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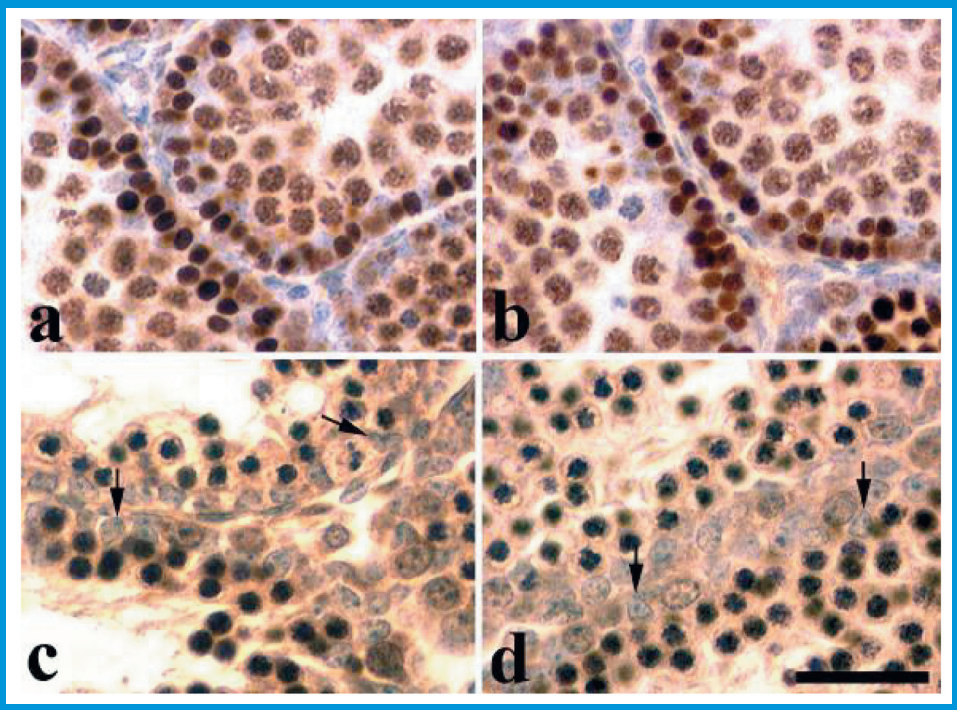

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Review Paper

REAL-TIME REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION TECHNIQUE: USEFULNESS AS AN ANIMAL VIRAL DISEASE DIAGNOSTIC

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Summary: Quick and early diagnosis of the causative agents is critical for countries which are enzootic to particular disease. In these diagnostics speed is paramount; Real-time Reverse Transcription PCR (rRT-PCR) is being utilized increasingly in novel clinical diagnostic assays in molecular biology. The combination of excellent sensitivity and specificity made this technique an alternative to cell culture and other laboratory testing methods for disease diagnosis. In this review, the usefulness or applications of rRT-PCR assays in the diagnosis of some of the important animal viral infections are summarized.

Key words: animal diseases; virus diseases - diagnosis; reverse transcriptase polymerase chain reaction - methods; RNA, viral - genetics

Introduction

Real-time Reverse Transcription Polymerase Chain Reaction (rRT-PCR) is a powerful tool for quantitative analysis of nucleic acids (1, 2). rRT-PCR techniques are increasingly used to quantify RNA viruses for diagnosis (3), the standard for the detection and quantification of RNA targets (4) and is firmly established as a mainstream research technology (5). This technique is a refinement of the original PCR developed by Kary Mullis and co-workers in the mid 1980ís (2, 6). The amplification is detected by using either probe (specific) or non-probe (non-specific) and is discussed elaborately (7). In rRT-PCR the amount of product formed is monitored during the course of the reaction by monitoring the fluorescence of dyes or probes introduced into this reaction which is proportional to the amount of product formed, and the number of amplification cycles required to obtain a particular amount of DNA molecules (2). The advantages of using rRT-PCR are as follows, 1] traditional PCR is measured at end-point, while rRT-PCR collects data in the exponential growth phase, 2] an increase in reporter fluorescent signal is directly proportional to the number of amplicons generated, 3] a permanent record amplification of an amplicon, 4] increased dynamic range of detection, 5] requirement of 1000-fold less RNA than conventional assays, 6] no-post PCR processing 7] detection ranges down to a 2-fold change 8] small amplicon size results in increased amplification efficiency and 9] less time-consuming. rRT-PCR can be applied to traditional PCR applications as well as new applications that would have been less effective with traditional PCR. With the ability to collect data in the exponential growth phase, the power of rRT-PCR has been expanded into applications such as, a] quantization of gene expression (8) including NK cell KIR gene expression (9), b] drug therapy efficacy/drug monitoring (10), c] Viral quantization (11), and d] pathogen detection (12). rRT-PCR when compared to ELISA, RT-PCR and virus Isolation in cell culture, have greater versatility, sensitivity and specificity (13, 14). The technique was and is being used in the diagnosis of a wide variety of diseases caused by RNA viruses in animals and birds and some of them are being summarized in this article.

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rRT-PCR In diagnosis of Animal Viral Infections

A. Positive-strand RNA viruses

rRT-PCR assays are also useful in the diagnosis of animal viral diseases which are economically and as well as zoonotically important. These include some of the viruses listed by the *Office International des Epizootics* (OIE). Foot and mouth disease (FMD) is the number 1 animal viral disease of OIE list and presently the rRT-PCR technique is being widely used in the diagnosis of FMD which causes huge economic crisis world wide. The technique has proven to be more efficient and sensitive than the conventional RT-PCR and virus isolation in cell culture. rRT-PCR assay was found to be very efficient for quantitation of Foot and mouth disease virus (FMDV) in porcine tissues (15); in detecting FMDV from experimentally affected animals (16, 17) and shows 100% specific diagnosis of all the 7 serotypes of FMDV in less than 2 hrs (18, 19). The assay is also used to diagnose swine vesicular disease virus and its differential diagnosis from other vesicular disease viruses like FMD, vesicular stomatitis and vesivirus (20). Another 1000-fold more sensitive TaqMan rRT-PCR was used for the specific detection of FMDV in both cell culture and clinical samples and also for the differential diagnosis of other vesicular diseases and bovine viral diarrhea (21). The two independent rRT-PCR techniques were studied and then compared to detect FMDV in clinical samples; the results suggested that the techniques could be used to enhance sensitivity of molecular methods for further FMDV detection (22). Surveillance and for field deployment another RT-loop mediated isothermal amplification technique was found to be very effective for rapid detection of FMDV (23).

The worldwide occurrence and re-occurrence of trans-boundary diseases like FMD or classical swine fever (CSF) indicates that there is a high need for the development of powerful, robust and high-capacity new diagnostic methods that are able to detect the causative agents before they could spread to large populations and cause tremendous losses (24). The rapid, powerful and internationally standardized molecular diagnosis contributes to the reduction of losses caused by the trans-boundary viral diseases to a larger extent (24). Thus, rRT-PCR forms an important assay in the detection and species-specific differentiation of pestiviruses like CSF virus (25). CSF in an experimentally infected swine will produce the same extent of disease that of natural outbreaks and its detection made easier by TaqMan rRT-PCR assays in less than 2 hrs thus providing a rapid method for the diagnosis of CSF virus on herd basis (26, 27) and also for the quantitative pathogenesis study of this virus (28).

Japanese encephalitis virus (JEV) is one of the most important zoonotic diseases of swine, in areas where it is endemic, such as East Asia (29). TaqMan rRT-PCR assay using fluorogenic probes was developed to distinguish JEV with West Nile virus (WNV) and the method was tested on experimentally infected animal tissues which showed clear discrimination between WNV and JEV (29). Quantitative detection of JEV by rRT-PCR using virus specific primers showed no cross-reactions with other swine viruses and bovine viral diarrhea virus (30). TaqMan rRT-PCR assay was also used for the detection of equine arteritis virus in seminal plasma and nasal secretions of infected horses (31) and then for differentiating the avian infectious bronchitis virus isolates in clinical samples thus by identifying the serotypes involved in disease outbreak (32).

B. Negative-strand RNA viruses

Typical influenza is an acute respiratory herd disease and commonly observed during autumn, winter and early summer (33). Influenza viruses affect all animal and avian species. Immuno-fluorescent techniques were used initially which showed evidence of viral antigen in bronchial epithelial cells within 2 hours after infection (34). Recently, a rRT-PCR assay was used to detect swine influenza A viruses and the test was found to be 100% specific and 88-100% sensitive in screening numerous nasal swab specimens and also very efficient and specific for the respective viral genes thus able to distinguish between their viral subtypes (35) and are largely used to detect and differentiate the North American swine influenza viruses (33). rRT-PCR based on LC technology was used to detect equine influenza virus over two influenza seasons, analyzing 171 samples and they could get a positive correlation between the quantitative rRT-PCR in both cases (36) indicating the high specificity of the assay. Rapid diagnosis of H5N1 influenza A virus was performed by using multiplex rRT-PCR from 75 clinical specimens isolated from both poultry and mammals. The results highlights that the assay could be feasible and very effective for large-scale screening during times of H5N1 outbreaks (37) and also as a good tool for the rapid screening of

flocks and live bird markets (38, 39). Simultaneous detection of influenza viruses A and B was carried out using TaqMan based rRT-PCR assay which was found to be more sensitive than the combination of viral culturing and shell vial culturing of influenza viruses (40).

Bovine respiratory syncytial virus (BRSV) causes respiratory disease in infected animals. Detection of BRSV was done by quantitative rRT-PCR assays based on fluorogenic probe using BioRad's iCycler iQ (41) was found to be 100 fold more sensitive than conventional RT-PCR used previously for BRSV diagnosis (42). Rabies is an enzootic fatal disease and is still a major problem in developing countries. Several RT-PCR methods have been reported for the detection of rabies and rabies-related viruses. Distinguishing the classical rabies virus and its genotypes was described in a single tube, non-nested rRT-PCR with TaqMan technology in real-time and found to be very useful in the detection and differentiation of members of the genus Lyssavirus (43).

Nipah and Hendra viruses belong to the novel genus Henipavirus of the family *Paramyxoviridae*. Its zoonotic circulation in bats and recent emergence in Malaysia with fatal consequences for humans who were in close contact with infected pigs has made the reinforcement of epidemiological and clinical surveillance systems a priority. TaqMan rRT-PCR assay has been developed targeting the Nipah nucleoprotein so that Nipah virus RNA in field specimens or laboratory material can be characterized rapidly and specifically quantitated (44). The method was able to detect virus from hamsters infected with Nipah virus and allows a rapid detection and quantitation of Nipah RNA both from field and experimental materials used for the surveillance and specific diagnosis.

Diagnosis of Newcastle disease virus (NDV) was recently carried out by using rRT-PCR from clinical samples and a positive correlation was obtained between these assays and detecting NDV by RT-PCR and virus isolation from clinical samples (45). To obtain a large diagnostic and surveillance sample workload, a high throughput rRT-PCR assay was developed during 2002-03 outbreaks of NDV occurred in California (46). And also a two-step rRT-PCR using SYBR Green I was designed for the screening large number of NDV specimens (47).

C. Retro viruses

Simultaneous calculation of the nucleic acid recovery rate along with the copy number of viral RNA molecules of equine infectious anemia virus in the plasma was obtained by a single tube rRT-PCR reaction using a fluorogenic probe (48). At the same time a robust, ultra-sensitive quantitative assay was developed for maedi-visna virus (MVV) RNA and DNA genomic sequences and mRNA expressed at various stages of lentiviral replication (49). The assay was designed based on PCR with real-time fluorescence resonance energy transfer measurements. The quantitative assay was found to have greater use in studying the role of genetic elements in MVV infection, pathogenesis, lentiviral vectors and packaging systems based on MVV. Quantitative rRT-PCR assay of MVV RNA in culture supernatants helped in obtaining the complete genomic sequence of a sheep lentivirus isolate that presents a slow/low phenotype (50).

