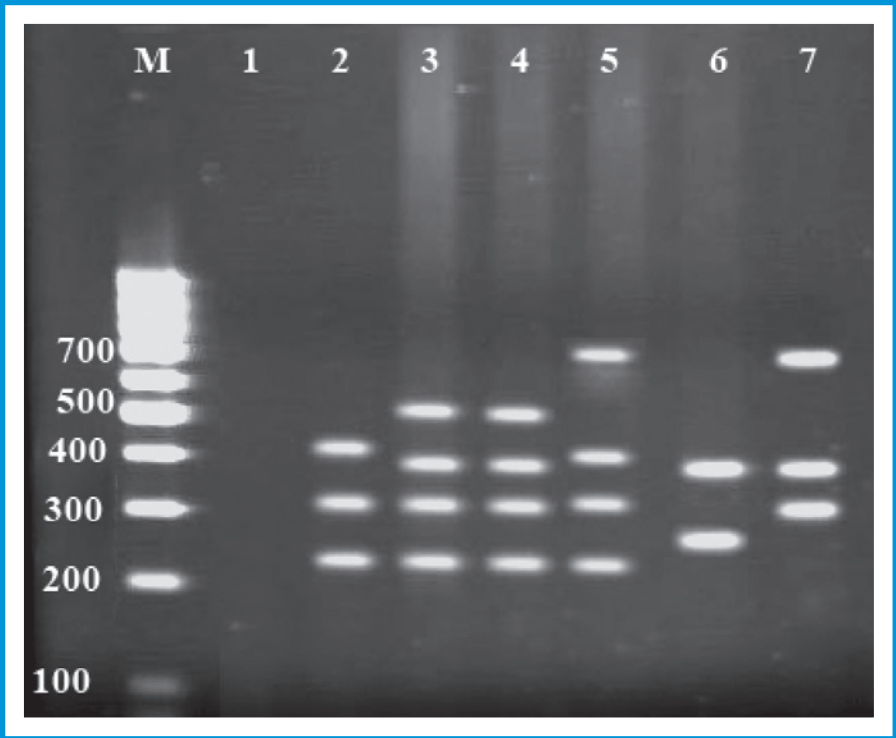

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Original Scientific Article

VIRULENCE PROPERTIES OF SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* ISOLATED FROM IRANIAN RAW MILK AND DAIRY PRODUCTS

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Summary: Shiga toxin (Stx)-producing *Escherichia coli* (STEC) strains are a diverse group of food-borne pathogens with various levels of virulence for humans. The main object of the present study was to determine virulence properties of STEC isolated from Iranian raw milk and dairy products. A total number of 300 samples, including sheep's (35), goat's (46), cow's (106), and buffalo's milk (21), soft cheese (42), butter (32) and ice cream (18), was obtained from farm bulk tanks, milk collection centres, and various supermarkets and retailer shops in different regions of Iran. Biochemical and molecular (PCR) method proved 26% of the samples to be *E. coli* positive, and among them, *Stx1* and *Stx2* genes were detected in 33 and 52 samples, respectively. But, *eaeA* and *sfaS* genes were not found in any of the sample. Beside *Stx2*, cnfI had the highest prevalence (42 isolates), and beside *eaeA* and *sfaS*, *fyuA* had the lowest prevalence of virulence genes. The results of this study demonstrate that there is a widespread distribution of potentially virulent *E. coli* strains in the environment and food that may be a cause of concern for human health.

Key words: STEC; *Escherichia coli*; virulence factors; raw milk

Introduction

Raw, unpasteurized milk is consumed directly by a large number of people in rural areas, and indirectly by a much larger segment of the population via consumption of several types of cheese. One of the main reasons why people prefer raw milk and its products may be their belief in their advantages or higher value compared to pasteurized milk (1). Many microorganisms, coliforms among them, can have access to milk and dairy products, and *Escherichia coli* are often used as marker organisms. Detecting and counting of *E. coli* is used as a reliable indicator of faecal contamination and indicates a possible presence of enteropathogenic and/or toxigenic microorganisms which constitute a public health hazard (2). *E. coli*, a gram negative bacillus, is naturally present in the gastrointestinal tracts of humans and animals as a part of natural microflora (3). While *E. coli* typically harmlessly colonizes the intestinal tract, several *E. coli* clones have evolved the ability to cause a variety of diseases within the intestinal tract and elsewhere in the host (4). Enterohemorrhagic *Escherichia coli* (EHEC) strains are a subset of Shiga toxin (*Stx*)-producing *E. coli* (STEC) strains that are

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isolated from human patients and are responsible for severe clinical symptoms, including diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome. These diseases are directly related to the virulence genes present in the causative agent (5,6). Four of the most commonly assayed virulence factors of STEC are the two phage-encoded cytotoxins, called Shiga toxin 1 and 2 (encoded by the *Stx1* and *Stx2* genes, respectively), the protein intimin (encoded by the chromosomal gene *eae*), which is responsible for the intimate attachment of the bacteria to intestinal epithelial cells, and the plasmid-encoded enterohaemolysin, also called enterohaemorrhagic *E. coli* haemolysin (EHEC-HlyA), encoded by the *ehxA* gene (3,7). Shiga toxin-producing *E. coli* can be transmitted through different routes, including food and water, person-to-person contact, and animal-to-person contact. Most human infections are caused by consumption of contaminated food. Domestic animals and wild ruminants, in particular cattle, are considered the main reservoir of STEC and the main source of contamination of the food supply (8,9). The presumed route of *E. coli* contamination of raw milk is via faecal contamination during milking (10). However, direct excretion of the organisms from the infected udder has also been reported (11).

There is a lack of information on virulence properties of Shiga Toxin-producing *Escherichia coli* contaminating Iranian milk and dairy products. Therefore, the main goal of the present study was to evaluate these properties.

Material and methods

Sample collection and identification of E. coli

From March 2010 to March 2011, raw bovine (n= 106), caprine (n= 46), ovine (n= 35) and buffalo (n= 21) milk samples were collected from farm bulk tanks and milk collection centres from several regions throughout Iran. Bovine and buffalo milk samples were collected in the above mentioned time period, whereas ovine and caprine milk samples were only available in the lactating periods of ewes and goats (i.e. from March through May, and from September to November of the subsequent year) within the same time frame. At each site, sampling of milk was performed according to the International Dairy Federation guidelines (IDF) (12). Samples (100 mL each, in sterile glass containers) were transported to the laboratory at ca. +4°C within 6–12 h after sampling.

Among dairy products, 42 samples of soft cheese, 32 samples of soft butter and 18 samples of soft ice-cream, all made of raw milk, were purchased at the supermarkets in the city of Shiraz, Isfahan and Shahrekord. All samples were kept under refrigeration and in plastic bags. Their information about dates of production and of assigned shelf-life was not presented. Dairy product samples were collected over a period of six months between May and November 2010, and were analyzed on the day of acquisition. Samples were transported under refrigeration (+4 to +6°C) in thermal boxes containing ice packs and were tested immediately after collection. A 25g portion of each sample was blended with 225 mL of nutrient broth (Merck, Germany) for two minutes, and at normal speed, using a Stomacher lab blender, and incubated at +37°C for 24h. A 1 mL sample of the nutrient broth culture was mixed with 9 mL of MacConkey broth (Merck, Germany) and further incubated at +37°C for 24h. One loop of each tube was streaked on MacConkey agar (Merck, Germany).

Four colonies from each plate with typical *E. coli* morphology were selected and examined by biochemical tests, including hydrogen sulphide, citrate, urease and indole.

All *E. coli* colonies were confirmed by molecular (PCR) method, determined by amplification of 16S rRNA gene region of *E. coli* as described by Sabat et al. (13). The identification of *E. coli* O157: H7 was also performed with the method described by Fode-Vaughan et al. (14). PCR conditions were optimized for DPCR by recommendations reported previously. The PCR conditions for amplification of *Stx1* and *Stx2* were the same as used for *pmoA*.

DNA isolation

Bacterial strains were grown overnight and at +37°C in the trypticase soy agar (TSA– Merck, German). One colony was suspended in 100 µL of sterile distilled water. The suspension was being boiled for 13 min, then frozen and subsequently centrifuged at 14,000 rpm for 15 min to pellet the cell debris (15). The supernatant was used as a template for amplification reaction.

Polymerase chain reaction (PCR)

The PCR assays, specific primer sequences and the predicted size of the amplified products for different pathogenic gene coding regions were employed as previously described (16-20). Details are shown in Table 1. For cycling, a DNA thermocycler (Eppendorf Mastercycler, Eppendorf-Nethel-Hinz GmbH, Hamburg, Germany) was used. The amplified products were visualized by ethidium bromide staining after gel electrophoresis of 10 µL of the final reaction mixture in 1.5% agarose. Strains of *E. coli* O157:K88ac:H19, CAPM 5933 and *E. coli* O159:H20, CAPM 6006 were used as positive controls.

Table 1: Sequences and predicted lengths of PCR amplification products of the oligonucleotide primers used in this study

Reference	Product size (bp)	Primer sequences (5'–3')	Primers name	Pathogenic factor
(16)	1105	ATCTTATACTGGATGGGATCATCTTGG GCAGAACGACGTTCTTCATAAGTATC	CNF1a CNF1b	Cytotoxic necrotizing factor 1 (*cnf1*)
(17)	543	AATCTAATTAAAGAGAAC CATGCTTTGTATATCTA	CNF2f CNF2r	Cytotoxic necrotizing factor 2 (*cnf2*)
(18)	366	AAATCGCCATTCGTTGACTACTTCT TGCCATTCTGGCAACTCGCGATGCA	Stx1f Stx1r	Shiga toxin 1 (*stx1*)
(18)	282	CGATCGTCACTCACTGGTTTCATCA GGATATTCTCCCCACTCTGACACC	Stx2f Stx2r	Shiga toxin 2 (*stx2*)
(19)	629	TGCGGCACAACAGGCGGCGA CGGTCGCCGCACCAGGATTC	EAE1 EAE2	Enteropathogenic attachment & effacement (*eaeA*)
(16)	430	AAATCACCAAGAATCATCCAGTTA AAATCTCCTGCAATCATCCAGTTTA	Cdt 1 Cdt 2	Cytolethal distending factor (*cdtB*)
(16)	720	ATGGCAGTGGTGTCTTTGGTG CGTCCCACCATACGTGCTCTTC	PapA-f PapA-r	P-Fimbriae (*papA*)
(16)	240	GTGGATACGACGATTACTGTG CCGCCAGCATTCCCTGTATTC	SfaS-f SfaS-r	S-Fimbriae adhesion (*sfaS*)
(16)	880	TGATTAACCCCGCGACGGGAA CGCAGTAGGCACGATGTTGTA	fyuA-f fyuA-r	Yersiniabactin (*fyuA*)
(16)	300	GGCTGGACATCATGGGAACTGG CGTCGGGAACGGGTAGAATCG	AerJ-f AerJ-r	Aerobactin (*iutA*)
(16)	290	GGTGTGGTGCGATGAGCACAG CACGGTTCAGCCATCCCTGAG	TraT-f TraT-r	Serum survival (*traT*)
(20)	432	CAATGCAGATGCAGATACCG CAGAGATGTCGTTGCAGCAG	Hly F Hly R	Haemolysin (*hlyA*)

Results

Eventually, 78 *E. coli* strains (representative of 78 different colony morphologies) (26%) were isolated from 300 samples (Table 2). Colony confirmation was performed by biochemical and molecular (PCR) methods. According to the obtained results, most pollution is related to cow's milk.

PCR assays were successfully developed for detection of twelve different virulence genes of genomic DNA of *E. coli* isolates from milk and dairy products. Agarose gel electrophoresis of PCR products revealed various bands that represent each virulence gene. In our research, 33 and 52 of *E. coli* isolates had *Stx1* and *Stx2*, respectively. By colony counting, 93.5% of independent isolates of *Stx*-positive *E. coli* was detected among 78 *E. coli* isolates; and, *Stx1* and *Stx2* genes were detected in 33 and 52 isolates, respectively. But, *eaeA* gene was not found in any of the samples.

The prevalence of samples containing *Stx*-positive and other virulence genes-positive *E. coli* is shown in Table 2. The prevalence of virulence genes was evaluated. 38.4% of the isolates contained only one virulence gene, 26.9% contained two, and, 34.6% contained three or more virulence genes. None of the samples in this study was positive on *E. coli* O157:H7 (the prevalence of *eaeA* gene was 0.0%). Our study showed that cow's milk (41.5%), cheese and ice cream (16.6%) had the highest prevalence of STEC. PCR-amplification and individually amplified fragments of gene detection were subjected to agarose gel electrophoresis (Figure 1 and 2).

