ÚSTAV ZEMĚDĚLSKÝCH A POTRAVINÁŘSKÝCH INFORMACÍ

VETERINÁRNÍ MEDICÍNA

Veterinary Medicine – Czech

ČESKÁ AKADEMIE ZEMĚDĚLSKÝCH VĚD

4

VOLUME 44
PRAHA
APRIL 1999
ISSN 0375-8427

VETERINÁRNÍ MEDICÍNA

Mezinárodní vědecký časopis vydávaný z pověření Ministerstva zemědělství České republiky a pod gescí České akademie zemědělských věd

VETERINARY MEDICINE – CZECH

An international journal published under the authorization by the Ministry of Agriculture and under the direction of the Czech Academy of Agricultural Sciences

World Wide Web (URL): http://www.clark.cz/vri/casopis.htm

Cíl a odborná náplň: Časopis Veterinární medicína uveřejňuje původní vědecké práce a studie typu review ze všech oblastí veterinární medicíny v češtině, slovenštině a angličtině.
Časopis je citován v bibliografickém časopise Current Contents – Agriculture, Biology and Environmental Sciences, a abstrakty z časopisu jsou zahrnuty v těchto databázích: Agris, CAB Abstracts, Current Contents on Diskette – Agriculture, Biology and Environmental Sciences, Czech Agricultural Bibliography, Toxline Plus, WLAS.

Periodicita: Časopis vychází měsíčně (12x ročně), ročník 44 vychází v roce 1999.

Přijímání rukopisů: Rukopisy ve třech vyhotoveních je třeba zaslat na adresu redakce: Ing. Zdeňka Radošová, vedoucí redaktorka, Ústav zemědělských a potravinářských informací, Slezská 7, 120 56 Praha 2, Česká republika. Tel.: +420 2 24 25 79 39, fax: +420 2 24 25 39 38, e-mail: editor@login.cz. Podrobné pokyny pro autory jsou v redakci a na URL adrese http://www.clark.cz/vri/Pokyny.htm.

Informace o předplatném: Objednávky na předplatné jsou přijímány pouze na celý rok (leden–prosinec) a zasílají se na adresu: Ústav zemědělských a potravinářských informací, vydavatelské oddělení, Slezská 7, 120 56 Praha 2. Cena předplatného pro rok 1999 je 696 Kč.

Aims and scope: The journal Veterinární medicína original publishes papers and reviews from all fields of veterinary medicine written in Czech, Slovak or English.
The journal is cited in the bibliographical journal Current Contents – Agriculture, Biology and Environmental Sciences, abstracts from the journal are comprised in the databases: Agris, CAB Abstracts, Current Contents on Diskette – Agriculture, Biology and Environmental Sciences, Czech Agricultural Bibliography, Toxline Plus, WLAS.

Periodicity: The journal is published monthly (12 issues per year), Volume 44 appearing in 1999.

Acceptance of manuscripts: Three copies of manuscript should be addressed to: Ing. Zdeňka Radošová, editor-in-chief, Institute of Agricultural and Food Information, Slezská 7, 120 56 Praha 2, Czech Republic. Tel.: +420 2 24 25 79 39, fax: +420 2 24 25 39 38, e-mail: editor@login.cz. Detailed instructions for authors are available in the editorial office and at URL address http://www.clark.cz/vri/Pokynya.htm.

Subscription information: Subscription orders can be entered only by calendar year (January-December) and should be sent to: Institute of Agricultural and Food Information, Slezská 7, 120 56 Praha 2. Subscription price for 1999 is 159 USD (Europe), 167 USD (overseas).

F 4884

A NEW LIPOID ADJUVANT: PREPARATION AND OBSERVATION OF ITS EFFECTIVENESS

NOVÉ LIPOIDNÉ ADJUVANS: PRÍPRAVA A SLEDOVANIE ÚČINNOSTI

J. Süliová, Z. Beníšek, Š. Švrček, R. Ondrejka, A. Ďurove

University of Veterinary Medicine, Košice, Slovak Republic

ABSTRACT: Authors prepared an experimental lipoid adjuvant (ELA) of the oil-in-water type, based on metabolisable isopropylester of palmitic acid (IPP) intended for veterinary use. Poloxamer 105 and Arlacel A were used as detergents to prepare a high quality stable emulsion. The observation of quality and stability of emulsion enabled to determine the optimum conditions of preparation and ratio of individual components of the adjuvant: IPP – 20%; detergent – 10% (7% Poloxamer 105 + 3% Arlacel A); water – 70%. Authors tested the potency-increasing effect of ELA on immunogenic and antigenic activity of inactivated, concentrated and purified rabies vaccine. The immunogenic activity of this vaccine, when used with ELA (in model experiments on mice), increased approximately twofold. The potentiating effect of ELA on the antigenic activity of rabies vaccine was observed by the authors on laboratory animals (guinea pigs) and on target animal species (domestic dogs and cattle). The level of rabies antibodies, determined by the ELISA method, served as a criterion. The results obtained indicated sufficient potency-increasing capability of ELA which was minimally comparable with that of commercial products.

adjuvant; emulsion of oil-in-water type; isopropyl palmitate; emulsion stability

ABSTRAKT: Autori pripravili experimentálne lipoidné adjuvans (ELA) typu olej vo vode na báze metabolizovateľného izopropylesteru kyseliny palmitovej (IPP), určené pre veterinárne použitie. Ako detergenty pre prípravu kvalitnej a stabilnej emulzie použili Poloxamer 105 a Arlacel A. Sledovaním kvality a stability emulzie stanovili optimálne podmienky prípravy a pomer jednotlivých zložiek adjuvans: IPP – 20 %; detergent – 10 % (7 % poloxamer 105 + 3 % Arlacel A); voda – 70 %. Autori testovali potencujúci účinok ELA na imunogénnu a antigénnu aktivitu inaktivovanej, koncentrovanej a purifikovanej antirabickej vakcíny. Imunogénna aktivita tejto vakcíny pri jej použití s ELA (v modelových pokusoch na myšiach) sa zvýšila približne dvojnásobne. Potencujúci účinok ELA na antigénnu aktivitu antirabickej vakcíny sledovali autori v porovnávacích pokusoch na laboratórnych zvieratách (morčatách), a tiež na cieľových druhoch zvierat (psoch domácich a hovädzom dobytku). Kritériom bola hladina antirabických protilátok, stanovená ELISA metódou. Výsledky ukázali, že ELA má dostatočnú potenčnú schopnosť, minimálne porovnateľnú s komerčnými výrobkami.

adjuvans; emulzia typu olej vo vode; izopropylpalmitát; stabilita emulzie

INTRODUCTION

For more than 70 years, adjuvants have been used to increase the effectiveness of various vaccines or antigens. In 1925 Ramon proved that the immune response to diphtheric and tetanus toxins can be stimulated specifically by an addition of some substances to a vaccine. Tapioca, agar, starch, lecithin and saponin were the first substances used as adjuvants.

The incorporation of an antigen into the water phase of water-in-oil emulsion for the purpose of increasing its immunization effect was first described by Freund et al. (1937). Freund's incomplete adjuvant (FIA) (Freund et al., 1948) has been widely used for experimental purposes and the Freund's complete adjuvant (FCA) exhibits so far unsurpassed capability to stimulate the production of antibodies (Edelman, 1980). Oil adjuvants (water-in-oil – W/O or oil-in-water – O/W emulsions) had played for a long time an important role in increasing the effectiveness of commercial veterinary vaccines. The oil component of emulsions consisted of mineral oils of varying structure. In essence, they all caused more or less serious postvaccination reactions in vaccinated individuals either of local character or with a total effect (Vanselow, 1987; Krejčí, 1988; Toman et al., 1992). Some forms of local reactions were eliminated by using less viscous emulsions (Kimura et al., 1978; Barteling and De Leeuw, 1980; Hubík et al., 1982) or metabolisable natural oils, e.g. soya (Reynolds et al., 1980) or sesame (Kimura et al., 1978). Commercial utilization has been pursued by research activities aimed at the study of adjuvant activity on non-ionic block copolymers which, when incorporated into oil emulsion prepared from natural lipids (e.g. squalane and squalene), induce strong antibody response and exhibit minimum toxicity (Hunter and Bennett, 1984;

Hunter et al., 1991; Takayama et al., 1991; Johnson, 1994).

Another possible way of decreasing the reactogenicity of lipoid adjuvants is evidently the use of fatty acid esters as an oil component of the O/W emulsion (Gall, 1966). They do not induce local chronic inflammation reactions and are fully metabolized (Bomford, 1981).

We prepared in our laboratory an experimental lipoid adjuvant (ELA) of the oil-in-water type using isopropyl-ester of palmitic acid (IPP) as an oil component. We consider this type of adjuvant to be a suitable diluent of inactivated lyophilised vaccines for domestic animals. We observed its potency-increasing effect on humoral immune response in model experiments on laboratory animals and on the most important target animal species – domestic dogs and cattle.

MATERIAL AND METHODS

Composition of ELA

Isopropylpalmitate (isopropylester of palmitic acid) of commercial name Isopropylpalmitate 7200 (Olefina, Belgium) was the oil component selected.

The following emulsifiers were used:

a) nontoxic well-resorbable emulsifier Poloxamer 105 (polyethylene-polypropylene glycol) with commercial name Synperonic PE/L35 (manufacturer ICI, England) of HLB = 18.5 (HLB: hydrophile-lipophile balance);
b) nontoxic and well-resorbable emulsifier Arlacel A (mannide monooleate – $C_{24}H_{46}O_7$; producer SERVA, Germany) of HLB = 3.3.

All emulsion components (water, oil component, emulsifiers) were sterilised at 100 °C for 1 hour.

The quantity of IPP – 20%, optimal for inducing the adjuvant effect when utilizing the oil-in-water system, was determined in preliminary experiments in guinea pigs (not published).

The optimum portion and relative proportions of detergents needed for obtaining a stable emulsion of the oil-in-water type were determined experimentally. An emulsifier of HLB value greater than 10 should be used to prepare O/W emulsion. Therefore we observed the quality of IPP emulsion with an addition of 2–12% of emulsifier Poloxamer 105. To attain better stability of emulsion we used mixtures with different proportions of both emulsifiers mentioned and evaluated their stability.

Preparation of IPP emulsions and checking their quality

Homogenization of all IPP emulsions (containing different ratio of detergents) in water was carried out in a homogenizer Omni-Mixer (DuPont Instruments, USA) at 16 000 r.p.m. Optimum homogenization conditions were determined experimentally by observation of the quality of homogenization as a function of time (1–5 min) and temperature (20–60 °C) based on emulsion of the following composition: 20% IPP + 10% emulsifier (7% Poloxamer 105 + 3% Arlacel A) + 70% water.

Determination of the amount of free palmitic acid in emulsions prepared at various temperatures allowed us to obtain the information about the measure of hydrolysis of isopropylpalmitate in the water medium. Palmitic acid was determined quantitatively by titration with alkali lye using phenolphthalein indicator according to Jureček (1950).

The quality of homogenization was determined by measuring of the diameter of emulsion particles by means of a measuring eyepiece with a micrometer screw of the type Meopta 57216 with 600x magnification. Because of increasing of inaccuracy of measurement below 0.4 μm, approximate values under this limit are presented.