D. Double-stranded RNA viruses

The outbreaks of bluetongue disease in sheep can be combated by extensive vaccination. In order to do so, a rapid and sensitive technique should be used to differentiate vaccine strains of bluetongue and the field strains. A new method for bluetongue virus differentiation using fluorescence resonance energy transfer probes with rRT-PCR assay was performed with LC system and described earlier (51). Infectious bursal disease virus (IBDV) causes an immunosuppressive disease in chickens and leads to severe economic losses in the poultry industry. Vaccination may not be effective if there is exposure of the vaccinated flock to a different antigenic subtype as a result of mutation in the VP2 protein upon which the major neutralizing epitopes are located (52) also they reported that the rRT-PCR assay could be a useful tool to assist in the development of more effective vaccines and control strategies of infectious diseases.

Conclusion

The newly established methods must be standardized to maintain high quality laboratory performance. Future challenges in the study of animal viral diseases include the application of modern techniques, such as nucleic acid chips, protein chips, proteomics and new biomarkers to avoid cross-reactivity among different samples, strains or serotypes, as well as development of internationally standardized guidelines to improve the quality of these laboratory tests.

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METODA OBRATNEGA PREPISOVANJA IN VERIŽNE REAKCIJE S POLIMERAZO V REALNEM ČASU: UPORABNOST V DIAGNOSTIKI ŽIVALSKIH VIRUSNIH BOLEZNI

R. Manojkumar

Povzetek: Hitro in pravočasno odkrivanje povzročiteljev bolezni je v državah z enzootijo za določene bolezni bistvenega pomena. Pri taki diagnostiki je hitrost odločilna in rRT-PCR (obratno prepisovanje in verižna reakcija s polimerazo v realnem času) se čedalje več uporablja kot sodoben test klinične diagnostike v molekularni biologiji. Zaradi odlične občutljivosti in specifičnosti predstavlja alternativo celičnim kulturam in drugim laboratorijskim testom za diagnostiko bolezni. V preglednem članku povzemamo uporabnost in aplikacije testov rRT-PCR v diagnostiki nekaterih pomembnih živalskih virusnih bolezni.

Ključne besede: živali, bolezni; virusne bolezni - diagnostika; polimerazna verižna reakcija z reverzno transkriptazo; RNA, virusna - genetika

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Original Research Paper

MICROBIOLOGICAL 5-PLATE SCREENING METHOD FOR DETECTION OF TETRACYCLINES, AMINOGLYCOSIDES, CEPHALOSPORINS AND MACROLIDES IN MILK

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Summary: Within the field of food hygiene and food control, the antibiotic residues in food of animal origin are analysed because their presence may have undesirable consequences. These include for example, allergic reactions in people, spread of resistance to antibiotics among microorganisms and damages in the food industry. Methods available for the detection of antibiotic residues in food are microbial, chemical and immunoassays. Microbial methods are used as screening methods and are always the first choice for this purpose.

The aim of our study was to develop a microbial method for the detection of antibiotic residues from the macrolide, aminoglicoside, cephalosporine and tetracycline families. The study involved investigation of bacterial test strains and establishment of the limits of detection (LOD) of antibiotics.

For cephalosporines and macrolides, the most appropriate sensitive strain proved to be *Micrococcus luteus* ATCC 9341, for aminoglicosides *Bacillus subtilis* BGA and for tetracyclines *Bacillus cereus* ATCC 11778. A significant component in our experiment were the so-called confirmation solutions. Magnesium sulphate inactivates aminoglicosides and can be used to confirm their presence when more than one antibiotic group can produce inhibition zones on the same plate. Cephalosporinase inactivates cephalosporines and was used to distinguish this group of antibiotics from macrolides. The LOD was at or below the allowed maximum residue level (MRL) for all tested antibiotic groups.

Key words: food analysis - methods; antibiotics; drugs residues - analysis; microbial sensitivity tests - methods; milk

Introduction

By definition, an antibiotic is either a natural product of a micro-organism, an identical synthetic product or a similar semi synthetic product, that inhibits the growth of other microorganisms (bacteriostatic effect) or destroys other microorganisms (bactericide effect). (1). The most common cause for the presence of antibiotic residues in food of animal origin is violation of withdrawal periods (2, 3, 4, 5). Other possible causes are overdosing of antibiotics and use of antibiotics banned for treatment of food producing animals (6, 7, 8). Also, antibiotic residues can be detected in bulk milk samples from a stable where individual animals are being treated for mastitis.

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In the field of food hygiene and food control we deal with analysis of antibiotic residues in food of animal origin due to the potential of unwanted consequences . Among them are sensitivity to antibiotics, allergic reactions and imbalance of intestinal microflora in people, spread of resistance to antibiotics in microorganisms and losses in the food industry where antibiotics can influence starter cultures used in the production of meat and milk products.

Microbial methods were the first choice of systematic detection of antibiotic residues in food in the past and are still mainstream screening methods. They allow determination of the presence of antibiotics in the sample and identification of specific antibiotic groups (9). Screening methods have acceptable false-positive result rates (10, 11) and allow detection of a wide spectrum of antibiotics (9, 12). Their other

advantages are the option to analyse a large number of samples simultaneously and the relatively short time needed for preparation of samples as no purification procedures are required (13, 14, 15, 16, 17, 18). As microbial methods can not be used to identify individual antibiotics a positive result should be confirmed with chemical or physical methods.

Tetracyclines are probably the most widely used therapeutic antibiotics in food producing animals because of their broad spectrum and cost effectiveness. In the United Kingdom and the Netherlands the amount of tetracyclines used for farm animals is nearly equal to the amount of all other antibiotics. Cephalosporines are used both for humans and animals. The first and second generation are approved worldwide strictly for treatment of mastitis infections in dairy cattle. A representative of the third generation, ceftiofur, and a representative of the fourth generation, cefquinome, have been developed strictly for veterinary use and approved in several countries for treatment of respiratory disease, foot rot mastitis in dairy cattle (9, 10, 15, 16, 19). Macrolides are used in veterinary medicine for the treatment of clinical and subclinical mastitis in lactating cows and for the treatment of chronic respiratory diseases (20). The aminoglycosides are broad-spectrum antibiotics also widely used for treatment of bacterial enteritis, mastitis and other infections. Aminoglycosides most commonly used as therapeutic agents are gentamicin, neomycin and streptomycin (21).

The most frequently used microbial method is based on the principle of inhibition of growth of testing strains which is known as the STAR five-plate method (22). It is used for detection of antibiotics from the macrolide, aminoglicoside, tetracycline and cephalosporine families (19, 23, 24, 25). Detailed procedures of these tests vary among laboratories and tests are not standardised for minimal detectable antibiotic concentrations, therefore comparison of results is difficult (26, 27, 28). The aim of our study was to develop a microbial method for detection of antibiotic residues from the four above mentioned families and to determine LOD for each tested antibiotic according the EU Regulation 2377/90 which prescribed maximum residue limits (29).

Material and methods

Microbiological methods are based on the measurement and evaluation of zones of inhibited bacterial growth on media. Two test strains are used to assess the presence of each antibiotic – one maximally sensitive and the other resistant to the tested substance. With the combination of different sensitive and resistant bacterial strains, specific antibiotic groups present in the sample can be identified. In our research we used the following strains with previously established sensitivity and resistancy profiles: *Bacillus cereus* ATCC 11778, *Micrococcus luteus* ATCC 2341, *Escherichia coli* ATCC 10536, *Staphylococcus epidermidis* ATCC 12228 and *E. coli* ATCC 10536 (manufactured by OXOID™).

Preparation of bacterial cultures and media

Bacterial strains stored as cultures in original bacterial loops (Culti loop) were applied to a test tube containing 1ml Trypton soya broth (TSB) medium and incubated at 37 °C for one hour. The culture was then inoculated on blood agar and incubated for further 16 hours at the same temperature. Afterwards the purity of bacterial colonies was assessed with a light microscope and pure colonies were stored in a fridge at temperatures between 2 °C and 8 °C for up to one month. To compose test plates, bacterial culture was diluted with normal saline containing peptone water to produce a suspension which was then incubated at 37 °C for one hour and afterwards added to the agar medium specified below. The suspension density was standardised with the Mc Farland method.

Basic media for preparation of test plates were antibiotic agar No. 1 (Merck™) and antibiotic agar No. 2 (Merck™). Antibiotic agar No. 1 was prepared as follows: 1000 ml of distilled water was added to 30, 5 g of the medium, left for 15 min and then heated to boiling point so that the medium was completely dissolved. The medium was then autoclaved at 121 °C for 15 min. For antibiotic agar No. 2 1000 ml of distilled water was added to 15, 5 g of medium and then the same procedure was followed. After autoclaving, the pH of the media was set to desired values: pH 8 for Er, I BGA, Kin and AC plates and pH6 for E plates.

Preparation of test plates

Test plates were marked according to the bacterial strain added to the medium: AC plate - Micrococcus luteus ATCC 2341, ER plate - Staphylococcus epidermidis ATCC 12228, I-BGA plate - Bacillus subtilis BGA, Kin plate - E. coli ATCC 10536 and E plate - Bacillus cereus ATCC 11778. The pH of the medium was maintained at 8.0 for AC, E and ER plates and at 6.0

for I-BGA and Kin plates. We defined the tolerance for the width of inhibition zone at (as) 8.5 mm – 0.5 mm wider than the width of the metal cylinder containing the sample. Inhibition zones between 8 mm and 8.5 mm wide were considered a non-specific reaction.

To prepare a test plate 0.45 ml of suspension of bacterial culture was added to 40 ml of basic medium and heated to 40 °C. Kin plate was an exception where 0.2 ml of suspension was added to 50 ml of medium. The mixture of medium and bacterial culture was poured into a petri dish (5 ml of mixture into each petri dish). At room temperature the petri dishes with silified medium were enveloped in a parafilm and stored in a fridge. The storage period of test plates was one week. Before application of samples to test plates, plates were warmed at room temperature for 20 to 30 min.

Preparation of milk samples

To test the sensitivity of our method, milk samples containing known concentrations of standard antibiotics were inoculated on test plates. Prior to the addition of antibiotics, milk was always tested for the presence of inhibitory substances.

As the initial step, standard antibiotic solutions were prepared using reference chemical composition and purity (Table 1). Standard antibiotics in powder were dissolved in appropriate solvents: tetracyclines in phosphate buffer with pH value 4.5, cephalosporines in phosphate buffer with pH value 6.0, aminoglicosides in phosphate buffer with pH value 8.0, and macrolides in methanol. Standard solutions were diluted to desired concentrations with UHT milk containing 1.6% fat (Ljubljanske mlekarne). These samples of milk with known concentrations of antibiotics were then poured into 10-ml test tubes and heated to 80 °C for 5 min to avoid later non-specific reaction on test plates. After heating, the samples were cooled and transferred to test plates in 8 mm wide cylinders. Test plates were incubated at 37 °C (I-BGA, AC, Er, Kin) or at 30 °C (E plate) for 18-24 hours.

For each antibiotic we used milk samples containing antibiotic concentrations equal to MRL and half the MRL for that substance. If at half the MRL the result was still positive, lower concentrations of antibiotic were applied until the minimal level of detection was reached.

Confirmation solutions

To confirm the presence of antibiotic groups or their individual representatives we used confirmation solutions. These solutions inhibit the action of certain antibiotics and can help to distinguish between antibiotic groups which cause inhibition zones on the same test plates. Magnesium sulphate ($MgSO_4$) was used to neutralise the aminoglicosides and cephalosporinase enzyme to neutralise the cephalosporines.

