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CULTURE, SELECTIVE BREEDING AND GENOME MANIPULATIONS OF TENCH IN THE CZECH REPUBLIC (A Review)

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ABSTRACT

This contribution provides a review of the information on the culture of tench (*Tinca tinca* Linnaeus, 1758) in The Czech Republic, and on the intensive experimental breeding work which has been performed at the Department of Fish Genetics and Breeding, The Research Institute of Fish Culture and Hydrobiology at Vodňany, The University of South Bohemia, České Budějovice since 1985. The genome-manipulated polyploid fast growing population of tench produced at this research department, has currently became a target of genetic, morphological, physiological, histological and biochemical analyses.

Key words: genome manipulations; genome polyploidy; *Tinca tinca*

INTRODUCTION

Apart from the common carp (*Cyprinus carpio* L.) as the main commercial species, Czech pond aquaculture focuses on other fish species, designated "supplementary species", cultivated in polyculture stocks with the common carp. The importance of polycultures is, above all, in the better utilization of the natural productivity of the pond environment due to the differing food and space requirements of the fish species cultivated (7).

Tench (*Tinca tinca* L.) is considered the most important supplementary cyprinid fish from the alimentary point of view. Historical records on tench culture in the present Czech Republic can be dated back to the 16th century. The stocking of tench in ponds for breeding began in the 17th century, and since the 19th century, tench has become a perennial part of the stocks in Czech pond-fish farming (23).

From the evolutionary point of view, the present tench originates with the highest probability from *Paleoleuciscus*, a primitive species of tertiary fish of the family Cyprinidae, superorder TELEOSTEI, which lived in the large lake systems of Central Europe (5).

From the geographical point of view, tench is considered a widespread species, living under natural conditions in the standing- and slowly-moving waters of Europe, Siberia and The Caucasus up to 61° of northern latitude (23), in Finland up to 61°30´, and according to Berg (4) even up to 65° of northern latitude (8). It was introduced into several countries of Africa, into Australia, Indonesia and other countries (23).

The wide geographical distribution of tench refers to its large genetic variability, due to which specialists consider tench one of most diverse cyprinid species (5).

TRADITIONAL CULTURE

Apart from The Czech Republic, tench is being produced in only a few European countries: Germany, France, Italy, Hungary and Spain, where it is a highly required species owing to its delicacy and excellent flavour (6) and where it is a target of intensive aquaculture (29).

In The Czech Republic, marketable tench is produced commercially on fish farms, using to culture the tench fry originating from natural spawning in ponds, or from the artificial propagation of our own broodstocks, mostly not genetically specified (19). Light-coloured, low-fat containing and very tasty, the flesh of tench is more appreciated by foreign consumers than by those in our country (2). According to some authors, from the point of view of consumer value, the tench flesh is superior to that of common carp (22). Nearly the entire Czech production of tench (about 350–370 tons of live weight annually) is exported abroad (1), and thus tench appears on the Czech market only sporadically.

Selective Breeding

From this point of view, The Czech Republic has an extraordinary position as the only country in the world dealing intensively with the selective breeding of tench. Breeding activities in The Czech Republic are carried out at the Department of Fish Genetics and Breeding, The Research Institute of Fish Culture and Hydrobiology at Vodňany, The University of South Bohemia, České Budějovice. The conservation of the genetic resources of commercially important fish species in The Czech Republic is another task of this workplace (19). Since 1996, this workplace has been co-ordinating a live gene bank of commercially important freshwater fish (common carp and trouts), complete with other species such as tench, wels, whitefish, and sturgeon one year later (17). Considering the tench, this gene bank currently keeps 6 autochthonous strains of the green phenotype (Vodňany, Hluboká, Tábor, Mariánské Lázně, Velké Meziříčí, Kož. 92), 3 strains collected from Hungarian, Romanian and German populations, 15 strain hybrids and 3 ornamental colour mutations (20).

Genome Manipulations and Characteristics of Polyploid Populations of Tench

Since 1985, the above-mentioned Department of Fish Genetics and Breeding has been carrying out an intensive experimental effort focused on genome manipulations of fish with the aim of developing rapidly growing polyploid populations (15, 26, 27, 28). This effort followed publications by various authors (in the world), which have appeared from various sources during the 1970's and 1980's, first describing induced polyploid fish populations. Foreign experiments have been carried out above all on rainbow trout, Atlantic salmon, brown trout, grass carp, common carp, wels, channel catfish and Nile tilapia.

Starting in 1985, the tench was chosen by the Czech researchers as a model freshwater species suitable for experimental genome manipulations. The results of these experiments up to the present were subsequently published and terminated successfully in 2000 with the elaboration and publishing of "*The Production of Triploid Tench"* (12), a technical manual for the culture of rapidly growing populations of tench.

The term triploidy is explained by the authors as the presence of three chromosome sets (3n = 72) in somatic cells of a triploid specimen of tench as opposed to two chromosome sets (2n = 48) in cells of a "normal" diploid specimen of tench. Triploid individuals tend to grow faster and gain bigger final size compared to diploids, with respect to sex and the degree of development of their gonads (their retardation associated with sterility). It mainly concerns fish species where the different growth rate results from sex dimorphism.

This characteristic phenotype trait of accelerated growth of triploids is due to the deviation from normal gonad development when nutritions and energy gained from food are utilized for somatic growth rather than for reproductive development. Production of polyploid fish is therefore reasonable in such fish species, which gain sexual maturity before gaining marketable weight. Triploid individuals gain higher slaughter value than diploids, as well as a standard high alimentary quality throughout the whole year, as they do not need to utilize energy sources from flesh during the reproductive season.

A proper method of growing tench in monoculture or in adequately chosen polyculture e.g. with herbivorous fishes is essential for manifesting the accelerated growth of triploids, due to feed competition reasons (12).

Spontaneous and Induced Polyploidy

Random disorders of meiosis in fish eggs (retention of the 2nd polar body) after fertilization with the male gamete cause spontaneous polyploidy reflected by the increased number of chromosome sets in somatic cells. Apart from factors like egg overmaturation or sudden changes of water temperature (14), a genetic predisposition was hypothesized as a recessive allele which might be responsible for the retention of the 2nd polar body during meiosis II.

Spontaneous triploidy might be caused also by a combination of the above-mentioned factors (16). According to the majority of authors reviewed by Beafey (3), spontaneous triploidy is a relatively frequent phenomenon in lesser vertebrates and it may occur in individuals within a species (autotriploidy) or in populations of interspecific hybrids (allotriploidy). Polyploid specimens of tench were registered in higher frequencies in populations originating from artificial propagation while no polyploid tench specimen has yet been registered from naturally-spawned populations (9).

Induced polyploidy can be achieved by means of physical treatment (cold- or heat shocks as well as sudden temperature changes; hydrostatic pressure shocks) or chemical treatment of the developing zygote, briefly after fertilization. The method of cold shock appeared to be the most advantageous one under practical conditions (easy to perform, inexpensive and 100 % effective).

There are also promising alternatives to solely induced polyploidy, based upon crossbreeding between tetraploid females and diploid males (21) or a highly sophisticated combination of approaches of uniparental inheritance, sex reversal, and induced polyploidy, finally producing monosex polyploid populations. According to Flajshans *et al*. (11), triploid all-female populations may be produced by cold-shocking the eggs of diploid females that were fertilized with spermatozoa (bearing X sex chromosomes only) produced from diploid gynogenic masculinized females.

METHODS OF PLOIDY LEVEL INVESTIGATION

The identification of the ploidy level was a crucial point of all the above approaches. It can be performed by means of a whole series of methods, differing in requirements for equipment, time spent on the analysis, and requirements for the age, category of the fish, and the number of specimens. Direct chromosome counting (karyotyping) is considered the most accurate method of ploidy-level identification. Somatic cells of diploid tench contain two chromosome sets (2n = 48), while 3 sets of chromosomes (3n = 72) are registered in the somatic cells of triploid tench.

A method of quantifying Nucleolar Organizer Regions (NORs) in interphase cells is another laboratory method used for the ploidy-level identification of swim-up fry or of blood samples from older fish. It is based upon microscopic visualization of the remnants of rRNA-protein complex after staining with $AgNO_3$ (18). One or two active NORs are present in cells of diploid fish, while 1, 2 or 3 (cca 40 % of the total count) active NORs are present in triploid cells. Ploidy level can be assessed from a blood smear also using computer-assisted analysis of a microscopic image (10, 33), based upon size differences in area of erythrocyte nuclei measured in mm^2. Mean value of nuclear area of a diploid erythrocyte of tench is $10.5 \pm 1.3\ mm^2$, compared to $16.5 \pm 1.7\ mm^2$ in triploid tench.

Flow cytometry for determining the relative DNA content in cells (25) appears to be an universal method which can be used for all age categories of fish, as well as for different types of tissues (blood, muscle, epithelium). It is based upon labelling the nuclear DNA of both the reference and analysed sample by a DNA-specific fluorescent dye, followed by fluorescence measurement resulting in the histogram distribution of the fluorescence intensity of labelled particles flowing through. Triploid tench have 1.5 fold more nuclear DNA per cell than a diploid tench, reliably enabling their differentiation from the histogram.

Apart from these direct methods of ploidy level identification it might be possible to use some other indirect methods such as e.g. the systematic recording of the external morphological traits and conditions, providing that there are sufficient numbers of specimens per sample. Traits related to growth and weight, as well as to the gonadosomatic index are considered most important (13, 24). These registered differences in the phenotype manifestation of growth of diploid and triploid tench are not the only existing differences between both fish populations.

Flajshans (10) described the differences in the histological composition of the gonads of females and males of diploid and triploid tench, Sedláček (30) compared the measurable indices of gametes related to sex and ploidy level. Svobodová *et al.* (32) dealt with intensive research into the differences in some haematological indices of diploid and triploid tench. Compared to diploids, triploids were found to have a highly a significant lower total protein concentration in blood plasma, a significantly lower erythrocyte count, and a lower haemoglobin concentration in erythrocytes. The blood profile comprised also mean corpuscular haemoglobin, haematocrit and leukocyte count. Based upon their results, the authors hypothesized a lower oxygenation capacity in the blood of triploid tench which might be associated with a lower level of non-specific immunity. Svobodová *et al.* (31) compared the leukocyte profiles of diploid and triploid tench.

CONCLUSION

The successful development and practical management of production of polyploid tench populations during the last decade of the 20th century at The Department of Fish Genetics and Breeding, The Research Institute of Fish Culture and Hydrobiology at Vodňany, The University of South Bohemia, České Budějovice represents a contribution to the five hundred years' tradition in the culture of this freshwater fish species in The Czech Republic. The current move from the field of breeding towards further scientific studies of these fish populations is a necessary prerequisite for their broader practical application in the new millenium.

There is a possibility of enhancing the annual volume of marketable tench production by means of rearing its polyploid form in closed aquaculture systems. It might not be only for enhancing exports but also for processing in the country of origin for direct sale in the market network. From the alimentary point of view, it would mean a positive contribution in terms of annually equalized nutritional quality of the flesh of tench, as there are no qualitative changes in its chemical composition associated with the reproductive period. Other possibilities for the utilization of triploid tench are in stocking, original angling areas, as well as other regions where tench is not autochthonous and where it is not possible to stock this species or any other fertile alien species due to the maintenance of the ecological balance. Production of triploid tench for the needs of angling targeted to big trophy fish might become an important future part of the system of triploid tench culture.

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THE APPLICATION OF THE PCR METHOD TO THE IDENTIFICATION OF MEAT SPECIES

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ABSTRACT

Pork, beef, chicken, mutton, goat meat and horse meat were identified using the molecular method of multiplex polymerase chain reaction. The DNA extracted from the respective meat species was subjected to the PCR analysis using a mixture of seven primers mixed together in the appropriate ratios and identified using one PCR reaction. The design of the forward primer was based on the known DNA sequences of the cytochrome b mitochondrial gene; the reverse primers were designed based on species-specific sequences depending on the meat species. The resulting fragments at the 157, 227, 274, 331, 398, and 439 bp levels corresponded to goat meat, chicken, beef, mutton, pork, and horse meat, respectively. The PCR products were subsequently evaluated using electrophoresis. PCR also made it possible to identify the respective species of both heat-processed and non-heat-processed meat.

Key words: DNA extraction; meat-species identification; PCR

INTRODUCTION

Meat species identification has recently become a topical issue. The number of producers increase, the range of meat products becomes wider and the international food trade grows. The issue of the real composition of a product and the composition identity with the data presented by the producer on the product packing has emerged. In some cases, the raw materials have been replaced or confused, which means that the real product composition is different from the composition declared. Most meat products are made from various raw materials, auxiliary substances and additives. These ingredients give the product its specific properties. For both sensory and technological reasons, most of the meat products do not contain only one meat species, but several, usually two or three meat species.

There are many methods of additive determination; however, the determination of the respective meat species or the determination of other foodstuffs of plant or animal origin is more complicated.

There is a wide range of methods that make it possible to identify the respective meat species. They include physico-chemical methods (electrophoresis, chromatography, enzyme analysis), immunological methods (methods based on serology, immunodiffusion, immunoelectro-phoresis, blotting techniques, use of radioisotopes and ELISA), methods based on lipid analysis and histological methods. However, these methods have certain limitations, mostly due to the problems connected with the detection of muscular tissue after heat processing.

Developmentes in molecular biology have made it possible to create methods based on precise molecular analysis of nucleic acids. These methods can be used for the identification of the respective meat species, in both farm animals and game. They can confirm the presence of a particular meat species or identify several meat species that were used in a particular meat product. To identify the meat species and type of meat products, DNA hybridisation has been used (2, 7). However, the methods based on DNA hybridization use complex and time-consuming procedures and the tests are relatively expensive. The development and introduction of the PCR method offered further possibilities to molecular biology and the method has also begun to be used for meat-species determination.

When applied to meat-species identification, the PCR method uses a part of the cytochrome b mitochondrial gene that is present in eukaryotic cells of vertebrates (5, 8). PCR (12) is a suitable method of meat identification, as it allows rapid and simple amplification of the template DNA target regions. The aim of this study was to verify the practical use

of the multiplex PCR method in the identification of both heat-processed and non-heat-processed meat.

MATERIAL AND METHODS

The study examined pork, beef, chicken, mutton, goat meat and horse meat. All meat samples were obtained through the regular market network. They were taken to the laboratory in a cooler and kept frozen until the examination. The meat samples were subjected to molecular analysis that included DNA extraction, testing using the multiplex PCR method and evaluation using electrophoresis on agarose gel. The study was divided into four independent phases.

Identification of the non-heat-processed muscular tissue

This part of the study examined six species of non-heat-processed meat: pork, beef, chicken, horse meat, mutton, and goat meat.

Identification of the heat-processed muscular tissue

All of the above-mentioned meat species were heat-processed using two procedures:

1) 100 °C/30 min (boiling, checked using a non-contact thermometer Raynger ST, Raytek);
2) 121 °C/30 min (autoclave, Vaposteri NAD 67).

Semi-quantification of a meat mixture

The semi-quantification of a meat mixture evaluated the pork, beef and chicken meat. Two-species of meat mixtures were prepared in certain ratios; the range was 100 to 4 % (100, 96, 75, 50, 30, 20, 10, and 4 %). The ratios were interdependent; a decreasing proportion of one meat species meant an increasing proportion of the other meat species.

The types of mixture examined:

— mixture of pork and beef,
— mixture of pork and chicken meat,
— mixture of beef and chicken meat.

Identification of meat species in meat products

Various types of meat products produced by the meat-processing industry were obtained through the regular market network. The testing of these products focused on the meat-species detected; the species detected were compared with the product-composition declared.

DNA extraction

DNA was extracted from the above-mentioned raw meat species using the following method. We weighed out 0.025 g of crushed meat and transferred it into a 1.5 ml Eppendorf test tube. Then we added 450 µl of TE buffer for extraction (Tris-HCl, EDTA, pH 8.0), 6 µl of proteinase K and 6 µl of Tween 20. The samples were incubated in a water bath at 50 °C for 1 hour. After adding 170 µl of 5 mol NaCl and 130 µl of chloroform, the samples were turned and centrifuged at 10 000 rpm for 5 min. We removed 500 µl of the upper water phase by suction, transferred this volume into a clean test tube and added 500 µl of phenol/chloroform/isoamyl alcohol in the ratio of 25 : 24 : 1 (Serva, Germany) and centrifuged at 8 000 rpm for 1 min. Next, step, we transferred 350 µl of the water phase into a clean test tube and centrifuged at 8 000 rpm for 1 min after adding 350 µl of chloroform. After that, we transferred 250 µl of the water phase into a clean test tube, added 25 µl of 3 mol sodium acetate and 625 µl of undercooled absolute ethanol, and incubated the test tube at –20 °C for 2 hours. After centrifugation at 10 000 rpm for 4 min, the ethanol was carefully removed by suction so that the pellet was left in place. Finally, we added 40 µl of the TE buffer for storage (Tris-HCl, EDTA, pH 8.0) and incubated the samples in a water bath at 60 °C for 1—2 hours. A refrigerator was used for short-term storage of samples; a freezer was used for long-term storage.

Polymerase chain reaction — multiplex PCR

The meat samples were tested using the multiplex PCR method. The oligonucleotides — primers — were designed for the purposes of amplification based on the known sequences of the beef, pork, chicken, mutton, goat, and horse cytochrome-b mitochondrial genes (1,9,6). The study used the common forward primer SIM and reverse primers G (goat), C (chicken), B (beef), S (sheep), P (pork) a H (horse). To obtain the SIM : G : C : B : S : P : H mixture, the primers were mixed in the ratio of 0.5 : 0.1 : 1.5 : 0.3 : 1.5 : 0.3 : 1.0. The ratio 1 meant 20 pmol of the primer/25 µl of the PCR solution (10). The primers were used together for simultaneous identification of a mixture of six meat species (Table 1).

Table 1. Overview of species-specific primers

Name	Sequence	Length (bp)
SIM	(5´- GAC CTC CCA GCT CCA TCA AAC ATC TCA TCT TGA TGA AA - 3´)	38
G	(5´- CTC GAC AAA TGT GAG TTA CAG AG GGA - 3´)	26
C	(5´- AAGATACAGATGAAGAAGAATGAGGCG - 3´)	27
B	(5´- CTA GAA AAG TGT AAG ACC CGT AAT ATA AG - 3´)	29
S	(5´- CTA TGA ATG CTG TGG CTA TTG TCG CA - 3´)	26
P	(5´- GCT GAT AGT AGA TTT GTG ATG ACC GTA - 3´)	27
H	(5´- CTC AGA TTC ACT CGA CGA GGG TAG TA - 3´)	26

SIM — forward primer
G — reverse goat primer
C — reverse chicken primer
B — reverse beef primer
S — reverse sheep primer
P — reverse pork primer
H — reverse horse primer

25 µl of the reaction mix contained: 10 mol Tris-HCl, pH 8.3, containing 50 mol KCl, 1.5 mol $MgCl_2$, 200 µmol dNTP mix, primer mix (4—60 pmol each), 2 µl of purified DNA, and 1.25 unit of Taq DNA polymerase (10).

PCR amplification: consisted of 35 cycles; we used the MiniCycler PTC-150 (MJ Research, USA) and the following protocol: denaturation 94 °C/0.5 min, annealing 60 °C/0.5 min, and extension 72 °C/0.5 min. A negative control using a non-template DNA was also prepared. We used GeneRuler™ 100 bp DNA Ladder Plus (MBI Fermentas, Lithuania) as a molecular marker in the PCR assay.

Electrophoresis

We analysed 5 µl of the PCR product using the electrophoresis on 3 % (w/v) agarose gel (Serva, Germany) at 130 V for 40 min in the TBE buffer (pH 8.3). Ethidium bromide (1 µg.l^{-1}) was used for staining; the PCR products were evaluated using the UV transluminator.

RESULTS

Identification of the non-heat-processed muscular tissue

The PCR products were amplified from goat meat, chicken, beef, mutton, pork and horse meat. The specific lengths of these products are 157, 227, 274, 331, 398, and 439 bp, respectively. Each species was identified on the specific length of the appropriate PCR product. It is obvious that the number of base pairs increases depending on the meat species. Due to the different lengths of their PCR products, the respective meat species can be detected easily.

The identification of pork and horse meat may be difficult in some cases as their PCR product lengths are similar. If goat meat or mutton were to be detected separately or if only one of these two meat species was mixed with another meat species, a PCR product of the length specific for the goat meat or mutton was identified (goat 157 bp, sheep 331 bp). However, if the assay used a mixture of goat meat and mutton, we detected two lengths of PCR products for each of these species (i.e. the specific product and also goat meat product for mutton and mutton product for goat meat). It was impossible to detect only the specific PCR product lengths for goat meat and mutton if a mixture of these meats was used.

Identification of the heat-processed muscular tissue

The respective meat species were heat-processed. Figure 1 shows the respective meat species following the heat processing performed using two methods – 100 °C/30 min and 121 °C/20 min and 30 min. The template for the PCR assay was the DNA extracted from 500 mg of each meat species. The PCR products were analysed on the 3 % agarose gel. As a results, we obtained specific fragments of lengths corresponding to the PCR products obtained for the respective meat species — i.e. goat 157 bp, chicken 227 bp, beef 274 bp, mutton 331 bp, pork 398 bp and horse 439 bp. The lengths specific for goat meat and mutton could not be detected in a mixture of these two meats, analogously to the non-heat-processed meat mixtures (Figure 1).

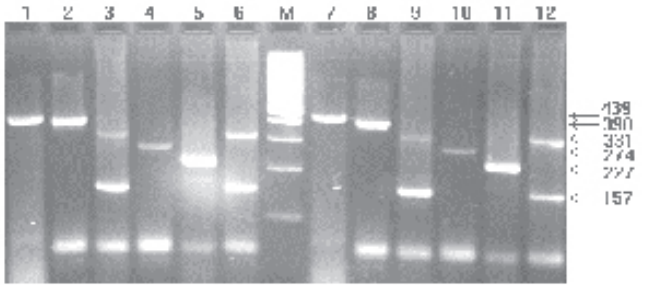

Fig. 1. Molecular identification of heat-processed meat
1, 7 — horse meat (439 bp); 2, 8 — pork meat (398 bp); 3, 9 — mutton meat (331 bp, 157 bp); 4, 10 — beef meat (274 bp); 5, 11 — chicken meat (227 bp); 6, 12 — goat meat (331, 157 bp); M — marker 100 bp DNA Ladder Plus

Semi-quantification of meat mixture

In this step we analysed a mixture of raw pork, beef and chicken. The DNA extracted from the above-mentioned mixed samples was used as a template for the reaction. The meat mixtures always contained two meat species. A variety of different meat ratios was prepared, ranging from 100 to 4 %. The sample type most suitable for the evaluation was a mixture of the P : C meats, regardless of the ratio tested, in which both meats produced the most intense signals. In most cases, the B : C mixture produced fragments giving signals of medium intensity. The P : B mixture analysis often resulted in the predominance of the very intense P fragments in comparison with the B fragments. The detection limit for meat mixture analysis was 4 %.

