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NEUTROPHIL IMMUNE FUNCTIONS OF BLAD HETEROZYGOUS ANIMALS*

IMUNITNÉ FUNKCIE NEUTROFILOV BLAD HETEROZYGOTNÝCH ZVIERAT

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ABSTRACT: Bovine leukocyte adhesion deficiency (BLAD) is a primary immune deficiency of cattle caused by absence of CD18 adherence molecule on neutrophils due to a single mutation. The consequence of this mutation for neutrophil functions of homozygous animals is well known. Our experiments were concentrated on some neutrophil immunological properties of heterozygous cows. The CD18 antigen expression was found 17% lower in average and the respiratory burst of neutrophils measured by quantitative iodenitrotetrazolium dye reduction (INT) test was significantly lower than those of healthy animals. The adherence of neutrophils to plastic surface was lower only in some animals.

cattle; leukocyte deficiency; respiratory burst; adherence

ABSTRAKT: Deficiencia adherencie bovinných leukocytov (BLAD) je primárnou imunodeficienciou hovädzieho dobytka, ktorá je spôsobená nepritomnosťou adherenčnej molekuly CD18 na neutrofiloch v dôsledku jednoduchej mutácie. Sprievodné príznaky tejto mutácie pre funkciu neutrofilov homozygotných zvierat boli popísané niekoľkými autormi. Naše experimenty sme preto zamerali na sledovanie niektorých imunologických vlastností neutrofilov heterozygotných krav. Zistili sme, že expresia molekuly CD18 bola v priemere o 17 % nižšia a respiračné vsplanutie neutrofilov merané kvantitatívnym stanovením jódnitrotetrazólium reduktázovej aktivity (INT testom) bolo významne nižšie ako u zdravých zvierat. Adherencia neutrofilov na plastikový povrch bola nižšia len u niektorých zvierat.

hovädzí dobytok; deficiencia leukocytov; respiračné vsplanutie; adherencia

INTRODUCTION

The interaction between β2 integrin (CD11/CD18) molecule on leukocytes and intercellular adhesion molecule (ICAM) on the endothelial cells is essential for the adherence and transendothelial migration of the leukocytes to a site of inflammation. Homozygous cattle with leukocyte adhesion deficiency (BLAD) due to the mutation of the gene encoding CD18 glycoprotein are unable to produce the complex between CD18 and the CD11 subunits. Neutrophils from affected calves had a function defect characterised by decreased adherence, chemotactic movements, phagocytosis, luminol-dependent chemiluminiscence response, and O_2 producing activities (Kehrli et al., 1990; Nagahata et al., 1994).

In heterozygous animals which are clinically normal (free of recurrent infections) a different degree of the decrease of CD18 molecule expression was recorded (Kehrli et al., 1990; Cox et al., 1997). Furthermore, the BLAD carriers are altered also in some morphological and physiological (production) features. The heterozygous bulls had significantly smaller average chromosomal areas and larger head areas of spermatozoa than normal bulls (Steinholt et al., 1994). In some populations, the carrier animals had lower values of some performance traits than those of normal animals (Powell et al., 1996; Tarlik, 1998).

The objective of this study was to evaluate if the carrier status (decreased expression of CD18) has some effect on such immunological functions as the adherence and phagocytic activity of the neutrophils of BLAD heterozygous animals.

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MATERIAL AND METHODS

Fifteen Holstein-Friesian BLAD heterozygous lactating cows selected from farms in the western part of Slovakia, aged 3–5 years in different lactation stages were included in this study. Nine normal age-matched cows free of BLAD served as controls. The heterozygous status of cows was confirmed by PCR-RFLP according to the procedure as described by Tammen et al. (1996).

Blood was obtained by jugular venipuncture and collected into heparin containing flasks (10 I.U./ml). Blood smears were prepared for differential WBC analysis, stained by May-Grünwald/Giemsa and 200 cells were differentiated into neutrophils, eosinophils, monocytes and lymphocytes. Total WBC counts were determined with a hemocytometer.

PMNC preparation

The collected blood samples were treated by red blood cells lysing solutions (recommended by Becton Dickinson for flow cytometry) to remove the erythrocytes. Then the cells were resuspended in Hanks balanced salt solution (HBSS) and layered onto Verografin (sp.gr. 1.078) and centrifuged at 1 500 g for 15 minutes at 20 °C. Thereafter, the cells (mainly lymphocytes) in the plasma Verografin interphase were isolated or removed and the pellet (PMNC) was resuspended and two times washed in cold HBSS.

Adherence

The PMNC ($1x10^6$/ml) were suspended in HBSS containing 10% autologous plasma. Two ml of cell suspension were allowed to settle in 60mm cell culture dish at 37 °C humidified with 5% CO_2. Each sample was used in 5 dishes. After one-hour incubation the supernatant was collected into tubes. The supernatant was replaced by HBSS and the dishes were washed twice under gentle shaking for 3 minutes to collect the non-adherent cells. At the end of the procedure, the non-adherent cells were counted and the percentage of the adherence was evaluated.

Iodenitrotetrazolium reductase activity (INT test)

Reductase activity of neutrophils was measured according to the previously published techniques by Procházková and John (1986). Briefly, 200 µl of PMNC suspension ($5x10^6$) was incubated with 100 µl 1% zymosan opsonized with a mixture of normal bovine serum and with 200 µl 0.1% solution of iodenitrotetrazolium for 45 min at 37 °C. The reaction was stopped by adding of HCl and dye was extracted with acetone. Absorbance was measured at 485 nm.

Luminol-dependent chemiluminiscence

1 ml of PMNC suspension ($1x10^6$) was incubated in luminometer with luminol (final concentration 10^{-4} M) at 37 °C. After 10 minutes, 40 µl of opsonised zymosan solution (10 mg/ml), prepared from normal bovine serum, was added. Chemiluminiscence was recorded by luminometer (Lumino M90a, Czech Republic) at intervals of 5 minutes during 30 minutes (Nagahata et al., 1994) and expressed as relative light units (RLU).

Flow cytometry

PBMC in HBSS + 0.2% NaN_3 were incubated with monoclonal antibody IVA35, diluted in RPMI medium conditioned with 5% foetal calf serum and 0.2 % NaN_3 for 30 minutes. All stainings were performed on ice. Cells were further incubated with phycoerythrin conjugated antimouse immunoglobulin (Dako) for 30 minutes. Following the washing, cells were fixed in 2% buffered formaldehyde and kept refrigerated in dark until examined. The FACSort flow cytometer (Becton Dickinson) was used to analyse the data. The mean fluorescence intensity (MFI) of positive cells was evaluated.

Monoclonal antibody

Monoclonal antibody IVA35 was prepared after the fusion of mouse myeloma cells SP2/0 with the spleen cells obtained from mice immunised with bovine PBMC. The CD11a/CD18 specificity of the antibody was confirmed in the Third Workshop of Ruminant CD antigens (Naessens and Hopkins, 1996).