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

Table 1: Antibiotic standards

ANTIBIOTIC	TRADE MARK	CATALOGUE NUMBER
Streptomyicin	Sigma - Aldrich	46754
sGentamicin	Sigma - Aldrich	46305
Neomycin	Calbiochem	4801
Cephalexine	Sigma - Aldrich	33989
Cephazoline	Sigma - Aldrich	22127
Cefoperazone	Sigma - Aldrich	22129
Chlortetracycline	Sigma - Aldrich	46133
Tetracycline	Sigma - Aldrich	46935
Erythromycin	Sigma - Aldrich	46256
Tylosin	Sigma - Aldrich	46992

Evaluation of results

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

Quantitatively the concentration of antibiotic can be assessed with microbial methods if the sample contains a known antibiotic or an antibiotic that has previously been identified qualitatively. In each case a calibration curve is required.

Results

We have confirmed sensitive and resistant bacterial strains for all antibiotic groups tested in our study (Table 2). Based on our results we chose to use *Bacillus cereus* ATCC 11778 (E plate) as the sensitive and *Micrococcus luteus* ATCC 9341 (AC plate) as the resistant strain for tetracycline and chlortetracycline from the tetracyclines group. For tylosine and erythromycine from the macrolides group *Micrococcus luteus* ATCC 9341 (AC plate) was chosen as the sensitive and *Escherichia coli* ATCC 10536 (Kin plate) as the resistant strain. For gentamycine, sterptomycine and neomycine from the aminoglicosides group *Bacillus subtilis* BGA (I-BGA plate) was chosen as the susceptible and *Staphylococcus epidermidis* ATCC 12228 (ER plate) the resistant strain. For cephalexine, cephoperasone and cephasoline from the sensitive group *Micrococcus luteus* ATCC 9341 (AC plate) was chosen as the susceptible and Staphylococcus epidermidis ATCC 12228 (ER plate) as the resistant strain.

We differentiated between antibiotic groups using a combination of five test plates (Table 3). To discriminate between aminoglicosides and macrolides we had to utilise used magnesium sulphate which inactivates the aminoglicosides. To discriminate between cephalosporines and macrolides we used the cephalosporinase enzyme.

Table 4 shows the limit of detection for milk samples containing standardised antibiotic solutions on selected test plates. The level of detection was at or below the MRL in all tested antibiotics.

Discussion

Microbial methods for detection of antibiotic residues in food of animal origin are used as a screening method in the majority of laboratories in Europe that deal with analyses of drug residues in food

Table 2: Sensitivity of bacterial strains:

ANTIBIOTIC	B.c/ E	M.l.1/ AC	B.s.BGA/ IBGA	S.e./ ER	E.c./ KIN
Cephalexine	-	+	+	-	-
Cephasoline	-	+	+	-	-
Cefoperazone	-	+	+	-	-
Gentamicin	+	-	+	-	+
Neomycin	+	-	+	-	+
Streptomycin	+	-	+	-	+
Erythromycin	-	+	+	-	-
Tylosin	-	+	+	-	-
Tetracycline	+	-	+	-	-
Chlortetracycline	+	-	+	-	-

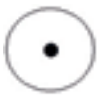
+	sensitive strain
-	resistant strain
B.c/E	Bacillus cereus ATCC 11778/ plate E
M.l.1/AC	Micrococcus luteus ATCC 9341/ plate AC
B.s.bga/IBGA	Bacillus subtilis BGA/ plate IBGA
S.e./ER	Staphylococcus epidermidis ATCC 12228/ plate ER
E.c./KIN	Escherichia coli ATTC 10536/ plate KIN

Table 3: Interpretation of results of 5-plate method:

Plate E	Plate Ac	Plate IBGA	Plate Er	Plate Kin	Antibiotics	Antimicrobial family
					Cephazoline Cephalexine Cefoperazone	Cephalosporins
					Gentamicin	Aminoglycosides
					Neomycin	
					Streptomyicin	
					Erythromycin	Macrolides
					Tylosin	
					Tetracycline Chlortetracycline	Tetracyclines

plate E	Bacillus cereus ATCC 11778
plate Ac	Micrococcus luteus ATCC 2341
plate IBGA	Bacillus subtilis BGA
plate Er	Staphylococcus epidermidis ATCC 12228
plate Kin	E. coli ATCC 10536
Sa + ceph	sample + confirmatory solution - cephalosporinase
Sa + MgSO4	sample + confirmatory solution – magnesium sulphate

sample with inhibition zone after incubation



sample withouth inhibition zone after incubation.

(30, 31, 32, 33). They are always the method of choice for screening purposes as they allow qualitative detection of antibiotics in the sample and identification of antibiotic groups. This facilitates subsequent confirmation of specific antibiotic residues with chemical methods. Microbial methods are relatively inexpensive, easy to use, do not require expensive equipment and can be efficiently adopted by laboratory staff. Although minimal expenditure is a significant factor of analyses, no test is valuable if it does not give reliable results (34, 35). We succeeded in developing a microbial method which is sensitive and meets the legislative requirements – to detect concentrations of antibiotics below the MRL. For some antibiotics the level of detection was at half the MRL or lower.

Microbial methods are semi quantitative, therefore any positive or suspicious result should be confirmed by chemical methods (36). In accordance with the EC 2002/657/EC regulation results of microbial methods are not reported as negative and positive, but as satisfactory or suspect when the MRL is exceeded.

Although the STAR five-plate test is the official method approved by the Community Reference Laboratory, many variations of microbial methods are

Table 4: Limit of detection and maximum residue levels (MRL) of antibiotics in milk

Antibiotic	Bacterial strain/ plate	LOD st.s. (µg/kg)	LOD milk (µg/kg)	MRL milk (µg/kg)
Cefalexin	M.l.1/ AC	50	50	100
Cefazolin	M.l.1/ AC	20	25	50
Cefoperazon	M.l.1/ AC	50	50	50
Tetracycline	B.c/ E	5	20	100
Chlortetracycline	B.c/ E	10	20	100
Erythromycin	M.l.1/ AC	20	20	40
Tylosin	M.l.1/ AC	20	10	50
Gentamicin	B.s.BGA/ IBGA	20	30	100
Neomycin	B.s.BGA/ IBGA	50	80	1500
Streptomyicin	B.s.BGA/ IBGA	80	100	200

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

used across the world and most laboratories apply a specific approach with a different number and types of bacterial strains and therefore a different number of test plates (31, 37, 38). Methods using between one and 18 plates have been described in the literature. There are also differences in incubation periods, pH values of media and the quantity of media on which the bacteria are cultured. Considering the length of time since the development of microbial methods it is perhaps surprising that relatively few studies have been published on this topic (33, 38). In this paper we have presented a method based on the STAR test but with additional use of two confirmation solutions and LOD for aminoglycosides below the MRL.

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MIKROBIOLOŠKA METODA PETIH PLOŠČ ZA UGOTAVLJANJE TETRACIKLINOV, CEFALOSPORINOV TER MAKROLIDNIH IN AMINOGLIKOZIDNIH ANTIBIOTIKOV V MLEKU

A. Kirbiš

Povzetek: Na področju higiene in nadzora živil se ukvarjamo z analitiko ugotavljanja ostankov antibiotikov v živilih živalskega izvora zaradi težav, ki jih lahko le-ti povzročijo. To so senzibilizacija in alergijske reakcije, širjenje rezistence na antibiotike med mikroorganizmi in ne nazadnje škoda, ki jo lahko povzročijo v živilski industriji, kjer lahko vplivajo na starterske kulture, ki se uporabljajo za proizvodnjo mlečnih in mesnih izdelkov.

Metode, ki se uporabljajo za ugotavljanje ostankov antibiotikov v živilih živalskega izvora, so mikrobiološke, imunoencimske in kemijske. Mikrobiološke metode se uporabljajo kot presejalne oziroma screenig metode in so vedno prvi izbor pri tovrstni analitiki.

Namen raziskave je bil uvedba mikrobiološke metode za ugotavljanje antibiotikov s testiranjem in uvedbo testnih sevov bakterij in ugotavljanje minimalne količine antibiotikov, ki jih je s posamezno metodo mogoče ugotoviti. Določili smo občutljive in odporne bakterijske seve za skupine makrolidnih, aminoglikozidnih antibiotikov, cefalosporinov in tetraciklinov v mleku. Za ugotavljanje cefalosporinov in makrolidnih antibiotikov uporabljamo bakterijski sev Micrococcus luteus ATCC 9341 kot občutljivi sev, za aminoglikozidne antibiotike bakterijski sev Bacillus subtilis BGA ter za tetracikline Bacillus cereus ATCC 11778.

V veliko pomoč pri poskusu pa so bile t. i. potrditvene spojine. Magnezijev sulfat inaktivira aminoglikozidne antibiotike. Uporabimo ga lahko pri sumljivih vzorcih za potrditev prisotnosti le-teh.

Druga potrditvena snov je bil encim cefalosporinaza, ki inaktivira cefalosporine. Uporabljamo jo, kadar imamo na plošči AC pozitiven rezultat, saj bi sicer lahko prišlo do zamenjave z makrolidnimi antibiotiki, ki jih cefalosporinaza ne inaktivira.

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

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Original Research Paper

DAMAGE CAUSED BY LARGE CARNIVORES ON DOMESTIC GRAZING ANIMALS IN SLOVENIA

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Summary: Brown bear *(Ursus arctos)*, wolf *(Canis lupus)* and Eurasian lynx *(Lynx lynx)* still occur in Europe but they are forced to live in highly fragmented and human-dominated areas. Like in other parts of Europe they are perceived as a major threat to domestic livestock in most of the Slovenia region, especially in places where cohabitation is unavoidable and conflicts are daily. Predators can threaten sheep, cattle and other farmed domestic and wild animals. For livestock producers and government, depredation can be frustrating and costly. There were over 1000 attacks by predators and killed more than 3500 domestic and breeding wild animals between 1995 and 2001. The most frequent predator was bear while the most frequent prey was sheep.
In this article we would like to present in detail the damage on livestock due to depredation between 1995 and 2001 and other findings concerning large carnivores in Slovenia.

Key words: animals, domestic; animals, wild; *carnivora*; predatory behavior; conservation of natural resources - economics; data collection; Slovenia

Introduction

Europe, which used to be a continent of various natural habitats ideal for large predators (wolf, brown bear and Eurasian lynx), offers today only the fragmented remains of the so-called wilderness suitable for large predators. Predators still occur in Europe but they are forced to live in fragmented and human-dominated areas (1). Today we witness a growing public interest in their preservation; however, their predatory way of living gives rise to conflicts with local economic activities, in particular with free-range breeding of domestic animals. The damage caused by the domestic animals depredation is as old as domestication of the animals themselves. Depredation is the most far-reaching problem in managing large carnivores and the main reason for their control or even extinction.

Their precise number of large carnivores in Slovenia is not known; it would, however, be reliable to say that there are approximately 400-600 bears, 50-80 wolves and around 70-100 lynxes. All three predator species are protected in Slovenia so that the hunting bag size for bear, wolf and lynx is determined quantitatively and structurally with a decree issued by the Ministry of Agriculture, Forestry and Food on an annual basis.

Owing to the uncontrolled grazing areas inadequately fenced in, lack of real, i.e. trained guard dogs and to the absence of shepherds, which results in increased costs of breeding, the damage caused by large carnivores and the costs reimbursed by the state covering this damage are continually going up. In addition to the direct material damage, to wit, the loss of a certain number of production animals, such damage undoubtedly causes secondary losses in live unhurt animals traumatised through stress. Moreover, each killing of sheep and goats is extensively covered by the media which results in creating a negative attitude to large carnivores.

