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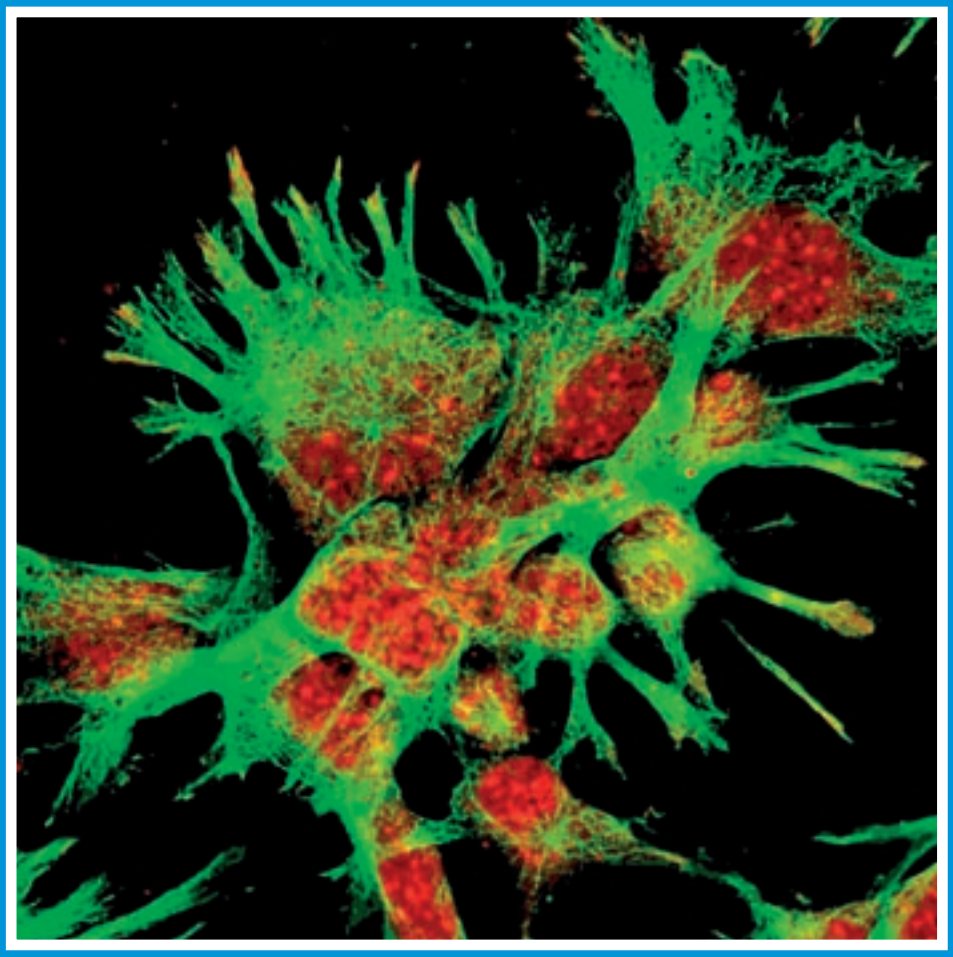

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Review Paper

AXOLOTL - A SUPERMODEL FOR TISSUE REGENERATION

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Summary: The process of regeneration - the regrowth of tissue or body part, have been focus of human attention for many centuries. Among vertebrates, the ability to regenerate lost tissue or organs is best developed in urodele amphibians, such as axolotl (Ambystoma mexicanum), which can replace lost tissue throughout adulthood. Axolotl is neotenic amphibian that can regenerate wide range of organs including limbs, tails, gills, heart, spinal cord, jaws. Among the organs they can regenerate, the limb has been most widely studied so that it provides the basis of knowledge about the mechanisms regulating tissue regeneration. Limb regeneration progresses through a series of steps: wound healing, dedifferentiation and re-differentiation. A number of genes involved in the activation of regeneration have been already identified and cloned, but to this moment, exact molecular mechanisms of regeneration process are not yet known. With the new techniques of functional genomics that will help us to identify candidate genes and testing their functions, there are good prospects to discover the whole mystery of regeneration and achieve the ultimate goal - regeneration in mammals.

Key words: regeneration-genetics; cell differentiation; gene expression regulation, developmental; *Ambystoma mexicanum*

Introduction

Regeneration is the process of restoring a missing part of a tissue or an organ. Complete regeneration is present in most of the lower species but among vertebrates, only urodele amphibians are capable of complete regeneration of number of different tissues such as limb, tail and brain even as adults (1, 2). Among these organs, the limb has been most widely studied and therefore provides the basis of our knowledge about the mechanisms regulating tissue regeneration (3).

Limb regeneration is a process that goes through a series of steps. It starts with wound healing as reserved stem cells of epidermis migrate to cover the wound surface within few hours. Cells that lie underneath start to dedifferentiate and migrate under the WE (wound epidermis) to become mesenchymal-like stem cells that form blastema. Blastema then gradually begins to proliferate. Cells re-differentiate (by the process similar to the processes during embryonic development) to replace a missing limb (3-5).

A number of important conclusions came from studies of the urodele limb regeneration. We now know that complete regeneration is indeed possible, and number of key regulators has been identified. But even though the regeneration of salamander limb was first reported in eighteen century by Spallazani (6), the exact physiological and molecular mechanisms are still not clear. Resent advances in a field of functional genomics and cell biology are raising new hopes. With the high throughput techniques for screening of genes such as DNA microarrays, techniques for functional analysis such as transgenic salamanders, more efficient use of in vitro cell cultures and possibility of genetic manipulation (5, 7, 8), there is a better opportunity now to identify cellular and molecular events responsible for regeneration that will hopefully in the future give us opportunity to induce regeneration in other vertebrates and will help us to achieve long-lasting goal of human regeneration (9).

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Mexican axolotl *(Ambstoma mexicanum)*

Mexican axolotl (Ambystoma mexicanum) is a urodele amphibian (Figure 1). They are neotenic, so they remain in its aquatic larval form even as sexually mature adults and are not undergoing metamorphosis into a terrestrial form. Metamorphosis is very rare and occurs as a response to an extreme habitat or as a result of iodine or hormone thyroxine injections. Most attempts at inducing metamorphosis lead to death, although some were successful but animal lifespan was shorten drastically. Axolotls live usually more than 12 years. A fully grown axolotl ranges in length from 15-45 cm. Their natural habitat is Lake Xochimilco in Mexico, but as laboratory animals, they are bred all over the world.

They have distinctive "fern-like" gills that are not covered, usually three stalks on each side of the head; the colour of the fern-like part varies although the usual colour is red. Axolotls also breathe through the skin and possess lungs. They have tiny teeth that are hardly noticeable and are used to grip food rather than to tear and chew it. They are carnivores consuming small prey such as worms, insects, and small fish. Axolotls live in salty water (the optimal mixture is called Hoeltfreter's solution and is a mixture of different salts) with optimum temperature around 18°C. Temperature above 23°C can lead to stress and increased appetite (10).

They are capable to regenerate wide range of organs including limbs, tails, gills, heart, spinal cord and jaws throughout their whole life (11). The regeneration is very rapid (usually a couple of weeks) and the animals are easy to handle, so they provide excellent model organisms for studying mechanisms of regeneration in vertebrates. Among organs that can regenerate, limb has been most extensively studied.

Limb regeneration

Regeneration of axolotl limbs occurs by the process of dedifferentiation. It is completed within a month. The timing of limb regeneration depends on axolotl size and age and it is very consistent. Usually older axolotls regenerate longer. There is no limit in the number of amputation of the same limb; each time the limb will regenerate completely. Regeneration progresses through a series of steps. Minimum requirements for limb regeneration are: a skin wound, adequate innervations and positional diverse blastema. This requirements separate the process into three distinct phases (Figure 2) (1, 2, 4). The first phase is wound healing, phase II is re-differentiation and phase III is dedifferentiation. The most unique is a second phase of re-differentiation while the dedifferentiation is a recapitulation of developmental phase with some differences (4). Summary of regeneration events is presented in figure 3.

Phase I: Wound healing

Within the first hour the epithelial cells from basal lamina begin to migrate to close the wound. Wound closure is incredibly fast; in young axolotls it is closed in 4 hours. Compared to mammals where it takes days, the speed is extraordinary. Outcomes of rapid wound healing include minimizing damage, infection and inflammation (12, 13). The wound is healed without a scar. Wound epidermis (WE) is formed which is crucial for the onset of regeneration and is essential for triggering expression of particular genes. If it is removed, the regeneration does not proceed. Covering the amputation wound surface with mature skin completely inhibits the regeneration response. The WE is crucial for epithelial/mesenchymal interaction (4, 8, 11). Extracellular matrix restructuring is initiated with MMP (matrix metalloproteinases) MMP-2, MMP-9 and MMP-13. Msx-2 gene is also expressed prior to wound closure, so it does not depend on WE as does expression of all other genes (11, 14). Recently, overexpression of Msx-1 was found to be important for inhibition of differentiation, suggesting that this gene may play a role in maintaining cells in a undifferentiated state (13, 15, 16), and it might be expressed at the same time as Msx-2. WE than begins to thicken and cells dedifferentiate to form AEC (apical ectodermal epithelium) that promotes limb regeneration. This stage is not dependent on nerves (4, 17). It is similar to wound healing in other vertebrates with some crucial exceptions; the inflammation response in axolotl is very mild and results in low cytokine synthesis whereas in mammals, there is apparently high concentration of interleukins 1 and 6 (IL-2 and IL-6) and some other cytokines that activate scaring and consequentially inhibit regeneration. Another difference is the presence of proteoglycan tenascin-C, which apparently helps to maintain differentiated state of cells (13).

Phase II: Dedifferentiation

This phase is unique to the process of regeneration. The phase II in regenerating limb is the period of limb regeneration when cells in the mature tissue start to dedifferentiate, losing their specialized functions and become migratory. Some days after amputation (day 2-3) cells begin to migrate under the WE and accumulate at the distal tip of the stump. At the same time as they dedifferentiate, they lose their specific function and than progress to proliferation. The final result is the genesis of population of undifferentiated, proliferated blastema cells (2, 4, 17) . Although overlying epidermis does not contribute to the blastema, it does influence the location at which blastema cells accumulate. Blastema arise from cells located 1-2 mm of the amputation plane. There is some evidence that all tissue participates in formation of blastema, several experiments demonstrated that the majority of cells that are essential for growth control and pattern formation in blastema are fibroblast of the connective tissue from amputated stump (2). The first divergence of phase II regeneration to normal skin regeneration is expression of Hoxa-9 and Hoxa-13 genes in the distal part of the stump 12-24h after amputation. Hox genes in general regulate morphogenetic events during regeneration. HoxA gene is responsible for specification of proximal-distal limb axis and may be the first transitional event on the limb regeneration pathway (18). Also HoxD (HoxD8, HoxD10) genes are expressed that play a role in re-expression of positional memory. Specification of the distal-most region of the pattern (autopod) is a consequence of co-expression of both 3' and 5' members of HoxA and HoxD, which is in contrast to the events during limb development (19). The early establishment of the distal tip of the limb ensures that the regenerate will always be a exact replacement of the amputated portion of the limb (4, 11, 17).

It is not yet clear, which are the factors responsible for activation of dedifferentiation and regeneration in generally but some of them definitely are connected to inervation. Phase II is dependent on nerves (4). If nerve supply is cut off, regeneration does not proceed. The nerves exert their effect through FGF-mediated pathway (11). Genes like fgf-1, fgf-2 and their receptors (fgfr-1, fgfr-2) are expressed in AEC. There is also a gene that is known to involve altered mechanisms of regulation in response to enervation. Dlx-3 (distal less) expression starts at early bud blastema and peaks at the transition from nerve dependency to independency of regeneration. Dlx-3 expression is up-regulated in blastemal stages of regeneration and is dependent on signals from nerves. Denervation inhibits Dlx-3 expression but addition of FGF sustains Dlx-3 expression and rescues regeneration (11, 20).

Phase III: Redevelopment

At the beginning of phase III, the large mass of undifferentiated cells (blastema) begins to differentiate and behave like a developing limb (4). Similar genes are expressed as in developing limb, but with some differences. Among expressed genes is sonic hedgehog (shh), which is expressed transiently in a small group of posterior cells and plays a crucial role in the establishment of the anterior-posterior limb pattern during both limb development and regeneration (21). Fgf-10 expressed in blastemal cells stimulate the expression of shh (22). In phase III genes that are characteristic of late stage regenerating limbs are expressed (HoxD11, Dlx-3). Expression of HoxC10 is specific for late phase blastema. Genes within HoxC complex are involved in specification of positional identity along the rostro-caudal axis. HoxC-10 is expressed in regenerating forelimbs and hind limbs, while in the development; it is expressed only in hind limbs, what indicates the presence of regeneration-specific signals (23). Cell differentiation that occurs in this phase is nerve-independent, but continued growth depends on nerves (17). In less than 6 weeks morphologically perfect limb is regenerated and from that moment on it only grows to reach its normal size.

Functional analysis

For many years studies concerning regeneration in urodels could not progress as wished due to the lack of tools for functional analysis of gene expression. In recent years, however, different approaches have been developed to achieve this goal. By far the most important approach in functional genomic analyses is knock-out and transgenic technology developed in mice. However, these technologies are very difficult to apply to axolotl models because of axolotl slow sexual maturity and lack of knowledge about their genome. Very recently, a big progress have been made in the development of transgenic salamander (7, 8). Transgenic axolotls were also generated by using I-Sce1 meganuclease method which is used to produce transgenic frogs and

fishes (24). Both methods resulted in the expression of the reporter green fluorescent protein in all tissues observed. In addition, tissue-specific promoters have already been shown to work in salamanders. By using this technology, one can study overexpression of cDNAs to increase protein expression or short hairpin RNAs to inhibit gene expression of a specific gene (8). There are other techniques for performing functional analysis such as overexpression of a gene introduced to a cell by electoporation, or shutting down a gene by antisense morpholinos (25). Furthermore, vaccine viruses have been used to introduce shh gene, that causes polydactyly (26). Different way to perform functional analyses is the pharmacological approach. Cyclopamine is a natural compound isolated from cabbage and is a potential inhibitor of hedgehog signalling. Treatment of axolotls with cyclopamine resulted in loss of a regeneration (27). All these tools will help us to answer questions pertaining to the functional roles of specific genes in different phases of regeneration.

Regeneration in mammals

Regeneration capabilities are very good in mammal embryos, but regeneration of body parts in adult mammals is much more limited. There is, however, continuous renewal of tissue in mammals as a part of the tissue homeostasis such as gametogenesis, skin-renewal, haematopoiesis, and in some other processes. Such homeostasis is achieved mostly by stimulation of stem cells and for this reason, most studies of regeneration focus on the role of stem cells (28). Some tissues in digestive system also have great potential for renewal. Liver and intestine are the most prominent organs. It is known that after partial hepatectomy (as much as one third of a liver), the remaining lobes of the liver enlarge to replace the missing tissue by the process of proliferation without any apparent dedifferentiaton or transdifferantiation (29).

There is also expanding evidence about olfactory-neuron regeneration and regeneration of new neurons in adult brain. Many attempts have been made to induce neuronal regeneration by applying FGF and integrins, but complete regeneration of part of CNS has yet to be achieved (30).

Many researchers have shown the plasticity and capability of adult and embryonic stem cells to repopulate tissues such as brain, heart, retina but so far no successful restoration of missing tissue or an organ has been documented (31). Apart from the focus on stem cells, the researchers should perhaps also focus their attentions more to one example of complete regeneration of tissue seen in mammals - regeneration of a digit tip in mice and in humans. The term “fingertip” refers to a part of the digit extending distally to interphalangeal joint (15). This phenomenon was first described for children but latter shown to extend to adults. If a wound after amputation is simply covered with sterile dressing and allowed to heal, it will result in perfect restoration of a portion of a finger after 5 weeks. While in mice, the process of digit tip regeneration goes through the formation of blastema, fibroblastic cells appear to be involved in the regeneration in humans. In mice, the capacity for digit-tip regeneration has been correlated to BMP (bone morphogenic factor) and Msx-1 expression, perhaps to maintain the dedifferentiation state of the blastema (28).

Prospects

It is a great interest of regeneration researchers to understand the difference between species that can regenerate lost tissue and those that can not. In case of humans and urodels, this is the matter of current interest in regenerative medicine. With the new techniques for identification of candidate genes and testing their functions, there are good prospects for enhancing regeneration in mammals, what is the ultimate goal of these studies. Axolotl model gave us a proof that regeneration is indeed possible. The fact that human embryos can regenerate more extensively and that some of those regeneration capabilities extend into an adult life, shows us that mechanisms are presents within us, we just have to find out how to activate and control them.

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AKSOLOTL – SUPERZVEZDA PRI REGENERACIJI TKIVA

M. Goršič

Povzetek: Razumevanje procesa regeneracije-ponovne rasti tkiva ali dela telesa ter njegova spodbuditev pri organizmih, ki tega sami niso sposobni, že več stoletij predstavlja izziv raziskovalcem. Med vretenčarji je sposobnost regeneracije tkiv in organov najbolje razvita pri repatih dvoživkah, kamor spada tudi aksolotl (*Ambystoma mexicanum*), ki regenerira tkiva skozi vse življenje. Aksolotl je neotenična dvoživka, sposobna regeneracije različnih organov, kot so okončine, rep, škrge, srce, hrbtenjača in čeljust. Med temi organi je najbolj proučena okončina, ki predstavlja osnovo znanj o regulatornih mehanizmih regeneracije. Proces regeneracije okončine poteka prek različnih stopenj: celjenje rane, dediferenciacija in rediferenciacija celic. Nakateri geni, vpleteni v aktivacijo regeneracije, so že znani in klonirani, natančni molekularni mehanizmi pa še niso jasni. S pomočjo novih tehnik funkcijske genomike in proteomike, ki nam bodo pomagale identificirati kandidatne gene in preveriti njihovo funkcijo, se odpirajo nove možnosti spoznavanja skrivnosti regeneracije in končno doseči najvišji cilj - regeneracijo pri sesalcih.

Ključne besede: regeneracija-genetika; celična diferenciacija; gensko izražanje, regulacija razvojna; *Ambystoma mexicanum*

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Original Research Paper

MICROBIOLOGICAL SCREENING METHOD FOR DETECTION OF AMINOGLYCOSIDES, β-LACTAMES, MACROLIDES, TETRACYCLINES AND QUINOLONES IN MEAT SAMPLES

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Summary: Antibiotics are natural products of a micro-organism, identical synthetic products or similar semi synthetic products that inhibit the growth of or destroy microorganisms. In veterinary medicine antibiotics are used for therapeutic, prophylactic, metaphylactic and nutritive purposes. The presence of antibiotics or their metabolites in food is potentially hazardous to health as it may cause allergic reactions in people and antibiotic resistance in pathogenic microorganisms. In addition antibiotics may influence starter cultures in food industry and lead to economic damage. The methods used to detect antibiotic residues in food of animal origin are microbiological, immuno-enzymatic and chemical. Microbiological methods are used as screening methods whose results direct the choice for more expensive and time consuming chemical confirmation methods. To serve this purpose the sensitivity of a microbiological method must be at or below the prescribed maximum residue level (MRL) for specific antibiotic group.

The purpose of our study was to develop a microbiological method for detection of macrolide, aminoglicoside, β-lactame, quinolone and tetracycline antibiotic families in meat by identifying the most appropriate set of bacterial test strains and to establish the limit of detection for each antibiotic group. *Micrococcus luteus* ATCC 9341 was selected as the most appropriate sensitive strain for detection of macrolides and β-lactams, *Bacillus subtilis* BGA for aminoglicosides, *Bacillus cereus* ATCC 11778 for tetracyclines and E. coli ATCC 10536 for quinolones. In addition magnesium sulphate was used to inactivate aminoglicosides β-lactamase to differentiate between macrolides and β-lactames. For all antibiotic groups the level of detection was below the MRL.