Table 2: Distribution of virulence factor genes in E. coli isolated from raw milk and dairy products

Sample	Sample No.	*E.coli* positive (%)	*stxI*	*stxII*	*eaeA*	*cnf1*	*cnf2*	*iutA*	*cdtB*	*papA*	*hlyA*	*traT*	*sfaS*	*fyuA*
Sheep milk	35	6 (17.1)	3	4	-	3	3	3	1	3	1	4	-	2
Goat milk	46	7 (15.21)	3	4	-	2	2	2	1	4	1	5	-	1
Cow milk	106	44 (41.5)	16	35	-	26	12	12	9	18	5	14	-	6
Buffalo milk	21	6 (28.5)	1	3	-	4	2	2	1	1	2	1	-	-
Cheese	42	7 (16.6)	6	5	-	2	1	1	1	4	2	1	-	1
Butter	32	5 (15.6)	3	1	-	4	3	3	-	-	1	4	-	-
Ice cream	18	3 (16.6)	1	-	-	1	-	-	1	-	-	-	-	-
Total	300	78 (26.0)	33	52	-	42	23	23	14	30	12	29	-	10

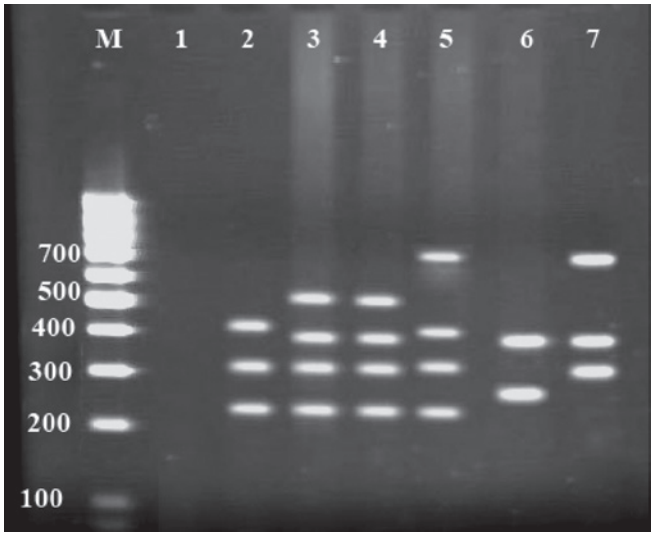

Figure 1: Agarose gel electrophoresis of PCR products amplified with PCR method for the *cnf2* (543 bp), *Stx1* (366 bp), *Stx2* (282 bp), cdtB (430 bp), papa (720 bp), *iutA* (300 bp) and *traT* (290 bp) genes in *E. coli* isolated from raw milk and dairy products

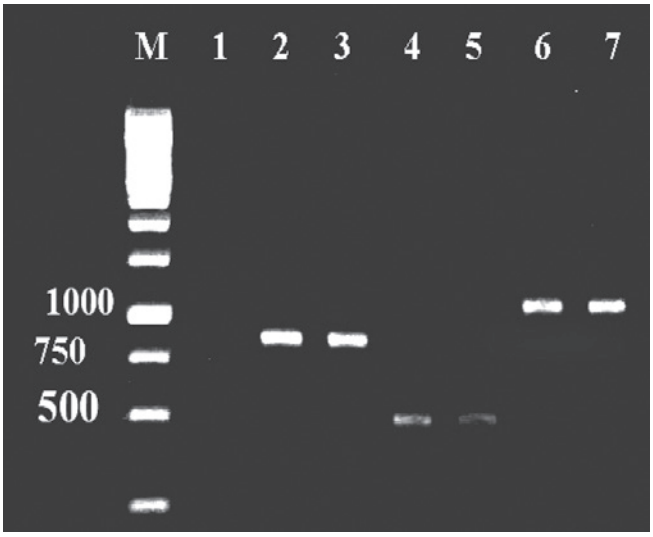

Figure 2: Agarose gel electrophoresis of PCR products amplified with PCR method for the *cnf1* (1105 bp), *fyuA* (880 bp) and *hlyA* (432 bp) genes in *E. coli* isolated from raw milk and dairy products

Discussion

Milk and dairy products can harbor a variety of microorganisms and can be an important source of food-borne pathogens. The presence of food-borne pathogens in milk is due to direct contact with contaminated sources in the dairy farm environment and to udder excretion of an infected animal. There are several reasons to be concerned about the microbial quality of dairy products: first, outbreaks of disease in humans have been traced back to the consumption of unpasteurized milk, and also to pasteurized milk; second, unpasteurized milk is consumed directly by dairy producers, farm employees, their families and neighbours, and raw milk advocates; third, unpasteurized milk is consumed directly by a large segment of population via consumption of several types of cheese produced from unpasteurized milk (21,22).

Nowadays, public health concern associated with microbial food safety is on the increase since *E. coli* is not only regarded as an indicator of faecal contamination but more likely as an indicator of poor hygiene and insufficient sanitary practices during milking. Furthermore, Shiga toxin-producing strains of *Escherichia coli* (STEC) are now recognized as an important agent of diarrhea and other foodborne-related illnesses through ingestion of contaminated food (23,24). Hence, the prevalence of Shiga toxin-producing *E. coli* isolated from milk and dairy products is evaluated in the present study. Our results indicate that 78 (26%) of 300 Iranian milk and dairy samples were contaminated with *E. coli*. 93.5% were found to be positive on two target genes, *Stx1* and *Stx2*. To our knowledge, the main reason for such high prevalence of E. *coli* and their virulence factors in milk and dairy products arises from contamination with faeces. Yet other reasons are: failure to comply with relevant rules, unsanitary milking, failure to control the staff of milking room and factories and/or workshops producing butter, ice cream and cheese, and finally, the use of raw animal milk for the production of dairy products.

The data of this study is older than the recent survey conducted for detection of prevalence and antimicrobial resistance of *E. coli* O157 isolated from Iranian traditional cheese, ice cream and yoghurt. The study reports about *E. coli* O157 being detected in 9 (3.1%) of the 290 samples tested, 5 isolated from traditional cheese and 4 isolated from traditional ice cream samples, whereas *E. coli* O157: H7 was not detected in any of the samples (25). One reason for this difference may be due to differences in the areas under study and the number of samples. Yet another study was conducted to investigate the presence of *E. coli* O157 and *E. coli* O157: H7 strains, and the presence of virulence genes *Stx1, Stx2, eaeA* and *ehlyA* isolates derived from some Iranian traditional dairy products and minced beef meat. *E. coli* non-O157, *E. coli* O157: NM and *E. coli* O157:H7 were isolated from 7%, 1.5% and 0.5% of the traditional butter, cream and kashk (a dairy product, similar to sour cream), respectively. Also, all *E. coli* O157:H7/NM isolates were positive on *eaeA* and *Stx1* and/or *Stx2*, and one *E. coli* O157:H7 isolate was positive on *ehlyA*. Of the 3 *Stx*-positive isolates, one and two isolates contained *Stx1* and *Stx2*, respectively (26).

Globally, several other studies reported the prevalence of *E. coli* and virulence properties of dairy products. For example in Italy, a polyphasic approach was evaluated for the detection of eight staphylococcal enterotoxin (SE)-encoding genes (*sea*, *sec*, *sed*, *seg*, *seh*, *sei*, *sej*, *sel*) and the *Escherichia coli* genes were most commonly associated with the virulence factors (*eae*, *elt*, *ipaH*, *Stx*) in traditional soft cheese, produced artisanally from raw milk in the Lombardy region. The results indicate that some of the artisanal cheese examined may constitute a potential hazard for the consumer health (27). According to the study of raw milk cheese production in Kerman, Iran, *Stx* genes were detected in 6.4% of the samples, but STEC strains were isolated in only 5 of them (4%) (28). Another study from Iran shows that about 21.8% of *E. coli* isolates from cattle were positive on *ehxA, Stx1*, and/or *Stx2* genes (29), but our study indicates that *Stx1*and *cnf1* with incidences of 42.3% and 53.84% had the highest frequency rate of virulence genes of *E. coli* isolates from raw milk samples. The results offer an answer to why food contamination from animal faecal sources is so common in Iran.

Yet another study of lamb food chain shows that all three virulence genes, *eae*, *Stx1*, and *Stx2* were the most prevalent genes in slaughterhouses (69%), processing plants (32%) and butcheries (9–10%) (30), but our results indicate that *Stx2* and *cnf1* had the highest prevalence of *E. coli* virulence genes in raw milk and dairy products.

Detection of bacteria in milk samples is still important, especially in more rural areas as it is easily available and economical. Due to bad climate conditions in some geographical places of Iran, oxen, ewes and goats have unsatisfactory survival conditions, so buffaloes present the main milk source for people. Buffalo's milk hygiene is therefore essential for human health, especially in arid and desert areas.

In United States, Van Kessel et al. (31) detected Shiga toxin genes enrichments in 15.2% of the bulk tank milk samples and in 51.0% filters by real-time PCR. These data confirm those from earlier studies showing significant contamination of bulk tank milk by zoonotic bacterial pathogens, and also, that the consumption of raw milk and raw milk products present a health risk. In Spain, occurrence of STEC in 'Castellano' cheese, a non-cooked and hard or semi-hard Spanish cheese made from ewe's milk, was reported. According to the report, three STEC strains were detected in two samples (2.4%) of 'Castellano' cheese, one with 2.5 and the other one with 12 month-ripening period. From those STEC isolates, two were identified as *E. coli* O14 and one third presented an O-specific polysaccharide not-groupable serologically (ONG) (32). In Bangladesh, the prevalence of Shiga toxin (*Stx*)–producing *Escherichia coli* was found in different types of food samples, and their genetic relation to STEC strains previously isolated from animal sources was investigated. In this study, approximately 10% of raw milk and 8% of fresh juice samples were positive for *Stx* (33).

All these data lead to conclusion that milk and dairy products represent a potential hazard for consumers, due to the potential presence of Shiga-toxin-producing *E. coli*, because of neglected sanitary measures during manufacture, handling and distribution of such fresh foods. Consequently, food manufacturers and specialists should design comprehensive programmes as good manufacturing practices (GMP) and implementation of HACCP system to ensure food free from the pathogens. In addition, effective heat treatments of food, providing information to food handlers and consumers, as well as implementation of strict hygienic measures during manufacture, storage and sale of these products are of significant importance in improving the quality of food and safeguarding the consumers against infections with such organisms. On the other hand, since many people still drink raw milk, especially in rural areas, this emphasises the need for educational efforts about health risks associated with consumption of raw, unpasteurized milk.

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VIRULENTNOST SEVA BAKTERIJE *ESCHERICHIA COLI*, KI IZLOČA ŠIGA TOKSIN, IZOLIRANE IZ SUROVEGA MLEKA IN MLEČNIH IZDELKOV

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Povzetek: Sevi bakterije *Escherichia coli*, ki izločajo šiga (Stx) toksin (STEC), so raznolika skupina patogenov, ki jih lahko najdemo v hrani in so različno nevarni za človeka. Glavni cilj raziskave je bil določiti virulentnost STEC, izoliranih iz surovega mleka in mlečnih izdelkov v Iranu.

Skupno število 300 vzorcev, ki so vključevali mleko ovac (35), koz (46), krav (106) in bivolov (21), mehki sir (42), maslo (32) in sladoled (18), je bilo pridobljenih iz zbiralnih rezervoarjev na kmetijah, zbirnih centrov za mleko, različnih trgovin in od trgovcev na drobno iz različnih področij Irana. Biokemijske in molekularne metode (PCR) so pokazale, da je bilo 26 % vzorcev pozitivnih na *E. coli* in med njimi smo gene *Stx1* in *Stx2* odkrili v 33 oziroma 52 vzorcih. Genov *eaeA* in *sfaS* nismo našli v nobenem vzorcu. Poleg gena *Stx2* je imel najvišjo prevalenco (42 izolatov) gen cnfI, poleg genov *eaeA* in *sfaS* pa je imel najnižjo prevalenco izmed virulentnih genov gen *fyuA*. Rezultati raziskave kažejo na to, da so potencialno virulentni sevi *E. coli* v okolju zelo razširjeni in da je hrana lahko vzrok za okužbo ljudi.

Ključne besede: STEC; *Escherichia coli*, virulentni faktorji; surovo mleko

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Original Scientific Article

CONTENT OF FIVE TRACE ELEMENTS IN DIFFERENT HONEY TYPES FROM KOPRIVNICA-KRIŽEVCI COUNTY

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Summary: Multifloral and unifloral honeys [black locust (*Robinia pseudoacacia* L.), chestnut (*Castanea sativa Mill.*), lime (*Tilia spp.*), indigobush (*Amorfa fruticoza* L.) and rapeseed (*Brassica napus*)] were collected from Koprivnica-Križevci County in northwestern Croatia during 2010 and 2011. The concentrations of Cd, Pb, Hg, As and Cu were determined and mean levels of elements (μg/kg) in honey samples measured were: in multifloral 1.26 for Cd, 163 for Pb, 135 for As, 1.35 for Hg and 11.7 for Cu; in black locust 1.52 for Cd, 182 for Pb, 23.2 for As, 0.46 for Hg and 7,697 for Cu; in lime 2.92 for Cd, 340 for Pb, 116 for As, 0.74 for Hg and 7,798 for Cu. Significant differences in Hg and Cu levels were observed between honey types. Average Cu levels found in lime and black locust honey types were much higher than those reported in other European countries. The highest element contents measured in different honey types were: Cd 4.0 μg/kg and As 502 μg/kg in rapeseed, Hg 6.11 μg/kg in chestnut, Pb 2,159 μg/kg in black locust and Cu 79,167 μg/kg in indigobush. Lead concentrations measured in all honey types were much higher than levels obtained in Italy, Slovenia, Poland, Romania and Turkey. These indicate that special attention should be paid to ensuring positions of hive in zones of bee forage that are more distant from highways and railways. The results presented indicate a differentiation of the trace element content of honeys of different botanical origin obtained from the same area.

Key words: different honey types; metals; As; Cd; Cu; Hg; Pb

Introduction

Honey is a sweet natural product, produced by honeybees (*Apis mellifera*) that collect nectar from flowers and turn it into a product considered to be a delicious food and known to be a healthier nutritional choice than sugar (1). It is mainly composed of fructose and glucose (65 %) and water (18 %), while protein contents are low (2). The mineral content in honey is low and ranges from about 0.04 % in pale honeys to 0.2 % in dark honeys (3). Honeybees are estimated to forage on plants growing over a relatively large area (more than 7 km^2) and when going from flower to flower, are also in contact with air, water and soil, branches and leaves. Therefore, honey is the result of a bio-accumulative process useful for collecting information about the environment and may be considered a bioindicator of environmental pollution (4).

The great variability of the trace element content in different honey types is closely related to botanical and geographical origin with regards to soil and climate characteristics (5). Environmental pollution factors that may contribute to the presence of metals in honey may be caused by non-ferrous metallurgy, industrial smelter and factories, leaded petrol, and agrochemicals such as cadmium-containing fertilizers, and organic mercury and arsenic-based pesticides still in use in some countries (6, 7).

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In addition to the environmental importance, the determination of trace metals is important regarding to quality control of honey and because the fact that today's total production of honey in the world is increasing. The European Union is the world's largest consumer of honey (1, 8).

In the past decade, different trace metals content have been determined in different honey types in European countries: France (9), Italy (10-12), Slovenia (13, 14), Poland (15), Czech Republic (16), Romania (6), Spain (17-19) and Turkey (20-23). In most of these studies, the botanical influence on honey composition has been neglected and only the differences among regions and the geographical origin of honey have been tested.

In Croatia, the most common unifloral honey types are black locust (*Robinia pseudoacacia* L.), chestnut (*Castanea sativa Mill.*) and lime (*Tilia spp.*), while the unifloral honeys indigobush (*Amorfa fruticoza* L.) and rapeseed *(Brassica napus)* are rarer. Unifloral honeys originating from *Lavandula stoechas* and *Salvia officinalis* are also rare and are indicative of the southern geographical origin in the country.