Identification of meat species in meat products

The respective meat species were identified in various meat products of different types using the multiplex PCR method. The meat species contained in these meat products were identified and compared with the composition specified by the producer (Table 2). According to the results, the composition of certain meat products corresponded to the producers' description of product composition on the packing. However, the composition of some products was different from the composition declared. Therefore, we may conclude that the meat species had been replaced or confused and the formula declared was not observed.

DISCUSSION AND CONCLUSION

In The Czech Republic, the issues of food marking are covered by Act No. 110/ 1997 of the Czech Collection of Laws, i.e. the Act on foodstuffs and tobacco products, as amended by the subsequent legislative acts. Apart from other duties, the law places on the producers

Table 2. Molecular identification of meat species in meat products

Product name (In Czech)	Meat species detected	Declaration	Products corresponding to the declaration
Salám lovecký	P, B	P, B	yes
Salám poličan	P, B	P, B	yes
Salám herkules	P, B	P, B	yes
Salám gothajský	P, B, C	P, B	no
Minišunka	P	P	yes
Salám žampionový	P, C	P, B	no
Tataráček	P	P	yes
Salám „Bratislava"	P, B	P, B	yes
Vídeňské párky	P, B, C	P, B	no
Debrecínské párky	P, B	P, B	yes
Jemné koňské párky	P, B	P, B, H	no
Luncheon meat	P, C	P, C	yes
Myslivecká paštika	P, C	P, B	no
Francouzská paštika	P, C	P	no
Mandlová paštika	P	P	yes
Paštika delicatesa	P, C	P, B, C	no

P — pork meat; B — beef meat; C — chicken meat; H — horse meat

the duty to declare the full product composition on the product packing. For foodstuffs without packing, this information must be available at the shop. The misleading description of meat products deceives the consumer. The consumer should know for certain that a product contains the raw materials declared on the packing, because a failure to comply with the formula declared can be accompanied by several risks. These risks include the health risks for people suffering from an allergy to various meat proteins or people who have to observe a diet prescribed by a physician.

Other aspects are the hygiene issues, in particular with respect to the occurrence of poultry separate in meat products. This raw material contains a certain percentage of bone fragments and also a high percentage of water, which makes it a substrate favourable for the propagation of pathogenic and food-spoiling micro-organisms. The religious and social aspects, i.e. excluding the meat species that are not acceptable to a particular population or individual because of religious customs or social traditions, are also important.

The adulteration of meat species and replacing the raw materials in meat products are phenomena common to many countries. The adulteration is performed mostly for economic reasons, which means that producers benefit from the replacement of an expensive and less available raw material with a cheaper and more available one. On the other hand, replacement of a raw material with a raw material of lower quality means a decrease in the quality of the final product and hence a deception of the customer. To check the meat species and the composition of meat products and to prove the adulteration, many methods have been developed (ELISA, PCR). These methods are commonly used and allow for the necessary raw material identification.

We used the multiplex PCR method to detect the muscular proteins in a mixture of six meat species and proved that the DNA was amplified from both raw and heat-processed meat. The mixing of primers in appropriate ratios made it possible to identify the species-specific DNA fragments using only one multiplex PCR assay.

The primers were designed with respect to the following requirements: they should provide fragments of different lengths for the six meat species tested and the efficiency of target sequence amplification for all primers should be the same. As each 1 % mismatching of bases in a double-stranded DNA reduces the melting temperature (Tm) by 1—1.5 °C (13), the forward primer SIM was designed to last longer than the species-specific primers. The designs of the reverse primers were based on species-specific regions. The designing of primers is an important step in the multiplex PCR technique, as the primer specificity and Tm play more important roles in this method than in a common PCR reaction. The multiplex PCR provided specific products from the target regions of the given meat species, without fragments produced by non-specific reactions.

All meat species tested were clearly distinguishable using the analysis of non-heat-processed meat. We focused on the identification of goat meat and mutton. However, if a mixture of goat meat and mutton was used for the assay, we detected two lengths of PCR products for each of these species (the specific product and also goat meat product for mutton and mutton product for goat meat). The fact that these two meat species affect each other is caused by the high homogeneity (92 %) of the PCR products in these two animal species (4). The homogeneity is particularly high in heat-processed meats. Another method that makes it possible to distinguish between *mutton and goat meat is the PCR-RFLP technique.*

The respective meat species detected using the multiplex PCR following the heat processing were amplified from both meat types — i.e. processing conditions 100 °C/30 min and 121 °C/30 min. Matsunaga *et al.* (10) reported that after heat processing at 120 °C for 30 min, the beef fragment was detected only as a very faint signal and the horse meat fragment could not be detected at all. The final fragments obtained from our samples of goat meat, chicken, mutton, pork and horse meat produced strong bands of almost identical intensity. This means that we were also able to detect the horse meat heat-processed at 121 °C for 20 minutes and 30 minutes. The exception was beef, which produced only a fragment giving a faint signal in comparison with the other species. Distinguishing between mutton and goat meat will be the subject of another study using the PCR-RFLP molecular technique.

The semi-quantification of a meat mixture evaluated the pork, beef and chicken. The reason for this choice was that these meat species are the most common ones used in meat products in both the domestic and foreign markets.

Samples of pork-and-beef, pork-and-chicken and beef-and-chicken mixtures were prepared in various ratios and analysed. We could observe a relationship between the DNA quantity and the intensity of the resulting bands. The semi-quantification method was described in a study dealing with the multiplex PCR (10). In this study, the DNAs extracted from beef and pork using separate processes were mixed together in the ratios of 88 : 12, 75 : 25, 50 : 50, 25 : 75, 12 : 88 and used as a template for the reaction. While the intensity of the bands produced by one of the meats increased, the intensity of the bands produced by the other meat fragments decreased.

The dependence of the resulting band intensity on the DNA quantity can also be observed in our results. However, we created the mixed samples containing different percentages of the respective meats prior to the extraction procedure, which meant that the intensity of the resulting bands might not have always corresponded to the meat percentages, as it could have been affected by the DNA quantity and character given by the particular extraction and PCR reaction. According to the literature (11) it may happen that all species are not equally represented in the final evaluation of a meat-mixture analysis – one species may be dominant and suppress the other species. In such cases, the suppressed species may be completely undetectable.

Within the framework of meat species identification in meat products, we evaluated the non-heat-processed, heat-processed and sterilised products. In a part of the meat products, the meat-species identified corresponded to the product description on the packing, the other part of the meat-products did not correspond to the description.

The composition of non-heat-processed meat products corresponded to the product description, failure to comply with the formula declared was observed mostly in heat-processed and sterilised products. In most cases, an expensive meat species was replaced by the cheaper chicken meat. Detection of meat species in meat product was also performed by Behrens *et al.* (3). These researchers used commercially available kits for DNA extraction and analysed the mixture of various meat species (pork, beef, mutton, goat meat, horse meat, chicken, turkey) and meat species in heat-processed meat products.

The results obtained imply that the enzymatic amplification of nucleic acids – PCR – can be used for the identification of the muscular protein of various animal species and for the detection of meat-species replacement. Using this method, the six meat species could be identified simultaneously, more easily and more sensitively than using the common methods. PCR meets the requirements for a simple, rapid and reliable analysis, which might be of great importance from the hygienic and economic points of view both today and in the future.

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PRIMERS FOR THE DETECTION OF *Escherichia coli* K88ab+

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ABSTRACT

The polymerase chain reaction (PCR) is a highly specific and accurate method for detecting of enterotoxigenic strains of *Escherichia coli* expressing K88 antigen. The tests to determine of optimum reaction temperatures, times, cycles and the concentration of $MgCl_2$ were done. We considered optimal those conditions for which the reaction times of individual steps were the shortest and, at the same time, we tried to minimize the number of cycles. Good results were obtained under conditions in which the concentration of $MgCl_2$ was equal to 2.5 mmol. The specificity which is ensured by the use of suitable primers was manifested in our study in the absence of false negative or false positive results in all of the cases. The time to detect *E. coli* using the conventional PCR was approximately 4—5 hours.

Key words: *Escherichia coli;* K88ab antigen; PCR

INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) strains are commonly associated with neonatal diarrhoea in man and domestic animals (13). ETEC are pathogenic because of two well recognized properties; the ability to adhere to the intestinal mucosa (which prevents the peristaltic elimination of the bacteria from gut) and the ability to produce enterotoxins. *E. coli* isolates from piglets with diarrhoea may express K88 (F4), K99 (F5), 987P (F6) or F41 antigens or additional fimbrial adhesins (colonisation factors). Of these, strains possessing K88 are the most common cause of diarrhoea and they usually produce LT (heat labile enterotoxin) in addition to STa or STb. According to Nagy (12) K88+ antigen may comprisse about 40—60 % of the *E. coli* strains causing diarrhoea in piglets, the non-K88 make up between 20—30 %. Post *et al.* (16) detected virulence factors in *E. coli,* they found that the predominant genotypes were K88, LT, STb or F18, STa, STb, respectively.

K88 fimbrial adhesins are long proteinnaceous appendages radiating from the surface of the bacterium to a length of 0.5—1.5 mm. They are peritrichously distributed in numbers of 100—1000 per bacterium (8). The genetic determinant for K88 fimbriae, which is located on a large, non-conjugative plasmid, has been cloned and sequenced (11).

There are a variety of in vitro techniques that detect fimbrial adhesins of ETEC including immunological and biological assays. The developments in molecular biology enabled the identification of genes coding for certain bacterial characteristics by the polymerase chain reaction (PCR). The PCR is a highly specific and sensitive method allowing the identification of specific segments of nucleic acids in a sample (18, 21). The aim of our experiments was to test an optimum procedure for the utilization of the PCR in the detection of enterotoxigenic expressing K88ab antigen.

MATERIAL AND METHODS

Bacterial strains: *E. coli* M1, *Salmonella typhimurium, Salmonella typhimurium* K88+ (4, 5), wild strains of *E. coli* and plasmid-free apathogenic *E. coli* HB 101 strain were used to optimise the conditions for determining *E. coli* possessing K88 encoding plasmid. The strains were cultivated in LB medium overnight at 37 °C with shaking.

The isolation of DNA: To optimise the PCR conditions we isolated the genomic DNA as follows. Bacterial cells were collected by centrifugation from the overnight culture (1.5 ml), washed with STE buffer and resuspended in 0.5 % SDS solution containing proteinase K in the final concentration

of 100 mg.ml^{-1} in the TE buffer. After one hour's incubation at 37 °C, we added 100 ml 5 mol NaCl, mixed the content, added 80 ml CTAB/NaCl solution and incubated the mixture for 10 min at 65 °C. The samples were then extracted with a mixture of chloroform and isoamylalcohol and again with phenol and chloroform and isoamylalcohol, precipitated with two volumes of ethanol, and washed with 70 % ethanol. The sediment obtained after the centrifugation was resuspended in the TE buffer, treated with RN-ase, washed with ethanol and resuspended again in the TE buffer.

The PCR reaction: For the PCR reaction, we used AmpliTaq DNA polymerase (Perkin Elmer) in the amount 0.5 U, 2 mM dNTPs, PCR buffer Perkin Elmer with $MgCl_2$ in a total volume of reaction mixture equal to 50 µl. The PCR conditions were tested within a temperature range and as a function of time:

The preliminary denaturation step 94—95 °C for the period of 1—4 min.

The denaturation step 92—95 °C, the annealing step 52—58 °C, the prolongation step, 70—73 °C in the time interval 0.3—0.6 min, number of cycles 25, 30, 35.

The prolongation step 72 °C, from 2 to 5 min.

Tests were carried out with $MgCl_2$ concentrations from 1.0 to 4.0 mM. The primers 318: 5'-AAA AAG ACT CTG ATT GCA CTG-3'; 320: 5'-CTT TAG TAA TAA GTT ATT GCT ACG-3' were designed in our laboratory from a well-known sequence encoding K88ab antigen (11) and were synthesised on ID-DLO Lelystad. Techne Genius was used as a thermocycler for the PCR.

The preparation of samples for the PCR determinations: To achieve the lysis of bacterial cells and the release of DNA, we subjected 0.5 ml of the sample to between 5 and 20-min boiling in a water bath and then carried out centrifugation at 14 000 *g* for 5 min at a refrigerator temperature (4 °C). The supernatant was transferred to a clean Ependorf tube. One or five µl of this supernatant was used for the PCR.

The detection of *E. coli* by means of the PCR was carried out using boiling for 5, 10, 15, and 20 min. The subsequent procedure for extraction and preparation of the reaction mixture was identical with the procedure described above.

Agarose gel electrophoresis: Agarose gel electrophoresis was used to identify PCR products. We used 1.5 or 2 % agarose gel in the electrophoretic TAE buffer. Visualisation of DNA was achieved by means of UV light following the staining with ethidium bromide (concentration 0.5 mg.ml^{-1}). We used λ DNA, cleft with restriction endonucleases *Hind*III and EcoRI, or λ DNA followed the cleft with *Hind*III, or 100 bp DNA ladder as molecular weight standards. Evaluation was carried out in "Kodak Digital Science" system.

RESULTS AND DISCUSSION

The need to determine the optimum conditions for the PCR reaction which serves to detect the germs results from differences in the published data which describe the conditions for this reaction (9, 10). This lack of uniformity results from the primers used in the reaction during which the length of an amplified fragment appears as a decisive factor, and on from the type of the PCR reaction used, the type of polymerase used, and also from the technical-material equipment, particularly the DNA thermocycler (1, 19).

From our point of view, the quality of the PCR products, detected by agarose gel electrophoresis, was the essential criterion for determining the optimum reaction temperatures and times. As the activity of the enzyme may not be always optimal during the reaction, an easy rule we applied successfully was to consider the time of reaction and the concentration of $MgCl_2$.

Many researchers use a 2—5 minutes first denaturing step before the actual cycling starts. This is supposed to help denaturing the target DNA better. Moreover, a final extension time of 5—10 minutes, is described in many papers (supposedly to help finish the elongation of many or most PCR products initiated during the last cycle). An annealing time of 15—60 seconds was sufficient for all primer pairs tested so far. The annealing temperature chosen can be based on the melting temperature of primers.

In our experiments in order to determine the optimum conditions for the PCR, we used DNA isolated from the bacterial strains *E. coli* by means of an alkaline denaturation method (2). The required quality of the products mentioned provided a distinct, clear and well defined band. The localization of this product on a gel depended on its molecular weight. One of the criteria for the optimization of the PCR conditions was the duration of the reaction which considerably affects the activity of DNA polymerase. For this reason, for the comparable quality of products detected, we considered optimal those conditions for which the reaction times of individual steps were the shortest and, at the same time, we tried to minimise the number of cycles.

The PCR conditions were tested for a range of temperatures and as a function of time:

1. The preliminary denaturation step 94—95 °C, for the period of 1—4 min.

2. The denaturation step 92—95 °C, the annealing step 52—58 °C, the prolongation step, 70—73 °C in the time interval 0.5—1.5 min, the number of cycles 25, 30, 35.

4. The final polymerization step, 2—5 min.

Similar tests were carried out using $MgCl_2$ in the reaction buffer in concentrations ranging from 1.0 mmol to 4.0 mmol.

Because of that we changed the reaction conditions and tested different concentrations of $MgCl_2$ in the reaction buffer. First of all, the time of individual steps of the PCR reaction was prolonged at different concentrations of $MgCl_2$. The best results were obtained under the reaction conditions shown in Table 1 at the concentration of $MgCl_2$ equal to 2.5 mmol.

In the case of DNA isolated from the *E. coli* M1 strain, the agarose gel electrophoresis method allowed us to detect a distinct band in the assumed region of molecular weight of 798 b.p. In the case of DNA isolated from bacterial strains which did not contain the DNA encoding

Table 1. Reaction conditions for K88ab PCR detection

Temperature	Time (min)	No. of cycles
95 °C	4	1 – preliminary
95 °C	1.0	
56 °C	1.0	30
72 °C	1.5	
72 °C	2.5	1 – final

the K88 adherence antigen (*Salmonella typhimurium, Salmonella enteritidis* and *E. coli* HB 101 – this strain was used as a recipient strain for the transformation of recombinant DNA encoding the K88 adherence antigen), the results were unambiguously negative.

Afterwards, we started with the PCR diagnosis of individual bacterial species without purification of the amplified DNA. Individual bacterial strains were first cultivated in the LB medium overnight (12—14 hours). Then the numbers of colony forming units in the bacterial cultures obtained were determined using the method of decimal dilution in the saline solution (6, 14). One millilitre of each dilution obtained in the described way (in the range of dilution from 10^{-1} to 10^{-9}) was

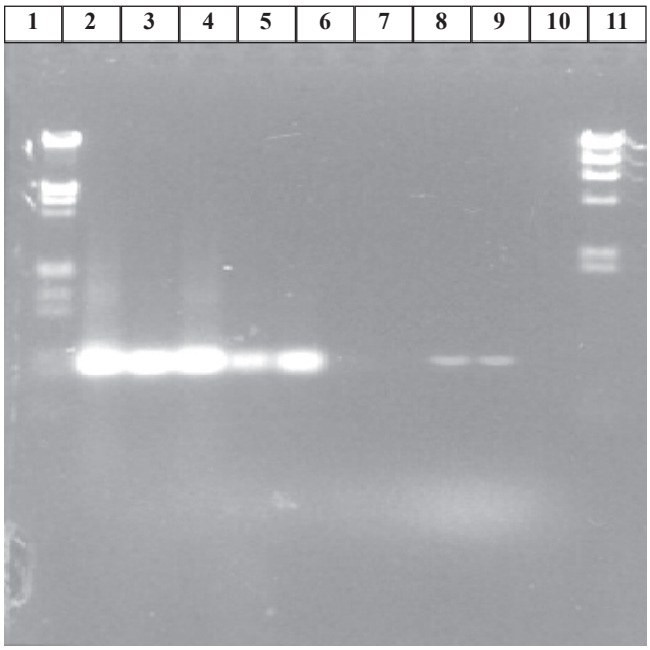

Figure 1. Agarose gel electrophoresis of the PCR products. Legend (from the left): 1 — Standard of molecular weight (λ *EcoR I/Hind III)*; 2—6 — PCR products K88 after lysis of bacteria: 2 — *E. coli* M1, 3 — *E. coli* G205, 4 — *E. coli* G7, 5 — *S. typhimurium* K88ab$^+$, 6 — *E. coli* pYA K88ab$^+$; 7 — *E. coli* HB 101; 8 — *S. typhimuriun;* 9—10 — Plasmid M1 DNA (positive control); 11 — Negative control; 12 — Standard of molecular weight (λ *Hind III*)

centrifuged and the sediment resuspended in 1 ml of the saline solution. Samples were transferred to a water bath preheated to the temperature of boiling (100 °C) for 5, 10, 15, and 20 min. After the corresponding exposure, the samples were centrifuged and an aliquot amount of supernatant was used for the PCR diagnosis under the reaction conditions for individual bacterial species. The quality of the PCR products investigated served again as a criterion in our evaluation.

It was observed that the 10 min exposure of the sample to 100 °C was sufficient to detect a positive fragment with primers 318 and 320 for enteropathogenic *E. coli* containing the gene encoding the K88 antigen. The prolongation of this time resulted in minimal differences in the quality of products and because of that, in an effort to shorten the time of examination, we considered this time as sufficient. Under the conditions mentioned, the detection limit ranged between the dilution 10^{-3} to 10^{-4} CFU.ml^{-1} of saline solution for *E. coli* M1. The results of the PCR in the case of *Salmonella typhimurium, Salmonella enteritidis,* and *E. coli* HB 101 were negative.

In order to speed up the diagnosis of individual bacterial species, we made an effort to determine the conditions of the PCR using the bacterial cultures directly without washing and resuspending the cellular suspension in a saline solution. Bacterial cultures of individual strains were cultivated overnight (12—14 hours). In this way, the bacterial culture obtained was exposed directly to 100 °C observing the PCR conditions for individual bacterial cultures mentioned above. The exposure of samples to 100 °C for various time intervals provided results similar to those in the saline solution (10 min exposure for *E. coli* and 15 min exposure for salmonellae), however, the detection limit was decreased down to 10^{-5} dilution for *E. coli*.

CONCLUSION

The use of PCR techniques in the detection of *E. coli* in the samples is justified by their specificity, sensitivity, rapidity in obtaining results and a whole range of other advantageous properties. The specificity which is ensured by the use of suitable primers was manifested in our study in the absence of false negative or false positive results in all of the cases. The time needed to detect salmonellae and *E. coli* using the conventional PCR (including the preparation of samples) was approximately 4—5 hours. This short time needed to obtain the results together with preservation of above mentioned specificity and sensitivity are the very factors which confirm of the suitability of the PCR method particularly in the cases which must be dealt with very quickly.

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THE LEVEL OF METHAEMOGLOBINAEMIA AND THE VALUES OF NITRATES AND NITRITES IN CALVES FED NITRATE RATIONS

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ABSTRACT

The postulate of increasing hectare yields of agricultural crop is conditional upon increased used of fertilisers. Such practice results in an increased content of nitrates in both the fodder and drinking water. Our experiment focused on the effect of nitrates on calves in the milk nutrition period. Six calves (mean weight 35 kg) were administered KNO_3 dissolved in water at a dose of 4 g.head.day^{-1} for 28 days. The mechanism of action of this substance was derived from the level of methaemoglobinaemia determined on days 1, 14, and 28 of the experiment. Measurements at these intervals showed that methaemoglobinaemia culminated one hour after the administration of KNO_3, decreased abruptly during the second hour, and then declined gradually to the initial values (within 24 h). Calves slaughtered 24 h after the last administration of KNO_3 exhibited higher values of nitrates than those slaughtered after 72 h ($P < 0.05$). The highest values of nitrate residues in internal organs were found in the kidneys and those of nitrites in the liver. Still higher levels were recorded in urine from the slaughtered animals which indicated good transrenal transfer of NO_3 in the experimental animals.

Key words: calves; methaemoglobinaemia; nitrates; nitrites; nutrition

INTRODUCTION

The increased use of chemicals in agricultural production brings about not only some positive aspects but also a risk to animal production and veterinary medicine in the form of intoxication and particularly the presence of residua. The chronic supply of subtoxic doses of nitrates results in metabolic depression (12) and the penetration of these xenobiotics into the products of the affected animals (4, 2).

The excessive application of artificial fertilisers is reflected in increased levels of extraneous matter not only in the fodder and grass cover (8) but also in ground water that serves as a source of drinking water (7, 3). After being taken up with feed and water, nitrates participate in biochemical reactions in animal bodies.

Rumen microflora of adult ruminants reduces nitrates to nitrites and subsequently to ammonia. In calves in the milk nutrition stage, the extent of microbial reduction is not the same as in animals with well developed ruminal activity. The enzymatic digestion prevails. A more intensive activity of the forestomaches occurs in association with its advanced development, approximately in the fifth or sixth week of age, depending on the way of feeding, particularly on the quantity of hay supplied (11).

MATERIAL AND METHODS

Our experiment was conducted on 8 calves approx. two weeks old. Six of them were supplied daily 4 g KNO_3 *per os,* dissolved in water. The treatment lasted 4 weeks. Two calves of similar age and weight (35 kg on average) served as a control. Both the drinking water and milk feed mixture Laktavit contained 18 mg nitrates per litre.