In addition to macroscopic observation, stability of the emulsions prepared was also determined by microscopic observation of changes in the diameter of IPP oil droplets dispersed in water by the method mentioned above. Emulsions were stored at 20 °C and 4 °C and observed for 4 months.

Dynamic viscosity of adjuvants was determined by an Oswald viscosimeter at 20 °C.

Verification of the effectiveness of ELA

The adjuvant effect of ELA was tested in experiments with inactivated purified concentrated rabies vaccine prepared in our laboratory (Süliová et al., 1997; Beníšek et al., 1998). The lyophilised vaccine was resuscitated with this adjuvant.

The potentiating capability of adjuvants on immunogenic activity of rabies vaccine was tested in model experiments on mice. This capability was determined by both the standard method NIH (Seligmann, 1973) and method NRLR (National Reference Laboratory for Rabies, Košice, Slovak Republic) developed in our laboratory (Švrček and Vrtiak, 1980). A vaccine with no adjuvant added was used as a control.

The adjuvant effect was also evaluated by testing the increase in antigenic activity of the rabies vaccine with ELA. This was evaluated first on laboratory animals – guinea pigs. In addition to that various types of commercial oil adjuvants were compared with the adjuvant activity of ELA. The level of rabies antibodies, determined quantitatively by the ELISA method using our own kit (Beníšek et al., 1989; Süliová et al., 1994), served as an indicator.

Additional experiments, carried out on target animal species – domestic dogs and cattle, consisted of vaccination of half of the experimental animals with a rabies vaccine potentiated with ELA and vaccination of the second half of animals (control group) with a vaccine with no adjuvant added. The level of rabies antibodies was also evaluated by means of the ELISA method mentioned above.

The statistical significancy of the rabies antibodies differences between the individual groups are evaluated by Mann-Whitney's *U*-test.

RESULTS

Determination of the optimum composition of ELA

The model experiments on guinea pigs allowed us to determine that 20% IPP emulsion in water should be used to reach the appropriate adjuvant effect. The increase of IPP proportion in the emulsion failed to further increase the adjuvant effectiveness.

The results of determination of the effect of emulsifier quantity on the quality of IPP emulsion are summarised in Tab. I which shows that 8% of the detergent is the minimum portion which must be ensured in order to obtain 0.2 μm microparticles in the emulsion.That is why further experiments were carried out using the following IPP – detergent – water proportions: 20%–10%–70%.

The influence of HLB of the mixture of emulsifiers Poloxamer 105 and Arlacel A on the quality and stability of emulsion is shown in Tab. II. The most suitable ratio of Poloxamer 105 and Arlacel A is 7 : 3 and the resulting value of HLB = 13.94. Tab. III shows the results of determination of dynamic viscosity of this mixture during 4 months observation. It is obvious that no changes in dynamic viscosity were recorded.

I. The influence of emulsion composition (ratio of IPP and Poloxamer 105) on its quality (diameter of droplets in μm)

Composition – % ratio		Diameter of droplets (μm)
IPP	Poloxamer 105	
20	2	2.0
20	3	0.8
20	4	0.4
20	5	cca 0.3
20	6	cca 0.3
20	7	cca 0.3
20	8	cca 0.2
20	9	cca 0.2
20	10	cca 0.2
20	11	cca 0.2
20	12	cca 0.2

Determination of the optimum conditions of emulsion preparation

Temperature of homogenization

Results of the influence of homogenization temperature on the quality of emulsion – size of oil droplets in μm – are presented in Tab. IV. This figure shows that the temperatures between 50 and 60 °C are suitable for preparation of fine emulsions.

Determination of the measure of IPP hydrolysis at elevated temperatures

The quantities of free palmitic acid in the emulsion at homogenization temperatures 20–60 °C are shown in Tab. V. The hydrolysis of IPP is very low, approximately 0.4%. An elevated temperature (up to 60 °C) during the preparation of emulsion causes no further hydrolysis of isopropyl-palmitate.

II. The influence of detergent HLB on the quality and stability of the emulsion

% ratio of detergents		HLB	Diameter of droplets (μm)	Stability of emulsion
Poloxamer 105	Arlacel A			
10.0	0.0	18.50	cca 0.2	creaming after 3 months
9.5	0.5	17.74	0.4	breaking after 2 months
9.0	1.0	16.98	0.8	breaking after 6 weeks
8.5	1.5	16.22	0.8	breaking after 2 months
8.0	2.0	15.46	0.4	creaming after 2.5 months
7.5	2.5	14.70	cca 0.2	stable after 4 months
7.0	3.0	13.94	cca 0.2	stable after 4 months
6.5	3.5	13.18	cca 0.2	stable after 4 months
6.0	4.0	12.42	0.4	breaking after 2 months
5.5	4.5	11.66	0.6	breaking after 2 months
5.0	5.0	10.90	1.0	breaking after 5 days
4.5	5.5	10.14	2.0	breaking within 48 hours
4.0	6.0	9.38	3.0	breaking within 48 hours
3.0	7.0	7.86	2.0	breaking within 48 hours
2.0	8.0	6.34	4.0	breaking within 24 hours
1.0	9.0	4.82	5.0	breaking within 1 hour

III. Dynamic viscosity of the emulsion prepared from 20% IPP, 10% detergents (7% Poloxamer 105 + 3% Arlacel A) and 70% water

Time after preparation	Dynamic viscosity (cP)
Immediately	5.245
1 month	5.193
2 months	5.296
3 months	5.268
4 months	5.305

V. Results of the free palmitic acid titration in emulsion containing 20% IPP, 10% detergents and 70% water as a function of homogenization temperature

Temperature of emulsion	Content of palmitic acid (mg)	% of hydrolysed IPP
20 °C	11.9	0.40
30 °C	12.0	0.41
40 °C	11.8	0.40
50 °C	12.1	0.41
60 °C	11.9	0.40

The time of homogenization

The optimum time of homogenization was determined at 50 °C. Several different emulsion compositions were investigated. Results presented in Tab. VI indicate that the 3 min homogenization period, used at 50 °C and 16 000 r.p.m., is sufficient to obtain emulsion of good quality.

Observation of emulsion stability

The data obtained by macroscopic observation of the stability of IPP emulsions of different composition together with the values of oil droplet size are shown in Tab. II. The observation of changes in the size of droplets during 4 months revealed that in the case of stable emulsions the number of "bigger" droplets (above 10 μm) in 20 viewing fields of the microscope was not higher than 25. In emulsions in which "creaming" occurred this number was equal to 25–45, and in the cases in which more than 45 "bigger" oil particles were present in 20 microscope viewing fields breaking of emulsions could be observed.

IV. The influence of homogenization temperature on the quality of emulsion (diameter of droplets in μm); emulsion composition: 20% IPP + 10% detergents + 70% water

Temperature (°C)	Diameter of droplets (μm)
20	0.8
25	0.7
30	0.6
35	0.5
40	0.4
45	cca 0.3
50	cca 0.2
55	cca 0.2
60	cca 0.2

Storage of prepared emulsions in plastic containers is preferred to the storage in glass vessels.

Verification of the effectiveness of ELA

Results of testing the influence of adjuvants on immunogenic activity of rabies vaccine using the methods NIH and NRLR are presented in Tab. VII. These results prove that adjuvants increase the effectiveness of rabies vaccines approximately twofold.

The experiments on guinea pigs compared various types of commercial oil adjuvants with ELA. Results presented in Tab. VIII point to sufficient potency-increasing capability of ELA, comparable with that of commercial products. Oil adjuvants of the water-in-oil type, i.e. FIA and Al-Span-Oil, show significant better effects on days 14 and 30 ($p < 0.05$ – FIA, or $p < 0.01$ – Al-Span-Oil).

The experiments on cattle (Tab. IX) and domestic dogs (Tab. X) showed pronounced capability of ELA to increase the antigenic activity of rabies vaccines ($p < 0.05$). As early as on day 14, the levels of rabies

VI. Inluence of homogenization time on droplet diameter (μm)

The time of homogenization in minutes	Ratio IPP/detergent – diameter of droplets (μm)				
	20/4	20/5	20/6	20/7	20/10
1.0	0.8	0.6	0.5	0.4	cca 0.3
1.5	0.6	0.6	0.4	cca 0.3	cca 0.3
2.0	0.5	0.5	0.4	cca 0.3	cca 0.3
2.5	0.5	0.4	cca 0.3	cca 0.3	cca 0.2
3.0	0.4	0.4	cca 0.2	cca 0.2	cca 0.2
3.5	0.4	0.4	cca 0.2	cca 0.2	cca 0.2
4.0	0.4	cca 0.3	cca 0.2	cca 0.2	cca 0.2
4.5	0.4	cca 0.3	cca 0.2	cca 0.2	cca 0.2
5.0	0.4	cca 0.3	cca 0.2	cca 0.2	cca 0.2

VII. The influence of ELA on immunogenic activity of a rabies vaccine; experiments on mice

Method	Animal group	ED_{50} (mm^3)	IU/cm^3	IU/vaccine dose
NIH	Vaccine	$5.794 \cdot 10^{-4}$	2.001	2.001
	Vaccine + ELA	$3.035 \cdot 10^{-4}$	3.818	3.818
NRLR	Vaccine	$5.375 \cdot 10^{-4}$	2.157	2.157
	Vaccine + ELA	$2.961 \cdot 10^{-4}$	3.913	3.913

NRLR – National Reference Laboratory of Rabies, Košice, Slovak Republic
ED_{50} (mm^3) – effective vaccine dose (expressed in mm^3) providing sufficient protection against subsequent rabies virus challenge to half of the animals
IU/cm^3 – vaccine effectiveness expressed in international units in 1 cm^3 vaccine

VIII. The influence of various adjuvants on the level of rabies antibodies in the serum of guinea pigs[1]

Adjuvant	Days after vaccination		
	7	14	30
Without adjuvant	$1 : 4^{xx}$	$1 : 8^{xx}$	$1 : 8^{xx}$
ELA	1 : 32	1 : 64	1 : 101.6
Oil adjuvant (Ivanovice na Hané)[2]	$1 : 6.3^{x}$	1 : 50.8	1 : 128
Oil adjuvant (Mevak Nitra)[3]	$1 : 2^{xx}$	1 : 40.3	$1 : 32^{x}$
Freunds incomplete adjuvant[4]	1 : 8	$1 : 256^{x}$	1 : 256
Al-Span-Oil (ÚSOL-Praha)[4]	1 : 12.7	$1 : 512^{xx}$	$1 : 512^{xx}$

1 = Titres of rabies antibodies were determined by means of the ELISA method. Five animals were examined in each group. Final values are 2^n; "n" is arithmetical mean from n_i in groups, where the titres are expressed $1 : 2^{ni}$ (i = 1–5)
2 = Oil adjuvant (oil-in-water type) produced by Bioveta Ivanovice na Hané (Czech Republic) contains: paraffin oil and lanolin
3 = Oil adjuvant (oil-in-water type) produced by Mevak Nitra (Slovak Republic) contains: Bayol and Tween 80
4 = Adjuvant of the water-in-oil type; x = $p < 0.05$; xx = $p < 0.01$
The group ELA was the basic group for comparison

IX. The potentiating effect of ELA on antigenic activity of rabies vaccine; experiments on cattle; results of antibody titration by means of the ELISA test

Group of animals[1]	Days after immunization		
	14	28	60
Vaccine	1 : 17.6	1 : 32.0	1 : 25.6
Vaccine + ELA	$1 : 36.8^{x}$	$1 : 98.6^{x}$	$1 : 55.2^{x}$

1 = The animals have not been vaccinated against rabies before. Another explanation see Tab. VIII. The number of animals was eight in each group (i = 1–8)
x = $p < 0.05$

X. The potentiating effect of ELA on antigenic activity of rabies vaccine; experiments on domestic dogs; results of antibody titration by means of the ELISA test

Group of animals[1]	Days after immunization		
	14	28	60
Vaccine	1 : 287	1 : 512	1 : 203
Vaccine + ELA	$1 : 1024^{x}$	$1 : 1448^{x}$	1 : 512

1 = The animals have not been vaccinated against rabies before. Another explanation see Tab. VIII. The number of animals was six in each group (i = 1–6)
x = $p < 0.05$

antibody titres were on average twice to three times higher after the application of an adjuvant vaccine in comparison with the levels in the control group. On day 60 post-vaccination, the difference was less pronounced, however, in cattle still considerable ($p < 0.05$).

