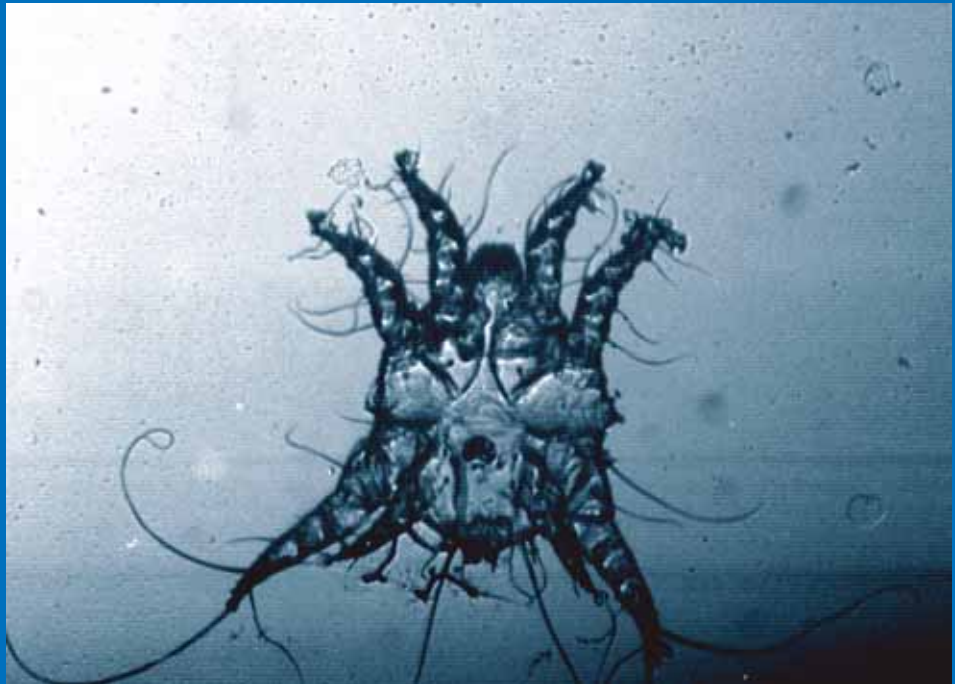


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SLOVENSKI VETERINARSKI ZBORNIK



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ISOLATION OF CATHEPSIN B FROM CANINE DISEASE-FREE LIVER AND ITS CHARACTERIZATION

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Summary: Cathepsin B (CB; EC 3.4.22.1) is one of the most widely investigated lysosomal cysteine proteases in humans. However, there has been no data on canine CB isolation and characterization published. The aim of this study was to isolate cathepsin B from canine disease-free liver, characterize it by means of Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), isoelectric focusing and N-terminal sequence analyses, and compare it to cathepsin B isolated from livers of other species.

Cathepsin B was isolated from the disease-free canine liver using single-step affinity chromatography on a semicarbazone of Gly-Phe-glycinal linked to sepharose 4B and elution with 2,2'-dipyridyl disulphide at pH 4.0. This method is called the Rich-Brown-Barrett's method. Using BANA as a substrate, cathepsin B-like activity was detected in effluent from an affinity column. Active fractions were combined and concentrated prior to characterization. Approximately 16 mg of cathepsin B was isolated from 2.93 kg of liver (5.46 mg kg⁻¹), applying 5 isolation procedures using the same affinity column. According to the results of the SDS-PAGE, isoelectric focusing and N-terminal sequence analyses, we assume that the proteins isolated from the canine liver can be recognized as the proform and mature form of cathepsin B with molecular masses of 40 kDa and 29 kDa, respectively.

Key words: canine cathepsin B; proteases; isolation; characterization

Introduction

Lysosomal cysteine proteases, generally known as the cathepsins, represent a major component of the lysosomal proteolytic repertoire and play an important role in intracellular protein degradation and turnover (1). The name cathepsin is derived from a Greek term meaning "to digest" (2). Cathepsins have also been implicated in proteolysis occurring within the endosomal system, in particular during antigen processing (3) and are thought to play an extracellular role in several physiological and pathological conditions like bone resorption (4), cartilage degradation in arthritis (5) and tumour invasion and metastasis (6, 7). The role of cysteine proteases in pathological conditions makes them attractive targets for synthetic inhibitor development (7).

Cathepsin B (CB; EC 3.4.22.1) is one of the most widely investigated lysosomal cysteine proteases in humans. Unlike most other enzymes of this family, it exhibits both endopeptidase and exopeptidase activities. In addition to cleavage within the peptide substrate, CB can also remove dipeptide units from the C terminus (peptidyl-dipeptidase activity). It belongs to the papain superfamily and shows high homology to cathepsins L, X, S and O, papain and actinidin, among others (8, 9, 10).

Methods have been proposed in detail for the purification of cathepsin B from liver using standard protein purification techniques including organomercurial-Sepharose chromatography. Kidneys, spleen and placenta have also been used as tissue sources (8). The development of immobilized Gly-Phe-Gly- and Phe-Gly-semicarbazones as affinity ligands represented a major advance in cathepsin B purification, effectively providing a

single-step purification of the enzyme following elution with pyridyl disulphide (11). With SDS PAGE (Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis) under reducing conditions, a mammalian proform of cathepsin B is observed as a 40 kDa band, a mature form of cathepsin B isolated from the previously mentioned tissues is usually observed as a 30 kDa band representing the single-chain enzyme and 25 kDa and 5 kDa bands representing the two-chain form. With isoelectric focusing, multiple components are observed with isoelectric point values in the range 4.5 – 5.5 (9). In addition to natural sources, several recombinant expression systems (12) have been used to prepare cathepsin B, including *E. coli*, *P. pastoris*, baculovirus systems and mammalian CHO cells. These systems have also facilitated the investigation of the role of specific residues by site-directed mutagenesis (9).

CB has been implicated in the progression of a variety of human tumours. Overexpression of CB mRNA, increased CB staining, elevated CB activities and altered localization have been found in different cancers, especially at the invasive edges, suggesting a role for CB in tumour invasion (13–15). Although human CB is one of the most widely investigated of all cathepsins, very little is known about canine CB. According to the available literature, there had not been any data published on the isolation and characterization of canine CB at the start of our research. However, elevated levels of CB activity in canine mammary tumours have already been shown (16). It has also been demonstrated that polyclonal antibodies against human CB, raised in rabbits, are not suitable for immunohistochemical studies on canine tissues (17).

The aim of this study was to isolate cathepsin B from canine disease-free liver, characterize it by means of SDS-PAGE, isoelectric focusing and N-terminal sequence analyses and compare it to cathepsin B isolated from the liver of other species.

Material and methods

Materials

The standard laboratory chemicals and reagents, unless otherwise stated, were obtained from Sigma, Germany. The reagents used for the SDS-PAGE were from Bio-Rad (Germany) and the

sequencing reagents were from PE Applied Biosystems (Foster City, CA, USA). The present study began with the collection of livers from healthy dogs that were euthanized at the Clinic for Small Animal Medicine and Surgery, Veterinary Faculty, University of Ljubljana, Slovenia.

Isolation of cathepsin B

Canine cathepsin B was isolated from the liver with single-step affinity chromatography (11) on a semicarbazone of Gly-Phe-glycinal linked to sepharose 4B and elution by 2,2'-dipyridyl disulphide at pH 4.0. Five isolations of cathepsin B were performed with the same affinity medium

Five grams (dry weight) of activated CH-sepharose 4B (Pharmacia, Sweden) was soaked overnight in 1 mM HCl (Merck, Germany) at 4°C and then washed with 0.1 M NaHCO₃. 100 mg of Gly-Phe-Gly-semicarbazone (Bachem U.S.; USA), a reversible inhibitor, was dissolved in 25 ml of methanol and 15 ml of 0.1 M NaHCO₃, pH 8.0 was added. The activated sepharose was agitated in the solution in a roller rack overnight at 20°C. The gel was collected on a sintered-glass filter, washed with 50% (v/v) methanol (Merck, Germany) and then with water, before being agitated over night with 6% (v/v) ethanolamine, adjusted to pH 9.0 (with HCl) for 4 h at 20°C, finally washed with water and stored at 4°C in 0.1% NaN₃ (Merck, Germany).

The canine disease-free livers were removed in the course of post-mortem examinations, minced and stored at -20°C until required. Crude cathepsin B was applied to the affinity medium and was prepared as follows: 1.) a portion of canine liver was thawed and homogenized in two parts (w/v) of an ice-cold solution of 1% (w/v) NaCl, 2% (v/v) butan-1-ol and 1 mM EDTA. 2.) The homogenate was centrifuged at 8000 rpm for 30 minutes at 4°C in a Sorvall centrifuge. 3.) The pH of the supernatant was then adjusted to 4.5 with 2 M HCl and acid activation at 37°C for 1 h followed. 4.) The mixture was centrifuged at 8000 rpm for 30 minutes and the supernatant used for either acetone (47–64%) or ammonium-sulphate precipitation (20–75%). 5.) The first precipitate was removed by centrifugation at 5000 rpm for 30 minutes. 6.) The second precipitate, containing cathepsin B, was collected after centrifugation at 5000 rpm for 30 min-

utes, redissolved in a minimum volume of 50 mM of a sodium-phosphate buffer, which contained 1 mM EDTA and had a pH of 6.0, before being dialysed, using the same buffer, overnight at 4 C.

The solution of prepared crude cathepsin B (brown solution) was made to 2 mM with respect to DTT and stirred with the affinity medium (gel) for 2 h at room temperature. The gel was then washed with 1.5 l of a 50 mM sodium-phosphate buffer (pH 6.0), containing 0.5 M NaCl, followed by the same volume of a 50 mM sodium format buffer (pH 4.0), before being packed into a column (18 cm x 1.5 cm dia.). Then 1.5 mM 2,2'-dipyridyl disulphide in a 50 mM sodium format buffer (pH 4.0) was run into the gel until the A_{280} measurements of the eluted fractions exceeded 2.5. The flow was then stopped and the column was left overnight at 4 C prior to the elution of the cathepsin B the next day. The elution of the cathepsin B from the gel was performed using 1.5 mM 2,2'-dipyridyl disulphide in a 50 mM sodium format buffer (pH 4.0).

The fractions being collected were assayed for cathepsin B activity using BANA (N-Benzoyl-DL-arginine-naphthylamide) as a substrate (18, 19). The substrate was hydrolysed, liberating 2-naphthylamine, and this assayed colorimetrically (A_{520}) by coupling it with a diazonium salt - Fast Garnet (4-diazo-2,3-dimethylazobenzene). The protein concentrations in the eluted fractions were determined with direct A_{280} measurements.

The active fractions (A_{520} 0.1) were combined, concentrated and transferred into a 20 mM sodium-acetate buffer (pH 5.2), containing 1 mM EDTA, by ultrafiltration on an Amicon YM-5 membrane (Millipore, USA).

The protein concentrations of the concentrated fractions of isolated cathepsin B were determined using a Bio-Rad Protein Assay (Bio-Rad, Munich, Germany) (20). The standard curve was obtained using bovine-serum albumin.

The cathepsin B samples were analysed using SDS-PAGE on 12 % polyacrylamide gels (21). Electrophoretic separations were performed on a Bio-Rad Mini-PROTEAN II electrophoresis cell (Bio-Rad; USA), following the manufacturer's instructions. The samples were reduced with 5 % 2-mercaptoethanol at 100 C for 5 minutes prior to electrophoresis. The proteins were visualised by Coomassie Brilliant blue R-250 staining. The molecular mass of the cathepsin B was deter-

mined by comparing the mobility of the isolated cathepsin B in polyacrylamide gels with those from the following standards: - Aprotinin 6.5 kDa; Lysozyme 14.4 kDa; Trypsin inhibitor 21.5 kDa; Carbonic anhydrase 31.0 kDa; Ovalbumin 45.0 kDa; Serum albumin 66.2 kDa; Phosphorylase b 97.4 kDa; -galactosidase 116.25 kDa; Myosin 200 kDa.

Isoelectric focusing was carried out on polyacrylamide plates with Pharmalyte carrier ampholines in the pH range of 3-10, using the PhastSystem apparatus (Pharmacia-LKB; Uppsala, Sweden), following the manufacturer's instructions. A mixture of standard proteins with pI ranging from 3.5 to 9.3 was run parallel with the samples. After fixation in 20 % trichloroacetic acid, the proteins were stained with Coomassie Brilliant Blue G-250.

For the determination of the N-terminal sequences, samples containing isolated cathepsin B were first subjected to SDS-PAGE. The proteins were electrophoretically transferred to a PVDF (polyvinylidene difluoride) membrane (Immobilon-P, Millipore, USA), using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad, USA) set at a constant voltage of 100 V for 45 minutes. The proteins were visualized with Coomassie Brilliant Blue G-250 stain. Components of interest were excised, destained with 50 % methanol and analysed directly from the membrane with an Applied Biosystems Procise sequencer system 492A. Phenylthiohydantoin derivatives were identified on-line using the attached Applied Biosystems 140C HPLC system.

Results

Cathepsin B was found expressed in a variety of tissues, with the highest level of expression observed in the kidneys, spleen and liver, and in a number of different types of tumours and cancer cell lines (22, 23, 24, 25, 26).

In our study, cathepsin B was isolated from canine disease-free liver by single-step affinity chromatography on a semicarbazone of Gly-Phe-glycinal linked to sepharose 4B and elution with 2,2'-dipyridyl disulphide (pH 4.0). During the isolation procedure, any cathepsin B-like activity in the eluted fractions was measured using BANA as a substrate. The distribution of the cathepsin B-like activity in the effluent of an affinity column is presented in Figure 1.

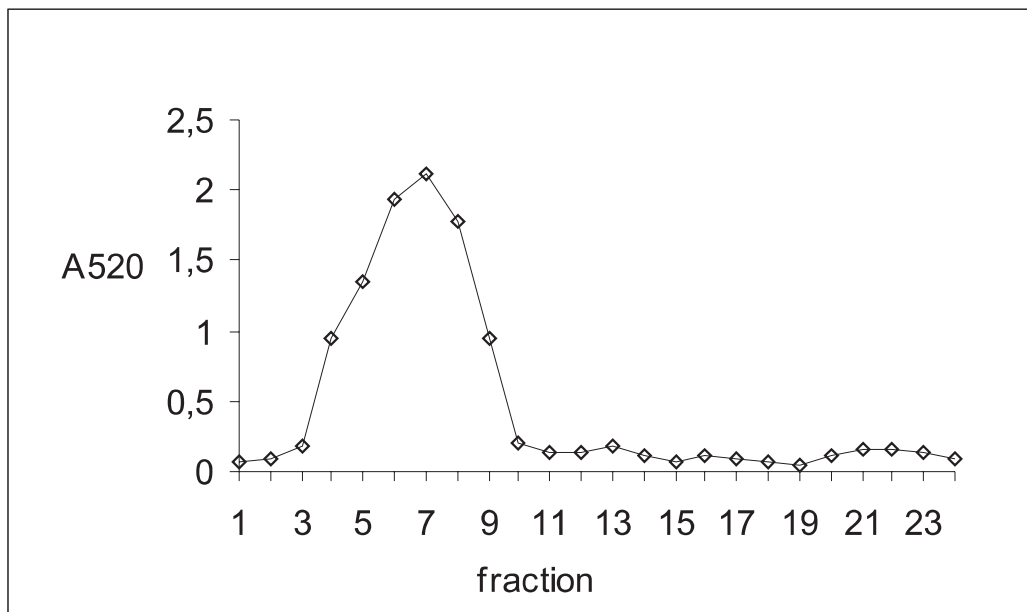


Figure 1: Distribution of cathepsin B-like activity in the effluent of an affinity column

Approximately 16 mg of cathepsin B was isolated from 2.93 kg of liver (5.46 mg kg^{-1}), applying 5 isolation procedures using the same affinity column.

Characterization of canine cathepsin B

SDS-PAGE

The molecular masses of the isolated proteins from the canine livers were estimated on SDS-PAGE gels under reducing conditions. Samples with cathepsin B-like activity after being concentrated, samples of crude cathepsin B preparations, and acetone and ammonium sulphate precipitations prepared from liver, were applied to 12 % polyacrylamide gels and electrophoresis performed as previously described. Two major protein bands with molecular masses of 29 kDa and 40 kDa were visible (Figure 2). We assumed that the 29 kDa and 40 kDa protein bands might represent the mature form and the proform of cathepsin B (procathepsin B), respectively (Figure 2; lines 3, 4, 5, 6). The results of the SDS-PAGE demonstrated that mature canine-liver cathepsin B exists only in a 29 kDa single-chain form like mature cathepsin B from sheep liver (27). The molecular mass of the mature cathepsin B matched a single-chain form of cathepsin B from human, rat, porcine, ox and rabbit liver, as well as cathepsin B from bovine spleen (28, 29).

There were no differences in the molecular masses of the mature form and the proform of cathepsin B when the affinity medium was used in conjunction with preliminary fractionation with ammonium sulphate or acetone.