The application of nitrate ended after 28 days. Three calves from the experimental group were slaughtered within 24 h of the last application of nitrate while the other three experimental animals and the two controls were killed 72 h after the last application. All the animals, experimental and control ones, were examined for residua of nitrates and nitrites in meat, internal organs and urine (Tab. 1).

The blood for determination of MtHb was taken at random from three calves one hour after application of the first KNO_3 dose. Additional samples of blood were withdrawn from the

same animals after 2, 4, 8, and 24 h. The same sampling procedure was repeated after 14 days and on the last day of the experiment (Fig. 1).

Blood samples taken from *vena jugularis* were used for the determination of haemoglobin by a colorimetric method employing potassium cyanide (*Veterinary Laboratory Examination Methods,* 1983). The levels of nitrates and nitrites were determined spectrophotometrically on a copper-coated reduction column (ISO method for meat and meat products).

RESULTS AND DISCUSSION

Conversion of haemoglobin to methaemoglobin (MtHb) results in a limited supply of tissues with oxygen and the associated consequences including a decrease in productivity. The information on the lower pathological limits of MtHb differ and range from one to five per cent of the total amount of circulating red blood pigment (5).

The majority of authors (10, 1) use the methaemoglobinaemia curve to assess the load of nitrates on the animal bodies. In our experiments with the application of 4 g KNO_3 per head and day, we were able to register the highest levels of MtHB one hour after the application of KNO_3 at all measurement intervals (Fig. 1). While on the first experimental day this value reached 4.3 % MtHb on average, examination on day 14 revealed 2.7 %, and by the end of the experiment, it had decreased to 1.8 % MtHb. Examination at 2 hours post-application showed an abrupt decrease in this parameter followed by gradual decline and stabilisation at initial level after 24 h. The level of MtHb in the controls did not increase above 1 %.

Our results do not fully agree with the conclusions of Jacková *et al.* (6). While the values of MtHb in our experimental calves reached the highest level within one

Fig. 1. Methaemoglobin levels on day 1, 14, and 28 of the experiment (n = 3 + 1)

hour post-application of KNO_3 the latter authors reported culmination of MtHb 2—3 hours after the treatment.

The values of residual nitrates and nitrites are presented in Tab. 1. This table shows that the highest residua were

Table 1. Nitrite and nitrate residual levels in the muscle, organs and urine of experimental and control calves (n = 3 + 1)

Calves	Muscles mg.kg^{-1}		Heart mg.kg^{-1}		Spleen mg.kg^{-1}		Kidneys mg.kg^{-1}		Liver mg.kg^{-1}		Urine mg.kg^{-1}		
	KNO_3	KNO_2	KNO_3	KNO_2	KNO_3	KNO_2	KNO_3	KNO_2	KNO_3	KNO_2	KNO_3	KNO_2	
1	29.09	0.22	21.90	1.05	14.16	1.96	35.14	0.54	24.16	1.22	44.33	0.14	Killed 24 h after the last application of KNO_3
2	24.12	0.11	18.24	0.09	20.09	2.53	32.09	0.22	31.90	2.14	35.07	0.25	
3	25.71	0.12	19.56	0.43	18.63	2.24	32.71	0.27	32.07	1.05	31.14	0.22	
4	19.62	0.10	16.48	0.92	11.92	2.07	33.01	0.12	22.17	0.76	30.04	0.01	Killed 72 h after the last application of KNO_3
5	23.19	0.21	12.72	1.14	12.66	1.78	34.14	0.32	19.36	1.09	28.12	0.07	
6	17.42	0.17	13.63	0.96	9.54	1.45	24.72	0.19	16.01	1.36	24.45	0.12	
C1	4.15	0.06	3.22	1.07	8.72	2.14	10.88	1.09	5.37	2.07	0.56	0.03	Killed together with the group 1–3
C2	2.69	0.14	1.65	0.82	7.69	1.89	12.24	0.54	6.19	1.61	1.12	0.93	

Calves No.: 1 to 6 — experimental; C_1 and C_2 — control

generally detected in the kidneys compared to muscles and other internal organs. The level of nitrate was the highest in the spleen and liver ($P < 0.05$). The values of both nitrates and nitrites were on average higher in calves killed 24 h after the last application of KNO_3 compared to the animals killed 72 h post-application. The levels of residua in urine exceeded those in muscles and internal organs which indicated a good transrenal transfer of NO_3 ions. A similar result was reported by Slanina *et al.* (10) in ruminants and Jacková *et al.* (6) in calves in the milk nutrition period. Considerably higher levels of residua were reported by Saladiová *et al.* (9) in fattening bulls in the period of feeding with spring mixed fodder.

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THE PROTECTION OF CATTLE AND PIGS IN SLAUGHTERHOUSES WITH REGARD TO HARMONISATION OF LEGAL REGULATIONS

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ABSTRACT

The present paper compares the levels of animal protection in slaughterhouses with regard to the differences between The Czech Republic and The European Union regulations. In 2000, in total 78 slaughterhouses were monitored for the following parameters: lairage for cattle and pigs in slaughterhouses, feeding, watering, use of electric prods, restraint of animals before stunning, methods of stunning used in slaughter animals. The results show that the slaughterhouses did not comply with EU regulations in the following aspects: feeding of animals 12 hours after their arrival to a slaughterhouse (highly significant for high-throughput slaughterhouses, statistically insignificant for low-throughput slaughterhouses), permanent access to water (highly significant for both high-throughput and low-throughput slaughterhouses), application of electric prods on the muscular surface of rear legs only (highly significant for high-throughput slaughterhouses, significant for low-throughput slaughterhouses), use of electric stunning device equipped with a measuring and regulating apparatus with visual display (highly significant both for high-throughput and low-throughput slaughterhouses). The fact of inadequate compliance with the aforementioned requirements suggests that changes will have to be implemented in the slaughterhouses in order to provide for the conformity with EU regulations.

Key words: animal protection; cattle; pigs; slaughterhouse

INTRODUCTION

The need to protect animals against cruelty has been increasingly acknowledged during the recent period also in slaughter animals. Expert knowledge and experience concerning the protection of slaughter animals have been reflected in national regulations as well as in EU regulations. The process of harmonising both legal systems requires that the differences between the two types of regulations are properly recognised. This paper compares the level of animal protection in slaughterhouses with regard to the differences between the respective Czech and EU regulations.

Kromka (12) pointed out the duty to protect the animals and discussed the need for animal protection from the aspect of "absolute ethic" expressed by Albert Schweitzer as "a reverence for life" (Ehrfurcht vor dem Leben). In this respect, he searched for a compromise between a humane existence of mankind and its need to use the animals. If the animals are used for production purposes, then the value of their life must be respected. Therefore, the main obligation of mankind in this field is to ensure the protection of the animals against cruelty.

Troeger (15) discussed the need to protect animals in slaughterhouses while Wenzlawowicz *et al.* (17) reported the connection between the handling of animals in slaughterhouses, the condition of animals before slaughter and meat quality. Schafer *et al.* (14) monitored the handling of animals in slaughterhouses and focused particularly on the use of electric prods in pigs. Orihuela and Solano (13) monitored stress levels in cattle at the beginning of the rushing area and during the passage of the animals through it. Chandra and Das (8) discussed the occurrence of contusion in cattle in slaughterhouses in connection with the way of handling of the animals.

In addition, Grandin (6) examined 24 slaughterhouses and evaluated the level of animal welfare with regard to the following parameters: number of cases of sliping and falling of the animals, use of electric prods, noise made by the animals, efficiency of stunning, level of unconsciousness of the animals achieved in the bleeding area.

The parameters used for electric stunning of the animals were reported by numerous authors, for example by Drawer and Gratz (3), Wormuth and Schuttabraham (19),

Fehrenberg et al. (5), Chang and Pearson (9), Haack et al. (7), Troeger and Nitsch (16), Wenzlawowicz *et al.* (18), Boosen and Roming (1). Drawer and Gratz (4) pointed out that expert knowledge on the protection of slaughter animals should be incorporated in the requirements of the respective regulations.

This was further discussed by Knierim (10) and Konigs (11) in connection with the requirements of the EU legislation. Csaba *et al.* (2) reported on harmonising the EU legislation with national legislations in the field of animal protection. The legal regulations on the protection of animals at slaughter are covered by *Directive 93/119/EC* (38) in the European Union, and by *Act No. 166/1999 Sb*. (25, 26, 27, 28, 29, 30, 31, 32), *Decree No. 287/1999 Sb.* (34, 35, 36, 37), *Act No. 246/1992 Sb. (20, 21, 22, 23, 24) and Decree No. 245/1996 Sb. (33) in The Czech Republic.*

MATERIAL AND METHODS

The Czech and EU regulations concerning handling of the animals in slaughterhouses were carefully studied in order to reveal the most important differences between both regulations. In 2000, in total 78 slaughterhouses (high-throughput and low-throughput slaughterhouses) were examined in order to find out whether they comply with the requirements of both legal regulations. The results obtained were subjected to the χ^2 test using a statistic program Unistat. The levels of the protection of cattle and pigs in slaughterhouses were evaluated with regard to their compliance with the Czech and EU regulations. The results indicate that changes have to be carried out in slaughterhouses for cattle and pigs if EU regulations are going to be implemented.

RESULTS

The Table 1 illustrates the differences in compliance with the Czech and EU regulations concerning the protection of cattle and pigs, as found in 78 slaughterhouses in The Czech Republic in 2000.

It can be concluded from the Table that both the Czech (CzR) regulations and European Union (EU) regulations contain the same requirement with regard to sufficient

Table 1. Compliance with selected requirements in slaughterhouses

Requirement	High-throughput slaughterhouses		Low-throughput slaughterhouses	
	Compliance		Compliance	
	Yes	No	Yes	No
CR: Provision of sufficient space to accommodate the animals	**43**	**7**	**28**	**0**
EU: Provision of sufficient space to accommodate animals	**43**	**7**	**28**	**0**
χ^2	–		–	
CR: Feeding of animals (24 hours after their arrival to the slaughterhouse)	3	3	0	0
EU: Feeding of animals (12 hours after their arrival to the slaughterhouse)	3	31	0	3
χ^2	++		–	
CR: Proper watering	50	0	28	0
EU: Permanent availability of watering sources	21	29	2	26
χ^2	++		++	
CR: Application of electric prods only on muscular areas	40	2	8	1
EU: Application of electric prods only on muscular areas of rear legs	24	18	3	6
χ^2	++		+	
CR: Ban on restraint by binding hind limbs	49	1	27	1
EU: Ban on restraint by binding any limbs	49	1	27	1
χ^2	–		–	
CR: Use of a measuring and regulating device with a visual display of the value of electric current used for stunning – not required	46	0	21	0
EU: Use of a measuring and regulating device with a visual display of the value of electric current used for stunning	10	36	0	21
χ^2	++		++	

Explanatory notes:

χ^2 — comparison of the statistic significance of differences between CzR and EU
++ — highly significant difference
+ — significant difference
– — insignificant difference
CzR — The Czech Republic
EU — The European Union

space for the accommodation of the animals. Out of 50 slaughterhouses observed, the requirements pursuant to both Czech and EU regulations were fulfilled in 43 cases. Out of 28 low-throughput slaughterhouses the requirements pursuant to both Czech and EU regulations were fulfilled in all 28 cases. Therefore, no statistically significant difference either for high-throughput or low-throughput slaughterhouses was found with regard to the compliance with CR and EU regulations.

According to the Czech regulations animals must be fed 24 hours after their arrival to slaughterhouse housing premises, while EU regulations require the feeding of the animals 12 hours after their arrival to a slaughterhouse. Out of 50 slaughterhouses, in 6 cases the animals were housed in the premises for more than 24 hours prior to slaughter. Three out of these 6 slaughterhouses fulfilled the requirements pursuant to the Czech regulations. In 34 cases the animals were housed at the slaughterhouse for the period of more than 12 hours. In three cases thereof the requirements pursuant to EU regulations were fulfilled. Neither of 28 low-throughput slaughterhouses provided housing of the animals for more than 24 hours prior to slaughter. In 3 cases the animals were housed in the premises for more than 12 hours. However, EU regulations were not fulfilled in either of the 3 aforementioned cases. In this respect, a highly significant difference for high-throughput slaughterhouses and statistically insignificant difference for low-capacity slaughterhouses were detected.

The requirement of the Czech regulations concerning proper watering of the animals was fulfilled in high-throughput slaughterhouses as well as in low-throughput slaughterhouses. EU regulations require permanent availability of watering for animals. Out of 50 slaughterhouses, watering of the animals complied with the Czech regulations in all 50 cases. EU regulations were fulfilled in 21 cases. The Czech regulations concerning watering of the animals were met in all 28 low-throughput slaughterhouses. EU regulations were met in 2 cases thereof. With regard to the compliance with the Czech and EU regulations, a highly significant difference was found for both high-throughput and low-throughput slaughterhouses.

The electric prods used to rush the cattle and pigs in slaughterhouses can be applied only on muscular areas on the surface of animal body according to the Czech regulations, or only on muscular areas on the surface or rear legs according to EU regulations. The electric prods were used in 42 out of 50 slaughterhouses. The requirements of the Czech regulations were met in 40 cases thereof and the requirements of EU regulations were met in 24 cases. Out of 28 low-throughput slaughterhouses the electric prods were used in 9 cases. The requirements of the Czech regulations were met in 8 cases thereof and the requirements of EU regulations were met in 3 cases. With regard to the compliance with CR and EU regulations, the differences were highly significant for high-throughput slaughterhouses and significant for low-throughput slaughterhouses.

While the Czech regulations contain the ban on the restraint of the animals by binding their hind limbs prior to slaughter, the EU regulations prohibit the binding of any limbs at all. In 49 out of 50 slaughterhouses the requirements of the Czech regulations concerning the restraint by means of binding the limbs were met. EU regulations were likewise met in 49 cases thereof. In 28 low-throughput slaughterhouses the Czech regulations were met in 27 cases and EU regulations also in 27 cases. No statistically significant differences were found for either high-throughput slaughterhouses or low-throughput slaughterhouses with regard to the compliance with the Czech and EU regulations.

Electrical stunning device equipped with a regulating apparatus using a visual display is not required by the Czech regulations. However, the EU regulations include this requirement. Electrical stunning of animals was carried out in 46 out of 50 slaughterhouses. In all 46 cases the Czech regulations were observed. EU regulations were observed in 10 cases. In low-throughput slaughterhouses electrical stunning was used in 21 cases and in all these cases the Czech regulations were observed. However, EU regulations were observed in neither of these cases. Accordingly, highly significant differences were found for both high-throughput and low-throughput slaughterhouses.

DISCUSSION AND CONCLUSION

The paper presents the information on the protection of cattle and pigs in slaughterhouses. In addition, the paper provides an overview of the requirements concerning the animal protection in slaughterhouses according to the Czech regulations, i.e. Act No. 166/1999 Sb. (25, 26, 27, 28, 29, 30, 31, 32), Decree No. 287/1999 Sb. (34, 35, 36, 37), Act No. 246/1992 Sb. (20, 21, 22, 23, 24) and Decree No. 245/1996 Sb. (33), and EU regulations, i.e. Directive No. 93/119/EC (38), and discusses the results from the viewpoint of harmonising both legal systems.

The paper further contributes to the studies previously reported by Drawer and Gratz (3), Knierim (10), Konigs (11), Csaba *et al.* (2). From the viewpoint of harmonising the legal systems this paper attempts to evaluate animal protection in slaughterhouses in connection with the following aspects: housing of cattle and pigs in slaughterhouses, feeding and watering of the animals, use of electric prods, restraint of animals before slaughter, stunning of animals. In addition, the present paper discusses the ways of use of electric prods and brings new facts on this topic previously studied by Schafer *et al.* (14), Grandin (6). The paper emphasises the fact that the electrical stunning devices used in slaughterhouses should be equipped with measuring and regulating apparatus with a visual display to control electric current used to stun the animals, which is in accordance with the reports published by Drawer and Gratz (3), Wormuth and Schuttabraham (19), Fehrenberg

et al. (5), Chang and Pearson (9), Haack *et al.* (7), Troeger and Nitsch (16), Wenzlawowicz *et al.* (18), Boosen and Roming (1).

The results are concluded as follows: As soon as the EU regulations become valid in the Czech Republic, it will be necessary either to reduce the period between the arrival to the slaughterhouse and the slaughter below 12 hours or to ensure that the animals are fed both in high-throughput and low-throughput slaughterhouses. Furthermore it will be necessary to change the system of watering of the animals by means of ensuring a permanent access of the animals to water both in typical and low-capacity slaughterhouses. The use of electric prods in slaughterhouses will have to be changed according to the requirements of The European Union. Both high-throughput and low-throughput slaughterhouses will have to be equipped and will have to use measuring, control and visual display devices in connection with electrical stunning of the animals.

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THE OCCURRENCE OF EMERGENCY SLAUGHTERS IN SELECTED SPECIES OF FOOD ANIMALS

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ABSTRACT

The numbers of emergency slaughters are among the important parameters of the health status of food animals inspected at slaughterhouses. The figures relating to this parameter were monitored at slaughterhouses during the periods of 1989—1994 and 1995—2000. The relative frequency of emergency slaughters decreased in different species during the period of 1995—2000 as follows: in cows from 23.38 % to 18.81 %, in heifers from 18.32 % to 7.37 %, in bulls from 10.63 % to 2.84 %, in calves from 50.11 % to 35.07 %, in pigs from 3.51 % to 1.90 %, in sheep from 6.67 % to 1.64 %, in goats from 46.09 % to 6.15 %, in horses from 86.09 % to 27.91 %, in chickens from 2.45 % to 0.02 %, in hens (and roosters) from 3.47 % to 0.69 %, in turkeys from 1.52 % to 0.09 %, in ducks and geese from 1.48 % to 0.05 % and in rabbits (and coypu) from 11.59 % to 4.08 %. The decrease was proved to be statistically significant in all species, production and age groups of food animals. Lowest values of relative frequency of emergency slaughters were found during the period of 1995—2000 in chickens and furthermore in the following species (ranked from lower to higher values): ducks and geese, turkeys, hens and roosters, sheep, pigs, bulls, rabbits and coypu, heifers and goats. The highest values were found in horses, cows and especially in calves. It can be concluded that the numbers of animals brought to slaughterhouses with confirmed or suspected disease or with injury have significantly decreased during the recent period in all species of food animals, which have been monitored. The lowest numbers of emergency slaughters were found in chickens, ducks and geese. On the other hand, highest numbers were found in horses, cows and especially in calves.

Key words: emergency slaughter; food animals; health status; slaughterhouse

INTRODUCTION

The inspection of meat and organs of slaughtered animals was already carried out in ancient times and any differences from the conditions usually found in healthy animals were detected. The experience gained from such inspections were reflected in religious traditions related to the slaughtering of food animals and in the decisions on the edibility of meat and organs. Legal regulations of the governing inspection of food animals and meat at slaughterhouses have been frequently based on numerous traditions mentioned above supported by scientific knowledge.

A significant majority of the animals that are currently slaughtered show no clinical alterations in health. However, there are still cases of emergency slaughter, which means the slaughter in animals with confirmed or suspected disease. The numbers of emergency slaughters in food animals may serve as one of the parameters of health status in food animals in general. The health status of food animals is reflected in the findings from the inspection at slaughterhouses and expressed in the numbers of carcasses classified as suitable for human consumption (edible), suitable for processing (conditionally edible) and condemned. Some authors (1, 3) emphasize the fact that findings at slaughterhouses truly reflect the general health status of the animals.

The findings in slaughtered cattle in 1994 were analysed by Lis (5). Total proportion of cases with pathological findings, which were identified in more than 1.7 million heads of slaughtered cattle, was 21.9 %. Out of more than 14 million pigs also slaughtered in 1994 the same author (6) found 36.57 % of cases with pathological findings. In another work Lis (7) compared the occurrence of findings within the framework of slaughter animals and meat inspections carried out in 1987 and 1997. In 1987 pathological findings were reported for 43.79 % of cases in cattle, 42.68 % in calves, 23.53 % in sheep, 33.21 %

in pigs and 21.21 % in horses. In 1987 the same values were 20.5 % in cattle, 2.85 % in calves, 48.96 % in sheep, 41.43 % in pigs and 18.10 % in horses.

Kofer *et al.* (4) analysed the findings from eight selected slaughterhouses. After the inspection of more than 66 000 pigs, carcasses in years 1999 and 2000 they found pneumonia at different stage in 43.7 % of cases, chronic pleuritis in 22.7 %, chronic pericarditis in 6.8 % and milk spots in liver in 45.6 %.

Any disease, either acute or chronic, affects feed conversion, weight gains and subsequently also meat quality. Schuh et al. (8) studied the relation between the occurrence of such findings and the economic effects in slaughter pigs. The frequency of different findings was analysed in the group of 6 250 slaughter pigs. The results showed that pneumonia was found in 83.3 % of the animals, pleuritis in 26.3 %, pericarditis in 2.6 %, milk spots in liver in 47.5 %. The dressing out percentage in slaughter pigs was principally lower in the animals with pathological findings compared to healthy animals.

Diseases of respiratory system seem to be the cause of the most frequent findings in slaughter pigs. Grest et al. (2) studied the occurrence of pulmonary lesions in 8 921 slaughter pigs at six large slaughterhouses. Most frequent findings were bronchopneumonia (21 %) and diffuse pleuritis (21 %).

The aim of the present work was to monitor the occurrence of emergency slaughters in selected species and production and age groups of food animals at slaughterhouses during the period of 12 years, to compare the occurrence of emergency slaughters in two consecutive time periods with the objective of determining the trend in the development of the numbers of emergency slaughters, as well as to identify the species, production and age groups of animals with highest and lowest occurrence of emergency slaughters.

MATERIAL AND METHODS

The numbers of all animals slaughtered and the numbers of emergency slaughters were recorded in selected species, production and age groups of animals in the periods of 1989—1994 and 1995—2000. Selected species, production and age groups included cows, heifers, bulls, calves, pigs, sheep (including lambs), goats (including kids), horses (including foals), chickens, hens (and roosters), turkeys, ducks and geese, rabbits (and coypu). Numbers of emergency slaughters in the period of 1989—1994 were compared with the period of 1995—2000. In selected species, production and age groups of food animals, the changes in the numbers of emergency slaughters were analysed. Furthermore the numbers of emergency slaughters in the periods of 1989—1994 and 1995—2000 were mutually compared among selected species, production and age groups of food animals. In this way the species, production and age groups with lowest and highest numbers of emergency slaughters were identified. Statistical calculations were carried out using χ^2 test (Unistat software).