DISCUSSION

The increase in the effectiveness of inactivated veterinary vaccines requires the use of adjuvants. The application of conventional adjuvants that contained mineral oils produced a number of anaphylactoid reactions, mostly in cattle (Krejčí et al., 1988; Toman et al., 1992). Because of the occurrence of undesirable local and total reaction, aluminium hydroxide is most frequently used in veterinary vaccines at present. This substance is however less effective and its subcutaneous administration to some recipients (cats) may induce formation of fibrosarcomas (Hendrick and Brooks, 1994).

Although the adjuvants of the water-in-oil type are undaubtedly very effective, their utilization for mass vaccination in practical medicine is limited (Woodard and Jasman, 1985). Besides inducing the inflammatory and alergic reactions mentioned above another of their disadvantages is their high viscosity and therefore limited applicability. That is why a number of various types of adjuvants (Cox and Coulter, 1997) is applied

at present and frequent use of O/W emulsions has been recorded (Woodard and Jasman, 1985; Playfair and de Souza, 1986; Allison and Byars, 1992; Valensi et al., 1994; Ott et al., 1995).

The use of metabolisable esters of fatty acids as an oil component of lipoid adjuvants of the oil-in-water type was investigated by Bomford (1981). The adjuvant effect of the substances mentioned on the length of chain as well as the degree of saturation of the fatty acid was observed. Of them, the adjuvant effect of isopropylpalmitate most closely resembled that of Freund's incomplete adjuvant. Therefore only aliphatic hydrocarbon chains with 16 or more carbon atoms exhibit sufficient adjuvant activity (Gall, 1966).

Our results obtained by comparing the effect of various adjuvants on antigenic activity of rabies vaccine in the experiments in guinea pigs point to good effectiveness of IPP. The results of quantification of rabies antibodies in the experiment comparing different adjuvants show the highest effectiveness of ELA on day 7 post vaccination; in the subsequent time intervals a stronger immunological response was achieved by means of FIA and Al-Span-Oil. Their higher potentiating effect can be ascribed, however, to the higher content of oil in them (water-in-oil system). The Titer Max adjuvant based on squalene, developed by Bennett et al. (1992), competing with its effect with the Freund's complete adjuvant belongs to the water-in-oil type, too.

The O/W emulsions contain 0.2 μm microdroplets of oil in water (Cox and Coulter, 1997). The HLB value of an emulsifier, ensuring development of high quality O/W emulsion, should be in general higher than 10 while the stability of emulsion is not directly related to the HLB value of the emulsifying mixture used (Hunter et al., 1981). Therefore it is necessary to test experimentally the ratio of the two emulsifying components of different HLB and find the optimum value. In our case (20% IPP and 10% detergents in water) – HLB = 13.94 for the mixture of emulsifiers ensured the highest emulsion stability. Macroscopic examinations supplemented with microscopic measurements of the diameter of oil droplets in the emulsion is one way of evaluation of the stability (Horie et al., 1978). The decrease in surface tension by replacing glass vials by polyethylene for storage of O/W emulsions can further increase the shelf life.

Besides a suitable adjuvant effect of the O/W emulsion an areactogenicity is another of its advantages. Experiments proving its areactogenicity have been carried out and are subject of other our studies (Beníšek et al., 1999).

REFERENCES

Allison A. C., Byars N. E. (1992): Syntex adjuvant formulation. Res. Immunol., *143*, 519–525.

Barteling S. J., De Leeuw P. W. (1980): Further experience with the use of concentrated footh-and-mouth disease virus stored at ultra low temperature. In: Rep. Sess. Res. Group Europ. Commis. FMD, Vienna, June 1980.

Beníšek Z., Süliová J., Švrček Š., Závadová J. (1989): ELISA test pre titráciu antirabických protilátok. Vet. správy, *III*, 25–27.

Beníšek Z., Süliová J., Švrček Š., Závadová J., Ďurove A., Ondrejka R. (1998): The effectiveness of inactivated purified and concentrated experimental rabies vaccine for veterinary use: antigenic activity. Experiments on domestic dogs. Vet. Med. – Czech. *43*, 45–49.

Beníšek Z., Süliová J., Švrček Š., Pauer T., Ďurove A. Ondrejka R. (1999): A new lipoid adjuvant: harmlessness and reactogenicity. Vet. Med. – Czech. *44*, 101–108.

Bennett B., Check I. J., Olsen M. R., Hunter R. L. (1992): A comparison of commercially available adjuvants for use in research. J. Immunol. Method., *153*, 31–40.

Bomford R. (1981): The adjuvant activity of fatty esters. The role of acyl chain length and degree of saturation. Immunology, *44*, 187–192.

Cox J. C., Coulter A. R. (1997): Adjuvants – a classification and review of their modes of action. Vaccine, *15*, 248–256.

Edelman R. (1980): Vaccine adjuvants. Rev. Infect. Dis., 2, 370–383.

Freund J., Casals J., Hismer E. P. (1937): Senzitization and antibody formation after injection of tubercle bacilli and paraffin oil. Proc. Soc. Exp. Biol. Med., *37*, 509–515.

Freund J., Thomson K. J., Hough H. B., Sommer H. E., Pisani T. M. (1948): Antibody formation and sensitization with the aid of adjuvants. J. Immunol., *60*, 383–398.

Gall D. (1966): The adjuvant activity of aliphatic nitrogenous bases. Immunology, *11*, 369–346.

Hendrick M. J., Brooks J. J. (1994): Postvaccinal sarcomas in the cat: histology and immunochemistry. Vet. Pathol., *31*, 126–129.

Horie K., Tanaka S., Akabori T. (1978): Determination of emulsion stability by spectral absorption. I. Relationship between surfactant type, concentration and stability index. Cosmet. Toilet., *93*, 53–62.

Hubík R., Flachsel P., Švec J., Horníček J. (1982): Výzkum vysoce účinných vakcín a metod praktické imunoprofylaxe se zaměřením na ochranu velkochovu prasat před slintavkou. [Průběžná zpráva.] Bioveta, Terezín, 69 s.

Hunter R. L., Bennett B. (1984): Studies on the adjuvant activity of nonionic block polymer surfactants. II. Antibody formation and inflammation related to the structure of triblock and octablock copolymers. J. Immunol., *133*, 1367–1372.

Hunter R. L., Strickland F., Kezdy F. (1981): The adjuvant activity of nonionic block polymer surfactants. I. The role of hydrophile-lipophile balance. J. Immunol., *127*, 1244–1250.

Hunter R., Olsen M., Buynitzky S. (1991): Adjuvant activity of nonionic block copolymers. IV. Effect of molecular weight and formulation on titre and isotype of antibody. Vaccine, *9*, 250–256.

Johnson A. G. (1994): Molecular adjuvants and immunomodulators: New approaches to immunization. Clin. Microbiol. Rev., *7*, 277–289.

Jureček M. (1950): Organická analýza. Praha, Československ. spol. chem.

Kimura J., Nariuchi H., Watanabe T., Matuhasi T., Okayasu I., Hatakeyama S. (1978): Studies on the adjuvant effect of water-in-oil-in-water (w/o/w) emulsion of sesame oil. I. Enhanced and persistent antibody formation by antigen incorporated into the water-in-oil-in-water emulsion. Jap. J. Exp. Med., *48*, 149–154.

Krejčí J., Toman M., Pinka K., Jurák E., Menšík P., Poláček R. (1988): Dosažené poznatky o anafylaktoidných reakcích skotu. [Dílčí zpráva.] Brno, Výzkumný ústav veterinárního lékařství. 35 s.

Ott G., Barchfeld G. L., Chernoff D., Radhakrishnan R., van Hoogevest P., van Nest G. (1995): Design and evaluation of a safe and potent adjuvant for human vaccines. In: Powell M. F., Newman M. J. (eds.): Vaccine Design. The Subunit and Adjuvant Approach. New York, Plenum Press. 227–296.

Playfair J. H. L., de Souza J. B. (1986): Vaccination of mice against malaria with soluble antigens. The effect of detergent, route of injection and adjuvant. Parasite Immunol., *8*, 409–414.

Ramon G. (1925): Sur l'augmentation anormale de l'antitoxine chez les chevaux producteurs de serum antidiphtérique. Bull. Soc. Centr. Méd. Vét., *101*, 227–234.

Reynolds J. A., Harrington D. G., Crabbs C. L., Peters C. J., di Luzio N. M. (1980): Adjuvant activity of a novel metabolisable lipid emulsion with inactivated viral vaccines. Infect. Immun., *28*, 937–943.

Seligmann E. B. (1973): The NIH test for potency. In: Kaplan M., Koprowski H. (eds.): Laboratory Techniques in Rabies. 3rd ed. Geneva, WHO. 279–286.

Süliová J., Beníšek Z., Švrček Š., Ďurove A., Závadová J. (1994): Kvantifikácia hladiny antirabických protilátok v sére vakcínovaných ľudí. Bratisl. Liek. Listy, *95*, 73–77.

Süliová J., Beníšek Z., Švrček Š., Ďurove A., Ondrejka R. (1997): The effectiveness of inactivated purified and concentrated experimental rabies vaccine for veterinary use: immunogenic activity. Vet. Med. – Czech, *42*, 51–56.

Švrček Š., Vrtiak O. J. (1980): Modifikacija količestvennogo metoda dľa opredelenija immunogennoj aktivnosti antirabičeskych vakcin v opyte na myšach. Acta Vet. Sci. Hung., *20*, 43–46.

Takayama K., Olsen M., Datta P., Hunter R. L. (1991): Adjuvant activity of non-ionic block copolymers. V. Modulation of antibody isotype by lipopolysaccharides, lipid A and precursors. Vaccine, *9*, 257–263.

Toman M., Krejčí J., Pinka K., Menšík P. (1992): Příčiny vzniku anafylaktoidních reakcí u skotu po aplikaci lipoidních preparátů. Vet. Med. – Czech, *37*, 417–426.

Valensi J. P. M., Carlson J. R., van Nest G. A. (1994): Systemic cytokine profiles in BALB/c mice immunized with trivalent influenza vaccine containing MF59 oil emulsion and other advanced adjuvants. J. Immunol., *153*, 4029–4039.

Vanselow B. A. (1987): The application of adjuvants to veterinary medicine. Vet. Bull., *57*, 881–896.