Statistical analysis

Results are expressed as the mean ± standard error. The obtained values in all experimental sets are normally distributed. Student's *t*-test was used to test data for interset differences. Values of $p < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

PCR-RFLP analysis of BLAD carrier animals previously selected on the basis of breeding records proved the presence of BLAD allele in all animals. The restriction analysis of PCR amplified DNA position 383 of the CD18 gene allowed discrimination between normal and heterozygous animals. The digestion with TaqI restriction endonuclease gave two DNA fragments (95 bp, 43 bp) in BLAD free animals and three fragments (138 bp, 95 bp, 43 bp) in BLAD carrier animals.

Some haematological values of BLAD homozygous animals are markedly altered. Total number of leukocytes (neutrophils) might be 5 to 20 times higher than in BLAD free animals (Nagahata et al., 1994). In Tab. I the leukocyte counts and the percentage of leukocyte types are compared. No significant differences were observed in total leukocyte or neutrophil counts between the two categories of cows. These results are in good agreement with the findings of Cox et al. (1997), therefore it seems that BLAD carriers are not leukocytic as the affected homozygotes.

I. Hematological values in control and BLAD heterozygous cattle

	Controls $n = 9$	BLAD heterozygotes $n = 15$
Total leukocytes (x $10^3/\mu l$)	6.6 ± 1.3	7.2 ± 1.3
Lymphocytes (%)	56.9 ± 5.9	53.0 ± 11.9
Neutrophils (%)	27.3 ± 4.4	32.3 ± 10.9
Eosinophils (%)	7.6 ± 4.8	6.2 ± 8.3
Monocytes (%)	6.2 ± 3.5	5.8 ± 2.5

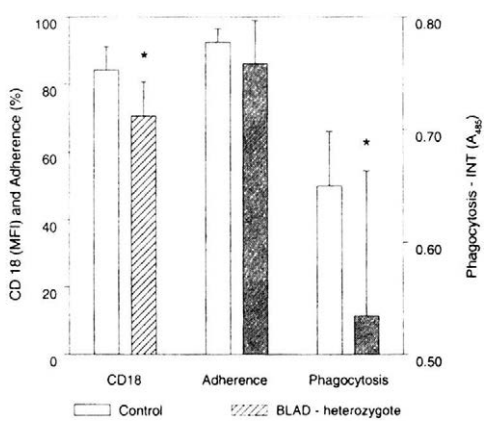
The samples from the PBMC suspensions, used for the functional tests, were analysed for the CD18 molecule expression by immunofluorescence flow cytometry using monoclonal antibody IVA35. The mean fluorescence intensity (MFI) of the CD18 positive cells in controls and BLAD heterozygotes ranged from 77.1 to 95.4, and 54.8 to 94.6, respectively. The mean MFI value was significantly lower in BLAD heterozygotes (70.6 ± 2.7) compared with controls (84.2 ± 2.3) (Fig. 1). However, the differences between normal and carrier animal are not clear-cut, some heterozygous animals had the same level of CD18 expression as controls. Therefore, the CD18 detection with the monoclonal antibody did not allow the direct identification of BLAD heterozygotes.

The neutrophil functions were characterised by phagocytosis and by adherence to the plastic dish. The phagocytic activity of PBMC was stimulated by opsonized zymosan and the oxidative burst was measured by INT test and by luminol-dependent chemiluminiscence (CL). The INT test showed significantly lower ($p < 0.014$) tetrazolium reductase activity of PBMC from BLAD heterozygotes in comparison to the controls (Fig. 1), however, some heterozygotes had similar values like controls (Fig. 2B). CL response of PBMC performed in selected cows showed lower phagocytic ability in BLAD heterozygotes (Fig. 3). Cow No. 2 with peak CL value similar to controls No. 1 and 3 was the exception. Contrary to this observation Sipes et al. (1999) have not recently found any differences in phagocytic activity of normal and carrier calves.

Our data revealed that the adherence of PBMC from the majority of tested BLAD heterozygotes to the plastic was similar to that from normal cattle. When the average percentages in controls and BLAD heterozygotes were compared, no significant differences were observed (Fig. 1). However, in three BLAD heterozygotes, the percentage of adhered cells was apparently lower than in all other animals (Fig. 2A). Adherence of PBMC to the plastic ranged from 57% to 96%, and from 85% to 98% in BLAD heterozygotes and in normal animals, respectively, indicating that the heterozygous genotype likely has no effect on adhesion functions of leukocytes.

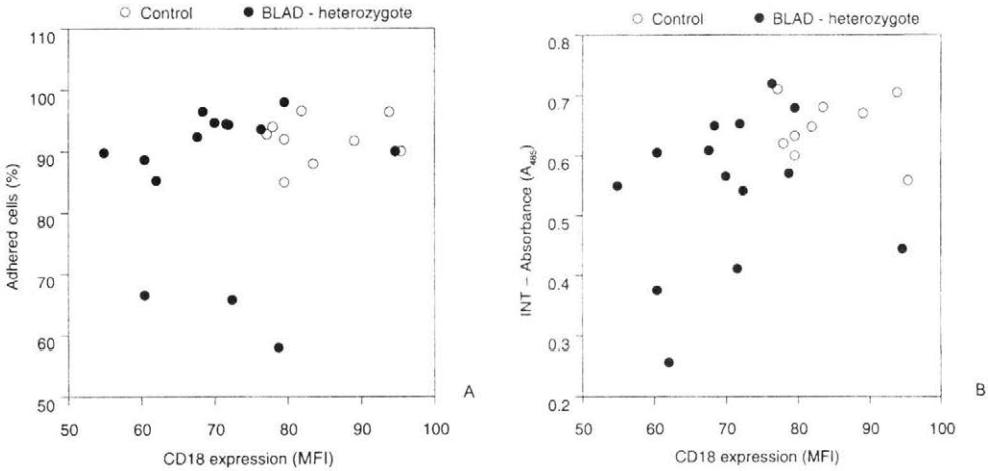
Fig. 2 simultaneously shows the relationship of the functions of PBMC and the level of the CD18 expression. The high level of the CD18 expression slightly correlated with higher tetrazolium reductase activity ($r = 0.36$). The very low correlation of the CD18 expression with adherence was also found ($r = 0.15$). However, it seems that there is no direct relationship between the CD18 expression and the functional properties of neutrophils.

The results of Kehrli et al. (1990), Cox et al. (1997) in agreement with our data revealed the lower expression of CD18 in BLAD carrier animals. These findings lead us to a speculation that the relationship between "changed" and "normal" allele for CD18 has an intermediate character. Full expression may be anticipated in the "healthy" homozygotes, 0 or very low expression in the affected homozygotes and middle or near to middle expression in heterozygotes similarly like it was found in human LAD patients (Anderson et Springer, 1987). However, the decrease of CD18 in carriers is not consistently observed. Sipes et al. (1999) have not found any differences in the expression of CD18 between heterozygous and normal calves. The lower expression of CD18 mostly has no consequence for the neutrophil functions.