Analytical Isoelectric focusing

Isoelectric focusing of the concentrated cathepsin B showed two major bands with pI of 4.8 and 5.0 (Figure 3; line 2), as estimated according to standard proteins that were run parallel with the samples. Similar bands were found as minor components in a crude cathepsin B preparation (Figure 3; line 3).

The results can be compared with the isoelectric focusing of six isoenzymes of human liver cathepsin B with pI ranging from 4.5 to 5.5 and the major peak of activity being between 5.0 and 5.2 (9, 30).

N-terminal sequence analysis

The N-terminal sequence analysis revealed the highest extent of homology of both protein bands from SDS-PAGE with the corresponding regions of bovine procathepsin B and mature form of bovine cathepsin B, as well as with cathepsin X. The N-terminal sequence of the mature form of canine cathepsin B (29 kDa) differs from the corresponding region in bovine cathepsin B in 4 out of 30 amino acids (Figure 4).

A comparison of the N-terminal sequences of procathepsin B (40 kDa) with the corresponding region of bovine procathepsin B is presented in Figure 5.

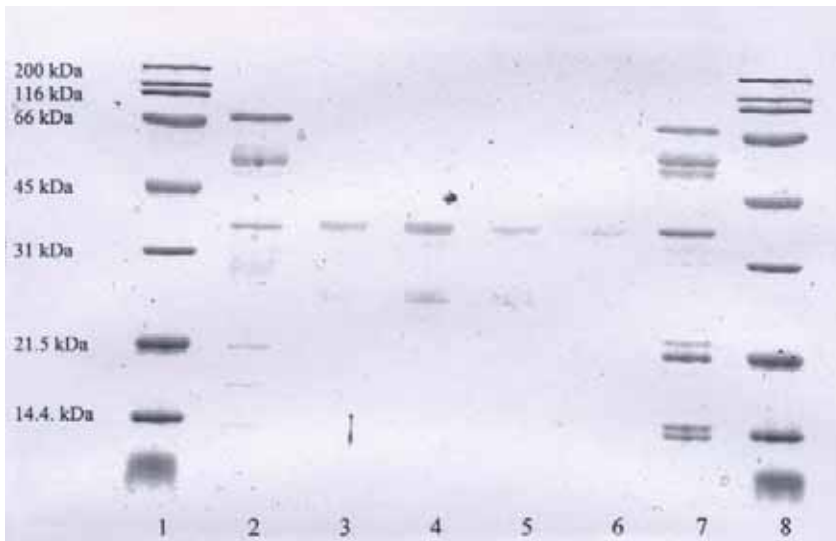


Figure 2: 1 & 8 = standards; 2 = acetone precipitate; 7 = ammonium sulphate precipitate; 3, 4, 5 & 6 = concentrated fractions eluted from an affinity column

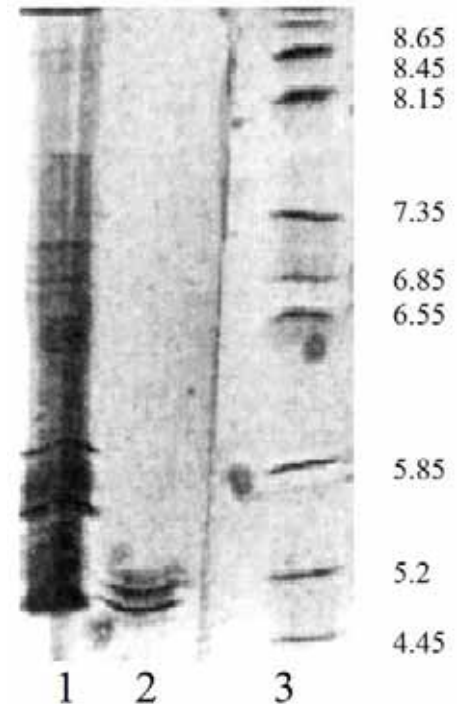


Figure 3: 1 = ammonium sulphate precipitate; 2 = concentrated cathepsin B after elution; 3 = standards

Isolated protein from canine liver (29 kDa):

VNVEVSAEDMLTCCGDQCGDGCNNGGFPAEA

Bovine cathepsin B: 129

VNVEVSAEDMLTCCGGE^{CGDGCNNGGFPSGA} 158

Figure 4: Comparison of the N-terminal sequences of the 29 kDa protein isolated from canine liver with the corresponding region in bovine cathepsin B. The homology between the amino acid sequences is shown by the shading

The alignment of the 40 kDa protein isolated from canine liver with the bovine procathepsin B matched with the procathepsin B region starting with residue 23, while the 29 kDa protein matched with the N-terminal part of the heavy-chain of the bovine cathepsin B, starting with residue 129. The 40 kDa protein isolated from canine liver matched with human procathepsin C and procathepsin X, starting with residues 231 and 62, respectively.

Discussion

Human cathepsin B is one of the most thoroughly studied lysosomal cysteine proteases (9, 30), most probably due to its involvement in several pathological conditions, notably tumour invasion and metastasis, cartilage degradation in arthritis and many others. Compared to human CB, little is known

about canine CB. When we began our study, we were unable to find any data on the isolation or characterization of canine CB in the literature available.

Several methods for the isolation of CB from liver have been reported in detail (8, 9). Besides its isolation from human liver, CB has also been isolated from rat, rabbit, ox, bovine and sheep liver (28, 29, 31). Kidneys, spleen and placenta from humans and from other species (bovine, sheep, rat) have also been used as tissue sources (9). A major advance in the purification of CB was the development of an immobilized reversible inhibitor – Gly-Phe-Gly-semicarbazone – as an affinity ligand, effectively providing a single-step purification process of this enzyme following elution with 2,2'-dipyridyl disulphide (Rich-Brown-Barrett's method) (11). In our study, the Rich-Brown-Barrett method was used for the isolation of CB from canine liver. Using BANA as

Isolated protein from canine liver (40 kDa):	
	LP KSWDWRNVNGVNY
Bovine procathepsin B:23	FPPLS D ELVNFVNKQ 39
Human procathepsin C: 231	LP TSWDWRNVHGINF 245
Human procathepsin X: 62	LP KSWDWRNV DGVNY 76

Figure 5: Comparison of the N-terminal sequences of the 40 kDa protein isolated from canine liver with the corresponding region in bovine procathepsin B, human procathepsin C and human procathepsin X. The homology between the amino acid sequences is shown by the shading

a substrate, cathepsin B-like activity was detected in effluent from the affinity column. Active fractions were combined and subsequently concentrated. The sample obtained was characterized by means of SDS-PAGE, analytical isoelectric focusing and N-terminal sequencing.

Under reducing conditions, the SDS-PAGE revealed two major protein bands; a 29 kDa band which we assumed to be the mature form of canine CB in a single-chain form and a 40 kDa band assumed to be the proform of cathepsin B (procathepsin B). Contrary to mature human, bovine, rabbit and ox liver mature CB that exist as a single-chain and two-chain forms, mature canine CB only exists in a single-chain form as is also the case for CB from sheep liver (28). Based on the purification of CB from many different normal tissues from numerous species, using SDS-PAGE under reducing conditions, mature CB exists in a single-chain form of 30 kDa or a double-chain form of 25 kDa (heavy chain) and 5 kDa (light chain) (9, 27). The mammalian proform of CB is observed as 40 kDa band (9). In addition to the single- and double-chain forms of CB detected in fractions from both normal human liver and human tumours, Moin et al. also detected two isoforms of the heavy chain of the double-chain form of CB as 25/26 kDa bands. The 1 kDa difference was due to N-linked high-mannose-type oligosaccharide moiety (32).

Using isoelectric focusing, both forms of canine CB, with pI of 4.8 and 5.0, are comparable with the isoelectric focusing of six isoenzymes of human-liver CB with pI ranging from 4.5 to 5.5 with a major peak of activity at 5.0 to 5.2 (9, 30).

N-terminal sequences were determined for both forms of the isolated proteins from canine liver and compared with the sequences that were

available for other cysteine proteinases. While the 29 kDa protein band from the SDS-PAGE was shown to have the highest extent of homology with the corresponding region of mature bovine CB, the 40 kDa band showed, to a lesser extent, homology with the bovine procathepsin B, which indicates that the isolated proteins may represent both the proform and the mature form of CB. However, a few months after the N-terminal sequence determination of the canine procathepsin B, a complete amino-acid sequence of human procathepsin X was published (33). After comparing the amino-acid sequences of the human procathepsin X with the canine procathepsin B, one may speculate that the isolated canine proteases might represent a canine cathepsin X and canine cathepsin B. On the other hand, Kos and co-workers (34) clearly demonstrated that only cathepsin B is involved in the degradation of extracellular matrix proteins, which supports our assumption that the isolated proteins, at least their major part, correspond to canine procathepsin B and the mature form of cathepsin B.

According to the results of the characterization, we assume the proteins isolated from canine liver can be recognised as the proform and the mature form of cathepsin B, with molecular masses of 40 kDa and 29 kDa, respectively.

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IZOLACIJA KATEPSINA B IZ ZDRAVIH JETER PSA IN NJEGOVA KARAKTERIZACIJA

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Povzetek: Kljub temu da je človeški katepsin B najbolj raziskana lizosomalna cisteinska proteaza, v literaturi trenutno ne moremo najti podatka o izolaciji ali karakterizaciji pasjega katepsina B. Cilj naše raziskave je bil izolirati katepsin B iz zdravih pasjih jeter, ga okarakterizirati z metodami poliakrilamidne gelske elektroforeze v prisotnosti natrijevega dodecil sulfata (SDS-PAGE), z izoelektričnim fokusiranjem in določitvijo aminokislinskega zaporedja N-terminalnega dela ter ga primerjati s katepsinom B, izoliranim iz jeter drugih živali. Katepsin B smo izolirali iz zdravih pasjih jeter z metodo Rich-Brown-Barrett. To je metoda afinitetne kromatografije, pri kateri smo kot ligand uporabili semikarbazon gly-phe-gly, ki je reverzibilni inhibitor katepsina B. Eluiranje katepsina B iz kolone je potekalo z 2,2'-dipiridil disulfidom pri pH 4. Z uporabo afinitetne kromatografije smo v eluiranih frakcijah z uporabo substrata BANA zaznali katepsinu B podobno encimsko aktivnost. Pred karakterizacijo izoliranih proteinov smo aktivne frakcije združili in skoncentrirali. S petimi izolacijami na isti afinitetni koloni smo iz 2.93 kg jeter izolirali približno 16 mg katepsina B (5.46 mg kg^{-1}). Na osnovi rezultatov karakterizacije predvidevamo, da sta izolirana proteina zrela oblika katepsina B z molekulsko maso 29 kDa in nezrela oblika katepsina B z molekulsko maso 40 kDa.

Ključne besede: pasji katepsin B; proteaze; izolacija; karakterizacija

EXPERIMENTAL AFLATOXIN B₁ TOXICOSIS IN YOUNG RABBITS - A CLINICAL AND PATHO-ANATOMICAL STUDY

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Summary: A feeding trial was conducted to assess the clinical, gross and histopathological alterations in various organs of New Zealand White rabbits fed an aflatoxin B₁ (AFB₁) contaminated diet. Aflatoxin extract was included in a toxin-free diet to provide the desired level of 0.5 ppm/kg of feed for *ad libitum* consumption by 16 young rabbits for a period of 50 days. Clinical signs of toxicosis were noticed from the 20th day onwards and were initially characterized by dullness, lethargy, reduced feed and water intake, hyperirritability, dyspnoea, oliguria and dehydration, which was followed by paralysis of the hind limbs, reduced heart rate and jaundice at the terminal stage of toxicosis. A decrease in body weight was observed in the treatment group. The carcasses of the rabbits in the toxin-fed group appeared emaciated and anaemic with subcutaneous oedema and gelatinization of fat. The liver and kidneys were the most affected organs followed by the stomach, intestines, lungs, heart, spleen, gonads and brain. Grossly, congestion and focal haemorrhages were observed in the affected organs in the initial stages. At the terminal stage of toxicosis, the liver was enlarged, icteric with greyish-white necrotic foci on all the lobes; nephrosis, catarrhal enteritis, pneumonia and mild testicular atrophy were also observed. Histopathology revealed vascular congestion, leucocytic infiltration and degenerative change in the affected organs during the initial stage of toxicosis. At its terminal stage, coagulative necrosis, perivascular and periductal fibrocellular reactions along with mononuclear-cellular infiltration and distortion of the hepatic chords were observed in the liver. Gastrointestinal ulcerations, hyalinization of the tubular epithelium and a widening of the glomerular capsules (Bowman's capsules) were also observed in the kidneys. The seminiferous tubules showed degeneration/denudation of the epithelium and a reduction in the number of mature spermatids. The study highlighted the toxic effects of a subacute dietary exposure of rabbits to AFB₁.

Key words: experimental toxicosis; aflatoxin B₁; pathology; rabbits

Introduction

Aflatoxin B₁ (AFB₁) is the most abundant and toxic metabolite produced by the *Aspergillus flavus* and *Aspergillus parasiticus* moulds, which are widespread contaminants of foods and feed in different parts of the world (1). In India, where the ambient temperature and humidity is high and long-term storage is often inadequate, high levels of AFB₁ in feed samples have been recorded (2). Animals, as well as human beings, are usually exposed to mycotoxins through their diet (3) and, depending on different factors such as age, sex,

route of administration and species involved, this can result in acute, sub-acute or chronic mycotoxicosis (4). In recent years large-scale rabbit farming has been taken up in India, both for meat production as well as for biomedical research purposes. Diseases are the major impediment to profitable rabbit farming. Rabbits are the most sensitive animals to aflatoxicosis, the LD₅₀ being only 0.3 mg/kg body weight, which is the lowest among all animal species (5). Because many of the clinical signs and clinico-pathologic changes of experimental aflatoxicosis in rabbits are similar to those reported in other species of animals, rabbits constitute an appropriate model for studying the mechanisms of AFB₁ toxic actions in food-producing animals (6). AFB₁ has been associated with

outbreaks of aflatoxicosis in Indian rabbit farms (7, 8). Experimental AFB₁ toxicosis is known to cause alterations in enzyme levels along with patho-anatomical changes in vital organs (9, 10, 11, 12). Dietary AFB₁ exposure causes immunosuppression in animals resulting in an increased susceptibility to infection (13, 14, 15). The metabolism of AFB₁ in the liver produces highly-reactive chemical intermediaries, which bind to DNA resulting in the disruption of transcription and abnormal cellular proliferation, leading to mutagenesis and carcinogenesis (16, 17). AFB₁ is a threat to an *in utero* developing foetus producing teratogenicity when administered to pregnant animals (18).

The aim of this study was to assess the sub-acute effects of AFB₁ through the clinical, gross and histopathological alterations in various organs of rabbits.

Materials and methods

An *Aspergillus parasiticus* (strain NRRL 2999) culture maintained at the Division of Nutrition and Feed Technology, Central Avian Research Institute, Izatnagar, India was used to produce aflatoxin on rice following a standard method (19). The determination of crude aflatoxin was carried out using thin-layer chromatography (20) followed by the quantification of toxin as per the standard spectrophotometric method (21).

Twenty-four 3-month-old New Zealand White rabbits of either sex were procured from the Laboratory Animal Research Division, Indian Veterinary Research Institute (IVRI), Izatnagar, India and were individually housed in stainless steel cages on a 12-h dark/12-h light cycle. These rabbits were maintained on a toxin-free base diet supplied by the Feed Processing Unit, IVRI, along with green fodder (Burseem) and water administered *ad libitum*, until they gained about 1 to 1.5 kg in body weight. The body weight of each animal was recorded and the rabbits were randomly divided into two groups, control and experimental, comprising 8 and 16 animals respectively. Before feeding, the basal diet was tested for any possible residual aflatoxin, using the Howell and Taylor method (22), and no detectable levels were found (detection limit 1 ppb kg⁻¹ feed). The AFB₁ was incorporated into the basal ration at the rate of 0.5 ppm/kg of feed. The control group was kept on a base diet only, while the experimental group was

fed the aflatoxin-mixed ration for a period of 50 days.

The clinical signs and feed and water intake of the rabbits were regularly monitored. At every 10-day interval, the body weight of all the animals was recorded and two animals from the treatment group and one from the control group were sacrificed. All the rabbits that were sacrificed or that died spontaneously were subjected to detailed post-mortem examinations. Representative tissues from the liver, kidneys, stomach, intestines, lung, heart, spleen, gonads (testes/ovaries), pancreas, adrenal and thyroid glands and brain were collected in 10 % neutral-buffered formal saline for detailed histological studies. The tissues were processed and paraffin sections (4-6 thickness) were stained with Haematoxylin and Eosin following the standard procedure (23).

Results

Clinical Signs

The control rabbits did not manifest any abnormal signs during the experimental period. In the AFB₁-treated group, the signs of toxicity were noticeable from the 20th day onwards and were initially characterized by dullness, lethargy, diarrhoea (in a few rabbits) and reduced feed and water intake. From the 30th day onwards, the rabbits showed hyperirritability, dyspnoea, oliguria (with dark yellowish urine), dehydration and emaciation. Towards the terminal stage of the experiment, bradycardia, jaundice and paralysis of the hind limbs were observed. The body weights of the intoxicated rabbits were lower than those measured in the control group. During the course of the experiment, two rabbits of treatment group died on the 25th and 28th days, respectively. At the end of the 50th day, the animals in the experimental group showed poor body condition compared to the control group.