Key words: food analysis-methods; antibiotics; drug residues-analysis; microbiological sensitivity tests-methods; meat

Introduction

Antimicrobials are classified according to their chemical structure. Each class is characterized by a typical core structure and various members of the class are differentiated by the addition or removal of secondary chemical structures (1, 2). Antimicrobials can also be classified as broad or narrow spectrum, depending on the range of bacterial species against which they are active, or as bacteriostatic or bactericidal on the basis of their mechanism of action. The latter fall into four categories: inhibition of cell wall synthesis, damage to cell membrane function, inhibition of nucleic acid synthesis or function, and inhibition of protein synthesis. The aim of antimicrobiological therapy is to rapidly produce and then maintain an effective concentration of drug at the site of infection for sufficient time to allow host specific and nonspecific defenses to eradicate the pathogen (3).

The most commonly used antimicrobials in food-producing animals are β-lactams, tetracyclines, aminoglycosides, quinolones, macrolides and sulfonamides. Antimicrobials are administered to animals by injections (intravenously, intramuscularly, or subcutaneously), orally in food or water, topically on the skin and by intramammary and intrauterine

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infusions (4). Theoretically, all these routes may lead to residues appearing in foods of animal origin such as milk, meat and eggs (5).

Acquisition of resistance to antimicrobial agents by consuming food of animal origin has been receiving increasing attention in the literature, also raising awareness of the importance of minimizing exposure to antibiotic residues in food (6, 7). The most common causes for the presence of antibiotic residues in food of animal origin are violation of withdrawal periods, overdosing of antibiotics and use of antibiotics banned for treatment of economic animals (8, 9, 10, 11). The 2377/90 Council Regulation (12) determines a limited number of drugs allowed in veterinary medicine and defines MRL for each drug. This Regulation represented a milestone in food control because in addition to mere identification it also requested quantitative determination of antibiotic residues, the strategy of analyses and subsequently the methods therefore had to be adapted to the new legislation.

Microbiological, immuno-enzymatic and chemical methods are used for detection of antibiotic residues in food of animal origin and the protocol of control is usually based on two steps: screening for presence of different antibiotic groups and confirmation with identification of specific antibiotic in the sample and more accurate quantitative analysis. An ideal screening method would detect all licensed antibiotics at or below their Marls and should be robust, rapid, simple and cost effective (13). Chemical methods are too specific to be applied as a first choice screening method for the high number of monitored substances. In addition chemical methods require more expensive equipment. Microbiological methods are better suited for the first step screening, but unfortunately a single bacterial inhibition test for all antibacterial residues does not exist (14).

Microbiological methods detect inhibitory substances diffusing from a piece of tissue (14, 15, 16) or from a paper disk soaked with tissue fluid (17) into an agar layer seeded with a susceptible bacterial strain. These methods are usually multiresidue screening tests able to detect several families of antimicrobial drugs and use one or more test plates which differ in bacterial strains, pH values of media and temperatures of incubation (15, 18, 19, 20, 21, 22, 23, 24). Ferrini et al (2006) reported a modification of such methods with the addition of neutralizing chemical substances that further help to narrow the spectrum of antibiotic families detected by a single test plate (24).

In our institution which is also the Slovenian National Reference Laboratory for detection of antibiotics in food of animal origin we have been using microbiological screening methods since 1968. These methods had to be adapted to the requirements of the above mentioned Council Regulation 2377/90. The aim of our study was to identify the appropriate combination of test strains for detection of aminoglycoside, macrolide, tetracycline, quinolone and beta-lactame antibiotic families and to determine the limit of detection (LOD) for each family in view of the prescribed MRLs for antibiotic residues. We also attempted to improve the specificity of different bacterial strains for different antibiotic groups by the addition of two neutralizing substances: β-lactamase and Mg SO_4.

Material and methods

The principle of method

Microbiological methods are based on measurement and evaluation of zones of inhibited bacterial growth on media. Two test strains are used to assess the presence of each antibiotic – one maximally sensitive and the other resistant to the tested substance. With the combination of different sensitive and resistant bacterial strains specific antibiotic groups present in the sample can be identified. These principles are followed in the STAR (screening test for antibiotic residue) protocol (22) on which our method is based.

Bacterial strains

In our research we used the following strains: Bacillus cereus ATCC 11778 (Remel, Lenexa, USA), Micrococcus luteus ATCC 9341 (Remel), Escherichia coli ATCC 10536 (TCS Bioscience, Buckingham, UK), Staphylococcus epidermidis ATCC 12228 (Remel) and Bacillus subtilis BGA(Merck, Darmstadt, Germany). For confirmation solutions we used β-lactamase (BioChemika, Seelze, Germany) at the concentration of 5 mg/ml and $MgSO_4$ (Sigma&Aldrich, Taufkirchen, Germany)

Antibiotics

Antibiotics used in the study are described in Table 1. The procedure or preparing the standard and working solutions are described in the section Preparation of working solutions and meat samples.

Table 1: Antibiotic standards

ANTIBIOTIC	PRODUCER	PURITY (%)
Chlortetracycline hydrochloride	Sigma – Aldrich/ Taufkirchen/Germany	83.0
Streptomycin sulfate	Sigma - Aldrich	95.8
Tetracycline hydrochloride	Sigma - Aldrich	97.3
Neomycin	Calbiochem/Darmstadt/Germany	67.8
Penicillin G potassium salt	Sigma-Aldrich	99.8
Cephalexin	Sigma - Aldrich	99.7
Enrofloxacin	Sigma - Aldrich	98.1
Ciprofloxacin	Sigma - Aldrich	98.5
Amoxicilin	Sigma - Aldrich	99.9
Tylosin	Sigma - Aldrich	90.8
Erythromycin	Sigma - Aldrich	99.1

Culture media

Basic media for preparation of test plates were antibiotic agar No. 1 (Merck) and antibiotic agar No. 2 (Merck). Antibiotic agar No. 1 was prepared as follows: 1000 ml of distilled water was added to 30,5 g of the medium, left for 15 min and then heated to boiling point so that the medium was completely dissolved. The medium was then autoclaved at 121 °C for 15 min. For antibiotic agar No. 2 1000 ml of distilled water was added to 15,5 g of medium and then the same procedure was followed.

Preparation of test plates

Bacterial strains stored as cultures in original bacterial loops (Culti loop) were applied to a test tube containing 1ml Trypton soy broth (Oxoid, Hampshire, UK) medium and incubated at 37 °C for one hour. The culture was then inoculated on blood agar and incubated for further 16 hours at the same temperature. Afterwards the purity of bacterial colonies was assessed with a light microscope and pure colonies were stored in a fridge at temperatures between 2 and 8 °C for up to one month. Before the composition of test plates a suspension of bacterial culture stored on blood agar was prepared and incubated at 37 °C for one hour. Density of the suspension was standardized with the Mc Farland method (Table 2).

Test plates were prepared as described in Table 2. To prepare each test plate 0,45 ml of suspension of bacterial culture was added to 40 ml of basic medium heated to 40 °C. Kin plate was an exception

Table 2: Assay plates

plate	bacterial strain	cfu/ml of agar	agar medium	pH	incub. temp. (°C)	McFarland standard
AC	Micrococcus luteus ATCC 9341	$5.6x10^6$	Antibiotic medium No 1	6.0	30	4
ER	Staphylococcus epidermidis ATCC 12228	$1.9x10^7$	Antibiotic medium No 1	8.0	37	0.5
I-BGA	Bacillus subtilis BGA	$1.9x10^7$	Antibiotic medium No 2	6.0	30	0.5
KIN	Escherichia coli ATTC 10536	$1.6x10^7$	Antibiotic medium No 2	8.0	37	1
E	Bacillus cereus ATCC 11778	$6.6x10^6$	Antibiotic medium No 2	6.0	30	0.5

where 0,2 ml of suspension was added to 50 ml of medium. The mixture of medium and bacterial culture was poured into a 90 mm diameter Petri dish (5 ml of mixture into each Petri dish) and after 15 min at room temperature the Petri dishes with solidified medium were enveloped in a parafilm and stored in a fridge. The storage period of test plates was seven days. Before application of samples to test plates, plates were warmed at room temperature for 20 to 30 min.

Confirmation solutions

To confirm the presence of antibiotic groups or their individual representatives we used confirmation solutions. They inhibit the action of certain antibiotics and can help to distinguish antibiotic groups which cause inhibition zones on the same test plates. Magnesium sulphate ($MgSO_4$) was used to neutralize the aminoglicosides and β-lactamase enzyme to neutralize the β-lactams.

25 µl of 20% $MgSO_4$ solution in water was added to the sample on E, AC and I-BGA plates where inhibition zones are produced by aminoglicosides, macrolides or tetracyclines. 25 µl of β-lactamase was added to samples on AC and I-BGA plates to identify cephalosporins.

Preparation of working solutions and meat samples

First standard antibiotic solutions had to be prepared from reference standard antibiotics of known chemical composition and purity (Table 1). Standard antibiotics in powder were dissolved in appropriate solvents: tetracyclines in phosphate buffer (Merck) with pH value 4,5, β-lactames in phosphate buffer with pH value 6,0, aminoglicosides in phosphate buffer with pH value 8,0, quinolones in pH 8 and macrolides in methanol (J.T. Baker, Deventer, Netherlands). Standard solutions were then diluted to desired concentrations with 1 mg/ml to create working solutions which were then added to meat samples as follows. One milliliter of working solution was added to 9g of minced beef formed into a sphere and left to diffuse throughout the meat. After 1 hour the meat sample with the disposed working solution was transferred to a 10-ml test tube and heated to 80°C for 5 min to avoid later non-specific reaction on test plates due to antagonizing micro flora in meat. This procedure was shown not to affect the concentration of antibiotic (25). After heating, the samples were compressed to obtain a liquid meat extract; 100ml of the extract were transferred to test plates in 8 mm wide cylinders. Detection levels were obtained by placing 100 µl meat extract obtained from meat sample containing the working solution with known concentration of antibiotic into cylinders. The range of working solutions is shown in table 2. For the evaluation of reversible concentrations, 75 µl of standard solutions were applied to cylinders with addition of 25 µl of confirmatory solution. Each concentration of antibiotics was tested 10 times.

Test plates AC, E, I-BGA were incubated at 30 °C and plate ER and Kin at 37 °C for 18-24 hours (20, 24).

Evaluation of results

Results of microbiological methods can be evaluated both qualitatively and quantitatively. Qualitative results are obtained by analyzing the effect of antibiotics on a combination of sensitive and resistant bacterial strains. When required neutralizing substances (confirmation solutions) can help to differentiate between antibiotics with similar action on test bacterial strains.

Results

We have identified sensitive and resistant bacterial strains for all antibiotic groups tested in our study. Based on our results we chose to use *Bacillus cereus* ATCC 11778 (E plate) as the sensitive and *Micrococcus luteus* ATCC 9341 (AC plate) as the resistant strain for the tetracyclines group. For macrolides group *Micrococcus luteus* ATCC 9341 (AC plate) was chosen as the sensitive and *Escherichia coli* ATCC 10536 (Kin plate) as the resistant strain. For the aminoglicosides group *Bacillus subtilis* BGA (I-BGA plate) was chosen as the susceptible and *Staphylococcus epidermidis* ATCC 12228 (ER plate) the resistant strain. For β-lactam group from the sensitive group *Micrococcus luteus* ATCC 9341 (AC plate) was chosen as the susceptible and *Staphylococcus epidermidis* ATCC 12228 (ER plate) as the resistant strain. For quinolones group *Escherichia coli* ATCC 10536 (Kin plate) was chosen as the sensitive strain.

Table 3 shows the limits of detection for meat samples containing standardized antibiotic solutions on selected test plates and also the limits of detection for pure standard solutions. The LOD was

Table 3: Detection levels, MRL, range of working solutions and diameters of inhibitions zones of antibiotics

Antibiotic	Bacterial strain/plate	LOD st.s. (µg/kg)	LOD meat samples (µg/kg)	MRL bovine meat (µg/kg)	Range of working solutions (µg/kg)	Diameters of inhibitions zones (mm)
Cephalexin	M.l.1/ AC	50	50	200	50-250	11.87-19.75
Tetracycline	B.c/ E	30	50	100	50-150	17.23-22.0
Chlortetracycline	B.c/ E	40	50	100	50-150	18.65-22.5
Erythromycin	M.l.1/ AC	50	50	200	50-400	10.45->25
Tylosin	M.l.1/ AC	30	100	100	100-200	11.14-13.5
Neomycin	B.s.BGA/ IBGA	50	100	500	100-1000	9.91->25
Streptomyicin	B.s.BGA/ IBGA	80	100	500	100-1000	11.37->25
Penicillin	M.l.1/ AC	4	20	50	20-100	10.87-18
Amoxicilin	M.l.1/ AC	4	20	50	20-100	11.31-19.3
Enrofloxacin	E.c./KIN	20	50	100	50-200	10.17->25
Ciprofloxacin	E.c./KIN	20	30	100	30-200	11.35->25

LOD st.s limit of detection of standard solution
LOD meat limit of detection in meat samples
MRL meat maximum residue level in meat samples
B.c/ E Bacillus cereus ATCC 11778/ plate E
M.l.1/AC Micrococcus luteus ATCC 9341/ plate AC
B.s.bga/IBGA Bacillus subtilis BGA/ plate IBGA
S.e./ER Staphylococcus epidermidis ATCC 12228/ plate ER
E.c./KIN Escherichia coli ATTC 10536/ plate KIN

Table 4: Mean diameter of inhibition zone (mm), recovery and standard deviation (SD) for each inoculated antibiotic

	number of samples	conc. (µg/kg)	mean inhibition zone at the limit of detection (mm)	recovery (%)	SD of inhibition zone (mm)
Cephalexin	10	50	11.87	94	0.17
Tetracycline	10	50	17.23	76	0.40
Chlortetracycline	10	50	18.65	98	0.23
Erythromycin	10	50	10.45	82	0.31
Tylosin	10	100	11.14	84	0.14
Neomycin	10	100	9.91	85	0.36
Streptomycin	10	100	11.37	96	0.22
Penicillin	10	20	10.87	90	0.59
Amoxicilin	10	20	11.31	90	0.43
Enrofloxacin	10	50	10.17	92	0.80
Ciprofloxacin	10	30	11.35	87	0.67

at or below half the MRL for all tested antibiotics, both for meat samples and for standard solutions. Determination of LOD in standard solutions is essential to assess the influence of matrix (in our case meat) on the sensitivity of the method. Knowing the LOD both in standard solutions and in meat samples allows calculation of the recovery which is one of the measures of the reliability of the method.

Table 4 shows the recovery and standard deviation of the inhibition zones at the LOD for each tested antibiotic. For all antibiotics the recovery was above 80% which is the limit set by the Directive EEC657/2002 (26). The only exception where the recovery was 76% was tetracycline.

Discussion

Microbiological methods for detection of antibiotic residues in food are used in practically every laboratory in Europe involved in controlling food of animal origin. Although the method is widely know as the "four-plate method", many variations are used and most laboratories apply a specific approach with a different number and types of bacterial strains and therefore a different number of test plates (19, 21, 24). Methods using between one and eighteen plates have been described in the literature. There are also differences in incubation periods, pH values of media and the quantity of media on which the bacteria are cultured, and, most importantly, differences in detection levels (27, 28, 29, 30).

Microbiological methods for detection of antibiotic residues in food are screening methods able to detect and differentiate only between antibiotic groups. Their results are used to minimize the number of chemical and immuno-enzymatic methods that are required to confirm the presence of antibiotics and identify specific substance within the antibiotic group (31, 32).

The sensitivity of a microbiological method must be high enough to allow detection of antibiotic residues level below the MRL prescribed in the current legislation (33). In some of the already published studies only working solutions were used instead of the real matrix investigated in routine analyses. This may lead to falsely low levels of detection. According to our experience the influence of matrix on the results should not be neglected, and this is especially true in the case of meat as a solid matrix whose preparation is especially troublesome. For this reason the recovery should always be calculated. In our method the values were within the values demanded by the Directive EEC 657/2002 (26) which confirms the reliability of our method. The standard deviations for inhibition zones are comparable with data reported by Ferrini et al(2006) and Myllyniemi et al(2001). Low values of standard deviations show the high accuracy of our method.

In several Scandinavian countries kidneys are used as the matrix from which the level of antibiotics in meat is assessed, because the concentration of antibiotics and therefore the MRL are higher in kidney tissue compared to meat. Using this approach a method that would otherwise have failed to reach MRL in muscle tissue was proved to be sensitive enough to detect antibiotic residues at half the MRL in kidney matrix and thus met the requirements of EEC 657/2002 (26). One of the problems of this approach is the rate of false positive results caused by natural inhibitors of bacterial growth such as lysozyme which are often present in kidneys. Ferrini et al (2006) managed to avoid their influence by placing a dialysis membrane between the growth media and the analyzed matrix (24). Despite some known advantages of analyses of kidney tissue, in Slovenia we opted for the use of meat in routine investigations.

The method developed in our study allows identification of b-lactame, aminoglicoside, macrolide, tetracycline and qionolone antibiotic groups at or below the MRL prescribed for meat. To improve the ability for differentiation between the listed antibiotic groups we used $MgSO_4$ which inhibits the action of tetracyclines, aminoglicosides and quinolones and enzyme b-lactamase which inactivates b-lactames but not macrolides which otherwise cause inhibition of growth on identical set of test plates. The drawback of b-lactamase is resistance of some synthetic b-lactames, for example newer generations of cephalosporins. A substance that would inactivate these groups of antibiotics would be an important step forward in the development of antimicrobiological screening methods.

Conclusion

The microbiological method described in this paper allows differentiation between five antibiotic groups and detection of antibiotic residues at or below the MRL prescribed for each group. The time required to perform the analysis is short (be-

tween 18 and 24 hours) and no expensive equipment is needed therefore the cost of investigation is relatively low. The drawbacks of the method are that any positive result must be confirmed by chemical methods and that the results are difficult to interpret quantitatively because a separate calibration curve would have to be constructed for each antibiotic. Altogether we consider this method as an appropriate and highly efficient screening method for detection of antibiotic residues in meat, especially for monitoring purposes where a high number of samples must be investigated for a high number of different antibiotics.

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MIKROBIOLOŠKA PRESEJALNA METODA ZA UGOTAVLJANJE AMINOGLIKOZIDNIH, β-LAKTAMSKIH, MAKROLIDNIH ANTIBIOTIKOV TER TETRACIKLINOV IN KINOLONOV V MESU

A. Kirbiš

Povzetek: Antibiotik je po definiciji naravni produkt mikroorganizmov ali naravnemu produktu enaka sintetična ali podobna polsintetična spojina, ki zavira razmnoževanje drugih mikroorganizmov in deluje bakteriostatično ali pa bakterije ubija, kar imenujemo baktericidni učinek. V veterinarski medicini se antibiotiki uporabljajo v terapevtske, profilaktične, metafilaktične in nutritivne namene. Ostanki antibiotikov in njihovi metaboliti v živilih so lahko nevarni za zdravje ljudi, saj lahko povzročajo alergijske reakcije oziroma vplivajo na nastanek odpornosti pri mikroorganizmih, povzročajo pa tudi gospodarsko škodo, saj delujejo zaviralno na štarterske kulture. Metode, ki se uporabljajo za ugotavljanje ostankov antibiotikov v živilih živalskega izvora, so mikrobiološke, imunoencimske in kemijske.

Mikrobiološke metode se uporabljajo kot presejalne oziroma screenig metode. Meja detekcije metode za posamezne skupine antibiotikov mora biti vsaj na meji MRL vrednosti oziroma pod njo. Rezultati, ki jih dobimo z zanesljivo presejalno metodo, so zelo dobra orientacija za potrjevalne kemijske metode, ki so zamudne in zelo drage.

Namen raziskave je bil uvedba mikrobiološke metode za ugotavljanje antibiotikov s testiranjem in uvedbo testnih sevov bakterij in ugotoviti minimalno količino antibiotikov, ki jih je s posamezno metodo mogoče ugotoviti. Določili smo občutljive in odporne bakterijske seve za skupine makrolidnih, aminoglikozidnih in β-laktamskih antibiotikov kakor tudi kinolonov in tetraciklinov v mesu. Za ugotavljanje β-laktamskih in makrolidnih antibiotikov uporabljamo bakterijski sev *Micrococcus luteus* ATCC 9341 kot občutljivi sev, za aminoglikozidne antibiotike bakterijski sev *Bacillus subtilis* BGA , za tetracikline *Bacillus cereus* ATCC 11778 ter za kinolone *E.coli* ATCC 10536. Pri poskusu smo uporabili potrditvene spojine, in sicer magnezijev sulfat, ki inaktivira aminoglikozidne antibiotike, ter predstavnike kinolonov in tetraciklinov, pa tudi encim β-laktamaza, ki inaktivira delovanje β-laktamskih antibiotikov. Rezultati, ki smo jih dobili, so pod MRL vrednostmi za posamezne predstavnike antibiotikov.