The High-Level Pan-European Conference on Agriculture and Biodiversity (2) put emphasis on the priority of the protection of wild animals in

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the areas characterised by the spread of breeding domestic animals and pointed out the necessity of cohabitation between wild animals and livestock breeders (2). At the European level, three statutory provisions govern the protection and preservation of animal species, namely the Convention on the Conservation of European Wildlife and Natural Habitats (3) and the related European directives (the Council Directive 92/43/EEC on the Conservation of Natural Habitats (4) and of Wild Fauna and Flora, and the Council Directive 79/409/EEC on the Conservation of Wild Birds (5)), which demonstrates how seriously Europe is interested in establishing cohabitation between domestic and wild animals.

The damage caused by the domestic animals depredation in Slovenia is year by year higher and may cause over € 300,000 expenses per year for government due to farmers' compensations claims. To reduce the damages and expenses caused by depredations it is in first place important to know as much as possible information's regarding predators, predator attacks, prey, pasture protection, etc. In view of these the aim of this study is to analyse the damage on domestic and breeding wild animals caused by predation and also to get other relevant information's related with this subject.

Material and methods

Between 1996 and 2001, the Ministry of Agriculture, Forestry and Food gathered data on over 1,000 incidents concerning attacks by bear, wolf and lynx on domestic grazing animals and game reared in pens in Slovenia. The data were collected due to farmers' compensations claims for grazing animals killed by protected predators. For analysis, data have been grouped according to predator, prey, month and year of attack and location of attack. We have arranged and analysed these collected data (groups) using basic tools for addition, arrangement and graph maker in computer program Excel (Microsoft Office Excel, 2003).

Results

Between 1995 and 2001 there were over 1000 attacks by predators on domestic grazing animals, pigs and fallow deer (Figure 1) with the pick of the attacks in 2001 (Figure 5). More than 3,500 animals were killed (Figure 1) among which by far the most frequent prey was sheep (Figure 3). More than one third (n=1005) of the animals were killed in 1998 (Figure 5). The most frequent predator was the bear with over 2,000 killed animals, followed by wolf and lynx (Figure 2). The number of attacks on domestic animals becomes substantial in the beginning of spring; the attacks reach their peak in summer and start to decrease by the end of the autumn months (Figure 4). The patterns of the predator attacks and attacked animals are the same in all examined years. In view of the obtained results, damage appears mainly in the west and south parts of Slovenia (Figure 6).

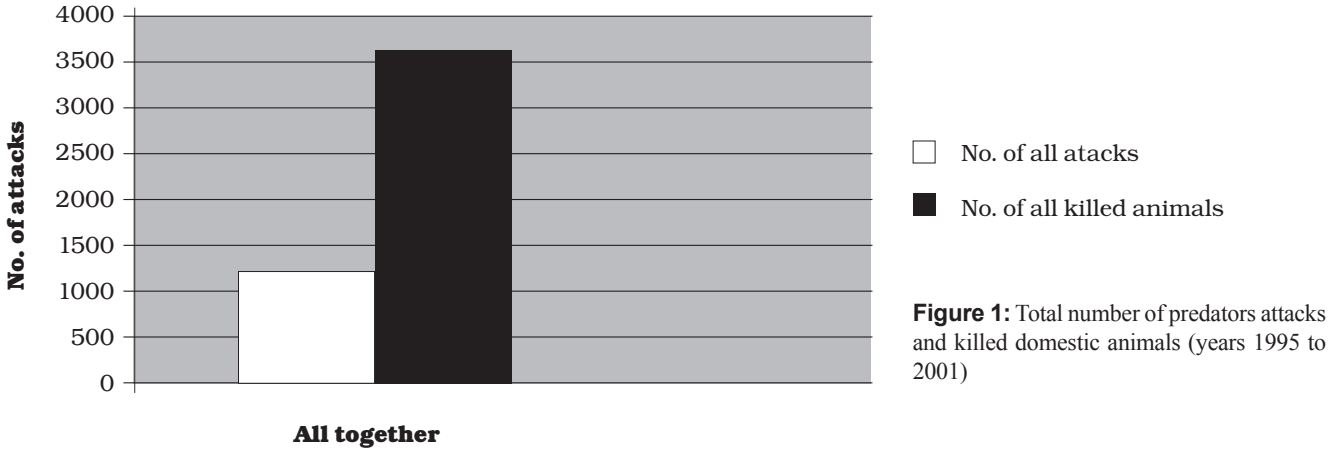

Figure 1: Total number of predators attacks and killed domestic animals (years 1995 to 2001)

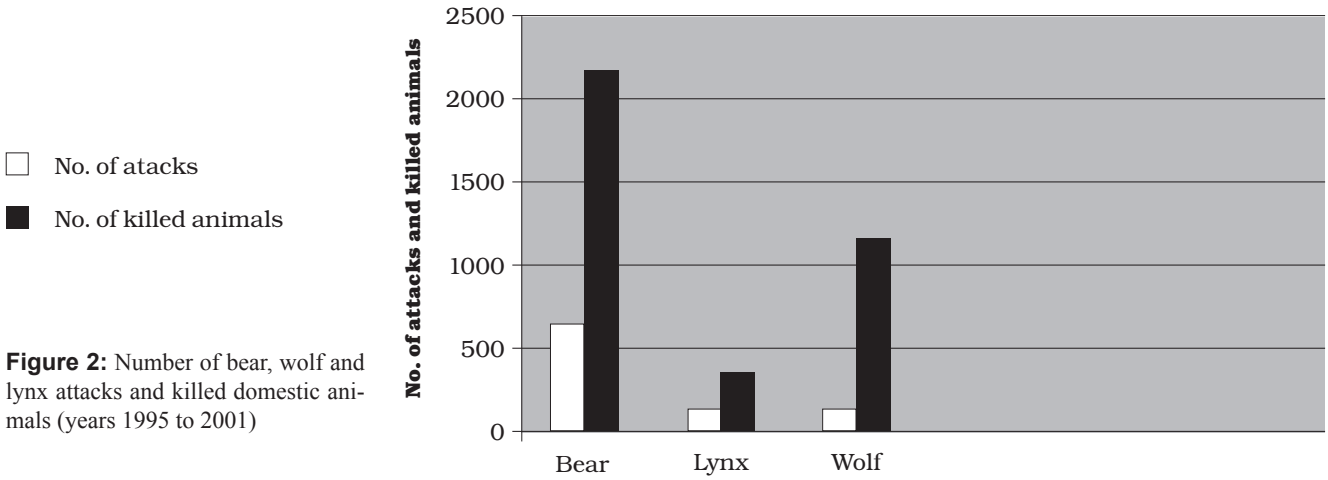

Figure 2: Number of bear, wolf and lynx attacks and killed domestic animals (years 1995 to 2001)

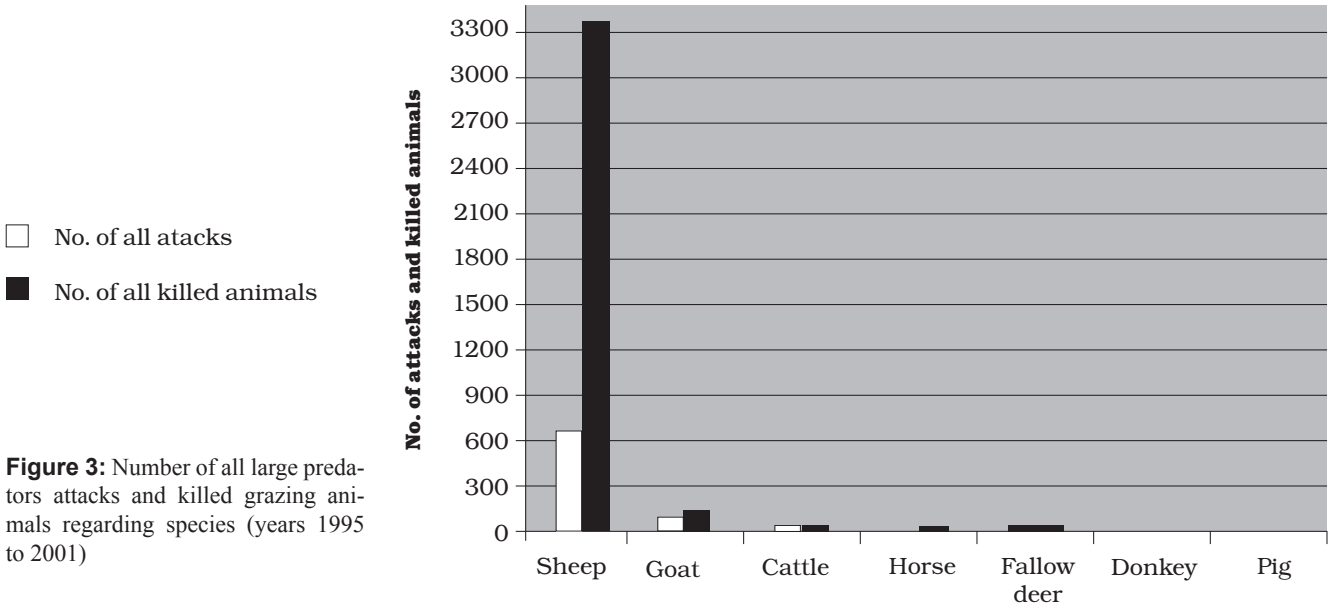

Figure 3: Number of all large predators attacks and killed grazing animals regarding species (years 1995 to 2001)

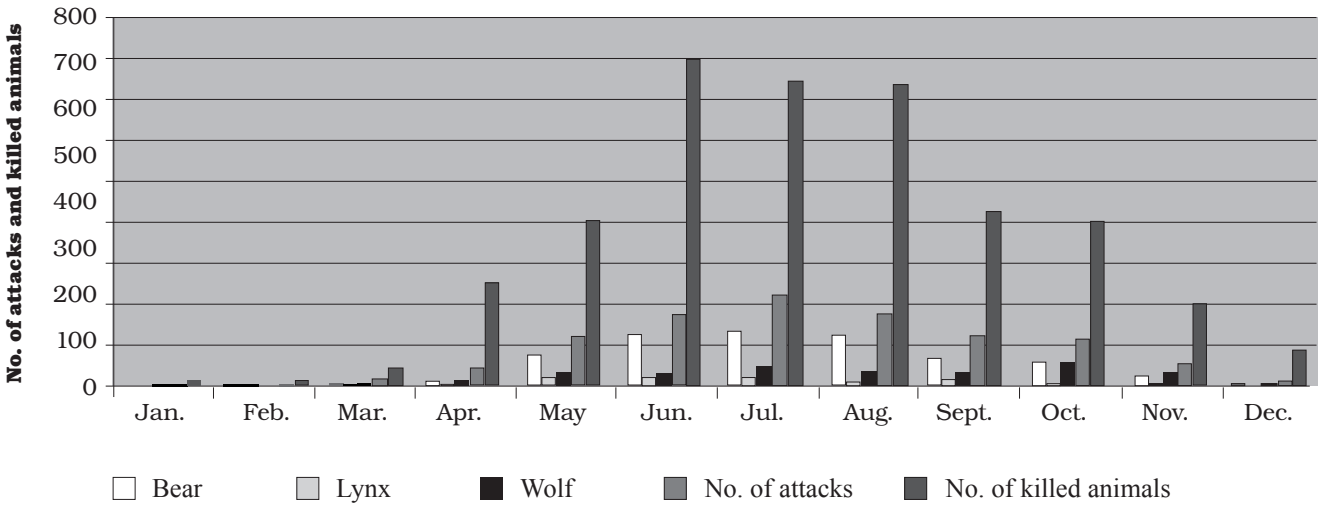

Figure 4: Number of bear, wolf and lynx attacks and killed animals regarding month (years 1995 to 2001)