Gross Pathology

The necropsy observations of the AFB₁-fed rabbits are presented in Table 1. All the visceral organs showed varying degrees of congestion with focal haemorrhages. The liver and kidneys were the most affected organs followed by the stomach, intestines, lungs, heart, spleen, gonads and brain. On the 50th day, the livers of the toxin-fed rabbits were enlarged, pale to icteric with multiple, variably-sized, prominent and greyish-white necrotic

Table 1: Necropsy findings of different organs in aflatoxin B₁ fed rabbits

Days Organ	Congestion					Haemorrhages					Oedema					Degeneration					Necrosis				
	10	20	30	40	50	10	20	30	40	50	10	20	30	40	50	10	20	30	40	50	10	20	30	40	50
Liver	+	++	++	+++	+++	-	-	+	++	++	-	-	-	-	-	-	+	++	++	+++	-	-	+	++	+++
Kidneys	-	+	++	++	+++	-	-	+	+	++	-	-	-	-	+	-	-	+	++	++	-	-	-	+	++
Stomach	+	+	++	++	++	-	-	+	+	++	-	-	+	++	++	-	-	+	++	++	-	-	-	-	+
Intestines	-	+	+	++	++	-	-	-	+	+	-	-	+	++	++	-	-	+	+	++	-	-	-	-	-
Lungs	-	+	+	++	++	-	-	-	+	++	-	-	+	++	++	-	-	-	-	-	-	-	-	-	-
Heart	-	-	+	++	++	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	
Spleen	-	-	+	++	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	

+→+++ = increasing severity of lesion

- = absence of lesion



Figure 1: Liver - enlarged with multiple variously-sized greyish-white nodular foci

foci on both surfaces of all the lobes (Fig. 1). The gall bladder was distended, with thick greenish-yellow bile. The kidneys were congested and showed a moderate degree of nephrosis. The urinary bladder was distended with thick yellowish turbid urine. The mucosa of the gastrointestinal tract (GIT) was thickened with focal areas of erosion in the gastric and duodenal regions. There were mild pneumonic changes in the lungs. The heart revealed epicardial congestion. Mild to moderate degrees of congestion were observed in the spleen, meninges, ovaries, pancreas, adrenal and thyroid glands in the later stage of toxicosis. The testes were pale and mildly atrophied. The AFB₁-fed rabbits that died during the experimental period were emaciated and moderate degrees of congestion were found in the liver, kidneys, GIT, lungs,

heart and meninges. The sacrificed animals showed emaciation, pallor of the mucus membranes, pronounced subcutaneous oedema and gelatinization of fat depots.

Histopathology

In the treated group, the liver revealed generalized vascular congestion, swelling of the hepatocytes, varying degrees of granular and hydropic degeneration along with mild fatty changes up to 30 days. From the 40th day onwards, marked fatty changes, areas of coagulative necrosis around the central veins, engorged portal areas, hyperplastic bile duct epithelium as well as perivascular/periductal mononuclear cellular infiltrations were observed. In the terminal stage of toxicosis, there

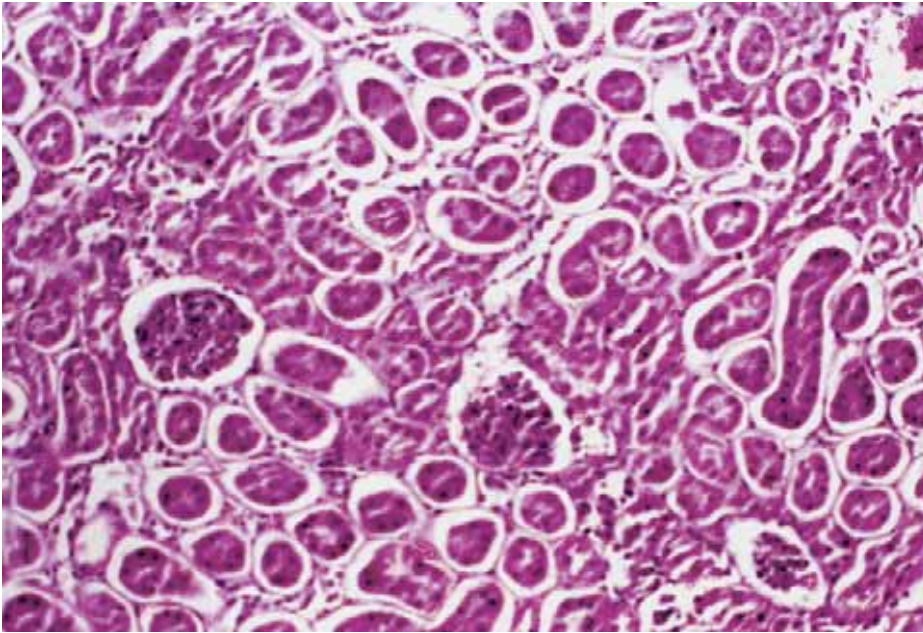


Figure 2: Kidney - degeneration of tubular epithelium. H&E x 190

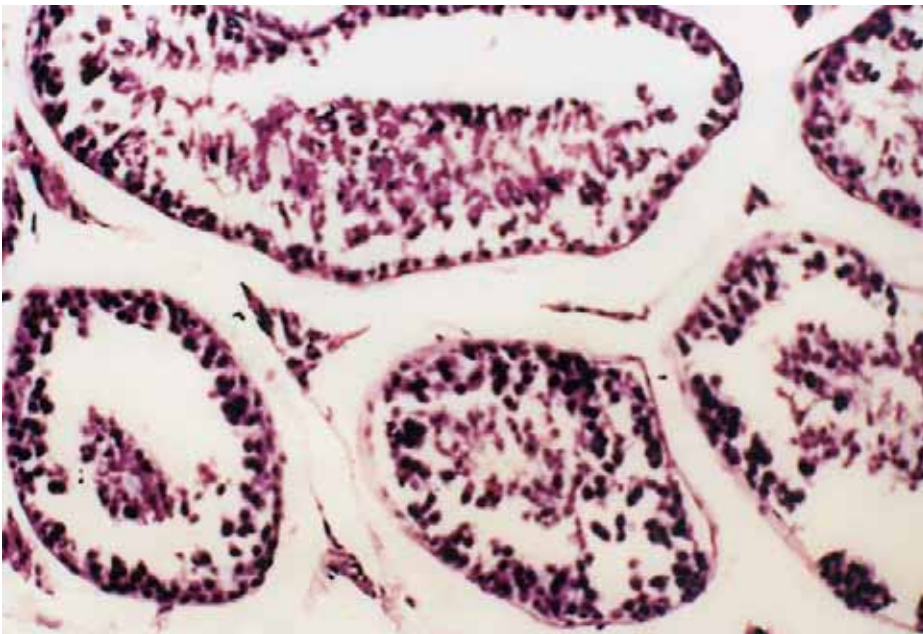


Figure 3: Testes - degeneration and desquamation of germinal layer/spermatogonial cells with a reduced spermatid population in the seminiferous tubules. H&E x 210

was extensive coagulative necrosis with disruption of the hepatic cords, moderate degrees of portal fibrosis along with focal mononuclear cellular infiltrations and bile duct proliferation. The mucosa of the gall bladder was oedematous.

In the kidneys, initially there was vascular congestion throughout the parenchyma followed by focal areas of haemorrhages and degeneration of the tubular epithelium up to the 30th day (Fig. 2). At the terminal stage of the experiment, the renal tubules showed a marked degeneration and hyalinization of the tubular epithelium along with widened Bowman's spaces of glomeruli.

The mucosa of the stomach and intestines initially showed vascular engorgement, focal areas of haemorrhages, thickening, hyperplastic mucus glands and a heterophilic inflammatory reaction that was followed by epithelial degeneration and desquamation up to the 40th day of toxicosis. On the 50th day, the superficial epithelium of the gastric and duodenal mucosae showed erosions along with mononuclear cellular infiltrations. In the spleen, the initially mild to moderate degrees of vascular congestion were followed by mild distension of the sub-trabecular sinuses, which was noticed after the 30th day of toxicosis. On the 50th

Figure 4: Brain - vascular congestion, widened Virchow-Robin space and mild perivascular oedema. H&E x 300

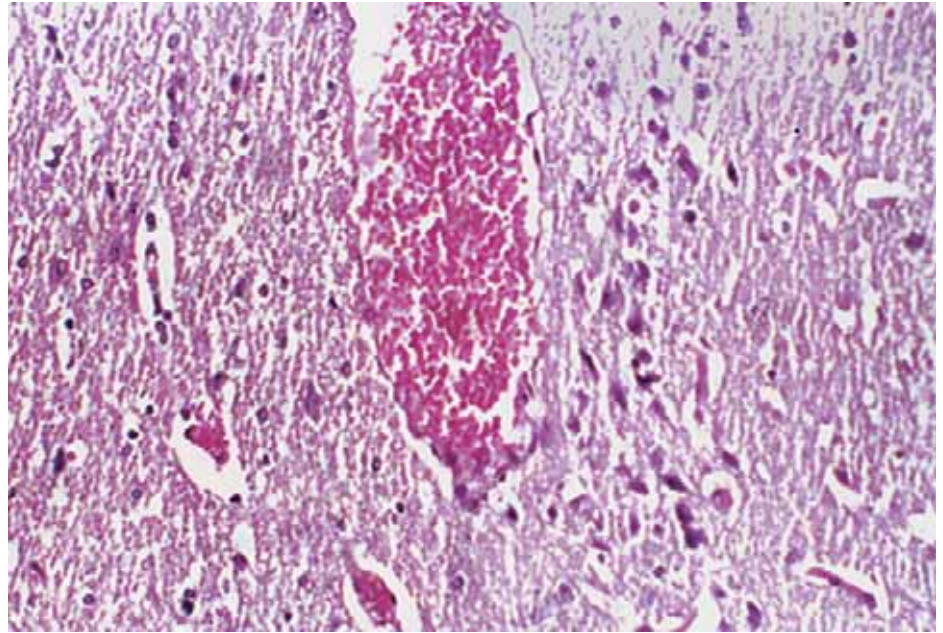
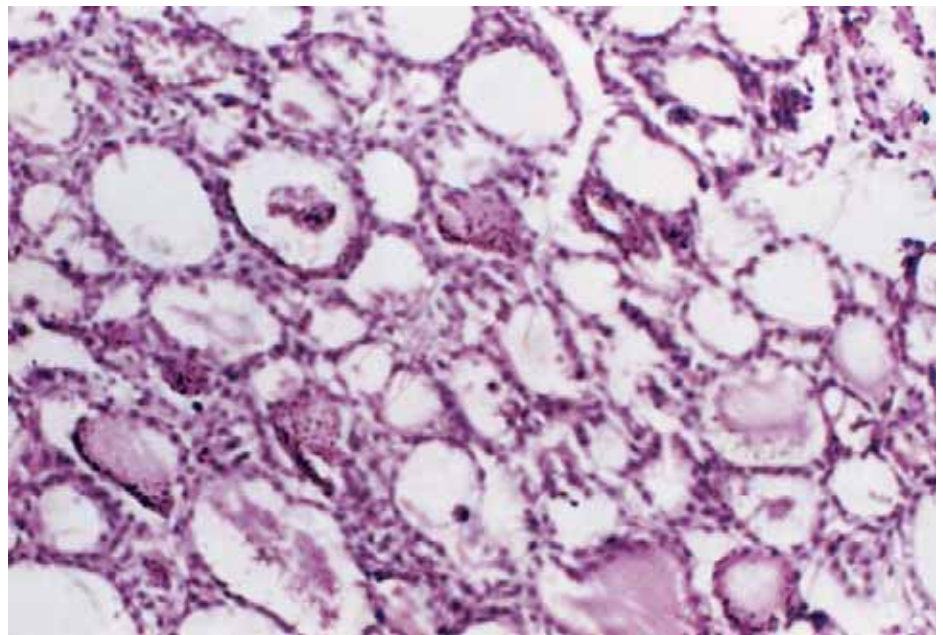


Figure 5: Thyroid gland - follicular degeneration with reduced colloid matter. H&E x 240



day of toxicosis, there was capsular sclerosis, a mild degree of depletion of the lymphoid follicles along with reticuloendothelial cellular hyperplasia.

During the first 30 days following the toxin's administration, the lungs developed alveolar congestion and focal areas of haemorrhages followed by varying degree of oedema and heterophilic and lymphocytic infiltration in the later stages. There was degeneration and desquamation of the bronchial epithelium and mild peribronchial lymphoid hyperplasia. Heart lesions comprised of congestion and mild degrees of haemorrhaging up to the 40th day of toxicosis. During the terminal

stage, mild fatty changes and focal mononuclear cell infiltrations along with a degeneration of muscular fibres were noticed.

The testes initially showed engorgement of the intertubular blood vessels followed by degeneration, detachment and denudation of the germinal and spermatogenic epithelium of the seminiferous tubules after the 30th day of toxicosis. The denuded spermatogenic cells accumulated in the lumen. The population of mature spermatids appeared to be reduced by the terminal stage of experiment (Fig. 3).

In the ovaries, up to the 30th day, mild to moderate degrees of vascular congestion were observed

followed by mild degenerative changes in the follicular epithelium by the 50th day of toxicosis.

The brain showed mild to moderate degrees of meningeal and parenchymal vascular congestion (Fig. 4) throughout the experimental period. After the 30th day of toxicosis, there was a focal mononuclear cellular infiltration along with a widening of the Virchow-Robin space followed by a perivascular cuffing, mild perivascular oedema, neuronal degeneration and gliosis by the terminal stage of experiment.

In the early stage of toxicosis, the adrenal glands showed vascular congestion and by the 50th day showed capsular sclerosis and vascular congestion in the cortex and medulla.

Mild to moderate degrees of vascular congestion followed by mild thickening of interfollicular space developed in the thyroid glands during the first 30 days of toxicosis. A reduction of colloids and microfollicular formations were observed in the terminal stage of the experimental toxicosis (Fig. 5). The pancreas revealed vascular congestion and mild degenerative changes in the Islet cells of the acini by the end of 50th day of toxicosis.

Discussion

Experimental aflatoxicosis was induced in young rabbits of either sex following their intake of a diet mixed with AFB₁ at a rate of 0.5 ppm/kg feed. Surveys in India have shown that the level of AFB₁ detected in animal-feed ingredients ranges between 0.4 – 8 ppm (2, 24).

In general, the AFB₁-fed animals showed emaciation and a decrease in body weight. The decrease in the body weights of the rabbits in the experimental group can be correlated to a reduction in their feed/water intake, which corresponds with earlier reports (10, 11, 25, 26, 27, 28). It has been reported that a decrease in body weight is one of the earliest indicators of clinical aflatoxicosis in animals (6).

The manifestation of diarrhoea, observed in a few animals in the early stages, may be due to the acute toxic effects of AFB₁. Studies on ileum isolated from guinea pigs have shown that aflatoxin can cause acute gastrointestinal effects by indirectly inducing a contractile effect through the cholinergic system following the stimulation of an acetylcholine release from the postganglionic-parasympathetic nerve endings (29).

The icterus observed in terminal stages may be

due to an increase in the levels of bilirubin as a sequela to hepatic necrosis and cholestasis. Similar findings have been recorded in the aflatoxicosis of rabbits dosed with 0.5 and 0.1 ppm/kg b.wt (25). Guerre et al. (25) attributed hyperbilirubinemia to liver damage resulting from decreased cytochrome P450, increased heme oxygenase and biliverdin reductase activities along with an increase in heme catabolism in the liver.

The vascular changes observed in the various organs and tissues in the present study are indicative of an AFB₁-induced endothelial injury. Most of the coagulation factors are synthesized in the liver, which is the target organ for aflatoxicosis. The haemorrhages observed in this study may be attributed to the impairment of coagulation. Blood coagulation defects involving the impairment of prothrombin, factor VII, X and possibly factor IX of the extrinsic pathway have been implicated as contributory factors for vascular changes in various organs following aflatoxicosis (12). Spontaneous haemorrhaging and bruising, in animals fed with aflatoxin, together with alterations in their coagulative profiles have been reported (30, 31).

The liver followed by kidneys were the most affected organs and the lesions observed in this study concurred with earlier findings following aflatoxicosis in rabbits (7, 8, 11, 26, 32, 33). Orally-ingested AFB₁ is most efficiently absorbed from the small intestine at the duodenum (34) and is metabolized in the liver by the cellular cytochrome-P450 enzyme system to form the reactive intermediate, AFB₁-8, 9-epoxide, which in turn reacts with macromolecules such as lipids and DNA, leading to lipid peroxidation and cellular injury (35). Shen et al. (36) suggested the role of reactive oxygen species (ROS), such as superoxide radicals, hydroxy radicals and hydrogen peroxide, in AFB₁-induced cellular injury. The fatty infiltrations/changes observed in the study could be due to impaired lipid transport rather than increased lipid biosynthesis. In addition, the mitochondrial damage following aflatoxicosis can result in a decrease in the oxidation of fats by these organelles, with a concomitant accumulation of lipids (37). The hepatomegaly observed in the AFB₁-fed rabbits appears to be associated with a higher lipid content. The necrotic changes, fibroblastic proliferation, bile duct hyperplasia along with the mononuclear cellular infiltration observed in this study are in accordance with those observed by Sahoo et al. (11).