RESULTS

Numbers of all food animals slaughtered and numbers of emergency slaughters in the periods of 1989—1994 and 1995—2000 are presented for selected species, production and age groups in Table 1. On the basis of the comparison of the numbers of all food animals slaughtered during the periods of 1989—1994 and 1995—2000 it can be concluded that the total numbers of slaughters decreased in the period of 1995—2000 in cows, heif-

Table 1. A comparison of occurrence of emergency slaughters in selected species of food animals

Species, production and age groups	Period of 1989–1994 Animals slaughtered in total numbers	emergency slaughters numbers	%	Period of 1995–2000 Animals slaughtered in total numbers	emergency slaughters numbers	%
cows	**2 468 441**	**577 070**	**23.38**	**1 327 544**	**249 725**	**18.81****
heifers	754 040	138 161	18.32	386 716	28 519	7.37**
bulls	2 341 935	248 841	10.63	1 489 000	42 287	2.84**
calves	558 573	279 916	50.11	160 569	56 315	35.07**
pigs	28 929 807	1 016 687	3.51	27 014 831	513 000	1.90**
sheep	537 254	358 32	6.67	47 573	779	1.64**
goats	6 607	3 045	46.09	14 335	882	6.15**
horses	3 739	3 219	86.09	5 034	1 405	27.91**
chickens	407 025 923	9 969 760	2.45	607 588 325	150 868	0.02**
hens	4 4702 794	1 553 358	3.47	3 5072 994	242 810	0.69**
turkeys	8 139 210	124 074	1.52	9 432 904	8 562	0.09**
ducks	18 483 332	272 714	1.48	10 876 471	5 271	0.05**
rabbits	8 775 206	1 016 727	11.59	7 986 762	325 602	4.08**

Explanations:
** — statistically highly significant difference in the numbers of emergency slaughters in the periods of 1989—1994 *vs* 1995—2000

Sheep — sheep and lambs; goats — goats and kids; horses — horses and foals; hens — hens and roosters; ducks — ducks and geese; rabbits — rabbits and coypu

Table 2. A descending ranking of the relative proportions of emergency slaughters in different species, production, and age groups of food animals

Period of 1989—1994		Period of 1995—2000	
Species, production and age group of food animals	Proportion of emergency slaughters in %	Species, production and age group of food animals	Proportion of emergency slaughters in %
horses	86.09	calves	35.07
calves	50.11	horses	27.91
goats	46.09	cows	18.81
cows	23.38	heifers	7.37
heifers	18.32	goats	6.15
rabbits	11.59	rabbits	4.08
bulls	10.63	bulls	2.84
sheep	6.67	pigs	1.90
pigs	3.51	sheep	1.64
hens	3.47	hens	0.69
chickens	2.45	turkeys	0.09
turkeys	1.52	ducks	0.05
ducks	1.48	chickens	0.02

Explanations:
Sheep — sheep and lambs; goats — goats and kids; horses — horses and foals; hens — hens and roosters; ducks — ducks and geese; rabbits — rabbits and coypu

ers, bulls, calves, pigs, sheep, ducks and geese, rabbits, while it increased in goats, horses, chickens and turkeys in comparison to the preceding period.

On the basis of the comparison of the numbers of emergency slaughters in food animals during the periods of 1989—1994 and 1995—2000 it can be concluded that the numbers of emergency slaughters decreased in the period of 1995—2000 in cows, heifers, bulls, calves, pigs, sheep, goats, horses, chickens, hens, turkeys, ducks and geese, rabbits in comparison to the preceding period. It was proved that the decrease was statistically significant in all species, production, and age groups of food animals included in the study.

The relative numbers of emergency slaughters in different species, production, and age groups of food animals, ranked in descending order for both periods monitored, are presented in Table 2. On the basis of data presented in Table 2 it can be concluded that numbers of emergency slaughters are different in separate species, production and age groups of food animals. In the period of 1989—1994 lowest numbers of emergency slaughters occurred in ducks and geese, followed by turkeys, chickens, hens, pigs, sheep, bulls, rabbits, heifers, cows, goats, calves, and horses with highest numbers. In the period of 1989—1994 lowest numbers of emergency slaughters in food animals occurred in chickens, followed by ducks and geese, turkeys, hens, sheep, pigs, bulls, rabbits, heifers, geese. Highest numbers were found in cows, horses and especially in calves. The differences in the results were highly significant.

DISCUSSION AND CONCLUSION

The total numbers of food animals slaughtered reflected the behaviour of meat consumers. The number of food animals slaughtered during the period of 1995—2000 showed a large decrease in cattle and partially also in pigs, while the total numbers in turkeys and chickens increased. The changes document a certain shift in consumer preferences from red meat towards poultry meat. The increased numbers of goats and horses slaughtered in 1995—2000 can be explained by closing down uneconomic goat and horse farms and by increasing their numbers in efficient farms. This assumption is also supported by the numbers of emergency slaughters in horses and especially in goats during this period. It can be concluded from the numbers of emergency slaughters that in these species mostly healthy animals were slaughtered.

Numbers of emergency slaughters and findings from the inspection of meat and organs of animals slaughtered are parameters of health status of food animals at slaughter. It can be concluded from the results of Lis (5, 6, 7), Kofer *et al.* (4), Schuh *et al.* (8), and Grest *et al.* (2) that pathological findings during the inspection of food animals and meat at slaughter are significantly frequent especially in organs. The works by Lis (5) in cattle, Lis (6) in pigs and Lis (7) in several species of food animals suggest that total proportion of carcasses of food animals classified as unsuitable for human consumption, condemned, and suitable for processing only, is less than 1 %. If the situation in years 1987 and 1997

is compared, the proportion mentioned above had further decreased. It means that the proportion of carcasses of food animals classified as suitable for human consumption had increased in 1997 compared to 1987. Such results also reflect the improved health status in food animals brought to the slaughterhouse.

We have detected statistically significant decreases in relative numbers of emergency slaughters in food animals during the period of 1995—2000 compared to 1989—1994 as follows: in cows from 23.38 % to 18.81 %, in heifers from 18.32 % to 7.37 %, in bulls from 10.63 % to 2.84 %, in calves from 50.11 % to 35.07 %, in pigs from 3.51 % to 1.90 %, in sheep from 6.67 % to 1.64 %, in goats from 46.09 % to 6.15 %, in horses from 86.09 % to 27.91 %, in chickens from 2.45 % to 0.02 %, in hens from 3.47 % to 0.69 %, in turkeys from 1.52 % to 0.09 %, in ducks and geese from 1.48 % to 0.05 % and in rabbits from 11.59 % to 4.08 %. It can be therefore concluded that health status in food animals at slaughter has significantly improved recently in all species, production and age groups that have been monitored. This can be attributed to several factors.

First of all, during the period of 1995—2000 the results of changes in veterinary regulations started to be manifested. The same applies to the changes brought about by the new act on the protection of animals against cruelty. The new regulations led to stricter enforcement of veterinary requirements in the farms of food animals, in particular as regards nutrition, husbandry, hygiene, etc. Furthermore the results of active surveillance in the area of protection of animals against cruelty also started to be apparent. This especially concerns the requirements for housing technologies, where cruelty and the suffering of animals are limited, as well as animal transportation.

More demanding requirements, their enforcement on farms and on sites of food animal handling, as well as reduced consumption of meat from food animals, led to closing down many farms of some species, production, and age groups of food animals mostly with unsatisfactory conditions. The result of such development was seen in the improvement of the health status of food animals during the period of 1995—2000.

During the recent period health status of food animals is best in chicken, ducks and geese, followed by turkeys, hens, sheep, pigs, bulls, rabbits, heifers and goats. The worst results were found in cows, horses and especially in calves.

The best health status in chickens, ducks and geese in comparison to other species, production and age groups of food animals monitored, is due to specialization in farming, sophisticated nutrition, observation of husbandry requirements, controlled hygiene and the strict control of the health status in these animals. The worst health status in horses is due to the fact that horses are principally not kept as food animals, and therefore they are mostly slaughtered because of disease, injury, low performance, etc. Therefore the proportion of emergency slaughters in horses is relatively high.

The situation in cows is somewhat similar, since dairy cows are slaughtered because of disease, injury, low production or reproductive disorders. Calves are also not principally kept to be slaughtered. Those that are found at slaughterhouses have been culled due to disease, injury or poor growth, and remain unsuitable to be kept further to produce milk or beef later on. Consequently also in calves the proportion of emergency slaughters from the total number of slaughters is high.

On the other hand, the species, production, and age groups of animals kept on a large scale to be slaughtered for meat show low numbers of emergency slaughters. This applies especially to bulls, pigs, turkeys, ducks and chickens. Low numbers of emergency slaughters in these species, production, and age groups of food animals may also reflect the direct interest of farmers in animal health and consequently in the quality of meat.

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DETERMINATION OF MALONDIALDEHYDE IN PORK BY HPLC AS THE 2,4-DINITROPHENYLHYDRAZINE DERIVATIVE

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ABSTRACT

An assay for the determination of malondialdehyde (MDA) levels in pork is described. The method involves the extraction of samples with 10 % trichloroacetic acid (TCA) and derivatization of TCA extract with 2,4-dinitophynylhydrazine (DNPH). MDA-DNPH complexes were extracted with pentane. After evaporation under of stream of nitrogen, aliquots of 20 µl in acetonitrile were detected by HPLC on reversed-phase C_{18} column (3 µm) with UV detection. The products were eluted isocratically with mobile phase containing acetonitrile : water : acetic acid (39 : 61 : 0.2; v/v/v). MDA-DNPH peak was identified by co-chromatography with peaks of authentic standards. Retention time for MDA-DNPH was 6.6 min and detection limit was 4.25 ng.g^{-1}. Specificity, speed, simplicity and economical costs determine this method for detection of lipid oxidation products.

Key words: HPLC; lipid oxidation; malondialdehyde; pork

INTRODUCTION

The fats take a very important place in human dietetics. The most important property is the presence of polyunsaturated fatty acids (PUFA). These components are mostly responsible for oxidation of fats and production of hydroperoxides in meat and meat products. Hydroperoxides decomposition produces a huge number of secondary metabolites. The most important product of PUFA decomposition is MDA (4, 6). In practice, the most often used method for MDA detection is thiobarbituric acid (TBA) test.

The MDA has been most commonly quantified spectrophotometrically as the complex formed after reaction with two molecules of TBA, which has an absorbance maximum at 532 nm (7, 9), or MDA is detected spectrofluorometrically. However, the TBA method is not absolutely specific for MDA detection, and the other TBA reactive products as proteins, DNA, deoxyribose absorb also significantly at 532 nm (2, 5, 6, 10). Therefore, the TBA test overestimates the amount of MDA and decreases specificity of TBA analysis (4). To overcome this problem a number of methods were developed to estimate MDA-TBA adducts using HPLC (3, 10). Although these results are more reliable compared to the spectrophotometric methods of TBA detection, this HPLC technique did not gain popularity because of the extremely complex nature of sample preparation and slowness of the technique (2).

MDA and DNPH reaction produces DNPH derivatives with intensive yellow colour and it makes HPLC detection easier. The other advantage is excellent elution and separation of decomposed aldehydes on HPLC column (5). These factors increase the specificity of MDA detection and they improve complex view on process of oxidation in sample (2). The aim of experiment was modifying of HPLC detection of MDA after derivatization with DNPH (2) and its application on MDA analysis in meat and meat products.

MATERIALS AND METHODS

Acetonitrile (ACN) was obtained from Merck (Germany); 1,1,3,3-tetramethoxypropan (TMP) was purchased from Sigma (Germany); pentane, hydrochloric acid (HCl), trichloroacetic acid (TCA) were purchased from LA-CHE-MA CZ (The Czech Republic). All other chemical reagents were of analytical or HPLC grade.

Butylated hydroxytoluene (BHT, Sigma, Germany) was prepared as 500 ppm in 95 % methanol. DNPH reagent (2,4-dinitrophenylhydrazine, Fluka, Switzerland) was prepared by dissolving 31 mg DNPH in 10 ml of 2 mol HCl.

Standards of MDA were prepared by acid hydrolysis of TMP (10). Ten microlitres TMP was accurately diluted to 10 ml with 0.1 mol.l^{-1} HCl in a screw-capped test tube and immersed into a boiling water bath for five minutes, then quickly cooled with tap water (solution A). A working stock solution of MDA was prepared by pipeting 1.0 ml of the hydrolysed acetal (solution A) into a 100 ml volumetric flask and diluting to volume with 0.1 mol.l^{-1} HCl. The resulting MDA stock solution of 4.37 µg.ml^{-1} was further diluted with water to yield final concentrations of 43.7, 17.5, 8.74 and 4.37 ng.ml^{-1} to get the standard curve. The fresh working standards were prepared from stock solutions daily.

The pork meat (thigh muscle) was obtained from retail market, then processed, grounded and stored at 4 °C for 48 hours. One gram of the sample was weighted to the test tube and 0.5 ml of 500 ppm BHT and 5 ml of 10 % TCA were added. The test tube content was homogenised 30 seconds at maximum rotation. After 30 minutes of incubation in water bath (85 °C) the samples were chilled and centrifuged at 8000 *g* for 10 minutes. Two millilitres of supernatant were used for HPLC analysis.

100 µl of DNPH reagent were transferred to a 2 ml of supernatant in 12 ml test tube, mixed and incubated 15 minutes at room temperature on the dark place. Then 5 ml of pentane were added and slightly mixed. After 15 minutes pentane phase was removed and the procedure was repeated one more time. Combined pentane extracts were dried under nitrogen at 40 °C and reconstituted in 500 µl of acetonitrile (mobile phase). Twenty microlitres of volume was injected onto the HPLC system.

Analytical HPLC separations were performed with Hewlett Packard machine (model 1050 HP) equipped with autoinjector and a variable-wavelength UV detector operated at 307 nm. The HPLC analysis of MDA-DNPH complex was carried out using 3 µm C_{18} reverse phase column (Nucleosil $C_{18,}$ 125 × 3 mm; SepServ.).The column was eluted isocratically with mobile phase ACN : water : acetic acid (39 : 61 : 0,2) at a flow rate 1 ml.min^{-1}.

RESULTS AND DISCUSSION

The chromatography records of standards and meat samples after MDA derivatization with DNPH are shown in Fig. 2.The complex MDA-DNPH was detected with an isocratic gradient at 6.6 minutes. Mobile phase (ACN : water : acetic acid; 39 : 61 : 0.2) was used for MDA-DNPH elution and the UV absorbance for all the samples with maximum response of peaks was observed at 307 nm. It appears that derivatization and separation of the MDA-DNPH resulted in a more accurate estimation of the concentration of MDA in animal tissues. Using this procedure, described detection limit for MDA was

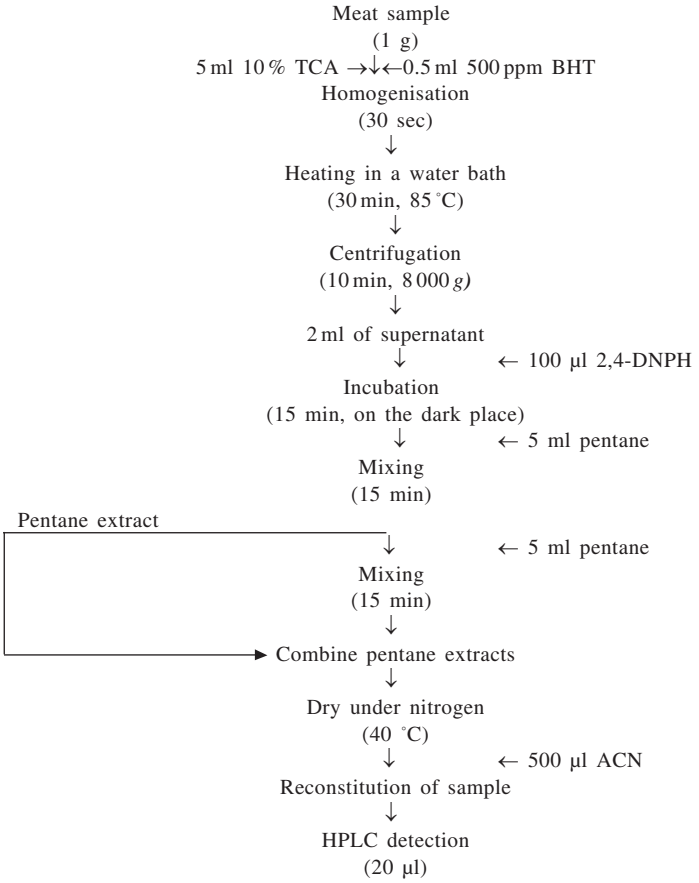

Fig. 1. Diagram indicating the steps involved in the aqueous acid extraction and derivatization with DNPH before HPLC detection of MDA

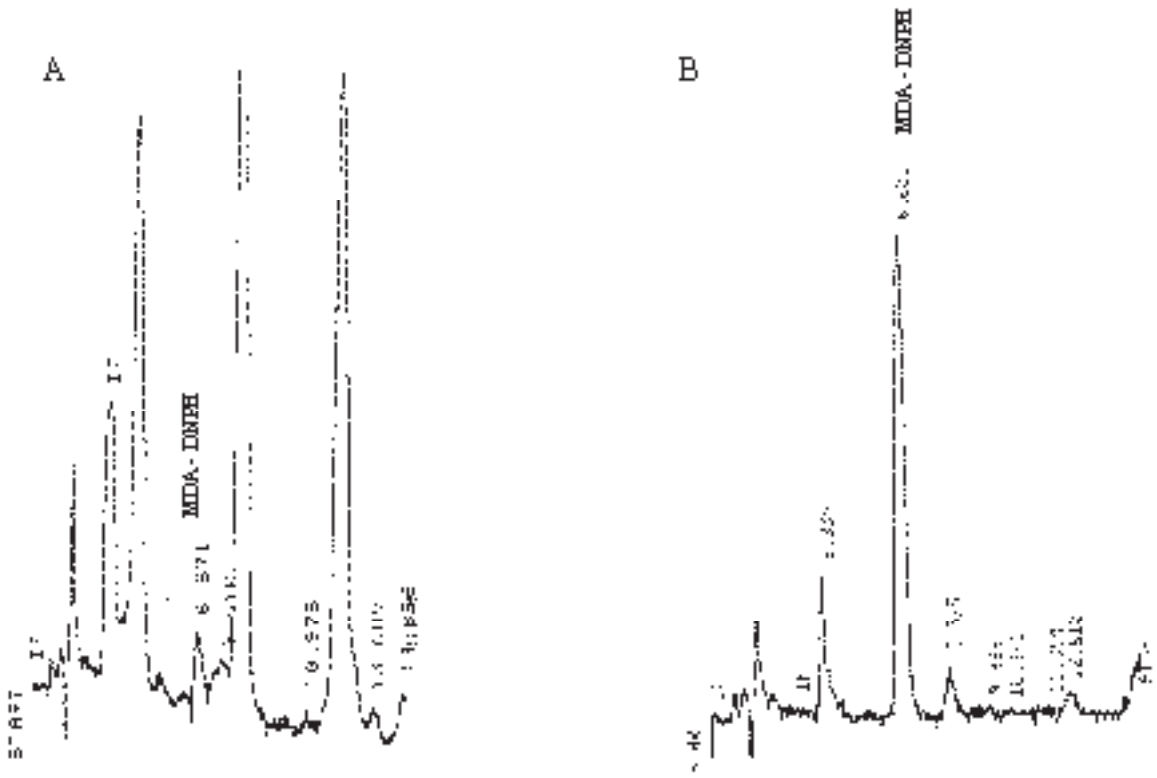

Fig. 2. Chromatographic separation of MDA: A) meat sample (0.11 µg.g^{-1}); B) standard of MDA (43.7 ng.ml^{-1})

4.25 ng.g^{-1}. The results indicated that the recovery average was c. 80 %.

The estimation of MDA from samples with complex biological matrix is difficult. Method of MDA extraction from the sample has a serious influence on the result of MDA detection. In biological matrices MDA exists both free and bound to -SH and -NH_2 groups of macromolecules such as proteins and nucleic acids (3, 5).

Draper *et al.* (3) reported that extraction of MDA with hot TCA produces higher values of the MDA than extraction with ice-cold TCA. The MDA in animal tissues occurs in bound forms from which it must be released by acid hydrolysis requiring heat. Bakalova *et al.* (1) introduced an alkaline hydrolysis for 30 minutes at 60 °C. Cordis *et al.* (2) recorded that using of NaOH caused a total loss of absorbance at the specific wavelength of 307 nm.

An important step in the using of higher temperatures is addition of adequate amount of antioxidants (too high concentration support oxidation) to protect oxidation and producing of MDA. The antioxidants must be added before homogenisation to decrease danger of oxidation with oxygen.

In our experiment a modificated method of MDA extraction, described by Draper *et al.* (3), was used. One gram of sample (thigh muscle) and 0.5 ml of 500 ppm BHT were mixed. After antioxidant and 5 ml of 10 % ice-cold TCA (4 °C) addition the samples were homogenised for 30 seconds. The final concentration of MDA is depended on the length and temperature of homogenisation. Centrifugation and filtration are important steps to remove matters (proteins) which can react with DNPH to form a complexes and peaks produced by them can interfere with MDA-DNPH peak.

Derivatization and HPLC detection of MDA were modificated according to method reported by Cordis *et al.* (2) For derivatization purposes 2 ml of supernatant were used. The reaction with DNPH was allowed to occur at room temperature, but samples must be protected from light. The light approximate 20 % decrease of height of peaks within first hour of derivatization and detection.

Quantitative extraction of MDA-DNPH is performed in organic solvents. Extraction MDA-DNPH complex with pentane before HPLC analysis removes products that can react with DNPH and also produces separated MDA-DNPH peaks on chromatogram. Instead of 10 ml of pentane only 5 ml were used for MDA-DNPH extraction and the time of extraction was 15 minutes. Instead of 30 °C recommended by Cordis *et al.* (2), 40 °C according to Pilz *et al.* (8) was used to make evaporation easier. MDA – DNPH was reconstituted with 500 µl of ACN. Recommended volumes of ACN for reconstitution of samples is 200 µl (2), 100 µl (6) and 60 µl (8). In comparison with them these volumes was not enough for complete reconstitution of sample.

MDA-DNPH peak was clearly separated by elution with mobile phase. Cordis *et al.* (2) recorded that the portion of ACN should be in the range of 34—40 %.

The other concentrations of ACN decrease sensitivity of detection. The HPLC columns were tested and column Nucleosil C_{18} was chosen (8). We observed good separation of MDA-DNPH complex by using mobile phase (39 : 61 : 0.2) in column. Apart from detection of MDA, Cordis *et al*. (2) also used this method for separation of aldehydes (acetaldehyde, formaldehyde, acetone) from lipid oxidation. The method is more sensitive and correct to detect the level of lipid oxidation then TBA test as it separates or detects another aldehydes.

Several methods are available for the quantitation of MDA in biological tissues. Among those, the measurement of TBA-reactive products has been frequently used because of its simplicity, although the method lacks specificity. To best of our knowledge the present is showing that MDA can be detected highly specifically by using HPLC.

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NON-SPECIFIC IMMUNE RESPONSE IN DOGS WITH DERMACOMYCOSIS

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ABSTRACT

In 10 dogs with malasseziosis (*Malassezia pachydermatis*) complicated with bacterial infection (*Staphylococcus intermedius, Staphylococcus haemolyticus*), the level of phagocytic activity of neutrophils and functional activity of lymphocytes were examined and compared with the parameters in 20 clinically healthy dogs. Clinical signs persisted for 2—3 months in dogs examined with the permanent formation of new lesions. A significant increase in the total leukocyte count, phagocytic and ingestion ability of neutrophils, non-significant lower values of metabolic activity of phagocytes, and a significant decrease in the blastogenic response of lymphocytes were found in the dogs affected.