Woodard L. F., Jasman R. L. (1985): Stable oil-in-water emulsions: preparation and use as vaccine vehicles for lipophilic adjuvants. Vaccine, *3*, 137–144.

Received: 98–04–07
Accepted after corrections: 99–01–12

Contact Address:

RNDr. Judita Süliová, CSc., Univerzita veterinárskeho lekárstva, Komenského 73, 041 81 Košice, Slovenská republika
Tel. +421 95 622 99 24, fax +421 95 632 36 66, e-mail: suliova@vsvnov.uvm.sk

ANNOUNCEMENT

XIIth DVG – Congress of Avian Diseases

Special Topics
- Pigeons, Breeding of Birds and Veterinary Medical Advice
March 1st – 4th 2000

Place of Conference:

Auditorium of the I. Medizinische Tierklinik
Veterinary Faculty of the Ludwig-MaximiliansUniversity München
Königinstraße 16
D - 80539 München, Germany

In addition for contricutions to the main topics, free papers may nbe submitted not later than October 15th, 1999. Only results not yet published should be presented. The manuscript of the pater to be given has to be at the disposal of the organizier for the preparation of the proceedings book at January, 1st, 2000!
Conference languages: German and english without simultaneous translation.

Conference Organizer:

German Veterinary Medical Society (DVG)/World Veterinary Poulty Association (WVPA)
and
Institute for Avian Diseases, Ludwig-Maximilians-University Munich

Registration and Information:

Prof. Dr. J. Kösters
Institute of Avian Diseases
Ludwig-Maximilians-University Munich
Veterinärstraße 3
D - 85764 Oberschleissheim
+49-89/218060-70 oder -80
E-Mail: gefluegelinstitut@avian.vetmed.uni-muenchen.de

A NEW LIPOID ADJUVANT: HARMLESSNESS AND LOCAL REACTOGENICITY

NOVÉ LIPOIDNÉ ADJUVANS: NEŠKODNOSŤ A LOKÁLNA REAKTOGENITA

Z. Beníšek, J. Süliová, Š. Švrček, T. Pauer, R. Ondrejka, A. Ďurove

University of Veterinary Medicine, Košice, Slovak Republic

ABSTRACT: Model experiments on laboratory animals (guinea pigs) were carried out to test the possible allergy reaction (possibility of senzitization) to the repeated administration of an experimental lipoid adjuvant (ELA) prepared on the basis of isopropylpalmitate. No significant differences were observed between the animals senzitized-provoked with ELA and the control animals. In order to evaluate the local tissue reactivity (local reactogenicity), also with regard to the process dynamics, to the administration of ELA and to carry out comparisons with other types of lipoid adjuvants, as well as aluminium hydroxide, comparative patho-anatomical and patho-histological examinations of tissues were carried out in the location of adjuvant administration. The examinations indicated very low local reactogenicity of the experimental lipoid adjuvant prepared in our laboratory.

lipoid adjuvant; isopropyl palmitate; senzitization; local reactogenicity

ABSTRAKT: V modelových pokusoch na laboratórnych zvieratách (morčatách) bola testovaná možnosť vzniku alergickej reakcie (možnosť senzibilizácie) na opakované podanie experimentálneho lipoidného adjuvans (ELA) pripraveného na báze izopropylpalmitátu. Neboli zistené signifikantné rozdiely medzi zvieratami senzibilizovane-provokovanými ELA a zvieratami kontrolnými. Pre posúdenie lokálnej tkanivovej reaktivity (lokálnej reaktogenity) aj v dynamike procesu, na podanie ELA a pre porovnanie tiež ďalších druhov lipoidných adjuvans ako aj hydroxidu hlinitého, boli vykonané porovnávacie patologicko-anatomické a patologicko-histologické vyšetrenia tkanív miesta aplikácie adjuvans. Aj na základe tohto vyšetrenia je možné súdiť o veľmi nízkej lokálnej reaktogenite nami pripraveného experimentálneho lipoidného adjuvans.

lipoidné adjuvans; izopropylpalmitát; senzibilizácia; lokálna reaktogenita

INTRODUCTION

Up to the present time many adjuvant substances have been described, capable of modifying immune reactions in the required direction. The substances mentioned can potentiate the induction of effective and long lasting humoral and cell-mediated immunity. Adjuvants can be of different origin, chemical composition and mode of action (Vanselow, 1987; Warren and Chedid, 1988; Johnson, 1994; Cox and Coulter, 1997). However only some of them are suitable for practical use (mainly for their potentiating effect on vaccines) either with regard to suitable administration and toxicity or mainly with regard to their reactogenicity.

Aluminium hydroxide is the compound most frequently used in veterinary medicine to exert a potentiating effect on vaccines despite its relatively low promoting effect in comparison with other adjuvants, e.g. oil adjuvants, and also despite the fact that its subcutaneous administration to some recipients (cats) can cause development of fibrosarcomas (Hendrick and Brooks, 1994; Lester et al., 1996). There was described creation of granulomas (which can necrotise or to change to cysts or steril abscesses) after subcutaneous application of aluminium compounds (WHO, 1976; Vanselow, 1987).

The oil adjuvants of the type of Freund's incomplete adjuvant (FIA) (Freund, 1948) find only minimum use at present despite the common knowledge of their substantial promoting effects. This results from the occurrence of undesirable postvaccination local reactions – development of granulomas, haemorrhagic lesions, serous infiltrations and long-term persistence of unmetabolized components of emulsions (oil) at the site of administration (Piretti et al., 1982; Bennett, 1992). Important and frequent are also the total reactions to the administration of oil adjuvants (Vanselow, 1987; Krejčí et al., 1988), development of metastatic granuloma and adjuvant arthritis (Amyx, 1987; Broderson, 1989; Kleinman et al., 1993). Broderson (1989) described the occurrence of lesions in the internal organs after intradermal, subcutaneous and intramuscular administration of FIA to rabbits and monkeys. Numerous subpleural granulomatous foci and pulmonary macrophage aggregates were observed in the lungs of the animals mentioned 4–12 weeks following the injection of FIA. Kleinman et al. (1993) described the develop-

ment of FIA-induced paresis in guinea pigs. Paresis occurred after the development of granulomatous lesions in the spinal cord channel after FIA injection to paraspinal muscles in guinea pigs. Another disadvantage of mineral oils is that they can be contaminated with carcinogenic polycyclic aromatic hydrocarbons and, in addition to that, persist in tissues for a long time (Piretti et al., 1982; Vanselow, 1987; Stone and Xie, 1990).

In 1987 and 1988 in the territory of the former Czechoslovakia total (anaphylactoid) postvaccination reactions were observed in cattle that was vaccinated with oil vaccines of Czechoslovak origin. According to Krejčí et al. (1988) the specific or non-specific hypersensitivity could result from the use of mineral oil contaminated with moulds, polycyclic aromatic compounds, short-chain carbohydrates, oxidation products of the type of peroxides, epoxides or aldehydes. In the authors' opinion the addition of emulgator Tween 80, a considerably toxic and potentially reactogenic substance, could represent one of the possible causes of anaphylactoid reactions. This fact was later experimentally confirmed (Toman et al., 1992).

The complications mentioned above can be probably caused either by the oil component – mineral oil, most frequently paraffin oil or its analogues known under commercial names Bayol, Marcol, and others, or by emulgators – Tween 80 (Toman et al., 1992).

The facts mentioned above necessitated the development of new types of lipoid adjuvants free of negative influences exerted by mineral oils. One of the possibilities is the substitution of mineral oil with esters of fatty acids (Bomford, 1981).

The reactogenicity of lipoid adjuvant containing isopropylester of palmitic acid as an oil component, was tested in laboratory animals (guinea pigs). The experiments were aimed at evaluation of its total reaction (organic) or local allergic reaction to the repeated administration of experimental lipoid adjuvant (ELA) – experiments A. In addition to that, patho-anatomical and patho-histological examinations at the site of administration were carried out in comparative experiments in guinea pigs to compare the tissue reactivity to the administration of ELA and other types of adjuvants in the process dynamics – experiments B.

MATERIAL AND METHODS

A) Study of possible senzitization to the administration of the experimental lipoid adjuvant (ELA)

An adjuvant of the oil-in-water type was prepared. The oil component was isopropylpalmitate (isopropylester of palmitic acid), commercial name Isopropylpalmitate 720 (Olefina, Belgium). Poloxamer 105, commercial name Synperonic PE/L35 (ICI, England) and Arlacel A (Serva, Germany) were used as emulsifiers. Detailed description of the preparation was presented by Süliová et al. (1999).

Experimental animals

Thirty laboratory guinea pigs of average weight 250 g were used.

Experimental scheme

Experimental animals were divided into 4 groups. Additional subgroups were formed within groups I and II, i.e. altogether 6 groups were formed, consisting of 5 animals in each.

The animals of the group (subgroup) Ia were administered ELA *i.m.* in the volume of 0.5 cm^3 and were provoked on day 14 using a volume of 0.1 cm^3, administered intradermally (*i.d.*) into the scapular region.

The group (subgroup) Ib served as a control for animals of subgroup Ia. The animals of this subgroup were not sensitized and were administered ELA on day 14 as the animals of subgroup Ia during their provocation.

The animals of the group (subgroup) IIa received ELA in the volume of 0.5 cm^3 *i.m.*, and were provoked on day 30 by administration of 0.1 cm^3, *i.d.*

The group (subgroup) IIb was the control for the subgroup IIa. Animals in this subgroup were not sensitized and received ELA on day 30 as those of the subgroup IIa at their provocation.

The group III of animals was administered ELA *i.m.* into a thigh muscle (sensitization) in a volume of 0.5 cm^3, and provocation on day 30 was carried out in the same way as sensitization.

The group IV of animals was sensitized in the same way as the group I and provoked on days 14 and 30.

The possible sensitization of animals was evaluated on the basis of animal behaviour – changes in behaviour and feed intake. Animals of groups III and IV were examined clinically every day for the period of one month following the last provocation. Groups (and subgroups) I and II were subjected to additional examinations for local allergic reactions carried out by measurement of the thickness of skinfold at the site of intradermal administration after 3, 24, 48 and 72 hours and 7 and 14 days. To eliminate the individual differences between the single animals the changes in the skinfold thickness (ST) were determined as multiples (reaction factor *f*) of its value before the *i.d.* administration of ELA:

$$f = \frac{\text{ST at the given time interval after administration}}{\text{ST before administration}}$$

B) Patho-morphological determination of tissue reactivity (local reactogenicity) to the administration of ELA and other types of adjuvants

The types of adjuvants used

a) ELA
b) commercial adjuvant supplied by Bioveta, Ivanovice na Hané, Czech Republic (paraffin oil, lanolin), oil-in-water type, adjuvant I
c) adjuvant supplied by MEVAK, Nitra, Slovak Republic (oil Marcol, Arlacel A, Tween 80), oil-in-water type, adjuvant N

d) aluminium hydroxide $Al(OH)_3$, saturated solution, adjuvant Al
e) physiological saline solution F – as a control.

Experimental animals

Sixty conventional guinea pigs of body weight 250 g were used. Each of the adjuvants mentioned as well as physiological saline solution were administered to groups consisting of 12 guinea pigs, intramuscularly to the left thigh using a medial approach. The volume of inoculum was 0.5 cm^3 for all animals.