The renal lesions are secondary to those observed in the liver and are in agreement with the findings of Sahoo et al. (11). Glahn et al. (38) reported that the target site of action of AFB₁ in the kidneys is the glomerular region.

Stetinova et al. (39) suggested that the effects of AFB₁ on the GIT were indirect following alterations in the liver's detoxification mechanism and a possible reduction in nutrient uptake.

The pulmonary inflammation and oedematous changes observed in the animals of the treatment group might also be due to the production of eicosanoids stimulated by the AFB₁ (40). This might have also contributed to the manifestation of dyspnoea in the early stages of toxicosis.

The testicular changes observed in the present study are in agreement with earlier reports (11, 31). Studies of adult male rats fed AFB₁ for prolonged periods, showed that AFB₁ caused regressive changes of different intensity in the germinal epithelium of the seminiferous tubules resulting in a severe dystrophic alteration of the spermatogenic epithelium along with oedematous changes in the interstitial tissue (41). Salem et al. (28) reported a relative decrease in testes weight and an increase in the number of abnormal/dead sperms following a 9-week administration of sub-lethal doses of AFB₁ to mature male rabbits.

The lesions observed in the brain corresponded with earlier findings following aflatoxicosis in rabbits (31). AFB₁ is known to alter the distribution of acetylcholine esterase (AChE) in the brain affecting cholinergic transmissions at the nerve endings and thus can result in manifestations of nervousness and behavioural deficiency (42). Sahoo et al. (11) reported vascular congestion and focal mononuclear infiltration in the meninges along with perivascular cuffing, mild neuronal degeneration and gliosis following the oral administration of AFB₁ to New Zealand White rabbits at the rate of 0.0625 mg/day/animal for a period of 30 days.

In conclusion, the prolonged feeding of AFB₁ in a diet, beyond 40 days, results in cumulative toxicosis, which is manifested by altered clinical signs as a result of lesions occurring in different vital organs.

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EKSPERIMENTALNO POVZROČENA TOKSIČNOST AFLATOKSINA B₁ PRI MLADIH KUNCIH – KLINIČNA IN PATOANATOMSKA ŠTUDIJA

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Povzetek: V študiji smo kuncem pasme beli novozelandski dajali krmo, okuženo z aflatoksinom B₁ (AFB₁), in nato ugotavljali klinične, patoanatomske in patohistološke spremembe v različnih organih živali. Izvleček aflatoksina smo dodajali krmi, ki je bila brez toksinov, v koncentraciji 0,5 ppm/kg krme. 16 mladih kuncev je imelo to krmo na voljo *ad libitum* 50 dni. Klinični znaki toksikoze so se pojavili po dvajsetih dneh, in sicer otopelost, letargija, zmanjšana poraba krme in vode, razdražljivost, oteženo dihanje, oligurija, dehidracija, kateri je sledila paraliza zadnjih okončin, upočasnjeno bitje srca in v končnem stadiju toksikoze še zlatenica. Živalim iz poskusne skupine se je v primerjavi s kontrolno tudi zmanjšala telesna teža. Trupla kuncev, ki so bili krmljeni s toksinom, so bila videti shujšana, anemična s podkožnimi edemi in želatinastim maščobnim tkivom. Najbolj prizadeti organi so bili jetra in ledvice, nato želodec, črevesje, pljuča, srce, vranica, spolne žleze in možgani. Na splošno so bile v začetnih stadijih bolezni najbolj opazne žariščne krvavitve in kongestija v prizadetih organih. V končnih stadijih toksikoze so bila jetra povečana in ikterična s sivobelimi nekrotičnimi žarišči na vseh režnjih,

opisali pa smo še nefrozo, kataralni enteritis, pljučnico in blago atrofijo mod. S histopatološko preiskavo smo v začetnih stadijih toksikoze ugotovili žilno kongestijo in degenerativne spremembe z infiltracijami levkocitov v prizadetih organih. V končnih stadijih toksikoze smo v jetrih ugotovili koagulativno nekrozo, perivaskularno in periduktalno fibrocelularno reakcijo skupaj z infiltracijo monocitov in spremenjenim položajem jetrnih trakov. Opisali smo tudi gastrointestinalne razjede ter hialinizacijo epitelija cevk in razširitev Bowmanovega prostora v glomerulih ledvic. Semenski kanalčki v modih so imeli degeneriran ali celo ogoljen epitelij, število zrelih spermatid je bilo zelo zmanjšano. Študija jasno poudarja toksične učinke subakutne izpostavljenosti kuncev AFB₁ v krmi.

Ključne besede: toksikoza; aflatoxin B₁; patologija; kunci

SCRAPIE-SUSCEPTIBILITY-LINKED POLYMORPHISMS OF THE PRION PROTEIN GENE IN ISTRIAN PRAMENKA SHEEP

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Summary: Scrapie is a transmissible degenerative disease of the central nervous system occurring naturally in sheep. It belongs to a group of prion diseases known as transmissible spongiform encephalopathies (TSE) and it is characterized by the accumulation of a proteinase-resistant prion protein mainly in the central nervous system. Three main scrapie-linked polymorphisms in the prion protein gene (*Prnp*) that modulate susceptibility to scrapie have been described and are located at the codon positions 136, 154 and 171. In order to evaluate and characterize the *Prnp* polymorphisms in Slovenian autochthonous sheep breeds and to evaluate the genetic susceptibility of these sheep to scrapie, we analyzed the *Prnp* of 41 sheep of the Istrian Pramenka breed from Slovenia. The polymorphisms at codons 136, 154 and 171 were determined by nucleotide sequencing of the *Prnp*. Four allelic variants and eight different genotypes were determined. At codon 136, two codon variants were observed, encoding amino acids alanine (A) and valine (V), 82.9 % of the sheep examined had AA, 2.5 % had VV and 14.6 % had AV. At codon 154, 90.2 % had arginine/arginine (RR) and 9.8 % arginine/histidine (RH); at the codon position 171, 46.3 % had amino acids glutamine/glutamine (QQ), 36.6 % had codon variant QR, and 17.1 % had RR codons. The most frequent genotype in the Slovenian population of Istrian Pramenka sheep is ARQ/ARQ (29.3 %). Animals carrying this genotype are moderately susceptible to scrapie, as shown by studies on other sheep breeds that were naturally or experimentally infected. The allelic variant VRQ, known to carry a very high risk of scrapie is only poorly represented in the population of the examined sheep (9.8 %). The more abundant allelic variant was ARR, which is typical to sheep resistant to scrapie (32.9 %).

Key words: Istrian Pramenka; sheep; scrapie; prion protein gene; polymorphism; susceptibility; sequencing

Introduction

Scrapie is a transmissible neurodegenerative disease of sheep and goats, showing characteristic brain pathology with vacuolated neurons, that generally manifests clinically with symptoms like pruritus, motor disorders and body deterioration. It belongs to a group of disorders known as transmissible spongiform encephalopathies (TSE) or prion diseases. Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS) fatal familial insomnia (FFI), fatal sporadic insomnia (FSI) and variant Creutzfeldt-Jakob disease (vCJD) in humans and bovine spongiform encephalopathy (BSE) in cattle also belong to this

group. Common to all TSE diseases is the accumulation of an abnormal prion protein (PrP^{Sc}) in the tissue of the central nervous system. The normal prion protein (PrP^c) is expressed in most tissues of the body, with the highest expression in the nervous tissues. Prions seem to be composed exclusively of a modified isoform of PrP designated PrP^{Sc}. The PrP^c is converted into PrP^{Sc} through a process whereby a portion of its α -helical and coil structure is refolded into β -sheet. This structural transition causes changes in its physicochemical properties that result in the accumulation of PrP^{Sc} in the cells. In its abnormal isoform, the PrP^{Sc} is infectious and proteinase-resistant (1, 2).

The occurrence of natural scrapie in sheep is a complex interplay between genetic susceptibility and different strains of the infectious prion (1,

2, 3). Scrapie-related polymorphisms in the coding region of the prion protein (PrP) gene, *Prnp*, have been studied in number of sheep breeds in various countries (4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14). While a range of amino-acid polymorphisms of the sheep *Prnp* have been described, not all of them have been found to be associated with disease susceptibility. However, the polymorphisms at codon 136 and 171 have been confirmed to be associated with scrapie susceptibility in experimental (10) and natural scrapie; valine at codon 136 and glutamine at codon 171 are often present in the genomes of sheep that are highly susceptible to scrapie (9, 11). For the polymorphism at codon 154, the evidence suggests that histidine at codon 154 is associated with a low susceptibility to scrapie in some breeds of sheep (12).

The Istrian Pramenka is a domestic, autochthonous sheep that was traditionally bred in the regions of Istria and the Karst (in Croatia and Slovenia). It originates from primitive domestic sheep known as "Zackel" sheep (13). This milk sheep is very tenacious, its long strong legs, distinct long-stepping walk and its slim snout enables its adaptation and survival in the rough conditions on the rocky terrain in the Karst region. In 2002, the estimated number of breeding females of Istrian Pramenka sheep in Slovenia was 770 animals, but this number is increasing (<http://www.tiho-hannover.de/einricht/zucht/eaap/descript/1617.htm>).

The *Prnp* genotypes of Istrian Pramenka sheep breed have not been examined previously. Therefore, we have studied the *Prnp* polymorphisms of the Istrian Pramenka that are known to be associated with scrapie susceptibility.

Material and methods

Animals

A total of 41 Istrian Pramenka sheep, from a larger flock of about 400 adult sheep located in Slovenia's Karst region were examined in the study. No clinical signs of scrapie were ever observed in the animals of the examined flock. The absence of the proteinase-resistant prion protein (PrP^{Sc}) in the obex tissue of the animals that either died or were slaughtered was confirmed by immunochemical screening using either the rapid post-mortem test Prionics Check Western

(Prionics AG) (western blot analysis) or an ELISA test Enfer TSE Kit (Abbott).

DNA isolation and *Prnp* amplification

Genomic DNA was isolated from blood leukocytes or from frozen brain tissue by a standard phenol-chloroform protocol as described by Sambrook et al. (18) or with a Wizard Genomic DNA Purification Kit (Promega).

The *Prnp*, including the whole open reading frame (794 bp), was amplified by a polymerase chain reaction (PCR) with set of primers: SPrP-1 (5'-CATCATGGTGAAAAGCCACATAGGC-3') and SPrP-2 (5'-GAAAACAGGAAGGTTGCCCTATCC-3') as described by Ikeda et al. (6). The conditions for the PCR amplification were: initial step at 94 °C for 2 min; denaturation at 94 °C for 1 min; annealing at 58 °C for 1 min; and extension at 72 °C for 2 min. The PCR products were electrophoresed in a 0.8 % agarose gel containing ethidium bromide (all Sigma) and visualised under ultraviolet light.

Sequencing

The PCR products were purified from the agarose gel using Wizard® SV Gel and a PCR Clean-Up System (Promega). The purified PCR products were directly sequenced using an ABI PRISM Dye Terminator Cycle Sequencing Kit with the primers: SPrP5: 5'-ATAAGCTGGGATTCTCTCT-3' SPrP-2: 5'-GAAAACAGGAAGGTTGCCCTATCC-3' as described by Gombojav (14). The sequencing was performed on an ABI Prism 310 autosequencer.

Data analyses

The DNA sequence data were analysed using Chromas (Technelysium).

Results

The western blot analyses and ELISA tests performed on the tissues of the animals that died or were slaughtered were always negative for the proteinase-resistant prion protein (PrP^{Sc}).

We observed dimorphisms at codons 136, 154 and 171; the different codon frequencies are shown in Table 1. At codon 136 – A and V were observed, at codon 154 – R and H were observed and at codon 171 – Q and R were determined. Four different allelic variants were observed along

Table 1: Codon frequencies for *Prnp* in Istrian Pramenka sheep

Codon position	136			154			171		
Codon variant	AA	AV	VV	RR	RH	HH	QQ	QR	RR
Number	34	6	1	37	4	0	19	15	7
Percentage	82.9	14.6	2.5	90.2	9.8	0	46.3	36.6	17.1

Table 2: Allelic frequencies for *Prnp* in Istrian Pramenka sheep

Allele (136/154/171)	Number of animals	Percentage
ARQ	43	52.4
ARR	27	32.9
VRQ	8	9.8
AHQ	4	4.9

Table 3: Genotypes of *Prnp* in Istrian Pramenka sheep associated with scrapie susceptibility

Scrapie susceptibility	Number of animals	Percentage
Low (ARR/ARR; ARQ/ARR; AHQ/ARR)	20	48.8
Moderate (ARQ/ARQ; ARQ/AHQ)	14	34.1
High (VRQ/VRQ; ARQ/VRQ)	5	12.2
Occasional occurrence (ARR/VRQ)	2	4.9

with eight different PrP genotypes. Tables 2 and 3 show the allelic and genotypic frequencies of the examined animals. The histidine observed at position 154 was never in the homozygous form.

The frequencies of the genotypes associated with susceptibility to scrapie are shown Figure 1. The most frequent genotype was ARQ/ARQ (29.3 %), which is moderately susceptible to scrapie. Only 12.1 % of Istrian Pramenka population were

shown to be highly susceptible to scrapie (ARQ/VRQ and VRQ/VRQ) (Table 3).

Discussion

The results of our study show the frequencies of different codons for the amino acids at positions 136, 154 and 171 of the PrP protein in the autochthonous Istrian Pramenka sheep breed,

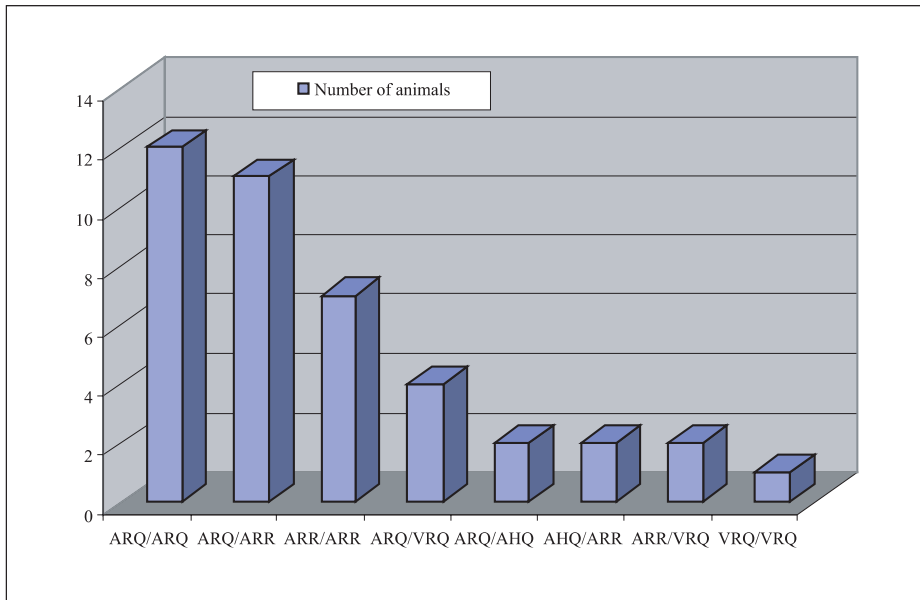


Figure 1: Graphic presentation of *Prnp* genotype frequencies in the examined Istrian Pramenka sheep

their allelic variants and genotypes. At codon 136 – A and V were observed, at codon 154 – R and H were observed and at codon 171 – Q and R were determined. As has also been the case for other sheep breeds, not all the theoretically possible allelic and genotypic variants were observed. Despite this, the Istrian Pramenka sheep belongs, in terms of *Prnp* genetics, to a group of breeds that are genetically complex having four allelic variants and eight different genotypes, similar to Cheviot, Swaledale and Shetland sheep that also have the same four *Prnp* alleles (19). The most frequent combination of the three codons in the Slovenian population of the Istrian Pramenka was ARQ (52.4 % allelic frequency; Table 2) and the most frequent genotype was ARQ/ARQ (29.3 %; Figure 1). The animals with this genotype are moderately susceptible to scrapie, as was shown by studies on other sheep breeds that were naturally or experimentally infected (9, 10, 11). Fortunately, the allelic variant VRQ, which is known to carry a very high risk of scrapie, was poorly represented in the population of the examined sheep (9.8 %). However, this ratio was still above a recently reported average of 3.7 % from a population of 430233 sheep from 243 different breeds (20). In the examined group of sheep, the homozygous form of the allele was only found in one animal (2.4 %). The allelic variant ARR, which is typical to sheep resistant to scrapie, was better represented in the examined sheep population (32.9 %).