Key words: blastogenic response of lymphocytes; dog; malasseziosis; phagocytosis

INTRODUCTION

Dermatomycoses in dogs are caused by dermatophytes belonging to genera *Microsporum, Trichophyton, Malassezia* and *Candida*. In recent years the incidence of the infections by *Candida* and *Malassezia* species has been on the increase (11). An increasing use of antibiotics, immunosuppressants, and cytostatics in small animal practice contributes to the development of these diseases. The occurrence of dermatomycosis is also associated with insufficient care and hygiene. Opportunistic fungi usually require a host that is immunosuppressed by metabolic problems, malnutrition, concurrent viral or bacterial infections or neoplasia. Generally, the predisposing factors limiting the development of dermatomycosis include any disease process that compromises the skin barrier and suppresses the immune response of a host (7).

Malasseziosis is caused by *Malassezia pachydermatis,* which inhabits, as a saprophyte, the ears and skin, and under optimals conditions becomes pathogenic. The main clinical signs are *otitis externa*, erythematous lesions, *alopecia*, *hyperkeratosis*. The condition is always accompanied by intense itching. In a case of mixed infections with gram-positive bacteria as well as other types of yeasts, the clinical signs are pronounced and the course of disease is prolonged.

The aim of our study was to evaluate some parameters of non-specific cellular immunity in dogs with malasseziosis complicated with secondary bacterial infection.

MATERIAL AND METHODS

Animals. Ten dogs with an average age of 4.7 years of different breeds and sexes with natural dermatomycosis confirmed by clinical and mycological examination. Dermatomycosis was complicated by bacterial infection verified by the BBL Crystal identification system (Becton Dickinson, USA). Twenty uninfected dogs with an average age of 3.1 years, different breeds and sexes served as control animals.

The evaluation of parameters of cellular immunity

Blastogenic response of blood lymphocytes to mitogens. Lymphocytes were separated from venous blood on the Ficoll density gradient (Pharmacia Biotech AB, Sweden). The viability of the isolated cells was determined by trypan blue exclusion and exceeded 97 %. Most (> 95 %) isolated cells were mononuclear cells. The cultivation (culture medium contained 10 % of autologous serum), mitogen stimulation and the measurement of blastogenic response of lymphocytes by the fluorescence method were performed according to Nakanishi *et al.* (9). Concanavalin A (Con A, Sigma Chemical Co., USA) was used for stimulation in the concentration 25 $mg.ml^{-1}$ (10). The level

of the blastogenic response of the lymphocytes was expressed as the stimulation index (SI). The SI was calculated according to the formula SI = (A – C)/(B – C), A = mean FI (fluorescence intensity) with mitogen, B = mean FI without mitogen, C = background FI. The FI was measured by a spectrofluorometer (Jasco FP-550, Japan).

The phagocytic activity of blood neutrophils was examined **as described by Větvička** *et al.* (12). 0.1 ml of fresh heparinized blood (5 U of heparin.1 ml^{-1} of blood) was mixed with 0.05 ml of 2-hydroxyethylmetacrylate particles (MSHP, diameter 1.2 mm, ARTIM Prague) and incubated for 1 hour at 37 °C with occasional shaking. The phagocytic activity (PA) of neutrophils (Ne) was expressed as the percentage of the neutrophils phagocytizing 3 and more MSHP, and as the index of phagocytic activity (IPA) representing the ingestion ability of neutrophils (the ratio of the number of phagocytized MSHP and the number of potentially phagocytizing Ne).

The metabolic activity of phagocytes was performed using INT (iodnitrotetrazolium) test as modified by Mareček and Procházková (8). The index of metabolic activity (IMA) was calculated from the metabolic activity of cells stimulated by zymozan and the metabolic activity of unstimulated phagocytes.

Total leukocyte count and differential cell count were determined using common haematological methods.

Statistical analyses. The data were characterized by the mean and standard deviation. The significance of differences was checked by Student's *t*-test.

RESULTS

In the dogs examined, mycotic infection (*Malassezia pachydermatis*) and bacterial infection (in most cases *Staphylococcus intermedius*, in 1 dog *Staphylococcus haemolyticus*, in 1 dog *Enterococcus faecium* and *E. coli*) were identified. Clinical signs persisted for 2—3 months, alopecic and erythematous lesions, scaling, and intense pruritus were dominant. A typical feature of this condition was a progressive spreading on the whole body and the permanent formation of new lesions. The results of the immunological examination are shown in Tab. 1. Leukocytosis was found in all dogs affected.

The parameters of phagocytic ability – the percentage of phagocytizing neutrophils and the index of phagocytic activity (ingestion ability) were significantly higher when compare to those in healthy dogs. The metabolic activity of phagocytes was lower in the affected dogs but without statistical significance.

The proliferation of dog peripheral lymphocytes to nonspecific mitogen, concanavalin A was significantly lower when compared to the control group.

DISCUSSION

When monitoring selected immunological parameters in dogs with malasseziosis the most significant changes were found in the values of phagocytic activity and the ingestion capacity of neutrophils. Similarly, an increase in the ingestion activity of phagocytes was presented in a study of trichophytosis in foxes (7), which was evident in the first weeks of the infection. When the disease proceeded to the chronic stage, as the result of immunosuppression due to galactomannans released from the cell walls of dermatophytes, in a cell-mediated immune response (5), this value significantly decreased. In the dogs in our investigation the clinical signs of the disease have persisted for 2—3 months with the permanent

Table 1. Haematological and immunological parameters in dogs with malasseziosis compared with those in healthy dogs

	DOG No.	Lc (x10^9. l^{-1})	%Ne	PANe (%)	IPa Ne	IMA	SI
	1	12.6	59	81	18.1	2.250	1.71
	2	10.6	53	84	16.7	2.103	1.14
	3	12.2	71	87	25.7	2.169	1.84
	4	9.2	51	88	21.8	2.477	1.25
	5	18.8	60	80	16.4	2.413	1.75
	6	21.2	58	81	19.5	1.546	1.35
	7	16.8	58	79	15.4	1.813	1.50
	8	17.2	60	82	18.1	2.110	1.62
	9	12.9	58	86	15.8	2.221	1.48
	10	14.9	60	83	19.1	2.330	1.24
mean		14.6	58.6	83.1	18.7	2.123	1.49
±SD		**± 3.6**	**± 5.0**	**± 2.9**	**± 3.0**	**± 0.258**	**±0.2**
Control mean		9.5	65.6	58.3	7.2	2.206	2.90
±SD		± 3.5	± 9.6	± 5.7	± 5.7	± 1.057	±1.2
t-test		*p < 0.005*	*NS*	*p < 0.005*	*p < 0.005*	*NS*	*p < 0.01*

Legends: Lc — total leukocyte count, Ne — neutrophils, PANe — phagocytic activity of neutrophils, IPANe — index of phagocytic activity of neutrophils, IMA — index of metabolic activity, SI — stimulation index of lymphocytes

formation of new lesions, which probably resulted in the high activity of the phagocytes found. Haematological findings – leukocytosis also corresponds with this fact. In the protection of organisms against dermatophytosis, cell-mediated immunity plays a decisive role.

In our cases acute inflammatory process on the skin has activated some immune functions, mainly a phagocytic activity of neutrophils. The metabolic activity of phagocytes was nonsignificantly lower, which agrees with the results of studies by Kostro *et al.* (7) and Ashbee *et al.* (1). Prolonged exposure of neutrophils to high concentrations of inflammatory mediators could result in deactivation of cellular function due to receptor-down regulation (4). So, a longer lasting chronic skin process can be accompanied by immunosuppression.

In our patients, where typical chronic skin disease has not been yet developed signs of the suppression of neutrophil functions were found only in the metabolic activity of neutrophils.

The proliferative activity of lymphocytes to ConA in dogs examined had decreased significantly.

A decrease in some parameters of cellular immunity in human patients suffering from dermatophytosis is reported in a study by Jautová *et al.* (6) Similarly DiSilverio *et al.* (3), DeMoraes-Vasconcelos *et al.* (2) have presented an alteration of cellular immunity especially of some functions of polymorphonuclear leukocytes and the proliferative response of lymphocytes in chronic dermatophytosis resistant to therapy.

The alteration of any cellular immunity function leads to a deterioration in the course of the disease and to secondary bacterial complications. In all the dogs examined secondary bacterial infection had developed, predominantly caused by *Staphylococcus intermedius.* We can not assess when the development of secondary pyoderma occurred because we did not examine the dogs repeatly during the disease process. Moreover, it is very difficult to explain the cause and consequence of immune function alteration. To clarify these connections further study is required including dogs in different stages of the disease.

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SOME OBSERVATIONS ABOUT THE PRESENCE OF THE SHEEP AND FARM HUSBANDRY SYSTEMS ON FOOT-AND-MOUTH DISEASE VIRUS INFECTION OF CATTLE IN AN ENDEMIC AREA

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ABSTRACT

The aim of this study was to determine whether the presence of sheep on the same farm influenced the prevalence of infection with foot-and-mouth disease virus (FMDV) in cattle and secondly whether the proportion of infected cattle varied with different farm husbandry systems in an endemic area. Farms were classified on the basis of cattle density, ovine/bovine ratio and cow/steer ratio. Adult bovine (n = 2285) and ovine (n = 1019) sera from farms were collected and tested by the agar gel diffusion test for the detection of antibodies to the virus infection associated antigen (VIAA). A total of 75 (7.32 %) ovine sera and 1451 (63.11 %) bovine sera gave positive reactions. No association was found between bovine VIAA prevalence and cattle density or steer/cow ratio. No significant differences were detected in cattle VIAA prevalence in different husbandry systems. No correlation was found between the evidence of infection in cattle and sheep, although a significant negative regression was found between the proportion of infected cattle and the ovine/bovine ratio.

Key words: epidemiology; farm husbandry; foot and mouth disease; sheep

INTRODUCTION

During the Foot and Mouth Disease Eradication Programme in Buenos Aires Province, Argentina, vaccination of cattle was compulsory but not of sheep. However, most of the farms in this area rear both species together. Sheep are susceptible to foot-and-mouth disease (FMD) although there is some controversy about their role in spreading the disease. FMD affects both domestic and wild cloven-hoofed mammals, however species differences in severity of clinical signs and susceptibility to different viral strains have been observed (8, 1). The disease in sheep is generally milder than in cattle. It may be clinically inapparent and lesions are found usually only after careful examination (8). Both sheep and cattle shed the virus in aerosols (13) and may become carriers for at least 9 months after the initial infection (6). Transmission of FMD virus (FMDV) from a carrier to an in-contact, susceptible animal has not, however, been documented.

A variety in the patterns of disease, known as FMD ecosystems, which follow regional husbandry systems, have been defined (4). The definition of a FMD herd health programme has been made on the basis of FMD ecosystems. But it is well known that farm husbandry systems may vary widely even within a region with consequences for the frequency of disease.

The aim of this study was to investigate whether the presence of sheep on the same farm influenced the prevalence of FMDV infection in cattle and whether the prevalence of FMDV infection in cattle varied with different farm husbandry systems within a FMD ecosystem defined as primary endemic area.

MATERIALS AND METHODS

This study was carried out in Ayacucho village, Buenos Aires Province during March and August 1991. Ayacucho is primarily a cow-calf operation area and was classified as a Primary Endemic Area for FMD, in spite of the fact that some farms also fatten steers and others have both sheep and cattle. Farms were classified according to farm husbandry systems from a database belonging to the FMD Local Agency for Eradication. Farms were classified by two variables at a time. The variables used to differentiate farm husbandry systems were animal density, ovine/bovine ratio and steer/cow ratio. Farms were classified as follows. For steer/cow ratio a ratio

less than 0.2 was classified as a cow-calf operation, from 0.2 to 0.6 as a mixed operation and more than 0.6 as a fattening operation. For ovine/bovine ratio three levels were used: no sheep, up to 0.5, and more than 0.5. Finally cattle density was calculated using an index called cow equivalent value (EV), which is defined as the average annual energy requirement for a beef cow that is pregnant and rearing a calf for 6 months. Farms were placed in three categories as less than 0.6 $EV.ha^{-1}$, 0.6 to 0.87 $EV.ha^{-1}$ and > 0.87 $EV.ha^{-1}$.

Bovine blood samples were obtained at least 1 year after the last FMD vaccination. Ovine samples were collected if the sheep had never been vaccinated for FMD. A total of 2281 bovine samples were collected from 86 farms and from 1019 adult sheep from 30 of those same farms and a questionnaire was completed at the same time. Sera were stored at –20 °C. The AGID test to detect viral infection associated antibody (VIAA) was carried out as previously described (11). Positive and negative reference sera and VIAA antigen were obtained from the National Animal Health Laboratory. Statistical Analysis System (*SAS Institute Inc., 1988) was used for analysis of the data. Analysis of variance, correlation coefficient and linear regression were used after data were transformed (angular transformation) and the basic assumptions for the tests were met.*

RESULTS

A total of 63.11 % (C.L. ± 10.1 %) bovine and 7.32 % (C.L. ± 9.3 %) ovine had VIAA The prevalence of positive reactions in the VIAA test in bovine and ovine sera on the various farms is presented in Tables 1 and 2.

The average steer/ cow ratio from all the farms was 0.38, SD 0.79 (CV = 207.07 %). The average animal density from all the farms was 0.78 E.V., SD 0.22 (CV = 28.26 %). No correlation was found between bovine VIAA prevalence and either steer/cow ratio (P = 0.06) or animal density (P = 0.40). The average ovine/bovine ratio from all the farms was 0.44, SD 0.41 (CV = 93.79 %). No correlation was found between bovine and ovine VIAA prevalence (P > 0.05). But a weak significant negative linear correlation was found between bovine VIAA antibody prevalence and ovine/bovine ratio (r = –0.22, P = 0.044) (Fig. 1). Only a very small portion (4.8 %) from the total variation in bovine FMDV infection can be explained by the ovine/bovine ratio on the farms. FMD outbreaks were recorded on five of the farms during the 12-month period before blood samples were collected. No differences were found between bovine prevalence (P = 0.2496) or ovine prevalence (P = 0.8689) from farms with and without outbreaks during the past year.

DISCUSSION

Serological evidence of viral infection among the cattle on these farms was high in spite of vaccination. However it is known that vaccination does not prevent viral replication or development of a carrier state (15). It is possible, but unlikely, that some of the positive reactions in the VIAA test in the cattle sera were due to vaccination. However it has been shown that the concentration of VIAA antibody after vaccination is low and does not persist after about 60 days (2). All samples were collected from cattle that had not been vaccinated in the previous year and from unvaccinated sheep. The occurrence of some outbreaks during the period after the samples were taken confirms that FMDV was circulating in this area but that protective immunity was good because there were few sick animals (data not shown). On the other hand ovine VIAA prevalence was low and it was did correlate with the prevalence of bovine VIAA reactions.

Table 1. The prevalence of reactions in the VIAA test on cattle sera from farms near Buenos Aires

Farm classification		**Sheep:cattle ratio**			**Total Positive**	**Total Sampled**
		0	**< 0.5**	**> 0.5**		
Animal density (EV/ha)	**No. farms**		**No. animals**			
< 0.6	**25**	**42**	**257**	**128**	**427**	**688**
0.6 to 0.87	**33**	**56**	**336**	**166**	**558**	**894**
> 0.87	**28**	**70**	**235**	**161**	**466**	**699**
Total	86				**1451**	**2281**
Steer:cow ratio						
< 0.2	46	150	361	252	763	1239
0.2 to 0.6	24	0	273	161	434	619
> 0.6	16	18	194	42	254	423
Total	86				**1451**	**2281**

Table 2. Prevalence of reactions in the VIAA test on sheep sera from farms near Buenos Aires

Animal density	(EV/ha)	No. farms	Sheep:cattle ratio	< 0.5	Total Positive > 0.5	Total sampled
		No. farms	No. animals			
	<0.6	8	4	13	17	268
	0.6 to 0.87	13	12	26	38	440
	>0.87	9	7	13	20	311
Total						**1019**
Steer:cow ratio						
	<0.2	15	23	19	42	506
	0.2 to 0.6	10	0	33	33	343
	>0.6	5	0	0	0	170
Total						**1019**

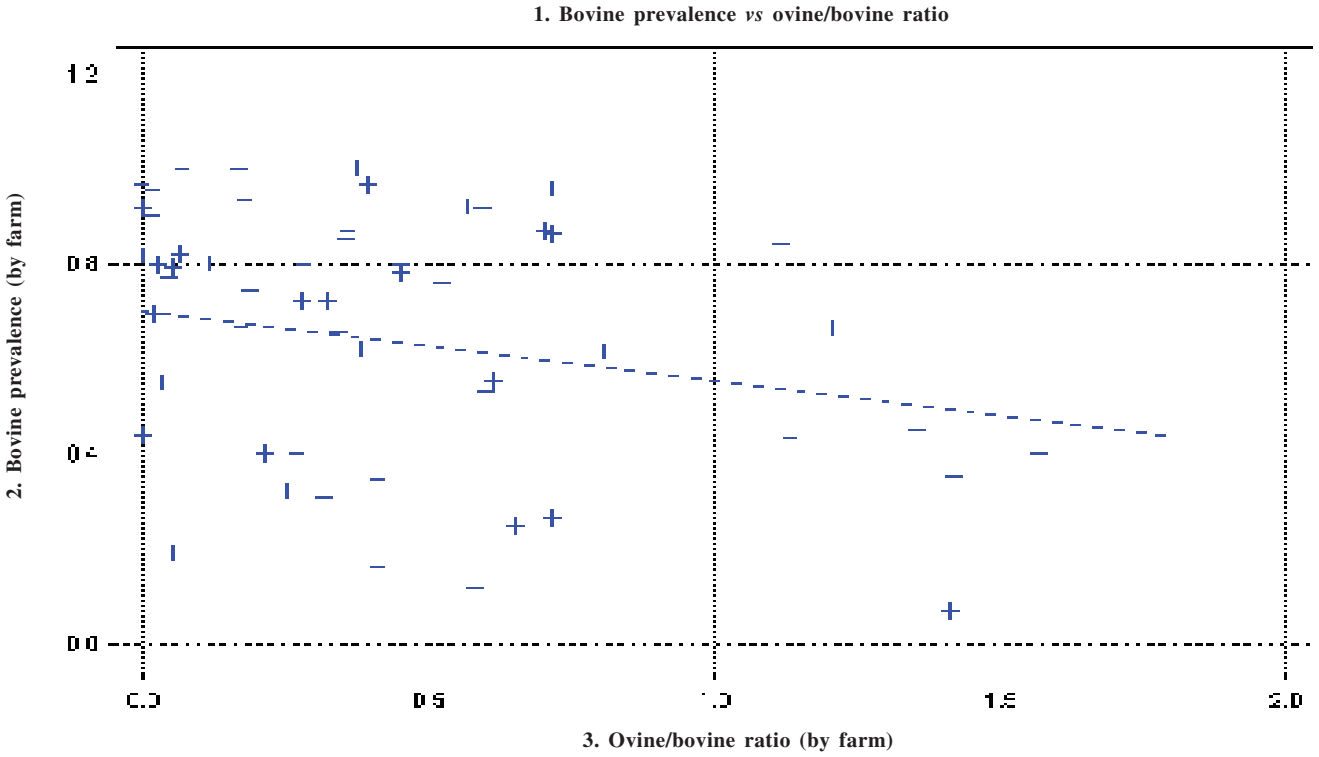

Fig. 1. Classification of farm husbandry systems — after three variables used in this study

Positive reactions in the VIAA test have been recorded in sheep after infection with FMDV (11). It seems that sheep are less susceptible to FMD since infection is frequently subclinical (5) or they show mild clinical signs of short duration (8, 7). Also they have a low infection rate during outbreaks (14). In some studies it was not possible to find sheep that were carriers of FMDV from highly infected regions (3).

It is important to highlight that animal movement was restricted in this area as part of the FMD eradication programme since this would be one of the possible explanations for the lack of correlation between bovine and ovine VIAA prevalence. A significant negative regression was found between bovine VIAA prevalence and ovine/bovine ratio. It could be postulated that because sheep are less susceptible to disease and excrete less virus when infected, the spread of infection on a farm is less efficient when more sheep are present (10).

Also sheep need a longer exposure time to become infected compared with cattle (5, 3) and, together with

shedding less virus when infected (12) may act as a 'dilution factor' for the infection on those farms that rear both species together. However, the ovine/bovine ratio only accounted for 4.8 % of the variation in bovine VIAA prevalence. This means that there are many other factors that are more important than this that contribute to the variation.

No differences were detected in bovine VIAA antibody prevalence between the different farm husbandry systems. The variables considered may be valid to identify differences between ecosystems but not for individual farm husbandry systems (10, 4). This study did not resolve this question. There was no evidence that the presence of infection in sheep was correlated with infection in cattle. This suggests that sheep do not significantly contribute to infection in cattle in an endemic situation.

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HISTOCHEMICAL STUDY OF THE ADRENERGIC INNERVATION OF THE HEART IN SOME MAMMALS

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ABCTRACT

The authors studied the innervation of the individual compartments of the heart in rabbits, pigs, and rats. Adrenergic nerve components were visualized by the glyoxylic histofluorescence method or by the method of formaldehyde-induced fluorescence of catecholamines and 5-HT. Several differences were observed in the distribution and density of adrenergic nerve profiles in the individual parts of the mammalian heart. The relative density of adrenergic nerve profiles was always higher in the auricles than in atria, and significantly lower in the ventricles. The innervation of the left half of heart was evidently lower than in the right one. The highest density of specifically fluorescent nerve fibres was found in the tricuspid valves, namely in the base compartments of individual cups. In pigs, the connection between nerve profiles lying in the valve alone and fibres running in the *chorda tendinea* or in the papillary muscle were especially notable. In rabbits, there is a particularly significant finding that in the narrow strip at the base of individial cups the thin-wall arterial profiles, which are partially and sometimes to a great extent bordered by adrenergic nerve fibres. These fibres are relatively abundantly represented always in the central and marginal parts of valves. The innervation of mitral valves was evidently modest. The relatively rich innervation of the epicardium was observed in the all animals examined, richest mainly in rabbits and sparce in rats. The authors suppose that richly represented adrenergic nerve components in the atrioventricular valves, auriculae and epicardium could play a significant role, especially in the regulation of noradrenaline concentration in the circulating blood.

Key words: adrenergic innervation; heart; rabbit; pig; rat

INTRODUCTION

In general, there it is no doubt that the actual demands of the body for the functions of organs and tissues are directly or indirectly ensured by the autonomic nervous system. Therefore, in a link to the traditional neuroanatomical and neurohistochemical examination procedures, and in the era of the neurotransmitter histochemistry, special attention is paid to the autonomic innervation of organs and tissues, inclusive of the heart.