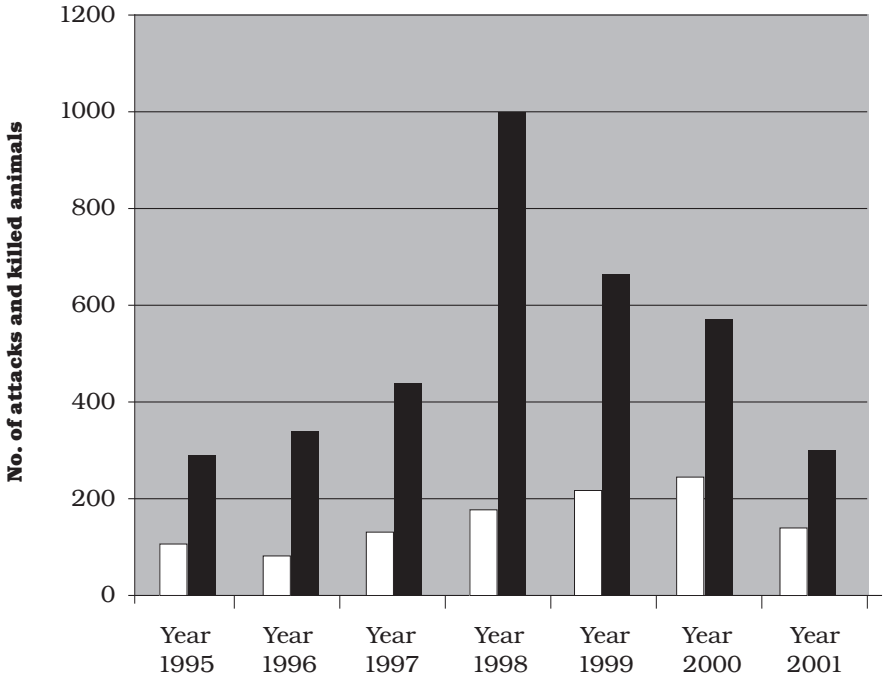

Figure 5: Number of large carnivores attacks and killed animals regarding years (1995 to 2001)

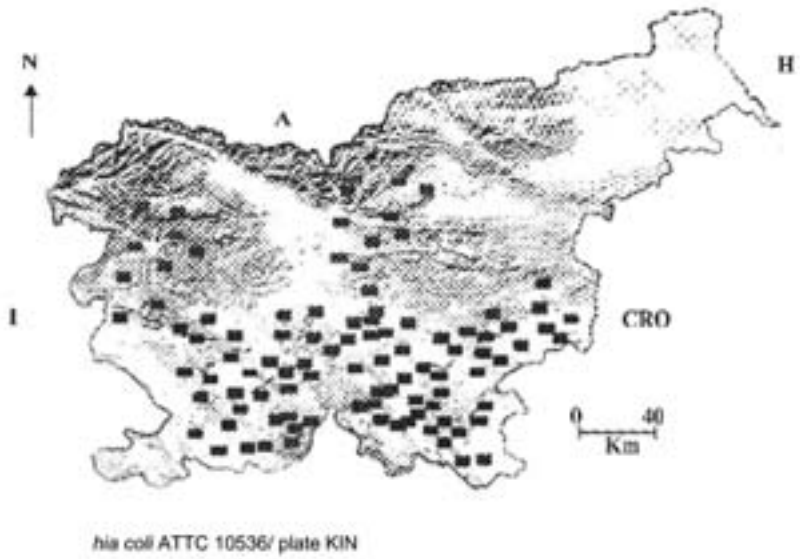

Figure 6: Locations (■) of bear, wolf and lynx attacks on domestic animals in Slovenia (years 1995 to 2001). I – Italy; A – Austria; H – Hungary; CRO – Croatia.

Discussion

In the past, damage was established on field crops and on individual domestic animals while more detailed and accessible data have been available for less than ten years. In Slovenia, the last decade has seen a rapid increase in the number of sheep and goats, for two reasons, i.e. an additional activity in agriculturally less developed areas and a national-level incentive to prevent the overgrowth of arable surfaces. In accordance with the data by the Statistical Office of the Republic of Slovenia (6, 7) the number of sheep increased from 22,000 in 1992 to 105,000 in 2004. At the same time certain changes were seen in the statutory treatment of the status of wild animals in Slovenia.

The brown bear *(Ursus arctos)* is a permanently present predator in Slovenia and since the end of WW II it has been considered under protection and its hunting bag size has been subject to strictly determined criteria and a limited number. At the same time hunting societies and organisations took care of additional foraging in the bear areas: this is how most bears were kept away from settlements and fields. In the last decade, however, due to statutory restrictions most feeding places have become dis-

used. Furthermore, owing to inadequate municipal infrastructure and the uncontrolled slaughter of domestic animals on farms, bears have started coming closer to the settlements and even entering them as food is plentiful. With sheep breeding increasing and spreading to the bear areas or to their vicinity, particularly to the poorly protected pastures, sheep became an easy prey, i.e. food, for the bear, which has also been reported by many foreign authors (8, 9, 10). In accordance with the latest information (11) bears may cause as much as over € 130,000 of damage on domestic grazing animals in a single summer, thus they of all predators cause most difficulty to livestock breeders and the state. Figure 2 clearly demonstrates that the number of killed animals is by far larger than the number of attacks on animals. Bears usually kill several animals in a single attack and is the only one among predators that breaks into night penning where livestock is kept.

In Slovenia, the wolf *(Canis lupus)* has had various statutory statuses over a relatively short period. In the last fifty years there were awards for wolves shot, poisoned, trapped and killed. Today it enjoys complete protection. Until 1990, the wolf had the status of a transitory animal which in winter migrated from neighbouring Croatia (Gorski Kotar) to the south of Slovenia. In this period losses among red deer in the regions of Kočevsko and Notranjsko were recorded. In the last decade, enjoying complete protection, the wolf has become a permanently present predator with documented lairs; at the moment it is present in Slovenia in the already mentioned approximate number.

As distinct from bear and lynx, damage on domestic animals caused by wolves is seen year-round with most frequent attacks on herds during the summer and autumn months (Figure 4). Similarly to bears, wolves also kill several animals in a single attack; consequently, the number of killed animals is twice the number of attacks (Figure 2), which is also shown in the damage caused in 2002 when it exceeded € 150,000 (11) and was the highest so far. Similar problems caused by wolves killing domestic animals are reported in Europe (12, 13, 14) and elsewhere in the world (15, 16).

The lynx *(Lynx lynx)* was reintroduced in Slovenia in 1973. According to certain information, prior to 1973 lynx were present in Slovenia until 1910. Its reintroduction was by far the most successful as far as Europe is concerned so that it is now present in the entire south-west of Slovenia. Before there were so many sheep in Slovenia, lynx caused damage mainly on roe deer. It causes approximately 20,000 of documented damage annually (11), which corresponds to its smaller number among the predators in Slovenia. Similarly to bears, it causes most damage on sheep and goats during the summer months (Figure 4).

There are some differences in number of attacks and killed animals between the years (Figure 5), however sheep as prey undoubtedly occupy by far the first place among domestic animals. It is followed by goats, other domestic animals and fallow deer (Figure 3). As mentioned above, the number of attacks by individual species of predators reflects their number in Slovenia, with bears being the most numerous predator. If the obtained results are analysed, damage occurs mainly in the west and south parts of Slovenia (Figure 6) where suitable conditions enabling the existence of large wild carnivores predominate and at the same time these are the areas where intense sheep-breeding has been brought back to life.

According to the data available to us there is no obvious difference between the attacks in protected and unprotected pastures, which shows how inadequate and insufficient fencing, i.e. protection of domestic animals while grazing, is. Protected pastures are considered to be all those pastures which are protected with any type of fence whatsoever, by a shepherd or a dog while unprotected pastures are those without any protection and control over the animals. In order to have a better insight into the above mentioned types of pastures we would urgently need the data relating to the size of pastures which, however, were not a part of the damage reports.

Most owners put up fences to prevent uncontrolled grazing of sheep and goats but they forget the protection against predators breaking in. The old method of control over grazing domestic animals by shepherds and dogs has become almost discontinued, while at the same time these pastures are irresistibly spreading to the forest edge and even into the forest, which additionally represents favourable conditions for the attacks by predators on domestic animals. The fact that in Slovenia most sheep stay out overnight, facilitates the attacks by predators.

At present, there is a project underway in Slovenia, the main purpose of which is to test various systems of protection of domestic animals, which would enable at first sight an absurd situation: a statutory protection of large predators and their

cohabitation with domestic grazing species, with preventing significant damage at the same time. It has been decided to solve the problem of cohabitation of domestic animals and predators living in the same area so as to observe European guidelines relating to the protection of large predators and the simultaneous encouragement of sheep-breeding. Such a decision is also supported by two studies, the first relating to bears conducted in Norway (8) and the second relating to lynx conducted in France (17), which report that hunting bag size of predators is not a solution, as their place is usually taken by a new predator, while Breitenmoser (18) sees the solution in a changed method of sheep and goat-breeding in the areas where pastures are mostly unprotected. It is, however, reasonable to also take into consideration the fact that in the areas with a high predator-related risk it will probably be necessary to grant priority to predators, i.e. protect them, and move grazing animals to less hazardous areas (8).

Acknowledgements

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PREGLED ŠKOD VELIKIH ZVERI NA DOMAČIH PAŠNIH ŽIVALIH V SLOVENIJI

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Povzetek: Rjavi medved *(Ursus arctos)*, volk *(Canis lupus)* in ris *(Lynx lynx)* se še vedno pojavljajo v Evropi, kjer pa so prisiljeni živeti v zelo razdrobljenih in z ljudmi poseljenih področjih. Tako kot v ostalih evropskih državah, predstavljajo velike zveri tudi v Sloveniji glavno grožnjo domačim pašnim živalim. Ta je še posebej evidentna v krajih kjer se sobivanju ni mogoče izogniti in prihaja do stikov vsak dan. Predatorji lahko ogrožajo ovce, govedo kot tudi druge domače in gojene divje pašne živali. Za rejce in vlado predstavlja predatorstvo veliko neprijetnost, ki je povrhu še zelo draga. Med leti 1995 in 2001 je bilo zabeleženo preko 1000 napadov in ubitih preko 3500 domačih in gojenih divjih živali. Najpogoste je napade povzročal medved, med tem, ko je bila najpogostejša žrtev napadov ovca.
V članku bi radi podrobneje predstavili škodo na domačih pašnih in gojenih divjih živalih, ki so jo med leti 1995 in 2001 v Sloveniji povzročili veliki predatorji.

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

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Original Research Paper

REDUCED SEMINIFEROUS TUBULE DIAMETER IN MICE NEONATALLY EXPOSED TO PERFUME

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Summary: Odors play important roles in rodent social behavior and reproduction. Pheromones, acting through vomeronasal organ, influence pubertal development, estrus cycling and implantation in female mice. Therefore, we examined whether constant exposure to strong odor (perfume) could influence postnatal development of male reproductive organs. A breeding pairs of mice (one male and one female) were constantly exposed to perfume in cages with filter tops for one week before mating, throughout pregnancy and until weaning of the pups. No difference was observed in litter size or time between pregnancies. Male pups were sacrificed at 6, 16, 19 and 50 days of life and their testes fixed in Bouin's solution. Separate group of sexually mature (60 days old) male mice was exposed to perfume for 12 days and blood collected on 4, 8 and 12 day of exposure. Immunohistochemical staining using antibodies against 3ß-HSD, antimullerian hormone and proliferating cell nuclear antigen did not reveal major differences in postnatal development of testicular cells. Testosterone levels in blood collected from sexually mature mice were lower in a group exposed to perfume in comparison to control group, but difference was not statistically significant. However, seminiferous tubule diameter was reduced ($p<0.05$) in group of male mice exposed perinataly to perfume in comparison to control group. Although the present study did not reveal a major effect of constant exposure perfume on reproductive function in mice, reduced tubule diameter could suggest moderate changes in hormone levels such as FSH, which is known to influence Sertoli cell proliferation.