The genetic data on the *Prnp* polymorphisms offers the possibility of controlling natural scrapie infections in the sheep population by selecting genotypes carrying scrapie-resistant alleles in breeding animals. Once the genotype is determined it may be possible to breed sheep resistant to scrapie by eliminating the *Prnp* genes with codon forms 136-V and 171-Q, which have been shown to influence scrapie-susceptibility (21). Breeding programmes for animals carrying genotypes that determine resistance and the elimination of animals with VRQ alleles are already underway in many European countries. The VRQ-allelic variant was poorly represented in the examined sheep breed, therefore its elimination could be possible. However, considering the high frequency of the ARQ-allelic variant in the Istrian Pramenka sheep population, eliminating all the susceptible sheep could be difficult and should be viewed as a long-term process. Additionally, atypical forms of scrapie have also been reported in sheep having genotypes that are known to be resistant to classical scrapie (22). Therefore, consideration should be given to the use of an appropriate breeding programme coupled with the use of diagnostic tests to control the possible onset of scrapie in our population of Istrian Pramenka sheep.

Scrapie in Istrian Pramenka sheep has never been diagnosed in Slovenia. The results of our study show that the majority of the Istrian Pramenka sheep population in Slovenia has the genotype which is moderately susceptible, however

some of them also carry the highly susceptible VRQ alleles. Considering the genotypes determined, we could expect the onset of scrapie in the Istrian Pramenka sheep population in Slovenia, therefore special attention should be paid to the monitoring for possible clinical signs of scrapie and to screen any suspect animals for the presence of PrP^{Sc}.

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POLIMORFIZMI GENA ZA PRIONSKI PROTEIN, ODGOVORNI ZA DOVZETNOST ZA PRASKAVICO PRI ISTRSKIH PRAMENKAH

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Povzetek: Praskavica je prenosljiva degenerativna bolezen centralnega živčnega sistema ovac, znana tudi kot oblika prenosljive spongiformne encefalopatije (transmissible spongiform encephalopathy - TSE), s katero se ovce okužijo po naravni poti. Spada v skupino prionskih bolezni, za katere je značilno, da se v celicah kopiči na proteazno delovanje odporne prion, kar se pojavlja predvsem v centralnem živčnem sistemu. Poznani so trije najpomembnejši polimorfizmi gena za prionski protein (*Prnp*), ki modulirajo dovzetnost ovac na praskavico; nahajajo se na kodonih 136, 154 in 171. V programu določanja polimorfizmov gena za PrP in ovrednotenja genetske dovzetnosti slovenskih avtohtonih pasem ovac za praskavico, smo analizirali *Prnp* pri 41 ovcah pasme istrska pramenka. Polimorfizme na kodonu 136, 154 in 171 smo določali s sekvenciranjem nukleotidnih zaporedij gena *Prnp*. Ugotovili smo štiri alelne različice in osem genotipov. Na kodonu 136 smo ugotovili dve različici aminokislin, alanin (A) in valin (V). Pri 82,9 % pregledanih živali smo ugotovili alanin/alanin (AA), 2,5 % pregledanih živali je imelo različico WV, 14,6 % pa AV. Na kodonu 154 smo pri 90,2 % ugotovili arginin/arginin (RR), pri 9,8 % živali pa arginin/histidin (RH). Na položaju 171 smo pri 46,3 % preiskovanih živali določili aminokislino glutamin/glutamin (QQ), 36,6 % je imelo različico QR, 17,1 % pa RR. Pri slovenski populaciji istrskih pramenk je najpogosteje zastopan genotip ARQ/ARQ (29,3 %), ki je značilen za ovce, srednje dovzetne za praskavico, kar so pokazale preiskave naravno ali poskusno okuženih ovac. Alelna različica VRQ, ki je značilna za ovce z visokim tveganjem obolevanja za praskavico, je le slabo zastopana v populaciji preiskanih ovac (9,8 %). Bolje je zastopana alelna različica ARR, ki je značilna za živali, odporne na praskavico (32,9 %).

Ključne besede: istrska pramenka; ovca; praskavica; gen za prionski protein; polimorfizem; dovzetnost; sekvenciranje

ECTOPARASITES: *OTODECTES CYNOTIS*, *FELICOLA SUBROSTRATUS* AND *NOTOEDRES CATI* IN THE EAR OF CATS

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Summary: In 1998-99 we examined the carcasses of 101 stray cats from the Ljubljana area for ectoparasites in the ear. The cats were transported to the pathology laboratory for examination after they had been euthanized for medical reasons, killed by hunters or on the roads or they had died as a result of various diseases. Swabs were collected from the auricle and deep inside the auditory canal of each cat using a circular motion and then immediately treated with 10 % KOH. We found that 34.65 % of the cats were infested with one or more of *Otodectes cynotis* (33 cats; 32.7 %), *Felicola subrostratus* (5 cats; 4.95 %) and *Notoedres cati* (2 cats; 1.98 %).

Key words: ectoparasites; ear; stray cats

Introduction

Stray cats may be a source of various diseases in their environment. They may transmit diseases directly to other animals and to humans via companion cats. These include some important parasitic diseases caused by ectoparasites, which are in many cases zoonotic and are not uncommon (1). Thus, the ectoparasites are a recurrent and important source for the transmission of various diseases; they provoke hypersensitivity and skin disorders in cats and, above all, they can cause serious, life-threatening anaemia in young animals.

The most common ear mite is *Otodectes cynotis*, which colonizes the external ear canal (auditory canal) and the outer ear (auricle) in dogs and cats. Sotiraki et al. (2) are of the opinion that *Otodectes cynotis* is responsible for at least half of the feline illness in the world. Mehlhorn believes that 80 % of Europe's stray cats carry this parasite (3). Cats and foxes are the most common carriers of *Otodectes cynotis* and may transmit the parasite to dogs and to humans who have close contact with an infested animal (4).

The parasitic *Notoedres cati* mite is relatively rare. It colonizes the facial area of cats and is especially frequent around the auricle. *Notoedres cati* is most frequently found on stray cats in particularly poor condition (1).

Additionally, the disease it causes is zoonotic and it most commonly affects humans in the area of the palm and the back of the hand. Young children are particularly at risk and it may cause transient dermatitis.

Felicola subrostratus belongs to the order of *Mallophaga*, chewing lice, which are rare in cats and usually only seen on the head, face, neck, back and auricle of elderly or chronically ill animals. They are more problematic in long-haired cats (3, 5) and can cause an unpleasant itch in humans.

Material and methods

The object of this study was to determine the type and degree of ectoparasitic ear infestations found in 101 stray cats from the Ljubljana area between 1998 and 1999. Swabs were collected from the auricle and from deep inside the auditory canal of both ears of the cats, whose carcasses had been brought to the pathology laboratory for examination. The swabbing was performed using a circular motion and the

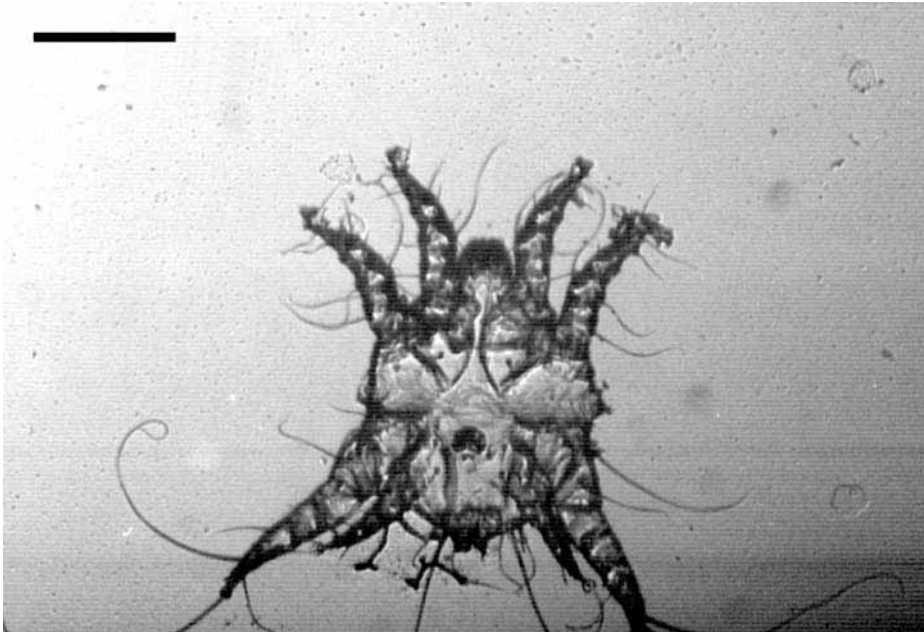


Figure 1: *Otodectes cynotis*, male, ventral view (scale = 160 μ m)



Figure 2: *Notoedres cati*, female, ventral view (scale = 120 μ m)

swabs were immediately treated with 10 % KOH. Treating the sample in this manner clears away any dark cerumen, debris and dirt and leaves a transparent sample, which allows any isolated parasites to be quickly observed and determined by their morphological characteristics. The 10 %-KOH treatment is commonly used in parasitology to observe ectoparasites, which are arthropods and whose exoskeletons are primarily composed of chitin. The KOH is used to make the chitin transparent and to dissolve keratin, hairs and debris (6).

A large number of the examined cats had dark cerumen, which indicated inflammatory distur-

tion of the auditory canal caused by *Otodectes cynotis*. *Felicola subrostratus* and *Notoedres cati* mites were also found in some of the cats with very dark cerumen, excessive content and crusts in the ear. These smears were intensely odorous. The swabs of the cats with clean ears, i.e. without dirty cerumen or crusts, were generally negative.

Results

The following ectoparasites were identified from the swabs: - *Otodectes cynotis*, *Felicola subrostratus* and *Notoedres cati*.

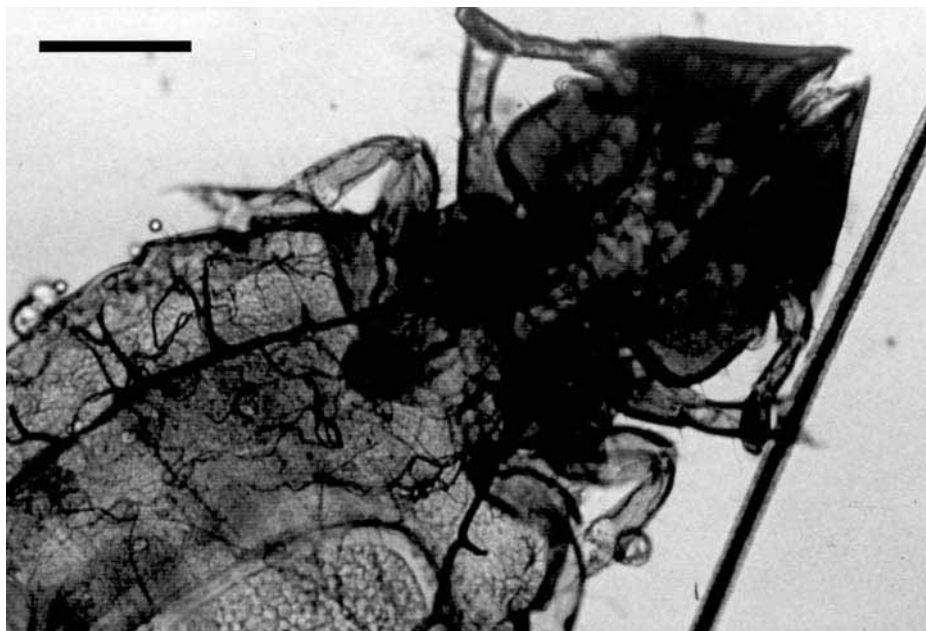


Figure 3: *Felicola subrostratus*, female, a proximal part of the body (scale = 200 μ m)

Table 1: Breakdown of the type and distribution of the ectoparasites identified in % (all cats n = 101)

Positive cats	<i>Otodectes cynotis</i>	<i>Felicola subrostratus</i>	<i>Notoedres cati</i>
35	33	5	2
34.6 %	32.7 %	4.9 %	1.9 %
	94.3 % of all positive	14.3 % of all positive	5.7 % of all positive

A large number of the cats had dark cerumen, which is symptomatic of an inflammation of the auditory canal, and in these cases the presence of *Otodectes cynotis* was confirmed.

The odour of many of the swabs was very strong. Where the ears were clean, i.e. without black-brown cerumen or scabs, the parasitological examinations were generally negative.

In our study, ectoparasites were found in the ears of 34.6 % of the examined cat carcasses. One cat was infested with both *Felicola subrostratus* and *Otodectes cynotis*; two others were infested with all three of the ectoparasites (*Otodectes cynotis*, *Notoedres cati* and *Felicola subrostratus*).

Discussion

Dermatitis, which is primarily caused by ectoparasites, can be located on the head area, neck and periauricular region. The *Otodectes cynotis* mites can migrate from the ear to the neck where it can cause alopecia. The parasites may

also migrate into the auditory canal, which is uncommon for the *Notoedres cati* and *Felicola subrostratus* mites. Animals may be without symptoms and may only be carriers.

Dirt, dark cerumen and debris in the auditory canal indicate the presence of parasites. In similar research performed by Raschka et al. in 1990-92, where 111 stray cats were examined, the results were comparable. They found *Otodectes cynotis* in 27.9 % of the cats, *Felicola subrostratus* in 12.6 % and *Notoedres cati* in 1.8 % cats, however, they examined the entire cat, not just the auditory canal as we did.

Sabolić wrote about 'otitis externa parasitaria' (inflammation of the external ear caused by parasites), which was established in 19.9 % of the cats examined, where the cause was *Otodectes cynotis* and the inflammations were purulent and eczematous (7).

Sotiraki et al. (2) found *Otodectes cynotis* in 41 out of 161 cats (25.5 %), with 9 of the cats (16.1 %) being heavily infested – more than 5 parasites

could be seen in the microscope's field of vision. They washed and massaged the ears with 1-2 ml mineral oil before aspirating the material from the ear for examination. They commented that washing the ears is more effective than taking a smear with cotton wool, especially if the infestation is only slight.

Otosopic examinations of 200 cats by Akucewich et al. (8) found that *Otodectes cynotis* was present in 45 cats (22.5 %). They reported that 74 cats (37 %) had infestations in both ears. In 1969, Vuković reported on an invasion of *Notoedres cati* in the former Yugoslavia that affected about 1.2 % of the country's carnivorous animals (9).

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ZUNANJI ZAJEDAVCI *OTODECTES CYNOTIS*, *FELICOLA SUBROSTRATUS* IN *NOTOEDRES CATI* V UŠESU PRI MAČKAH

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Povzetek: V letih 1998-99 smo pregledali 101 potepuško mačko iz ljubljanske regije na prisotnost zunanjih zajedavcev v ušesu. Trupla potepuških mačk, ki so bile usmrčene iz medicinskih vzrokov, ki so jih ustrelili lovci ali pa so poginile zaradi posledic prometnih nesreč ali različnih bolezni, so bila prepeljana na oddelek za patologijo. Odvzeli smo brise iz ušesnega kanala, kjer smo z vatirancem obrisali notranjost obeh ušes s krožnimi gibi. Ušesni brisi so bili takoj obdelani z 10 % KOH, bombažni bris z vzorcem se je nekaj minut kuhal v 10 % KOH. Ugotovili smo, da je bilo 65,3 % mačk neinvadiranih, 34,65 % pa invadiranih. *Otodectes cynotis* smo ugotovili pri 33 mačkah (32,7 %), *Felicola subrostratus* pri 5 mačkah (4,95 %) ter *Notoedres cati* pri 2 mačkah (1,98 %).

Ključne besede: zunanji zajedavci; uho; potepuške mačke

INFLUENCE OF COLOSTRUM QUALITY ON THE HEALTH STATUS AND GROWTH OF CALVES

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Summary: Thirty six calves were monitored from birth through to the age of 24 weeks. The Ig content of their dam's colostrum was measured via a colostrometer and indirectly by measuring the enzyme activity of the gamma-glutamyl transferase (GGT) or the concentration of total-serum protein (TSP) in the calves' sera during their first week of life. The concentration of albumin (Alb) and leukocyte count (WBC) in calf serum were also measured. The health status and chest girths of the calves were also regularly recorded.

The mean chest girths of the ill and healthy calves did not differ significantly. The Ig concentration in the colostrum had a statistically significant influence on the TSP and GGT but not on the Alb and WBC. The interval between birth and the first intake of colostrum had a statistically significant influence on GGT and WBC, but not on the TSP and Alb. In the calves that fell ill the only statistically significant influence their illness had was on WBC. The correlation between the Ig concentration in colostrum and TSP and GGT in a calf's serum was highly significant, as was the correlation between the activity of GGT and the TSP and WBC.

Key words: calves; colostrum; immunology; health status; growth

Introduction

Calves are born without immunoglobulins (Ig), because the bovine epitheliochorial placenta is impermeable for protein macromolecules. They get Ig with the first intake of colostrum. The calf intestine is able to absorb macromolecules, like Ig, for 24 hours after birth. During this time enzyme activity is minimal, therefore Ig are not decomposed in the small intestine. It is crucial for the health of a calf to get an adequate amount of colostrum as soon as possible after birth. Calves that do not get an adequate supply of colostrum more often than not end up with an infection and have a higher mortality than other calves.