Both the regulation and systematic coordination of the heart activity in the body are ensured by the *n. vagus* and sympathetic nerves. A more complex view on the adrenergic innervation of the myocardium, papillary muscles, and coronary arteries of the heart has been described by several authors in dogs Zhao *et al.* (19), Doležel *et al.* (7, 8), in guinea-pigs and cats Zhao *et al.* (19) and in people Danihel *et al.* (6), Brodde and Michel (1), Chow *et al.* (3). The development of this innervation in the intrauterine and postnatal period was mapped in people by Danihel *et al.* (6), and in dogs by Doležel *et al.* (8). According to other literary data it is apparent that morphological pictures of the nerve supply of the heart testify to the irregular distribution and density as adrenergic as cholinergic and NADPH-d positive nerves in individual parts of the heart (20, 1, 12, 14).

It is known that catecholamines play a significant role as at physiological regulation of the cardiovascular activity as well as in the pathogenesis of heart insufficiency (20, 9). Therefore, we have made an effort to investigate the adrenergic innervation of individual compartments of the heart in rats, rabbits and pigs with a special regard to the existing species-dependent differences.

MATERIALS AND METHODS

Clinically healthy adult animals of both sexes were used in the study. The hearts of 25 rats (Wistar, body mass 350—450 g), 18 rabbits (Chinchilla, body mass 2.5—3.5 kg) and 8 pigs (*Sus scrofa* f. *domestica* – Brno White miniature pig, body mass 20—30 kg) were examined. The animals were killed by thiopental (50—60 mg.kg^{-1}, i.p.). Constantly, multiplice tissue specimens were examined. Adrenergic nerve components were visualized by the method of formaldehyde-induced fluorescence of catecholamines and 5-HT (10). The tissue was frozen, lyophilized, condensed with formaldehyde and embedded in paraffin; or by the histofluorescence method (17), where tissue samples or cryostat sections were incubated in glyoxylic acid solution. Preparations of the epicardium and heart valves were processed using the technique of "whole mount or stretch preparation" on the microscope slide. Individual sections were mounted in a non-fluorescent medium. The fluorescent examinations and photographic documentation were performed using Jenalunar 2 (Zeiss).

RESULTS

In all the animals examined the richest density of specifically fluorescent adrenergic nerve profiles was found in the auriculae of the atrium. Here, they form characteristic plexiform aggregations round arterial or arteriolar vessels, while next individual nerve fibres are very closely adherent to the endothelium of trabeculae and cardiomyocytes. In the heart atria, the total number of visualized adrenergic nerve components clearly exceeds their number in the heart ventricles, whereas the total number of nerve components in the right atrium is always greater than that in the left one (Fig. 1). In addition, in the animals examined, a slightly increased density of adrenergic nerve fibres was observed also in the areas of sinoatrial and atrioventricular "pacemakers".

It is remarkable that the distribution of specifically fluorescent nerve fibres, especially in the musculature of the left ventricle is apparently irregular (patchy). The nerve fibres in the focuses of more densely innervated parts occur mostly within the perivascular plexuses and only rarely as solitary nerve profiles lying between cardiomyocytes or accompanying fine thin-wall arterial branches or capillaries, with which they form very tight anatomical contacts. Innervation of the internal compartments of both heart ventricles deserves a special mention. Specifically fluorescent nerve profiles are also present in more trabeculae, and individually varicose nerve fibres sometimes lie in the right ventricle especially, and also close under the endothelial lining (Fig. 2). In addition, they also occur in the papillary muscles, and in pigs they were often observed in apparent connection with nerve fibres running through the *chordae tendineae* from the innervated margins of atrioventricular valves.

As far as the innervation of atrioventricular valves is concerned, the relatively high density of specifically fluorescent and plexiformly arranged nerve fibres was found in the base compartments of the tricuspid valve in rabbits and pigs, while in rats there is apparently less density of nerve supply. In pigs especially, the above-mentioned connections between nerve profiles lying in the valve itself and fibres running in the *chordae tendineae* (Fig. 3) are notable. In rabbits in the narrow strip at the base of individual cusps the presence of more thin-wall arterial profiles, which are bordered by adrenergic nerve fibres, was recorded (Fig. 4). The plexiform nerve profiles in the rat valves appear to be relatively the thinnest. In all the animals, including rats, however, the adrenergic innervation of mitral valves is evidently poorer than that in tricuspid valves.

The next striking feature of the histological findings is a remarkable density of nerve profiles in the epicardium (Fig. 5), which is not only in the shape of periarterial or periarteriolar nerve borders, but also plexiform, relatively rich, and irregularly distributed nerve aggregations in all three species of animals.

According to our results of the study of adrenergic innervation of the heart in rats, rabbits, and pigs by the fluorescent histochemical methods, no more substantial species-specific differences have been found.

DISCUSSION

It is known, that mammalian heart displayed regionality in the distribution and density of autonomic innervation, because the branches of sympathetic and parasympathetic nerves influence specific cardiac regions to a different extent (15). Functional studies in the canine heart (4) demonstrated differences in the site of action between right and left-sided branches of efferent vagal and sympathetic cardiac nerves, providing further evidence that four chambers of the mammalian heart recive a differential autonomic supply. Evidence from electrophysiological (16), scintigraphic (5) and immunohistochemical studies (13, 2) support the findings that within the heart are specific regions where the supply of the individual nerve subpopulations predominates.

There are data (13) that AChE-containing nerves dominate in endocardial, myocardial and atrial epicardial tissues. For example, in the atrioventricular nodal tissue AChE-containing nerves representing 60—70 %, while tyrosine hydroxylase (TH)-immunoreactive nerves only 37 % of the total pattern of innervation. Similarly, rich AChE-population of ganglionic cell bodies were observed within the musculature of compact atrioventricular nodal regions in the porcine heart. On the other hand, only a minor population of ganglionic cell bodies displyed TH-immunoreactivity in this region (4).

More facts emphasize the relevance of discussion to the problem of our contribution. In the first place it is a fact that even if the left heart auricula in mammals has been defined as a special and richly innervated endocrine organ, the statement of Forssman

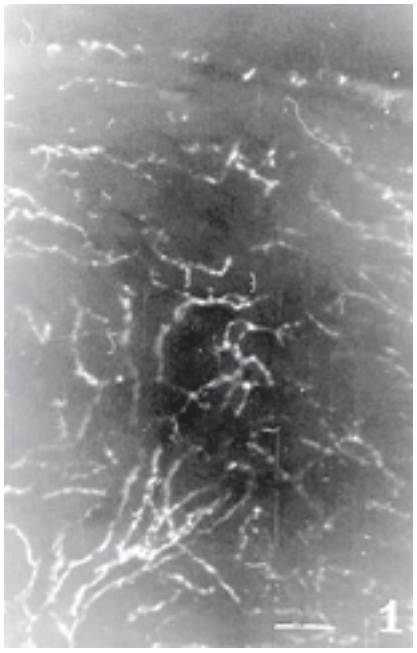

**Fig. 1. The relative numerous presentation of adrenergic nerve profiles in the pig's right auricle.
Bar represents 30 μm**

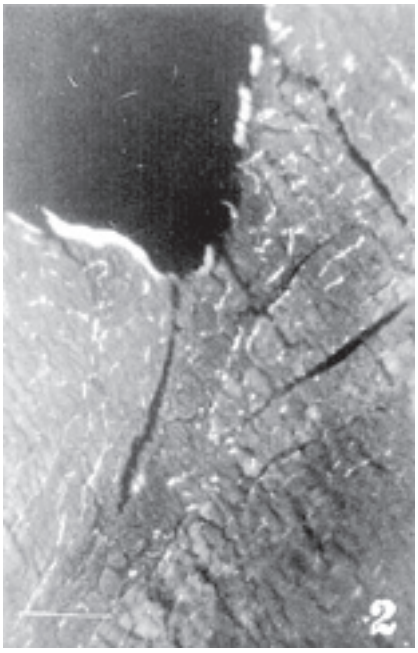

**Fig. 2. The focal accumulation of relatively numerously presented adrenergic nerve fibres lying on the inner surface of the right ventricle septum in the pig.
Bar represents 30 μm**

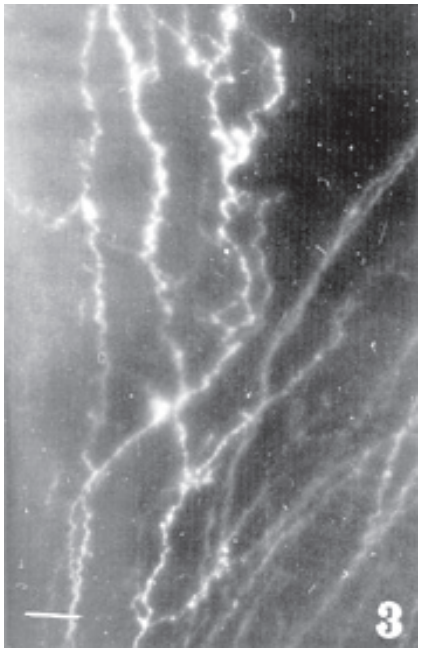

**Fig. 3. Specifically fluorescent thinner and mostly varicose nerve fibres in the tricuspid valve of the pig.
Bar represents 1 μm**

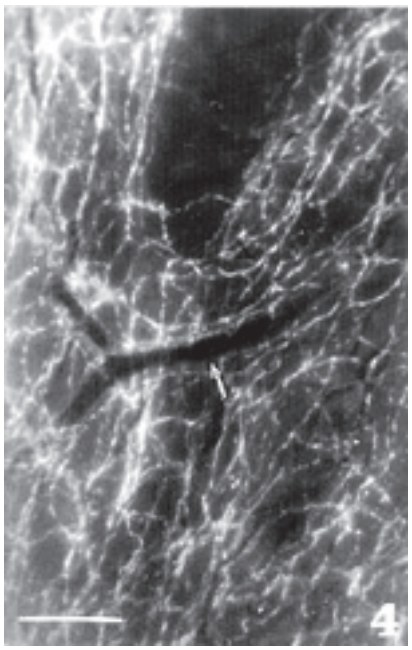

**Fig. 4. The dense aggregation of specifically fluorescent adrenergic nerve profiles of the rabbit's tricuspid valve. Thin-walled vascular branches (arrow) are bordered by typical varicose nerve fibres in parts.
Bar represents 30 μm**

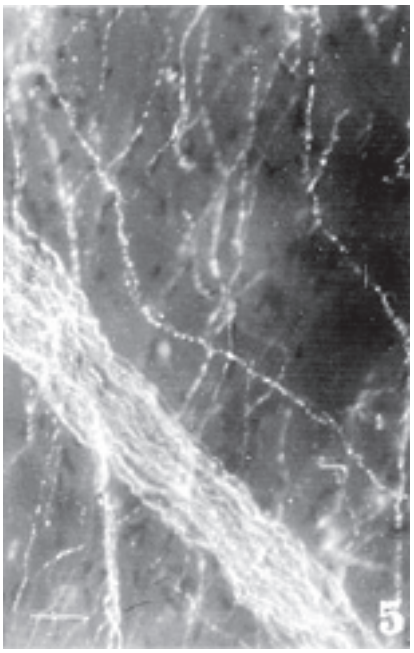

**Fig. 5. The thinner plexiform aggregation of solitary nerve fibres in the rabbit epicardium. Partly perivascularly running profiles as well as free nerve fibres forming thicker bundles dominate in the picture.
Bar represents 1 μm**

(11): *"The functional implication of these nerves concerning the secretory status of myoendocrine cells is not established"* has not lost its validity. In the right auricula along with the adrenergic populations and AChE-positive nerve fibres there are more subpopulations of immunoreactive peptidergic nerve components (VIP, NPY, CGRP), including fibres containing the P substance, which are considered the components of afferent systems (20, 14). So the right heart auricula appears, at least from the morphological view, as a complicated neuroendocrine organ, which in the cardiovascular system has a strategic position, which, in continual contact with circulating blood already at its entering the heart, can register more significant chemical and mechanical stimuli, and then react to them promptly. The reaction may be reflexive through sympathetic and vagus efferent and afferent components of the reflex arch as well as by secretion of very efficient chemical signal molecules that after release from myocytes diffuse into the blood arteries or directly into the blood through the endothelial

lining of trabeculae in order to get from the left heart into the systematic circulation and by coronary arteries, by a relatively short way, also to the heart itself (8).

However, it seems that for a deeper understanding of the significance of the richly represented adrenergic nerve components of auriculae rely on their traditional classification as motoric nerves with responses of target cells of the type "yes" – "no" (i.e. as regulators of vaso- or secretory and mechanical activity of cardiomyocytes). Peripheral adrenergic neurones not only produce, store noradrenalin, but also take it up from their surroundings, and this runs independently of the origin of this neurotransmitter (18).

Therefore, in connection with some more complex aggregations of adrenergic nerve fibres, which do not have in their close vicinity manifestly reacting target components (e.g. in the fibrous trabeculae of the spleen as well as in the fibrous cortico-medullar partition and the round fascicles of the *vasa recta* in the kidney medulla in dogs) there is an opinion (7) that they could play – according to the body's need – a double role: either as "emergency reservoirs" of the neurotransmitter or sources of endogenous noradrenalin in the blood or also as very efficient "uptakers" of this monoamine from the nerve vicinity and "inactivators" of excessive catecholamines by the effect of intraneural monoaminooxidase.

In the hearts of mammals such functions are suggested mainly for the adrenergic nerve components of the atrioventricular valves, however, an analogous role could be also played by all the nerve fibres lying close under the endothelial lining of the trabeculae in the auriculae in atriums and ventricles. Finally, it cannot be excluded that rich nerve plexuses in the epicardium could also fulfil the role of next endogenous source of noradrenalin (18).

Some think, that varicose nerve fibres (together with nerve trunks) observed throughout the endocardial and epicardial plexuses were in close physical contact with surrounding endothelial and mesothelial cells and some regulatory factors released by epicardial mesothelial cells can alter cardiac neurotransmission and myocardial contractibility (13). Each serous membrane has a notable resorption capacity and an ability to carry away the accepted components either by blood or lymphatic pathway.

In conclusion it may be stated that the richly represented adrenergic nerve components in the auriculae, atrioventricular valves and epicardium can play a significant role in the regulation of the noradrenalin concentration in the circulating blood and the activity of the heart itself.

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MORPHOLOGICAL ALTERATIONS OF SOME STRUCTURES IN THE DIABETIC HEART VALVES

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SUMMARY

Numerous clinical reports have confirmed that the atherosclerosis of the coronary arteries, macroangiopathy, and autonomic neuropathy of human diabetics are a frequent cause of the cardiovascular dysfunction. In this study we have demonstrated the presence of possible morphological alterations in vessels of the anterior cusp of the rat *mitral valve* in experimental *diabetes mellitus* (DM). A histochemical technique was used to investigate neuronal and vascular structures in this valve cusp, that show NADPH-diaphorase (NADPH-d) activity. Arterioles and fine capillaries were localized in the attachment part of the examined cusp. Perivascular nerve fibres were identified running in *tunica adventitia*. Dilated vessels were seen during DM in comparison with control samples. No NADPH-d positive nerve fibres were observed in *tunica adventitia*. It can be presumed that metabolic changes in vessel walls in diabetes reflect modified neurotransmission of NO by means of its excessive overproduction of endothelial synthase in endothelial cells.

Key words: *diabetes mellitus*; innervation; *mitral valve*; rat; NADPH-d; vessels

INTRODUCTION

It is well known that heart failure is accompanied by changes of reactivity of the coronary and systemic vessels (17). Diabetic microangiopathy and macroangiopathy are the principal causes of morbidity and mortality. Neural tissue and vessels show after affecting by some of chemical agents many function and structure alterations (28). *Diabetes mellitus* (DM) is one of the main causes of many neurovascular – biochemical, functional and ultrastructural abnormalities, which affect vascular and neural structures of living organisms, disturbing their metabolic and enzymatic balances (1). Diabetes is the activator of sclerotic alterations and ischaemia of coronary arteries. Under influence of permanently deteriorating of the metabolic conditions in the vessel wall and high glycaemia DM lead to their regression. Increased glucose concentration induces the production of oxygen free radicals in diabetes by increased activity of polyol metabolic pathway as well as by its own auto-oxidation (23). Oxidative stress contributes so to the development of neural and vascular complications in experimental diabetes (7).

Rats are the animal model commonly used and are rendered diabetic by treatment with streptozotocin (STZ), a nitrosoamine which is toxic to the pancreatic beta-cells (22).

The anterior cusp of *mitral valve* is in the area of atrioventricular ring nourished by terminal branches of coronary arteries (13, 14). By damage of endothelial cells (ECs) by running pathological process is the protection mechanism against oxygen free radicals impaired. These radicals if not are absorbed damage the vessel endothelium and neutralize the NO (7).

Nitric oxide (NO) derived from endothelial cells plays an important role as a coronary vasodilator. NO participates in regulation of coronary blood flow and tension of vessel wall. The regulator of NO production are the physical and chemical stimulations transmitted by the vessel wall. It is released not only from endothelial cells but also may serve as a nonadrenergic, noncholinergic neurotransmitter in cardiac innervation. In the vascular and cardiac tissue NO is constitutively produced in modest amount from L-arginine by endothelial (eNOS) or neuronal (nNOS) NO synthase, which has been shown to be identical with neuronal NADPH-diaphorase under condition of tissue fixation (10) what corresponds with our laboratory conditions – valve cusp were fixed by paraformaldehyde.

In the heart nNOS is produced in cells of peripheral autonomous nervous system, in heart nerve fibres, and eNOS is produced by ECs in two types: cytosol and membraneous types; it is produced by cardiomyocytes and also exists in the endocardium lining the cavities (15, 20, 30, 29).

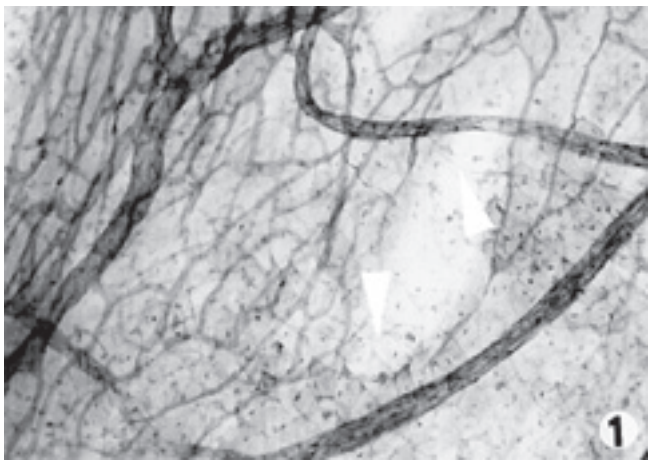

Fig. 1. The dense network of NADPH-d positive vessels and of fine nerve fibres in the attachment part of the valve cusp. In the back endothelial cells (ECs) are seen (arrows)

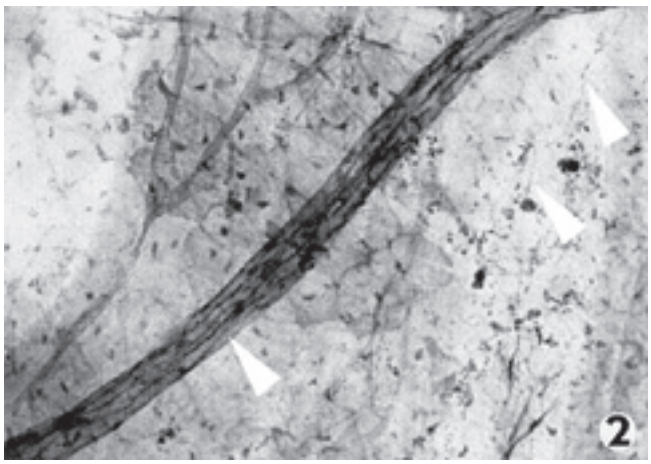

Fig. 2. NADPH-d positive nerve fibres in the attachment part running along the vessels and producing adventitial plexuses. Detail of the blood vessel with dark coloured nerve fibres in *tunica adventitia* and in the perivascular localization (arrows)

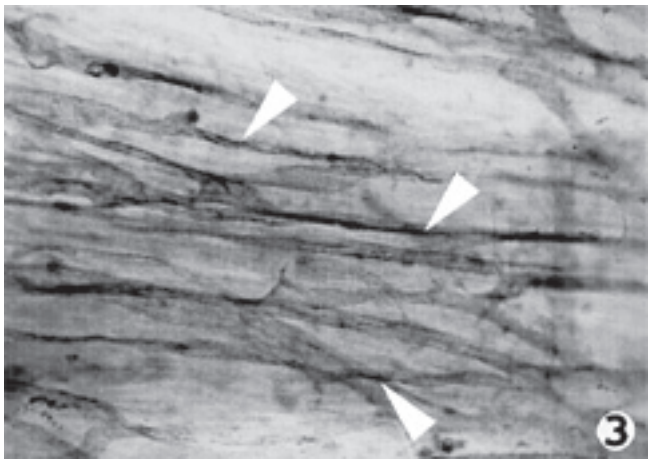

Fig. 3. NADPH-d positive nerve fibres in *tunica adventitia* of healthy blood vessels (arrows)

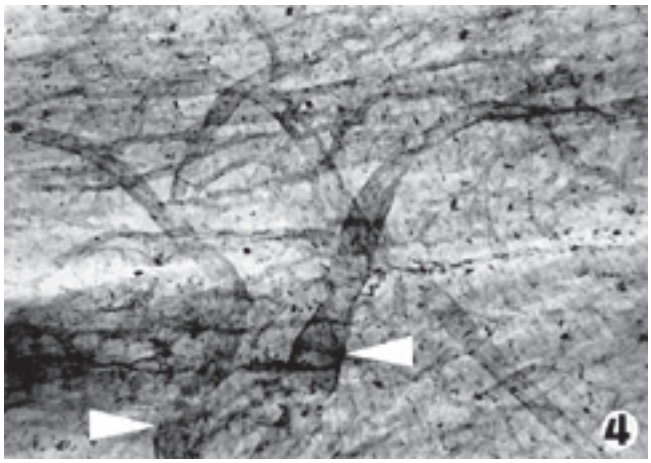

Fig. 4. NADPH-d positive vessels are dilated in the attachment part of the diabetic *mitral valve* (arrows)

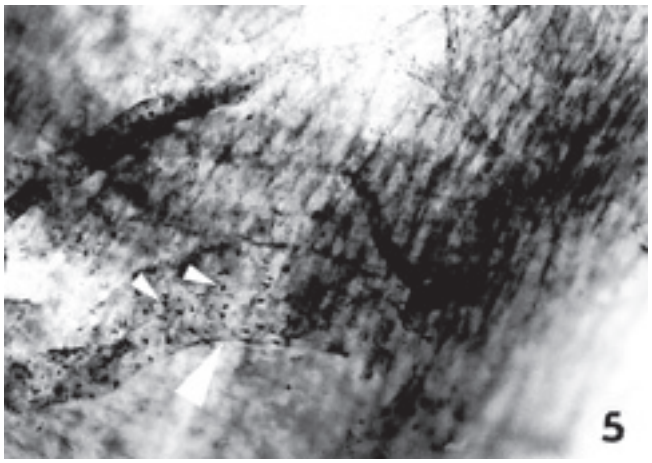

Fig. 5. One of many dilated blood vessels in the diabetic valve. No nerve fibres are seen in *tunica adventitia*. In the vessel wall ECs are seen only (arrows)

In response to some cytokines and bacterial products (cells stimulated by *E. coli*, macrophages, neutrophiles) an inducible form of NOS (iNOS) can be expressed in vascular and heart tissue (20, 3, 11). This synthase has been identified in different types of cardiac failure (such as dilated and inflammatory cardiomyopathy, ischaemic heart and valvular heart diseases) and it is occurred as a response to permanent activation by cytokines (12, 19).