Key words: pheromones - analysis; perfume - adverse effects; testis - growth and development - cytology; mice

Introduction

Odors, or more specifically pheromones, play important roles in rodent reproduction. Pheromones and functional vomeronasal organ are necessary for mating behavior, sex discrimination, and also aggressive behavior in rodents. Exposure to adult male pheromones triggers early onset of puberty in juvenile female mice and similarly, exposure to male pheromones would induce cycling in anestric female mice (reviewed in (1-3). Pheromones act through vomeronasal organ, which has direct connections to hypothalamus through accessory olfactory bulb (3). Pheromones have, at least in rodents, profound effects on hormone secretion. One of the most interesting effects of pheromones is so called Bruce effect (4). Female mice exposed to different male during first four days of pregnancy (before implantation) will abort as a consequence of exposure to pheromones of male that did not impregnate her. In this case, male pheromones influence dopamine release from hypothalamus, which inhibits prolactin secretion from pituitary and these reduced levels of prolactin could not sustain corpus luteum, what would finally cause miscarriage (3). Pheromones could also influence sperm motility in male mice as reported in study by Koyama and Kamimura (5). Pheromones in vomeronasal organs act through specific receptors, divided into two distinct families, V1R and V2R and are encoded by approximately 300 genes (6-8). Although several pheromones were identified in recent years, the chemical structure of many pheromones is not yet known. Some studies have shown that VNO neurons are very sensitive to different chemical stimuli and furthermore, they also respond to some volatile substances (9-11).

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Postnatal development of the testis is a critical period for function of this organ in adult life (12). Sertoli cells proliferate only for a limited period during prenatal and postnatal life and by day 18 postnatally, when Sertoli cell proliferation cease, their numbers are final for the rest of life in most animals (13). Sertoli cell proliferation is hormonally regulated; FSH was reported to stimulate Sertoli cell proliferation while thyroid hormones T3 and T4 are most likely involved in cessation of Sertoli cell proliferation (13, 14). During first postnatal days, fetal Leydig cells disappear from the testis and are gradually replaced by adult type of Leydig cells, which differ from their fetal counterparts in many aspects. Although these processes are not well understood, it is most likely that this change of Leydig cell populations is also influenced by reproductive hormones (15, 16). Therefore, the proper function of hypothalamic – pituitary – gonadal axis in this period is extremely important for proper development of testis and any potential disturbances in this period could have profound effects on function of adult testis.

Perfumes are ubiquitously used chemicals with strong odor in humans that act through main olfactory epithelium. However, many different substances with strong odors influence also vomeronasal organ and/or accessory olfactory bulb and this could modulate secretion of pituitary hormones. Therefore, the aim of the present study was to determine whether constant exposure to perfume could influence reproductive function and pre- and postnatal development of the testis, possibly by disturbing normal pheromonal signaling.

Material and methods

Animals

Sexually mature (60 – 70 days old) BALB/c male and female mice were bred in cages with filtertops. Minisart filters (Sartorius, Goetingen, Germany) were soaked with perfume of undisclosed producer and placed in the cage with individual male and female mice. Minisart filters were chosen as preferred way of exposure as they are encapsulated in plastic frame that prevented direct access of animals to perfume, but enabled constant evaporation of perfume into environment. Every third day, exactly 0.5 ml of widely used perfume from undisclosed producer was added to the filter. Male and female mice were kept under influence of perfume for one week separately, after which one male and one female mouse was joined in a single cage, containing one Minisart filter soaked with perfume. Mice were exposed to perfume throughout pregnancy and lactation period until weaning at 21 days. Pups were sacrificed at 6, 16, 19 and 50 day of age. 50 days old mice were exposed to perfume only until weaning. 6 days old mice were sacrificed by CO_2 exposure followed by cervical dislocation. Mice old 16, 19 and 50 days were anaesthetized by a mixture of ketamine (2.5mg/animal), Xylazine (0.25mg/animal) and Acepromazine (0.05mg/animal) and perfused with Bouin's solution. Testes were extracted and post-fixed in Bouin's solution for 4 to 20 hours depending on size. Subsequently, testes were processed into paraffin wax using standard procedures. Separate group of 5 sexually mature males was exposed to perfume for 12 days and blood was collected from saphenous vein on day 4, 8 and 12 of experiment. For all experiments, control groups were bred in identical conditions in filter top cages but in a separate room to prevent any cross-exposure to perfume. All animal work was done according to EU directive and NIH guidelines and with permission from Veterinary commission of Slovenia.

Immunohistochemistry

Sections (5 microns) were mounted on slides coated with 3-aminopropyl triethoxy-silane (TESPA; Sigma, Taufkirchen, Germany) and dried overnight at 50°C. Before incubation with primary antibodies, sections were dewaxed, rehydrated in graded ethanols, washed in water and phosphate buffer saline (PBS) followed by blocking endogenous peroxidase by incubating the section for 30 min in 1% H_2O_2 in PBS. Sections used for immunostaining with antisera against antimullerian hormone (AMH) and PCNA were subjected to antigen retrieval by microwaving in 0.01M citrate buffer (pH 6.0) on full power for 20 min, and thereafter left standing for 20 min without disturbance. Sections were then washed for 5 min in PBS and blocked using normal rabbit (3ß-HSD and AMH) or mouse (PCNA) serum (Dako, High Wycombe Bucks, UK) diluted 1:5 in PBS. Polyclonal rabbit antibodies directed against AMH (gift from Dr. Nathalie Josso, France) were used at a dilution of 1:100, polyclonal rabbit antibodies against 3ß-HSD (gift from Dr. Ian Mason, Edinburgh, Scotland) were used at a dilution of 1:500 and monoclonal mouse antibodies against PCNA (Dako) were used in 1:100 dilution. All primary antibodies were diluted in PBS containing 20% normal goat (AMH, 3ß-HSD) or rab-

bit (PCNA) serum. Sections were incubated with primary antibodies overnight at 4°C in humid chamber. The following day coverslips were removed, sections washed twice in PBS (5 min each wash), incubated for 30 min with goat anti-rabbit (AMH, 3ß-HSD) or rabbit anti-mouse (PCNA) immunoglobulins (Dako) diluted 1:100 in PBS and then washed again in PBS (2 times 5 min). For detection of bound antibodies, sections were first incubated with rabbit (AMH, 3ß-HSD) or mouse (PCNA) peroxidase-antiperoxidase complex (Dako) for 30 min and washed 2 times in PBS (5 min each). Color reaction product was developed by incubating sections in a mixture of 0.05% (w/v) 3,3'-diaminobenzidine tetra-hydrochloride (DAB, Sigma) in 0.05M Tris-HCl, pH 7.4 and 0.01% hydrogen peroxide. After 5-15 min, sections were washed in distilled water, counterstained with hematoxylin, dehydrated in graded ethanols, cleared in xylene and coverslipped using Pertex mounting medium (CellPath plc, Hemel Hempstead, UK). Specificity of the antibodies was controlled by using non-immune rabbit serum instead of primary antibodies.

Tubule diameter measurements

Testes from 50 days old mice (5 experimental and 5 control mice), exposed neonatally to perfume, were cut on a microtome at 5 µm and stained with hematoxyline and eosin using standard procedures. Seminiferous tubule diameter was measured at 40x magnification using measuring eyepiece. Diameter of 50 randomly chosen tubules was measured in each testis from each animal. Only tubules considered perfectly round by eye examination were used for diameter measurement to make sure there was no error due to oblique cutting of tubules.

Testosterone measurements

Blood was collected from all animals from saphenous vein at 12.00 am exactly. Heparinized blood was centrifuged at 2000 rpm for 3 minutes. Plasma was removed and stored at -20°C until use. Testosterone was measured at Department for clinical biochemistry, Clinical center, Ljubljana using Testosterone direct radioimmunoassay kit from Diasorin (Saluggia, Italy).

Statistical analyses

Microsoft excel was used for all statistical analyses. ANOVA was performed to determine whether there are significant differences between groups. Where implicated, student T-test was additionally used to confirm difference between two groups and $p<0.05$ was considered as significant.

Results

Litter size and time between pregnancies

All together, 8 females with total of 16 litters were included in control group and 7 females with total of 16 litters were included in experimental group. Both litter size and time between pregnancies did not differ significantly between control and experimental groups. Average litter size in control group was 7.19 ± 0.58 and 8.06 ± 0.71 (mean ± S.E.) in perfume exposed group. Time between pregnancies was 35.87 ± 4.40 days in control group and 32.24 ± 2.25 days in perfume exposed group (mean ± S.E.).

Immunohistochemical staining

Testes from 6 days old animals were used for immunohistochemical staining with antibodies against antimullerian hormone and 3ß-HSD. No major difference was observed between control and perfume exposed groups (Fig. 1). Testes from 16 and 19 days old mice were used for immunohistochemical staining with antibodies against 3ß-HSD (not shown) and PCNA (Fig. 2). Again, no obvious difference was observed between control and perfume exposed groups.

Seminiferous tubule diameter

Measurement of seminiferous tubule diameter revealed small, but statistically significant ($p = 0.013$) difference between control and experimental group. Tubule diameter was 169.45 ± 1.48 µm in control group and 164.52 ± 1.30 µm in perfume exposed group (mean ± S.E.).

Testosterone measurements

Testosterone levels measured in plasma were lower in experimental group in comparison to control group, although the difference did not reach statistical significance. Testosterone levels in control group were 23.6 ± 8.3 nMol/L and 13.88 ± 7.25 nMol/L in perfume exposed group (mean ± S.E.).

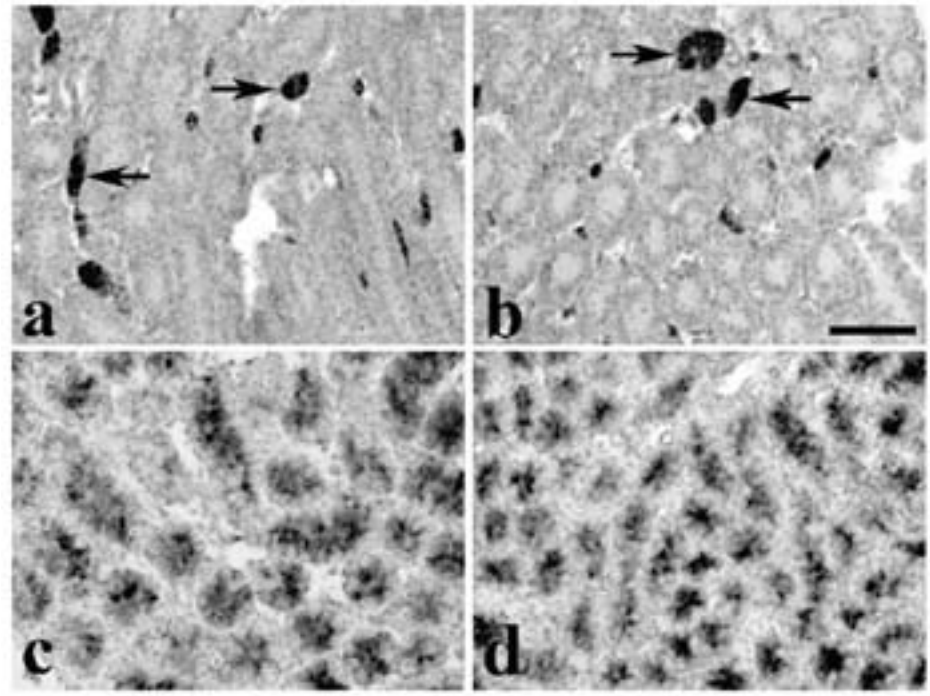

Figure 1: Immunostaining with antibodies against 3ß-HSD (a, b) revealed the presence of grouped fetal Leydig cells (arrows) and there was no difference observed between control (a) and perfume exposed (b) group at 6 days of age. Immunostaining using antibodies against AMH (c, d) showed that majority of Sertoli cells within the testicular cords still expressed AMH at 6 days of age and again, there was no difference between perfume exposed (d) and control (c) group of mice (bar = 50μm).