Morbidity and mortality in neonatal calves have complex aetiologies. Factors often linked to outbreaks of a disease are associated with an insufficient intake of colostrum, exposure to a potentially virulent pathogen, inadequate hygiene, and suboptimal nutritional support (1). Ma-

ny studies have been performed to investigate the connection between immunoglobulin status and the health of calves (1, 2, 3) and the connection between colostrum quality and immunoglobulin concentrations in calves sera (4, 5).

The volume and Ig concentration of the ingested colostrum and the interval between birth and the first intake influenced the absorption of immunoglobulin. The latter is most important for Ig2G and IgM absorption (6, 7). The Ig concentration in colostrum can be measured with a colostrometer (8). It can also be indirectly estimated in a calf's serum by measuring the activity of the enzyme gamma-glutamyl transferase (GGT), which is highly concentrated in colostrum, and like Ig, is absorbed with passive transfer (9, 10). Another indirect way to estimate serum Ig is by measuring the amount of total-serum protein (TSP) (11).

For normal development and body-weight gain it is very important that calves are healthy. Calves that are ill gain body weight slowly, which has a negative influence on the cost efficiency of breeding (12).

Table 1: Mean chest girths (cm) in calves with regard to age

Age in weeks	n	\bar{x}	SD	CV (%)	Min.	Max.
1	36	79.37	2.78	7.71	73.00	86.00
2	36	79.43	3.20	10.25	73.00	89.00
3	36	79.47	3.06	9.39	72.00	85.00
4	36	82.24	3.91	15.30	73.00	92.00
5	36	84.72	3.89	15.12	75.00	93.00
6	36	87.17	4.45	19.80	79.00	98.00
8	36	93.43	4.94	24.36	82.00	103.00
12	36	104.94	4.46	19.88	97.00	112.00
16	36	115.23	5.50	30.29	106.00	130.00
20	36	126.00	9.80	96.00	117.00	177.00
24	36	130.23	6.37	40.58	120.00	154.00

Table 2: Basic statistical data for the sera values - GGT, TSP and Alb; Ig in colostrum and WBC in blood

Parameter	n	\bar{x}	SD	CV %	Min.	Max.
GGT in U/L	36	437.17	406.47	85.90	30.00	1,762.00
TSP in g/L	36	54.13	7.04	13.02	41.60	74.40
Alb in g/L	36	26.70	1.90	7.11	23.10	32.60
Ig in mg/ml	35	93.28	33.41	35.81	20.00	155.00
WBC x 10 ⁹ /L	36	9.20	3.99	43.31	4.60	20.60

Table 3: Mean values for the activity of GGT and the concentrations of TSP and Alb in the sera and Ig in the colostrum of healthy and ill calves

Parameter	Healthy calves			Ill calves		
	n	\bar{x}	SD	n	\bar{x}	SD
GGT in U/L	24	564.48	457.41	12	345.33	290.07
TSP in g/L	24	55.87	7.34	12	51.69	6.01
Alb in g/L	24	26.14	1.47	12	27.49	2.19
Ig in mg/ml	24	96.50	7.01	12	89.00	9.46
WBC x 10 ⁹ /L	24	8.78	3.49	12	9.78	4.66

The aim of our research was to assess what influence that the interval between birth and the first intake of colostrum, and its quality, has on the health of a calf and the difference in weight gain between healthy calves and calves which were ill within their first 24 weeks of life.

Materials and methods

Thirty six dairy calves, 19 male and 17 female, which were born between October and November 2003 to a herd of 200 dairy cows, were monitored from birth until they were 24

weeks of age. The calves were kept in individual boxes for the first week after birth and in groups of 10 thereafter. Straw was used as litter throughout the study and it was regularly changed. For the first four days of life they took in 1-1.5 litre of their dam's colostrum and milk, they were fed three times a day. From the fifth day they were fed up to 4 litres of milk twice a day, from cows treated with antibiotics. During first week they suckled milk from a nipple pail. Thereafter, they drunk from a trough and had free access to the starter and hay. They were weaned at four months of age.

Table 4: Correlation coefficients for the investigated parameters

Parameter	Ig in colostrum mg/ml	GGT U/L	TSP g/L	Alb g/L	WBC x10 ⁹ /L
Ig in colostrum mg/ml	1.0000	0.3862 *	0.6328 ***	-0.1250 ns	0.1455 ns
GGT U/L		1.0000	0.3958 *	-0.2195 ns	0.3924 *
TSP g/L			1.0000	-0.0376 ns	0.0773 ns
Alb g/L				1.0000	-0.2008 ns
WBC x 10 ⁹ /L					1.0000

* p < 0.05

*** p < 0.001

ns not significant

Table 5: F-values, coefficient determination-R² and presentation of the significance of influence on the investigated parameters

Sources of variability	F-values			
	GGT U/L	TSP g/L	Alb g/L	WBC x 10 ⁹ /L
Sex	2.56 ns	0.04 ns	8.70 **	4.29 *
Health status	0.06 ns	1.47 ns	2.99 *	4.54 *
Interval to first intake of colostrum	4.08 *	0.01 ns	0.00 ns	8.40 **
Ig concentration in the colostrum	4.45 *	14.29 ***	3.84 *	0.50 ns
R ² (%)	28.73	37.29	45.47	29.65

* P < 0.05

** P < 0.01

*** P < 0.001

ns not significant

The calves received 1-1.5 litres of their dam's colostrum almost immediately after birth. A record was kept for each calf, noting its first intake of colostrum and the colostrum's quality, which was measured at 22 °C using a colostrometer (Bergophor, Germany) (8, 13). Throughout the study, a veterinarian, who was constantly in attendance, monitored the health of the calves and recorded the details (date, duration, type, etc.) of any disease afflicting them (scours, respiratory disease, etc.) or the death of the animal.

The gain in body-weight was estimated by measuring each animal's chest girth once a week for the

first 6 weeks, again in the 8th week and then in the 12th, 16th, 20th and 24th weeks of their lives.

Vacuum tubes (Venoject®) were used to take a blood sample from the vena jugularis of each calf when it was two or three days old. A Cobas Mira biochemical analyser (La Roche) was used to measure the activity of the GGT, the TSP and the albumin concentration of the serum and an ABC Vet haematological counter was used to determine the number of leucocytes (WBC) in the blood.

The data were statistically processed using version 8 of the SAS/STAT programme. We assessed the statistical parameters – mean value

(x), standard deviation (SD), coefficient of variability (CV) – of the colostrum, blood and serum characteristics that we were investigating. We also determined the correlations between the Ig concentration of the first colostrum and the levels of WBC, TSP, albumin and the activity of GGT.

For the analysis of variance we used the statistical model:

$$Y_{ijklm} = \mu + S_i + H_j + T_k + C_l + e_{ijklm}$$

μ = mean value of the model

S_i = influence of sex ($i = 1, 2$)

H_j = influence of health status ($j = \text{ill, healthy}$)

T_k = influence of time of first intake of colostrum ($c = 0.5 \dots 6.5$ hours)

C_l = influence of Ig concentration in first colostrum ($l = 20 \dots 145$ mg/ml)

e_{ijklm} = accidental error of the model (rest)

Results

The mean chest girth in the first week was 79.37 ± 2.78 cm and changed very little until the calves were four-weeks old. By the fourth week it was 82.24 ± 3.91 cm, then it slowly increased by 2-3 cm per week and was 130.23 ± 6.37 cm by the 24th week.

We compared the gain between the calves that were ill at least once and the healthy calves and determined that the gain in the former group was slightly less than the latter, however, the difference was not statistically significant. The duration of any illness in the calves was usually between one and four days.

The majority of the calves (88.89 %) suckled colostrum within three hours of birth. The activity of GGT was below 200 U/L in six calves and 10 had a TSP concentration that was less than 50 g/L.

The correlations between the monitored parameters of the calves are represented in Table 4. We determined a statistically significant correlation between the Ig concentrations in the colostrums and the amount of TSP and GGT activity in the sera of the calves. There was a statistically significant correlation between the GGT activity and the quantity of TSP and WBC in the blood. There were no other statistically significant correlations established.

With analysis of variance we assessed the influence of sex, health status, interval to the first intake of colostrum and the Ig concentration in colostrum on the levels of GGT activity, TSP

and Alb in serum, and WBC in blood and showed the degree of variance. With our statistical model we were able to account for 45.47 % variability in the level of Alb, 37.29 % variability in TSP, 29.65 % variability for WBC and 28.73 % variability for GGT activity. The interval between the first intake of colostrum and its Ig concentration had a statistically significant ($P < 0.05$) influence on the level of GGT activity. The influence of the colostrum Ig concentration on the level of TSP amount was also statistically significant ($P < 0.001$). The level of Alb in the serum was influenced by sex, health status and the colostrum Ig concentration, whereas the latter was the only source of variability not to influence the level of WBC in the blood.

The calves that fell ill did so within their first 6 weeks; the mean age at the first recognisable sign of disease was 16.3 ± 3.06 days. The Ig content in colostrum had a statistically significant influence on the level of GGT activity ($P = 0.041$), as well as on the quantity of TSP ($P = 0.0008$). It also had slightly less influence on the level of Alb ($P = 0.0601$) and an insignificant influence on the level of WBC ($P = 0.0421$). The most common diseases were diarrhoea ($n = 7$) and respiratory diseases ($n = 5$). The faeces samples from calves with diarrhoea indicted the presence of *Cryptosporidium* and rotavirus.

The interval between birth and the first intake of colostrum had a statistically significant influence on GGT activity ($P = 0.0532$) and the WBC level ($P = 0.0072$), but not on the levels of TSP ($P = 0.9258$) and Alb ($P = 0.9814$).

Discussion

Our research established that the chest girth of calves increased very slowly until they were 4-weeks old and then increased steadily beyond their 24th week (the duration of the study). The calves that fell ill during the study had smaller chest girths than the healthy calves. Caldwell et al. (12) established a statistically significant difference in body weight gain ($P < 0.001$) between healthy and sick calves. In our study, the periods of disease were very short, a matter of days, which is why they had such little impact on weight gain.

We established lower levels of GGT activity and TSP in the calves that fell ill, but again, the difference was not statistically significant. Naylor et al.

(11) reported a higher morbidity in calves that had plasma protein concentrations under 60 g/L than in calves with plasma protein concentrations above 60 g/L, which was statistically significant ($P < 0.001$). This implies that plasma protein concentration is as reliable as serum Ig for predicting a calf's susceptibility to disease during its first five weeks of life. Tyler et al. (14) also compared methods for measuring the serum Ig concentration in clinically ill calves and reported that GGT activity above 50 U/L and a TSP level above 55 g/L indicates that there is a sufficient level of Ig in the serum. However, Perino et al. (15) reported that these values were 200 U/L for GGT and 42 g/L for the TSP. Braun et al. (10) recorded higher levels of GGT activity in sera during the first and second day after birth (370 – 5000 U/L). We also recorded a wide variance in the levels of GGT activity.

In this research, we were unable to establish any statistically significant correlation between the levels of GGT activity, TSP and the health of the calves. Other authors also reported similar findings; they were not able to identify any statistically significant correlations between IgG in plasma, mean daily gains and morbidity (3, 12, 16). Other studies have shown that calves with low values of IgG are twice as likely to be frequently ill and/or die than calves with high serum IgG (2, 3). We are of the opinion that the calves that fell ill during the course of our study had adequate levels of Ig in their blood, as only 6 calves had GGT-activity levels below 200 U/L and only 10 had concentrations of TSP below 50 g/L. Therefore, we have attributed the causes for their illnesses to the other factors.

Tyler et al. (1) reported that 39 % of calf mortality was due to inadequate levels of serum Ig, and they attributed the rest to other influences. Factors often linked to outbreaks of disease are associated with inadequate amounts of colostrum, exposure to a potentially virulent pathogen, inadequate hygiene and less than optimal nutritional support. Rajala and Castren (4) were unable to explain the appearance of diarrhoea by only using Ig concentrations in calf serum as an indicator.

The mean Ig concentration in colostrum that we determined in our research was a little higher than the 76.2 mg/ml reported by Rajala and Castren (4). Muller and Ellinger (17) reported that there were differences in the levels of Ig concentration in colostrum between different breeds.

We established that the influence of the Ig concentration in colostrum on the level of GGT activity and TSP was statistically significant, which agrees with the findings of Nocek et al. (18). Rajala and Castren (4) also established a statistically significant correlation between the Ig concentration in colostrum and the Ig level in calf serum ($P < 0.05$), while Erhard et al. (5) also concluded that there was a correlation between the two ($r = 0.37$).

Conclusion

We are of the opinion that the calves used in our study had adequate levels of Ig in their blood, as only 6 calves had a GGT-activity level below 200 U/L and only 10 had concentrations of TSP below 50 g/L.

With our statistical model we were able to account for a 37.29 % variance in the levels of TSP and 28.73 % variance for GGT activity, so we attribute the causes for the recorded illnesses more to other factors (management, exposure to virulent pathogen and nutrition).

The calves that fell ill during this study had lower levels of GGT activity and TSP and gained weight more slowly than the other calves.

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VPLIV KAKOVOSTI KOLOSTRUMA NA ZDRAVSTVENO STANJE IN PRIRAST TELET

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Povzetek: V raziskavo smo vključili 36 telet črno-bele pasme, ki smo jih spremljali od rojstva do starosti 24 tednov. Določali smo koncentracijo Ig v kolostrumu, v serumu pa aktivnost encima gama glutamil-transferaze (GGT), koncentracijo celotnih serumskih beljakovin (CSB) in albuminov (Alb) ter število levkocitov (L) v prvem tednu starosti. Pri teletih smo redno spremljali zdravstveno stanje in prirast s pomočjo merjenja prsnega obsega.

Povprečni prirasti pri obolelih teletih so bili nižji kot pri zdravih, vendar razlika ni bila statistično značilna. Koncentracija Ig v kolostrumu je statistično značilno vplivala na koncentracijo CSB in na aktivnost GGT, ne pa na koncentracijo Alb in število L. Čas prvega pitja kolostruma je statistično značilno vplival na aktivnost GGT in število L, ne pa na vsebnost CSB in količino Alb. Pri bolnih teletih smo ugotovili statistično značilen vpliv samo na število L. Ugotovili smo visoko statistično značilni korelaciji med količino Ig v kolostrumu ter vsebnostjo CSB in aktivnostjo GGT. Aktivnost encima GGT je v statistično značilni korelaciji z vsebnostjo CSB in številom L v krvi.

Ključne besede: teleta; kolostrum; veterinarska medicina; imunologija; zdravstveno stanje; prirast

PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME (PRRS) IN SLOVENIA: EVALUATION OF SEROLOGY

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Summary: Between 1995 and 2003, 27,925 pig sera were tested for PRRS virus antibodies using the Herd Check ELISA test (IDEXX) to determine the PRRS status of pig herds in Slovenia. The prevalence of seropositive samples was low and annually ranged from 1 to 7 % and the average hovered between 2 and 3 %. No clinical signs associated with a PRRS viral infection have been reported by any of the farms. Under field conditions, false positives with this assay may run up to 3 %. Based on the absence of clinical signs, the recorded levels of false positives associated with the assay and the persistently low frequency of serological signs, the presence of PRRS can not be determined.

Key words: PRRS; serology; monitoring

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is characterized by the reproductive failure of sows as well as respiratory distress in piglets and growing pigs and has a significant economic impact on the pig industry (1).

The disease was first seen in the United States and Canada in 1987 and was first reported in Europe in Lower Saxony in June 1990. Since then it has been reported in other parts of Europe – the United Kingdom, France, Spain, Denmark, Luxembourg, Italy, Malta, Poland – and in Japan, Korea, the Philippines and South America (2). Only Australia, Sweden and Switzerland have documented their virus-free status (2, 3, 4). The PRRS virus is currently classified as a member of the newly established order of *Nidovirales*, family *Arteriviridae*, genus *Arterivirus* (5). Clinical signs such as an increase in stillbirths, pre-weaning mortality and in abortions, as well as the development of respiratory diseases, the presence of lesions during post-mortem examinations and histological findings could indicate the presence of PRRS. This can be confirmed by immunofluorescence or by immuno-

peroxidase and by a reverse polymerase chain reaction. Virus isolation can also be performed (6). The detection of serum antibodies to the virus is the most commonly used method to confirm a diagnosis and to monitor the disease (5).

Serological methods used to determine PRRS include the following: ELISA tests, immunoperoxidase monolayer assays (IPMA), indirect fluorescent antibody tests (IFA) and virus neutralisation tests (VNT) (6, 7). Slovenia first detected animals that were seropositive to PRRS, in a quarantine station in 1994. Every pig at the station was immediately destroyed. Prior to that event PRRS had been unknown in Slovenia, although there had been no clinical or serological monitoring for the disease. In 1995, the Slovenian Government introduced strict guidelines governing the monitoring of PRRS. It became compulsory for farmers to test their breeding-age pigs for the disease on an annual basis. These tests are fully subsidized by the government (8, 9).