Ključne besede: hrana, analize-metode; antibiotiki; zdravila, ostanki-analize; mikrobni občutljivostni testi-metode; meso

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Original Research Paper

MUTATION OF *MDR1* GENE ASSOCIATED WITH MULTIDRUG SENSITIVITY IN AUSTRALIAN SHEPHERDS IN SLOVENIA

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Summary: The multidrug-resistance 1 (MDR1) transport protein plays an important protective role at blood-tissue barriers by limiting the entry of MDR1 protein substrates to brain, testis, fetus and other tissues. For subpopulation of Collies and related dog breeds increased susceptibility to neurotoxic side effects of several drugs including ivermectin, moxidectin and loperamide was detected. It was demonstrated that in ivermectin susceptible dogs the deletion mutation in *MDR1* gene produces a frame shift resulting in the production of severly truncated non-functional MDR1 protein. In order to evaluate the occurrence and frequency of the mutated *mdr1-1*Δ allele in the population of Australian Shepherds in Slovenia we have screened 10 dogs representing approximately one third of the Slovenian population. The results of our study indicate very high frequency of *mdr1-1*Δ allele (70%) in Australian Shepherds in Slovenia. In 40% of studied population homozygous mutated genotype was determined. Considering the important role of MDR1 protein in drug disposition and blood-brain barrier protection, testing of the MDR1 genotypes and *MDR1* genotype-based breeding programs are recommended for improving the safety of drug therapy with MDR1 protein substrates in Australian Shepherds.

Key words: molecular biology-genetics; P-glycoprotein-genetics; ivermectin-adverse effetcs; genes, MDR-genetics; neurotoxicity syndromes-etiology-genetics; pedigree; dogs-genetics

Introduction

The multidrug-resistance 1 (MDR1) transport protein is a large transmembrane P-glycoprotein (P-gp) encoded by the *MDR1* gene. MDR1 is a member of the ATP-binding cassette (ABC) superfamily of transporters that use the energy derived from ATP hydrolysis to export various molecules including a variety of drugs across cell membranes from the cytosol to the extracellular medium and therefore performing the protective role in cells. MDR1 is expressed in a variety of tissues with excretory function, e.g. small intestine, liver, kidney, and at blood-tissue barriers, such as blood-brain barrier, blood-testis barrier and placenta. This protein is thought to have an important role in removing toxic metabolites from the cells, it limits the absorption of orally administered drugs, promotes drug elimination into bile and urine and protects various tissues, e.g. brain, testis and fetus (1, 2, 3, 4).

Unbalanced level of MDR1 protein expression in the cells can cause disorders in the availability of MDR1 substrates in the cells. The expression of the *MDR1* gene is frequently amplified in multidrug-resistant cells, resulting in a large overproduction of the MDR1 protein. If these cells are exposed to toxic compounds they can develop resistance to several drugs. This is well documented in humans, where the over-expression of human MDR1 protein in tumor cells is causing resistance of these cells to various chemotherapeutic drugs (5). The opposite, lack of MDR1 protein is observed in dogs – because of the mutation in the *MDR1* gene the functional MDR1 protein is not expressed and the protective role is impaired.

More than 20 therapeutic drugs are known substrates of MDR1 protein, one of these is ivermectin that is used extensively in veterinary medicine as parasiticide toxic for nematodes and arthropod

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parasites, in dogs it is often used for prevention and therapy of heartworm (*Dirofilaria immitis*). In some Collies and Collie related dog breeds ivermectin treatment causes neurotoxicity (6, 7). In one study 1/200 of the lethal dose of ivermectin for beagles was lethal for Collies (6). Mealey et al. (8) discovered that the affected dogs were homozygous for a 4-bp deletion of the fourth exon of *MDR1* gene. This mutation causes a frameshift causing premature stop codon, presumably resulting in truncated MDR1 protein with loss of function and therefore defective brain-blood barrier.

MDR1 deletion mutation associated with the ivermectin sensitivity has been reported in Collies and related breed dogs in the northwestern United States (9) and in France (10), in herding breeds in Australia (11), Japan (12) and in Germany (13). *MDR1* deletion mutation was observed in many pure-breed dogs: in Australian Shepherds, Collies, English Shepherds, Longhaired Whippets, McNabs, Old English Sheepdogs, Shetland Sheepdogs and Silken Windhounds (14).

Population of Australian Shepherds in Slovenia is relatively small, it comprises only about thirty dogs. In Slovenia it is a relatively new breed, the dogs are imported from different countries. The aim of our study was to determine if *mdr1-1*Δ allele is present in our population of Australian Shepherds.

Material and methods

Animals

13 dogs were included in the study (10 Australian Shepherds, 3 mixed breed dogs). Samples were taken from dogs whose owners were interested in determining the *MDR1* genotype of their dogs or with the consent of the owners to use the samples for research. Owners were informed about the study mainly through announcements made at the meetings of the owners of Australian Shepherds. Samples from mixed breed dogs were taken from a repository at the Laboratory for Molecular Biology and Molecular Genetic at the Veterinary Faculty in Ljubljana.

DNA isolation

Genomic DNA was isolated from blood samples collected in tubes containing anticoagulant (EDTA or acid citrate dextrose solution B) by a standard phenol-chloroform protocol as described by Sambrook et al. (15) or by commercially available Wizard Genomic DNA Purification Kit (Promega).

MDR1 gene amplification

MDR1 gene was amplified by use of primers (5' - GGC TTG ATA GGT TGT ATA TGT TGG TG - 3' and 5' - ATT ATA ACT GGA AAA GTT TTG TTT C - 3') described by Neff et al. (14) in polymerase chain reaction (PCR). The primers bracketed the reported 4 bp deletion in *MDR1*. The PCR consisted of 35 cycles with denaturing (20 seconds at 93°C), primers annealing (20 seconds at 55°C) and primers extension (1 minute at 72°C) in thermocycler (Biometra).

PCR product analyses

PRC products were separated by capillary electrophoresis on the ABI PRISM 310 apparatus to detect the size of the PCR products and analyzed by the programme GeneMapper 3.7.

Results

The allele *mdr1-1*Δ was found in 7 dogs of Australian Shepherd breed. The samples originated from five dogs with one known parent that was shown to be homozygous for the *mdr1-1*Δ allele. All five offspring had *mdr1-1*Δ allele, three were homozygous for this allele, two were heterozygous. In another family the parent was shown to be homozygous for *mdr1-1*Δ allele as well as its single offspring that was tested. The results of genotyping in Australian Shepherds are shown in Table 1 and in Figure 2.

In addition to Australian Shepherds some non-pure breed dogs were tested, one sample was obtained from the dog related to Collie breed. This dog was heterozygous for *MDR1/mdr1-1*Δ. Two other samples obtained from mixed breed dogs were homozygous for the wild type *MDR1* allele.

Discussion

Ivermectin sensitivity connected to *mdr1-1*Δ homozygous genotype in dogs was first described in dogs of Collie breed (16, 17). Initial studies performed to determine the frequencies of *mdr1-1*Δ allele responsible for ivermectin toxicity were done on Collies (9, 10), however, recent studies include also other dog breeds of Collie lineage. The *MDR1* gene frequencies in dogs of Australian Shepherd breed have already been determined in some countries. In

Table 1: Observed allele and genotype frequencies of gene encoding MDR1 protein in studied dogs of the Australian Shepherds breed

		Allele %		*Genotype %*		
Breed	*No. of dogs*	*MDR1*	*mdr1-1Δ*	*mdr1-1Δ/ mdr1-1Δ*	*mdr1-1Δ/ MDR1*	*MDR1/ MDR1*
Australian Shepherd	10	45	55	40	30	30

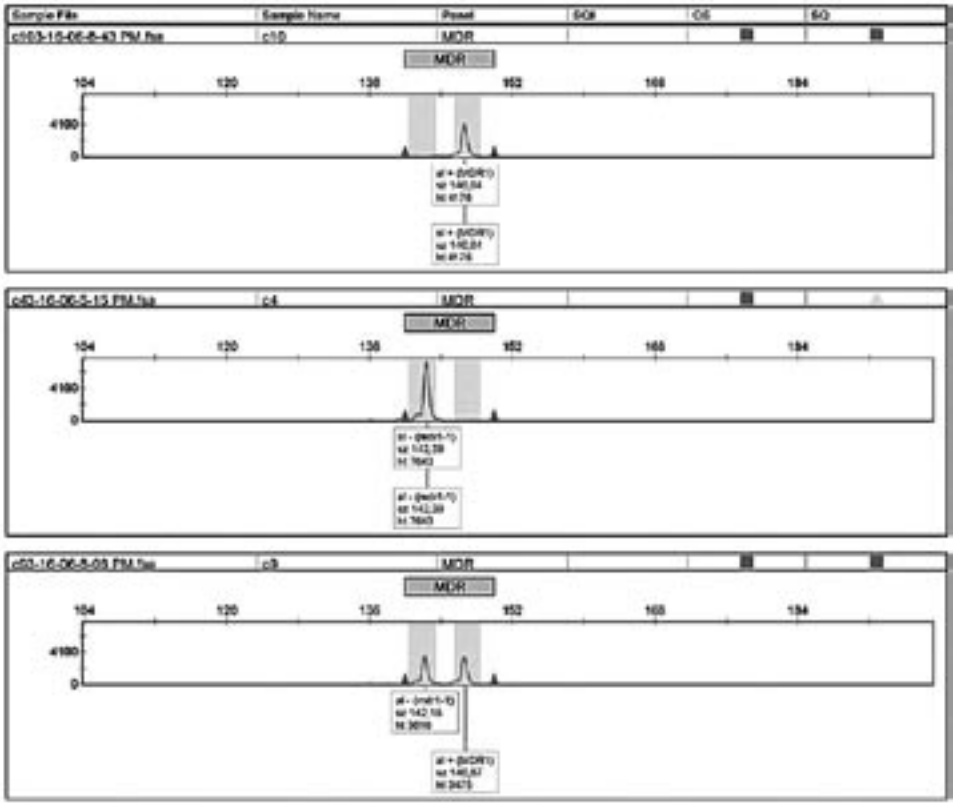

Figure 1: Electropherogram of PCR products after PCR amplification using the primers neighbouring the 4 bp deletion in *MDR1* gene. Top – homozygous wild type genotype *MDR1/MDR1*; middle - homozygous mutated genotype *mdr1-1Δ/mdr1-1Δ*; bottom – heterozygous genotype *mdr1-1Δ/ MDR1*

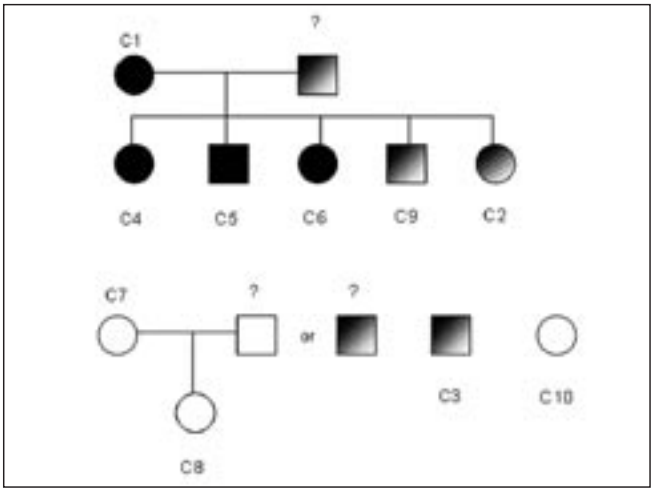

Figure 2: The results of genotyping for the *MDR1* and *mdr1-1Δ* alleles in Australian Shepherds dogs. The samples originated from two families and two non-related dogs. The examined dogs represented approximately one third of all Slovenian dog population of Australian Shepherds breed (*MDR1/MDR1* – white; *mdr1-1Δ/mdr1-1Δ* – black; *mdr1-1Δ/MDR1* – shadowed). Symbols labeled with question-marks represent the dogs that were not tested, the genotypes were only predicted

the United States 178 dogs were examined, obtained *mdr1-1*Δ allele frequency was 16.6 % (14). In Japan 9 Australian Shepherds were tested and the obtained *mdr1-1*Δ allele frequency was 33.3 % (12), in Australia, 17 Australian Shepherds were tested for the same mutation in the *MDR1*, the *mdr1-1*Δ allele frequency was 42.86 % (11). The most extensive study that included 1500 dogs from 7 different dog breeds was performed in Germany. Frequency of the mutated genotype was the highest in Collies (54.6%), followed by Shetland Sheepdog (30.0%) and Australian Shepherd (19.5%). In this study 333 Australian Shepherds were included (13). We have included in our study 10 Australian Shepherds, this is small sample, however, it represents about 30% of Slovenian population of this breed. A very high frequency of the *mdr1-1*Δ allele was detected (55%), 40% of examined animals were homozygous for the *mdr1-1*Δ allele and 30% were carriers of *mdr1-1*Δ allele. Only a minority of studied dogs, 30%, was free of the mutated *mdr1-1*Δ allele, responsible for the multidrug sensitivity (Table 1, Figure 2).

MDR1 gene is displaying the pattern of Mendelian inheritance, the mutation was detected also in some pure breeds related to Collies as well as in non-pure breeds that previously did not have *mdr1-1*Δ allele (11, 12, 13, 14). In our study we have observed one *mdr1-1*Δ allele in one sample obtained from non-pure breed dog that was descendent of Collie. In this dog, most probably the mutated allele was obtained from the Collie parent.

Sensitivity of Collies to ivermectin was initially studied in the United States in the regions where heartworm *Dirofilaria immitis* is endemic and therefore ivermectin was widely used in low preventive doses and in high doses for therapy of heartworm in dogs. Neurotoxicoses were often observed in some Collies after the application of ivermectin in therapeutic doses (7). In Slovenia, heartworm *Dirofilaria immitis* is present in Primorska region and in the neighbouring Italy and Croatia, the preventive application of ivermectin is indicated for the dogs living or visiting these regions.

Ivermectin is the most studied substrate of the transmembrane transporter P-gp encoded by the *MDR1* gene, however, other drugs used in therapy of dogs are also MDR1 protein substrates. MDR1 protein substrates in previously documented interactions with canine *mdr1-1* Δ are antimicrobial agents (erythromycin, grepafloxacin), anticancer agents (doxorubicin, vincristine), immunosuppressants (cyclosporin A, tacrolimus), steroids (dexamethasone, hydrocortisone), gastrointestinal drugs (loperamide, domperidon), cardiac drugs (quinidine, digoxin) (14). Probably there are many more MDR1 protein substrates that can cause neurotoxicoses if the blood-brain barrier is not efficient because of non-functional product of *mdr1-1*Δ allele.

Based on the determined frequency of *mdr1-1*Δ allele in Australian Shepherds in Slovenia we would suggest the verification of the presence of the *mdr1-1*Δ allele before using therapeutic doses of ivermectin or other MDR1 protein substrates. The method used in our study to detect the mutated allele is reliable, fast and affordable. According to the known presence of the *mdr1-1*Δ allele in some dog breeds in countries where the allele frequencies in different dog populations were already determined (9, 10, 11, 12, 13, 14) the same practice would be suggested for dog breeds related to Collie.

In conclusion, the results of this study indicate that very high percentage of Australian Shepherds in Slovenia (70%) contains at least one *mdr1-1*Δ allele responsible for sensitivity to ivermectin and other substrates of P-gp transporter. Considering the important role of *MDR1* protein in drug distribution to the cells and in particular for blood-brain barrier protection special care should be taken when treating Australian Shepherds with drugs that are P-gp substrates. Detection of dogs with mutated *mdr1-1*Δ allele in Slovenia based on MDR1 genotyping are recommended to increase the safety of drug therapy with P-gp substrates and to prepare specific breeding programmes to lower the frequencies of the homozygous *mdr1-1*Δ allele in a dog population.

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MUTACIJA GENA MDR1, POVEZANA S PREOBČUTLJIVOSTJO NA RAZLIČNA ZDRAVILA PRI AVSTRALSKIH OVČARJIH V SLOVENIJI

M. Cotman, J. Zabavnik

Povzetek: Transportni protein MDR1 (multidrug-resistance 1 protein – protein za odpornost proti različnim drogam) igra pomembno zaščitno vlogo v različnih krvno-tkivnih pregradah in s tem omejuje dostop MDR1-substratov do možganov, testisov, zarodka in drugih tkiv. Pri deležu populacije škotskih ovčarjev in njim sorodnih pasmah je bila opažena povečana pojavnost nevrotoksikoz ob obdelovanju z različnimi zdravili – z ivermektinom, moksidektinom in loperamidom. Pri psih s povečano občutljivostjo na ivermektin je bila ugotovljena delecijska mutacija gena (alel *mdr1-1*Δ). Zaradi mutacije pride do zamika bralnega okvira gena, kar povzroči proizvodnjo zelo skrajšanega nedejavnega proteina MDR1. Z raziskavo smo želeli ugotoviti pojavnost in frekvenco mutiranega alela *mdr1-1*Δ v populaciji avstralskih ovčarjev v Sloveniji. Preiskali smo 10 psov pasme avstralski ovčar, kar predstavlja približno eno tretjino populacije te pasme v Sloveniji. Rezultati naše študije kažejo zelo visoko frekvenco alela *mdr1-1*Δ (70%) v slovenski populaciji avstralskih ovčarjev. V 40 % proučevanih avstralskih ovčarjev se je mutirani alel pojavil v homozigotni obliki. Glede na pomembno vlogo proteina MDR1 pri razporejanju drog v organizmu in pri zaščiti v krvno-možganski pregradi priporočamo ugotavljanje genotipov *MDR1* in uvedbo rejskega programa za usmerjeno izbiranje genotipov *MDR1*, kar bi povečalo varnost ob obdelovanju avstralskih ovčarjev z zdravili, ki so substrat proteina MDR1.

Ključne besede: molekularna biologija-genetika; P-glikoprotein-genetika; ivermektin-škodljivi učinki; gen MDR-genetika, nevrotoksični sindromi-etiologija-genetika; rodovnik; psi-genetika

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Original Research Paper

THE SOURCE OF CONTAMINATION OF GROUND MEAT FOR PRODUCTION OF MEAT PRODUCTS WITH BACTERIA *STAPHYLOCOCCUS AUREUS*

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Summary: In a plant for beef-slaughtering we established the sources of contamination of beef carcasses and ground meat, made from meat of beef carcasses and which are the most important places of contamination in the production process. We took altogether 250 smears from the surface of beef carcasses. The specimens were taken on five different areas on beef carcasses. The bacteria *S. aureus* was isolated on the thorax in 78 % (39/50) of the specimens, 62 % (31/50) on the front legs, 58 % (29/50) on the abdomen wall, 14 % (7/50) on the thigh and 10 % (5/50) on the neck. The established contaminations of workers' hands was 50 % of specimens before the beginning of the work and 58.33 % of specimens taken after the handling of five slaughtered carcasses and are an important source of contamination of meat.
For differentiation of separate strains of bacteria *S. aureus* we used the RAPD-PCR method and four different oligo-nucleotide primers OPJ5, OPJ6, E7 and E8. With genotypification of oligo-nucleotide primers OPJ%, E8 we proved the correlation between ground meat, hands, equipment and beef carcass.
Because the surfaces of beef carcasses are contaminated with the same type of bacteria
S. aureus as the hands of workers, establishing contamination of the surface of beef carcasses with bacteria *S. aureus* on the slaughtering line can be the indicator hygiene of work among workers on the slaughtering line.
The major source of contamination of ground meat are the hands of workers (contamination of workers' hands with bacteria *S. aureus* was 58.33 % of the specimen taken after the handling of five beef carcasses) and the contaminated surface of beef carcass (44.4 % of smears of surface of beef carcasses was contaminated with bacteria *S. aureus*).
The contaminated surface of beef carcasses is crucial for contamination of ground meat as the meat goes to grinding.