The aim of the present study is to examine the concentrations of the toxic trace metals Cd, Pb, Hg, As and Cu in different honey types collected in the same geographical region, in Koprivnica-Križevci County in northwestern Croatia.

Materials and methods

Sample collection

A total of 14 different honey type samples were collected: 3 multifloral, 5 black locust (*Robinia pseudoacacia* L.), 3 lime (*Tilia spp.*), 1 chestnut (*Castanea sativa Mill.*), 1 indigobush (*Amorfa fruticoza* L.) and 1 rapeseed *(Brassica napus)*. Honey samples were produced and collected during 2010 and 2011 from individual beekeepers near the towns Križevci, Đurđevac, Koprivnica and Virovitica in Koprivnica-Križevci County (Table 1).

Koprivnica-Križevci County covers an area of about 1,746 km^2 and the urban centres Križevci, Đurđevac, Koprivnica and Virovitica each have populations of 20,000 to 55,000. The region is relatively clean, as economic activities are primarily agricultural with cultivated fields and woods, and a high representation of vineyards. The region has no pronounced industrial activities and vehicular traffic is rather low in comparison with European standards and the more populated, urbanized and industrialized Centre region around the capital city, Zagreb.

Following collection, honey samples (500 g) were placed into clean glass bottles, labelled and brought to the laboratory and stored at 4–8°C until analysis.

Table 1: Geografical and botanical origin of honey samples

Sample no.	Sample identification	Honey type (common name)	Geographical origin	Year
1	BL 1	Black Locust	Đurđevac	2010
2	BL 2	Black Locust	Đurđevac	2011
3	BL 3	Black Locust	Đurđevac	2011
4	BL 4	Black Locust	Koprivnica	2011
5	BL 5	Black Locust	Križevci	2011
6	L 1	Lime	Đurđevac	2011
7	L 2	Lime	Đurđevac	2010
8	L 3	Lime	Koprivnica	2011
9	MF 1	Multifloral	Koprivnica	2011
10	MF 2	Multifloral	Đurđevac	2011
11	MF 3	Multifloral	Križevci	2011
12	C	Chestnut	Đurđevac	2010
13	I	Indigobuch	Virovitica	2010
14	R	Rapeseed	Koprivnica	2010

Standard preparation

Acid used, HNO_3 and HCl were of analytical reagent grade (Kemika, Croatia). Ultra high purity water processed through a purification system NIRO VV UV UF 20 (Nirosta d.o.o. Water Technologies, Osijek, Croatia) was used for all dilutions. Calibrations were prepared with element standard solutions of 1 g/l of each element (Perkin Elmer, USA). Stock solution was diluted in HNO_3 (0.2 %). In the preparation of Hg standards, 1 ml of HNO_3 conc., 0.1 ml 10 % $K_2Cr_2O_7$, and 0.1 ml HCl conc. were added to all working standards and prepared in brown glass volumetric flasks. As matrix modifiers in each atomization for As, Cd, Cu and Pb, 0.005 mg $Pd(NO_3)_2$ and 0.003 mg $Mg(NO_3)_2$ (Perkin Elmer, USA) were used.

Plastic and glassware were cleaned by soaking in diluted HNO_3 (1/9; v/v) and by subsequent rinsing with high purity water and drying prior to use.

Sample preparation

Honey samples (0.5 g) were digested with 4 ml HNO_3 (65 % v/v) and 2 ml H_2O_2 (30 % v/v) with a Multiwave 3000 microwave closed system (Anton Paar, Germany). A blank digest was carried out in the same way. The digestion programme began at a power of 500 W, ramped for 1 min and hold for 4 min. The second step began at a power of 1000 W ramped for 5 min and hold for 5 min. The third step began at a power of 1400 W, ramped for 5 min and hold for 10 min. Digested samples were diluted to a final volume (50 ml) with double deionised water.

Determination of metals

Atomic absorption spectrometry by AAnalyst 800 and AAnalyst 600 (Perkin Elmer, USA) equipped with an AS 800 and AS 600 autosamplers (Perkin Elmer, USA) was used for measurement of As, Cd, Cu and Pb concentrations. Argon was used for graphite furnace measurements. Pyrolytic-coated graphite tubes with a platform were used. The atomic absorption signal was measured in peak area mode against a calibration curve.

Mercury in honey samples were quantified using the AMA-254 (Advanced Mercury Analyzer, Leco, Poland) without acid digestion by direct combustion of the sample in an oxygen-rich atmosphere. The operating parameters for working elements are presented in Table 2.

Detection limits for the five metals were determined as the concentration corresponding to three times the standard deviation of twenty blanks. All samples were run in batches that included blanks, a standard calibration curve, two spiked honey samples, and one duplicate. In order to determine the method accuracy and to calculate the recovery percentage, ten honey samples were spiked with known amounts of Cd, Pb, As, Hg and Cu analytical standards.

Data analysis

Statistical analysis was performed using the software package Statistica 6.1 (StatSoft® Inc., USA). One-way analysis of variance was used to test for differences in honey metal concentrations. Data were log-transformed to improve normality prior to analysis to meet the underlying assumptions of the analysis of variance; the values given are therefore geometric means. The differences between the metal concentrations in different honey types were analyzed using the *t*-test. A probability level of $p \leq 0.05$ was considered statistically significant.

Results and Discussion

In this study, concentrations of heavy metals and Cu were determined in six different honey types from the same geographical origin under the same climatic conditions.

The quality of data was checked by analysis of the recovery rate with spiked honey samples for Cd, Pb, Hg As and Cu and showed good accuracy, with recovery rates for metals of 95.9 % to 99.2 % (Table 3). The limits of detection (LODs, μg/kg) were: Cd 1.0, Pb 4.7, Hg 0.1, As 5.0 and Cu 1.2. The concentrations of the five elements in multifloral and unifloral honey samples are reported in Table 4. Statistical analyses by one-way ANOVA showed a significant difference in Cu levels ($p<0.01$). However, no significant differences were observed in Cd, As, Hg and Pb levels. It has to be point out that element levels results presented for honey types chestnut, rapeseed and indigobush is for one sample only.

In the present study, Cd content in all honey samples range from 1 to 5 μg kg^{-1}. No significant differences were observed between different honey types. Literature values for different honey type originating from European countries are given in Table 5. Cadmium multifloral levels observed were similar to contents obtained in different geographical regions of Turkey (23), Macedonia (3.63 μg/kg; 24) and to previously reported levels in multifloral honey samples from different regions in Croatia (1.51 μg/kg; 25). In general, Cd levels in all honey types were 2 to 10 times lower than those reported in Italy (12) and Turkey (21, 22) and more than 100 times lower than the high Cd levels found in mixed flower honey from Bologna, Italy (11).

Lead is one of the most widespread metal pollutants and, like Cd, can reach humans through air, water and food. This metal had no beneficial role in human metabolism and produces a progressive toxicity and can cause health disorders such as fatigue, sleeplessness, hearing and weight loss. A provisional tolerable weekly intake (PTWI), as acceptable levels of major toxic elements that can be ingested on a weekly basis and may accumulate in the body established by WHO (The World Health Organization) and FAO (Food and Agriculture

Table 2: Instrumental conditions for atomic absorption spectrometry and mercury analyzer and graphite furnace program (temperature and time) for Pb, Cd, Cu, As and Hg determination in honey samples

Conditions for graphite furnace atomic absorption spectrometry				
	Lead	Cadmium	Copper	Arsenic
Wavelength (nm)	283.3	228.8	324.8	193.7
Argon flow (ml/min)	250	250	250	250
Sample volume (μl)	20	20	20	20
Modifier volume (μl)	5	5	5	5
Heating pogram tempetature °C (ramp time (s), hold time (s))				
Drying 1	110 (1, 30)	110 (1, 30)	110 (1, 30)	110 (1, 30)
Drying 2	130 (15, 30)	130 (15, 30)	130 (15, 30)	130 (15, 30)
Ashing	900 (10, 20)	700 (10, 20)	1200 (10, 20)	1600 (10, 20)
Atomisation	1850 (0, 5)	1550 (0, 5)	2000 (0, 5)	2000 (0, 5)
Cleaning	2450 (1, 3)	2450 (1, 3)	2450 (1, 3)	2450 (1, 3)

Conditions for determination on mercury analyzer	
Wavelength (nm)	253.65
Drying time (s)	60
Decomposition time (s)	150
Wait time (s)	45
Weight / volume of sample	100 mg / 100 ml
Working range	0.05 – 600 ng

Table 3: Trace metal concentrations and recoveries in spike honey samples

Element	Spiked value (μg/kg)	Measured value (μg/kg)	Recovery (%)
Cd	10	9.67 ± 0.33	96.7
Pb	50	48.9 ± 6.45	97.8
Hg	10	9.92 ± 0.14	99.2
As	10	9.59 ± 0.27	95.9
Cu	50	49.4 ± 5.23	98.8

Table 4: Concentrations of trace elements Cd, Pb, As, Hg and Cu (mg/kg) in honey samples of different botanical origin from Koprivnica-Križevci County

Sample identification	Cd (mg/kg)	Pb (mg/kg)	As (mg/kg)	Hg (mg/kg)	Cu (mg/kg)
BL 1	4.0	270	11.0	0.90	318
BL 2	1.0	134	10.1	0.62	1091
BL 3	1.0	62.1	12.2	0.13	1272
BL 4	2.0	2159	499	0.53	124
BL 5	1.0	41.3	10.0	0.51	665
Geometric mean	1.52	182	23.2	0.46	515 [ab]
L 1	5.0	303	512	0.75	47.5
L 2	2.0	364	11.1	1.21	195
L 3	2.5	358	279	0.45	86.1
Geometric mean	2.92	340	116	0.74	92.7 [ac]
MF 1	2.0	438	185	2.41	10.1
MF 2	1.0	134	118	0.83	12.1
MF 3	1.0	73.2	112	1.22	13.2
Geometric mean	1.26	163	135	1.35	11.7 [bc]
C	1.0	1637	30.1	6.11	53.4
I	1.0	159	11.1	1.62	154
R	4.0	180	502	1.11	29.2

Significant differences between honeys: [a] BL:L $p < 0.05$; [b] BL:MF $p < 0.01$; [c] L:MF $p < 0.01$;

Table 5: Overview of the element contents in different honey types from different countries

	Country Honey type (reference)			
Element (µg/kg)	Italy MF1-mixed floral (11) C-chestnut (11) MF2- multifloral (12) MF3- multifloral (10)	Slovenia MM-monofloral (13)	Spain MM-multifloral + monofloral (18)	Turkey MF1 -multifloral (21) MF2 -multifloral (22) MF3-multifloral (23)
Cd	MF1 305 C < 50 MF2 4.25	-	-	MF1 10.9 – 21.2 MF2 1.1 – 17.9 MF3 0.38 – 2.03
Pb	MF1 620 C < 50 MF2 76.4	MM 1.86 - 4.3	-	MF1 17.6 – 32.1 MF1 8.4 – 106 MF3 1.54 – 36.7
Hg	MF2 < 2	-	-	-
As	MF2 6.59	MM 1.24 to 1.49	-	-
Cu [*] mg/kg	MF1 890 C < 50 MF2 647 MF2 0.31[*]	MM 1.4 to 2.7 [*]	MM 0.1-1.73[*]	MF1 0.25 – 1.1[*] MF2 0.23 – 2.41[*] MF3 9.97 – 29.5

Organization): Cd (7 μg/kg b.w.), Pb (25 μg/kg b.w.), As (15 μg/kg b.w.) and Hg (5 μg/kg b.w.) (26-28). In 2000 the maximum residue limit (MRL) values of 0.1 mg/kg for Cd and 1 mg/kg for Pb were proposed for the European Union. However, until today there are no established MRL values for heavy metals in honey. In Croatia there is not established maximum permitted levels for the Cd, Hg, Pb, As and Cu. According to the published data, only in Macedonia maximum permitted value for Cd and Cu in honey is set to be 0.03 mg/kg for Cd and 1 mg/kg for Cu (24, 29). In the present study, obtained mean concentrations of Cd and Pb were below MRL values proposed by EU.

Lead contents decreased in the following order: chestnut > black locust > lime > multifloral > rapeseed > indigobush. The lowest and highest Pb concentrations obtained were 159 μg/kg in indigobush honey and 2,159 μg/kg in black locust honey. Mean Pb concentrations measured in black locust, lime and multifloral honeys were much higher (in some cases for more that 4,000 times) than levels obtained in other countries: Italy (12), Poland (70 μg/kg; 15), Turkey (21, 22) and Romania (0.07 μg/kg; 6). However, only in multifloral honey were Pb levels for 3.8-time lower than those found in mixed flower honey from Italy (11). With regards to fact that studied region has no pronounced industrial activities and vehicular traffic is rather low it is not clear why higher concentrations of Pb were determined. It is only be assume that may be due to the position of hives in zones near highways and railways during, which is often the case. Other factor that may contribute is that in soils, and suspended air particulates, concentrations of Pb were influenced by distance from highway and direction of prevailing winds. Also it is demonstrated that Pb accumulation in and on plants next to highways were caused principally by aerial deposition and not by, at least to any great extent, absorption by the plant from contaminated soil (30). On the other hand, Pb is relatively unavailable to plants when the soil pH level is above 6.5. In case of pH values less than 6.5 there is actual increase of Pb uptake by the plant itself from soil. Recent study of soil metal content in Croatia shown that Pb is present in much higher amounts in soil (25.3–27.0 μg/g d.w.) than for example Cd (0.2 μg/g d.w.) (31).

In this study, As levels ranged from the lowest level of 11.1 μg/kg in indigobush honey to 502 μg/kg in rapeseed honey. In comparison to the very few literature data, As levels obtained were higher than those found in Siena County, Italy (12), but more than 9 time lower than levels in Slovenia (13).