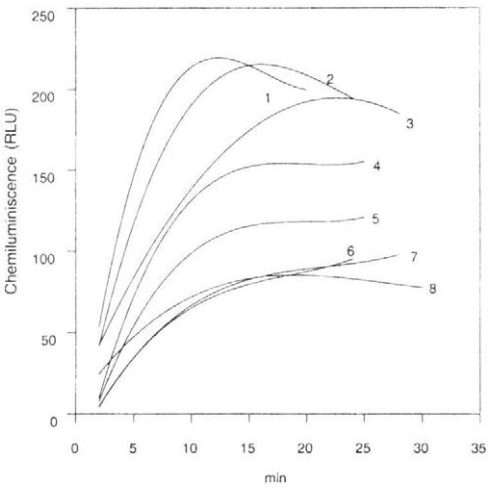
1. Comparison of neutrophils – CD18 expression, adherence and phagocytosis – of heterozygous ($n = 15$) and control animals ($n = 9$). Results are expressed as the mean ± standard error

*$P < 0.05$, MFI = mean fluorescence intensity, INT = quantitative iodenitrotetrazolium dye reduction test



2. The relationship of neutrophil adherence (A) and INT test values (B) with CD18 expression in individual cows

MFI = mean fluorescence intensity, INT = quantitative iodenitrotetrazolium dye reduction test



3. Luminol-dependent chemiluminiscence of neutrophils of some BLAD heterozygous and control cows (No. 1 and 3 – controls, others – BLAD heterozygotes). Values in curves were recorded 10 minutes after the addition of zymosan

RLU = relative light units

Moreover, the expression of CD18 is influenced by a number of other factors of the immune system. Consequently, the experiments testing the functional properties, such as the respiratory burst of neutrophils, could give different results also depending on the stimulus and measurement protocol used.

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Změna publikačního jazyka ve vědeckých časopisech ČAZV

Předsednictvo České akademie zemědělských věd přijalo na zasedání dne 6. 4. 2000 usnesení, kde mj. doporučuje změnu publikačního jazyka ve vědeckých časopisech vydávaných pod gescí ČAZV. Předsednictvo navrhuje Vydavatelské radě ČAZV zavést angličtinu jako jediný jazyk ve všech vědeckých časopisech od 1. 1. 2001. Redakce časopisu Veterinární medicína (Veterinary Medicine – Czech) přijímá od 1. 7. 2000 příspěvky psané pouze v angličtině.

A change of publication language in Scientific Journals of the Czech Academy of Agricultural Sciences

At its session on the 6th April 2000, the Presidium of the Czech Academy of Agricultural Sciences adopted a resolution recommending, among other things, to change the publication language in scientific journals published under the Academy patronage. The Presidium proposes to the Publishing Board of the Academy to introduce English as the only language in all scientific journals from the 1st January 2001. The papers written exclusively in English are accepted by the editor's office of the journal Veterinární medicína (Veterinary Medicine – Czech) from the 1st July 2000.

EFFECT OF CADMIUM ON THE RUMEN PROTOZOAN POPULATION IN SHEEP*

VPLYV KADMIA NA BACHOROVÉ CILIÁTY OVIEC

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ABSTRACT: The effect of increased cadmium doses on the rumen ciliate population in sheep was investigated. The experiment was carried out on 18 adult Merino sheep fed a diet composed of meadow hay and barley meal for 3 weeks prior to and during the experiment. The cadmium content of the diet (control value) was 0.09 mg/kg dry matter of feed (DM). Increased doses were 5 mg Cd/kg DM and 10 mg Cd/kg DM. Protozoa in the ruminal fluid of sheep were examined throughout seven weeks. *Entodinium* spp., *Dasytricha ruminantium*, *Isotricha* spp., *Ophryoscolex caudatus tricoronatus*, *Polyplastron multivesiculatum* and the total number of ciliates were counted. Short-time supplementation of cadmium (day 20) increased the total number of ciliates and the number of all protozoan groups examined. No significant effect on the protozoan population can be seen after long-term supplementation of the cadmium doses tested (on day 47).

ruminants; heavy metal; rumen ciliate protozoa

ABSTRAKT: Sledovali sme vplyv zvýšeného príjmu kadmia na bachorové prvoky oviec. Použitých bolo 18 zvierat plemena Merino kŕmených lúčnym senom a jačmenným šrotom. Obsah kadmia v krmive bol 0,09 mg/kg sušiny krmiva (DM). Zvýšený príjem kadmia v experimentálnych skupinách 5 mg/kg DM a 10 mg/kg DM bol realizovaný formou síranu zamiešaného do šrotu. Experiment trval sedem týždňov. Vyšetrovaný bol počet obrvených prvokov (ciliát) v bachorovej tekutine rodu *Entodinium* spp., *Isotricha* spp., druhov *Dasytricha ruminantium*, *Ophryoscolex caudatus tricoronatus*, *Polyplastron multivesiculatum* a celkový počet ciliát na 20., 33. a 47. deň podávania kadmia. Krátkodobé podávanie kadmia (20. deň) zvýšilo celkový počet ciliát a všetkých vyšetrovaných skupín prvokov. Po dlhodobom podávaní kadmia (47. deň) neboli zaznamenané štatisticky významné zmeny v počte sledovaných ciliát oproti kontrolám na začiatku pokusu. Možno usudzovať, že bachorové ciliáty sa postupne adaptovali na mierne zvýšený príjem kadmia. Citlivosť bachorových ciliát na kadmium pravdepodobne závisí aj na zvyšovaní pufračnej kapacity bachorového prostredia postupnou selekciou baktérií schopných detoxikovať kadmium.

prežúvavce; ťažké kovy; bachorové prvoky

INTRODUCTION

Industrial utilization of compounds containing cadmium has accelerated the mobilization and transport rates of cadmium which highly exceed the rates of natural cycling processes. These rates have led to increased deposition of cadmium in the biota (Babich and Stotzky, 1977). Ruminant animals can be exposed to toxic concentrations of heavy metals by consumption of contaminated feeds and water. The microorganisms present in the reticulorumen initially contact materials consumed by the animal and a number of interactions may occur. Heavy metals can be inhibitory to both fermentative activity and growth of microbes, decreasing thereby productivity of the animals by transformation of the element to a more toxic form, e.g. methylation of mercury (Norseth and Clarkson, 1971). Alternately microbes may also modify the toxicity of the elements to the animal by decreasing the toxicity by precipitation of the heavy metals (Suttle, 1991; Ivan et al., 1986). Ciliate protozoa form 18–32% of the total microbial biomass in the rumen (Michalowski, 1990). Hence, they could contribute significantly to the interactions of rumen microbes with heavy metals.

The present paper reports on the effect of cadmium upon the rumen ciliate population in sheep. It is that part of work in which the effect of increased cadmium intake on the parameters of rumen fermentation and its cumulation in sheep tissues was investigated. (Sviatko and Zeleňák, 1993).

*Supported by the Grant Agency for Science of the Slovak Academy of Sciences (No. 2/ 702920).