Key words: food inspection; food contamination; meat-microbiology; *Staphylococcus aureus*-isolation and purification

Introduction

Staphylococci are ubiquitous in nature. *S.aureus* is the most important species in the group as some strains are capable of causing human foodborne intoxication. The primary reservoir is on the skin and mucous membranes of mammals and birds. *S. aureus* is frequently isolated from ground meat. Enterotoxigenic strains of *S. aureus* in ground meat can grow to sufficient level to allow a toxic dose of enterotoxin to be produced prior to consumption. Enterotoxin is thermo-stable and is not destroyed with heat treatment. Because of the enterotoxin thermo-stability, level of contamination of meat is to be kept as low as possible during the production process. The initial contamination of meat occurs during slaughtering. Hygiene deficiencies cannot be compensated for even by the most rigorous hygiene measures during later production process. Microbiological hygiene measures in meat production aim at protecting the consumer against pathogenic agents. To prevent contamination of meat with *S. aureus*, sources of *S. aureus* must be determined and well known.

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Molecular test, Random Amplified Polymorphic DNA (RAPD-PCR), is a useful method for differentiation of strains of *S. aureus*. Several authors reported that a strain of S. aureus isolated from cows with mastitis can be differentiated using a PCR method with random primers. This method is preferable over biotyping, as it is a much more specific method (1). Molecular methods such as PCR-based DNA fingerprinting may be useful for epidemiological purposes (2).

Material and methods

Collection of samples

Swabs: Swabs were taken with sterile cotton sticks in mannitol salt broth (Mannitol salt broth-Biolife Italiana S.r.l.) from a surface of 25 cm^2. We limited the area with a sterile, paper model.

Swabs were taken from surface of equipment on the slaughter line and in the cutting room, from hands of workers on the slaughter line and in the cutting room and from the surface of carcasses on five different areas on the carcass; thigh, abdomen wall, thorax, front leg and neck.

Cough up air samples: Samples were taken directly on Baird –Parker broth during coughing of workers.

Ground meat samples: Samples were taken in a sterile plastic bag immediately after grinding.

Isolation and identification of S. aureus

Swabs were incubated in mannitol salt broth (Mannitol salt broth- Biolife Italiana S.r.l.) at a temperature of 37°C for 24 hours. 1g of ground meat is diluted in 9 ml of mannitol salt broth.

Mannitol salt broth was spread on Baird –Parker agar (Baird –Parker agar Staphylococcus Selective Agar base acc. to Baird –Parker) and incubated at 37°C for up to 48 hours. Colonies which exhibited typical morphology (grey-black shiny convex colonies, 1-1.5 mm in diameter with a narrow white entire margin surrounded by a zone of clearing, 2-5mm) were spread on blood agar

(Blood Agar Base N°2+5% blood) and incubated at 37°C for up to 48 hours. Colonies were tested for coagulase production using rabbit blood plasma (BBL ™ coagulase plasma, rabbit with EDTA). Additional phenotypic traits were used with a commercial kit (BBL CRYSTAL ™ GP Identification System, Gram-Positive ID Kit).

DNA extraction

DNA from bacterial cultures was extracted by the PROMEGA method. (Wizard® Genomic DNA Purification Kit) according to the manufacturer instructions. For destruction of the bacteria cell wall lisozim and lisostafin were used.

DNA amplification

Amplification was performed with RAPD-PCR. For differentiation of separate strains of bacteria *S. aureus* we used the RAPD-PCR method and four different oligo-nucleotide primers OPJ5, OPJ6, E7 and E8 (2).

Results

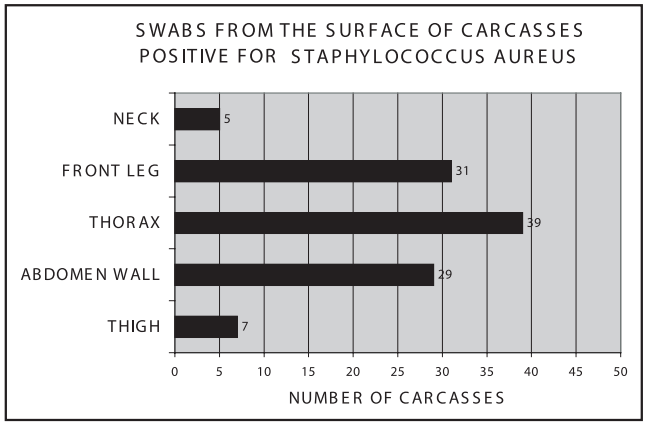
For establishing the hygiene status of the establishment 140 swabs were taken from surfaces of equipment. Established contamination (> 100 micro-organisms/cm^2) was 6.42% (9/140) of specimens. For establishing contamination before the beginning of work with *S. aureus* on surfaces of equipment we took 40 specimens. *S. aureus* was isolated from 5% (2/40) of specimens. In addition we took 41 specimens from equipment after the handling of five beef carcasses. *S. aureus* was isolated from 46.34% (19/41) of specimens.

For establishing contamination of workers hands, 24 swabs were taken before the beginning of the work and 24 swabs after handling five beef carcasses. S. aureus was isolated from 50% (12/24) of specimens and 58.33% (14/24) of specimens, respectively. From 10 specimens of coughing up air of workers, S. aureus was recovered from 10% (1/10) of specimens.

From the surface of beef carcasses samples were taken from five different areas on the carcass. *S. aureus* was isolated from 78% (39/50) of specimens taken from the thorax area, 62% (31/50) of specimens taken on the front leg, 58% (29/50) of specimens taken on the abdomen wall, 14% (7/50) of specimens taken on the thigh and 10% (5/50) of specimens taken from the area of the neck. Altogether, S. aureus was isolated from 44.4% (111/250) of specimens taken from surfaces of beef carcasses.

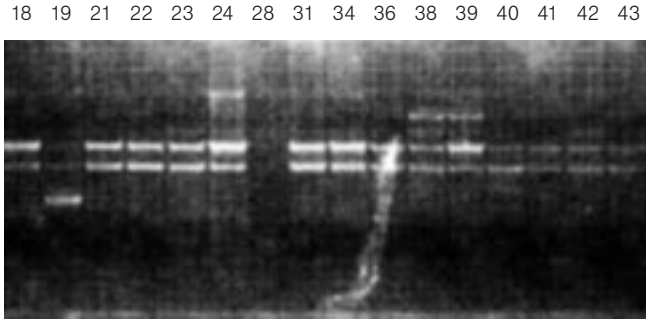
From ground meat specimens *S. aureus* was recovered from 62.5% (10/16) specimens. Results of genotyping of S. aureus, isolated from specimens taken in slaughterhouse by the RAPD-PCR method.

Table 1: Swabs taken from the surface of carcasses positive for *S. aureus*



With oligo-nucleotide primer OPJ 5 we got 4 different profiles of DNA, designated as A, B, D, C profile. Profile A: We got the same profiles of DNA of *S. aureus* isolated from workers hands before the beginning of work, knife and surface of carcasses. Profile B: We got the same profile from specimens taken from humans, bovine and surface of beef carcasses. Profile C: We got the same profile from specimens taken from hands of workers before the beginning of work, from hands of workers taken after handling five beef carcasses, specimens taken from the surface of carcasses and specimens of bovine origin. Profile D: We got the same profile from specimens taken from hands of workers after handling five beef carcasses and specimens of bovine origin. With oligo-nucleotide primer OPJ 6 we did not obtain any results.With oligo-nucleotide primer E 7 we got different profile of DNA.

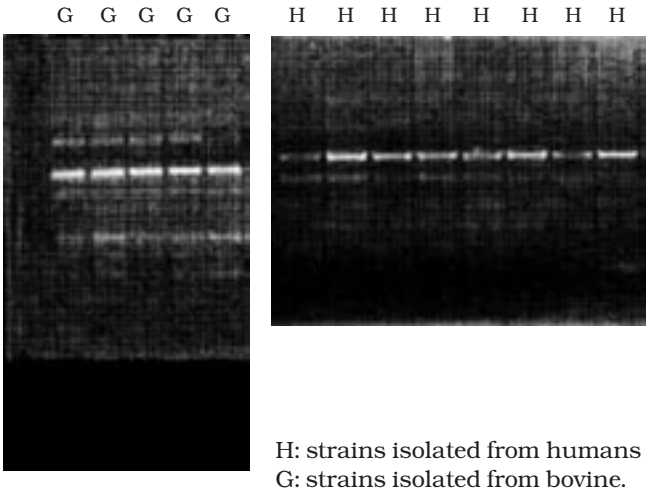
Figure 1: Results of genotyping of S. aureus, isolated from specimens taken in slaughterhouse with the RAPD –PCR method, with oligo-nucleotide primer E 7



38, 39: strains isolated from ground meat

The profile of DNA of the strain isolated from the hands of workers (sample 21, 22, 40) was the same as the profile of DNA of strains isolated from the surface of beef carcasses (sample 23, 31, 34) The profile of DNA of strains isolated from ground meat was different from the strains isolated from humans and bovine. We got a different profile of DNA isolated from humans and bovine.

Figure 2: Results of genotyping of *S. aureus*, isolated from specimens taken in slaughterhouse with the RAPD –PCR method, with oligo-nucleotide primer E 7.



H: strains isolated from humans
G: strains isolated from bovine.

Strains isolated from humans and bovine have a different profile of DNA.

With oligo-nucleotide primer E8 we got 2 different profiles of DNA. The profile of DNA of the strain isolated from the hands of workers was the same as the profile of DNA of strains isolated from the surface of beef carcasses. *S. aureus* isolated from the knife have the same profile of DNA as strains isolated from humans and from ground meat. Strains isolated from ground meat have the same profile as strains isolated from humans and bovine.

Discussion

S. aureus is frequently isolated from food samples. The high incidence of staphylococci on beef carcasses is of concern, since they can act as a source of contamination to other foods.

S. aureus can present a risk to other foods especially those that are not subject to a bactericidal process.

The objective of our study was to establish the correlation between sources of bacteria *S. aureus* and ground meat and determine weather the strains

of *S. aureus* are of human or animal origin.

Equipment in the slaughterhouse was contaminated before the beginning of work. Although this contamination may to a certain extant be difficult to avoid, the level of contamination can be substantially increased or decreased by poor or good slaughter procedures, respectively. Contamination of muscle tissue during the slaughter process may occur as a result of direct or indirect contact with e.g. faeces, skin, contaminated tools and equipment, personnel and clothing. In this study 5 % of samples from equipment were contaminated before the beginning of work, 46.34 % of samples were contaminated after handling five carcasses. During the process of meat production the contamination was raised. According to these results, the probability of contamination of carcasses had been raised during the production process.

Contamination of workers hands was high, 50 % (12/24) of samples, already before the beginning the work, and it was raised after handling five carcasses to 58.33 % (14/24) of samples. The hands of workers are an important primary source of contamination of products with *S. aureus* during meat processing (3). *S. aureus* is present on the skin of hands, and hands are also contaminated through contact with surfaces that are rich in bacteria, such as the skin of slaughtered animals, intestinal contents, abscesses. Especially, hands can be a source of contamination if infected cuts and sores are present. During evisceration, and to a lesser extent trimming, workers have considerable contact with - and have to handle - the carcasses around the brisket and the flank. The hands of evisceration workers and trimming staff at the slaughterhouse were shown to be heavily contaminated with coagulase positive staphylococci (CPS), while the hands of non-meat workers from the same slaughterhouse were shown to be infrequently contaminated (4). Hands of evisceration workers were continually wet and were subjected to rough physical activities. Their hands had signs of skin damage and this poor skin condition could have led to increased colonisation by staphylococci, accounting for the high numbers present on hands at the commencement of work (4). Hand washing has a variable effect on the reduction of bacteria on hands, depending on the mechanical action and the duration, together with the type of soap or sanitisers used (5).

Contamination of the surface of carcasses was examined with 250 smears from the surface of meat on five different areas. The bacteria *S. aureus* was isolated on the thorax in 78% (39/50) of the specimens, 62% (31/50) on the front legs, 58% (29/50) on the abdomen wall, 14% (7/50) on the thigh and 10% (5/50) on the neck. Contamination was shown to be the highest on the thorax and front leg of carcasses. The area of the abdomen wall was still high, and areas of thigh and neck were contaminated to a lesser extent. Underlying tissue of the hide in a healthy animal is sterile. The initial contamination of meat occurs during slaughtering. The level of contamination is in correlation with working operations on the slaughter line. Working operations in the production of meat: hide removal, evisceration, splitting of carcasses, trimming and washing of surface, handling of carcasses all contribute to contamination of the meat. The level of contamination is apparently in correlation with working operations on the slaughter line and the time and temperature of holding the meat.

The process of hide removal results in some microbiological contamination of the underlying carcass tissue, and the subsequent extent of the contamination depends on the technique used and the level of contamination on the hide (6). Contamination with CPS was increased after evisceration and to a lesser extent after trimming (4).

Hygienic practice was found to be associated with the carcass contamination level, especially disinfection frequency. Designing slaughtering lines so as to make hygienic work possible is very important (7).

Chilling temperatures are not low enough to prevent growth of *S. aureus*. Production of enterotoxins is related to growth and it is unlikely that the growth of CPS during weekend chilling of beef carcasses poses an immediate health risk. A longer period of refrigeration would be required before enterotoxins were detected (4).

S. aureus was isolated also out of 10% (1/10) of specimens of cough up air of workers on the slaughtering line. Samples of cough up air suggests that the workers can contribute to contamination also in this way. The environment may also contribute to bacterial cross-contamination of carcasses because of the presence of CPS in air samples (4).

Associations between the microbiological contamination of air and carcasses with the movements of workers were found (7).

The presence of *S. aureus* in ground meat was found in 62.5% (10/16) of specimens. The presence of *S. aureus* in ground meat is not an immediate health risk. Storage of such contaminated ground

meat in an inadequate environment for a longer period of time could enhance the growth of bacteria and production of enterotoxins, especially in meat products that are not subject to a bactericidal process.

Animal biotypes were isolated from workers in the slaughterhouse. Humans could act as a reservoir for both human and animal biotypes (8).

For differentiation of separate strains of bacteria *S. aureus* we used the RAPD-PCR method. We proved a correlation between beef carcasses, hand equipment and ground meat, and we established differences in RAPD-PCR patterns between isolated strains of *S. aureus* from human and animal origin. In the slaughterhouse we isolated both types of strains from workers hands, equipment beef carcass and ground meat .

These findings support the results of other studies, that coagulase positive staphylococci isolated from workers in slaughterhouses have similar phage patterns to strains isolated from meat products (3).

According to the findings from our study the hands of workers are a source of contamination of the surface of beef carcasses with bacteria *S. aureus*. The surface of carcasses frequently is comes into contact with hands during operations on the slaughter line.

The contaminated surface of beef carcasses is crucial for contamination of ground meat as the meat goes to grinding. To prevent the contamination of carcasses it is essential that the incidence and counts of *S. aureus* be reduced. To achieve this, more care needs to be exercised in removing the hide, and viscera, and in carefully trimming the surface of carcasses prior to chilling. Washing of the carcass may have resulted in redistribution of the contamination (3). It is also important, according to our findings, to avoid contact between meat and the hands of workers. Several studies have shown that elimination of carriage in the anterior nares, the principal reservoirs of *S. aureus*, reduces the incidence of *S. aureus* infection (9). Because the surfaces of beef are contaminated with the same type of *S. aureus* as the arms of workers, establishing contamination of the surface of beef carcasses with *S. aureus* on the slaughtering line can be the indicator of work hygiene among workers on the slaughtering line.

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VIR ONESNAŽENJA MLETEGA MESA ZA PROIZVODNJO MESNIH IZDELKOV Z BAKTERIJO STAFILOKOKUS AUREUS

B. Podpečan

Povzetek: V obratu za klanje govedi in predelavo mesa smo ugotavljali vire kontaminacije klavnih trupov in mletega mesa, pripravljenega iz mesa klavnih trupov, in najpomembnejša mesta v proizvodnem procesu, kjer prihaja do kontaminacije.
Odvzeto je bilo skupaj 250 brisov površine govejih klavnih trupov. Vzorci so bili odvzeti na petih različnih mestih na trupu. Bakterijo *S. aureus* smo izolirali na prsih iz 78 % (39/50) vzorcev, na prednji nogi iz 62 % (31/50) vzorcev, na trebušni steni iz 58 % (29/50) vzorcev, na stegnu iz 14 % (7/50) vzorcev in na vratu iz 10 % (5/50) vzorcev. Roke delavcev so pomemben vir kontaminacije mesa. *S. aureus* smo ugotovili pri 50 % (12/24) vzorcev, odvzetih z rok delavcev pred začetkom dela, in pri 58,33 % (14/24) vzorcev, odvzetih z rok delavcev potem, ko so obdelali pet klavnih trupov.
Za diferenciacijo posameznih izolatov bakterije *S. aureus* smo uporabili metodo RAPD-PCR in štiri različne začetne oligonukleotide OPJ5, OPJ6, E7, E8. Ugotovili smo štiri različne genotipe bakterij. Pri tipizaciji izolatov z začetnim nukleotidom OPJ 5 smo ugotovili zvezo med sevi bakterije *S. aureus* z rok, opreme in pribora in sevi s klavnega trupa. Z genotipizacijo z začetnim oligonukleotidom E 8 smo dokazali povezavo med mletim mesom, rokami delavcev, priborom in klavnim trupom. Ker so področja klavnega trupa kontaminirana z enakim tipom bakterije *S. aureus* kot roke delavcev, je ugotavljanje kontaminacije površine klavnih trupov z bakterijo *S. aureus* na klavni liniji lahko pokazatelj higiene dela delavcev.
Glavni vir kontaminacije mletega mesa so roke delavcev (pred začetkom dela je bilo 50 % vzorcev, po obdelavi petih klavnih trupov pa 58,33 % vzorcev kontaminiranih z bakterijo *S. aureus*) in kontaminirana površina klavnega trupa (44 % brisov s površine klavnega trupa je bilo kontaminiranih z bakterijo *S. aureus*).
Za kontaminacijo mletega mesa je kontaminirana površina klavnega trupa odločilna, ker gre tako meso v mletje.

Ključne besede: živali, domače; živali, divje; carnivora; predatorsko vedenje; naravni viri, varovanje-ekonomija; podatki, zbiranje; Slovenija

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Original Research Paper

ECHOCARDIOGRAPHIC DIASTOLIC INDICES OF THE LEFT VENTRICLE IN NORMAL DOBERMAN PINSCHERS AND RETRIEVERS

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Summary: The aim of our study was to evaluate diastolic function of the left ventricle assessed by Doppler echocardiography in normal Doberman pinschers (DPs; n=39) and to study the effects of gender, age, body weight and heart rate on diastolic values. Diastolic echocardiographic values obtained in DPs were compared to the diastolic values in a group of Retrievers (n=20). The following correlations were found in DPs: a negative correlation between heart rate and mitral E/A ratio, a negative correlation between E-wave velocity and s/d ratio of pulmonary vein flow and a positive correlation between E-wave velocity and deceleration time of mitral E wave (DTE) and a negative correlation between left ventricular posterior wall in diastole (LVPWd) and DTE. Age, body weight and sex had no influence on the left ventricular diastolic indices in DPs. In Retrievers the following correlations were found: a negative correlation between heart rate and IVRT, a positive correlation between age and s/d ratio, a positive correlation between left ventricular dimension in diastole (LVDd) and mitral E/A ratio, a positive correlation between interventricular septal thickness in systole (IVSs) and DTE, a positive correlation between LVPWd and DTE and a positive correlation between LAD and s/d ratio. Body weight had no influence on the left ventricular diastolic indices in Retrievers. There was no gender influence on diastolic parameters in either group of dogs.
We found statistical significant difference in several of the obtained diastolic indices in DP comparing to the Retrievers: d - wave ($P = 0,0316$), s – wave ($P = 0,0035$), Ar – wave ($P < 0,001$) and mitral A – wave ($P = 0,0179$) were significantly higher and DTE ($P < 0,001$) was significantly longer in DP. Differences were also found in comparison to a group of dogs of different breed and boxers from the literature.
Results show that Doberman pinschers have some differences in diastolic parameters present that may be inherent for the breed, but may be also influenced by physiologic conditions such as heart rate or due to variability in sampling.