Environment pollution by mercury may be caused by industrial activities, mining and combustion and pollution from agricultural sources (32). Several reports were available for Hg concentrations in honey samples. Mercury levels ranged from 50 to 212 μg/kg in contaminated and from 1 to 3 μg/kg in uncontaminated areas in Slovakia (33). In multifloral and other honey types examined in Siena County in Italy, Hg levels were lower than the quantification level of 2 μg/kg (12). In the present study, Hg ranged from a minimal of 0.13 μg/kg in black locust honey to a maximum of 6.11 μg/kg in chestnut honey.

As an essential element, Cu may influence growth, skin pigmentation, bone mineralisation, gastrointestinal and heart function. However, Cu may also generate toxic effects such as dermatitis, liver cirrhosis and neurological disorders, while acute Cu poisoning causes symptoms of nausea, vomiting and abdominal and muscle pain (34). The provisional permitted daily intake for Cu determined in an average adult with 60 kg body weight is 3 mg (35). Copper can enter the food chain and also honey through the mineralisation of crops and due to its application on a large number of crop pests as a fungicide, bactericide and herbicide in the vicinity of the hives locations (36).

In the present study, Cu concentrations ranged from the lowest concentration of 10.1 μg/kg measured in multifloral honey to the highest of 1,272 μg/kg in black locust honey. Mean Cu levels determined in multifloral honey were significantly lower than those of lime and black locust honey types ($p < 0.01$, both). Also, significantly higher Cu concentration was determined in black locust than in lime honey ($p < 0.05$). Differences in the element content between different honey types have also been reported in previous studies (12, 16, 23, 37).

In previous studies, upper Cu limit determined in other parts of the world was around 2 μg/g (38). Average Cu levels found in multifloral honey (11.7 μg/kg) were more than 25 lower than those reported in previous studies from Italy (10, 11, 12), and more than 110 and 230 times lower than the highest level measured in Turkey (21, 22), Spain (18) and Slovenia (13). Also, the mean Cu level determined were more than 90 times lower than previously obtained results for multifloral honey samples from different regions in Croatia (1074 μg/kg; 25). On the other hand, the results

obtained were similar to levels found in the Black Sea region in Turkey (23).

Copper levels determined in chestnut honey in this study are higher than those from Bologna, Italy (11) but for 8 times lower than those found in chestnut honey from Anatolia, Turkey (0.42 mg/kg; 39). On the other hand, Cu levels determined in monofloral honeys black locust and lime was lower that those found in Slovenia (13) and Spain (18). A soil metal study in Croatia showed low Cu levels raging form 10 to 25.4 μg/g d.w. in region studied (31).

The quantities of heavy metals in honey are usually so small that even 100 g eaten daily would not contribute appreciably to dietary requirements. However, if in the environment is increased one or more elements these increased content of it in honey which then has a significant impact. As a result of present study, trace element levels of Pb and Cu determined in multifloral honey were lower than in unifloral honey black locust, lime and chestnut honeys obtained in the same geographical region. Accordingly, these results represent a differentiation of the trace element content in honeys of different botanical origin obtained from the same geographical region.

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VSEBNOST PETIH ELEMENTOV V SLEDOVIH V RAZLIČNIH VRSTAH MEDU IZ OKROŽJA KOPRIVNICA-KRIŽEVCI

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Povzetek: V okrožju Koprivnica-Križevci na severozahodnem delu Hrvaške so bili med leti 2010 in 2011 zbrani vzorci medu, nabrani na več rastlinah, ali sortnega medu robinje (*Robinia pseudoacacia* L.), kostanja (*Castanea sativa Mill.*), lipe (*Tilia spp.*), grmaste amorfe (*Amorfa fruticoza* L.) in kapusnice (*Brassica napus*). Koncentracije vsebnosti Cd, Pb, Hg, As in Cu ter srednje vrednosti elementov (µg/kg) v vzorcih medu so bile: v medu, nabranem na več rastlinah: 1,26 za Cd, 163 za Pb, 135 za As, 1,35 za Hg in 11,7 za Cu, v medu z robinje: 1,52 za Cd, 182 za Pb, 23,2 za As, 0,46 za Hg in 7,697 za Cu ter v medu z lipe 2,92 za Cd, 340 za Pb, 116 za As, 0,74 za Hg in 7,798 za Cu. Opažene so bile značilne razlike v nivoju Hg in Cu med vrstami medu. Povprečna raven Cu v medu lipe in robinje je bil bistveno višji kot v ostalih evropskih državah. Najvišja izmerjena vrednost elementov v različnih vrstah medu je bila: Cd 4,0 µg/kg in As 502µg/kg v medu kapusnice, Hg 6,11 µg/kg v medu kostanja, Pb 2,159 µg/kg v medu robinje in Cu 79,167 µg/kg v medu grmaste amorfe. Vsebnosti svinca, izmerjenega v vseh vrstah medu, so bile bistveno višje kot izmerjene vsebnosti v Italiji, Sloveniji, Poljski, Romuniji in Turčiji. Podatki kažejo na to, da je potrebno pozorneje izbrati lokacije čebelnjakov v področjih, kjer čebele nabirajo med. Pomembno je tudi, da so oddaljena od avtocest in železnic. Rezultati kažejo na razlike v vsebnosti elementov v sledovih v vzorcih medu, ki imajo različno botanično poreklo, pridobljeno na istem področju.

Ključne besede: različni tipi medu; kovine; As; Cd; Cu; Hg; Pb

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Original Scientific Article

COMPARISON OF MORPHOLOGICAL CHARACTERS BETWEEN WILD AND CULTURED STERLET (*ACIPENSER RUTHENUS* L.)

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Summary: Sterlet (*Acipenser ruthenus* L.) populations in the Danube River had experienced severe decline during the 20th century, and have become dependant on stocking measures in significant area of their distribution. Despite the current wide use of stocking, there are few studies dealing with efficiency of stocking efforts, especially with impact of rearing conditions on fitness of released individuals. This study tried to assess existence of morphological changes in reared sterlet that could impact their swimming performance and thus reduce survival of stocked fish. Wild sterlet (*n=45*) from the Danube River were compared with sterlet from aquaculture (*n=20*), originating from wild Danube spawners. Statistical comparison of 15 morphological traits revealed that samples significantly differed in 11 traits, as well as that reared sterlet had significantly shorter pectoral fins and stockier body than those from the wild. Additional investigation is needed to determine if these morphological differences could affect adaptability and survival of reared sterlet after their release into the river. Period of adaptation in lotic environment, prior to stocking, could probably alleviate the influence of aquaculture rearing on the fitness of released fish.

Key words: sturgeon; *Acipenseridae*; Danube River; Fulton factor; pectoral fin; biometry

Introduction

Populations of sterlet (*Acipenser ruthenus* L.) in the Danube River have experienced serious decline during the 20th century (1), and sterlet has been almost extirpated from the German and Austrian section of the Danube River, where its presence depends on continuous stocking efforts (2). It has a limited distribution in the basin of the Middle and the Lower Danube (3), and there are ongoing stocking activities with the majority of fish released by Hungary, and to a small extent by Slovakia and Bulgaria (4-6). Estimated quantity of stocked juveniles in Hungary declined from 10-100 thousands of annually released specimens during the 1980s to only sporadic stocking activities in 1990s and 2000s (3). According to Guti and Gaebele (3), there were 60.000 specimens released in 2002 in the Hungarian section of the Danube River.

While the stocking has been employed as a sturgeon management and rehabilitation tool for several decades, only limited information exists about the long-term effects of stocking on natural sturgeon populations (7). Significant attention of the scientific community was recently focused on the negative genetic effects of stocking on the wild sturgeon populations (8, 9), while the impact of aquaculture rearing conditions on fitness of

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stocked individuals was not addressed in greater extent. Hatchery-cultured fish typically encounter conditions very different from those encountered by their wild counterpart, which may result in behavioural, morphological, and physiological differences (10). Hanson et al. (11) provided one of the first evidences from the wild that the morphology is correlated with the swimming activity. Swimming performance is probably the main trait determining fitness in many species of fish and other aquatic animals (12).

Although the comparisons of the morphology between the reared and wild salmon stocks have been already conducted by a number of authors (13-15), such investigations are still lacking for sturgeon species. The effect of the hatchery rearing on body morphology tends to increase with the time the fish spent in the hatchery (14). In this study, we tried to determine possible morphological differences between the reared and wild juvenile sterlet.

Materials and methods

Sample origin and rearing conditions

A total number of 45 sterlet were caught by professional fishermen during November 2002 in the Serbian part of the Danube River, near Belgrade (44° 50' 36.85"N, 20° 25' 15.83"E), with the average total length 32.3±2.0 cm. Age of sterlet was determined from pectoral fin spine sections using a method of Stevenson and Secor (16), modified further by Lenhardt et al. (17). Average monthly temperatures of the Danube water during that period were: April 11.4° C, May 19.2° C, June 22.4° C, July 25.1° C, August 21.6° C, September 19.3° C, October 13.3° C and November 8.6° C. Sterlet were frozen immediately following the capture and biometric measurements were performed on defrosted material. Analysis of pectoral fin sections showed that all wild specimens were young-of-the-year. Since the late April and early May is the spawning period for sterlet in the Danube, specimens were approximately 6 months old at the time they were captured. Diet analysis, performed by Lapkina et al. (18), showed that in July it consisted mainly of chironomids, while the leeches were dominant in August and September (70% and 100%, respectively). Daily growth in weight was 1.8 g / day in July, and 2.2 g / day in September (18).

Sterlet (n=20) reared in aquaculture (Rideg & Rideg fish farm) at Homokmegy, Hungary (46° 29' 43.28"N, 19° 04' 03.75"E), with the average total length 33.4±1.4 cm, were also used for the analysis. These specimens originated from artificially fertilized eggs of adult specimens (6 females and 4 males), which were taken from the natural population in the Danube River, near Budapest. Hatching was performed between 15 and 17 April 2004, and specimens were reared on the average temperature of approximately 20° C, due to the use of underground water of the same temperature for basins supply. Fish were initially fed with tubifex worms and, thereafter, only dry feed was used. Specimens were fed *ad libitum*. In early October sterlet were moved to an outdoor basin with the ambient temperature. The main purpose for sterlet rearing at this farm is ornamental fish production for aquariums and ponds. Before the biometric analysis was performed on 3 November 2004, live specimens were placed in a water tank and anaesthetized with few drops of oleum caranfilium in water. These specimens were about 200 days old.

The distance between the sites of origin of the two fish samples (i.e., the wild fish and the broodstock that the aquaculture specimens originated from) was around 460 km of the river flow. Nevertheless, sterlet are known to move regularly over long distances of 300 km or more (19), and the recent genetic studies confirmed that the Danube sterlet should be considered as a single, panmictic population (20). As a result, we believe that the distance between the two locations produced no bias with regard to the genetic background of the studied specimens.

Since the cultured sterlet were measured fresh, while those from the wild were frozen prior to measurements, additional experiment was conducted to determine if the freezing has an impact on morphological measurements. Fifteen specimens were kept frozen for one month, and morphological measurements were performed on both fresh and defrosted material. Mann-Whitney U test showed that there were no significant differences ($p>0.05$) in any of the measured characteristics.

Laboratory and statistical analysis

In addition to body mass (M) and total length (L_t), biometric analyses of wild and cultured sterlet included 15 morphometric traits, nine of them in the head region, and three meristic traits (Figure 1). Morphological variable distributions were evaluated using the Kolmogorov-Smirnov test for normality, as well as with the Shapiro-Wilk test, due to to the small sample size. As they lacked normality of distribution, Mann-Whitney U test was applied.

It is usual to standardize morphometric measurements related to the head region as a proportion relative to head length, and those not related to the head region as proportions of the total length, if the growth is isometric (21-24). Therefore, a regression analysis was performed on studied specimens using allometric growth formula $y = ax^b$, described by Huxley (25), where a and b (slope - relative growth rates of variables) are constants. In isometric growth, the growth curve has a slope b=1. When slope b is smaller than the isometric slope, it is identified as a negative allometric growth, while it is identified as positive when b>1.

Hypothesis about the equality of slopes of the pectoral fin length related to L_t, between wild and reared specimens, was determined by formula $t = (b_1 - b_2)/S_{b1-b2}$ where b_1 and b_2 are regression coefficients of the two samples, and S_{b1-b2} is a standard error of the difference between regression coefficients (Figure 2) (26).

Fulton's body condition (FC) was estimated as FC = (M / L_t^3) x 100 and Kolmogorov-Smirnov test was used to compare FC between groups (26, 27).

Results

Wild specimens ranged in M between 83.5 and 278.2 g and in L_t between 30 and 37.7 cm. Cultured specimens ranged in M between 80 and 136 g, and in L_t between 30.2 and 36.7 cm. Allometric growth formula $y=ax^b$ (25) showed that wild sterlet displayed negative allometric growth in seven morphometric traits and positive allometric growth in one trait, "Head width at barbel base". Reared sterlet displayed negative allometric growth in 10 morphometric traits (Table 1). As a result, four traits that had isometric growth within both groups were standardized prior to statistical analysis, while the comparison of the remaining traits was performed using original measurements (see Table 1).

Wild and cultured specimens differed significantly (P<0.05) in eight morphometric traits, as well as in one meristic trait (Table 1). Wild sterlet revealed a significantly higher value of the regression slope for pectoral fin length (L_t; Figure 2), and the average length of pectoral fin, expressed as a percentage of the total body length, was 16.1% and 14.1% for wild and reared sterlet, respectively. Six length related traits (measurements 4-9 in Figure 1) were significantly larger in wild specimens, while two width related traits (distance between the eyes and head width at the base of the barbel) were significantly larger in cultured specimens.

Range of body mass was wider in the sterlet from wild population (145.8±38.4 g) than in the reared specimens (111.2±15.7 g). Fulton's body condition in wild specimens (0.42±0.06) was significantly higher than in the cultured ones (0.30±0.02; Kolmogorov-Smirnov test P<0.01).

Discussion

Significantly smaller number of ventrolateral scutes in cultured sterlet, obtained in this study, could be explained by different water temperatures during early rearing. The scute number in sturgeon may vary due to water temperature during early rearing (28), and retarded growth during larval development (i.e., due to a low temperature) can result in a higher number of serial features, such as scutes, because they are allowed more time to form (29).