The role and functions of NO, mainly vasodilatation one are very important for clinical practice in specifical areas of coronary blood supply, especially in valve. The aim of this study is therefore to characterize possible alterations of vessel and neural structures of the anterior cusp of the *mitral valve* during developed DM.

MATERIAL AND METHODS

Twenty male white *Wistar* rats, of the some age (3 months), 250—350 g, were used in this study. All animals were allowed food and water *ad libitum*, during the experiment the rats were kept on *Larsen's* diet. DM was induced in a group of 10

animals, i. p. administration of streptozotocin (STZ, Zanosar, UPJOHN; 65 mg.kg^{-1}) while rats were under light ether anaesthesia. Control rats, in the number of 10, were injected with citrate buffer only. The diabetic animals were observed from 8 to 12 weeks without the insulin care. During the period of observation (one of weekly) the level of blood glucose and weight were determined. All experiments on laboratory animals were performed in accordance with:

1. The Act on protection of animals No. 115/1995 Coll., as amended;

2. Decree of the Ministry of Agriculture of The Slovak Republic No. 231/1998 Coll., On breeding of domestic animals, wild animals, dangerous animals and on protection of experimental animals.

The rats of both groups were anaesthetized with pentobarbital (50 mg.kg^{-1}).

A midline abdominal incision, followed by two lateral costal margin incisions, exposed the diaphragm which was then incised. The thorac cavity was opened and the heart was perfused with a saline solution and 4 % paraformaldehyde with 0.1 % glutaraldehyde in 0.1 mol phosphate buffer (PB), pH 7.4. The heart was removed, the left ventricle was rapidly opened and the valves were cut from the atrioventricular ring. The sample material was placed in the same fixative for 2 hours. The fixed tissue was transfered overnight into 30 % saccharose in the same PB at 4 °C.

The investigation of NADPH-diaphorase activity vessels of the anterior cusp of the *mitral valve* was carried out using an histochemical technique according to Scherer-Sing-ler *et al*. (27) modified by Kluchová *et al*. (16). The valves were incubated for 1 h at 37 °C in a solution containing 1.5 mmol nitro blue tetrazolium, 1.0 ß-NADPH, 10 mmol L-malic acid (all Sigma Chemicals) and 0.5 % Triton X-100. After incubation, valves were rinsed in 0.1 mol PB (pH 7.4) and processed by a *whole mount stretch* technique by which they were mounted on glass slides, dried overnight and covered with Entellan (Merck, Germany). The preparations were examined under a light microscope NIKON and photographs were obtained by means of a photomicrographic apparatus *Labophot-2*, MICROFLEX, HFX-DX, NIKON (Japan).

RESULTS

Under physiological condition with various intensity, the ECs of the endocardium, dense network of vessels and fine nerve fibres in the attachment part of the researched valve cusp (Fig. 1) were coloured to blue shadows and dark-blue up to black reaction product as an evidence of the reduction of nitro blue tetrazolium (NBT) to the formazan insoluble in water.

Heavy NADPH-diaphorase activity was manifested by the ECs edges and probably, the perinuclear cytoplasm as well. The cytoplasm of ECs showed the colour intensity from medium dark up to very light. Expressively were coloured those nerve fibres which run perivascularily in *tunica adventitia* (Figs. 2, 3). The nerve fibres travel a long distance along these capillaries, can provide innervation their musculature and form a network around them.

In the period of 8—12 weeks after experimental induction of diabetes rats involved in the experiment, the glycaemia values of this group were about 21.5 mmol.l^{-1} in average. The glycaemia values of the control group were about 5.2 mmol.l^{-1}. Glucose level of experimental animals increased so almost by nine times and weights of diabetic rats were reduced proportionally to glycaemia increase by approximately 10 %.

The NADPH-d activity was retained by structures investigated by us in the period from 8 to 12 weeks after experimental induction of diabetes. Many vessels located in the cusp attachment part manifested the expansion signs e. i. possible dilatation during it (Fig. 4). Through their wall the contoured ECs were seen, only (Fig. 5). The nerve fibres were located in the perivascular location in limited range. Their presence was not seen in tunica adventitia of vessels (Figs. 4, 5).

By means of the histochemical method, the NADPH-d activity of endocardial ECs of the anterior cusp of the mitral valve, endothelium of vessels presented in it and nerve fibres (in limited range also in diabetic tissue) was confirmed by indirect determination of the activity eNOS and nNOS synthases.

DISCUSSION

The NADPH-d activity in terminal branches of coronary arteries in the area of attachment cusp, which was in this study determined is the evidence of nourishing assurance in this valve area. NO produced by isoform nNOS co-operates, when maintaining the homoeostatic balance of the inner space, with NO which is produced in endothelium by the isoform nNOS and participates in vessel dilatation by SMCs activation (19). The evidence of adventitially located NADPH-d positive nerve fibres indicate that the NO fulfils the vasodilatory function not only from the vessel inside by releasing from ECs (by means of eNOS, by diffusion into SMCs), but also under influence from adventition.

According to results in this experimental study we expect that with possible damage of *tunica intima,* when no damage of *tunica media* is occurred, under influence of metabolic diabetic alterations, the NO molecules may act to SMCs by means of releasing from plexuses of nerve fibres in *tunica adventitia*. These plexuses are formed mainly by sympathetic, postganglionic, adrenergic and also efferent cholinergic nerve fibres. Plexuses represent the axone bundle which are unmyelinized and typically varicose (32). A smaller group is represented also by nitrergic fibres (NADPH-d positive, NOS-immunoreactive) which are branched, anastomozed and formed the plexuses around the blood vessels (8, 25). These fibres are considered to be postganglionic parasympathetic fibres with vasomotoric function (14, 24). The above mentioned is confirmed by the statement that small branches of coronary arteries (arterioles and fine

capillaries) are innervated by means of *n. vagus* (32). NO produced by ECs together with NO produced by diaphorasis (nitrergic) nerve fibres represents the main endogenous vasodilatory system (4).

Production of nNOS in SMCs of the wall of *arteria carotis,* spontaneously hypertension rats, however showed the possibility of the non-endothelial activity of this synthase (6). Its presence was proved also in nervous terminals around coronary arteries of a rat and in myocardium (15). Klimaschewski *et al*. (15) and Ursell and Mayes (30); Andries *et al*. (2) also stated the strong activity of NADPH-d in ECs of coronary arteries and arterioles and generally in the vessel endothelium.

High glucose level increases the expression of eNOS in ECs of capillaries (4) and following the NO production. Non-desired event which cause the origin of many cardiovascular diseases is, under influence if this, the over-production of NO (26, 29). It is possible to expect that partial or total damage such as axonal degeneration of nervous sympathetic as well as parasympathetic fibres (24) is occurred as a consequence of the increased production of NO (by activating the ECs under influence of metabolic diabetic alterations) into the vessel lumen either by means of eNOS or iNOS and under influence of its cytotoxic activity what is manifested by their reduction or absence. NO neurotransmission is probably locked by its own excessive production participating in substantial vessel vasodilatation by influencing SMCs so that they were seen in this experiment. Shortage of L-arginine or actual shortage of co-factors necessary for normal activity of NOS may be responsible for abnormal synthesis of NO in diabetes (22).

Rösen *et al*. (24) described the morphometric alterations of fine capillaries in the myocardium of diabetic *Bio-Breeding* (BB*)* rats. Alterations are manifested by enlargement of capillaries diameter and volume density under influence of their re-construction. Pieper (21) characterized that increased blood flow in tissues in early stages of DM may be caused by hypoxic vasodilatation. Mompeo *et al*. (18) also described the hyperplastic coarsened *lamina basalis* of SMCs of diabetic coronary vessels with multiple layering, but Warley *et al.* (31) stated the strong surfacial reduction of capillary occurrence with STZ-influenced rats.

Occurrence of iNOS in vessels activated by lipopolysaccharides (LPS) or some cytokines suggests the excesive NO production with pathological consequences resulting in vasodilatation and hypotension. The cytotoxic function of NO is also manifested by excessive concentration of glutamate which is a very important activator of receptors and neurons producing NO. These release lethal amounts of NO causing the degeneration of target neurons (5). It may also be manifested by locking the above mentioned transmission by means of NO as characterized above. It may be the question of its own form of protection mechanism which may be alternated to pathological after reaching a certain critical state (5). NO produced by adventitial cells activated by iNOS, regarding to Muller *et al*. (19), does not functionate to SMCs immediately but gradual releaseing it may assure long-term protection influence in *tunica media*.

Results obtained in this work showed, and also from the above mentioned it is resulted that the NO overproduction may play a very destroiyng or protecting role in blood vessels what is to be researched furthermore because the mutual relationship of NO and vasculopathy is not still clear (9). Therefore we can conclude that metabolic changes in vessel walls in diabetes reflect modified neurotransmission of NO by means of its excessive overproduction of eNOS in endothelial cells.

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THE INFLUENCE OF *Rhaponticum carthamoides* Willd. ON THE DEVELOPMENT OF THE REPRODUCTIVE ORGANS AND WEIGHT GAIN OF PIGLETS

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ABSTRACT

A model experiment conducted on growing pigs. The feeding diet consisted of 10 % of *Rhaponticum* hay meal. After 30 days of feeding the body weight of the male and female piglets was higher than in the control group, which were fed with the classical feed. The investigation of the internal organs showed an increase in the weight and size of the uterus and ovaries, an enlargement in the weight and size of the testes and a sufficient increase in the area of the sperm ducts and in spermatogenesis. The feeding improved the health and condition of the experimental animals. All the above-mentioned effects persisted for 40 days when the animals were returned to their classical diet. In the work the result of the feeding experiment is presented. There were two groups of animals fed with different diets. The animals in the control group A (20 male and 20 female piglets) were fed with a complete feed mixture for growing pigs. Animals in the experimental group B (20 male and 20 female piglets) were fed with a diet composed of 90 % of the same diet as group A and 10 % of hay meal *Rhaponticum carthamoides* Willd.. After the 30th day of the feeding experiment, we started to observe statistically significant differences in weight gain. Animals in the experimental group were better in reproduction organ development and health than the control group.

Key words: growing pigs; pigs internal organs; *Rhaponticum carthamoides* Willd.; 20-hydroxyecdysone

INTRODUCTION

The simulative substances, which play important roles were in agricultural practice from the disease prevention and health points view, by increasing the dynamics of metabolic processes, the intensity of growth and immunity system to infections, and improving the animal constitution, form a separate chapter in animal nutrition. Among their natural sources are some medicinal plants, which can enrich the structure of vegetable production and contribute positively to the improvement of economy and efficiency in animal production. One of these plants is *Rhaponticum carthamoides* Willd. (RcW).

This plant is very interesting because it contains a whole range of organic compounds (flavonoids, saponins, antocyanides, phenolic substances and ecdysteroides). The substance 20-hydroxyecdysone has been considered as the active ingredient in this plant. It has a multiple effect. Syrov and Kurmukov (11) ascertained its neurotropic effect by EEG measurements after its administration to laboratory animals. Syrov *et al.* (13) and Malikov *et al.* (5) observed its effect on the levels of saccharides, fats and cholesterol after its parenteral application. Japanese scientists (21, 9) described the influence of 20-hydroxyecdysone on protein synthesis. Syrov *et al.* (12, 21) observed a more pronounced increase in the weight of growing mice rather than adult ones compared to the control, during their studies of anabolic manifestations. Similar effects were also observed after the administration of extracts obtained from RcW roots (7, 22). On the basis of these studies the extract obtained from RcW roots has found multiple use in human medicine.

There is little knowledge about the utilization of the shoot (aboveground phytomass) of this plant. Šelepcová (15) and Šelepcová *et al.* (17) did some tests on the hay meal obtained from this plant by feeding it to mice and observed the effect of graded doses on selected morphological, qualitative and health parameters of mice. The diet with 10 % content of the tested hay meal appeared most effective.

The purpose of the present investigation was to examine the effect of the 10 % content of RcW hay meal in the diet, on

weight gain, health, growth and development of the reproductive organs in growing pigs.

MATERIALS AND METHODS

The experiment was carried out on 40 animals of the domestic breed of Slovak-white meat-breed, average age of approx. 96 (± 5 days). Twenty male piglets and 20 female piglets were divided into four experimental groups, 10 animals in each. The genetic and weight uniformity of the biological material was assured. All animals were housed individually in boxes in the same environmental conditions.

Time schedule of the experiment

1st phase 14 days for adaptation of animals
2nd phase 30 days for feeding on two different diets
3rd phase 40 days of feeding on the classical diet

The basic ration was composed of the classical feed — Šelepcová *et al.* (18) for growing pigs. But 10 % of RcW hay meal was added with the classical feed to the experimental animals.

Experimental diet

Group A 10 males Group C 10 females
Classical diet
Group B 10 males Group D 10 females

Five animals from each group were slaughtered after the 30th day of the feeding phase.

Parameters examined after the slaughter

Female piglets

The weight and size of ovaries, uterus, liver, spleen, empty stomach, empty caecum, small intestine and kidneys.

Male piglets

The weight and size of testes, spleen, liver, empty stomach, caecum and small intestine.

In the 3rd phase animals were housed in four boxes in groups of the same sex and fed with the classical diet.

All animals were weighed weekly. During all three experimental phases, the daily consumption of feed was registered. Water was given *ad libitum.* All zoo-veterinary conditions were strictly handled during the whole experimental period.

RESULTS AND DISCUSSION

The first phase of the experiment was phase of adaptation (14 days).

The second phase of the experiment

The statements of numerous authors (4, 3, 2) and the breeding knowledge indicate that the rearing period of piglets is important for each individual animal. During this period the locomotive and reproductive apparatuses of the animals developed. It is therefore obvious that this is exactly the period during which the basis for future reproductive and productive performance is being laid.

For this reason our experiment was aimed at the investigation of the effect of diet with 10 % content of RcW on the overall health, growth and development of growing animals.

The weight of animals feeding on two different diets was determined weekly and the consumption of feed was recorded on a daily basis.

Weight gain and consumption of feed

Comparison of measured weight gains between groups revealed that animals of both sexes feeding on the diet with 10 % content of RcW hay meal (groups A, C) reached higher weight gains than those feeding on the classical diet. However the dynamics of the increase of weight in the experimental animals was different for the two sexes, which corresponds to the results obtained by Šelepcová (15, 14, 17, 20, 16) and Šelepcová *et al.* (18) in the experiment on mice. During the first week of the experiment a decrease in weight was observed in male piglets fed with the experimental diet. During the subsequent weeks the increased weight gains were recorded in the experimental piglets in comparison with the control (Figs. 1A and 1B).

The female piglets of the experimental group C (10 %) and the control group D (0 %) had almost identical growth curves during the first two weeks of the experiment. In the third week a growth depression was observed in the

Table 1. The average daily weight gain of piglets in grams during the second phase of experiment

	1st week	2nd week	3rd week	4th week	Average
Male piglets					
Group A	0.600	0.670	1.014	1.2	0.883
Group B	0.770	0.670	0.940	0.844	0.810
Female piglets					
Group C	*0.628*	*0.600*	*0.657*	*1.077*	*0.799*
Group D	*0.628*	*0.587*	*0.914*	*0.855*	*0.750*

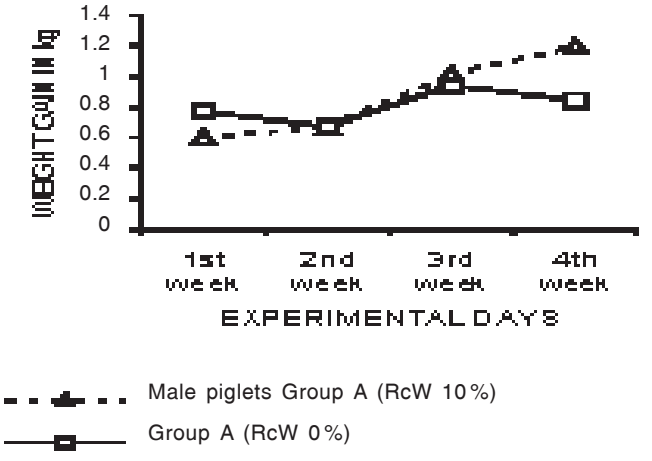


Fig. 1A. The average weight gain of the male piglets in g.day^{-1} during the whole experiment

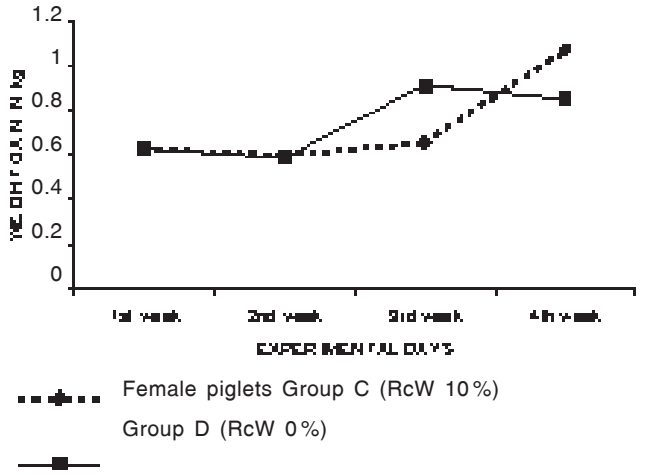


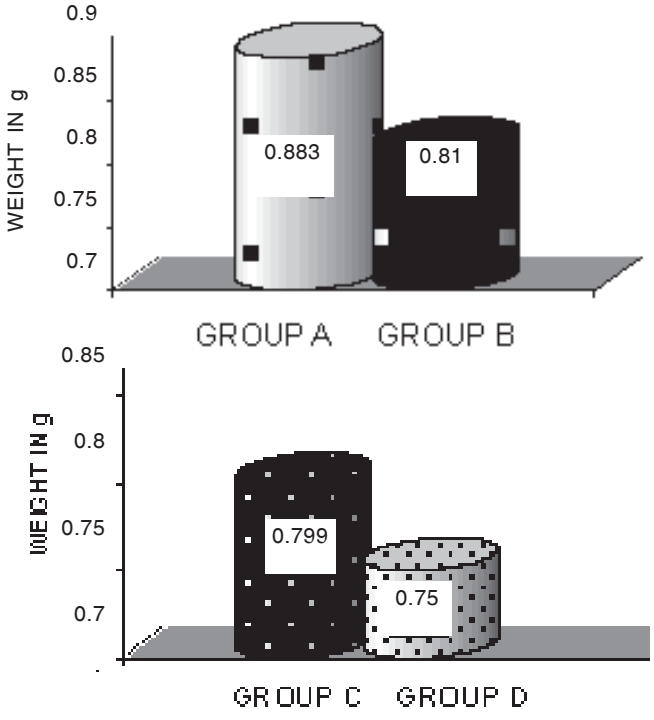
Fig. 1B. The average weight gain of the female piglets in g.day^{-1} during the whole experiment

experimental piglets followed in the fourth week with a rapid increase in the weight of piglets of group C compared to the control group D (Table 1, Figs. 1A, 1B).

The data obtained as well as results of the experiment on rats (20) suggest the suitability of prolonging the experimental period.

The values of the feed consumption measured per kg weight gain, also point to the benefit of the experimental diet (Figs. 2A and 2B).

Feokistova (1) described the favourable effect of extracts obtained from RcW roots on the metabolism of N-substances through the improved utilization of energy reserves of an animal organism which can also explain the results obtained in our experiment.



Figs. 2A and 2B. The weight gain dynamic during the 30 days of the feeding experiment of the male and the female piglets in grams

Table 2. The consumption of feed per one kg of weight gain

Male piglets		Female piglets	
A	B	C	D
2.629	2.842	2.797	2.897

The overall evaluation of the weight gains and consumption of feed measured per kg weight gain in both sexes, the internal organs showed the positive influence of the experimental diet. It is likely that the utilization of the diet with 10% content of RcW hay meal by animals was more economical which points to a better conversion of nutrients. The results are in accordance with those of Tadahiko *et al.* (21) on anabolic effects of ecdysterons.

Internal organs

The determination of the weight of liver, stomach, caecum, and large intestine failed to detect differences between groups, which is also in accordance with the observations of Šelepcová (15) and Šelepcová *et al*. (17).

Kidneys

Table 3 shows the results measured the weight of kidneys was higher in animals fed with the experimental diet than in those, which consumed the classical diet. Saratikov *et al.* (8) conducted experiments with extracts obtained from RcW and found out that in addition to other effects they also exhibited diuretic and vasodilatation effects. This could affect our experimental values. However the values determined for all kidneys were within the reference range.

Neither the *post mortem* macro- nor the microscopic examination detected any pathogenic effects of the diet on this or on other organs examined.

Table 3. The average weight of kidneys in grams

Male piglets		Female piglets	
A	B	C	D
160.7	140.2	119.8	137.6

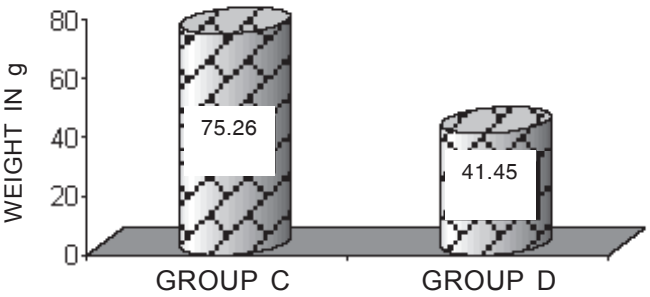


Fig. 3. The weight of the uterus of the piglets after the 2nd phase of the experiment

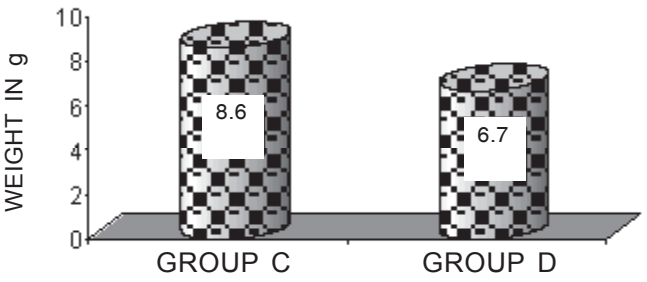


Fig. 4. The weight of the ovaries of the piglets after the 2nd phase of the experiment

Table 4. The average weight of the uterus and ovaries in grams

Uterus		Ovaries	
C	D	C	D
75.26	41.45	8.6	6.7

Reproductive organs

The investigation into the weight of reproductive organs revealed a highly significant increase in the weight of ovaries and uterus in female piglets of group C in comparison with the control animals (Figs. 3 and 4).