Key words: veterinary medicine; cardiology-veterinary; ventricular function, left – physiology; diastole – physiology; echocardiography; dogs

Introduction

Within the last few decades there has been a growing realization that heart failure can occur in the presence of normal systolic function. (1) More studies defining indices of diastolic function were performed by development of sophisticated echocardiographic techniques. (2,3) We know that some indices of left ventricular systolic function can differ in various breeds. (4,5) For example in Doberman Pinschers it was found that systolic echocardiographic parameters were different from dogs of other breeds. (5) We were interested if also diastolic indices can vary in Doberman Pinschers. An influence of several factors such as body weight, heart rate and gender on echocardiographic diastolic indices was found in other breeds. (6, 7, 8) We assumed that age, body weight, heart rate and gender of examined dogs have some influence on the left ventricular echocardiographic diastolic indices, similar as in other author's studies.(6,7,8) The influ-

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ence of age, body weight and heart rate on diastolic indices can explain up to 51% of the differences in Doppler echocardiographic values. (8)

The aim of our study was to evaluate diastolic function of the left ventricle assessed by Doppler echocardiography in normal Doberman pinschers and to study the effects of gender, age, body weight and heart rate on diastolic values in comparison to a group of large breed dogs. For the comparison of measured diastolic values in the same time period and by the same observer, a group of 23 Retrievers was also examined.

Material and methods

A physical examination, electrocardiography and echocardiography were performed in 47 Doberman pinschers of various body weights and age, both male and female animals and in a group of 23 various Retrievers (14 Labrador retrievers - LR, 6 Golden retrievers - GR, 2 Flat-coated retrievers - FCR and one LRxGR mixed), also of various body weights, age and both sexes. All dogs were healthy according to the owner's report and randomly selected from the population (either of Doberman pinschers or Retrievers) in Slovenia. Inclusion criteria for dogs to be included were normal history, normal physical examination, normal electrocardiogram (ECG) (only applicable for DP) (9) and normal echocardiographic dimensions (4) (left ventricular systolic and diastolic dimension, interventricular septum dimension in systole and diastole, left ventricular posterior wall thickness in systole and diastole, dimension of the left atrium, dimension of aorta, fractional shortening, ejection fraction, end systolic and end diastolic volume of left ventricle calculated by Teicholz formula.

Electrocardiographic measurements were performed only in DP as we wanted to exclude any possible arrhythmias, which the DPs are known for. Standard nine-lead electrocardiogram was recorded in right lateral position in all DP. All dogs had simultaneous ECG recordings during the echocardiographic study.

The echocardiographic measurements were performed in the right and left parasternal and left apical window (10). Left ventricular wall and cavity dimensions were measured in diastole and systole in M-mode and aorta with left atrial diastolic dimension were measured in two-dimensional right short axis view.

Color Doppler echocardiography was used to examine all the valve areas from the right parasternal and left apical view. Pulsed wave Doppler was used to measure the following parameters from the left apical view: pulmonary vein flow velocity from the left or right pulmonic vein (systolic, diastolic and atrial reverse flow), mitral inflow maximum velocity (E and A wave, E wave deceleration time, the isovolumic relaxation time). Mitral flow was measured at the tips of open mitral leaflets in the left ventricle. To measure pulmonary vein flow velocities the sample volume was placed approximately 2 to 5 mm distal to the entrance of the pulmonary vein into the left atrium. The sample volume size used had an axial dimension of 4 mm. Velocities were measured in at least three cardiac cycles. Values of three cardiac cycles were averaged for quantitative data analysis, irrespective of respiratory phase.

Statistics

Descriptive statistics was calculated and the correlation coefficients between age, body weight, heart rate and systolic and diastolic indices in both groups were calculated. Data were reported as average value ± 1 standard deviation. Analysis of variance (ANOVA) was used to evaluate differences in Doppler-derived indices between females and males in both groups. Pearson's correlation coefficients were calculated to determine correlation between age, body weight, and heart rate and individual systolic and diastolic parameters in DPs and Retrievers. For comparison of the values of diastolic indices in DPs and the values of diastolic indices in Retrievers from our study and other dog breeds from the literature Student's T-test was used. Differences with $P< 0,05$ were considered to be significant and differences with $P< 0,01$ were considered to be highly significant.

Results

Data were obtained from 47 clinically normal Doberman Pinschers. Eight Doberman Pinschers were excluded from the study for the following reasons: dilated cardiomyopathy (2 dogs), ductus arteriosus persistens (1 dog), ventricular premature complexes (1 dog); three dogs had the end systolic and/or end diastolic dimensions of the left ventricle deviating more then two standard deviations from normal values (4); one dog was excluded from the study because he died two years after the examination without known cause. Among 39 dogs included in the study 22 were females and 17 were males, age from 1 to 11 years (4,31 ± 2,38 years), body weight ranged from 26 to 53 kg. The 39 Doberman Pinschers represented approximately 6,5% of the Slovene population of Doberman Pinschers.

There were 23 dogs of various Retrievers examined in the study for comparison to DP. One dog (LR) was excluded from the study because of the systolic murmur due to mitral regurgitation and two other dogs were excluded due to left ventricle deviating more then two standard deviations from normal values. (4) Among 20 Retrievers included in the study there were 13 females and 7 males, age from 1 to 9 years (3,93 ± 2,11 years) and body weight ranged from 26 to 37 kg.

Physical examination and Electrocardiography

The results of physical examination and electrocardiography in DP were the following: heart rate (114±19 beats per minute), normal sinus rhythm (64,1% dogs), sinus arrhythmia (35,9% dogs) and normal heart sounds. Electrocardiographic parameters of all DP were normal. Sixteen dogs (42%) had mean electrical axis < 40° which is considered specific and normal for the breed. (11)

The results in Retrievers: heart rate (107,32 ± 16,8 beats per minute), normal sinus rhythm or sinus arrhythmia and normal heart sounds.

Echocardiography

The indices of systolic function of the left ventricle in DP and in Retrievers from our study are presented in Table 1.

Table 1: The indices of systolic function of the left ventricle, heart rate, body weight, dimension of aorta, left atrial dimension and LV mass in normal DP and normal Retrievers and comparison of these parameters between both groups of dogs

	DP	DP	Ret	Ret	DP : Ret
Parameter	N	$\overline{x}$ ± SD	N	$\overline{x}$ ± SD	P
Heart rate	39	114 ± 19	19	107,32 ± 16,79	0,181
BW	39	35,1 ± 5,41	20	29,95 ± 2,89	0,000
LVDd (mm	39	48.2 ± 5.4	19	46,34 ± 3,36	0,112
LVDs (mm)	39	33.3 ± 4.2	19	30,38 ± 3,82	0,011
IVSd (mm)	39	8.3 ± 1.9	19	9,29±1,89	0,069
IVSs (mm)	39	12.6 ± 2.2	19	13,61±2,34	0,125
LVPWd (mm)	39	8.4 ± 1.1	19	8,95 ±1,49	0,163
LVPWs (mm)	39	11.7 ± 1.6	19	13,03 ±2,39	0,0370
LAD (mm)	36	37.8 ± 5.0	19	37,59 ±3,87	0,861
Ao (mm)	37	25.2 ± 2.7	19	25,15±3,12	0,952
FS (%)	39	30.7 ± 5.1	19	34,54±5,65	0,017
EF tz	39	0.57 ± 0.07	19	0,63±0,07	0,004
EDV tz (ml)	39	110.6 ± 29.5	19	99,84±17,07	0,085
ESV tz (ml)	39	46.5 ± 14	19	37,03±10,96	0,007
LV Mass (g)	39	160.15 ± 48.6	19	165,75±49,07	0,684

Legend: DP – Doberman Pinschers , Ret – Retrievers, N – the number of examined dogs, SD – standard deviation, $\overline{x}$– mean value, BW – body weight, LV – left ventricle, LVDd – left ventricular diastolic dimension, LVDs – left ventricular systolic dimension, IVSd – interventricular septum dimension in diastole, IVSs – interventricular septum dimension in systole, LVPWd – left ventricular posterior wall thickness in diastole, LVPWs – left ventricular posterior wall thickness in systole, LAD – dimension of the left atrium, Ao – dimension of aorta, FS – fractional shortening, EFtz – ejection fraction caculated by Teicholz formula, EDVtz – end diastolic volume of left ventricle calculated by Teicholz formula, ESVtz – end systolic volume of left ventricle calculated by Teicholz formula,

Diastolic echocardiographic parameters

Pulmonary venous flow. Pulmonary venous flow was possible to record in 19 DP, in the rest image quality was too low due to restlessness of dogs, panting or low signal. In 15 (79%) DP pulmonary venous systolic flow was visible as monophasic. In 4 (21%) DP pulmonary venous systolic flow was biphasic (early and late systolic flow, s1- and s2-wave). In 18 DP the systolic pulmonary vein velocity was lower than diastolic velocity. In one DP the systolic pulmonary vein flow velocity was higher than diastolic pulmonary vein flow velocity (s-wave: 0,67 ms-1, d-wave: 0,63 ms-1).

Pulmonary vein systolic to diastolic velocity ratio (s/d) in 18 DP was 0,58 to 0,92. In one DP the s/d ratio was 1,2. The velocity of pulmonary vein reversal flow (Ar-wave) was -0,35 ± 0,12 ms-1.

A good quality signal of pulmonary venous flow was obtained from 17 dogs from the group of Retrievers. In 14 Retrievers (82,3 %) the obtained pulmonary venous systolic flow was monophasic and in 3 (17,7 %) Retrievers pulmonary venous systolic flow was visibly biphasic. Systolic pulmonary vein flow velocity was lower than diastolic velocity in all Retrievers. Pulmonary vein systolic to diastolic velocity ratio (s/d) in Retrievers ranged from 0,6 to 0,97.

Echocardiographic diastolic parameters of pulmonary venous flow in both groups of dogs (DP and Retrievers) are presented in table 2.

Transmitral flow. Good quality signal of transmitral flow was obtained in 38 DP. All dogs had biphasic mitral flow with visible early (E) and atrial (A) wave. The E-wave peak velocity in 35 DP was higher than A-wave peak velocity, hence the E/A ratio was more than 1. Four (10,5%) DP had E-wave peak velocity lower than A-wave velocity, hence the E/A ratio in these dogs was below 1 (0,87 – 0,95).

The value of mitral E wave deceleration time in 38 DP was 142 ± 27 ms.

Transmitral flow was recorded in 20 Retrievers. All Retrievers had biphasic mitral flow (E – and A – wave). Mitral E – wave peak velocity in all Retrievers was higher than A –wave peak velocity and the the E/A ratio more than 1.

Echocardiographic diastolic parameters of transmitral flow in both groups of dogs (DP and Retrievers) are presented in table 2.

Table 2: Echocardiographic diastolic parameters of pulmonary venous and transmitral flow in normal DPs and normal Retrievers and comparison of these parameters between both groups of dogs

	DP	DP	Ret	Ret	DP:Ret
Parameter	N	$\overline{x}$ ± SD	N	$\overline{x}$ ± SD	P
BW	39	35,1 ± 5,41	20	29,95 ± 2,89	0,000
HR	39	114 ± 19	19	107,32 ± 16,79	0,181
s (m/s)	19	0,48 ± 0,08	17	0,39 ± 0,09	0,0035
d (m/s)	19	0,63 ± 0,11	17	0,53 ± 0,15	0,0316
Ar (m/s)	19	-0,35 ± 0,12	17	0,25 ± 0,06	0,000
s/d	19	0,78 ± 0,15	17	0,75 ± 0,10	0,349
E (m/s)	38	0,82 ± 0,11	20	0,77 ± 0,15	0,300
A (m/s)	38	0,66 ± 0,11	20	0,58 ± 0,12	0,0179
E/A	38	1,26 ± 0,19	20	1,34 ± 0,24	0,205
DTE (ms)	38	142 ± 27	20	94,1 ± 29,80	0,000
IVRT (ms)	38	58 ± 14	19	60,7 ± 16,76	0,549

Legend: DP – Doberman Pinschers , Ret – Retrievers, N – the number of examined dogs, SD – standard deviation, $\overline{x}$ – mean value, BW – body weight, HR – heart rate, s – systolic pulmonary venous flow velocity, d – diastolic pulmonary venous flow velocity, Ar – atrial reversal flow, s/d – ratio between systolic and diastolic pulmonary venous flow velocity, E – early ventricular filling velocity (E-wave), A – late ventricular filling velocity (A-wave), E/A – ratio between mitral A- and E-wave, DTE – mitral deceleration time, IVRT – isovolumic relaxation time,

Correlations between echocardiographic parameters and BW, HR and age in DP and Retrievers

The correlation coefficients were calculated between age, body weight, heart rate and systolic and diastolic echocardiographic indices of examined DP and Retrievers. Among independent variables in DP a negative correlation between heart rate and mitral E-wave to A-wave ratio was found. In Retrievers a negative correlation between heart rate and IVRT and a positive correlation between age and s/d ratio was found. Heart rate, age and body weight did not affect any other diastolic parameter in DPs and Retrievers.

Among individual systolic and diastolic parameters the following correlations were found in DP: a positive correlation between mitral E-wave and mitral deceleration time, a negative correlation between mitral E-wave and pulmonary venous s/d ratio and a negative correlation between the LVPWd and DTE. The following correlations among individual systolic and diastolic parameters were found in Retrievers: a positive correlation between LVDd and mitral E/A ratio, a positive correlation between EDVtz and mitral E/A ratio, a positive correlation between IVSs and DTE, a positive correlation between LVPWd and DTE and a positive correlation between LAD and s/d ratio.

ANOVA showed no differences in values of diastolic indices between females and males in DP as well as in Retrievers.

The results of positive or negative correlations between heart rate, age, body weight and individual systolic and diastolic parameters in DP and Retrievers are presented in Table 3 and Table 4.

Table 3: Correlations between heart rate as well as individual systolic and diastolic parameters in DP.

Variable	P	r
Heart rate E/A	0,036	-0,360
LVPWd DTE	0,015	-0,392
E –wave DTE s/d	0,045 0,003	0,325 -0,657

Legend: E – w ave - early ventricular filling velocity , LVPWd - left ventricular posterior wall thickness in diastole, E/A – ratio between mitral A- and E-wave, DTE – mitral deceleration time, s/d – ratio between systolic and diastolic pulmonary venous flow velocity

Table 4: Correlations between heart rate, age as well as individual systolic and diastolic parameters in Retrievers

Variable	P	r
Heart rate IVRT	0,037	- 0,496
s/d age LAD	 0,002 0,019	 0,704 0,517
E/A LVDd EDVtz	 0,043 0,040	 0,469 0,474
DTE IVSs LVPWd	 0,010 0,012	 0,577 0,566

Legend: IVRT – isovolumic relaxation time, s/d – ratio between systolic and diastolic pulmonary venous flow velocity, LAD – left atrial dimension, E/A – ratio between mitral A- and E-wave, LVDd – left ventricular dimesion in diastole, EDVtz – end diastolic volume calculated by Teicholz formula, DTE – mitral deceleration time, IVSs – interventricular septum thickness in systole, LVPWd – left ventricular posterior wall thickness in diastole

The comparison of diastolic indices in DP, Retrievers, and other dog breeds from the literature

The results of comparison of the values of diastolic indices in DP and the values of diastolic indices in Retrievers and other dog breeds from the literature are presented in table 1, table 2 and table 5.

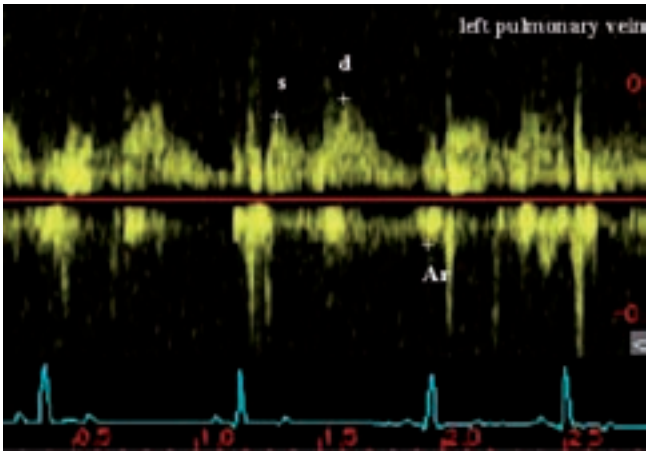

Figure 1: Pulmonary venous flow in the left pulmonary vein

Legend: s – systolic pulmonary venous flow (s-wave), d – diastolic pulmonary venous flow (d-wave), Ar – pulmonary venous reversal flow (Ar-wave)

Table 5: The reference values of the left ventricular systolic and diastolic achocardiographic parameters from the literature and comparison of our DP to referenced population

Parameter	N	Boxers*	N	Dogs of various breeds **	N	DPs from the literature***	DP:Dogs of various breeds**	DP: Boxers*
		X ± SD		X ± SD		X ± SD	P	P
BW	66	30 ± 4			10	36,1 ± 4,4		0,000
HR	66	100 ± 21	14	85 ± 16	10	113 ± 12	0,000	0,001
LVDd (mm)	66	43,5 ± 4,7			10	38,4 ± 2,3		0,000
LVDs (mm)	66	29,7 ± 3,6			10	29,2 ± 3,7		0,000
IVSd (mm)	66	9,7 ± 1,5						0,000
IVSs (mm)	66	13,3 ± 2,0						0,098
LVPWd (mm)	66	9,7 ± 1,5						0,000
LVPWs (mm)	66	13,8 ± 2,2						0,000
LAD (mm)	66	33,7 ± 5,4						0,000
Ao (mm)	66	21,7 ± 2,6						0,000
FS (%)	66	32 ± 6	14	31 ± 4	10	24,0 ± 7,2	0,843	0,260
EF tz			14	58 ± 7			0,649	
s (m/s)	57	0,42 ± 0,12	14	0,39 ± 0,14	10	0,50 ± 0,59	0,026	0,046
d (m/s)	57	0,62 ± 0,12	14	0,56 ± 0,14	10	0,49 ± 0,42	0,118	0,749
Ar (m/s)	57	-0,23 ± 0,05	14	-0,20 ± 0,08	10	0,27 ± 0,44	0,000	0,000
s/d	57	0,72 ± 0,28	14	0,70 ± 0,16	10	1,0 ± 0,1	0,151	0,376
E (m/s)	63	0,76 ± 0,13	14	0,73 ± 0,11	10	0,76 ± 0,13	0,012	0,019
A (m/s)	63	0,53 ± 0,12	14	0,48 ± 0,16	10	0,55 ± 0,10	0,000	0,000
E/A	63	1,49 ± 0,34	14	1,63 ± 0,47	10	1,4 ± 0,3	0,000	0,000
DTE (ms)	55	80 ± 14	14	81 ± 17	10	131 ± 24	0,000	0,000
IVRT (ms)	58	53 ± 8	14	60 ± 20	10	83 ± 14	0,687	0,028

Legend: * –; Schober, Fuentes, Baade, Oechtering, 2002; ** – Schober. Fuentes. McEwan, French, 1998; *** - O'Sullivan, O'Grady, Minors, 2007;
N – the number of examined dogs, Ret – Retrievers, SD – standard deviation, BW – body weight, HR – heart rate, LVDd – left ventricular diastolic dimension, LVDs – left ventricular systolic dimension, IVSd – interventricular septum thickness in diastole, IVSs – interventricular septum thickness in systole, LVPWd – left ventricular posterior wall thickness in diastole, LVPWs – left ventricular posterior wall thickness in systole, LAD – dimension of the left atrium, Ao – dimension of aorta, FS – fractional shortening, EFtz – ejection fraction caculated by Teicholz formula, s – systolic pulmonary venous flow velocity, d – diastolic pulmonary venous flow velocity, Ar – atrial reversal flow, s/d – ratio between systolic and diastolic pulmonary venous flow velocity, E – early ventricular filling velocity (E-wave), A – late ventricular filling velocity (A-wave), E/A – ratio between mitral A- and E-wave, DTE – mitral deceleration time, IVRT – isovolumic relaxation time.