Examination of guinea pigs

After 24 h and subsequently after 7, 21 and 35 days after the administration of adjuvants, three animals from each group were killed with ether and the site of administration was subjected to patho-anatomical examination. A sample of muscular tissue of dimensions 0.5 x 0.5 x 0.5 cm was taken from the site of administration and examined histologically.

The patho-anatomical examination consisted in macroscopical examination of local changes in tissues and changes in corresponding lymph nodes. The samples of tissues (excisions) from the sites of administration of adjuvants or saline solution and of corresponding inguinal lymph nodes, obtained for patho-histological examination, were fixed in cold formalin, embedded in paraffin and the sections were stained with haematoxylin-eosin or van Gieson method, respectively.

Samples of tissues and lymph nodes taken from the right thigh of the respective animals served as a control.

RESULTS

Experiment A

The possible sensitization to the administration of the experimental lipoid adjuvant was tested in model tests on guinea pigs. Some differences in the skinfold thickness were observed between the animals sensitized and provoked on day 14 or 30 and the unsensitized animals that received the respective dose of the adjuvant at the time when the provocation was carried out. The differences were however insignificant. This was evident not only from the results at provocation (reaction factor "f") three hours following the administration but also on day 7 and 14 post-administration of adjuvants. The reaction factor was identical in the cases mentioned or even higher in the unsensitized animals. Results of skin reactivity are presented in Tab. I.

No changes in behaviour or feed intake were observed in experimental animals of groups III and IV during the entire experimental period.

Experiment B

Comparative tests in model guinea pigs were used to test the local tissue reactivity (local reactogenicity) to the administration of ELA, two oil commercial adjuvants N and I, adjuvant Al and solution F. They were evaluated on the basis of results of patho-anatomical and patho-histological findings in the process dynamics.

Patho-anatomical examination

Slightly oedematous subcutaneous and intramuscular tissue around the site of injection was observed in 24 hours following the injection of ELA. The superficial inguinal lymph nodes were slightly enlarged and grayish-pink in colour. Similar findings were observed also in animals administered adjuvants N and I. No changes were observed in animals administered the Al adjuvant or control injection of physiological saline solution F.

After 7 days, the animals administered the lipoid adjuvant exhibited more intensive reaction at the site of administration. The least intensive reaction was observed in the case of administration of ELA; no changes were observed for the Al adjuvant.

After 21 and 35 days, the animals administered either ELA or Al exhibited no microscopical changes

I. Changes in the skinfold thickness at the site of intradermal application of ELA: expressed as multiples of the thickness before the application (f)

Time of evaluation after challenge	Guinea pig groups			
	Ia	Ib	IIa	IIb
	a multiple of skinfold thickness			
3 hours	2.10 ± 0.23	2.12 ± 0.19	2.01 ± 0.10	1.95 ± 0.15
24 hours	1.84 ± 0.09	1.81 ± 0.17	1.81 ± 0.16	1.70 ± 0.20
48 hours	1.74 ± 0.10	1.67 ± 0.10	1.71 ± 0.12	1.62 ± 0.14
72 hours	1.63 ± 0.13	1.59 ± 0.12	1.52 ± 0.10	1.50 ± 0.08
7 days	1.52 ± 0.07	1.49 ± 0.09	1.22 ± 0.10	1.21 ± 0.10
14 days	1.25 ± 0.08	1.26 ± 0.09	1.14 ± 0.07	1.09 ± 0.09

Guinea pig groups (5 animals in each group)
Ia group – sensitization: 0.5 cm^3 *i.m.*
provocation: on 14th day, 0.1 cm^3 *i.d.*
Ib group – sensitization none
provocation: on 14th day, 0.1 cm^3 *i.d.*
IIa group – sensitization: 0.5 cm^3 *i.m.*
provocation: on 30th day, 0.1 cm^3 *i.d.*
IIb group – sensitization: none
provocation: on 30th day, 0.1 cm^3 *i.d.*

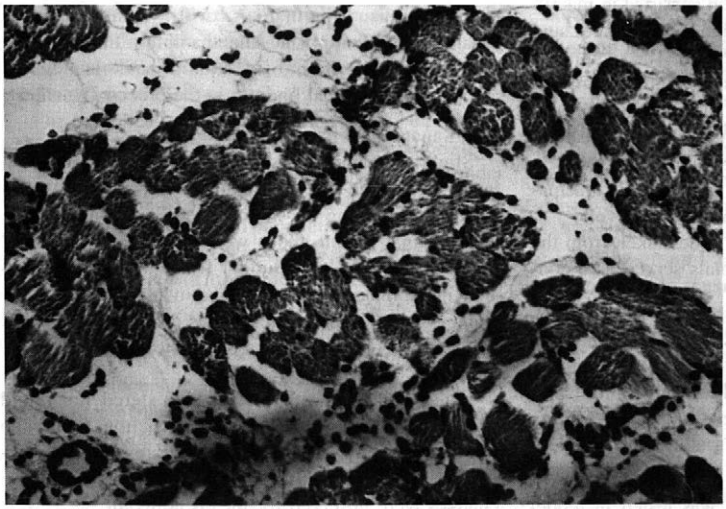
at the site of their administration. The animals treated with N and I adjuvants exhibited proliferation of intermuscular tissue in the time intervals mentioned. However, no enlargement of the corresponding lymph nodes was observed.

Patho-histological examination

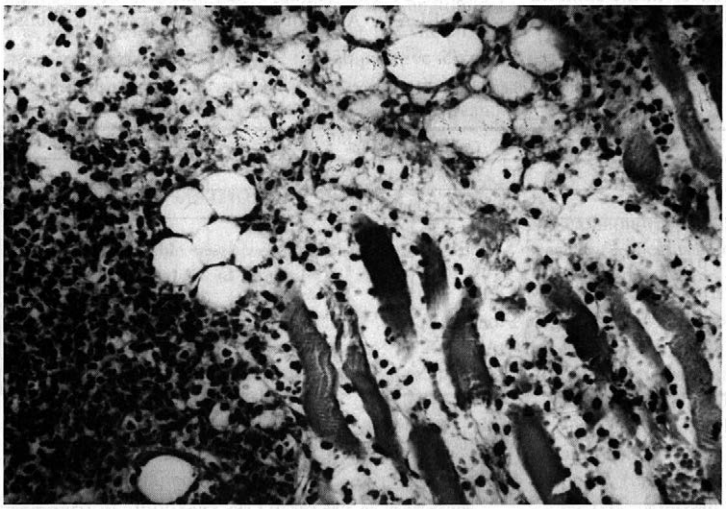
Observations carried out 24 h following the ELA administration showed marked dilatation of vessels and oedematous changes in the connective tissue at the site of injection (Fig. 1). Inflammation-cellular infiltration of oedematous connective tissues with predominance of polymorphonuclear leukocytes and admixture of eosinophils appeared in the foreground. This infiltrate penetrated as narrow bands between muscular fibre bundles. Inguinal lymph nodes showed slight hyperplasia although retaining their original structure. Similar changes were observed following the administration of N and I adjuvants. As a result of oedema, the bundles of muscular fibres were separated and muscular cells exhibited homogenization and the loss of striation. Necrotic decomposition of tissue was observed at some places (Fig. 2). The inguinal lymph nodes exhibited hyperplasticity with extended subcapsular and intermediary sinuses.

The histopathological findings after administration of Al were similar but not intensive.

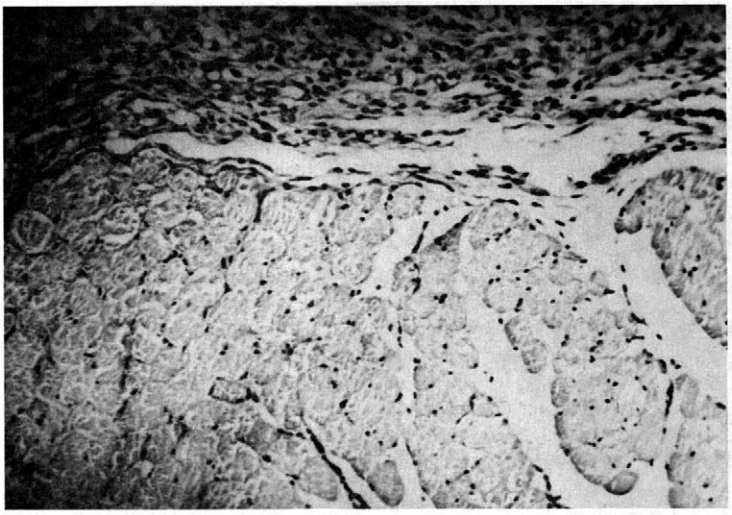
Seven days after the administration of adjuvants, the oedematous and regressive changes in muscular cells were alleviated considerably. The polymorphonuclear infiltrate was reduced and replaced by mononuclear cells. The connective tissues of animals treated with N and I adjuvants contained many optically empty vacuoles around which neutrophil leukocytes tended to concen-



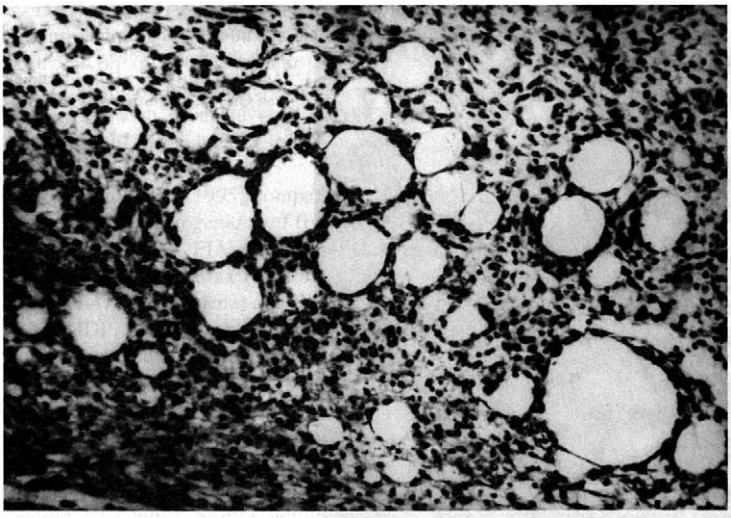
1. Cell infiltration with polymorphonuclear leukocytes, oedema and extravasation in intermuscular connective tissue. Adjuvant ELA after 24 hours. Staining: haematoxylin-eosin; magnification: 63 times



2. Dystrophy changes in muscular tissue, oedematisation and cellular infiltration between muscular bundles. Adjuvant N after 24 hours. Staining: haematoxylin-eosin; magnification: 63 times



3. Reduction of polymorphonuclear cells in intermuscular tissue, moderate oedematisation. Adjuvant ELA after 7 days. Staining: haematoxylin-eosin; magnification: 63 times



4. Creation of the granular tissue in the intramuscular connective tissue with oil residua in vacuoles. Adjuvant I after 7 days. Staining: haematoxylin-eosin; magnification: 63 times

trate. The vacuoles formed as residua after the oil components of the adjuvants administered (Figs. 3, 4 and 5). Such a picture was not observed in the ELA treated animals.

After the elapse of 21 and 35 days, considerable consolidation of reactions could be observed. The more pronounced mononuclear infiltration also regressed. Some granular tissue was formed and the considerable number of fibroblasts suggested that fibrotization had occurred, particularly in the time interval of 35 days following the administration of adjuvants. The extent of changes in the group of animals treated with ELA was smaller (Fig. 6). However, more pronounced formation of collagen fibres, oriented parallelly to the muscular fibres, was observed.