MATERIAL AND METHODS

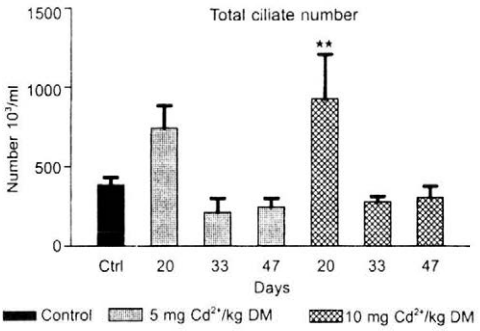
Eighteen Merino sheep of 40 – 45 kg of live weight were used in the experiment. The animals were fed 1 kg of meadow hay and 0.2 kg of ground barley twice daily. Water was available *ad libitum*. The animals were given the diet three weeks before the experimental period started. Then the rumen fluid was sampled from six animals as control values. Other 12 animals were divided into two experimental groups. The first experimental group was supplemented 5 mg of cadmium and the second experimental group received 10 mg of cadmium per kg dry matter (DM) of feed per day and animal throughout seven weeks. Cadmium supplements as sulfate was mixed with ground barley. The cadmium content of the diet (hay, barley) was 0.09 mg/kg of dry matter. Rumen fluid was collected three times at two week intervals with a tube via the oesophagus. Rumen fluid was collected before the morning feeding. Rumen fluid was thoroughly mixed and 5 ml were fixed with an equal volume of 8% formalin solution. The protozoa were identified according to Dogiel (1927) and Ogimoto and Imai (1981). The *Entodinium* spp., *Dasytricha ruminantium*, *Isotricha* spp., *Ophryoscolex caudatus tricoronatus*, *Polyplastron multivesiculatum* and the total number of ciliates were counted. The organisms were counted as described by Coleman (1978) and the results expressed as the means ± standard error of the mean (SEM). One-way analysis of variance was performed to determine significant differences. Probability values of $P < 0.05$ or less were considered as significant.

RESULTS

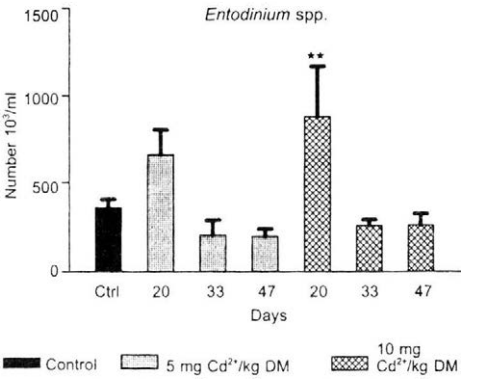
The effect of cadmium upon the number of *Entodinium* spp., *Polyplastron multivesiculatum*, *Ophryoscolex caudatus forma tricoronatus*, *Dasytricha ruminantium*, and *Isotricha* spp. was evaluated. Medium-size ciliates (*Diplodinium*, *Diploplastron*, and *Enoploplastron*) occurred only in few experimental animals and therefore no statistical analysis could be made. The results are shown in Figs. 1–6.

A short-time increase in the total number of ciliates and *Entodinium* spp. was observed at the beginning of the experiment (by 140% at the dose of 10 mg Cd). It was observed that the counts of *Entodinium* spp. and there after the total counts of rumen protozoa decreased by the end of the experiment (Figs. 1, 2). Both cadmium doses decreased the *Entodinium* population after long-term cadmium supplementation.

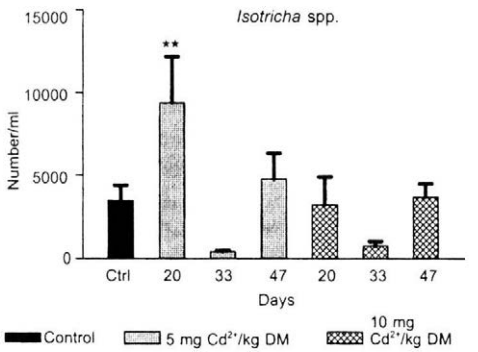
A significant short-time increase of *Isotricha* population (on day 20) was observed at the dose of 5 mg Cd (by 170%, Fig. 3). The population of Trichostomatid ciliates (*Isotricha* and *Dasytricha*) decreased after both doses on day 33 of the experiment (Figs. 3, 4). By the end of the experiment the number of *Trichostomatids* recovered to the initial level.



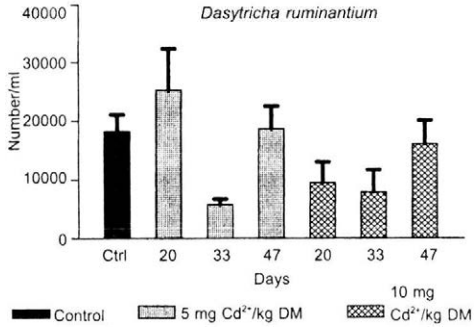
1. Effect of cadmium supplementation on the total ciliate number; values are means ± SEM; experimental groups were compared to control group; probability value is $P < 0.05$ (**)



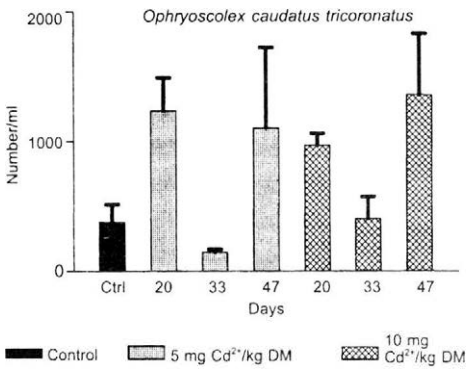
2. Effect of cadmium supplementation on the number of *Entodinium* spp.; values are means ± SEM; experimental groups were compared to control group; probability value is $P < 0.05$ (**)



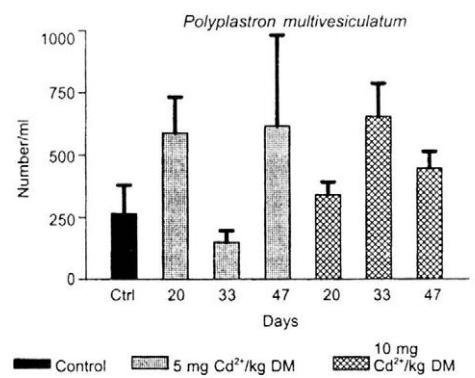
3. Effect of cadmium supplementation on the number of *Isotricha* spp.; values are means ± SEM; experimental groups were compared to control group; probability value is $P < 0.05$ (**)



4. Effect of cadmium supplementation on the number of *Dasytricha ruminantium*; values are means ± SEM; experimental groups were compared to control group



5. Effect of cadmium supplementation on the number of *Ophryoscolex caudatus tricoronatus*; values are means ± SEM; experimental groups were compared to control group



6. Effect of cadmium supplementation on the number of *Polyplastron multivesiculatum*; values are means ± SEM; experimental groups were compared to control group

A similar course of population changes was observed in the large Entodiniomorphids (*Polyplastron* and *Ophryoscolex)* with a tendency to increase in number by the end of experiment (Figs. 5, 6).

DISCUSSION

Toxicity of cadmium has been well documented (Flick et al., 1971). Ruminants tolerate a higher oral intake of cadmium (40–160 mg/l) in contrast to other animals (Powell et al., 1964; Doyle et al., 1973; Sviatko and Zeleňák, 1993). However, little is known about the role of the individual participants of the rumen microbial consortium in cadmium detoxication. Forsberg (1978) tested the effect of heavy metals on eight species of rumen bacteria. He found a different susceptibility of the bacteria to the tested dose scale of heavy metals. He observed decreased fermentation activity of mixed rumen bacteria at cadmium concentration of 175 mg/l. In addition, rumen protozoa were more resistant than rumen bacteria *in vitro* (Forsberg, 1978). Lauková (1994) found most of the rumen staphylococci to be heavy metal ion polyresistant strains.