Material and methods

Sera: Blood was taken from every boar and sow of breeding age from each piggery with more than 1,000 breeding sows as well as from a selection of small farms. According to the figures, the number of

Table 1: Sera samples tested for PRRS between 1995 and 2003

Year	No. of samples tested	Positive	Negative
1995	4,537	53	4,484
1996	4,943	170	4,773
1997	4,395	107	4,288
1998	3,176	91	3,085
1999	4,352	89	4,263
2000	1,932	32	1,900
2001	1,471	97	1,374
2002	1,582	30	1,552
2003	1,537	70	1,467

samples varies each year, however, a total of 27,925 samples were tested between 1995 and 2003.

Test: The Herd Check PRRS (IDEXX Laboratories Inc, Westbrook, Maine, USA), which is an enzyme immunoassay for the detection of antibodies to PRRS in swine serum using PRRS and normal host cell (NHC) antigens, was used to test all the sera. The PRRS and NHC antigens are coated on alternating columns on a microplate. The test sample is introduced into the coated wells and upon incubation antibodies specific to PRRS form a complex with the viral antigen coating. The coating of NHC antigens is used to assess whether immunoglobulins against tissue-culture components, present in vaccines, are contributing to the test results. After washing away any unbound material from the wells, an anti-porcine horseradish-peroxidase conjugate is added which binds to any porcine antibody attached to the wells. In the final step of the assay, unbound conjugate is washed away and enzyme substrate and a chromogen are added to the wells. The extent of any host-cell contribution to the total signal is assessed by relating the PRRS activity to the NHC reactivity. For the assay to be valid, the positive-control mean for PRRS, which is determined by the formula $PC:PRRS - NC:PRRS$, must be greater than or equal to 0.150. The presence or absence of PRRS antibodies is determined by calculating the S/P ratio. If the S/P ratio is less than 0.4, the sample is classified as negative for PRRS. If the S/P ratio is greater than or equal to 0.4, then the sample is classified as positive for PRRS antibodies.

Results

Number of samples positive for PRRS antibodies was low in all years. The seroprevalence to PRRS is from 0.01 to 0.07. From all tested sera in this period 2.6 % were positive to PRRS. The only evidence of PRRS in Slovenia is based on serology.

Discussion

Serologic tests are very important tools in control and prevention programmes. The United States uses the ELISA, as we do in Slovenia. Under experimental conditions the test is essentially 100 % specific and 100 % sensitive when testing serum from acutely infected swine. In general, the range of the S/P ratio for false negatives is about 0.2 to 0.4. Under field conditions, false positives may run up to 3 % with this assay (10).

The testing of sera from 9 PRRSV-infection-free farms at the University of Minnesota's Veterinary Diagnostic Laboratory found that 0.89 % of the sera tested positive to PRRS. Adult animals had a higher prevalence of false positive results than younger animals (1.3 %). Twenty-four of the positive sera from the ELISA were retested using an immunofluorescence assay (IFA); only one sample tested positive and the herd history subsequently confirmed this sample as negative (11). In developing and validating an ELISA test for the detection of antibodies directed against the European or the North American strain of the PRRS virus, swine sera from countries free of PRRS (New Zealand and Switzerland) were used to gauge its specificity. This ELISA test established that out of

a total of 277 sera tested, 266 were negative, 6 were doubtful and 5 were positive (12). The testing and removal protocol for PRRS, detected 74 false positives (approx. 2.2 %) out of the 3,408 samples tested, which were taken from the farms during the monitoring periods (13).

The prevalence of PRRS antibodies detected in Slovenia ranges from 1 to 7 %, with the average between 2 and 3 %. As only pigs of breeding age are tested, the percentage of false positives can be higher than when only younger animals are tested. The herd history of Slovenia's eight largest pig farms indicates that there have been no typical signs of PRRS since monitoring began in 1995. These farms are now governed by strict biosecurity guidelines; replacement animals for the breeding herds usually come from the same farm and if they are imported they are quarantined and tested for PRRS with only seronegative pigs being introduced to the farm.

Despite nine years of serologically testing adult animals without clinical signs of PRRS, it is still unclear if our large pig herds are PRRS free. Only further testing and continued vigilance will provide any certainty in this regard.

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PRAŠIČJI REPRODUKCIJSKI IN RESPIRATORNI SINDROM (PRRS) V SLOVENIJI: VREDNOST SEROLOGIJE

Z. Valenčak

Povzetek: V obdobju od 1995 do 2003 smo pregledali 27.925 prašičjih serumov s testom ELISA Herd Check proizvajalca IDEXX Laboratories Inc, Westbrook, Maine, ZDA, in sicer na prisotnost protiteles proti virusu PRRS, da bi ugotovili stanje glede te bolezni na farmah prašičev v Sloveniji. Število pozitivnih vzorcev je bilo med 1 in 7 %, večinoma pa med 2 in 3 %. Na nobeni od farm ni bilo kliničnih znamenj, ki bi kazale na okužbo z virusom PRRS. Pri uporabi istega testa ELISA so ugotovili od 2 do 3 % lažno pozitivnih reaktorjev. Ker pri nas niso bila opisana klinična znamenja bolezni ter glede na opisan odstotek lažno pozitivnih reaktorjev v svetu in dolgoletno nizko prevalenco serološko pozitivnih reaktorjev ostaja status slovenskih farm prašičev v zvezi s PRRS še vedno nejasen.

Ključne besede: PRRS; serologija; monitoring

DOCTORAL DISERTATIONS AT THE VETERINARY FACULTY, UNIVERSITY OF LJUBLJANA - SUMMARIES

Marko Cotman: INTRACELLULAR TRANSPORT OF LANOSTEROL 14 α -DEMETHYLASE (CYP51) AND NADPH CYTOCHROME REDUCTASE IN MAMMALIAN CELLS

Lanosterol 14-demethylase (CYP51) is enzyme belongs to the superfamily of cytochromes P450. CYP51 is involved in postsqualene part of cholesterol biosynthesis. CYP51 removes 14-methyl group from lanosterol forming FF-MAS (follicular fluid meiosis activating sterol). In somatic cells FF-MAS and T-MAS (testis meiosis activating sterol - the product of the sterol 14-reductase) are intermediates in of cholesterol biosynthesis, while they in the germ cells acumulate. FF-MAS and T-MAS stimulate the reinitiation of meiosis in mouse oocyte *in vitro* and are belived to have important roles in fertilisation. High expression of CYP51 mRNA and protein are detected in rat and pig posmeiotic male germ cells. This data coincide with data about MAS accumulation in germ cells. Mammalian CYP51 is ubiquitously expressed microsomal cytochrome P450. Many endoplasmic reticulum (ER) resident protein are transported from the ER and then retrived. Signal motif KDEL or KKXX or RRXX on C-terminus are normally responsible for retriveal retention. Some proteins are ER resident retention protein and are not involved in recyclisation through *cis*-Golgi compartment. Transmembrane region or linker region is probably responsible for resident retention. In cytochromes P450 mechanism of resident retention are unknown.

Expression of CYP51 protein was determined in mouse male germ cells and compared with expression of CYP51 in mouse hepatocytes. CYP51 protein are detected using immunohistochemistry with fixed mouse tissues and western analysis of particular isolated organeles. Expression of CYP51 protein in different cells was determined using fluorescent antibodies and confocal laser microscope. Expression of CYP51 on organelles was determined using antibodies

labelled with gold conjugate and electron microscope. Particular organelles were isolated using ultracentrifugaton of tissue homogenate on sacharose gradient. Microsomes and different Golgi fractions were isolated from hepatocytes while, germ cells, Leydig cell and microsomes were isolated from testicular tissues, respectively. Acrosomal membranes were isolated from bull and ram semen. Particular isolate of organelle were loaded on SDS-PAGE gel and then transferred on nitrocelulose membrane. Proteins on membrane were labelled using specific antibodies and detected with ECL method. Influence of exogene cholesterol on CYP51 protein trafficking were determined by comparison of western analysis of particular Golgi fractions in normal fed mice and mice fasting for 24 hours.

In the mouse liver, CYP51 resides primarily on membranes of the smooth endoplasmic reticulum (SER). Significant amount of the protein was also detected in Golgi apparatus (GA) but not on plasma membrane, suggesting retriveal of CYP51 from Golgi to ER in the liver. Di-arginin motif KRRS is present in C-terminus of CYP51, that might be responsible for retriveal of CYP51 protein back to ER. In mouse haploid male germ cells, CYP51 resides not only on SER and GA but also on outer and inner membranes of the secretory Golgi-derived organelle acrosome. CYP51 was detected during all phases of acrosome development (15 days in the mouse), including most mature spermatids that lack cytoplasmic organelles. It was also detected on acrosomal membrane from ejaculated bull and ram sperm, indicating evolutionary conserved mechanism for its longterm storage/stabilization. The expected 53 kDa immunoreactive CYP51 predominates in the liver microsomes. However on *cis*- , *medial*- and *trans*- Golgi 70 kDa form was also detected. In mouse after 24-hours fasting, 70 kDa form disappered, and only 53 kDa form was present. On acrosomal membrane isolated from ram and bull sperm, 50 kDa, 60 kDa, 70 kDa, 90 kDa and 120 kDa were detected bands of different intensity. Both 70 kDa immunoreactive CYP51 from mouse liver Golgi fractions and ram and bull acrosome are likely formed in the Golgi. The 70 kDa form appear not to be ubiquinated but glycosilated.

These data show for the first time that cell type-specific mechanisms and post-translational

modifications may influence trafficking of cytochrome P450 proteins. Localisation of CYP51 protein on acrosome is the first identification of cytochrome P450 on acrosome membrane in mammals. The importance of CYP51 protein on acrosome is not yet known, it is possible that CYP51 on acrosome is involved in production of MAS that could be delivered by sperm to the oocyte where it would trigger the conclusion of second meiotic division.

The research work was performed at the Institute for health care of pigs, Veterinary Faculty in Ljubljana and at the Medical centre for molecular biology, Medical Faculty in Ljubljana.

The date of public defense: April 22, 2003.

Mentor: Prof. Dr. Danjana Rozman, co-mentor: Assist. Prof. Gregor Majdič.

Leon Ščuka: ENROFLOXACIN – THE META ANALYSIS OF THE EFFICACY OF DISEASE TREATMENT FOR DOMESTIC ANIMALS

Meta-analysis is the process of using statistical methods to review and combine the results of different independent clinical studies. Glass first used the term meta-analysis in 1976, when he and his coworker Mary Lee Smith have statistically combined the results of 375 studies that evaluated efficacy of psychotherapy. Through meta-analysis, the scientists are able to integrate results and findings from different studies. This analytical method is of particular importance in the assessment of therapeutic efficacy when individual studies do not provide an overview over all studies on a topic. As their samples are too small, individual studies cannot provide a quantitative evaluation of the effect of treatment, nor can they test null hypothesis. Prior to meta-analysis, the traditional method was a narrative discourse on previous findings, which, however, could be misleading and subjective.

In the past few years, meta-analysis has been increasingly used in all fields of science. This is particularly evident for the medical science, where two other methods are used as well –the Systematic Review and Evidence Based Medicine, while especially in medicine, the Decision Analysis and Cost-Effectiveness Analysis have developed. All methods are connected, and the last two are the Upgrade of the first two. Systematic reviews are exact summaries of the best evidences related to exactly specified clinical dilemmas. Special centers, like Cochrane collabo-

ration have been organized in different places around the world, where systematic reviews of scientific literature and their own findings are published in databases collecting data on most appropriate therapies of individual illnesses. These reviews support the synthesis of best evidence for treatment or establishment of best medical practice. In this case, meta-analysis has a broader impact and is not just, as usually, a statistical method for collecting study results. This knowledge in veterinary medicine is only just beginning to develop but centers of this kind have not been established yet.

Our research of literature revealed 21 cases of meta-analysis in veterinary medicine; all of them carried out after 1990. In comparison to more than 1000 meta-analyses carried out only in the year 1997 in human medicine; this is a very small number.

About 16 years ago, fluoroquinolones were introduced to clinical medicine and they promised a new era in the development of antimicrobial agents. Due to its broad spectrum of action, excellent clinically significant pharmacokinetics properties and low toxicity -this being great advantages over other groups of antibiotics -they were classified as almost ideal antimicrobial agents.

Enrofloxacin is a chemotherapeutic from the fluoroquinolone group that was developed exclusively for the use in veterinary medicine. Low concentrations of the substance exert a rapid bactericidal effect against gram-negative and gram-positive bacteria, while the substance also very effective against *Mycoplasma* spp. It acts against bacteria in their reproductive phase and microorganisms in their latent state. It acts in the presence of oxygen and due to this phenomenon does not damage the indigenous anaerobic intestinal micro flora.

We also reviewed literature describing the use of enrofloxacin in domestic animals. In addition to the meta-analysis, we made a systematic review and in the end combined the general findings. We reviewed and evaluated the applicability and efficacy of enrofloxacin in the health care of pigs, poultry and cattle. Individual studies were collected by reviewing databases available on CD ROMs or Online. We also reviewed references in different published studies and data bases on the internet. We first collected many different studies. We obtained 919 articles for the studies for the first

selection, and after review and careful consideration of the studies, we chose for a closer review 110 studies on health care in pigs, 67 in ruminants and 60 in poultry, i.e. a total of 237 studies.

We reviewed and evaluated the efficacy of the treatment of various infections with enrofloxacin for individual animal species. A special meta-analysis was carried out and graphically presented for the treatment of each disease. In most cases, we chose the odds ratio to present the size of the effect. By following a systematic way of reviewing, we ensured repeatability of our meta-analyses in case this would be done by other investigators. In heterogeneous meta-analyses, we calculated the total size of the effect according to a random calculation model for total effect size. Additionally the homogeneity of studies was graphically evaluated with funnel plots. In addition to clinical studies, we reviewed and combined data on bacterial in vitro susceptibility to enrofloxacin. These results were also considered in the final opinion about individual meta-analysis of efficacy of enrofloxacin. In a systematic review, we compared efficacy of enrofloxacin and other antimicrobial agents.

We prepared 19 meta-analyses about different uses of enrofloxacin in various diseases in pigs, poultry and domestic ruminants (mainly cattle), while in 7 cases we also calculated the individual effect size (odds ratio) for a specific parameter.

It is evident from the results that enrofloxacin is potently effective in the treatment of respiratory infections in all domestic animals ($P < 0.01$). Enrofloxacin is very effective in the treatment of all coli and salmonella infections in pigs and poultry ($P < 0.001$), while additional studies about colibacillosis and salmonellosis in cattle would be necessary. In cattle, in vitro resistance to enrofloxacin was established in 11.8 % of *E. coli* strains ($n = 195$), 1.8 % *E. coli* strains, isolated in the udder ($n = 1695$), and 8.4 % if salmonella strains ($n = 1211$).

After taking into account all findings (in vivo and in vitro), it was revealed that enrofloxacin is effective in the treatment of mycoplasma infections in poultry and pigs, while additional studies would be necessary in cattle.

A meta-analysis in poultry revealed that administration of enrofloxacin is effective in pasteurellosis in turkeys ($P < 0.001$), and in infectious coryza ($P < 0.001$), staphylococcosis ($P < 0.001$) and *R. anatipestifer* infection in ducks ($P < 0.001$).

These results are also confirmed by findings of high in vitro susceptibility to enrofloxacin of the pathogens of these diseases.

In pigs, treatment with enrofloxacin was significantly more effective in the trial group than in the control group for MMA syndrome ($P = 0.002$), urinary tract infections ($P < 0.05$) and streptococcal infections (fewer deaths, $P = 0.045$). These results are also confirmed by findings of high in vitro susceptibility to enrofloxacin of the pathogens of these diseases. For Glässer's disease the difference, in comparison to the control group was not significant ($P = 0.25$), however the pathogen (*H. parasuis*, $n = 124$) was 100 % susceptible to enrofloxacin. In greasy pig disease, there is a high in vitro susceptibility of *S. hyicus* to enrofloxacin (98.3 %, $n = 744$).

To be able to answer the complex questions about mastitis in cattle, one or more additional studies with enrofloxacin would be necessary, as our results indicate that enrofloxacin is not more effective than drugs in control groups (fixed model: odds ratio = 0.3; $P = 0.5$, random model: odds ratio = 1.19; $P = 0.79$). However, the in vitro results on susceptibility of mastitis pathogens to enrofloxacin are good. An additional study would also be necessary for the treatment of endometritis in cattle, since the difference between the trial and the control group was not statistically significant ($P = 0.9$), although the results were in favor of the treatment with enrofloxacin.

In the thesis most of the aims were reached and the majority of tasks necessary for the investigator during the process of meta-analysis were successfully performed: descriptive survey, guidelines for further research, diagnostic survey and transfer of our findings into practice. We reviewed the available studies and could assess sufficiently and insufficiently analysed parameters. Some studies revealed statistically significant results and some not. It occurred in some cases that studies that lacked significant results, due their weight, had a greater impact on the analysis than those with significant results. It was this part of our research that revealed one of the greatest difference between meta-analysis and the narrative comparison of the literature.

Our findings can be considered useful for investigators, doctors of veterinary medicine in practice and for the breeders, as well as for the manufacturers of veterinary medicines and governmental authorities. Our work has a great economic impact too, since it offers an overall survey

of the problem and provides guidelines for further research of the topic.

The research work was performed at the Veterinary Faculty in Ljubljana and Krka d.d., Novo mesto.

The date of public defense: June 27, 2003.