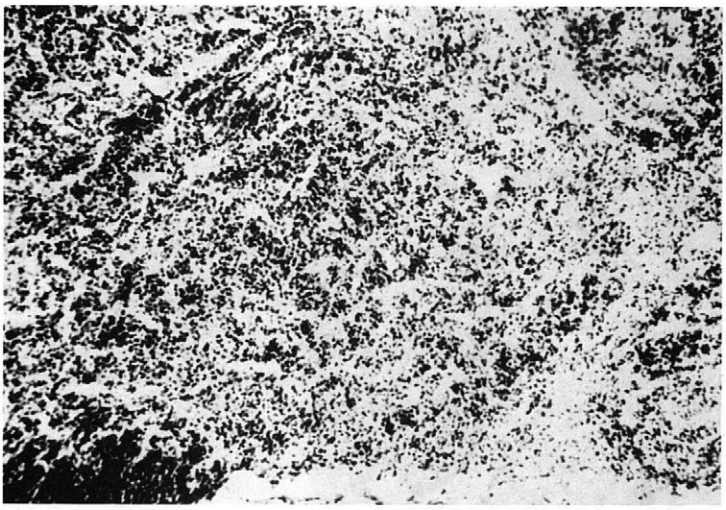
The process of consolidation was observed also in the lymph nodes.

DISCUSSION

The occurrence of undesirable postvaccinal reactions (local and total) after administration of lipoid adjuvants in animals led us to develope a new type of areactogenic lipoid adjuvant. The isopropylpalmitate was used as the oil componenent. The use of Tween 80 as emulsifier was avoided.

The experimental lipoid adjuvant (ELA) was submitted to tests of harmlessness and areactogenicity. The study of the possible senzitization and local tissue reactivity (reactogenicity) to administration of ELA and other types of adjuvants was carried out in extremely sensitive model animal species, the guinea pig.

The differences in the skinfold thickness (reactive factor) between sensitized and subsequently provoked animals and those which were not sensitized but pro-



5. Dilatation of subcapsular and intermediary sinuses and follicular hyperplasia of inguinal lymph node. Adjuvant N after 7 days. Staining: haematoxylin-eosin; magnification: 20 times

6. Differentiation of granular tissue with creation of collagenous fibres. Adjuvant ELA after 35 days. Staining: van Gieson; magnification: 252 times

voked in the respective time intervals were insignificant. This points to the areactogenicity of the main component of the experimental lipoid adjuvant, isopropylpalmitate, and of the emulgators, Poloxamer 105 and Arlacel A, used at an optimum ratio. The possibilities of sensitization (and subsequent provocation) in the case of repeated use of the adjuvant mentioned were taken into consideration.

Patho-anatomical and patho-histological examinations documented relatively low local tissue reactogenicity of ELA in comparison with "conventional" oil adjuvants represented by N and I adjuvants, used in experiments. Important was the observation of the absence of ELA residua at the site of administration as early as after 7 days and rapid consolidation of changes in the tissues.

The administration of aluminium hydroxide did not cause a rise of macroscopical changes in the site of the application. It is responsible to low intensive finding at the pathological-histological examination. The formation of a small granuloma is inevitable with alum-precipitated vaccines and is considered as a necessary requirement for effective adjuvant action (WHO, 1976). Our result is in accordance with the facts published in the world literature (Ramanthan et al., 1979; Edelman, 1980).

The experimental lipoid adjuvant is an emulsion of the oil-in-water type. From the point of view of practical application (as a diluent for lyophilised veterinary vaccines), the smaller viscosity of this type of emulsion in comparison with water-in-oil (e.g. Freund incom-

plete adjuvant FIA; Freund, 1948) emulsions appears as an advantage.

Several replacements of mineral oils can be used in the preparation of effective and harmless lipoid adjuvants besides of esters of fatty acids (Bomford, 1981). We can mention, for example, terpene oils, squalane and squalene, which are used predominantly in water-in--oil emulsions (Stone and Xie, *1990*). They can be combined with other immunopotentiating substances, e.g. sulphopolysaccharides (Hilgers et al., 1994a, b) or block copolymers (Bennett et al., 1992). The local reaction to the administration of compounds mentioned above had mostly character of a mild inflammation, which prevailed a different long time. The reduced incidence and duration of muscle lesion from the terpene oil vaccines used in trials probably reflects their natural compatibility with tissues, because they are normal components of skin and other tissues. On 25th day after injection (squalene) wasn't detected any signs of inflammation in the site of adjuvant application (Stone and Xie, 1990).

Deeb et al. (1992) describe only mild multifocal granulomatous myositis in the application site of RIBI adjuvant (mixture of monophosphoryl lipid A, trehalose dimycolate, mycobacterium cell wall skeleton and Tween 80, with squalene – oil-in-water emulsion) in contrast to FIA which caused marked multifocal granulomatous myositis.

Robuccio et al. (1995) comparised the adjuvant effect and local reactogenicity of five lipoid adjuvants (in different doses) – FIA, Freund's complete adjuvant (FCA), RIBI, Titer Max (mixture of block copolymer CRL-89-41 and squalene) and Syntex adjuvant (it contains MDP, squalene, block copolymer and pluronic acid). They found out that Freund's adjuvants at high and low doses and RIBI adjuvant at high dose caused significantly greater swelling at the inoculation site than control (saline). Titer Max adjuvant at high and low doses caused minor swelling at the inoculation site, but it was not significantly different from the swelling caused by control. Syntex adjuvant at high and low doses. RIBI at low dose caused no swelling after inoculation. Histologically Freund's adjuvants caused granulomatous myositis, Syntex – histiocytic lymphocytic myositis, RIBI – pyogranulomatous myositis (in high dose) and Titer Max necrotizing pyogranulomatous myositis (also in high dose).

Bomford (1981) investigated the local reaction at the site of injection of classical adjuvant (FIA) and some fatty acid esters. The same degree of local reaction (footpad swelling) was caused by emulsions of ethyl caprate alone or mixed with 10% butyl stearate. The reaction to the esters was more severe than to FIA up to 14 days after injection but afterwords disappeared more quickly.

Our experiences with IPP are not fully (absolutely) identical to experiences of Bomford. The local reaction to isopropylester of palmitic acid was less severe than that to "classical" adjuvant prepared from mineral oil, and the rapidity of the changes consolidation in the tissues was also greater.

With regard to the above mentioned considerations and results obtained by testing the supportive effect of isopropylpalmitate as well as results of tests presented in this study, we can unambiguously recommend the use of this metabolisable adjuvant for the purpose of potentiating the effect of lyophilised veterinary vaccines without the risk of development of postvaccination complications in target animal species.

REFERENCES

Amyx H. L. (1987): Control of animal pain and distress in antibody production and infectious disease studies. J. Amer. Vet. Med. Assoc., *191*, 1287–1293.

Bennett B., Check I. J., Olsen M. R., Hunter R. L. (1992): A comparison of commercially available adjuvants for use in research. J. Immunol. Meth., *153*, 31–40.

Bomford R. (1981): The adjuvant activity of fatty acid esters. The role of acyl chain length and degree of saturation. Immunology, *44*, 187–192.

Broderson J. R. (1989): A retrospective review of lesions associated with the use of Freund's adjuvant. Lab. Anim. Sci., *39*, 400–405.

Cox J. C., Coulter A. R. (1997): Adjuvants – a classification and review of their modes of action. Vaccine, *15*, 248–256.

Deeb B. J., DiGiacomo R. F., Kunz L. L., Stewart J. L. (1992): Comparison of Freund's and RIBI adjuvants for inducing antibodies to the synthetic antigen (TG)-Al in rabbits. J. Immunol. Meth., *152*, 105–113.

Edelman R. (1980): Vaccine adjuvants. Rev. Infect. Dis., 2, 370–383.

Freund J., Thomson K. J., Hough H. B., Sommer H. E., Pisani T. M. (1948): Antibody formation and sensitization with the aid of adjuvants. J. Immunol., *60*, 383–398.

Hendrick M. J., Brooks J. J. (1994): Postvaccinal sarcomas in the cat: histology and immunochemistry. Vet. Pathol., *31*, 126–129.

Hilgers L. A. T., Platenburg P. L. I., Luitjens A., Groenveld B., Dazelle T., Ferrari-Laloux M., Weststrate M. W. (1994a): A novel non-mineral oil-based adjuvant. I. Efficacy of a synthetic sulphopolysaccharide in a squalane-in-water emulsion in laboratory animals. Vaccine, *12*, 653–660.

Hilgers L. A. T., Platenburg P. L. I., Luitjens A., Groenveld B., Dazelle T., Weststrate M. W. (1994b): A novel non--mineral oil-based adjuvant. II. Efficacy of a synthetic sulphopolysaccharide in a squalane-in-water emulsion in pigs. Vaccine, *12*, 661–665.

Johnson A. G. (1994): Molecular adjuvants and immunomodulators. New approaches to immunization. Clin. Microbiol. Rev., *7*, 277–289.

Kleinmann N. R., Kier A. B., Diaconu E., Lass J. H. (1993): Posterior paresis induced by Freund's adjuvant in guinea pigs. Lab. Anim. Sc., *43*, 364–366.

Krejčí J., Toman M., Pinka K., Jurák E., Menšík P., Poláček R. (1988): Dosažené poznatky o anafylaktoidních reak-

cích skotu. [Dílčí zpráva.] Brno (Czech Republic), Výzkumný ústavveterinárního lékařství, pp. 35.

Lester S., Clemett T., Burt A. (1996): Vaccine site associated sarcomas in cats: clinical experience and a laboratory review (1982–1993). J. Amer. Anim. Hosp. Assoc., *32*, 91–95.

Piretti M. V., Franchini A., Zanatello T. (1982): Investigation of the hydrocarbons found in the tissues of chickens injected with inactivated oil adjuvant vaccine. Z. Lebensm. Unters. Forsch., *175*, 245–248.

Ramanthan V. D., Badenoch-Jones P., Turk J. L. (1979): Complement activation by aluminium and zirconium compounds. Immunology, 37, 881–888.

Robuccio J. A., Griffith J. W., Chroscinski E. A., Cross P. J., Light T. E., Lang C. M. (1995): Comparison of the effects of five adjuvants on the antibody response to influenza virus antigen in guinea pigs. Lab. Anim. Sci., *45*, 420–426.

Stone H. D., Xie Z. (1990): Efficacy of experimental Newcastle disease water in-oil oil emulsion vaccines formulated from squalane and squalene. Avian Dis., *34*, 979–983.

Süliová J., Beníšek Z., Švrček Š., Ondrejka R., Ďurove A. (1999): A new lipoid adjuvant: preparation and observation of its effectiveness. Vet. Med. – Czech, *44*, 93–99.

Toman M., Krejčí J., Pinka K., Menšík P. (1992): Příčiny vzniku anafylaktoidních reakcí u skotu po aplikaci lipoidních preparátů. Vet. Med. – Czech, *37*, 417–426.

Vanselow B. A. (1987): The application of adjuvants to veterinary medicine. Vet. Bull., *57*, 881–896.

Warren H. S., Chedid L. A. (1988): Future prospects for vaccine adjuvants. Crit. Rev. Immunol., *8*, 83–101.

WHO (1976): Immunological Adjuvants. Technical Report Series, No. 595. Geneva, 40 pp.