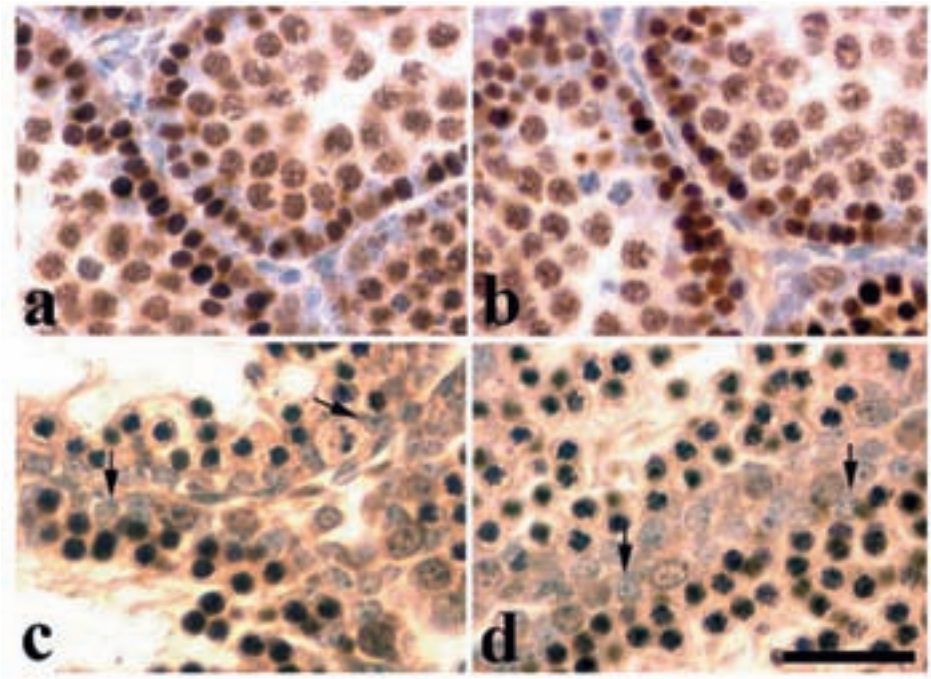

Figure 2: Immunostaining with antibodies against PCNA revealed positive reaction in majority of tubular cells including Sertoli cells from both control (a) and perfume exposed (b) testes at 16 days of age. At 19 days of age, Sertoli cells (arrows) were negative for PCNA expression in both control (c) and perfume exposed (d) groups suggesting that Sertoli cell proliferation stopped (bar = 50 μm).

Discussion

In many mammals, smell and not vision is the main sensory input influencing animal life. In rodents, odors are important for animal orientation, recognizing their environments and also for social interactions. Social interactions and especially sexual encounters are thought to be mainly influenced by pheromones that are detected by vomeronasal organ (reviewed in 2, 3). Although it is not clear whether pheromones are important also in primates and especially humans, they have profound roles in regulating reproductive function in rodents. Pheromones produce several significant effects in female mice such as Whitten, Bruce and Vanderbergh effects (reviewed in 3), at least partially through modulating LH and prolactin secretion (3). Two classes of putative pheromone receptors have been

identified and it is now established that rodents poses about 300 different pheromone receptors, suggesting that many different substances could acts as pheromones (6-8, 17). Although pheromones and odors are usually different substances, it is possible that some strong odors might also act through vomeronasal organs or influence pheromone receptors, and some studies have shown that volatile compounds indeed stimulate vomeronasal neurons (10, 11). Pheromones are necessary for normal sexual behavior and reproductive function in mouse and functional vomeronasal organs are needed for normal mating and aggressive behavior in mice. In our study, pairs of male and female mice were housed together in presence of strong odor from perfume to examine whether constant exposure to volatile odor could influence reproductive performance. Neither litter size nor time between pregnancies differed significantly between control and perfume exposed mice, suggesting that perfume used in our experiment did not disturb normal pheromonal signaling through vomeronasal organ, at least not enough to disturb normal reproductive behaviors such as estrus detection and mating in mice.

Vomeronasal neurons project directly to the accessory olfactory bulb, which has connections to the hypothalamus, a master center for regulation of most endocrine processes in mammalian organism (1, 3). Some pheromone effects are directly connected to differential secretion of pituitary hormones such as LH, FSH and prolactin (18-20). For example, Bruce effect is a result of reduced prolactin levels that prevent implantation and reduced prolactin secretion is caused by increased dopamine secretion from hypothalamus (20). Pheromone effects are best known in female rodents. However, since pheromones in females could modulate gonadotropin and prolactin secretion, we hypothesized that constant exposure to strong odors might also influence testis development in postnatal period. This is a period when important changes occur in testis, with both Sertoli and Leydig cells differentiating into adult cells. Pituitary hormones are involved in these processes and careful hormonal regulation is necessary to ensure proper development and differentiation of different testicular cells (12, 15). In the present study, both Sertoli and Leydig cell differentiation was monitored by immunochemical staining with markers for either Sertoli or Leydig cell differentiation. No changes were observed either in AMH or PCNA immunostaining, suggesting that both postnatal maturation of Sertoli cells and cessation of their proliferation were not greatly affected by constant exposure to strong odor. Similarly, the intensity of staining as well as numbers of Leydig cells (as determined by qualitative observations) was not obviously different between experimental groups. However, interestingly, seminiferous tubule diameter was moderately, although statistically significantly reduced in animals perinataly exposed to perfume. Reduced tubule diameter could reflect either reduced Sertoli cell number or defective spermatogenesis. We did histological analyses of testes at 50 days of age, 2 days after first wave of spermatogenesis should be completed. No differences in spermatogenesis between both groups were observed, suggesting that first cycle of spermatogenesis was normally completed in both groups of animals as we observed tubules of all stages in all 50 days old samples. Therefore, reduced tubule diameter could reflect reduced numbers of Sertoli cells. Sertoli cells proliferate only during postnatal life and their proliferation normally cease by day 18 (13). To determine whether there are any differences in Sertoli cell proliferation, we used immunohistochemical staining with antibodies against PCNA, marker of proliferating cells (21). On day 16 postnatally, all Sertoli cells were still positive for this marker and on day 19, all Sertoli cells were negative. This would suggest either that there is no change in timing of cessation of Sertoli cell proliferation or changes were so subtle that we did not observe them with experimental design. Since differences in tubule diameter were small, it is possible that only short difference, perhaps few hours, occurs in cessation of proliferation, or, perhaps more likely, that the rate at which Sertoli cells proliferate was slightly different between experimental groups. This later possibility might be more plausible knowing that FSH, secreted from pituitary has effect on Sertoli cell proliferation and several studies have shown that pheromones in mice could modulate FSH secretion (22, 23). However, in the present study we did not measure FSH levels to be able to confirm any changes in secretion of this hormone.

In conclusion, our study demonstrated that there is no major effect of constant exposure to perfume on mouse reproductive performance. This could suggest that perfumes do not contain substances that would strongly influence function of vomeronasal neurons or perhaps that vomeronasal organ does not importantly affect secretion of any reproductive hormones in normal breeding conditions. However, small difference in seminiferous tubule

diameter suggests that constant exposure to perfume might produce moderate effects on postnatal development of reproductive organs.

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ZMANJŠAN PREMER SEMENSKIH CEVK PRI MIŠKAH, IZPOSTAVLJENIH PARFUMU OB ROJSTVU DO ODSTAVITVE

K. Kovačevič, T. Budefeld, G. Majdič

Povzetek: Vonji imajo pri glodavcih zelo pomembno vlogo pri socialnem obnašanju in obnašanju, povezanem z razmnoževanjem. Feromoni, ki jih organizem zaznava z vomeronazalnim organom, vplivajo na začetek pubertete, delovanje spolnega ciklusa in vsaditev zarodkov pri miših. Zaradi pomembne vloge vonjev za delovanje spolnega sistema smo v opisani raziskavi ugotavljali, ali lahko stalna izpostavljenost močnemu vonju ga oddaja parfum, vpliva na razvoj moških spolnih organov po rojstvu. Pari miši (en samec in ena samica) so bili stalno izpostavljeni parfumu v kletkah s filtrskimi pokrovi, in sicer en teden pred parjenjem, med parjenjem in do odstavitve mladičev. Med kontrolno (skupina brez parfuma) in poskusno skupino ni bilo razlik v številu živorojenih mladičev. Moške potomce smo žrtvovali na 6., 16., 19. in 50. dan starosti in njihova moda učvrstili v bouinovem učvrščevalcu. Posebna skupina spolno zrelih živali (starih 60 dni) je bila izpostavljena parfumu 12 dni, v tem času pa smo jim 4., 8., in 12. dan odvzeli kri. Imunohistokemično barvanje z uporabo protiteles proti antimulerjevemu hormonu, 3beta-hidroksi steroidni dehidrogenazi in antigenu delečih se celic ni pokazalo nobenih očitnih razlik v razvoju mod med kontrolno in poskusno skupino. Raven testosterona je bila v skupini, izpostavljeni vplivu parfuma sicer nižja kot v kontrolni skupini, vendar pa razlika ni bila statistično značilna. Ugotovili pa smo statistično značilno razliko v premeru semenskih cevk pri 50 dni starih živalih. Premer je bil manjši ($p<0.05$) pri samcih, ki so bili v obdobju po rojstvu izpostavljeni vplivu parfuma v primerjavi s samci iz kontrolne skupine. Čeprav v opisani raziskavi nismo ugotovili pomembnih razlik v dozorevanju mod po rojstvu med kontrolno in poskusno skupino pa manjši premer semenskih cevk pri samcih izpostavljenih vplivu parfuma, kaže na možnost motnje v delovanju nekaterih hormonov, kot je FSH, ki vplivajo na deljenje sertolijevih celic in s tem na kasnejši premer semenskih cevk v zgodnjem obdobju po rojstvu.

Ključne besede: feromoni - analize; parfum - škodljivi učinki; testis - citologija - rast in razvoj; miši

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IZDELKI ZA MOLEKULARNO BIOLOGIJO

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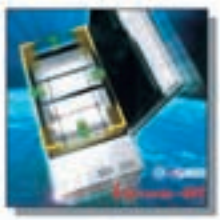

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DIAGNOSTIKA MIKOPLAZEM IN LEGIONEL

HPLC in GC POTROŠNI MATERIAL

SLOVENSKI MEDICINSKI SLOVAR

TRETJA RAZŠIRJENA IZDAJA

Slovar je nastal kot plod četrtstoletnega sodelovanja okrog 150 avtorjev, urednikov, jezikovnih svetovalcev in računalniških strokovnjakov. **Knjižna izdaja 2002** obsega preko 1000 strani ter vsebuje več kot 60.000 biomedicinskih terminov. Namen je dati uporabnikom navodila za pravilno pisanje, izgovarjanje, pregibanje in kratko razlago pomena teh terminov. Med drugim zajema slovenske, poslovenjene in izvirno zapisane grško-latinske termine, generična imena zdravilnih učinkovin, mednarodno sprejete krajšave, osebnosti iz domače in svetovne zgodovine medicine.

Kot plod sodelovanja med Medicinsko fakulteto v Ljubljani in farmacevtsko družbo Lek, Verovškova 57, Ljubljana, je **2003 izšla tudi druga, elektronska izdaja** slovarja. Več informacij je na www.lek.si/slovar. Prednost elektronske izdaje je predvsem preprosto iskanje gesel, tudi njihovih kombinacij, možnost iztiskovanja in vstavljanja lastnih zabeležk. Tiskana verzija pa omogoča udobnejšo čitljivost in preglednost obsežnejših geselskih člankov.