Cultured specimens in this study had a significantly shorter pectoral fin (P<0.05). This is in accordance with findings of other authors (30,31) that cultured fish exhibit shorter fins than the wild fish of similar size. Kalmykov et al. (32) found three sterlet subpopulations within the Lower Volga which showed correlation between the water velocity and the pectoral fin length: in the Volga River sections with water velocities 0.36-0.76m/s, 0.76-1.00m/s and 0.84-1.23m/s, length of pectoral fins (expressed as a percentage of the total body length) was 15.59-15.69, 16.32 and 16.51-16.58, respectively.

Fulton's body condition for wild sterlet (with L_t ranging from 14.2 cm to 42.5 cm) varied from 0.27 to 0.79 throughout an annual cycle, with the highest value recorded in June (33). In

Table 1: Mean values ± SD of 15 morphometric characters and three meristic traits, correlation coefficients (R2) and slopes (b) and results of Mann-Whitney U test, for the studied wild and cultured sterlet samples

Morphometric and meristic traits (see footnote)	Wild sterlet (*n*=45)		Cultured sterlet (n=20)		Mann-Whitney U test	Wild sterlet (n=45)	Cultured sterlet (n=20)
	R^2	*b*	R^2	*b*	*P*	mean±SD	mean±SD
Standard length	0.88	1	0.84	0.82	0,702134	26.5±1.7	26.4±1
Fork length	0.91	0.99	0.81	0.84	0,622832	28.5±1.8	28.5±1.1
Preanal length	0.87	1	0.77	0.81	0,000793	19.3±1.3	18.1±0.7
Predorsal length	0.81	1	0.86	0.89	0,000569	20.6±1.3	19.3±0.9
Prepectoral length	0.58	0.92	0.53	0.95	0,000710	7.6±0.6	7.1±0.4
Pectoral fin length	0.91	1	0.81	1	0,000000*	5.2±0.4	4.7±0.2
Head length	0.66	0.89	0.46	0.87	0,000154	7.4±0.4	6.9±0.4
Preorbital length	0.54	0.91	0.59	1	0,000884	3.7±0.4	3.5±0.3
Preoral length	0.75	1	0.76	1	0,098007**	4.4±0.5	4.2±0.3
Prebarbel length	0.56	1	0.57	1	0,120252**	2.9±0.3	2.7±0.3
Mouth width	0.67	0.91	0.64	0.8	0,827056	1.3±0.1	1.3±0.06
Distance between eyes	0.57	0.9	0.7	0.84	0,029066	1.7±0.1	1.8±0.08
Maximum head width	0.68	1	0.68	1	0,332208**	3.3±0.3	3.1±0.2
Head width at barbel base	0.69	1.1	0.72	0.72	0,004776	1.5±0.1	1.6±0.06
Head width at mouth level	0.49	0.84	0.5	0.95	0,213410	2.4±0.2	2.3±0.1
No. of dorsal scutes					0,180403	13.6±0.6	13.1±1.1
No. of lateral scutes					0,837093	61.8±3.9	62.1±1.8
No. of ventrolateral scutes					0,000002	14.2±0.9	13±0.9

* Variable standardized prior to statistical comparison as a proportion of the total length
** Variables standardized prior to statistical comparisons as a proportion relative to head length

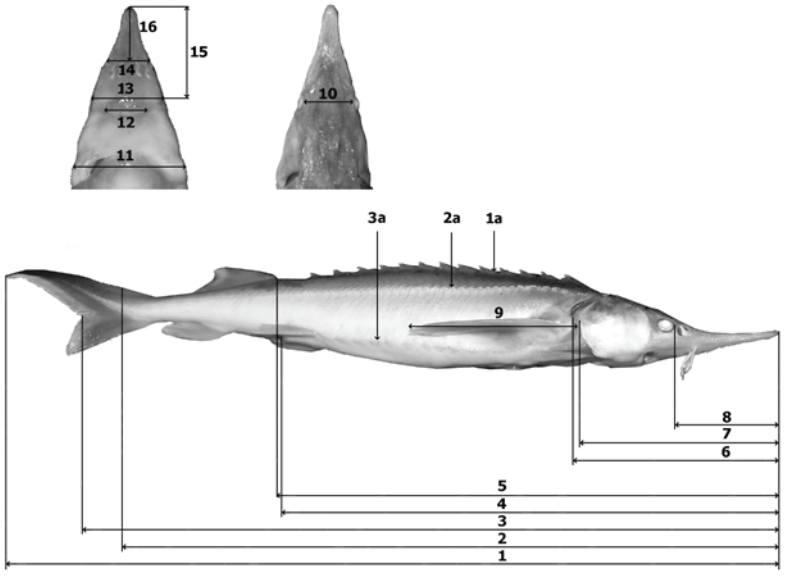

Figure 1: Sterlet morphometric and meristic traits used in analysis: 1 – Total length, 2 – Standard length, 3 – Fork length, 4 – Pre-anal length (from tip of rostrum to anterior margin to anus), 5 – Predorsal length, 6 – Prepectoral length, 7 – Head length, 8 – Preorbital length, 9 – Pectoral fin length, 10 – Distance between eyes, 11 – Maximum head width, 12 – Mouth width, 13 – Head width at mouth level, 14 – Head width at barbel base, 15 – Preoral length, 16 – Prebarbel length, 1a – Number of dorsal scutes, 2a – Number of lateral scutes, 3a – Number of ventrolateral scutes

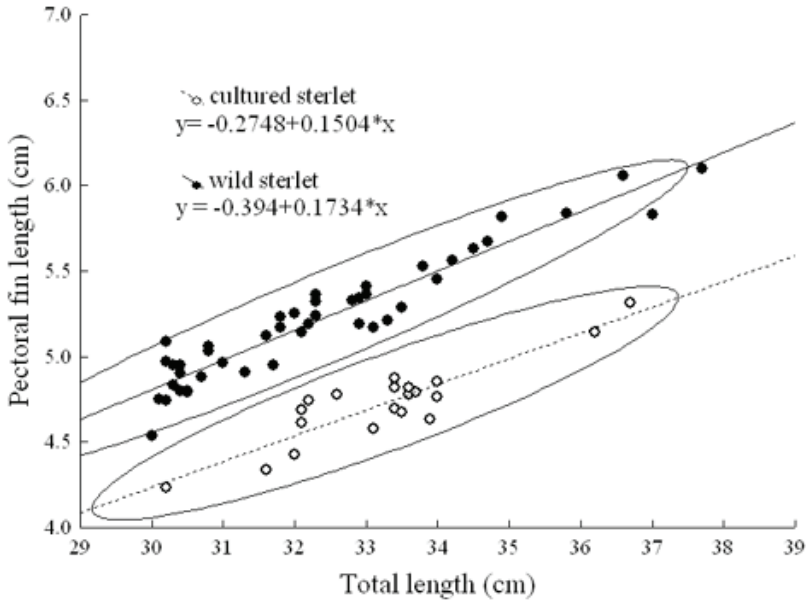

Figure 2: Pectoral fin length of the two groups (wild and reared sterlet), presented as a function of the total length. Regression: $Y_{cultured}$ = -0.2748 + 0.1504 x; Y_{wild} = -0.394 + 0.1734 x; ellipses represent 95% confidence limits

the analysis of six months old sterlet reared in warm water culture, the average value of the FC was 0.45±0.07 (ranging from 0.34 to 0.55; M. Prokeš, pers. comm.). Data obtained within this study showed that the wild specimens had a higher value of FC (0.42) than the cultured ones (0.30). This could be explained by the transfer of cultured sterlet to an outdoor basin with a lower temperature one month before sampling, as well as by a significant daily increase in wild sterlet weight during August and September, caused by a diet based on leeches (18).

There is a need for research that would be focused on the best conditions of sterlet rearing. As stated by Vehanen and Huusko (34), a simple hatchery environment may delay or modify the development of morphometric characteristics that are important in a natural river environment. Differences caused by the aquaculture environment might be probably alleviated if individuals could be released in certain small and confined parts of the natural habitat, before the actual stocking, to enable their adaptation to natural conditions. Chebanov et al. (35) presented a comprehensive system of guidelines for sturgeon hatcheries, including those for the juvenile rearing for release into natural waterbodies. According to these guidelines, the key parameters that would have to be taken into consideration are the illumination regime that mimicks a natural photoperiod, thermal regime, sufficient water flow and the use of live feeds (35). Holčik et al. (6) recommended that all juveniles reared in the fish farms should be adapted to the conditions in natural water bodies before their stocking, mostly through feeding with natural diet and residing in facilities with lotic environment.

Comprehensive literature exists about morphological divergence between cultured and wild juvenile salmon, and results indicated that it may affect the success of cultured specimens after their release into the wild (13, 14, 36, 37). Wild Atlantic smolts differed in the shape from hatchery-reared smolts, and this difference was less pronounced but still statistically significant when wild adults were compared with hatchery-reared adults after a year spent in the sea (14). Furthermore, Svåsand et al. (37) reviewed morphological and behavioural differences between reared and wild individuals of the Atlantic cod and European lobster, while Arechavala-Lopez et al. (38) found a clear morphological differentiation between farmed and wild individuals in a number of Mediterranean fish species. According to Sarà et al. (39), the key parameters that influence morphology of reared fish are stock density, container volume, reduced swimming performance and the food quality. Although there were some attempts to assess the performance of the stocked specimens of sturgeon species, such as the one that compared the growth between the wild and stocked European sturgeon (*Acipenser sturio*) juveniles (40), such studies are unfortunately still scarce. This study was the first attempt to compare morphology of the wild and reared sterlet, and it revealed the presence

of morphological differences. Nevertheless, this investigation had a number of limitations, especially when bearing in mind a small, but still realistic possibility that the analyzed specimens from the wild could originate from stocking activities in Hungarian section of the Danube. Such problems could be alleviated by further studies, which would involve assessment of a more representative sample and also include comparisons of adult individuals. Additional research should also deal with the assessment of inter-annual differences between cohorts in a culture system, as well as of differences among wild populations living in conditions with different water velocities.

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PRIMERJAVA MORFOLOŠKIH LASTNOSTI DIVJE IN GOJENE KEČIGE (*ACIPENSER RUTHENUS* L.)

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Povzetek: Populacije kečige (*Acipenser ruthenus* L.) v reki Donavi so v 20. stoletju doživele veliko padcev in postale odvisne od stopnje gojenja v značilnem področju njihove razširjenosti. Kljub trenutni široki uporabi gojenja rib se malo raziskav ukvarja z učinkovitostjo uspeha gojitve, še posebej z vplivom rejskih pogojev na izpuščene osebke. Naša raziskava poskuša ugotoviti obstoj morfoloških sprememb pri gojenih kečigah, ki bi lahko vplivale na kakovost plavanja in tako zmanjšale preživetje gojenih rib. Divje kečige (*n=45*) iz reke Donave smo primerjali s kečigami iz akvakulture (*n=20*), ki izvira iz divjih ikrnic na Donavi. Statistična primerjava 15 morfoloških značilnosti je pokazala, da se vzorci razlikujejo v 11 značilnostih, poleg tega pa so imele gojene kečige značilno krajšo prsno plavut in bolj čokato telo v primerjavi z divjimi. Potrebne so dodatne raziskave, da bi se ugotovilo ali proučevane značilnosti lahko vplivajo na prilagodljivost in preživetje gojenih kečig po izpustu v reko. Obdobje prilagajanja v okolju pred gojitvijo lahko najbrž zmanjša vpliv akvakulturne reje na preživitvene sposobnosti izpuščenih rib.

Ključne besede: kečiga; *Acipenseridae*; reka Donava; Fultonov faktor; prsna plavut; biometrija

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Original Scientific Article

DETECTION OF *CHLAMYDIA PSITTACI* IN CAGE BIRDS IN SLOVENIA BY REAL-TIME PCR

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Summary: Avian chlamydiosis is a zoonotic disease of birds caused by the bacterium *Chlamydia psittaci*. The highest infection rates are found in psittacine birds (Psittacidae) which are the most common cage birds. *C. psittaci* causes infections of the conjunctiva, respiratory tract, and digestive tract, with or without clinical signs in birds. Infected birds can shed chlamydiae through respiratory tract excretions and in faeces. Transmission of *C. psittaci* primarily occurs by close contact of infected bird to the susceptible bird or human.

To determine the prevalence of *C. psittaci* in cage birds in Slovenia, oropharyngeal and cloacal swabs from 125 cage birds were examined by *Chlamydiaceae*-specific real-time polymerase chain reaction (real-time PCR). Two lovebirds (2/12) and a budgerigar (1/44) were positive for *Chlamydiaceae* by real-time PCR and were also positive for *C. psittaci* by an *omp*A-based real-time PCR assay specific for *C. psittaci*. Multiple loci variable number of tandem repeats analysis (MLVA) identified *C. psittaci* of genotype A in the positive budgerigar and *C. psittaci* of genotype B in the two positive lovebirds. The infected birds had no significant clinical signs of avian chlamydiosis on clinical examination. Using real-time PCR, the study showed a low prevalence (2.4 %) of *C. psittaci* in Slovenia.

Key words: *Chlamydia psittaci*; real-time PCR; MLVA; cage birds; Slovenia

Introduction

Chlamydia psittaci (*C. psittaci*) is a member of the *Chlamydiaceae* family and is an obligatory intracellular, gram-negative bacterium, which can infect many avian species as well as a wide range of mammalian hosts (1). The disease caused by *C. psittaci* is designated avian chlamydiosis, originally termed psittacosis or parrot fever in psittacine birds and ornithosis in other bird species (2, 3). The clinical disease depends on the chlamydial strains and avian host. Symptoms in the affected bird can include respiratory distress, keratoconjunctivitis, sinusitis, mucopurulent nasal discharge, diarrhea (yellow-green droppings are common), polyuria, dullness and affection of the central nervous system. Asymptomatic infections can occur with any strain of *C. psittaci*. A large number of chlamydiae cells can be found in respiratory secretions and faecal material of infected birds. Transmission of *C. psittaci* predominantly occurs through inhalation of contaminated material and sometimes through ingestion, from an infected bird to a susceptible bird. Humans can become infected by inhaling an organism shed by infected birds, mouth-to-beak contact or by handling the

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plumage and tissues of infected birds. In humans *C. psittaci* most often causes a respiratory infection and fever (3, 4). Person-to-person transmission of avian chlamydiosis is possible, but it is believed to be rare (5, 6, 7, 8).