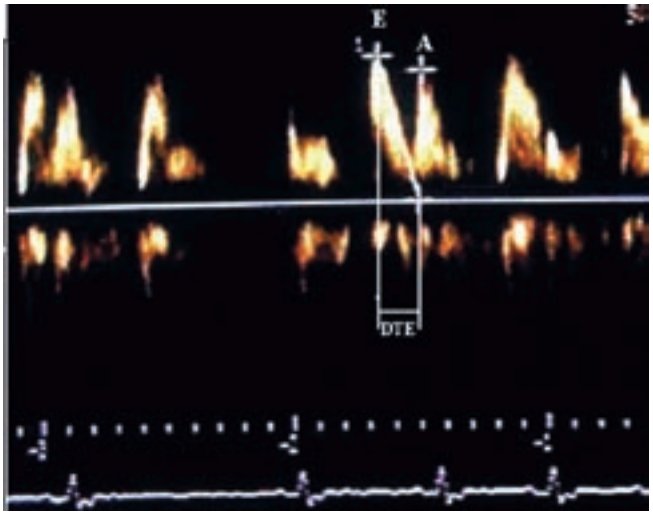

Figure 2: Transmitral flow (E – early mitral flow; A – mitral flow at the time of atrial contraction; DTE – mitral deceleration time

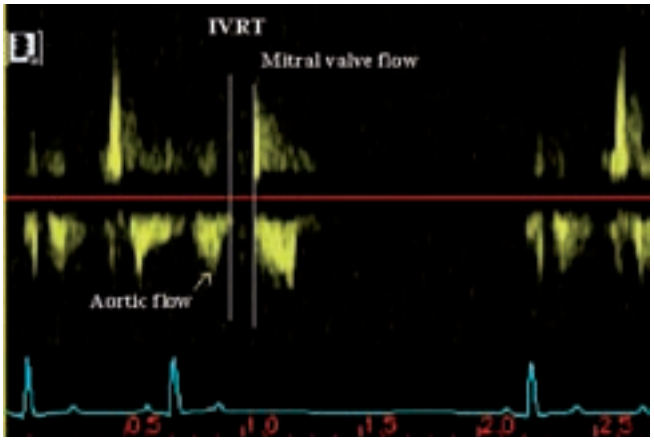

Figure 3: Isovolumic relaxation time (IVRT) measured between closure of aortic valve and before opening of mitral valve

Discussion

Presently it is accepted that diastolic dysfunction can cause heart failure by itself, therefore knowing normal values of diastolic function can help to determine its function. However it is also known that echocardiographic parameters of systolic function can vary in different breeds of dogs. Especially some larger breeds of dogs like Doberman Pinschers are found to have lower fractional shortening than most of other dogs of the same size. (5) It was our hypothesis that if systolic variables can differ in this breed in comparison to other breeds than also diastolic parameters might have some differences. Our interest was to define any gender, age or heart rate influence on these parameters as well. Other authors have shown that diastolic abnormalities can be found in dogs with a normal systolic function and clinical signs can either accompany sole diastolic dysfunction or not. By the same authors good correlation was found between diastolic parameters and clinical signs of diseased dogs. (12).

Pulmonary venous flow was not perfectly laminar in all examined dogs (DP and Retrievers), but this was the case also in other study (13). A difficulty by obtaining pulmonary venous laminar flow was reported by other authors as well. (7)

Systolic pulmonary venous flow velocity values in DP were similar to values in DP from O'Sullivan et al's study (13) but were significantly different from systolic PV flow in Retrievers, (Table 2) and other dog breeds from the literature. (6,7) Pulmonary venous flow is dependent on intrinsic myocardial diastolic properties as well as external factors such as loading condition, left atrial and left ventricular systolic function, heart rate and rhythm. (8) We can speculate that differences in left ventricular systolic function have some influence on s flow velocity of PV between DPs and other breeds. We did not measure left atrial systolic function so we cannot discuss effect of possible influence of this on s flow velocity. Looking at heart rate (HR) in O'Sulivan et al's study where the HR were lower than in our study, we can assume that HR had no major effect on s wave. (13)

Clearly seen biphasic pulmonary venous systolic flow was evident in 4 (21%) DPs and in 3 (17,7 %) Retrievers. Other dogs showed monophasic pulmonary venous systolic flow. In one of the Schober's study the systolic flow was biphasic in 93% of healthy dogs without cardiovascular disease. (7) High heart rates can cause merging of pulmonary venous early (s1) and late (s2) systolic flow (heart rate in DPs was 114 ± 19 and in Retrievers 107,3 ± 16,8).(7)

Peak pulmonary venous diastolic flow velocity in DPs was significantly higher in comparison to Retrievers (Table 2) and also to the DPs from another study, but there were no significant differences in these values in comparison to dogs from two other studies (Table 5).(6,7,13) This variations may be due to different sample volumes and depth of the sample (inter observer variability).

The s/d ratio in ours DPs did not statistically differ from the s/d ratio in Retrievers as well as DPs from O'Sullivan et al's publication and dogs from the literature. The values of the s/d ratio in dogs with normal left ventricular filling pattern are between 0,5 and 1,0 (12). In all Retrievers the s/d ratio

value was less than 1 (0,36 – 0,89). In Schober's study s/d ratio values in all dogs of various breeds were between 0,5 and 1,0.(7) O'Sullivan et al in their study of diastolic function in DPs report of 50 % values of s/d ratio < 1 and 50 % > 1. (13) We observe s/d ratio > 1 only in one DP.

Atrial reverse velocity in pulmonary vein of DP was significantly higher in relation to Ar-wave velocity in Retrievers (Table 2) and other breeds. (Table 5), but was similar to normal DPs from another study.(6,7,13) Atrial reverse flow in pulmonary vein is produced by left atrial contraction in late diastole and is influenced by left ventricular compliance, left ventricular mid diastolic pressure, left atrial contractility and HR, which was higher in our DPs than in other dogs from literature. (2,7,8) Severely increased Ar-wave velocity could be a sign of restrictive filling of the left ventricle. At the same time an increased mitral E-wave to A-wave ratio appears. Values of the mitral E- to A-wave ratio were normal in DP from our study. Schober and Fuentes in one of their studies found an increased Ar-wave velocity in dogs with various cardiovascular diseases. The highest values were found in dogs with dilatative cardiomyopathy (12) In the study of diastolic function in normal DPs and DPs with dilated cardiomyopathy authors found no such connection. (13) High left atrial filling pressures also influence greatly on the increase of the Ar-wave velocity. The compliance of the left atrium in normal circumstances is greater than the compliance of the pulmonary vein system. At the left atrium filling pressure > 6 mmHg atrial reversal flow begins. Its velocity increases by increasing left atrial filling pressure. (14)

The average value of the mitral E-wave velocity in DP were higher than the values of the E-wave velocity in other breeds and boxers (Table 5), but did not differ significantly from the values of the E – wave in our group of Retrievers and DPs from another study. (6,7,13) Although we found no correlation between HR and E wave velocity it is known from other studies that higher heart rates increase E wave velocity therefore we can conclude that this was the case in our DP. (13) Mitral A-wave velocity in DP differentiate significantly from the values of A-wave velocity in Retrievers and from the values of A-wave velocity in other breeds and boxers (Table 5) and also DPs from the literature (6,7,13) Most likely the reason for higher E and A wave velocity were high heart rates, which is known to increase E and A wave velocity. O'Sullivan et al found that increase in HR result in an increase in mitral E – and A – wave. (13) DPs in our study had higher values of HR than dogs from other studies. (6,7,13)

The E-wave to A-wave ratio was lower in DP than in dogs from the literature, but yet in normal ranges. The E/A ratio in DP did not differ to the E/A ratio in Retrievers and DPs from the literature. (6,7,13)

Four DP (10,2%) had A-wave velocity higher than E-wave velocity and in these dogs E-wave to A-wave ratio was less than 1,0. The values of the E-wave to A-wave ratio less than 1,0 were also found by other authors in dogs more than 6 years old. (8) Our DP with the E/A < 1,0 were 4,5; 5, 6 and 8 years old.

Deceleration time of mitral E wave was in DP significantly longer in comparison to Retrievers (Table 2), other breeds and boxers (Table 5) (6,7) Similar DTE values as in our study were also found by authors assessing diastolic function of LV in DPs. (13) Mitral deceleration time reflects LV compliance and therefore viscoelastic properties of the LV, which are determined also by LV walls. Prolonged mitral deceleration time can indicate an impaired relaxation of the left ventricle. (12) The time interval of filling of the left ventricle can be prolonged also due to thinner left ventricular walls. The results in our study as well show the difference in the thickness of the left ventricular wall in DP and, Retrievers and other breeds. (6,7) The average left ventricular posterior wall thickness in systole in DP was significantly lower in comparison to Retrievers and to boxers, and the average interventricular septum thickness in diastole in DP was also significantly lower in comparison to boxers but not to our Retrievers. (6,7) The average body weight in DP was 35,1 kg, in Retrievers 29,95 kg and in Schober's boxers 30 kg of average body weight. (6)

Thinner left ventricular walls among other factors influence the left ventricular filling. The calculation of the Pearson's correlation factors also showed significantly negative correlation between the left ventricular posterior wall thickness and mitral deceleration time - thinner the wall, longer the mitral deceleration time. In contrary the Retrievers showed a positive correlation between DTE and IVSs and LVPWd. Similar values of the mitral deceleration time in normal DP were obtained by other authors. (13)

The isovolumic relaxation time values in DP were similar to those in Retrievers and in dogs of various breeds and normal DPs from the literature but were significantly longer from values of boxers (Table 5). (6,7,13) A short LV IVRT indicates an earlier mitral valve opening and can be seen in normal young individuals. The population of the examined boxers from Schober's study was younger (2,8 ± 1,7 years) than the population of examined DP (4.31 ± 2,38 years) and Retrievers (3,97 ± 2,07) in our study. That could

explain the difference in IVRT values between DP and boxers.

Gender in our DP and Retrievers had no influence on the echocardiographic indices. A smaller number of examined DP and also Retrievers in our study than the number of examined boxers could be a reason for the difference in gender influence on echocardiographic indices between these studies. In boxers there was found higher diastolic pulmonary venous flow velocity and lower s/d ratio in females than in males. (6)

Age in our study had no influence of any echocardiographic diastolic indices in DP. In Retrievers we found a positive correlation between age and s/d ratio. Schober found significantly positive correlation between age and isovolumic relaxation time, but solely in the group of older dogs, not in the average of the whole group. Our DP were in average 4,31 years old, so there is not to expect the age to significantly impact the isovolumic relaxation time. The same goes for all the other parameters on which age in our study had no influence. (7) In DPs from the literature age negatively correlated with mitral E – wave and mitral E/A ratio and positively correlated with mitral DTE and IVRT. (13)

Body weight in our study also did not correlate with any of the diastolic indices in DPs nor in Retrievers. Insignificant influence of body weight on the diastolic indices in our study could be due to the narrow range of the body weight values in DP (26-53 kg) and also Retrievers (26 – 36 kg) in comparison Schober's study (2 – 43 kg) (8) Schober also states that most of the Doppler echocardiographic indices are not body weight dependent. (6) In DPs from the literature BW positively correlated with mitral E/A ratio, mitral DTE and IVRT. (13)

Heart rate was found to have much greater effect in other studies of diastolic function in dogs. (8) We found only a negative correlation between heart rate and mitral E- to A-wave velocity ratio in DP and a negative correlation between heart rate and IVRT in Retrievers. (Table 2) A negative correlation between heart rate and IVRT was also found in DPs from the literature. (13) At higher heart rates E-wave velocity is lower due to shorter duration of the early diastole. There is also shorter time period between the closure of the aortic valve and closure of the mitral valve (IVRT). Heart rate did not influence any other diastolic parameters in our study; this could be due to a smaller group of dogs of the same breed, with narrower range of heart rates. Other studies included dogs of various sizes with a large scale of heart rates. (8)

Correlations between individual echocardiographic indices in our study showed a negative correlation between E-wave velocity and the pulmonary venous s/d ratio in DPs (Table 3). At the high values of the atrio-ventricular pressure gradient the velocity of early filling of the left ventricle is high, so the correlation is logical. Due to strong ventricular suction in early diastole also the velocity of pulmonary venous diastolic flow is high. At higher E-wave velocity the s/d ratio decreases due to higher d-wave velocity. In Retrievers a positive correlation between LAD and s/d ratio was found. When the LAD is greater also the pressure gradient between pulmonary veins and left atrium in LV systole is greater and therefore the greater s/d ratio values.

In Retrievers also a positive correlation between mitral E/A ratio and LVDd and a positive correlation between mitral E/A ratio and EDVtz were found (Table 4). When the LVDd is greater also the transmitral pressure gradient is greater and therefore the greater E/A ratio values.

In conclusion we can say that DPs show some differences in pulmonary venous flow and deceleration time of mitral E comparing to Retrievers which can be most likely due to breed specifics as it was shown for systolic parameters in this breed. (5) Some differences of course may be a consequence of physiologic conditions such as heart rate and also to inter observer variability due to different sample volumes and different depth of sampling in the pulmonary vein which can change conditions affecting these parameters.

In summary we can conclude that this normal sample of DP and Retriever populations were chosen carefully and that these echocardiograpic values of diastolic function can serve as a basis for further studies.

Study limitations

Larger groups of DP and Retrievers with a wider age range would enable us to see age and weight related influence on diastolic echocardiographic parameters. To get more exhaustive information of diastolic function more parameters could be evaluated such as mitral A wave duration, mitral E at A wave velocity, pulmonary vein atrial reverse flow duration, the relation between pulmonary venous Ar wave and mitral A wave duration. Tissue Doppler analysis of diastolic function would give us even more insight into LV diastolic properties in examined dogs. This should be a challenge for further studies.

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EHOKARDIOGRAFSKI DIASTOLIČNI INDEKSI LEVEGA PREKATA PRI ZDRAVIH DOBERMANIH IN PRINAŠALCIH

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Povzetek: Namen študije je bil ovrednotiti diastolično funkcijo levega prekata s pomočjo doplerkse ehokardiografije pri zdravih dobermanih (DPs; n=39) in ugotoviti učinek spola, starosti, telesne mase in srčne frekvence na diastolične vrednosti. Diastolične vrednosti dobermanov smo primerjali z enakimi parametri pri skupini prinašalcev (n=20). Pri dobermanih smo našli naslednje korelacije: negativno korelacijo med srčno frekvenco in razmerjem mitralnega pritoka (E/A), negativno korelacijo med hitrostjo E vala mitralnega pritoka in pojemalnim časom mitralnega E vala (DTE) in negativno korelacijo med prosto steno levega prekata v diastoli in DTE. Starost, teža in spol pri dobermanih niso vplivali na diastolične indekse levega prekata. Pri prinašalcih smo našli naslednje korelacije: negativna korelacija med premerom levega prekata v diastoli in razmerjem mitralnega pretoka E/A, pozitivno korelacijo med debelino medprekatnega pretina v sistoli (IVSs) in DTE, pozitivno korelacijo med prosto steno levega prekata v diastoli in DTE in pozitivno korelacijo med dimenzijo levega atrija in sistolično-diastoličnim razmerjem pljučnega venskega pritoka. Telesna masa pri prinašalcih ni prav nič vplivala na diastolne indekse levega prekata. V obeh skupinah tudi spol ni vplival na diastolne parametre.

Statistično značilne razlike v diastolnih parametrih med dobermani in prinašalci so bile naslednje: diastolni val pljučnega pritoka (P = 0,0316), sistolni val pljučnega pritoka (P = 0,0035), atrijski reverzibilni val pljučnega pritoka (P < 0,001) in mitralni val A (P = 0,0179) so bili pri dobermanih značilno večji in tudi DTE (P < 0,001) je bil značilno daljši. Razlike smo našli tudi v primerjavi s skupino različnih psov in bokserjev v literaturi.

Ugotovitve kažejo na to, da so pri dobermanih možna nekatera odstopanja od diastoličnih parametrov v primerjavi z drugimi pasmami, kar je najverjetneje pasemsko pogojeno, oziroma je razlika lahko v vrednostih diastoličnih parametrov posledica razlik v fiziološkem stanju pasem, npr. različnih srčnih frekvenc ali pa posledica različnega vzorčenja.

Ključne besede: veterinarska medicina; kardiologija, veterinarska; levi prekat, delovanje-fiziologija; diastola-fiziologija; ehokardiografija; psi

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4TH MEETING OF YOUNG GENERATION OF VETERINARY ANATOMISTS

Supported by European Association of Veterinary Anatomists - EAVA
Ljubljana, Slovenia, 8 - 10 July 2007
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4. SREČANJE MLADIH VETERINARSKIH ANATOMOV – YGVA

Pod pokrovitelstvom Evropske zveze veterinarskih anatomov EAVA
Ljubljana, Slovenija, 8. - 10. julij 2007
Sofinancirala Javna agencija za raziskovalno dejavnost Republike Slovenije

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Invited lectures – Vabljena predavanja

CONFOCAL MICROSCOPY: PRINCIPLES AND APPLICATIONS

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Background

The laser scanning confocal microscopy (LSCM) is an essential tool for many biomedical imaging applications at the level of the light microscopy. It enables multi dimensional imaging and optical sectioning of fluorescently labeled thick specimens and living cells. Argon ion and Helium/Neon laser beam of different wavelength is commonly used to excite fluorochrome present in the specimen rapidly, point by point in the x-y plane. The emitted fluorescent light of longer wavelength originating from the excited dye is then collected by the objective, directed through a small pinhole which rejects out of focus photons and reduces out of focus light. In this way thin and high quality optical section is generated with Z-resolution significantly improved compared to the conventional light microscopy. By changes in the focal plane serial of optical sections can be obtained from thick specimens and displayed as a digitalized images that allows subsequent 3-dimensional reconstruction (XYZ).

Material and methods

Liver cryo-sections and in vitro cultured rabbit embryos and in human embryonic kidney (HEK-293) cell line (European Collection of Animal Cell Cultures (Salisbury, UK)) were studied in a Leica multispectral LSCM (Leica TCS NT). The sequential detection of the microtubules labelled with indirect immunofluorescence, rhodamine-phalloidin-labelled actin filaments and the nuclei labelled with TO-PRO-3 iodide was achieved with the use of argon and helium-neon laser with excitation lines at 488, 543 and 633 nm. The data from the channels were collected sequentially using an oil immersion objective lens (Leica, Planapo 40◊N.A.=1.25) with fourfold averaging of single frame scan at a resolution of 1024 x 1024 pixels. When appropriate, Z-series were generated by collecting stacks of optical slices by using a step size around 1 μm in the Z-direction. Acquired images were analysed and presented by Leica Confocal Software (Lite Version, Leica Microsystem, and Heidelberg, Germany) and Adobe Photoshop 7.0 computer software, respectively. For the three-dimensional (3-D) reconstructions stacks were also exported and analyzed in Silicon Graphics by Imaris 3.0 (Bitplane).

Results and discussion

In our laboratory, we primarily use confocal microscopy to study cell's cytoskeleton distribution pattern in tissue samples as well as in in vitro cultured rabitt embryos (1) and in cell lines stably expressing individual members of membrane-bound G protein-coupled receptors (2). Examples of actin and microtubules cytoskeleton visualization are shown on Fig. 1. Technique of direct fluorescence using rhodamine-phalloidin demonstrated actin cytoskeleton in frozen liver tissue sections (Fig. 1a) and in paraformaldehyde fixed and with Triton X-100 permeabilised rabbit embryos (Fig. 1b). Indirect immunofluorescence using a mouse monoclonal anti-tubulin antibody was used to visualise microtubules distribution in HEK-293 cell line (Fig. 1c). The cell nuclei were stained with To-Pro-3 (Molecular Probes, Oregon, USA) for 30 min. Actin filaments are located under the cell membrane of hepatocytes (Fig. 1a) and blastomeres (Fig. 1b). In the cultured HEK-293 cells, the microtubule radiates through the cells from microtubule organization centers (Fig. 1c).

Over of the past decade, technological advancements in the LSCM have mainly encompassed improvements in the photon efficiency of the LSCM and continued development in digital imaging methods, laser technology and the availability of brighter and more photostable fluorescent probes. Such advances have made possible novel experimental approaches for multiple label fluorescence, live cell imaging and multidimensional microscopy. In conclusion, advantages of confocal microscopy which includes greater spatial

resolution over conventional fluorescence microscopy, optical sectioning of examined samples, 3-D images reconstruction and multi channels acquisition enabled widespred use of confocal microscopy in the cell biology imaging.