Tretja, razširjena izdaja bo obsegala okrog 1100 strani ter bo vsebovala za okrog 10 % več gradiva kot druga. Je rezultat dela okrog 60 avtorjev, urednikov in svetovalcev, deloma novih deloma starih. Tretja izdaja pomeni aktualizacijo gradiva. Izid je predviden konec leta 2006.

Slovar bo prispeval k boljši izobraženosti širokega kroga uporabnikov (biomedicinskih strokovnjakov, študentov, novinarjev in drugih). Slovar je nepogrešljiv za študente in diplomante medicine, stomatologije, veterine, farmacije, biologije in drugih biomedicinskih ved, uporabljajo pa ga tudi drugi delavci v zdravstvu (medicinske sestre, zdravstveni tehniki), prevajalci, novinarji in pacienti.

Naročam _____ izvod(ov) 3. izdaje Slovenskega medicinskega slovarja.
Cena knjige po izidu bo 30.000 tolarjev (125,19 evra). Do izida pa veljajo prednaročniške cene. Kupci prve knjižne izdaje, ki to dokažejo z iztrganim prvim listom iz knjige, imajo poseben popust pri prednaročilu – 20.000 tolarjev (83,46 evra). Plačal bom (označite način plačila):

- ☐ po položnici v enem obroku 24.000 tolarjev (100,15 evra)
- ☐ v treh obrokih po 9.000 tolarjev (37,56 evra)
- ☐ po položnici v enem obroku 20.000 tolarjev (83,46 evra) – sem kupec prve knjižne izdaje

Opomba: Zneski v evrih so informativnega značaja in so preračunani iz tolarskih zneskov po centralnem paritetnem tečaju (1 evro = 239,640 tolarja).

Ime in priimek: _____

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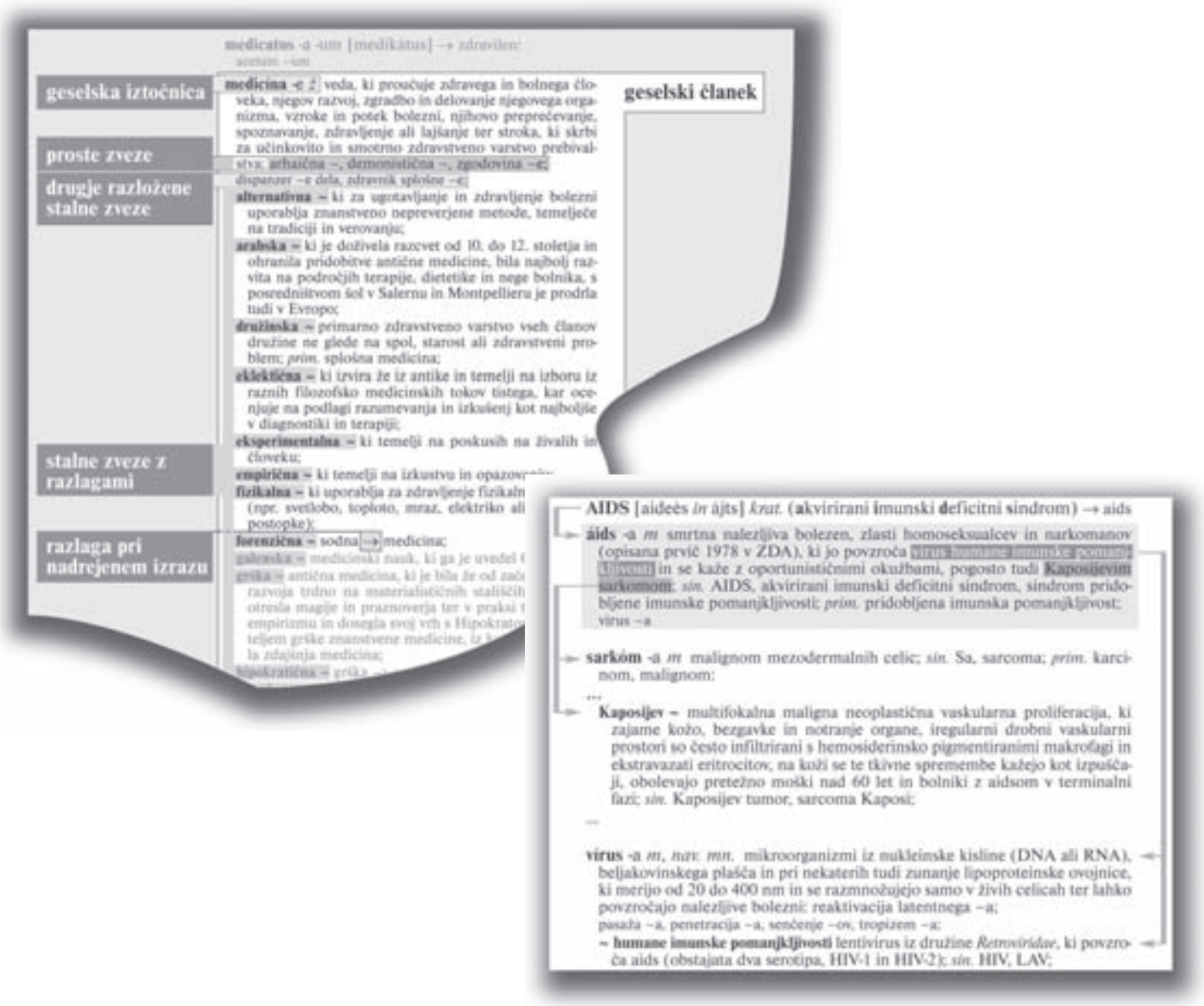

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Contributions should be written in English and should not exceed 12 pages (27 lines per page, approx. 75 characters per line). They should be submitted electronically (preferably to E-mail address, slovetres@vf.uni-lj.si), written in any word processor for Windows. Authors are requested to provide names of three potential reviewers. The text should be double spaced and the lines should be numbered on the left-hand side. The margin on the left-hand side of the page should be 4 cm.

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Under 'Keywords:' (after the colon), keywords should be given. Individual words or word combinations should be separated by semicolons. Scientific papers and papers which present the author's research and findings should also include the following obligatory headings assigned by the author to appropriate parts of the text: Introduction, Materials and methods, Results, Discussion, and References. Review articles should consist of an introduction, sections logically titled according to the content, and references. Information on fund-providers and other matters important for the paper (e.g. technical assistance) should be supplied under 'Acknowledgements', which should be placed before the references. Figure legends should follow the references.

Tables, graphs and diagrams should be logically incorporated in the text file. Original photographs or drawings should be sent as separate files in bmp, jpg or tif format. They should be referred to by type and using Arabic numerals (e.g. Table 1:, Figure 1:, etc.). The colon should be followed by the text or title. All references cited in the text should appear in the References. They should be numbered in the text in the order in which they appear, marked with Arabic numerals placed in parenthesis. The first reference in the text should determine the number and order of the respective source in the References. If the author refers again to a source which has already been used in the text, he should cite the number the source had when it was referred to for the first time. Only works which have been published or are available to the public in any other way may be referred to. Unpublished data, unpublished lectures, personal communications and similar should be mentioned in the references or footnotes at the end of the page on which they appear. Sources in the References should be listed in the order in which they appear in the text. If the source referred to was written by six authors or less, all of them should be cited; in the case of seven or more authors, only the first three should be cited, followed by 'et al.'.

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Examples of references

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

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

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

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

NAVODILA AVTORJEM

Slovenski veterinarski zbornik (Slovenian Veterinary Research) objavlja izvirne prispevke, ki še niso bili objavljeni oz. poslani v objavo drugam. Za vse navedbe v prispevkih so odgovorni avtorji. Uredniška politika obsega publiciranje znanstvenih člankov, preglednih znanstvenih člankov, strokovnih člankov, povzetkov disertacij in drugih prispevkov, kot so kritične presoje o vsebini razprav, objavljenih v zborniku, kratke znanstvene prispevke, pisma uredniku in drugo. Avtorji pošljejo prispevke na naslov uredništva. Glavni urednik pregleda vse prispevke. Za vse članke je obvezna strokovna recenzija, za katero poskrbi uredništvo.

Prispevki naj bodo napisani v angleškem jeziku, z naslovom, povzetkom in ključnimi besedami tudi v slovenščini. Obsegajo naj največ 12 strani, kar pomeni 27 vrstic na stran s približno 75 znaki v vrstici. Prispevki naj bodo poslani v elektronski obliki v katerem koli urejevalniku besedil za okensko okolje. Zaželjena je uporaba elektronske pošte (slovetres@vf.uni-lj.si) in avtorji naj predlagajo tri možne recenzente. Besedilo naj ima dvojni razmik med vrsticami, pri čemer naj bodo vrstice na levi strani oštevilčene. Besedilo naj bo na levi strani od roba oddaljeno 4 cm.

Naslovna stran prispevkov se začne z naslovom, sledi ime in priimek avtorja. Kadar je avtorjev več, jih ločimo z vejicami. V naslednjih vrsticah je v rubriki Addresses of authors: za dvopičjem treba navesti polno ime in priimek ter naslov(e) avtorja(ev), tj. ustanovo, ulico s hišno številko, pošto in kraj. Vse navedene podatke ločujejo vejice. Sledi vrstica, kjer je treba navesti ime ter elektronski (E-mail:) in poštni naslov ter telefonsko številko (Phone:) odgovornega avtorja.

Sledi besedilo povzetka Summary v obsegu 16 do 20 vrstic (približno 1000 do 1500 znakov). V naslednji rubriki Key words: se za dvopičjem navedejo ključne besede. Posamezne besede ali sklopi besed morajo biti ločeni s podpičjem.

Znanstveni članki in tisti, ki so prikaz lastnih raziskav in dognanj, morajo vsebovati še naslednje obvezne rubrike, s katerimi avtor sam naslovi ustrezne dele besedila v prispevku: Introduction, Material and methods, Results, Discussion in References. Pregledni članki naj vsebujejo uvod, poglavja, ki so glede na vsebino smiselno naslovljena, in literaturo. Podatke o financerjih ali drugih zadevah, pomembnih za prispevek, npr. o tehnični pomoči, avtorji navedejo v rubriki Acknowledgements, ki se uvrsti pred rubriko References. Za rubriko References sledijo spremna besedila k slikam.

Priloge, kot so tabele, grafikoni in diagrami naj bodo smiselno vključene v besedilo. Slikovni material naj bo poslan posebej v obliki bmp, jpg, ali tif.

Priloge in slike morajo biti poimenovane z besedami, ki jih opredeljujejo, in arabskimi številkami (npr. Table 1:, Figure 1: itn.). Za dvopičjem sledi besedilo oziroma naslov. Vsi navedki (reference), citirani v besedilu, se morajo nanašati na seznam literature. V besedilu jih je treba oštevilčiti po vrstnem redu, po katerem se pojavljajo, z arabskimi številkami v oklepaju. Prvi navedek v besedilu opredeli številko oziroma vrstni red ustreznega vira v seznamu literature. Če se avtor v besedilu ponovno sklicuje na že uporabljeni vir, navede tisto številko, ki jo je vir dobil pri prvem navedku. Citirana so lahko le dela, ki so tiskana ali kako drugače razmnožena in dostopna javnosti. Neobjavljeni podatki, neobjavljena predavanja, osebna sporočila in podobno naj bodo omenjeni v navedkih ali opombah na koncu tiste strani, kjer so navedeni. V seznamu literature so viri urejeni po vrstnem redu. Če je citirani vir napisalo šest ali manj avtorjev, je treba navesti vse; pri sedmih ali več avtorjih se navedejo prvi trije in doda et al.

Da bi se morebitni popravki lahko objavili v naslednji številki, jih morajo avtorji pravočasno sporočiti glavnemu uredniku.

Načini citiranja

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

Poglavje ali prispevek v knjigi: Baldessarini RJ. Dopamine receptors and clinical medicine. In: Neve KA, Neve RL, eds. The dopamine receptors. Totowa: Human Press, 1996: 475-98.

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