Avian chlamydiosis occurs world-wide. To date, *C. psittaci* infections have occurred in at least 467 bird species, belonging to 30 different bird orders (9). The highest infection rates are found in psittacine birds (Psittacidae) and pigeons (Columbiformes) (4). Psittacidae represent major reservoirs of chlamydiae, especially under captive conditions. Psittacine cage birds should therefore be considered to be potential reservoirs of *C. psittaci* (4, 9).

Diagnosis of chlamydial infection in birds is still a considerable challenge. Clinical changes are not pathognomonical and persistent infections can also occur. The infection can only therefore be confirmed by direct identification of the agent or indirectly by detection of specific antibodies. PCR is currently the method of choice for diagnosis of chlamydial infection. Specific and sensitive PCR methods to detect *C. psittaci ompA* gene have been developed (2, 10, 11).

Serotyping of *C. psittaci* isolates, which was previously performed with a panel of serovar-specific monoclonal antibodies in an immunoflurescence test, allowed their classification into six avian serovars (A through F) (12, 13, 14). Serovar A is repeatedly isolated from psittacine birds, serovar B from pigeons and psittacine birds, serovar C from ducks and geese, serovar D from turkeys, serovar E from pigeons and ratites, and serovar F from psittacine birds and turkeys (13, 14, 15). All serovars appear to be transmissible to humans (4). Analysis of the gene encoding outer membrane protein A (*omp*A) is most often used to characterize avian *C. psittaci* strains into genotypes (16). Nine genotypes of *C. psittaci* are currently accepted (A-F, E/B, WC and M56) (2, 16, 17). Using comparison of serotyping results and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) patterns, Vanrompay et al. (12) were able to demonstrate that serovars have genetic equivalents in the corresponding genotypes (16). Other molecular tools, such as microarray analysis, multiple loci variable number of tandem repeats analysis (MLVA) and multilocus sequence typing (MLST) are also used for genotyping *C. psittaci* (16, 18, 19).

The aim of our study was therefore to establish the excretion rate of *C. psittaci* in oropharyngeal and cloacal swabs from cage birds by real-time PCR in Slovenia. Samples were first verified as *Chlamydiaceae* and positive samples for *C. psittaci* were then detected and genotyped.

Material and methods

Clinical examination

Cage birds were clinically examined at the Department for Birds, Small Mammals and Reptiles of the Institute for Health Care of Poultry, Veterinary Faculty in Ljubljana. Anamnesis was taken and different diagnostic procedures (microscopic examination of faeces and crop swabs, x-ray, molecular investigation to avian bornavirus and circovirus) were performed (data not shown) in some birds after agreement had been obtained from the owners of the birds.

Clinical samples

Oropharyngeal and cloacal swabs were taken during clinical examination of 125 cage birds sampled randomly, in 2010 and 2011. The order, family, genus and species of the examined birds are given in Table 1. Samples were collected with Dacron swab and stored in one millilitre of 2-sucrose-phosphate based transport medium (2SP) at -20°C until processing.

DNA extraction

Total DNA was extracted with a QIAamp® Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's blood and body fluid spin protocol. Each swab was vortexed vigorously before DNA extraction. Two hundred microliters of each sample were used as starting material for the DNA extraction. Finally, DNA was eluted with 100 µl of AE buffer and stored at -20°C until examination.

Detection of chlamydiae DNA by real-time PCR

Generic real-time PCR was used for the amplification of the partial 23S RNA gene of *Chlamydiaceae*. For the amplification, the following primers Ch23S-F (5'-CTG AAA CCA GTA GCT TAT AAG CGG T-3') and Ch23S-R (5'-ACC TCG CCG

TTT AAC TTA ACT CC-3'), and probe Ch23S-p (FAM-5'-CTC ATC ATG CAA AAG GCA CGC CG-3'-TAMRA) were used (20). The reaction mix contained 4.0 µl of nuclease-free water (Promega, Madison, WI, USA), 7.5 µl of 2x QuantiFast probe PCR master mix with ROX, and 0.6 µl of each primer (0.4 µM), 0.3 µl of specific probe and 2 µl of extracted DNA. The reaction was performed by ABI 7300 thermocycler (Applied Biosystems, Foster City, CA, USA). The cycling profile included initial denaturation at 95°C for 3 min, followed by 50 cycles of denaturation at 95°C for 5 s and 60°C for 30 s. The cycle threshold (Ct value) was calculated automatically. All positive samples were tested in two repeats before they were further examined by the primers CppsOMP1-F (5'-CAC TAT GTG GGA AGG TGC TTC A-3'), CppsOMP1-R (5'-CTG CGC GGA TGC TAA TGG-3') and probe CppsOMP1-S (FAM-5'-CGC TAC TTG GTG TGA C-3'-TAMRA) corresponding to the region of the *omp*A gene specific for *C. psittaci* (21). The real-time PCR reaction mix and cycling conditions were the same as for the 23S RNA gene.

DNA-based characterization - multiple loci variable number of tandem repeats analysis (MLVA)

C. psittaci positive samples were examined by MLVA. For the variable number of tandem repeats (VNTR) amplifications, PCR was performed in a total volume of 15 µl containing 2 µl of sample DNA, 1.5 µl 10 x PCR reaction buffer, 0.1 µl (1 U) of Hot start Taq DNA polymerase (Qiagen), 0.15 µl (200 µM) of each deoxynucleotide triphosphate, and 1.5 µl (0.3 µM) of each flanking primer (18).

The initial denaturation step at 95°C for 15 min was followed by 40 cycles consisting of denaturation at 95°C for 30 s, annealing at 56°C for 45 s, and elongation at 72°C for 45 s. The final extension step was at 72°C for 10 min. Nine microliters of amplification product were loaded onto a 4 % standard agarose gel. Gels stained with EZ-Vision® (Interchim, Montluçon, France) were visualized under UV light and photographed. A 100 bp ladder marker was used (Mbi, Euromedex, Souffelweyersheim, France).

Amplicon sizes were determined manually by a recently described methodology (18). The resulting data were compared with known MLVA types (10) for determining the genotype of *C. psittaci*.

Statistical analyses

All statistical analyses were performed with GraphPad Prism 6.0 software (GraphPad Prism 6.0 software, San Diego, California, USA). Fisher's exact test was used to calculate a p-value with statistical significance considered at $p < 0.05$.

Results

A total of 125 cage birds were sampled in 2010 and 2011 during clinical examination (Table 1). Ninety-seven birds (77.6 %) had various clinical problems. Fifteen birds (12 %; quail, two cockatiel, two African grey parrot, Senegal parrot, two Amazon parrot, Superb parrot, four budgerigars and two Hill myna) from among them had a suspected clinical signs of having *C. psittaci*. While the other 28 birds (22.4 %; two cockatiels, one amazon parrot, nine lovebirds, twelve budgerigars, two canaries, one zebra finch and one java sparrow) were apparently healthy (data not shown).

Three cage birds, two lovebirds and a budgerigar, were positive for *Chlamydiaceae* by real-time PCR for the detection of the 23S RNA gene of *Chlamydiaceae* and were also positive for *C. psittaci* by real-time PCR for the detection of the *omp*A gene specific for *C. psittaci*. The two positive samples from lovebirds showed MLVA types (ST) identical to ST 10 and the positive sample from a budgerigar was identical to ST 12. ST 10 is characteristically equivalent to genotype B, whereas ST 12 is equated with genotype A. Detailed information about the results of different diagnostic methods and clinical signs of positive cage birds are given in Table 2.

In order to determine whether budgerigars and lovebirds are more susceptible to the infection with *C. psittaci* in a given sample, the Fisher chi - squared test was used. There was statistically significant presence of *C.psittaci* for lovebirds with $p = 0{,}028$, but no for budgerigars ($p = 1$).

Discussion

C. psittaci is a very important bacterial pathogen in veterinary and human medicine. It can cause a clinical disease called avian chlamydiosis in many avian and mammalian species. Birds,

Table 1: Order, family, genus and species of examined birds. The total number of examined birds and the number of birds that tested positive for *C. psittaci* are shown

	Order	Family	Genus	Species	Birds examined (No)	*C. psittaci* positive birds (No)
Fowl (n=1)	Galliformes	Phasianidae	*Coturnix*	Quail*	1	0
Parrots (n=112)	Psittaciformes	Cacatuidae	*Nymphicus*	Cockatiel	17	0
			Eolophus	Galah	4	0
		Psittacidae	*Psittacus*	African grey parrot	9	0
			Poicephalus	Senegal parrot	1	0
			Pionites	Black-headed parrot	1	0
			Amazona	Amazon parrot	7	0
			Cyanoliseus	Burrowing parrot	1	0
			Aratinga	Sun parakeet	2	0
			Nandayus	Nanday parakeet	1	0
			Eclectus	Eclectus parrot	1	0
			Alisterus	Australian king parrot	1	0
			Polytelis	Superb parrot	2	0
			Psittacula	Ring-necked parakeet	6	0
				Alexandrine parakeet	2	0
			Agapornis	Lovebird	12	2
			Melopsittacus	Budgerigar	44	1
			Cyanoramphus	Yellow-crowned parakeet	1	0
Songbirds (n=12)	Passeriformes	Sturnidae	*Gracula*	Hill myna	2	0
		Fringillidae	*Serinus*	Canary	6	0
		Estrildidae	*Taeniopygia*	Zebra finch	3	0
			Padda	Java sparrow	1	0
Total No					125	3

*kept as a pet birds

Table 2: Details of positive chlamydiae test results of cage birds with clinical signs and results of parallel diagnostic investigation from Slovenia

Place of origin	Bird species	Real-Time PCR *Chlamydiaceae* (Ø Ct value)		Real-Time PCR *C. psittaci* (Ø Ct value)		MLVA Pattern	Genotype of *C. psittaci*	Clinical signs	Results of parallel diagnostic investigation
		OS	CS	OS	CS				
Ljubljana	Lovebird	33.3	32.97	33.94	33.44	10	B	Depression, weight loss, anorexia, abdominal mass.	nd
Ljubljana	Lovebird	neg	30.2	neg	33.34	10	B	With no clinical signs.	nd
Ljubljana	Budgerigar	34.75	27.02	neg	30.25	12	A	Progressive weight loss.	*Macrorahbdus ornithogaster* found in faeces.

OS oropharyngeal swab; *CS* cloacal swab; *neg* negative; *nd* not done

especially under captive conditions, are the major reservoir of *C. psittaci* (10). Most chlamydia infected cage birds are parrots, especially African grey parrots, Amazon parrots, lovebirds, cockatiels and budgerigars (4, 22). The results of our study, in which the three *C. psittaci* positive birds (two lovebirds and a budgerigar) belong to Psittaciformes, are in agreement with this. The birds examined in our study were from the orders Galliformes (0.8 %), Psittaciformes (89.6 %) and Passeriformes (9.6 %). In general, Psittaciformes are the most frequently investigated for chlamydial infections (9). It is still not known whether Psittaciformes are more susceptible to *C. psittaci* than other bird species. It is possible that the clinical signs of avian chlamydiosis are more easily noticed in these birds and infection therefore more often diagnosed (9, 23, 24).

Avian chlamydiosis can also occur in Passeriformes, especially in canaries and finches, but the infection is rarely diagnosed (4, 9, 22, 25). Passeriformes is the largest bird order and contains more than 4000 species (9). Nevertheless, chlamydia has been detected in only 2 % of song birds in comparison to a 45 % prevalence of chlamydial infections in parrot birds (9). Based on our experience, it is very likely that parrots are the largest group among cage birds. For example, during the period of the present study, 399 cage birds (80.7 % parrots and 7.8 % song birds) had the first clinical examination at the Department for Birds, Small Mammals and Reptiles.

In our study, 125 cage birds were sampled during clinical examination, in order to detect chlamydia infections by real-time PCR diagnostic methods. Oropharyngeal and cloacal swabs were taken from each bird, because infected birds can shed chlamydiae through respiratory tract excretions and in faeces (3, 4). Birds were sampled before any antibiotic treatment because antibiotic drugs can prevent shedding of chlamydiae (27). Serological monitoring was not done because the aim of our study was to establish the excretion rate of *C. psittaci* in cage birds through oropharyngeal and cloacal secretions.

C. psittaci infections in birds may range from subclinical to infections with diverse clinical signs, such as lethargy, anorexia, ruffled feathers, ocular or nasal discharge, conjunctivitis and greenish faeces (3, 4). Among the tested birds, twenty-eight (22.4 %) showed no clinical signs, while the other 97 birds (77.6 %) had a variety of clinical signs. In a budgerigar that showed progressive weight loss and was positive for *C. psittaci*, *Macrorhabdus ornithogaster* was also found by parallel diagnostic investigation. Only one of the two lovebirds that were positive for *C. psittaci* showed clinical signs not typical for infection with *C. psittaci.* The other bird appeared clinically healthy (Table 2). Anamnesis revealed that the lovebirds had been in close contact before they were tested. In the lovebird with clinical signs, *C. psittaci* was detected in oropharyngeal and cloacal swabs, while in the clinically healthy lovebird *C. psittaci* was detected only in the cloacal swab. The explanation of this may be that the shedding of *C. psittaci* occurs intermittently and healthy carrier birds might not excrete bacteria for extended periods (5).

Three cage birds (2.4 %) were positive for *Chlamydiaceae* and *C. psittaci* by real-time PCR. Oropharyngeal and cloacal swabs were positive for *C. psittaci* in the lovebird with clinical signs, while in the other two birds, the clinically healthy lovebird and the budgerigar with progressive weight loss, only cloacal swabs were positive. In the budgerigar, the oropharyngeal swab was positive for *Chlamydiaceae* but negative for *C. psittaci*. The reason for this may be that the amount of investigated DNA of *C. psittaci* was under the detection limit of the method.