Received: 98–04–07
Accepted after corrections: 98–12–28

Contact Address:

MVDr. Zdenek B e n í š e k, CSc., Univerzita veterinárskeho lekárstva, Komenského 73, 041 81 Košice, Slovenská republika
Tel. +421 95 622 99 24, fax +421 95 632 36 66, e-mail: benisek@vsvnov.uvm.sk

HISTOCHEMICAL STUDY OF MASTITIC MAMMARY GLAND IN LACTATING COWS*

HISTOCHEMICKÉ ŠTÚDIUM ZÁPALOVO ZMENENEJ MLIEČNEJ ŽĽAZY LAKTUJÚCICH DOJNÍC

Ľ. Lenhardt[1], E. Švický[1], E. Dudriková[1], Š. Mozeš[2]

[1]*University of Veterinary Medicine, Košice, Slovak Republic*
[2]*Institute of Animal Physiology, Slovak Academy of Sciences, Košice, Slovak Republic*

ABSTRACT: The activity of alkaline phosphatase, adenosine triphosphatase, acid phosphatase, and succinate dehydrogenase in the epithelial cells of the chronic, inflammatory, mammary gland in the lactating cows was determined by a densitometric analysis. In comparison with the intact mammary gland, the inflammatory disease led to expressive changes in all the enzyme activities. Significantly increased activity ($p < 0.001$) of alkaline phosphatase, adenosine triphosphatase, acid phosphatase, and succinate dehydrogenase was detected in the epithelial cells. The results of the experiment refer to a narrow relathionship between the enzymatic activity and inflammatory process in the mammary gland during the lactation period.

alkaline phosphatase; adenosine triphosphatase; acid phosphatase; succinate dehydrogenase; histoenzymatic study; mammary gland; cows

ABSTRAKT: Denzitometrickou analýzou bola stanovená aktivita alkalickej fosfatázy, adenozíntrifosfatázy, kyslej fosfatázy a sukcinátdehydrogenázy v epitelových bunkách mliečnej žľazy dojníc s chronickým zápalom mliečnej žľazy. Pri porovnaní s intaktnou mliečnou žľazou, zápalové ochorenie viedlo k signifikantnému zvýšeniu aktivity ($p < 0.001$) všetkých sledovaných enzýmov v epitelových bunkách. Výsledky experimentu poukazujú na úzky vzťah medzi enzymatickou aktivitou a zápalovým procesom mliečnej žľazy počas laktácie.

alkalická fosfatáza; adenozíntrifosfatáza; kyslá fosfatáza; sukcinátdehydrogenáza; histochemické štúdium; mliečna žľaza; dojnice

INTRODUCTION

Nowadays, mastitis is still the most relevant and most costly infectious disease of dairy cows. The principal microorganisms (*Streptococcus agalactiae, Staphylococcus aureus, Streptococcus uberis, Streptococcus dysgalactiae, Escherichia coli*) cause expressive clinical and morphological changes (Philpot and Pankey, 1978) which can be determined biochemically and histochemically. Up to the present, research has been directed at the detection of clinical and morphological changes in the inflamed mammary gland (Vasiľ, 1994). Still, there is little data about the influence of infection on the enzyme activity in the cells of mammary gland. For the possibility of using a histochemical method, the work of Lenhardt et al. (1994) should be consulted. In this paper the alkaline phosphatase (AP), adenosine triphosphatase (ATP-ase), acid phosphatase (AcP), and succinate dehydrogenase (SDH) activities were detected in the healthy mammary glands of lactating cows.

The objective of this study was to determine the influence of chronic mastitis on the activity of alkaline phosphatase, adenosine triphosphatase, acid phosphatase, and succinate dehydrogenase in the parenchyma of mammary gland.

MATERIAL AND METHODS

Sixty lactating cows slaughtered at the slaughterhouses in Košice were used in the experiment. Material for histochemical evaluation was obtained from the parenchyma of 25 cows with clinically healthy mammary glands and from 35 cows with mastitis, with positive findings in bacteriological examination (*Staphylococcus aureus, Streptococcus dysgalactiae*, and *Streptococcus uberis*) detected in the parenchyma of mammary gland.

Samples sized 1 x 1 x 0.5 cm, were taken from the pars glandularis sinus lactiferis of the mammary gland.

* Supported by the Slovak Academy of Sciences (Grant No. 2/3005/97).

Samples were taken within 10 minutes of slaughter, frozen, and kept in a liquid nitrogen until processed.

On the day of experiment, a segment of the frozen tissue was cut (7 μm) in the cryostat at –21 °C and the tissue slices were transferred to glass slides and air-dried. From each tissue segment, seven sections were cut for different enzyme assays.

Demonstration of alkaline phosphatase activity was performed by using a modified simultaneous azo-coupling method (Lojda et al., 1979). The incubation medium contained naphthol-AS-MX-phosphate (Fluka, Germany), and stable diazonium salt Fast Blue BB (Sigma, USA), veronal acetate buffer (pH 9.2). The incubation was performed at 37 °C for 30 min.

Adenosine triphosphatase activity was demonstrated by the lead salt method according to Lojda et al. (1979). The incubation medium contained adenosine triphosphate-sodium salt (Sigma, Germany), lead nitrate and magnesium sulphate (Aldrich, Germany), tris-maleate buffer (pH 7.2). The sections were incubated for 20 min at 37 °C.

Determination of acid phosphatase activity was performed according to Lojda et al. (1979). The incubation medium contained naphthol-AS-BI-phosphate (Fluka, Germany), hexazotized new fuchsin (Serva, Germany), veronal acetate buffer (pH 6.0). The incubation lasted for 10 hours at 37 °C.

Succinate dehydrogenase activity was performed according to Lojda et al. (1979). The medium consisted of succinate-disodium salt (Fluka, Germany), nitro BT, tris-HCl buffer (pH 7.2). The incubation lasted for 30 min at 37 °C. After incubation, sections were washed with distilled water to remove the incubation medium and to stop any reactions. Postfixation of the sections was performed in a solution of 4% (v/v) formaldehyde for 10 h at 20 °C. The sections were rinsed in distilled water and mounted in glycerin jelly.

Enzyme activity of the epithelial cells was cytophotometrically analysed with a Vickers-M-86 microdensitometer. The measurements were performed using a x 40 objective and effective scanning area of 28.3 μm^2 and scanning spot 0.5 μm. The integrating absorbance was measured at a wavelength of 480 nm for alkaline phosphatase and succinate dehydrogenase, 590 nm for adenosine triphosphatase, 520 nm for acid phosphatase.

The enzyme activity was evaluated as the absorbance values recorded by the instrument/ $min/\mu m^3$ epithelial cells ± *SD*. The density of enzymes investigated was determined in six sections of each sample at 10 sites corresponding to the epithelial cells in the mammary gland. Statistical analyses were done by *t*-test.

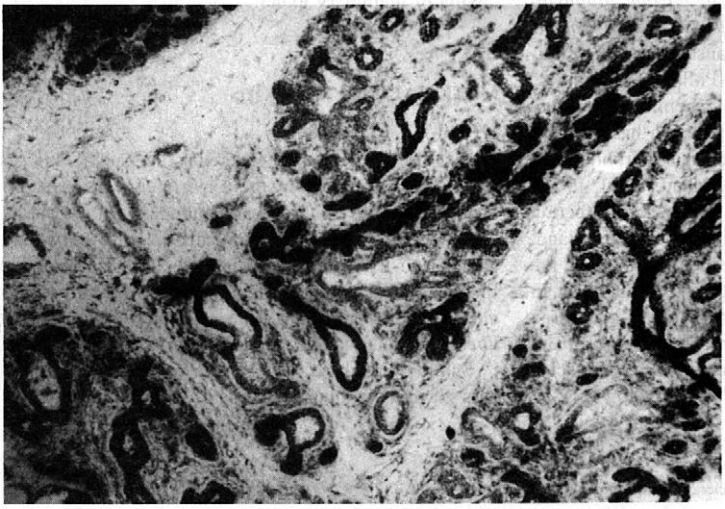
RESULTS

Tab. I summarizes the measurements of enzyme activities in the epithelial cells of healthy and diseased mammary glands of lactating cows. In comparison with intact mammary glands, mastitis led to a significant increase in the activity of all the enzymes investigated;

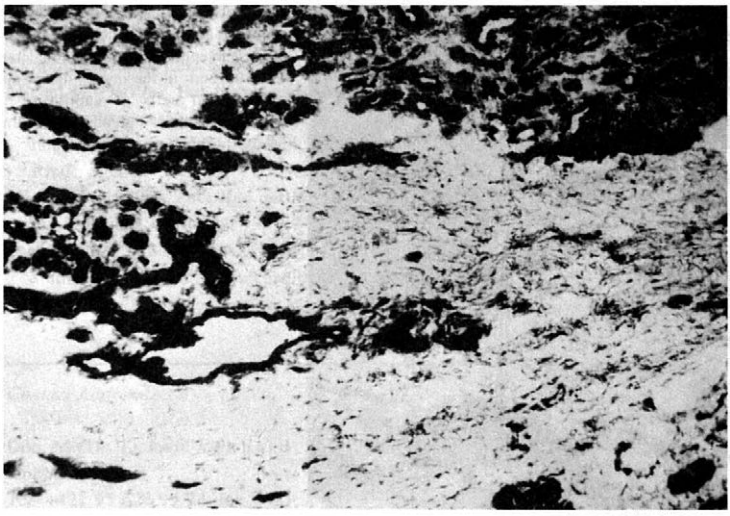
I. Densitometric analysis of alkaline phospatase, adenosine triphosphatase, acid phosphatase, and succinate dehydrogenase in the epithelial cells of healthy and diseased bovine mammary glands

Enzyme	Bovine mammary gland	
	healthy ($\bar{x} \pm SD$)	diseased ($\bar{x} \pm SD$)
Alkaline phosphatase	15.66 ± 1.14	38.87 ± 3.40[a]
Adenosine triphosphatase	15.70 ± 1.02	44.07 ± 1.19[a]
Acid phosphatase	23.27 ± 1.85	50.60 ± 2.68[a]
Succinate dehydrogenase	12.10 ± 0.94	33.64 ± 2.99[a]

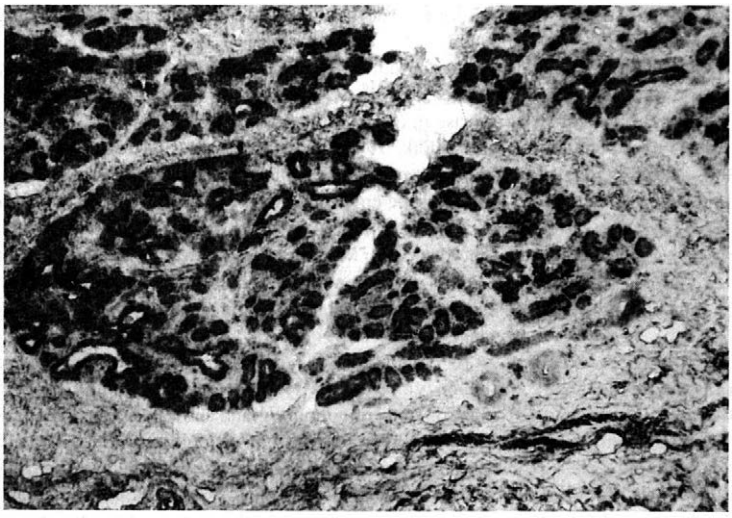
[a]significantly different from control $p < 0.001$



1. A histochemical picture of uniformly noticeable activity of alkaline phosphatase in the epithelial cells, lower activity in myoepithelial cells and in the endothelial cells of the capillaries in mastitic mammary gland sections of lactating cows (obj. 6.3; eyepiece 6.3)



2. Marked activity of adenosine triphospatase in the epithelial cells, and moderate activity of this enzyme in the myoepithelial alveoli cells. A very vigorous activity is observed in the endothelial cells of capillaries in mastitic mammary gland sections of lactating cows (obj. 6.3; eyepiece 6.3)



3. A histochemical picture of the acid phosphatase activity in diseased mammary glands of mastitic cows. Anspicous activity in the epithelial cells, and moderate activity in the myoepithelial cells. The endothelial cells of capillaries did not react (obj. 6.3; eyepiece 6.3)

ranging from 1.0 (AcP) to 3.0 times (SDH) higher than with intact glands.