Rumen ciliates represent a population of physiologically and metabolically diverse organisms. This is reflected in their reaction to increased cadmium intake by the host animal. Gradual saturation of the rumen environment was evident during the short-time period (up to 20 days) when cadmium seemed to behave as an essential microelement and number of ciliates increased. The other observations are needed to confirm this assumption. No data are available on the essentiality of cadmium for rumen ciliates. However, another phenomenon can be included in the increased number of ciliates. The number of almost all ciliate groups was increased. *Entodinium* spp. is the most numerous group of rumen ciliates, representing about 90 % of the rumen ciliate population and therefore the total number of rumen ciliates reflected the number of *Entodinium*. *Entodinium* spp. appeared to be sensitive to the tested cadmium doses. Their growth was reduced after long-term supplementation of cadmium (on day 47). On the other hand, the *Trichostomatids* appeared to be most resistant. Their growth was suppressed during the adaptation period (about day 33). However, growth was recovered at the initial level after the long-term supplementation of cadmium (by day 47). The growth of large *Entodiniomorphids* varied during the experiment and no significant changes could be observed. However, the course of growth was similar to that of *Trichostomatids*. The results are consistent with those obtained in the artificial rumen (Jalč et al., 1994) when the ciliate *Dasytricha* grew well after cadmium supplementation up to 20 mg/kg DM of feed. Buffer capacity of the rumen bacterial population seems to play an important role in the cadmium resistance of some genera of rumen ciliates. *In vitro* experiments on both *Entodinium caudatum* and *Ophryoscolex caudatus tricoronatus* tube monocultures showed

a toxic effect of cadmium at a dose of 1.5 mg/l media under conditions with reduced bacterial population (Kišidayová, 1996). Precipitation by inorganic and chelation by organic material as well as particulate matter are also important for the reduction of cadmium toxicity (European Inland Fisheries Advisory Commission, 1978).

As can be concluded from the results the examined rumen ciliate genera and species revealed a different sensitivity to the tested cadmium doses. Sensitivity to cadmium depended on the buffer capacity of the rumen environment, which may probably be caused by gradual selection of bacteria capable to detoxify cadmium and adaptation of the protozoa.

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A SEROLOGIC SURVEY OF HEMAGGLUTINATION-INHIBITION ANTIBODIES TO HUMAN TYPE A AND B INFLUENZA VIRUSES IN WILD PIGS IN CROATIA

SÉROLOGICKÝ PŘEHLED HEMAGLUTINAČNĚ-INHIBIČNÍCH PROTILÁTEK VŮČI VIRŮM CHŘIPKY U LIDÍ TYPU A A TYPU B U DIVOKÝCH PRASAT V CHORVATSKU

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ABSTRACT: The method of hemagglutination inhibition on U microtitration plates was used to examine 101 wild pig sera for the presence of antibodies to three strains of human influenza virus A: A/Johannesburg/82/96, subtype H1N1; A/Nanchang/933/95, subtype H3N2; and A/Wuhan/359/95, subtype H3N2; and human influenza virus B/Beijing/184/93-like strain. All influenza virus strains used in the study have been circulating in most European countries for some twenty years. A positive titer of hemagglutination inhibition antibodies (≥1 : 20) for virus A/Johannesburg/82/96 (H1N1) strain was recorded in 19 (18.81%), for virus A/Nanchang/933/95 (H3N2) and A/Wuhan/359/95 (H3N2) strains together in 88 (87.12%), and for virus B/Beijing/184/93-like strain in 68 (67.32%) wild pig sera. The sera were collected from wild pig hunt in February 1999 and February 2000. Results of the study provide preliminary data on the presence of antibodies to human influenza A (subtypes H1N1 and H3N2) and B viruses in wild pigs in Croatia.

influenza; human influenza virus; antibodies; wild pigs

ABSTRAKT: Metodu hemaglutinační inhibice na mikrotitračních plotnách tvaru U jsme použili k šetření 101 séra divokých prasat za účelem zjištění výskytu protilátek proti třem kmenům viru chřipky u lidí typu A: A/Johannesburg/82/96, podtyp H1N1; A/Nanchang/933/95, podtyp H3N2; A/Wuhan/359/95, podtyp H3N2 a kmeni viru chřipky u lidí podobného typu B/Beijing/184/93. Všechny kmeny chřipkových virů použité v této studii se v evropských zemích vyskytují opakovaně asi dvacet let. Pozitivní titr hemaglutinačně-inhibičních protilátek (≥1 : 20) pro kmen viru A/Johannesburg/82/96 (H1N1) jsme zjistili u 19 (18,81 %), pro kmeny virů A/Nanchang/933/95 (H3N2) a A/Wuhan/359/95 (H3N2) dohromady u 88 (87,12 %) a pro kmen viru podobného B/Beijing/184/93 u 68 (67,32 %) sér divokých prasat. Séra jsme získali při lovu divokých prasat v únoru 1999 a v únoru 2000. Výsledky této studie přinášejí předběžné údaje o výskytu protilátek vůči virům chřipky u lidí typu A (podtyp H1N1 a H3N2) a typu B u divokých prasat v Chorvatsku.

chřipka; virus chřipky u lidí; protilátky; divoká prasata

INTRODUCTION

The widespread presence of human (H1N1, H3N2) influenza viruses in swine herds has been demonstrated in numerous studies (Tumova et al., 1980; Miwa et al., 1986; Zhang et al., 1988, 1989; Shiraishi et al., 1989; Roy et al., 1991; Goto et al., 1992; Ewald et al., 1994; Chatterjee et al., 1995; Katsuda et al., 1995; Zhou et al., 1996), strongly suggesting that the swine were infected with human H1N1 or H3N2 virus during the respective epidemics in humans (Hirano et al., 1985; Miwa et al., 1986; Goto et al., 1988, 1992; Zhou et al., 1996). Furthermore, it is known that swine play an important role in the ecology of influenza viruses (Shortridge and Stuart-Harris, 1982) and as 'mixing vessles' to produce new human pandemic strains by genetic reassortment (Scholtissek et al., 1985; Webster et al., 1992, 1995; Claas et al., 1994; Scholtissek, 1995; Brown et al., 1998; Ito et al., 1998; Zhou et al., 1999).

In recent years, however, the literature dealing with the prevalence of antibodies to human type A and B influenza viruses in wild pigs (Kawano et al., 1978; Dedek et al., 1990; Teuffert et al., 1991; Brown et al., 1995; Markowska-Daniel and Pejsak, 1999) has been scanty. The present report provides preliminary data on the prevalence of antibodies to human influenza A (subtypes H1N1 and H3N2) and B viruses in wild pigs in Croatia.