Real-time PCR is one of the favored methods for the detection of DNA of *Chlamydiaceae* as well as for further detection of *C. psittaci* DNA. The method proved to be rapid and have good specificity and sensitivity as reported by Geens et al., Ehricht et al. and Pantchev et al. (2, 20, 21). The prevalence of *C. psittaci* in cage birds of different species detected by real-time PCR described by Ehricht et al. and Pantchev et al. (20, 21) was, in our study, 2.4 % (3 of 125). One of the reasons for the low prevalence of *C. psittaci* infections detected in the present study may be in randomly sampled cage birds as only 12 % (15 of 125) of them have had clinical signs that could be related to *C. psittaci* infection and no sampled birds have had history of previous infections with *C. psittaci.* The prevalence of the infected birds detected in the present study also correlates well with the results of previous serological monitoring of *C. psittaci* infection in breeding flocks of parrots, canaries and finches (25, 26). Antibodies were detected in 6.2 % of parrots, 0.8 % of canaries and 0.9 % of finches breeding flocks in the period from 1991 to 2001 (25, 26). In our study, most

of the sampled birds (112 of 125) were parrots, of which three (2.7%) were positive for *C. psittaci* DNA. In canaries (4.8% of sampled birds) and in finches (3.2% of sampled birds) no infections with *C. psittaci* were detected.

The *C. psittaci* detected in cloacal swabs samples from the two lovebirds and the budgerigar was typed by the MLVA method. *C. psittaci* of genotype A was detected in the budgerigar and *C. psittaci* of genotype B was detected in both lovebirds. These results correlate with previous findings, whereby genotypes A and B have been predominantly found in psittacine birds (18, 28). Surprisingly, only genotype A was detected by MLVA in samples from lovebirds in a recent study (18).

Even though the statistical analyses showed that lovebirds are more susceptible to the infection with *C. psittaci* in a given sample it should be mentioned that both positive lovebirds were from the same household. In this case, it is very likely that this was the main reason for the infection of both birds rather than the susceptibility of the lovebirds as a species.

For an avian practitioner, *C. psittaci* infections in cage birds, especially in the absence of clinical signs, can be difficult to diagnose. Generally, avian chlamydiosis should be suspected in any parrot that shows respiratory or gastrointestinal or neurological signs or has been in contact with infected birds. Real-time PCR is a diagnostic tool that can be used to determine infection by *C. psittaci*. In addition, the MLVA method can be used for typing the microorganism. If possible, serological analysis should be still used to complement the molecular diagnosis. The infected birds should be correctly treated and the transmission of the pathogen to susceptible animals and to humans should be prevented.

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UGOTAVLJANJE BAKTERIJE *CHLAMYDIA PSITTACI* PRI SOBNIH PTICAH V SLOVENIJI S PCR V REALNEM ČASU

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Povzetek: Aviarna klamidioza je obolenje ptic, ki jo povzroča bakterija *Chlamydia psittaci*. Okužbe s to bakterijo najpogosteje ugotovimo pri papigah (Psittacidae), ki so hkrati tudi najštevilčnejše predstavnice sobnih ptic. Okužba ptic s *C. psittaci* zelo pogosto poteka brez kliničnih znakov. Pri obolelih pticah pa lahko opazimo apatičnost, neješčnost, našopirjenost, očesni in nosni izcedek ter vodene temnozelene iztrebke. Okužene ptice izločajo povzročitelja najpogosteje v presledkih z očesnimi in nosnimi izcedki ter z iztrebki. Prenos okužbe s *C. psittaci* je neposreden in je možen med različnimi vrstami ptic in tudi s ptic na ljudi.
Za ugotavljanje prisotnosti *C. psittaci* pri sobnih pticah v Sloveniji smo odvzeli brise žrela in kljunske votline ter brise kloake pri 125 pticah. Vzorce smo najprej pregledali na prisotnost bakterij iz družine Chlamydiaceae z metodo PCR v realnem času. Okužbo s *C. psittaci* smo dokazali pri dveh agapornisih (2/12) in eni skobčevki (1/44). Za ugotavljanje okužb s *C. psittaci* smo uporabljali PCR v realnem času, specifičen za gen *omp*A. S hkratno analizo večjega števila lokusov z variabilnim številom tandenskih ponovitev (MLVA) smo pri skobčevki določili *C. psittaci*, genotip A, pri obeh agapornisih pa *C. psittaci*, genotip B. Z metodo PCR v realnem času smo ugotovili nizko prevalenco (2,4 %) okužb s *C. psittaci* pri sobnih pticah v Sloveniji.

Ključne besede: *Chlamydia psittaci*; PCR v realnem času; MLVA; sobne ptice; Slovenija

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Original Scientific Article

BONE METABOLISM MARKERS AND BLOOD MINERALS CONCENTRATION IN DAIRY CATTLE DURING PREGNANCY AND LACTATION

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Summary: Biochemical markers of bone metabolism are substances in blood and urine that indicate how intensively bone is forming or degrading at the time of sample (blood or urine) collection. The aim of the study was to establish concentration of total calcium (Ca), inorganic phosphate (iP), magnesium (Mg), C-terminal telopeptide crosslinks of collagen I (CTx) (biochemical marker of bone resorption) and activity of total alkaline phosphatase (ALP) and bone specific alkaline phosphatase (BALP) (biochemical marker of bone formation) in blood serum of clinically healthy mature dairy cows from dry period until peak lactation. Holstein-Friesian cows (n=54) with high milk yields (average 8,463 kg/previous lactation) were enrolled in the study. Cows were divided in 5 groups according to stage of lactation: 1. approximately 1 month before calving (AP) (early dry period) (n = 10), 2. 10 to 1 day AP (close-up dry period) (n = 10), 3. 0 – 48 hours after calving (PP) (n = 10), 4. 10 – 20 days PP (n = 10) and 5. peak of lactation (35 – 55 days in milk) (n = 14). The nadir of mean Ca and iP were in the third group and significantly lower than in other groups ($P<0.05$). Mean Mg was higher in groups 3 and 5 compared to groups 2 and 4 ($P<0.05$). The lowest mean value of BALP was established in group 1. The difference was statistically significant compared to all other groups. The highest values of CTx were measured in groups 4 and 5. These values were also significantly higher compared to groups 1, 2 and 3. The lowest CTx values were measured in the 1. group. Results of the study indicate that bone tissue is the most catabolically active 10 to 20 days after calving and the most anabolically active during the dry period. Strong negative correlation ($P<0.01$) was established between BALP and minerals Ca and iP.

Key words: biochemical markers; CTx; BALP; ALP; cows; mature

Introduction

Bone metabolism in physically mature animals is a continuous dynamic process of bone resorption coupled with bone formation, so called bone remodeling. This allows adaptation of bones quality (flexibility and firmness), to physical load and during periods of minerals shortage, bone can be the main source of especially calcium (Ca) (1). In mature dairy cattle bone metabolism is especially active due to Ca requirement from parturition to peak lactation when Ca demand quickly rises to its highest. Enough Ca for maintenance and production from ration is typically very hard to obtain and they have to use also their body Ca reserve in bones to maintain normocalcemia. Ca metabolism is connected to bone metabolism and closely hormonally regulated. During bone resorption, inorganic phosphorus (iP) and magnesium (Mg) are also released, but their blood concentration is not hormonally regulated (2-4).

Biochemical markers of bone metabolism are substances in blood and / or urine that indicate the intensity of bone formation or resorption at the time of sample collection. During bone formation (anabolic activity), osteoblasts are more

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metabolically active. Byproducts of osteoblast activity are markers of bone formation, eg. bone specific enzyme bone alkaline phospahatase (BALP) in blood serum. During bone resorption (catabolic activity), osteocalsts are metabolically more active. Byproducts of osteoclast activity are markers of bone resorption, eg. C-terminal telopeptide crosslinks of collagen I (CTx) in blood serum. Markers of bone metabolism can indicate if bone metabolism is more anabolically, catabolically or in general more or less active (1, 5). In high yielding dairy cows we expect increased catabolic bone metabolism at the beginning of lactation when absorbable Ca demand suddenly increases from about 20 g to around 60 g or more per day because of colostrum and milk production concurrent with a relatively low dry matter (nutrient) intake (3, 6). Lactational osteoporosis, where around 13 % Ca can be lost from bones, can result as significant proportion of Ca is resorbed from bones at the beginning of lactation (7). This finding was confirmed also in a study conducted by *Beighle* (8) where cortical bone Ca concentration decreased as milk production increased. When milk production starts to decline body stores of Ca are replenished and bone metabolism is more anabolic (9). If these processes are not functioning properly bone disease and Ca metabolism disorders develop (hypocalcaemia and milk fever) which affect animal welfare, result in suboptimal production and other diseases in cattle (3). Very little is known about dynamics of biochemical markers of bone metabolism in dairy cattle during lactation and dry period.

The aim of this study is to investigate bone metabolism in mature dairy cows in intensive dairy production by measuring serum bone resorption marker CTx and bone formation marker BALP along with the classic mineral metabolism panel (Ca, iP, Mg and total alkaline phosphatase (ALP)) from dry period until peak lactation.

Material and methods

Herd and animals

The study was carried out in a heard of Holstein-Friesian cattle in intensive dairy production in farm setting. Clinically healthy skeletally mature cows (n=54) in appropriate body condition for their physiological period and at least in 4th lactation (range 4th to 7th lactation; mean age 7 years, median age 7 years, range 5 to 10 years) were enrolled in the study. Cows were sampled from February until mid-April when all animals were exclusively kept indoors, in tie-stall type housing system on short stalls. Average annual milk yield of investigated cows in previous lactation was 8,463 kg (rage from 7,128 kg to 10,215 kg) milk with 3.7 % milk fat and 3.17 % crude milk protein. All the cows included in the study were in dry period for 50 ± 3 days.

Cows were divided in 5 groups according to their stage of lactation:

1. 35 – 25 days before calving (AP) or at early dry period, n = 10,
2. 10 – 1 day AP (end of close-up period – mammary gland is regenerating and secreting colostrums), n = 10.
3. 0 – 48 hours after calving (PP) (the most critical time for development of milk fever (3)), n = 10,
4. 10 – 20 days PP (direct influence of calving on bone metabolism is low; milk production is steeply rising), n = 10,
5. peak lactation (35 – 55 days in milk), n = 14,

Ration

Cows were fed usual winter total mix ration (TMR), adapted to high productivity and their stage of lactation during the study, according to NRC (6) recommendations. The TMR was based on home produced forages: grass silage, maize silage, hay and straw. Anion salts were not added to feed in our study and forages were not treated in any way to reduce DCAD. DCAD of TMR was estimated to be in the range from +200 to +300 mEq/kg DM.

Composition of TMR is presented in table 1.

The ration for lactating cows was suitable for 30 kg milk yield per day. Fresh cows received about 15 kg DM of ration for peak lactation cows and hay ad libitum.

Blood sampling and analytical methods

Venous blood samples were collected in evacuated tubes (10 mL) without any additives (Venoject, plain silicone coated, Terumo Europe N.V., Belgium) from *v. caudalis mediana* according to the protocol between 9 and 11 a.m., to avoid daily fluctuations in analytes. Cows were

Table 1: Composition of TMR fed to cows during lactation and dry period

TMR ingredient	Peak lactation	Early dry period	Close-up dry period
Grass silage (33.1 % DM)	14 kg	14 kg	5 kg
Corn silage (39.2 % DM)	20 kg	7 kg	7 kg
Hay (85.3 % DM)	1 kg	/	3,5 kg
Straw	/	3 kg	/
Wheat	3 kg	/	1 kg
Commercially prepared concentrated feed (26% crude protein, 3% protected fats)	3 kg	/	1 kg
Commercially prepared energy feed (9 MJ NEL, 14% crude protein)	/	/	1kg
Rape skins	1 kg	0.5 kg	0.3 kg
Beet pulp	1 kg	/	0.3 kg
Soya bean skins	0.3 kg	/	0.1 kg
Mineral-vitamin supplement for lactating cows (Ca 18%, P 6%, Mg 4%, D_3 80.000 IE/kg)	0.1 kg	/	/
Mineral-vitamin supplement for lactating cows (Ca 19%, P 4.5%, Mg 2.5%, D_3 125.000 IE/kg)	0.1 kg	/	/
Mineral-vitamin supplement for dry cows (Ca 3%, P 8%, Mg 7%, D_3 125.000 IE/kg)	/	0.1 kg	/
Grounded calcium carbonate	0.06 kg	/	/
$NaHCO_3$	0.12 kg	/	/
Dry matter intake by animals	22 kg	11 kg	10,5 kg
Ca	165.69 g	49.99 g	58.74 g
P	86.14 g	39.54 g	33.70g
Mg	47.75g	25.53 g	43.28 g

sampled once or twice a week, during 14 sampling sessions. During this time at least 10 animals were randomly obtained for each group. Only one blood sample was taken from each animal. After blood clotting, samples were centrifuged at 3000 rpm for 10 minutes then supernatants were centrifuged again at 3000 rpm for 10 minutes at room temperature. Harvested blood serum was stored at -20° C until analyses.

Blood serum Ca, aP and Mg concentration and activity of total ALP were measured with automatized biochemical analyzer RX Daytona (Randox, Ireland) according to manufacturer's instructions. Blood serum BALP activity was measured using Alkphase-B kit (Metra Biosystems, USA) by enzyme imunoanalysis according to manufacturer's instructions on Immulite 2500 analyser (Siemens, Germany). The absorbance at the end of reaction was measured with optical reader Humareader (Human, Egypt) at 405 nm wave length. Cross reactivity of the test was validated for use in cattle (1). CTx concentration in blood serum was measured by electrochemiluminiscent imunoanalysis ECLIA. The test was conducted using Elecysis 3 – CrossLaps kit on Elecys 1010 analyzer (Roche Diagnostics, USA) according to manufacturers' instructions.

Statistical analysis

Data were statistically analyzed using SPSS verson 15.0. software (SPSS, USA). Mean and standard deviation were calculated for measured parameters. Influence of physiological period on parameters was analyzed with analysis of variance (one-way ANOVA). All obtained values were previously normalized according to Box-Cox. When statistical differences were discovered between groups, they were ascertained with Tukey's algorithm of multiple comparisons. Person's correlations were calculated for all parameters. Statistical significance was set at $P<0.05$.

Results

Descriptive statistics for Ca, iP, Mg, ALP, BALP, CTx and statistically significant differences according to stage of lactation (group) are presented in Table 2. Dynamics of investigated parameters from dry period until pick lactation are demonstrated in Graph 1 and 2.

Values of Ca and iP were statistically significantly lower in 3. group compared to other groups and under physiological range for adult cattle (reference blood serum Ca of adult dairy cattle is 2.2 – 2.5 mmol/L and iP 1.61 – 2.26 mmol/L (3)). All the animals had Mg within reference range for adult dairy cattle, > 0,82 mmol/L (3). The lowest values of BALP were measured in 1. group. The difference was statistically significant compared to all other groups. The highest values of CTx were measured in 4. group.