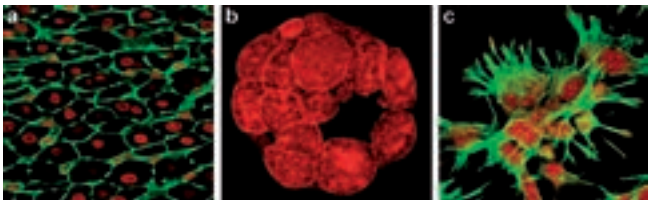

Figure 1: Confocal images of actin and microtubules cytoskeleton organisation. (a) confocal image of liver tissue section shows cortical actin organisation of rat hepatocytes (green signal) (b) actin cytoskeleton distribution in blastomeres of three days old rabbit embryo shown in red (c) cultured HEK-293 cells showing normal distribution of microtubule filaments (green signal). TO-PRO-3 iodide stained nuclei (a, c) are shown in red.

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MITOCHONDRIAL TRIGGERING OF CELL DEATH AND CONFOCAL MICROSCOPY

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The laser scanning confocal microscope detects images of multiple labelled fluorescent samples. One can follow intracellular distribution of the protein under investigation by tracing the location of fluorescently labelled protein or fluorescent antibodies directed against the protein under investigation and of marker proteins for cellular compartments. This is useful to localize the protein under investigation and even more to follow the movements of a particular protein within the living cells. Here we present an example of procaspase-9 movements during the early stages after triggering apoptosis, before its activation can be detected by other biochemical methods.

Apoptosis is a process that controls the number of cells and their quality. Procaspase-9 is the inactive form of one of the main apoptotic initiators, caspase-9. It is activated as a consequence of mitochondrial damage and can be also activated directly or indirectly by other initiator caspases. There were contrasting reports that caspase-9 is in different cellular compartments, i.e. in the cytosol, the nucleus and in the mitochondria. We have determined that procaspase-9 is located in the cytoplasm in physiological conditions in rat neurocrine cells and rat hepatocytes, by transfecting the cells with DNA encoding the fluorescent fusion protein between the caspase-9 and enhanced green fluorescent protein (EGFP) and by immunocytochemistry. However, upon the induction of apoptosis, procaspase-9 is translocated to mitochondria. This shift depends on an activated caspase, other than caspase-9. The colocalization signal of caspase-9 and of mitochondria observed under the confocal microscope does not tell us whether the caspase-9 is associated with mitochondria or it is located closely to the mitochondrial outer membrane. Through biochemical methods, like cellular fractionations, in vitro import of proteins into mitochondria, mitochondrial fractionations and protease treatments of mitochondrial membranes, we determined that procaspase-9 is attached to the outer surface of the mitochondrial outer membrane shortly after the initiation of apoptosis.

MEDCIAL IMAGE ANALYSIS

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Over the last decades, medical imaging has witnessed a diversification of image formation methods, which has led to a rich palette of modalities providing information on many aspects of human anatomy, physiology, and pathology. In order to use the vast amount of available image information efficiently, the relevant image content needs to be extracted, analyzed, and interpreted. For a human operator, it is by no means trivial to interpret the images accurately in a limited amount of time. In addition, such an interpretation is subjective and generally irreproducible. Accordingly, a number of image analysis techniques have been introduced to assist the human expert in a broad variety of tasks, such as image restoration, image segmentation, image registration, motion tracking and change detection, and measurement of anatomical and physiological parameters. Image analysis techniques, which have expanded the role of medical imaging beyond mere visualization, are nowadays used increasingly throughout the clinical track of events, not only within diagnostic settings, but also prominently in the areas of planning, performing, and evaluating surgical and radiotherapeutical procedures.

Oral presentations: abstracts – Predavanja: izvlečki

COMPARATIVE MORPHOFUNCTIONAL ORGANIZATION OF THE ENTERIC NERVOUS SYSTEM IN MAMMALS

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The gastrointestinal (GI) tract fulfils a variety of functions such as transport of luminal content, secretion, absorption of ions, water and nutrients, blood flow, defence against pathogens and elimination of waste material. The enteric division of the autonomic nervous system (i.e., the enteric nervous system (ENS), the brain in the gut, the small brain) organizes and coordinates these activities in a dynamic way through interaction with different cell systems, including the interstitial cells of Cajal, the enteric glia, the smooth musculature, and the vascular, immune and mucosal epithelial systems. The ENS is composed of enteric neurons and glial cells which arise from vagal and sacral precursors cells of the neural crest line. The ENS extends along the entire GI tract and contains an estimated 108 neurons which are situated between two major layers in two interconnected ganglionated plexuses: the myenteric plexus

(MP), between the longitudinal and circular muscle, and the submucosal plexus (SP) associated with the mucosal epithelium. Both plexuses are composed of ganglia (contain neurons and glial cells) and interconnecting nerve fibre strands, which consist of the axons of myenteric neurons, the axons of extrinsic neurons that project to the gut wall and glial cells. Over the last decades, several studies dealing with the ENS of different species have revealed that the architecture of the enteric plexuses is more complex in larger animals, including man, than in small animals. The MP forms a continuous network that is continuous around the circumference and extends from the upper oesophagus to the anal sphincter. Its texture and ganglionic density show regional differences in the same individual, and differences between species. The submucous plexus exhibits a limited number of neurons in the oesophagus and gastric compartments, with a more complex intramural structural organization in the ruminant forestomach, and a continuous plexus in the intestine, that is situated on one plane in small animals, and multilayered and functionally distinct in large animals.

GI neurons release a plethora of substances that are chemically different but only partially have been identified functionally.

Combined morphological, electrophysiological, pharmacological, neurochemical and retrograde labelling, has led to identification of GI neurons into different functional classes, i.e., sensory neurons, interneurons, excitatory and inhibitory motor neurons. These neurons are interconnected by chemical synapses into intrinsic neuronal circuits that generate functional reflexes: they are partly independent of the central nervous system (CNS). In the intestine reflex functions arise even if the segment has been isolated from the body.

FLOURESCENT IMMUNOCYTOCHEMISTRY – A METHOD FOR STUDYING GENE EXPRESSION IN A MOUSE BRAIN

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Fluorescent microscopy techniques are excellent tool for studying cell identity and micro-circuitry in the brain. We are using Steroidogenic factor 1 knockout mice (SF-1 KO) and mice expressing GFP under the influence of SF-1 promotor as models to study neuroendocrine brain development. In the SF-1 KO mice, a very specific disorganization of the ventromedial hypothalamic nucleus (VMH) occurs, with all other parts of the brain being intact.

For studying gene expression in the mouse brain, immunocytochemistry on free-floating sections is used. To obtain brain tissue, mice are perfused with 0.05M PBS and 4% paraformaldehyde. 50?m thick coronal brain sections are cut on an Integraslice vibrotome (Campden instruments) and further processed for immunocytochemistry. For the present study, primary antibodies against calbindin D-28k raised in mouse, estrogen receptor alpha and green fluorescent protein both raised in rabbits, were used. For fluorescent detection of bound antibodies, sections were incubated with secondary antibodies conjugated with Cy2 or Cy3 fluorophores. Bound Cy2 and Cy3 flourophores were visualized under specified excitation wavelengths using confocal microscope. Primary mouse and rabbit antibodies (anti GFP/anti calbindin, anti ERalpha/anti calbindin) were used simultaneously while labelling of GFP/ER alpha coexpressing cells was performed by sequential incubation with each antibodies.

Immunocytochemistry with all three antibodies produced a strong fluorescent signal. Examination of sequential sections revealed that calbindin and ER-alpha are expressed in the same cells both in WT and SF-1 KO mice, even though the location of these cells is altered in SF-1 KO mice, while GFP cells (SF-1 expressing cells) do not co-express either ER-alpha or calbindin.

THE INFLUENCE THROUGH FEEDING ON THE FAT PADS IN THE BOVINE DIGITAL CUSHION

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The effect of an intensive respectively extensive feed on the fat content and the fatty acid profile of the bovine digital cushion was tested by examining the claws of 32 feedlot animals. In addition, it was examined if, respectively how the fatty acid profiles can affect the claw health. Samples from subcutaneous adipose tissue and the claws of 9 cows served as comparison. Furthermore, the microscopic structure of the fat pads was analyzed and the results were compared with those from previous studies.

The fat pads as well as the subcutaneous adipose tissue showed obvious differences in the fat content and the fatty acid profile between the two different feeding-groups. The fat pads of the intensive fed animals contained a lot less fat and noticeable more omega-6-fatty acids, above all Linoleic and Arachidonic acid. In addition, these animals showed the highest proportion of Eicosapentaenoic acid (EPA) and Docosahexapentaenoic acid (DHA), two omega-3-fatty acids. The extensive fed animals contained more omega-3-fatty acids, mainly α- Linoleic acid. Also the subcutaneous adipose tissue of the intensive fed animals showed a much higher proportion of omega-6-fatty acids, whereas the extensive fed group had a higher proportion of omega-3-fatty acids. The differences in the fatty acid profile are for sure due to the different composition of the feeds.

The claws of the intensive fed animals showed post mortem a significant better claw health than the extensive fed group.

THE SENSA COW, A HAPTIC MODEL FOR RECTAL EXPLORATION TO BRIDGE THE GAP BETWEEN ANATOMY AND CLINICAL WORK: PRESENTATION OF THE WORK OF THE COWBOYS EMMA PROJECT GROUP

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In this presentation the results of the co-operation between the School of Arts and the Faculty of Veterinary Medicine to develop a haptic interface to practice rectal examination will be shown.

The goal of the project was to develop a device that can be used between the classes of our regular course of topographical anatomy and the rectal examination in the clinical phase of the veterinary school teaching program. The major learning goal was to achieve the 3D orientation in the cow. The result is the Sensa cow which has wax elements with sensors. After evaluation it appears that Sensa is very useful in learning the first 3D orientation in the cow.

DIFFERENCES IN SKIN COMPONENTS INSIDE REPTILIAN AND AMPHIBIAN GROUPS

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Reptiles and amphibians became increasingly popular pets. In recent years the knowledge on medicine of these animals is improving; however there is a gap between general knowledge on morphology and detailed studies of certain organs on selected species. Comparative study of integument of conventionally kept species, dissected at student practical work was performed. Both reptiles and amphibians demonstrate skin shedding or slough and commonly histology slides show the upper, shedding layer of skin.

While amphibians in terrestrial phase show slightly more prominent keratinisation, the skin is very thin. In dermis there are prominent poison (serous) and mucous glands, and secreting Leydig cells are occasionally encountered in epidermis. While axolotl was not known to posses toxic or irritating skin secretion, we found prominent poison glands. Buffonidae are supposed to have poison glands concentrated on warts. We did find numerous poison glands also on other parts of the body but the size of them increased from abdomen through legs and was greatest at warts on dorsum. In Ranidae the size of poison and mucous glands was approximately the same. In aquatic species Leydig cells were more numerous.

While snakes have similar strength and distribution of scales, in lizards seemingly the skin toughness varies a lot. However, the epidermis on flank skin (excluding ornamental scales) was only twice as thick in green iguana (or tortoise red-eared slider) compared to leopard chamelon, tocke gecko and leopard gecko. The main difference is in dermis. Geckos are colloquially known as scaleless lizards, nevertheless, typical overlaps and hinges were also found.

The black and white subcutaneous glandsî that students found on the neck region in tocke gecko and Rana frog turned out to be at least in part lymphatic tissue.

EPIDERMAL SHEETS - PREPARATION, QUALITY CONTROL, IMUNOHISTOCHEMISTRY AND VISUALISATION BY CONFOCAL MICROSCOPY

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Epidermal sheets are often used for studies regarding exclusively the outer skin layer. They can be prepared with inorganic salt solutions or enzymes and used subsequently e.g. for tissue culture and immunohistochemistry. The aim of this study was to test selected methods for preparation of epidermal sheets and to assess conservation of histological structure, stainability including immunohistochemical staining and the possibility of visualisation of staining results by confocal microscopy. Punch necropsy samples (diameter 3 mm) of shaven neck skin of an eight days old piglet euthanised for another study were taken and stored in a moist chamber at 4°C before processing. Epidermal sheet preparation was attempted after incubation with 2 M CaCl2 solution (20 min, 37°C), with 20 mmol/l EDTA-solution (3 hours, 37°C), or with 0.1% trypsin solution (30-120 min, 37°C). Whole mount immunohistochemistry and/or nuclear staining with DAPI was performed without permeabilisation of the sheets, after permeabilisation with chilled acetone, or after permeabilisation with 0.1% Triton X-100. Staining results were visualised using a laser scanning confocal microscope. For quality control, selected samples were embedded in paraffin and epoxy resin for light and electron microscopy, respectively. The easiest and least time consuming method for epidermal sheet preparation was incubation in a CaCl2 solution. The epidermis was firm enough to handle and peeled of the corium without difficulties, including epithelial root sheaths of hairs. Preparation of epidermal sheets with trypsin was unsuccessful, even after prolonged incubation. Only a 0.5†mm margin of the epidermis could be detached from corium, both corium and epidermis were very brittle. CaCl2 as well as EDTA sheets stained well without differences regarding different pretreatment methods. Morphology of epithelium and corium was conserved satisfyingly in all samples. Interestingly, basement membrane material (laminin, PAS-positive material) could be found on both epithelium and corium, indicating a splitting of the membrane itself during preparation. Connective tissue did never remain on epidermal sheets. If the basement membrane was split incompletely during preparation, basal epithelial cells remained on the surface of corium. Confocal microscopy could be used successfully to visualise individual cell layers of the epidermal sheets. However, epithelial root sheaths of hairs caused a wavy appearance of the epidermis and impaired the assessment of e.g. cell numbers and stratification. In conclusion, skin epidermis can be easily detached from corium after incubation in a CaCl2 solution. Whole mount immunohistochemical staining as well as routine histology and staining of sections are possible without disruption of the sheets. For hairy skin removal of root sheaths from the epidermis should be attempted for confocal microscopy.

CONFOCAL MICROSCOPY – A TOOL TO STUDY 7TM RECEPTOR CHIMERAS OF GHRELIN RECEPTOR WITH GABAB RECEPTOR TAIL-SWAP

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Seven transmembrane receptors (7TM-Rs) also designated as G protein-coupled receptors (GPCRs), were traditionally thought

to act as monomers, but this idea has been challenged over the past several years by accumulating pharmacological and biochemical data about the association of many GPCR types into higher-ordered oligomers. Many different approaches were employed e.g. co-immunoprecipitation of differentially-tagged expressed receptors, sucrose density gradient fractionation, Western blot, functional complementation of two inactive mutant receptors, atomic force microscopy, and proximity assessment of receptor proteins in cell membranes using bioluminescence and fluorescence resonance energy transfer (BRET, FRET) techniques to show that 7TM-R can form either homo- or heterodimers.

Heterodimerization in family C 7TM-R has been most extensively studied and demonstrated. Therefore, these receptors represent a good model for studying the functional relevance of 7TM-R dimerization. GABAB receptor, which is a member of class C 7TM-Rs, is an obligatory heterodimer composed of two distinct subunits, $GABA_{B1a}$ (GB1a) and $GABA_{B2}$ (GB2). During evolution, a system has been developed to ensure that only the functional heterodimer reaches the cell surface. GB1 subunit contains an endoplasmic reticulum (ER) retention signal in its intracellular tail, preventing it from reaching the cell surface as a monomer. Only when associated with GB2, this subunit can reach the cell surface and function. Although no covalent linkage between the subunits has been observed, these dimers are likely to be very stable due to the coiled-coil interaction. Consequently, our approach to study dimerization of family A member ghrelin receptor (ghR) was based on engineering ghrelin receptor (ghR) constructs with swapped GB1a (ghR-GB_{1a}) or GB2 (ghR-GB_2) C-terminal tails, which should selectively lead to formation of heterodimers. Constructs were tested with the classical pharmacological tools and the results confirmed by the means of confocal microscopy. To detect cellular localization of ghR-GB_{1a} chimera indirect immunofluorescent staining in non-permeabilized and permeabilized cells was employed. Co-localization experiments with an ER resident chaperone protein calnexin were employed to detect distribution pattern of the chimeric protein.

On the basis of obtained results, it could be suggested that the ghR-GB1a chimeric construct was not completely retained in the ER in the absence of the ghR-GB2 chimeric construct. On the contrary this chimera was capable of targeting to the cell surface, binding, and signaling. Therefore this system cannot be considered for studying dimerization of the ghR, a member of family A of 7TM-Rs, or adapted to other families of 7TM-Rs for which the functional significance of dimer formation is still unknown.

LASER SCANNING CONFOCAL MICROSCOPY IN CELL CYTOSKELETON AND APOPTOSIS STUDIES

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Fluorescence-based imaging including laser scanning confocal microscopy (LSCM) is extensively used in the field of biomedical research. In our laboratory effects of some toxic substances on cytoskeleton organisation and apoptosis have been studied by LSCM. Rabbit embryos and whole embryo cultures were examined with a multispectral laser scanning confocal microscope (Leica), using an Argon ion laser beam of wavelength 488 nm and a helium–neon laser with wavelengths of 543 and 633 nm. Immunofluorescence and fluorescence methods were used to stain microtubules, actin filaments and nucleic acids. Additionally, apoptotic cells (programmed cell death) based on the TUNEL method were determined by LSCM. Applications of LSCM and procedures that have been used to stain and visualize the cytoskeleton in rabbit embryos, embryo cell cultures and apoptosis will be introduced.

EVALUATION OF G PROTEIN-MEDIATED ACTIN CYTOSKELETON REARRANGEMENT PATTERN USING CONFOCAL MICROSCOPY

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Reorganization of the actin cytoskeleton could coincide with the activation of several G protein coupled receptors (GPCRs). The small GTPase RhoA plays a central role in GPCR stimulated actin polymerization and stress fiber formation. RhoA is activated through various GPCRs and it has been well established that G proteins of the $G\alpha_{12}$ and $G\alpha_{13}$ family can link GPCRs to RhoA. However, several controversies exist as to the exact role of $G\alpha_{q/11}$ and $G\alpha_s$ in this process. While several reports clearly demonstrate the exact role of $G\alpha_{q/11}$ in this process others show no such involvement. The role of $G\alpha_s$ is still under debate.

Therefore the aim of our study was to examine the changes in actin cytoskeleton rearrangement pattern in cells after the activation of the $G\alpha_{q/11}$- and $G\alpha_s$-coupled GPCRs. We have also monitored the status of actin cytoskeleton in cells expressing different constitutively active mutants of G-protein α-subunits.

To study the role of different G-proteins in actin cytoskeleton rearrangement autofluorescently-tagged β-actin (pEYFP-actin) was co-expressed together with receptor constructs (neurokinin type 1 receptor (NK1-R) and β_2-adrenergic receptor β_2-AR)) or constitutively active mutants of $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{12}$, $G\alpha_{13}$ and $G\alpha_s$ in the HEK 293 cells. Evaluation of the autofluorescently-labeled actin filaments was performed with the use of confocal microscope.

Our findings show that the $G\alpha_{q/11}$-coupled NK1-R activation as well as the expression of different constitutively active mutants of $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{12}$ and $G\alpha_{13}$ caused changes in cell morphology, enhancement in the cortical actin signal and stress fiber formation. In contrast, neither the β_2-AR activation nor constitutively active mutant of $G\alpha_s$ caused any apparent changes in actin cytoskeleton status in the HEK-293 cells. Based on these findings it could be assumed that only $G\alpha_{q/11}$-coupled receptors activation coincides with the robust changes in the actin cytoskeleton organization.

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DEMONSTRATION OF CONNEXINS IN CELL CULTURES OF BOVINE PLACENTA

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Connexins (cx) are the subunits of hexagonal connexons which form gap junctions when docking to each other. Signals may not

only be transduced through gap junctions but also through the mere presence of connexons or even connexins. The expression of cx differs according to their location and function. In human and rodent reproduction, deficiencies in cx expression lead to inadequate embryo implantation and trophoblast invasion and also characterize stages of malignancy in endometrial carcinogenesis. Therefore, cell-cell communication via gap junctional cx may be essential for the restricted trophoblast invasion performed by migrating trophoblast giant cells (TGC) in the synepitheliochorial placenta of the cow. TGC fuse with single caruncular epithelial cells forming mostly trinucleated feto-maternal hybrid cells which deliver hormonal products to the maternal compartment.