MATERIAL AND METHODS

Virus

Three reference strains of human type A influenza virus, i.e. A/Johannesburg/82/96 (subtype H1N1); A/Nanchang/933/95 (subtype H3N2); and A/Wuhan/359/95 (subtype H3N2); and a reference strain of human type B influenza virus B/Beijing/184/93-like were used in the study. The viruses originated from the World Health Organization Collaborating Center for Influenza, Center for Disease Control, Atlanta, Georgia, USA. The viruses were multiplied in the allantoic cavity of fertilized fowl ova with advanced embryos, and the allantoic fluid of the ova was used as antigen.

Sera

Upon the wild pig hunt, a total of 101 blood samples of wild pigs from the Garjevica hunting ground on Moslavačka gora were collected. Sixty-six blood samples were collected in February 1999, and 35 blood samples in February 2000. The blood mostly derived from male and female piglets, with 17 samples obtained from boars and 6 from sows. Blood samples were collected into sterile test tubes by heart puncture from killed animals with open thoracic and abdominal cavity, and transferred in a refrigerator at 4 °C to the laboratory. Blood sera were separated by a standard procedure and stored frozen at –20 °C until analysis.

Preparation of sera

Immediately before analysis, the sera were thawed off at room temperature and prepared for analysis, strictly following the instructions of Palmer et al. (1975).

Virus hemagglutination and hemagglutination inhibition

Determination of the virus hemagglutination titer and hemagglutination inhibition test were performed in plastic U microtitration plates (Greiner disposable plates, Cooke system), in accordance with the instructions of Palmer et al. (1975). A hemagglutination inhibition (HI) titer of ≥1 : 20 was considered positive.

RESULTS

Out of 101 wild pig sera examined, a positive titer (≥1:20) of HI antibodies to virus A/Johannesburg/82/96 (H1N1) strain was recorded in 19 (18.81%) sera; to both virus A/Nanchang/933/95 (H3N2) and A/Wuhan/359/95 (H3N2) strains in 88 (87.12%) sera; and to virus B/Beijing/184/93-like strain in 68 (67.32%) sera. Out of 66 sera examined upon wild pig hunt from February 1999, 19 (28.78%) sera were positive for virus A/Johannesburg/82/96 (H1N1) strain, 54 (81.81%) sera for virus A/Nanchang/933/95 (H3N2) strain; and 38 (57.57%) sera for virus B/Beijing/184/93-like strain. Among 35 sera examined upon wild pig hunt from February 2000, none of the sera was positive for virus A/Johannesburg/82/96 (H1N1) strain, while 34 (97.14%) and 30 (85.71%) sera were positive for virus A/Wuhan/359/95 (H3N2) and B/Beijing/184/93-like strains, respectively (Tab. I).

In positive wild pig sera, the HI antibody titer for virus A/Johannesburg/82/96 (H1N1) strain ranged from 1 : 20 to 1 : 80; for virus A/Nanchang/933/95 (H3N2) strain from 1 : 20 to 1 : 1 280; for virus A/Wuhan/359/95 (H3N2) strain from 1 : 20 to 1 : 160; and for virus B/Beijing/184/93-like strain from 1 : 20 to 1 : 5 120 (Tab. II).

I. Number and percentage of hemagglutination inhibition antibodies to human A and B influenza virus strains in wild pig sera in Croatia

Wild pig hunt time	Total number of examined sera	Virus strain		
		A/Johannesburg/82/96 (H1N1)	A/Nanchang/933/95 (H3N2) A/Wuhan/359/95 (H3N2)*	B/Beijing/184/93-like
		Number (%) of sera with ≥1 : 20 antibody titer		
February 1999	66	19 (28.78)	54 (81.81)	38 (57.57)
February 2000	35	0	34 (97.14)*	30 (85.71)
Total	101	19 (18.81)	88 (87.12)	68 (67.32)

II Number of wild pig sera according to the titer of hemagglutination inhibition antibodies to influenza virus A/Johannesburg/82/96 (H1N1), A/Nanchang/933/95 (H3N2), A/Wuhan/359/95 (H3N2) and B/Beijing/184/93-like strains

Antibody titer	Human influenza virus strain			
	A/Johannesburg/82/96 (HINI)	A/Nanchang/933/95 (H3N2) A/Wuhan/359/95 (H3N2)*		B/Beijing/184/93-like
	(*n*)	(*n*)		(*n*)
<10	71	10	1*	28
10	11	2		5
20	16	28	3*	15
40	2	11	5*	4
80	1	4	12*	11
160		1	14*	8
320		5		12
640		4		7
1 280		1		6
2 560				3
5 120				2

DISCUSSION

Swine are an animal reservoir for influenza viruses capable of causing disease in humans. Genetic and biologic observations suggest that pigs may serve as 'mixing vessels' for the generation of human-avian influenza reassortants. The concept is additionally supported by numerous reports on the finding of antibodies to human influenza virus type A, subtypes H1N1 an H3N2, in pig sera. Zhang et al. (1988) report on the presence of antibodies to influenza virus A, subtypes H1N1 and H3N2, in pig sera in Germany, detected by single radial hemolysis and HI. Zhang et al. (1989) also report on the presence of antibodies to virus A/Singapore/6/86 (H1N1) strain in 1.6% and 3.0%, to A/Victoria/1/75 (N3N2) strain in 22.6%, to A/Hong Kong/1/68 (H3N2) strain in 14.4%, and to A/Philippines/2/82 (H3N2) strain in 10.6% of pig sera in Germany. In the same year, Shiraishi et al. (1989) report from Japan on the presence of HI antibodies to virus A/Shimane/1/80 (H3N2) strain in 54.4% of pig sera. Chambers et al. (1991) describe their finding of antibodies to virus A/Los Angeles/2/87 (H3N2) strain in pigs from the United States, while in the same year Roy et al. (1991) examined 112 pig sera in India and found them to contain antibodies to some of the above mentioned influenza virus A strains. So, antibodies to A/Pune/2/78 (H1N1) were found in 5 (7.7%) and to A/Pune/6/78 (H3N2) in 15 (23.1%) of 65 Large White pig sera; also, antibodies to the two influenza virus A strains were detected in 14 (29.8%) of 47 country bred pig sera each. In the former East Germany, Teuffert et al. (1991) found antibodies to influenza virus subtype H3N2 in 57% of fattening pigs and 62% of breeding pigs. Having examined a total of 2 115 fattening pig serum samples in Germany, Ewald et al. (1994) recorded HI antibodies to virus A/Philippines/2/82 (H3N2) and virus A/Port Chalmers/1/73 (H3N2) in 20.6% and 5.1% of the samples, respectively. Brown et al. (1995) report on the finding of antibodies to influenza virus subtype H3N2 in 39% of pigs from Great Britain. Chatterjee et al. (1995) detected antibodies to influenza virus subtypes H1N1 and H3N2 in 43% and 46% of pigs, respectively, from the area of Calcutta, India. In the same year, Katsuda et al. (1995) from Japan report on the finding of HI antibodies to influenza virus subtypes H1N1 and H3N2 in pig sera. The findings of HI antibodies and neuraminidase inhibition antibodies, described by Zhou et al. (1996) indicate that the swine from Nanchang and its surroundings in southeastern China were infected with the influenza virus subtypes H1N1 and H3N2.