Mentor: Prof. Dr. Jože Drinovec, co-mentor: Prof. Dr. Peter Lazar.

Janez Posedi: GENETIC MARKERS FOR SHEEP GASTROINTESTINAL STRONGYLIDS SUSCEPTIBLE TO BENZIMIDAZOLE ANTIHELMINTICS

This study defines the genetic markers in rDNA for sheep gastrointestinal strongylids. In the study, parasites of species *Trichostrongylus axei*, *Trichostrongylus colubriformis*, *Teladorsagia circumcincta*, *Cooperia curticei* and *Chabertia ovina* are included.

Previous to molecular analyses, the susceptibility to benzimidazole anthelmintics in investigated population of gastrointestinal strongylids was determined. Egg hatch assay and faecal egg count reduction test for determination of resistance to anthelmintics were performed.

The results of duplicate egg hatch assay showed LD₅₀ to be between 0.046 and 0.054 µg/ml thiabendazole. Therefore, the investigated population of gastrointestinal strongylids is susceptible to benzimidazoles, as it has been shown that resistance occurs when the LD₅₀ value is equal to or greater than 0.1 µg/ml thiabendazole. Albendazole was used in the faecal egg count reduction test. It was determined that reduction of egg output was 100 %. The faecal egg count reduction test confirmed the result of egg hatch assay and definitely detected the population of gastrointestinal strongylids as susceptible to benzimidazoles.

For all species included in study, the nucleotide sequence for ITS2 and 5.8S region of rDNA was determined. Additionally, for *C. ovina* the nucleotide sequence for ITS1 region was determined. For this purpose, PCR was performed for ITS1, 5.8S and ITS2 region in three individuals of investigated species. Then, PCR products were cloned and nucleotide sequences of the ITS2 region in three plasmids were determined. The length of the 5.8S sequence was 153 bp for all species. The length of the ITS2 sequence ranged from 233 bp to 248 bp.

The length of the ITS2 sequence was 237 bp for *T. axei*. The G+C content was between 31.22 and

31.65 %. Polymorphism was detected at 2, 33, 130 and 189 positions. In our study, intraindividual variation for ITS2 sequence for *T. axei* ranged from 0 to 1.3 %, intraspecific variation was 1.7 %. In all clones obtained from *T. colubriformis*, 100 % identical nucleotide sequences for ITS2 region were determined. The length of the sequence was 238 bp, and the G+C content was 31.09 %. A comparison of all nucleotide sequences for *T. axei* and *T. colubriformis*, showed interspecific variation to be between 3.8 and 4.2 %.

The length of the nucleotide sequence of ITS2 region was 246 bp or 248 bp for *T. circumcincta*. The G+C content was between 33.47 and 34.55 %. The ITS2 sequence in *T. circumcincta* contained 9 polymorphic sites. Intraindividual variation ranged to a high of 2.1 %, and the intraspecific to 2.9 %.

For *C. curticei*, the intraindividual and intraspecific variation ranged to a high of 3.8 %. 13 polymorphic sites were detected, of which 4 had not been discovered before. The length of the ITS2 sequence was 242 bp for *C. curticei*. The G+C content was between 30.17 and 32.23 %.

For *C. ovina*, the length of the ITS2 sequence was 233 bp in five clones and 235 bp in four clones. The G+C content was between 44.26 and 45.06 %. The deletion of two nucleotides at positions 142 and 143 in the sequences of the length 233 bp was determined. Polymorphic positions were also detected at positions 56, 60, 62 and 80. The intraindividual and intraspecific variation of nucleotide sequences for ITS2 region ranged to a high of 2.6 %. The length of the sequence ITS1, 5.8S and ITS2 was 852 bp or 854 bp for *C. ovina*. The length of the ITS1 sequence was 370 bp. Polymorphic sites were detected at positions 258 and 303.

A comparison of the consensus nucleotide sequences for the ITS2 regions for all examined species was performed. It revealed 94.9 % identity between species *T. colubriformis* and *T. axei*. The identity between *T. colubriformis*, and *T. axei* and *T. circumcincta* was 75.5 % and 77.7 %, respectively. The similar percentage of identity was observed between the species of the genus *Trichostrongylus* and *C. curticei*. Thus, the identity between *T. colubriformis* and *C. curticei* was 74.4 %, and between *T. axei* and *C. curticei* 73.8 %. The identity between *T. circumcincta* and *C. curticei* was 71.1 %. The consensus nucleotide sequence of the ITS2 region of *C. ovina* revealed

60 % identity to the sequence of *T. axei*, 61.7 % identity to the sequence of *T. colubriformis*, 24.7 % identity to the sequence of *T. circumcincta* and 54.9 % identity to the sequence of *C. curticei*.

The research work was performed at the Institute for microbiology and parasitology, Institute for anatomy, histology and embryology and Institute for health care and breeding of wild animals, fish and bees, Veterinary Faculty in Ljubljana.

The date of public defense: July 8, 2003.

Mentor: Prof. Dr. Andrej Bidovec.

Malan Štrbenc: MYOSIN HEAVY CHAIN TRANSITIONS IN DOG SKELETAL FIBRES DURING POSTNATAL DEVELOPMENT

The contractile characteristics of individual fibre types are strongly correlated with the types of myosin heavy chain isoforms (MHC) they possess. At least 9 different MHC isoforms, coded by different genes, were shown to exist. The expression and distribution patterns of MHC vary greatly among animal species and this expression pattern in the skeletal muscles of the dog has not been previously described. The ability of skeletal muscle fibres to adapt to environmental changes and hormonal influences is well known; and the MHC-isoform transitions are a part of this mechanism. Similarly, MHC transitions occur during postnatal development, where developmental transform into adult forms of myosin.

The aim of this study was to establish a clear pattern of muscle fibre types in a few skeletal muscles of dogs of different ages. Special emphasis was put on MHC isoform content and their transitions during development using immunohistochemical methods.

Samples of longissimus dorsi, rhomboideus, triceps, extensor carpi radialis, semitendinosus, sartorius, rectus femoris, tibialis cranialis, diaphragm and masseter muscles were studied in this research. The samples were obtained after the routine euthanasia of 6 adult dogs and 18 puppies whose ages ranged from late prenatal to 6 months. Eleven monoclonal antibodies were used in the immunohistochemical demonstration of MHC: MHC-s (slow isoform), MHC-f (fast isoforms), MHC-d (developmental isoform) and MHC-n (neonatal isoform) from Novocastra, and A4.74 (detects MHC-IIa), F113.15F4 (detects Ila and Iib), F185.4C10 (neonatal isoform), F1.652 (embryonal isoform) and F88-12F8 (MHC- α) from Alexis Biochemicals. Donated BF-F3 (detects

MHC-IIb in rats) and BF-35 (reacts with all isoforms except MHC-IIx) antibodies were also used. The isoforms were separated by SDS-PAGE and the identity of the gel bands was confirmed by Western-Blotting. The results of the immunohistochemical staining were compared using the classical fibre classification system, which is based on mATPase activity and the activity of the metabolic enzymes SDH and α -GPDH that was established in the same fibres.

In adult dog muscles three major fibre types were ascertained according to mATPase activity technique and MHC content: type I with MHC-I isoform, IIA with MHC-IIa, and IIDog (IIA/X), which contained a mix of MHC-IIa and MHC-IIx. A smaller percentage of IIC fibres, which are hybrid fibres containing MHC-I and MHC-IIa, was also shown. The percentage of hybrid fibres, especially the Ila/Iix combination, varied greatly among the individual animals, although a very high percentage of IIA/X fibres was always noted in the masseter muscles (84 %). The electrophoretic separation indicated the presence of a different MHC in the masseter muscle, however, a special masticatory isoform could not be confirmed using the available antibodies. We have demonstrated that, due to the liability of mATPase, classical fibre-typing can only be performed by using several pre-incubation solutions. We believe that the major cause for the discrepancies between previous studies in the classification of dog fibre types lies in the varied content of the MHC isoforms in hybrid fibres and a relatively high percentage of the hybrid fibres.

Fast fibres could be distinguished from the slow ones by their higher α -GPDH activity but SDH activity was relatively high in all types of fibres, indicating the adaptation of dog muscles to endurance exercises.

In the perinatal and early postnatal periods a prevalence of developmental isoforms (MHC-emb and MHC-neo) was demonstrated. A primary fascicle in an undeveloped muscle has a centrally located fibre with a greater diameter and a prominent MHC-I expression, which is representative of a primary slow fibre. During the first ten days after birth, the quantity of embryonal isoform decreased, as did that of the neonatal isoform during the second and third weeks. Correspondingly, the expression of MHC-IIa, and later, that of MHC-I increased. After six weeks of age the presence of MHC-IIx became obvious, but

at first only in the masseter muscles. Based on the increasing number of fibres in the primary fascicles and the slow increase of the average fibre diameter, we have assumed that the last generation of fibres form as late as the perinatal or early postnatal period. It was also noted that there was a lengthy retention of the MHC-neo isoform in some small fibres in the triceps, rectus femoris, longissimus dorsi and masseter muscles; up to six months of age while it disappeared in other muscles. Enzyme-histochemical differentiation was not possible in puppy muscles since they possess developmental MHC isoforms with different mATPase characteristics. Both the enzyme-histochemical and immunohistochemical reactions came into general agreement by 2 months of age.

The transitional pattern was similar in all muscles except in the rhomboideus, where secondary slow fibres were present before birth. In the other muscles the secondary slow fibres evolved from developmentally-fast fibres between the second and third week of age. The maturation of muscles was not simultaneous; the fastest developing muscles were the masseter muscles and the diaphragm. These muscles are already fully functional at birth. Development followed in locomotory muscles: rhomboideus, extensor carpi radialis and tibialis cranialis, and later, the longissimus dorsi, triceps brachii, semitendinosus and sartorius muscles. Comparatively, m. rectus femoris was the least developed muscle at birth, however, its development reached the same level as the other muscles during the postnatal period. A pronounced swap from developmental to adult isoforms was noted between the 4th and 6th week of age. At this age puppies are weaned and begin playing intensively and investigating their surrounding.

Comparing our results with those from research in other domestic animals we have ascertained that canine skeletal muscles develop relatively slowly.

The research work was performed at the Institute for anatomy, histology and embryology, Veterinary Faculty in Ljubljana and Institute for anatomy, Medical Faculty in Ljubljana.

The date of public defence: October 28 , 2003.

Mentor: Assoc. Prof. Dr. Gregor Fazarinc.

Vesna Kadunc Kos: MATRIX METALLOPROTEASES IN SYNOVIAL FLUID AS DIAGNOSTIC MARKERS FOR DETECTING

OSTEOARTHRITIS IN DISTAL INTERPHALANGEAL JOINT IN HORSES

The purpose of the study was to evaluate the results of arthroscopic and x-ray examination of horses which were lame because of pain in the interphalangeal joint and healthy horses and to evaluate the presence of different forms MMP-2, MMP-9 and MMP-3 in the synovial fluid. Single diagnostic parameters were evaluated and the contribution of the determination MMP in synovial fluid to the diagnostic of OA was estimated.

Clinical examination was performed on forty horses, which were classified in five groups based on arthroscopically visible lesions on the cartilage surface of the hoof joint, and lesions visible on x-rays:

- group 1: extensive cartilage damage, no x-ray visible changes
- group 2: mild cartilage damage, no x-ray visible changes
- group 3: extensive cartilage damage, changes visible on x-ray
- group 4: cartilage damage because of a chip fracture, changes visible on x-ray
- group 5: no cartilage damage, no x-ray visible changes

More horses were lame on one or both of their fore legs (87.5 %) than on one of their hind legs. For the first and second groups, in which mild cartilage damage was visible arthroscopically while the x-rays showed no visible lesions, the difference in the degree of lameness compared to the third group, in which arthroscopic examination showed extensive cartilage damage, was not statistically significant ($P > 0.05$). However a statistically significant difference in the degree of lameness was found between the third group, in which extensive cartilage damage was visible arthroscopically, and the fourth group, in which arthroscopic examination showed a cartilage fragment in the joint cavity ($P = 0.002$). The correlation between arthroscopy results and the toe bending test was not statistically significant ($P > 0.05$).

In 28 joints of horses from the first, second and third groups, in which arthroscopy showed cartilage damage in the palmar area of the hoof joint, x-rays confirmed OA only in 60.7 % of cases. The correlation between x-ray results and arthroscopic results was not statistically significant ($P = 0.926$). More horses were lame on one or both of their fore legs (87.5 %) than on one of their hind legs.

Gelatinase activity of MMP-2 and MMP-9 in the synovial fluid of all horses was tested in NaDS-polyacrylamide gel with copolymerised substrate. Inhibition of gelatinase activity using EDTA confirmed that the detected enzymes were matrix metalloproteases. The presence of the monomeric forms of MMP-2 (64 kDa) and MMP-9 (94 kDa) was found in all control samples and in all samples from diseased joints. In addition to monomeric forms, the synovial fluid of horses with OA also contained the dimeric form of MMP-9 (225 kDa) in 50 % of cases and above all the active form of MMP-2 (59 kDa) in 60.7 % of cases and the active form of MMP-9 (84 kDa) in 64.2 % of cases, which were present to a significantly greater extent in OA samples than in control samples. In the samples of the fourth group of horses, on the other hand, the active forms appeared in only 20 % of cases and the dimeric form of MMP-9 in only 40 % of synovial fluid samples. Diagnostic parameters such as sensitivity, specificity, accuracy, predictive value for the positive result and predictive value for the negative result were calculated for the x-ray method and for determination of active forms of MMP-2 and MMP-9, while arthroscopy served as the diagnostic criterion. Sensitivity was the highest in the case of presence of the active form of MMP-9, which indicates that in 64.2 % of cases OA can be predicted on the basis of the active form of MMP-9. The specificity of the x-ray method was 100 %, because in all healthy joints of the control group, in which arthroscopy did not show any joint cartilage changes, x-ray also yielded a negative result. However, some healthy samples also contained the active form of MMP-2 and MMP-9, therefore, the specificity of this method was slightly lower (88.2 % and 82.3 %, respectively). The sensitivity for the x-ray method was only 42.8 %, which indicates that OA was rarely confirmed with the x-ray method. A method's accuracy, i.e. the average between its specificity and sensitivity, was the highest (74.4 %) for the determination of the active form of MMP-2 in the synovial fluid. Our results indicate that in cases where the x-ray shows lesions characteristic of OA, determination of MMP in the synovial fluid does not contribute to diagnosis. But if arthroscopically visible changes in the joint cannot be confirmed with an x-ray, OA can be predicted in 81.8 % of cases with the presence of the active form of MMP-2 and in 76.9 % of cases in the presence of the active form

of MMP-9 in the synovial fluid. The difference in the presence of both active forms of MMP-2 and MMP-9 between samples with mild or extensive cartilage damage, was not significant, so it can be concluded that their presence does not depend on the severity of the disorder.

Multiple logistic regression confirmed that there is a correlation between the arthroscopy method and the determination of the active form of MMP-2 ($P = 0.006$), as well as between the arthroscopy method and determination of the active form of MMP-9 ($P = 0.007$). No correlation was found between the arthroscopy method and the x-ray method using multiple logistic regression ($P = 0.926$). The Western immunoblotting method was used in the determination of various forms of MMP-3. Immunogenic bands with molecular weights of 23 kDa, 26 kDa, 28 kDa, 50 kDa and 74 kDa appeared in both the control samples and in positive samples. This shows that the determination of individual forms of MMP-3 does not have a diagnostic significance for the detection of OA, at least not by using above mentioned method.

The research work was performed at the Clinic for reproduction and horses, Veterinary Faculty in Ljubljana and Horse clinic in Zorneding, Germany.

The date of public defense: December 9, 2003.

Mentor: Prof. Dr. Marjan Kosec.

INSTRUCTIONS FOR AUTHORS

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Tables, graphs, diagrams, figures, etc. may be enclosed separately or logically incorporated in the text file. They should be referred to by type and using Arabic numerals (e.g. Table 1.; Figure 1.; etc.). The colon should be followed by the text or title. On the reverse side of these items the name and surname of the first author, the title of the paper, and the name and number of the item should be supplied. Indicate, if necessary, where the item should be placed (top, bottom). All references cited in the text should appear in the References. They should be numbered in the text in the order in which they appear, marked with Arabic numerals placed in parenthesis. The first reference in the text should determine the number and order of the respective source in the References. If the author refers again to a source which has already been used in the text, he should cite the number the source had when it was referred to for the first time. Only works which have been published or are available to the public in any other way may be referred to. Unpublished data, unpublished lectures, personal communications and the like should be mentioned in references or notes at the end of the page on which they appear. Sources in the References should be listed in the order in which they appear in the text.

If the source referred to was written by six authors or less, all of them should be cited; in the case of seven or more authors, only the first three should be cited, followed by 'et al.'.

Any errata should be submitted to the editor-in-chief in good time after publication so that they may be published in the next issue.

Examples of references

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

Chapter or article in a book: Baldessarini RJ. Dopamine receptors and clinical medicine. In: Neve KA, Neve RL, eds. The dopamine receptors. Totowa: Human Press, 1996: 475-98.

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

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

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