To study the potential role of connexins during placentomal development we localized cx26, cx32 and cx43 in frozen sections from day 90-210 of pregnancy and primary cell cultures by immunofluorescence and confocal laser scanning microscopy. The presence of the corresponding cx proteins was confirmed by Western blot analysis.

Although cx26 was present in tissue sections (trophoblast cells) it could not be detected in placentomal cell cultures. Surprisingly cx32 was localized in nuclei of cultured caruncular epithelial cells, whereas in placentomal sections it was found in caruncular stroma and caruncular epithelium specifically at the tips of maternal septa. In contrast to in vivo material, only cultured fibroblastoid cells were positive for cx43. In tissue sections also mononuclear trophoblast cells showed an apical-lateral cell membrane associated cx43 expression. In TGC, cx43 signals differed depending on the localization within the placentome. In the centre of the placentome cx43 was associated to the cell membranes whereas at the base of the fetal villi TGC additionally showed cytoplasmatic cx43 specific fluorescence. In contrast, TGC which were about to fuse with uterine epithelial cells and hybrid cells were negative.

We may conclude that apical cx43 localization supports the hypothesis that cx43 connexons may be involved in the regulation of cell proliferation without forming gap junctions. The correlation of TGC invasion with the loss of cx43 suggests that cx43 plays an important role for the differentiation and migration of TGC. The unexpected finding of cx32 in nuclei of cultured caruncular epithelial cells cannot be explained up to date. The loss of cx26 and cx43 during cell culture of epitheloid cells may be due to suboptimal culture conditions. Funded by the German Research Foundation (DFG).

APPLICATION OF COMPUTER ASSISTED THREE-DIMENSIONAL VISUALIZATION TECHNIQUES IN HISTOLOGY, MEDICAL COMPUTER TOMOGRAPHY AND NUCLEAR MAGNETIC RESONANCE IMAGING

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Over the last years, three-dimensional imaging has become more and more prevalent in biomedical and material science studies. For almost every highly specialized application, different specific 3D-software solutions have been designed. Although the 3D visualization package amira ResolveRTTM has primarily been developed for confocal microscopy, it has also proven to be a valuable instrument in different other applications. In recent studies performed at our department, this stand-alone software pack turned out to be a rewarding tool in morphometrical examinations of the equine semicircular canals starting from medical CT images, ex-vivo haemodynamic studies in porcine and human livers observed by MRI and micro-CT, and histological investigations on the developing digestive system in sea bass larvae and juveniles. The major benefits of this versatile application include the fact that image segmentation is not necessarily based on pixel value thresholds, its ability to deal with unaligned or lost slices and its capacity to render different types of real 3D stereo images or movies, starting from a wide range of input data types. Although the user can easily intervene in almost every automated process such as image alignment or labelling, many of these manual corrections and adaptations are rather time-consuming. Another inconvenience is that the broad potential and complexity of the program causes a substantial load of the internal and graphical memory of the system. Notwithstanding these disadvantages, investment in this software is certainly paying off as it can offer unparalleled representations of complex structures as a basis for the development of new insights in various morphological domains.

Poster presentations: summaries Povzetki posterjev

ELEMENTS OF THE ANATOMY OF TWO STURGEONS (ACIPENSER STELLATUS PALLAS, 1771 AND *ACIPENSER BAERII* BRANDT, 1869): OSTEOLOGY AND RADIOLOGY

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Most of the studies†that have been carried out on†the Acipenseridae concern evolution, phylogeny, diversity, conservation and reproduction, notably concerning their economic importance for caviar production (Billard, 2002). However, information about the peculiar osteology of this family†is relatively rare. The only osteologic data that can be found in the literature (Grassé, 1958 ; Findeis, 1997) are difficult to use in the diagnosis of the bone remains discovered on archaeological sites and are only known for a few species (Radu, 2003). Consequently, a study associating dissections and radiology observations has been attempted in order to gather anatomical information.

Two individuals of two different species have been studied here: a Siberian sturgeon Acipenser baerii and a stellate sturgeon Acipenser stellatus. The first species is potamodromous (migration only in fresh water) while the second is diadromous (migration between salt water and fresh water).

This work is a preliminary contribution to the knowledge of the osteology of these two species of sturgeons and we hope that it will be developed in a near future.

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MORPHOLOGY OF THE STIFLE MENISCI IN DOGS: PRELIMINARY STUDY

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Introduction

In dogs, ultrasonography can be realised to assess soft tissue and bony surfaces. Little is known about ultrasonographic appearance of canine meniscal lesions and their histological appearance and signification. Meniscal lesions are common in dogs and are generally associated with rupture of the cranial cruciate ligament. The medial meniscus is more often and more severely injured than the lateral one.

Objectives

The aims of this study were to set the technique for the histological examination of the dog menisci, to describe the normal echographical and histological appearance and to present 2 pathological specimens of injured menisci.

Methods

Sound menisci were taken from the stifles of an 8 month old mixed breed dog and a 9 years old Bernese mountain dog. Two injured medial menisci were also evaluated: from a 9 years old rottweiler and a 7 years old shepherd dog.

The menisci were examined in situ and after post-mortem excision in a water bath with a linear 7.5 MHz ultrasonographic transducer. Three zones were examined: Zone 1: cranial horn, Zone 2: body of the meniscus, Zone 3: caudal horn, near the collateral ligament.

Vertical sections were made. These are plane perpendicular sections to a given horizontal plane. Two kinds of sections were tested. The isolated menisci of the 8 month old dog were put on the dissection table (horizontal plane) and cut from the cranial to the caudal horn (the sections were triangular in shape, with thin axial border and thick abaxial border), or cut into 4 quarters that were then cut tangential from the abaxial border to the axial border. Menisci of the 8 month old dog were embedded in paraffin whereas the other menisci where embedded in methyl metacrylate and cut with a vertical diamond saw. The sections were stained either with toluidine blue, PAS/ hematoxylin or safranin O.

Results and discussion

Ultrasonography

The normal menisci appeared triangular and homogenously echogenic. The injured menisci were more heterogeneous and contained hypoechogenic areas. In horses, hypoechogenic defects were associated with fibre disruption and collapse, oedema, or degenerative processes such as fibroplasias or necrosis.

Histology

Normal menisci were more fibrous in the middle, with a regular architecture composed of collagen trabeculae in two main directions: circumferential or cranio-caudal direction and radial or abaxio-axial direction. The periphery showed more chondrocytes and more matrix organised in several layers.

In this study, hypoechoic defects or heterogeneous areas were associated with fibrillation, major degenerative changes and modification of internal architecture.

MORPHOMETRIC STUDY OF INTERPHALANGEAL JOINTS IN ARDENNER HORSES WITH JUVENILE OSTEOARTHROPATHY

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Introduction

Little information is available about the morphometrical aspect of osteochondral tissues in horses though osteo-articular pathologies may cause pain, handicaps and also important economic loss. Attempts are made to rehabilitate Ardenner horses but they may develop juvenile osteoarthropathy which lead to precocious cast. This disease, also called osteoarthritis, may be considered as a group of degenerative disorders characterized by a common end stage: progressive deterioration of the articular cartilage accompanied by changes in the bone and soft tissues of the joint.

Objectives

The aim of this study was to improve knowledge about bone and cartilaginous tissues in Ardenner horses with osteoarthropathy, regarding proximal and distal interphalangeal joints.

Material and methods

Two Ardenner geldings aged 4 years were included in this study. One was euthanized because of dangerous behavior and the other for ataxic problems after an accident. Both of them presented radiographic signs of osteoarthropathy with entheseophytes at the dorsal border of the middle phalanx. Fore and rear digits were dissected. Four sampling sites were used for each digit: distal articular surface of the proximal phalanx (P1D), proximal (P2P) and distal (P2D) articular surfaces of the middle phalanx and articular surface of the distal phalanx and navicular bone (P3P). A standardized squaring was carried out using a graduated rubber band giving place to 9 intake points by articular surface. Osteocartilaginous samples, of a fixed diameter of 5 mm over a length of 8-10 mm, were taken owing to a surgical motor with a bell mill. The samples were embedded in methyl metacrylate without previous decalcification. Sections were stained with toluidine blue or methylene blue and were imaged with a microscope provided with a video camera connected to a computer. Five measurements were made with the imaging analysis system (Leica): maximal thickness of full cartilage (TC), maximal thickness of calcified cartilage

(CC), maximal thickness (TSB) and porosity (PSB) of subchondral bone and porosity of cancellous bone (PCB). Maximal thickness of non calcified cartilage (NCC) was calculated as well as the ratio between CC and TC. For each variable, the effect of the following factors was studied: member, articular level, site 1 (type Anterior, Middle and Posterior), site 2 (type Internal, Middle and External) and the interaction between site 1 and site 2.

Results and discussion

Significant differences were observed for the variables listed in the table below.

	Member	Level	Site 1	Site 2	Site 1* Site 2
CC		****	****	****	**
NCC	****	****	****	**	
TC	****	****	****	****	
CC/TC	***	****	****		
TSB	****	****	****		***
PSB		**			
PCB				**	

TC and NCC were thicker for the fore digit, with a smaller ratio CC/TC and smaller TSB. This could be related either to the pathology or to the different distribution of body weight and efforts during locomotion. TC, CC and NCC were thicker for P3P and smaller for P1D. TSB was thicker for P2P and smaller for P2D whereas PSB was higher for P1D. For site 1, CC, NCC, CT and TSB were larger for site M whereas CC/TC was higher for site P. For Site 2, CC, NCC and CT were also larger for site M, as well as TSB, PSB and PCB. The results are related to different strains zones within and between joints. To better understand the results, itís necessary to compare them with aged-matched sound horses.

MORPHOLOGICAL DIFFERENCES BETWEEN THE KIDNEYS OF SHEEP AND DOG

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Introduction

The kidneys of horse, ox, pig, sheep and dog belong to the same group of organs and it is not difficult to identify them. However, the kidneys of dog and sheep are very similar to each other. Our aim, therefore, was to find some differentiating between the organs using certain parameters, which would help identification to which animal do the individual kidney belong.

Material and methods

We collected sheep kidneys during the year 2006 from a few slaughterhouse surrounding sarajevo and sacrificed three sheep for the organs studying in situ. We took kidneys of dogs of different sexes, ages, and breed from the dog-pound in Sarajevo. The number of organs in total was 25 left and 25 right sheep kidneys and 15 left and 15 right dog kidneys. We performed transverse and longitudinal sections for observation of the number and form of lobes and their mutual relationship.

Results

During the examination of the kidneys, some parameters were used. So we compared the dorsal and ventral surfaces (Facies dorsalis et ventralis), cranial and caudal extremities (Extremitas cranialis et caudalis) as well as the borders of organ (Margo lateralis et medialis). Special attention was paid to medial border (renal hilus). These examinations were done separately on the left and on the right kidneys of both, sheep and dogs. Our observations are presented in the table below which shows the differences in some parameters between the sheep and dog kidneys.

Discussion

In various references, including different anatomy books, we did not find detailed morphological description of kidneys of dog and sheep as it is done here considering all the relevant parameters (surfaces, poles and borders including renal hilus). Ellenberger-Baum mentions that the kidneys of small ruminants are bean shaped, while the dog's have similar shape as kidneys of pig, but are thicker and rounded, whereas the pig's are flatter. Our observations brought us to another conclusion. On the other side, Niel D.S. May, while examining the kidneys of dogs and sheep, concluded that Dog kidneys are somewhat longer and have darker color in comparison to sheep kidneys, which are more bean- shaped. Our observations correspond with his statements. Sisson mentions the elliptical form of sheep kidneys with convex surfaces and round borders which are bean shaped, while those of dogs are somewhat longer and have more convex ventral surfaces in comparison to the dorsal surface. However, our observations show that both sheep kidneys have heart-like shape, while the surfaces correspond with the statement of Sisson.

Conclusion

Comparing all the parameters of left and right kidneys separately of both animals, we can say that the similarities are great. That is why it is difficult to differentiate which kidney belongs to which animal.

On the basis of morphological data the most obvious differences of sheep and dog kidneys are connected with the form of the organ. While sheep kidneys are bean shaped, dog kidneys have more the shape of a flat bean. There are also differences in the borders of the kidneys, the Sheep's are more bent, while the Dog's are less bent, especially the medial border which is more straight. Dorsal surface (Facies dorsalis) of Dogs is almost flat, while the sheep's is convex. Kidney poles of sheep and dogs differ in the sense that the cranial extremities more round in comparison to the posterior extremity which is more flat on both kidneys. While the anterior extremity of sheep is somewhat narrower compared to the posterior extremity, dog's extremities have almost equal diameters.

Parameters	Sheep	Dog
Colour	Bright reddish brown	Dark reddish brown
Form	Heart- shaped	Bean- shaped and flat
Facies dorsalis Facies ventralis	Less flat Convex	More flat Convex
Relation Margo lat et med.	More bent	Less bent
Extremitas cranialis Extremitas caudalis	Narrower-rounded Wider - rounded	Wider-rounded Less-rounded and flat
Hilus renalis	Shallow hilus	Deeper hilus

DIFFERENCES OF THE INITIAL PART OF THE URINARY ORGANS (PELVIS RENALIS WITH RECESSES) AND A. AND V. RENALIS, WITH THEIR BRANCHES IN THE KIDNEYS OF SHEEP AND DOG

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Introduction

Morphological studies which have included comparison of dorsal and ventral surfaces (Facies dorsalis and ventralis), lateral and medial borders (Margo lateralis et medialis), as well as comparison of cranial and caudal extremities (Extremitas cranialis et caudalis) of both left and right kidneys of sheep and dogs did not provide us with relevant data by which we could differentiate with certainty the sheep kidney from the dog kidney. That is the reason why we decided to examine by corrosion techniques initial parts of the urinary organs (renal pelvis with their recesses), renal arteries and renal veins (a. et v. renalis) and their mutual relationship.

Material and methods

For the presentation of initial parts of the urinary organs and blood vessels, we used the corrosion technique with the ſVinilyte'. For the sake of examination we used 11 pairs of sheep kidneys and 14 pairs of dog kidneys. The age of the animals was from 1-3 years. For renal pelvis (pelvis renalis) and recesses we used yellow and for arteries and veins red and blue vinilyte respectively. After the injection and when the time needed for hardening of vinilyte was over (8-12 hours) , we put the kidneys in the adequate acid (36% HCl) for the purpose of maceration. 48 hours after the corrosion we washed off the kidneys so we could examine the initial part of the urinary organs and blood vessels as well as their mutual relationship.

Results

By the corrosion preparation of sheep and dog we studied the size and the form of renal pelvis and also the size, form and number of recesses. The distribution of renal artery and vein and their branches also were studied:

Pelvis renalis

Both animals, sheep and dogs, have the same number of recesses. The most obvious difference is that the pelvis walls of dogs are unequal. The dorsal wall from which the dorsal recesses come out is longer then the ventral one. Renal pelvis of sheep on the other side has equal dorsal and ventral wall. Recesses of sheep come closer to their end (dorsal and ventral recesses) than those of dogs. The ureter exit of dog differs in the fact that its initial part is a funnel - shaped, while the sheep's is triangular in form.

The distribution of the renal artery in sheep and dog

Renal artery (a. renalis) of the right kidney of sheep and dog differ in the position. Right renal artery (a. renalis dextra) is divided into two branches, one dorsal and one ventral.

The division of right renal artery in dog is much prior to the hilus, while the sheep's division is just before the hilus. Ventral branch of renal artery in the right kidney of dog is much stronger then the dorsal branch and it provides more interlobar arteries which even run over to facies dorsalis of the cranial pole. The number of interlobar arteries of sheep corresponds with the number of recesses in the kidney and we have the same situation in the left kidney of dog. This is not the case in the right kidney, because the ventral interlobar arteries run into the dorsal recesses. According to this, it is very difficult to differentiate between the sheep and dog kidneys since the vascularity is quite similar in both animals. Any anastomoses between the dorsal and ventral branches, as well as interlobar arteries and their branches have not been noticed.

Table 1: Number of aa. interlobares of the right kidney in sheep and dog

	DOG	SHEEP
Aa. interlobares (dorsales)	6-7	6-8
Aa. interlobares (ventrales)	7-8	6-7

Table 2: Number of aa. interlobares of the left kidney in sheep and dog

	DOG	SHEEP
Aa. interlobares (dorsales)	6-7	5-7
Aa. interlobares (ventrales)	7-8	6-7

The distribution of the renal vein in sheep and dog

Renal veins of both sheep and dog are much prior to renal hilus into two branches, one dorsal and one ventral. From the dorsal branch, at the entrance to renal hilus, two branches run in separate ways and inside of the sinus of the kidney both of them divide into two more branches in sheep, and into 2-3 branches in which they join interlobar veins in dogs.

The ventral branch is stronger than the dorsal and prior to hilus it is divided into three branches, while the fourth branch appears in the renal sinus. These branches give off interlobar veins (mostly 7-9) from the ventral side.

In both left and right kidneys of sheep and dog, there are anastomoses between two neighboring interlobar veins and between the ending branches which are separated from the dorsal and ventral branch of renal vein. The number and position of branches in Dogs differ, and there are also differences between the right and left kidney which can be seen in the table 3 and 4 below:

Table 3: Number of vv. interlobares of the right kidney in sheep and dog

	DOG	SHEEP
Vv. interlobares (dorsales)	4	4-5
Vv. interlobares (ventrales)	8-9	7-9

Table 4: Number of vv. interlobares of the left kidney in sheep and dog

	DOG	SHEEP
Vv. interlobares (dorsales)	6	4-5
Vv. interlobares (ventrales)	6-7	7-8

Conclusion

According to the analysis of the corrosion preparation of pelvis and his recesses, and renal artery and vein and their mutual relationship it can be concluded that it is possible to differentiate not only

the kidneys of the sheep and dog, but also the right and left kidneys of the animals.

The most interesting observation in our investigation is that the interlobar arteries vascularise just the half of two neighbouring lobes. A part of the renal parenchyma in the centre of which lays aa. interlobares is actually the physiological lobus. That is why it would be proper to rename interlobar arteries into lobar arteries which are placed in the centre of the physiological lobus. We also noticed that the anatomical lobus does not coincide with the physiological lobus and also that anastomoses do not exist between the neighbouring interlobar arteries. These information we could not find in the literature. The differences of sheep and dog kidney are mainly connected with the form of renal pelvis and the initial part of ureters, and with the number of interlobar arteries and veins which can be seen in the tables.

STUDY OF THE WILD FAUNA OF FRENCH ZOOLOGICAL GARDENS: WORKSHOP OF AN ORIGINAL ASSOCIATION

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Dead wild animals are a primary source of information for scientific purposes. Then, a cooperation of different institutions for comparative anatomy studies is a promising approach. In 2006 (Guintard et al.), we have created a working group of dissection (an open association located in Nantes) which continues this goal with new members (Paris, Constantine). Thanks to a more efficient support from zoological gardens of the western part of France, three recent dissection sessions (2006-2007) have been performed and are exposed here: an Orfe (Leuciscus idus L., 1766), a Boa (Boa constrictor L., 1758), and an Hippopotamus (Hippopotamus amphibius L., 1758). This project gathers the advantages of a total traceability: the precise origin of the animals (the zoological gardens) and the removed parts (skin, skeleton or plastinated organs) is exactly known. This incipient association welcomes every person or institution dealing with comparative anatomy (technicians, veterinarians, researchers, Medicine faculties, Anatomy departmentsÖ) and biodiversity conservation (museological collections, genes banks Ö).

Reference

Guintard et al. A study of the wild fauna of French zoological gardens: the birth of an original association, Messina, 19th-22th July 2006, XXVIth Congress of the European Association of the Veterinary Anatomists).

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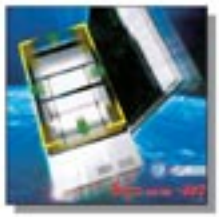

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Article in a journal or newspaper: Fuji J, Otsu K, Zorzato F, et al. Identification of mutation in porcine ryanodine receptor asociated with malignant hyperthermia. Science 1991; 253: 448-51.

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: Veterinary Faculty 1995: 83-6.

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