Data on the presence of antibodies to human influenza virus type A in wild pigs and on their role as ‚mixing vessels' are insufficient. Dedek et al. (1990) from the former East Germany examined 397 wild pig sera by use of serum neutralization and HI, and found 4 sera positive for influenza virus subtype H3N2. Teuffert et al. (1991) report on 3.7% of positive sera for the same virus subtype among 161 wild pig sera examined in the same country. Markowska-Daniel and Pejsak (1999) from Poland examined 440 wild pig sera and report on 6.8% of positive sera for influenza virus subtype H3N2.

Data on the findings of antibodies to human influenza virus type B in swine, especially in wild pigs, also are quite scanty. So, Takatsy et al. (1967) from Hungary report on the presence of this type of influenza virus in 8% to 24% of pigs, while Kawano et al. (1978) found 0.1% of pig sera to be positive for it. Brown et al. (1995) from Great Britain recorded it in 7 pigs. The latter group of authors consider this type of influenza virus to be transmitted to the swine without any further dissemination.

In our study, we used human influenza virus type A, subtypes H1N1 and H3N2, and human influenza virus type B, circulating for some 20 years in most European countries. The sera with HI antibody titer of ≥1 : 20 were considered positive, which is consistent with the studies of Dedek et al. (1990) and Youzbashi et al. (1996), however, Katsuda et al. (1995) set the limit of positivity at ≥1 : 8, Kawano et al. (1978) at ≥1 : 32, and Shiraishi et al. (1989), Chambers et al. (1991), and Markowska-Daniel and Pejsak (1999) at ≥1 : 40. With the HI antibody titer of ≥1 : 20 as a measure of serum positivity, we found 18.81% and 87.12% of pig sera to be positive for influenza virus subtype H1N1 and H3N2, respectively, whereas 67.32% of pig sera were positive for infuenza virus type B. Wild pigs are sociable animals living in herds, with frequent migrations, thus coming in contact with other wild or even domestic animals. Such a way of life greatly contributes to the exposure of wild pigs to infection with influenza virus and to the virus maintenance in the wild pig population.

The results of the study suggest that wild pigs can be infected by a number of influenza viruses, some of which may play a role in the epidemiology of human influenza.

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IDENTIFICATION BY ELISA OF POULTRY, HORSE, KANGAROO, AND RAT MUSCLE SPECIFIC PROTEINS IN HEAT-PROCESSED PRODUCTS*

VYUŽITÍ ELISA METODY K ROZLIŠENÍ SPECIFICKÝCH PROTEINŮ DRŮBEŽÍ, KOŇSKÉ, KLOKANÍ A POTKANÍ SVALOVINY V TEPELNĚ OPRACOVANÝCH MASNÝCH VÝROBCÍCH

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ABSTRACT: Indirect competitive ELISA for specific identification of heat-processed poultry, horse, kangaroo, and rat muscular tissue with a sensitivity of 1–5% has been developed. The method can identify meat species in heat-processed products and differentiate heat-stable antigens in phosphate buffered saline (PBS) extracts. The ELISA is based on the use of species-specific polyclonal antibodies prepared by immunisation of rabbits with heat-stable antigens extracted from visible fat-free muscular tissue and heated to 100 °C or 120 °C for 30 min. Adulterations in terms of declared product compositions were demonstrated by this method in some of the 62 tested commercial products.

ELISA; heat-processed meat; heat-stable antigen; poultry; horse; kangaroo; rat

ABSTRAKT: Byla vyvinuta nepřímá kompetitivní ELISA metoda pro druhové rozlišení tepelně opracovaného masa drůbežího, koňského, klokaního a potkaního s citlivostí od 1 do 5 %. Touto metodou lze prokázat druhový původ masa použitého při výrobě tepelně opracovaných masných výrobků a rozlišit termostabilní antigeny připravené jednoduchou extrakcí fosfátem pufrovaným fyziologickým roztokem (PBS). ELISA metoda je založena na využití druhově specifických polyklonálních protilátek připravených imunizací králíků termostabilními antigeny extrahovanými z libové svaloviny a zahřátými na teplotu 100 °C a 120 °C po dobu 30 minut. Bylo vyšetřeno 62 vzorků tepelně opracovaných masných výrobků z tržní sítě. U některých výrobků bylo zjištěno porušení deklarovaných receptur.

ELISA; tepelně opracované maso; termostabilní antigen; drůbež; kůň; klokan; potkan

INTRODUCTION

Identification of meat species is an important task of food quality control. Intentional adulteration of meat products with other-than-declared meat species can bring the manufacturer considerable economic profit. Moreover, adulteration is associated with a hazard of allergic reactions in sensitive consumers. The significance of species composition of meat-and-bone meals fed to ruminants has risen dramatically in association with the emergence of bovine spongiform encephalopathy which necessitated the implementation of effective methods for checking the adherence to rules of correct heat processing (Hofmann et al., 1996, 1999; Holst et al., 2000).

Meat species can be identified by serological (Hayden, 1977; Shaw et al., 1983), histological (Tremlová, 2000) or electrophoretic methods (Hayden, 1981; Swart and Wilks, 1982; Mageau et al., 1984; Manz, 1987; Cutrufelli et al., 1987; Reddy et al., 2000).

The procedures of the identification of raw meat species by ELISA or electrophoretic methods are rather simple (Patterson and Whittaker, 1984). Most of the commercial antisera intended for species identification are prepared against blood proteins and are therefore suitable only for raw meat species differentiation.

The difficulties in the preparation of species-specific antisera against heat-processed proteins, as described by Kang´ethe and Lindqvist (1987) and Kang´ethe and

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Gathuma (1987), result from thermal denaturing of proteins (Hofmann et al., 1996). Therefore, antibodies to heat-stable soluble proteins, which retain their antigenicity after autoclaving at 120 °C for 30 min, must be prepared. Such proteins are present especially in adrenal tissues (Milgrom et al., 1963) and in small amounts also in striated muscles (Hayden, 1977; Hofmann, 1977).

The objective of our investigations was to develop a simple and reliable method based on the demonstration of small amounts of soluble muscle proteins, such as the low-molecular actin, myosin, and troponin (Chin-Sheng Cheng and Parrish, 1979; Sherikar et al., 1993; Hofmann et al., 1996), which are present in extracts of heat-processed meat.

MATERIAL AND METHODS

Solutions

1. binding carbonate/bicarbonate buffer 0.05M pH 8.6
2. washing buffer – PBST (physiological saline – NaCl buffered with 50 mM sodium phosphate and supplemented with 0.1 % TWEEN 20, pH 7.2, Sigma, USA)
3. blocking solution (PBST, pH 7.2, containing 0.5% of casein hydrolysate, (IMUNA, Slovakia)
4. phosphate buffered saline 0.05 M, pH 7.2

Antigens

Immunisation antigens were prepared from samples of visible fat-free muscular tissues of the chicken, horse, kangaroo and rat. The samples were homogenised in a blender with equal parts of 0.05M phosphate-buffered saline, pH 7.2 (PBS); the homogenates were heated for 30 min either in a water-bath at 100 °C, or in an autoclave at 120 °C, gauze-filtered and centrifuged at 10 000 × g and 4 °C for 15 min. The protein content in the supernatants was determined using the Bicinchininic Acid kit (Sigma, USA). The antigen concentration was adjusted to 2 mg per ml.