Similarly, male piglets of the group A exhibited higher weight and size of testes than the animals in group B, however the difference was not as significant as that observed in female piglets (320.3 g compared to 358.9 g),

This observation may in part explain the differences in dynamics and the overall effect of the experimental diet on the total weight gain between sexes. Male piglets showed a higher increase in body weight whereas the female piglets showed an increase in the weight and size of reproduction organs.

Evaluation of the histological structure of he testes and spermatogenesis has shown that an increase in the relative area of spermatogenic tubules and a lower numbers of pathologically changed and unripe sperms in group A in comparison to group B.

The 3rd phase of the experiment

In the 3rd phase of the experiment the animals (5 + 5 male piglets in groups A and B, and 5 + 5 female piglets in groups C and D) were fed the classical diet for an additional 40 days. Even during this phase the animals previously fed with the experimental diet had higher weight gains than the control animals (Table 5).

Table 5. The average total weight gains of piglets in kg in the 3rd phase of the experiment

Male piglets		Female piglets	
A	B	C	D
43.8	39.6	34.6	31.76

The results presented for organs show that the experimental diet had a long-term effect on animals, which indicates better utilization of feed.

Adaptation activity and increased performance was observed in mice and rabbits after the application of extracts obtained from RcW roots (8, 11, 6). Similarly, an increased activity and adaptability of animals of groups A and C in comparison with those of the control groups was also observed.

The evaluation of results obtained in the 2nd and 3rd phases of the experiment showed that the diet with 10 % content of RcW hay meal had better nutrition-dietetic properties than the classical diet. This was confirmed by its positive effect on the health constitutional state of the animals and their weight gains. The quantitative as well as qualitative growth of reproductive organs could lay the basis for the improvement of reproductive properties of animals. However, until we gain knowledge of the mechanism leading to the increased growth of reproductive organs we recommend using RcW hay meal only for fattening animals.

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CANINE N-RAS ONCOGENE-MUTATION IN A CASE OF MALIGNANT MELANOMA IN THE BRAIN (A Short Communication)

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ABSTRACT

Tumours localized in the brain of dogs were subjected to molecular genetic analyses. In codon 61 of the N-ras oncogene in a malignant melanoma metastase an A→T (CAA→CTA) point mutation giving rise to an amino acid substitution glutamine to leucine was detected.

Key words: brain; dog; melanoma; mutation; N-ras

INTRODUCTION

Melanocytes are derived from neural crest cells and a malignant melanoma is a tumour that shows melanocytic differentiation and which has a known and proven potential for metastasis. They often arise from the skin and mucous membranes, less frequently from other sites (5).

K-ras, N-ras and H-ras protooncogenes are members of the ras-gene family. The ras protooncogenes represent 21kD proteins and are therefore referred to as p21. Their oncogenic activity is very often associated with point mutations in codon 12 or 13 in exon 1 or codon 61 in exon 2.15—20% of all human malignancies encompass ras mutations (12). Human N-ras mutations have often been detected in myeloid disorders, melanomas, thyroid tumours, certain forms of sarcomas and seminomas (3, 2, 12, 13, 14).

In the domestic dog almost no N-ras mutations have been reported to date. The only exception is a point mutation on N-ras codon 13 in a malignant lymphoma (4). In other investigations no N-ras mutations have been found (9, 17).

In the present study we investigated 10 canine brain neoplasms for N-ras mutations.

MATERIAL AND METHODS

Ten canine patients diagnosed with brain tumours were studied for the presence of N-ras alterations at the Veterinary University in Vienna. The patients were 3 to 14 years old and the neoplasms were four meningeomas, three gliomas, two glioblastomas and one melanoma. Genomic DNA was extracted from the tumours in accordance with standard methods (11). For polymerase chain reaction (PCR), we designed primers for parts of exons 1 and 2 including the mutation hotspot codons 12, 13, and 61. The sense primer for exon 1 was 5'-TACAAACT-GGTGGTGGTTGGAGC-3', the antisense primer for exon 1 was 5'-CTATGGTGGGATCATATTCATCTAC-3'. The sense primer for exon 2 was 5'TCTTACCGAAAACAGGT-GGTTATAG-3', the antisense primer for exon 2 was 5'GTCCT-CATGTATT-GGTCTCTCATGGCAC-3'. PCR and DNA-sequencing analysis conditions were as described earlier (10).

RESULTS

Only one of the ten investigated patients showed an N-ras mutation. It was found in a five-year-old castrated female wire-haired Dachshound five months after a melanoma resection in the skin of the mammary region. One day before the euthanasia the dog suffered a convulsion; on the day of the euthanasia, she showed penetrate-moving, set going and sideposition. The tumour was a poorly pigmented malignant melanoma of the spindle cell type (Fig. 1) with extensive necroses and haemorrhage in the cerebrum. A CAA→CTA transversion (A→T) giving rise to an amino acid substitution Q→L (Q61L, glutamine→leucine) at codon 61 was detected. This point mutation was not found in control peripheral blood lymphocytes, thus suggesting its somatic nature.

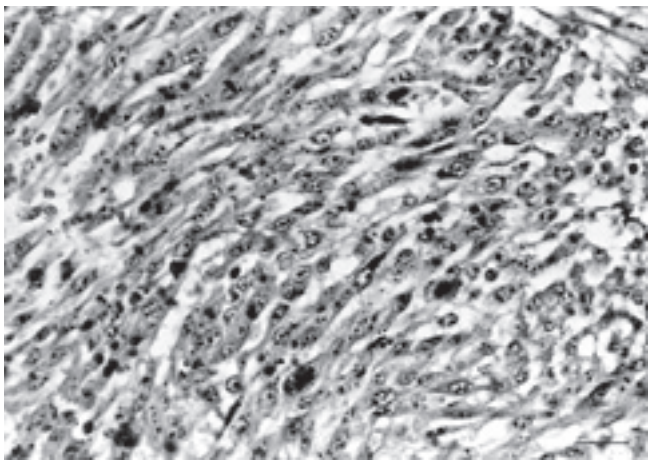

Fig. 1. Malignant melanoma, spindle cell type, poorly pigmented with extensive necroses and haemorrhage in the cerebrum; swarm of irregular spindle shaped cells with poor melanin content, prominent nucleoli and high mitotic activity. Bar represents 60 µm

DISCUSSION

Several reports concerning ras mutations in human brain tumours are available in literature. Most of the screened neoplasms were gliomas, glioblastomas and neuroblastomas. In most cases very low ras-incidences or their absence were reported (1, 3, 7, 8, 6, 15). To date no ras data is available on melanomas in human and canine brains.

Unfortunately the primary melanoma on the skin of the mammary region was not available for us for a molecular genetic analysis. The issue of a presence of the N-ras-mutation on the primary tumour would have been of considerable interest with respect to the molecular development of the putative metastasis.

Malignant melanomas often recur and show metastatic spread. The majority of tumours shows lymph node participation and spread to the lungs. Distant sites for metastases include the brain, heart, liver, kidney and spleen (5).

Our N-ras point-mutation represents the first mutation detected in a brain tumour in the domestic dog. Given the extreme gene-sequence similarities in the ras-family between domestic animals and man (16) this result may be interesting for both veterinary and human medicine.

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CHRONICLE

Academician ALEXANDER KOTLÁN (1887—1967)

Academician A. Kotlán — the founder of Hungarian veterinary parasitology, organizer of parasitological research, world-wide known researcher, university professor, Dr.h.c., Member of Hungarian Academy of Sciences. Together with his teachers F. Hutyra and J. Marek, he contributed to the international fame of the Veterinary University in Budapest.

A. Kotlán was born on the 14th July, 1887, in Smolenice, district Trnava (The Slovak Republic). His father was a practical veterinarian at the estate of the count Pálfy. His mother, nee Špačková, came from Moravia — her grandmother lived in Rájec by Blansko. He attended a secondary school in Trnava where he also passed his final exam in 1904. His decision to study veterinary medicine was probably made as a result of his family environment. In 1906 he enrolled in The Veterinary College in Budapest. He completed his college studies in 1911 and obtained a veterinary diploma. He was one of a group of excellent students and owing to that, after obtaining absolutorium, he won a scholarship at the Institute of Pathologic Anatomy where he worked under the leadership of Prof. Rátz. In 1912 Dr. Kotlán was promoted to an assistant. Rumour had it that he had to keep his interest in parasitology in secret.

In 1912 he entered military service. The World War One interrupted the successfully developing career of the young scientist. During the war he was a military veterinarian and even then he paid attention to his special parasitological interests. At the beginning of 1918 he returned to Budapest. In the same year he was summoned to Veterinary College in Vienna by Prof. Zwick. There he finished his doctor's thesis and returned back in October. In the same year he defended the doctor's thesis on the theme *"Sclerostomids occurring in Hungarian horses with special respect to the genus Cyclostomum"* and was promoted to Doctor of Science. In October 1918 he joined again the staff of the Institute of Pathological Anatomy and devoted his time to parasitology. Soon he published his first papers dealing with parasitology in specialised and scientific journals. His earnestness and determination was witnessed by the fact that despite his teaching and scientific activities he enrolled as a student in the Faculty of Natural Sciences where he took courses in general zoology, botany, and mineralogy.

Since 1921, as an adjunct, he read lectures in zoology and parasitology and established a parasitological laboratory which developed later to an institute. He was the head of this educational and scientific establishment for many years. Dr. Kotlán was an excellent organizer of Hungarian veterinary parasitology. There was probably no section in the field of parasitology that did not interest him.

In 1924 A. Kotlán went to the U.S.A. (Michigan State College) to veterinary department to continue with his study of parasitoses. His stay at this department broadened his horizons and allowed him to draw experience from American specialists. During the stay he obtained new impulses for scientific research at his own department in Budapest. As a head of the Hungarian laboratory International Corn Born Investigations he proved his diverse capabilities and met with considerable success that was apprised also abroad. He presented his results at international conferences abroad (Paris in 1928, Warsaw in 1929).

Dr. Kotlán paid attention to several branches of parasitology but his research focused particularly on helminthology and was based on new modern approaches. He got involved in the research concerning taxonomy of helminths and paved new ways in this respect by using histology. He passed always from the pure theory to the needs of practice and contributed to considerable progress in diagnostics and therapy of diseases of farm animals caused by parasitic helminths. His scientific studies served as a basis for development of prophylactic measures that helped to control serious parasitoses in domestic animals in Hungary. The experience gained in this process was used also in other countries. Not less important was his contribution to the development of veterinary medicine in general, enjoying universal respect of specialists from different branches. From the early twenties to late sixties of the past century he was considered a leading scientific personality not only by wide veterinary circles but also by natural scientists and biologists.

Remarkable results of his long-time scientific research allowed him in 1931 to put across the idea of including parasitology in the syllabus as a separate subject, independent of general zoology. In 1929 he was appointed an adjunct professor and in 1935 a full professor. From that time on his establishment was included among exceptional institutions. In the period of 1947—1951 he held the function of dean and vice-dean. Only during this period, when he acted as an academic functionary, he successfully put through parasitology as an equivalent and important subject of veterinary medicine.

In 1946 he was elected a member correspondent of the Hungarian Academy of Sciences and in 1952 he became its full

member. His activities were apprised by 2 high state distinctions. He was a founder and first chairman of the Hungarian Parasitological Society and a member of several foreign and international societies. He was also a founder and the head of the Parasitological department of the Zoological Research Institute of the Hungarian Academy of Sciences in Budapest.

His international reputation, scientific studies, and organisational talents were such that important scientific societies abroad elected him an honorary member: Helminthological Society in Washington (1927), Society of Polish Parasitologists (1927), Moscow Federal Helminthological Society (1958). In 1963 he was awarded a plaque of Theodor Kitt of the Munich Veterinary Society and was asked to collaborate in editorial boards of important scientific journals (*Folia parasitologica, Helminthologia*).

With his diligence and enthusiasm he served as an example for young co-workers. He tutored many capable students at the College and in branch research who carried out and published sound, even top parasitological studies used also abroad. Results of their research are well known and have been cited also in Slovakia. L. Nemeséri and F. Holló published in 1950 a manual: *"Állatorvosi parazitológiai diagnosztika"* (Budapest). Its second edition was published in 1961; this edition was then translated to German and published in German Democratic Republic in 1964, and to Spanish and published in 1965 in Zaragoza (Spain). Ľ. Pellérdy published 2 books named *"Eimeriides" and "Coccidia and Coccidiosis"* (1965).

Academician Kotlán published more than 100 studies and papers in Hungarian and foreign scientific and specialised journals. He enriched Hungarian and world-wide veterinary science by excellent books — monographies and textbooks that have been used also abroad. He published a textbook *"Parasitology"* (1944), with second edition in 1953 and third edition in 1961. However, his basic book is *"Helminthology",* that was published in 1960 in Budapest. Due to its profoundness and new approaches to taxonomy, morphology, and determination of parasitic helminths, this book will serve for many years to come as a valuable aid in diagnostic and research establishments. As a well-informed specialist he participated in preparation of nomenclature of parasites. He also published a book about the history of veterinary education in Hungary (*A magyar állatorvosképsész tőrténete*, 1987—1937, Pátria, Budapest, 1941).

Praiseworthy were his editorial activities in specialised and scientific Hungarian journals, such as: *Állatorvosi lapok* (later *Magyar állatorvosok lapja*) and *Acta veterinaria Hungarica*.

He could speak very well Greek and Latin and used fully his language skills within specialised terminology in the field of parasitology and veterinary medicine in general.

As a native of Slovakia he had very hearty relationship to his home country which he criss-crossed many times when he was young. Even later he liked to visit Slovakia on various occasions, e.g. scientific conferences or other events. We appreciate very much his friendly relationship with our University and also with other Slovak scientific institutions and their personnel. His first visit to our College took place on the 3rd December, 1954, when he read a lecture in an assembly hall for teachers, students, and many practical veterinarians. He visited our College the second time as a member of Hungarian delegation on the occasion of the IIIrd Scientific conference in July 1956. It should be mentioned that the presence of guests from abroad, including those from the College in Budapest (it was the first international conference), built up considerably the reputation of our College. In conclusion of the conference academician Kotlán stated: *"I am very thankful to the dignitaries of this Faculty who made it possible that this Conference could take place with the participation of the Hungarian delegation. I can declare that it was fully successful. I wish to your Faculty, which is one of the youngest in Europe, more and more success in this direction and close my speech with the acclamation: Vivat, crescat et floreat Facultas Veterinaria Cassoviensis"*.

He visited our College for the third time as the head of the delegation from the Veterinary College in Budapest that participated in the jubilee scientific conference organised on the occasion of the 10th College anniversary in September 3—9, 1959. His last visit took place during the VIIIth Scientific conference organised in June 20—21, 1963. Throughout his life he used his mother language and his discussion with Slovak professionals took place in Slovak which he handled very well. Also the letter-of-thanks sent to the dignitaries of our Faculty for their congratulations to his 80th birthday anniversary was written in Slovak.

The personal qualities of Academician Kotlán were exceptional. He was interested not only in his favourite scientific branch but also in art, history, culture, and nature, and was a great example for other, particularly the young co-workers. We admired the exceptional vitality of Academician Kotlán which did not leave him up to the advanced age. His working ardour, persistence, and consistency will serve as a stimulus of further development of educational art and scientific research, and will help to practice not only in the field of parasitology but also in other scientific branches.

In 1962, on the occasion of the 175th anniversary of founding the veterinary education in Hungary, the College in Budapest conferred a honorary doctorate to Academician Kotlán.

We bow to the life-long work of our compatriot who lifted so high and progressively the torch of veterinary science far beyond the borders of his home country.

He died on December 22nd, 1967, at the age of 80 years in Budapest, and was also burried there.

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MVDr. SAMUEL ADAMAŤ (1904—1976)

Dr. Samuel Adamaťi — a long-term member of staff of the Czechoslovak and Slovak Veterinary Administration in Prague and Bratislava. In 1948—1951 he was the head of veterinary services in Slovakia. In 1949 he was one of the initiators of establishing the Veterinary College in Slovakia.

He was born on the 25th August, 1904, in the village Bošáca, on the slopes of White Carpathian Mountains, district Nové Mesto nad Váhom (The Slovak Republic). He obtained his veterinary diploma in Brno on the 10th December, 1929, where he also graduated on the 8th February, 1930, on the basis of successful defending of dissertation thesis *"The study of stimulation of cell divisions with salts of heavy metals and organic acids"*. Shortly after his graduation he joined the cavalry regiment in Košice where he did his military service in the period of 1930—1931.

In 1931 he returned as a practical veterinarian to Stará Turá. From 1933 till 1936 he was employed as a veterinarian draftsman at the Veterinary Section of the Regional Office in Bratislava from where he was transferred in 1936 as a Slovak to the Veterinary Section of the Ministry of Agriculture in Prague where he held the post of ministerial commissary. In March, 1939, he returned to Bratislava and joined the newly established Veterinary Section of the Ministry of Economy. In 1944 he was promoted from the position of chief veterinary commissary to a sectional counsellor.

In 1948 Dr. Adamaťi was became a head of the Veterinary Section of the former Commissionary for Agriculture and Land Reform. It was a very difficult period for veterinary services. The infectious situation was highly unfavourable. Farm animals herds were exposed to risk of dangerous infections. In 1949 agricultural co-operative farms were formed and animals were concentrated to large herds. This increased even more the existing risk of infections. Because of the very complicated situation in veterinary practice the Veterinary Section organised a work conference for all Slovak veterinarians in Bratislava on the 14th February, 1949. Other participants of this conference were Dr. M. Falťan, the Commissionary for Agriculture and Land Reform, Dr. Bubenička from the Ministry of Agriculture in Prague, and representatives of students of the Veterinary College in Brno.

Dr. Adamaťi of this conference was the principal speaker. He pointed to the unacceptable animal health conditions in Slovakia and suggested measures for the improvement. He presented a request to establish a central veterinary diagnostic institute in Bratislava with branch institutes in district towns, to set up a veterinary research institute and network of veterinary hospitals and sanitation institutes and to start with the production of vaccines. In his speech he raised again the issue, that had been discussed repeatedly after the World War Two, concerning the need for establishing another veterinary college, this time in Bratislava.

In addition to other arguments he said the following: *"The chapter referring to the establishment of veterinary institutes would not be complete without stressing the need for establishing Veterinary College in Bratislava which is justified by the fact that the Veterinary College in Brno, one school of its kind in the Republic, has more than 300 Slovak students. The College ensures veterinary education for twice as many Czech students which allows us to presume that another veterinary college will eventually be established. With such a high number of students even the maximum effort of teachers and students themselves cannot ensure presentation and mastering of the required knowledge by all these students in one single establishment. There have been calls for establishing second veterinary college in Prague and some steps have already been taken toward this goal. With regard to the fact that the infectious situation in Slovakia is worse than in the Czech part of the Republic, establishing of veterinary college in Slovakia would mean better supply of material for practical training of students and allow teachers to ensure better education of students with special orientation on Slovak conditions.*

The concentration of teachers on the problems encountered in Slovakia would be reflected also in the field of scientific research and could contribute to better handling of infectious situation in Slovakia. After careful consideration of all questions related to the establishment of veterinary institutes and a veterinary college in Slovakia, I take the liberty of claiming that their realisation will inevitably lead to obtaining sufficient number of qualified professionals needed for diagnostics and research of animal infections and that this is the only way how to extricate Slovakia from the highly unfavourable infectious situation. It is not possible to ensure sanitation of the infected and scientifically unmapped areas without collaboration of a sufficient number of scientists and executive veterinary police organs and because of that it is justified to call for establishment of veterinary, research, and diagnostic institutes, and veterinary college in Slovakia."

The requirements presented by Dr. Adamaťi were summarised in 11 paragraphs. In the fourth paragraph of the conference resolution Slovak veterinarians requested *"to establish a veterinary college in Slovakia focused on economical, educational and infectious conditions with special stress on the health of animals in Slovakia"*.

On the request of the Commissionary for education Dr. Adamaťprepared a report about *"The number of veterinarians in Slovakia on 1st March, 1949"*. According to this report the State Veterinary Administration had 146 state veterinarians and out of that 10 was on the staff of PPPR, 10 was employed by the State Diagnostic Institute, 18 by Regional National Committees (KNV), and 108 by District National Committees (ONV). Eight veterinarians were involved in the state breeder's service and 77 in town, communal, and private veterinary service. The State Veterinary Administration needed at least 600 veterinarians so it lacked 389 of them. On the basis of this report the Commissionary for education L. Novomeský established a "Preparatory committee for founding the Veterinary college" which included also Dr. Adamaťt. All this points to the important role that Dr. Adamaťplayed in the establishing of the veterinary college in Slovakia which had been frequently unjustifiably underestimated.

In 1951 Dr. Adamaťwas recalled from his post, obviously for political reasons, and was replaced by Juraj Baláž, a political nominee, originally a blacksmith. From 1951 till 1971 Dr. Adamaťwas employed by the hygienic veterinary service at the Municipal Slaughterhouse in Bratislava. He retired in 1971.

During his active employment, after he returned from Prague to Bratislava in 1939, he was also involved in the activities of Slovak bee-keepers. As a veterinarian, he held the post of a health officer. He paid attention to the health problems of bees and his contribution in this respect was considerable on a nation-wide level. He acted for two terms as a chairman of the Regional Branch of Slovak Association of Bee-keepers. He himself kept 25 bee families.

Dr. Adamaťcontributed to various professional and popular-specialised journals. He published the following papers: Adamať, S.: *Activities and goals of veterinary service in Slovakia* (In Slovak), Čas. čsl. veterinářů, 4, 1949, 154—157; Adamať, S. and Nižnánsky, F.: *Report about the visit to serological institutes in Budapest* (In Slovak), Čas. čsl. veterinářů, 5, 1950, 52—55. He published also a booklet *"Suppression of Serious Diseases of Bees"* (In Slovak), and Adamať, S.: *How to improve the control of mite infestation in Slovakia* (In Slovak), Včelářství, 90, 1956, No. 2, 26—27; Adamať, S.: *Importance of sampling and laboratory examination of winter dead bees* (In Slovak), Včelár, 2, No. 2, 1958; Adamať, S.: *Suppression of bee infections in Slovakia* (In Slovak), Včelár, 5, 1961, No. 9; Adamať, S.: *Health inspection of migratory sites* (In Slovak), Včelár, 37, 1974, No. 6.

He was awarded a gold medal for his devotion toward improvement of Slovak bee culture.

Dr. Samuel Adamaťdied on the 23rd December, 1976, at the age of 72 years.

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E R R A T U M

In FOLIA VETERINARIA, 46, 1: 39—41, 2002 the article by Lakkawar, A. W., Kumar, R., Nair, M. G., and Varshney, K. C. appears incorrectly titled "Anencephaly and associated malformations in a (sic) ovine foetus (A case report)". It should have read "Anencephaly and associated malformations in a **bovine** foetus (A case report)". The editorial board regrets and inconvenience concerned.

Doc. MVDr. Emil Pilipčinec, PhD.
Executive Editor