Statistically significant positive Pearson's correlations were found between total ALP and BALP ($r=0.585$, $P<0.01$) and also between Ca and iP ($r=0.671$, $P<0.01$) (Graph 1 and 2). Strong negative correlation was ascertained between Ca and BALP ($r=-0.372$, $P<0.01$), and between iP and ALP ($r=-0.191$, $P<0.05$) / BALP ($r=-0.352$, $P<0.01$). Negative correlation between iP and Mg was close to significance ($r=-0.162$, $P=0.08$). Ca was also positively correlated with CTx but not statistically significanty ($P>0.05$). All the other correlations were statistically insignificant.

Discussion

Sampling methodology used in this study employed original approach compared to other studies in biochemical bone markers research, which achieved minimal changes in environmental conditions despite very long production cycle in cattle (ideally of 1 year, but usually much more than a year) in farm settings. It also includes different animals, sample more general population, physically comparable though in contrast to other studies that were monitoring the same animals through the lactation. This way we wanted to test the use of biochemical markers of bone metabolism in more applied clinically relevant settings that wanted to prove their dynamics in different groups of healthy cows in appropriate body condition.

The highest mean CTx concentration was established in the 4. group in our study. This finding is in accordance with *Holtenius and Ekelund* (9) who report that CTx concentration in dairy cows was highest in the first week after calving and then decreased evenly over the next 33 weeks. *Filipović et al.* (10) also observed the highest CTx values in 21 Holstein-Friesian cows 10 days after calving compared to 14 days before calving and 30 days after calving. Similarly reported *Liesegang et al.* (11) in a study performed in 30 Brown Swiss cows when they measured bone resorption marker ICTP. The highest CTx and ICTP concentrations first week after calving are in accordance with general finding that Ca resorption from bones is the most intensive during this period (3). The highest mineral demand of cows is from calving until peak lactation, what is demonstrated in more intense bone resorption, since enough Ca can not be provided from the ration alone in this period.

Our findings suggest that bone is minimally catabolically active (very low CTx), and still has high anabolic activity (relatively high BALP) during the dry period, which indicates that there is a net gain in mineral reserves in bone tissue. We believe that reserve gained during dry period is very important for maintenance of normocalcaemia at the beginning of next lactation. It is well known that peak production is followed by maximal consumation of dry matter in dairy cow by about 2 – 3 weeks (12). During this period the deficit of nutrients (including Ca) between those required for production of milk and those available from consumed feed has to be mobilized from body reserves (3).

Liesegang et al. (11) found out that blood osteocalcine (OCN), a bone formation marker, concentration markedly decreased after calving and then started to slowly rise until the second month of lactation when it reached a plateau, then slowly decreased until calving. Similar findings about OCN were reported by *Holtenius and Ekelund* (9) and *Iwama et. al.* (13). *Filipović et al.* (10) measured the highest values of BALP 14 days before calving, but not statistically significantly higher than 10 days after calving. Our findings are not completely in agreement with findings of mentioned authors since we ascertained the highest BALP activity within 48h after calving and then in 4. and 2. group. This means that bone remodeling is very intensive around calving (both bone resorption and formation are very high). High activity of BALP PP shown in our study is negatively correlated with serum total Ca concentration

Table 2: Mean values and standard deviations of Ca, iP, Mg, ALP, BALP and CTx at each stage of lactation and statistical significance

Group	n	Ca (mmol/L)	iP (mmol/L)	Mg (mmol/L)	ALP (U/L)	BALP (U/L)	CTx (ng/L)
1	10	2.43 ± 0.12[3]	2.22 ± 0.33[3,4]	0.97 ± 0.08[2]	32.4 ± 5.9[2.3]	11.64 ± 1.64[2.3.4,5]	0.089 ± 0.044[4,5]
2	10	2.44 ± 0.09[3]	2.04 ± 0.19[3]	0.84 ± 0.05[1.3.5]	48.1 ± 7.4[1]	16.29 ± 2.1[1.3]	0.201 ± 0.131[4,5]
3	10	1.96 ± 0.27[1.2.4.5]	1.37 ± 0.30[1.2.4.5]	1.02 ± 0.13[2,4]	52.1 ± 10.7[1]	21.75 ± 4.35[1.2.4.5]	0.386 ± 0.132[4,5]
4	10	2.30 ± 0.14[3,5]	1.87 ± 0.17[1.3]	0.88 ± 0.10[5]	41.9 ± 14.7	16.91 ± 3.12[1.3]	1.174 ± 0.582[1,2,3]
5	14	2.57 ± 0.13[3.4]	1.94 ± 0.23[3]	1.05 ± 0.06[2.4]	44.1 ± 12.3	16.14 ± 4.03[1.3]	0.828 ± 0.352[1,2,3]

Legend: 1,2,3,4,5 – statistically significant differences for parameters between groups at P<0.05

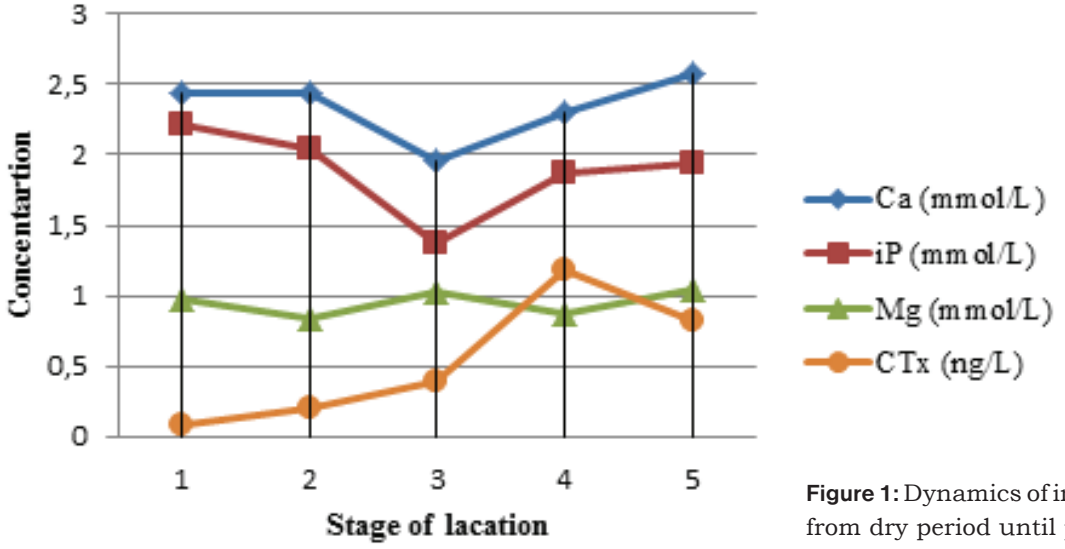

Figure 1: Dynamics of investigated parameters from dry period until pick lactation

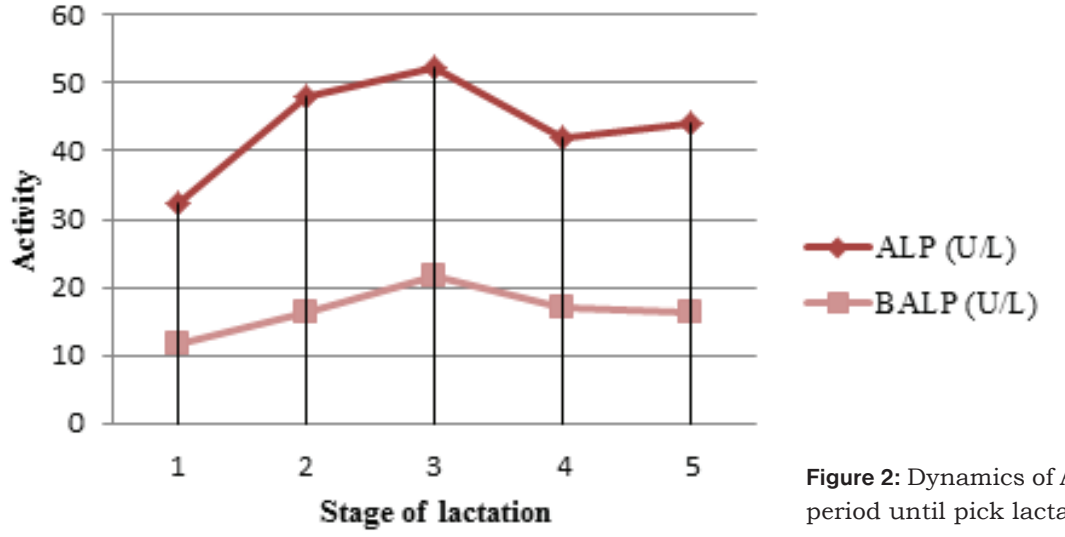

Figure 2: Dynamics of ALP and BALP from dry period until pick lactation

and indicates inappropriate response of bone metabolism to hypocalcaemia. The mechanism for maintaining normocalcaemia is likely overridden by other mechanisms, associated with calving in dairy cattle beside enormous Ca drain for milk production. Cows with milk fever had lower CTx and higher BALP than healthy cows in a study by *Starič and Zadnik* (14). Adding anionic salts to the ration would increase bone resorption (CTx) and the concentration of ionized calcium in blood (3). High BALP activity can be associated with bone metabolism that was unprepared to provide Ca for the steep increase in demand or responded inappropriately to hypocalcaemia. *Filipović et al.* (10) ascertained statistically significant ($P<0.001$) negative correlation between estradiol and CTx. Estradiol reaches the highest values before calving (15). Anabolic and osteoprotective effect of estradiol on bone tissue is well known, which can significantly contribute to the development of periparturient hypocalcaemia in dairy cows (5, 9, 10, 16). Estradiol contributed probably to the highest mean BALP activity within 48 hours after calving in this study.

Total ALP correlates with BALP and can be used in assessing bone metabolism, provided that there is no liver pathology present.

Results of Ca and iP dynamics in our study were in accordance with results of other authors (17, 18, 19). Almost all cows experience a certain level of hypocalcaemia during the first days after calving in our study and in mentioned studies. It was suggested that this is physiological in high yielding cows due to the very abrupt rise in Ca demand for milk production. After few days the homeostatic mechanisms for Ca adapt to higher Ca and iP demand and maintain blood Ca and iP concentration within the normal range until the next calving (17, 18, 19).

Mean Mg concentration was within normal range for dairy cattle in all test groups in our study which confirms appropriate Mg supply. Mg dynamics resemble those of *Bigras-Paulin and Tremblay* (17) and *Riond et al.* (20). Higher mean Mg values were also ascertained in the 3. and 5. group in our study. Higher Mg concentration around calving is consequence of hypocalcaemia and high PTH, which stimulates Mg resorption in kidney tubules. High PTH concentration lowers Mg secretion in urine and thus elevates blood Mg concentration. PTH also stimulates iP excretion via kidney and salivary glands (3), which was the reason for almost statistically significant negative correlation between Mg and iP in our study.

Further studies in bone metabolism are needed to evaluate and extend current knowledge particularly in bigger animal sample and in groups treated by different means that elevate blood Ca concentration around calving and influence bone metabolism, like anion salts.

Conclusion

Dynamics of biochemical markers of bone metabolism in groups of cows from early dry period until peak lactation were demonstrated. Results of the study indicate that bone tissue is the most catabolically active 10 to 20 days after calving and the most anabolically active during the dry period. Strong negative correlation ($P<0.01$) was established between BALP and minerals Ca and iP. Our results suggest that biochemical bone markers can be used as precalving tool for detection of cows that are at risk of having low blood Ca and iP after calving, which could be an important novelty in detection of cows at risk of milk fever. From the results of this study we can speculate that the dry period is important for filling up Ca reserves in bone tissue.

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KAZALCI METABOLIZMA KOSTI IN KONCENTRACIJA MINERALOV V KRVI PRI KRAVAH MOLZNICAH MED PRESUŠITVIJO IN LAKTACIJO

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Povzetek: Biokemijski kazalci metabolizma kosti so snovi v krvi in/ali urinu, ki kažejo kako intenzivno se kostnina izgrajuje oz. razgrajuje v času odvzema krvnih oz. urinskih vzorcev. Namen raziskave je ugotoviti gibanje celotnega kalcija (Ca), anorganskega fosfata (aP), magnezija (Mg), celotne alkalne fosfataze (ALP), kostno specifične alkalne fosfataze (BALP) kot biokemijskega kazalca izgrajevanja kostnine in C-terminalnih prečno povezanih telopeptidov kolagena I (CTx) kot biokemijskih kazalcev razgrajevanja kostnine v krvnem serumu odraslih krav molznic v času od presušitve do viška laktacije. V raziskavo je bilo vključenih 54 klinično zdravih črno-belih krav z visoko prirejo mleka (povprečno 8,463 kg v prejšnji laktaciji). Krave so bile razdeljene v 5 skupin glede na fazo laktacije: 1. približno 1 mesec do poroda (AP) oz. sredi presušitve (n = 10), 2. 10 do 1 dan AP (n = 10), 3. 0 do 48 ur po porodu (PP) (n = 10), 4. 10 do 20 dni PP (n = 10) in 5. višek laktacije (n = 14). Vrednosti Ca in aP so bile pri kravah v 3. skupini nižje kot pri tistih v ostalih skupinah ($P<0,05$). Višje vrednosti Mg smo izmerili pri kravah v 3. in 5. skupini glede na 2. in 4. ($P<0,05$). Najnižjo vrednost BALP smo izmerili v 1. skupini. Razlika je bila statistično značilna glede na ostale skupine ($P<0,05$). Najvišje vrednosti CTx so bile izmerjene v 4. in nato v 5. skupini. Statistično značilno so se razlikovale od vrednosti v 1., 2. in 3. skupini. Rezultati raziskave kažejo, da je kostno tkivo katabolično najbolj aktivno 10 - 20 dni PP, anabolično pa med presušitvijo. Ugotovljena je bila močna negativna korelacija ($P<0,01$) med BALP in rudninama Ca in aP.

Ključne besede: biokemijski kazalci; CTx; BALP; ALP; odrasle krave

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