Solid-phase antigens for specificity tests of ELISA were prepared by the same procedure from chicken, turkey, ostrich, horse, swine, sheep, goat, kangaroo, and rat muscular tissues.

Animals

New-Zealand White rabbits, three months old. Three rabbits for each type of antigen and each treatment temperature were used.

Immunisation

For the first dose, the immunisation antigen was mixed 1 : 1 with complete Freund's adjuvant (Sigma, USA) and administered intradermally. The dose of 0.2 ml (0.4 mg protein) was distributed to 10 sites on the back. The immunisation was repeated 28 days later when the same dose of the antigen was mixed, but complete Freund's adjuvant was replaced with Al-Span-Oil adjuvant (USOL, Czech Republic) and the dose was administered subcutaneously at two sites. The latter procedure was repeated three times at 10-day intervals. After the last dose, the rabbits were bled by cardiac puncture. Sera were separated by centrifugation (1000 × g at 4 °C for 20 min) and stored at –20 °C.

Serum sensitivity tests were done using twofold dilution series of homologous antigen extracts within the range 10 to 1.25%.

Sample processing

Samples of 100 g of meat and meat products (salamis, frankfurters, cooked ham, sausages, pork and beef in natural juice, canned ham, fresh meat, boiled pressed meat, canned sandwich spreads, and canned meat) were processed in a blender with 100 ml of PBS, the homogenate was centrifuged at 10 000 x g and 4 °C for 15 min and the supernatant was used for analysis.

ELISA

Indirect competitive ELISA in a 100-µl system was done using 96-well microtitre plates (GAMA, Czech Republic) and the following protocol:

1. treatment of plates with ethanol for 3 h, removal of ethanol and drying at room temperature
2. application of dilution series of solid-phase antigen in the binding solution (range 20 to 100 µg of protein per 1 ml) and incubation at room temperature for 2 h, or in a refrigerator overnight
3. triple washing with PBST
4. application of blocking solution for 30 min
5. addition of sample extracts, twofold dilution series (10% to 1.25%) of antigen homologous with the respective solid-phase antigen (extracts of chicken, horse, kangaroo, or rat muscles), and the respective antiserum
6. incubation at room temperature for 45 min followed by triple washing
7. addition of peroxidase-labelled conjugate – swine anti-rabbit antibody (SwAR-Px supplied by USOL, Czech Republic)
8. incubation at room temperature for 45 min followed by triple washing
9. addition of substrate (8 mg of 5-aminosalicylic acid and 20 mg of a mixture of potassium phosphates and monosodium citrate at a ratio adjusting pH to 5.3 per 100 ml) and incubation at room temperature for 30 min
10. measurement of absorbance at 492 nm (Labsystems iEMS Reader MF); decrease in extinction to one half or less of the original value was interpreted as a positive result

RESULTS AND DISCUSSION

The best results in terms of specificity and sensitivity were obtained with the antisera RACh 100/7, RAH/100, RAKa 100/7, and RARa 120/1. Except for the first, the antisera have not shown any cross-reactivity and hence no saturation was necessary. The sensitivity of ELISA for the detection of chicken, horse, kangaroo, and rat protein, tested with dilution series of the immunisation antigens, was 1.25, 5, 2.5, and 0.5%, respectively.

The specificity of the antisera is expressed in terms of cross-reactivity with heterologous antigens in Tab. I. While the reactions of the antisera to horse, kangaroo, and rat antigens were strictly species-specific, chicken antiserum yielded false positive reaction with the extract of a product containing kangaroo meat and processed at 120 °C.

Sixty-two commercial heat-processed meat products (salamis, frankfurters, cooked ham, sausages, pork and beef in natural juice, canned ham, fresh meat, boiled pressed meat, canned sandwich spreads, and canned meat) were tested by this method. In most cases, the test results were consistent with the declared composition. However, chicken protein was demonstrated in four and kangaroo protein in two products declared as containing beef and/or pork only. Rat protein was not detectable in any of the products. Out of keeping with the declared ingredients, no horse protein was detected in one product. As explained later by the manufacturer, the declaration was incorrect since this type of product was actually free of horse meat.

Specificity tests revealed cross-reactivity of the antiserum to chicken protein with ostrich and turkey proteins (Tab. I). Hence, the method is not suitable for the differentiation of the three phylogenetically related species.

The quality of antisera is of crucial importance for obtaining correct results in ELISA. Their specificity and sensitivity depend on the choice of the immunisation antigen, the donor species, and the immunisation scheme. Most of the commercially available antisera are prepared against blood proteins and their use for species identification is limited to raw meat. Like Berger et al. (1988), Patterson and Jones (1989), and Andrews et al. (1992), we used extracts of heat-treated muscles of the individual animal species as the immunisation antigens.

So far, no generally applicable immunisation scheme has been suggested. Most of the authors prepared their antisera in rabbits (Swart and Wilks, 1982; Cutrufelli et al., 1987; Martin et al., 1988), but some of them, such as Mageau et al. (1984) preferred goats and sheep. Sherikar et al. (1988) prepared anti-bovine sera by immunisation of the phylogenetically related buffalo calves.

To a certain extent, cross reactions can be avoided by saturation of antisera with the respective antigen(s) followed by centrifugation and/or affinity chromatography in CNBr-activated Sepharose 4 B (Martin et al., 1988). Alternatively, the donor animals can be immunised with antigens purified by fractionation with ammonium sulphate (Berger et al., 1988; Martin et al., 1992). However, even

I. Antisera specificity

Antiserum	B		Sw		H		O		G		Ch		T		Os		Ka		Ra	
	100 °C	120 °C	100 °C	120 °C	100 °C	120 °C	100 °C	120 °C	100 °C	120 °C	100 °C	120 °C	100 °C	120 °C	100 °C	120 °C	100 °C	120 °C	100 °C	120 °C
RACh 100/7	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	–	±	–	–
RAH 100	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
RAKa 100/9	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–	–
RARa 120/1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+

RACh – Rabbit anti chicken antiserum
RAH – Rabbit anti horse antiserum
RAKa – Rabbit anti kangaroo antiserum
RARa – Rabbit anti rat antiserum

B – Bovine antigen
Sw – Swine antigen
H – Horse antigen
O – Ovine antigen
G – Goat antigen

Ch – Chicken antigen
T – Turkey antigen
Os – Ostrich antigen
Ka – Kangaroo antigen
Ra – Rat antigen

a combination of all the procedures may fail to yield strictly species-specific antisera (Kang'ethe and Gathuma, 1987). A review on cross-reactivity of commercial antisera was published by Pickering et al. (1992). In our experiments, the specificity competitive ELISA with unsaturated antisera was high enough to distinguish among phylogenetically distant species.

It can be concluded from the results of our experiments that the indirect competitive ELISA is a simple and sensitive method for the qualitative identification of species-specific proteins in heat-processed meat products.

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