Differences in the enzyme activities in other structures of the mammary gland (myoepithelial cells and endothelial cells of the capillaries) are presented in Figs. 1–4.

Against the very noticeable activity of alkaline phosphatase in the epithelial cells, the activity of this enzyme was lower in the other structures investigated (Fig. 1).

Fig. 2 shows the presence of the reaction product of the membrane adenosine triphosphatase in the epithelial cells. Similarly, the vigorous activity of this enzyme was observed in the myoepithelial cells, and in the endothelial cells of capillaries.

Epithelial cells showed a marked activity of acid phosphatase. The reaction product of this enzyme was also observed in the myoepithelial cells; hardly any final reaction was found in the endothelial cells of capillaries (Fig. 3).

When tissue slices were incubated to demonstrate succinate dehydrogenase, an increased amount of the reaction product was detected in the epithelial cells in comparison with myoepithelial and endothelial cells (Fig. 4).

DISCUSSION

The results of our experiment showed the close relathionship between enzyme activities and chronic mastitis in the cow's mammary gland during lactation. Alkaline phosphatase is found primarily in the cell membranes of the cow's mammary gland where the active transport processes take place. In agreement with this, we detected the presence of AP in all the structures

4. A histochemical picture of the pronounced activity of succinate dehydrogenase in epithelial cells, and moderate activity both in the myoepithelial and endothelial cells of capillaries in mastitic mammary glands of lactating cows (obj. 6.3; eyepiece 6.3)

investigated both in the mastitic mammary gland as well as in the healthy udder (Lenhardt et al., 1994). Similar results were obtained by Korfmeier (1976): he detected positive reactions to alkaline phosphatase in the epithelial cells of the mammary gland in mice. He related the level of this enzyme activity to the coherent functioning of the mammary gland. The higher levels of alkaline phosphatase detected in our experiment during mastitis suggest a changed dysfunctioning of the mammary gland. Similarly, an increased activity of alkaline phosphatase in the proliferated fibroblasts around carcinomas was observed by Packdaman and Stain (1963).

Cell membrane adenosine triphosphatase is responsible for the physiological degradation of adenosine triphosphate and takes place in the transport of potassium and sodium across membranes. In healthy cows the enzymatic activity of membrane-bound ATP-ase was high in the epithelial cells (Tab. I). In agreement with this Korfmeier (1976) described a marked activity of ATP-ase in all structures of murine mammary gland investigated during the lactation period. Like in the case of AP, mastitis led to a noticeable increase in the ATP-ase activity in the investigated structures.

In the mammary gland of healthy cows, the acid phosphatase showed a marked activity in the epithelial cells and a moderate activity in the myoepithelial cells. Hardly any final reaction product was found in the endothelial cells of capillaries. Similar results were obtained by Michel (1979b), Longauer and Bilčík (1980, 1993), Lenhardt et al. (1994). In the diseased mammary gland in our experiment, a significant increase of AcP was found in the epithelial cells. The increased level of AcP probably reflects the raised lysosomal activity during chronic mastitis. This is in agreement with the observation of Kotz et al. (1978), who described a significant increase of AcP in the epithelial cells during acute mastitis and recommended the use of this reaction for the identification of mastitis intensity in the mammary gland.

In comparison with clinically healthy mammary glands, where a low activity of succinate dehydrogenase in all cellular structures was detected (Longauer and Bilčík, 1980; Lenhardt et al., 1994) in our experiment the most noticeable increase of this enzyme was found in the epithelial cells. In other structures, a lower activity of SDH was detected. This is in agreement with the results of Michel (1979a), where the activity of SDH detected in myoepithelial cells of the alveoli, stroma, and capillaries in the mastitic changed mammary gland was very low.

In conclusion, the densitometric quantification of the enzymatic activity between healthy and chronic mastitis in the mammary gland showed marked changes in the enzymatic activity of the epithelial cells. The use of this histochemical method should be one of the tools for the precise histochemical examination of acute and chronic mastitis as well as the picture of the functional status of the mammary gland.

REFERENCES

Korfmeier K. H. (1976): Involution of the mammary gland. Stuttgart, New York, Gustav Fischer Verlag. 57.

Kotz J., Basmadj K., Nadej J. A. (1978): Aktywność lizozymu oraz fosfatazy kwasnej przy zapaleniu gruczolu mlekowago. Med. Vet., *34*, 89–91.

Lenhardt L., Dudríková E., Švický E. (1994): A histoenzymatic study of healthy mammary gland of dairy cows. Folia Vet., *38*, 103–106.

Lojda Z., Gossrau R., Schiebler T. N. (1979): Enzyme histochemistry. A laboratory maual. Berlin, Heidelberg, New York, Springer-Verlag. 339.

Longauer F., Bilčík P. (1980): Enzymes in blood capillaries of the breast. Histochemical study. Bratisl. Lek. Listy, *74*, 361–372.

Longauer F., Bilčík P. (1993): Enzymes in tumors of the breast. Histochemical study of the stroma in breast carcinomas. Bratisl. Lek. Listy, *94*, 192–200.

Michel G. (1979a): Das histochemische Verhalten der Sukzinatdehydrogenase und Laktatdehydrogenase sovie der Ribonukleinsaäure im Epithel der Milchgänge und Alveolen des Rindereuters. Arch. Exp. Vet.-Med., *33*, 745–751.

Michel G. (1979b): Zum Bau der Milchgänge des Rindereuters. Mh. Vet.-Med., *34*, 133–137.

Packdaman P., Stein A. A. (1963): Enzyme histochemical studies on surgically resected breasts. Arch. Surg., *86*, 593–595.

Philot W. N., Pankey J. W. (1978): Control of mastitis by hygiene and therapy. Dairy Res. Report. Hommer Louisiana, USA, 17–40.

Vasil M. (1994): Treatment of clinically apparent forms of mastitis in lactating cows using Sulpha-mycin (Polfa, Poland), an intramammary preparation. Vet. Med. – Czech, *39*, 511–517.

Received: 98–06–26
Accepted after corretions: 98–12–08

Contact Address:

Doc. MVDr. Ľudovít Lenhardt, Csc., Univerzita veterinárskeho lekárstva, Komenského 73, 041 81 Košice, Slovenská republika
Tel. +421 95 622 99 24, fax +421 95 632 36 66, e-mail: dudrik@uvm.sk

RESISTANCE TO ANTIBIOTICS IN *STAPHYLOCOCCUS AUREUS* ISOLATED FROM DAIRY COW MASTITIS, MILK, UDDER SMEARS, AND MILKING INSTALLATION

REZISTENCIA K ANTIBIOTIKÁM U *STAPHYLOCOCCUS AUREUS* IZOLOVANÉHO Z MASTITÍD DOJNÍC, MLIEKA, STEROV VEMENA A DOJACIEHO ZARIADENIA

M. Vasiľ

Research Institute of Veterinary Medicine, Košice, Slovak Republik

ABSTRACT: During the period from 1993 to 1997, the sensitivity to 8 antibiotics in 320 strains of *Staphylococcus aureus* was studied by means of disc diffuse method. The *S. aureus* strains had been isolated from both clinical and latent dairy cow mastitis, milked milk, smears taken from the skin of dairy cow udder, milking installation, and environment. The occurrence of resistant *S. aureus* strains was as follows: 22.5% with penicillin, 6.9% with ampicillin, 1.9% with oxacillin, 7.2% with tetracycline, 2.2% with chloramphenicol, 14.7% with streptomycin, 1.6% with neomycin, and 0.6% with erythromycin. A total of 63.4% of *S. aureus* strains were sensitive to the antibiotics observed. 24.7% of strains, however, were resistant only to one antibiotic, 5.6% of strains were resistant to two antibiotics at the same time, 4.1% of strains to three, 1.6% to four, and 0.3% of strains were resistant to five and six antibiotics at the same time.

resistance and sensitivity to antibiotics; *Staphylococcus aureus*; penicillin; ampicillin; oxacillin; tetracycline; chloramphenicol; streptomycin; neomycin; erythromycin

ABSTRAKT: V priebehu rokov 1993 až 1997 bola diskovou difúznou metódou sledovaná citlivosť k osmi antibiotikám pri 320 kmeňoch *Staphylococcus aureus* izolovaných z klinických a latentných mastitíd dojníc, nadojeného mlieka, sterov odobratých z kože vemena dojníc, dojacieho zariadenia a prostredia. Výskyt rezistentných kmeňov *S. aureus* bol nasledovný: 22,5 % pri penicilíne, 6,9 % pri ampicilíne, 1,9 % pri oxacilíne, 7,2 % pri tetracyklíne, 2,2 % pri chloramfenikole, 14,7 % pri streptomycíne, 1,6 % pri neomycíne a 0,6 % pri erytromycíne. Celkove bolo 63,4 % kmeňov *S. aureus* citlivých k sledovaným antibiotikám, ale 24,7 % kmeňov bolo rezistentných iba k jednému antibiotiku, 5,6 % kmeňov vykázalo súčasne rezistenciu k dvom antibiotikám, 4,1 % kmeňov k trom, 1,6% k štyrom a 0,3 % kmeňov bolo rezistentných súčasne voči piatim a šiestim antibiotikám.

rezistencia a citlivosť k antibiotikám; *Staphylococcus aureus*; penicilín; ampicilín; oxacilín; tetracyklín; chloramfenikol; streptomycín; neomycín; erytromycín

ÚVOD

Staphylococcus aureus patrí k významným patogénom, tak v humánnej, ako aj veterinárnej medicíne. Podstatou patogenity tohoto patogéna je jednak invázia do makroorganizmu (zápalové ochorenia, lézie, abscesy, sepsy apod.), resp. nežiaduce pôsobenie v potravinách v dôsledku produkovaných toxínov, hlavne enterotoxínov (Benda a Vyletělová, 1995).

Rozšírené používanie antibiotík pre liečbu bovinných mastitíd a realizáciu programov kontroly mastitíd vyzýva k obozretnosti pri terapii mastitíd. V súčasnosti primárne miesto v antibiotickej terapii mastitíd dojníc zaberajú *Staphylococcus aureus* a environmentálni pôvodcovia, hlavne koliformné zárodky. Mastitídy dojníc spôsobené *S. aureus* sú veľmi často terapeuticky ťažko zvládnuteľné, a to hlavne pre výraznú schopnosť tohoto patogéna nadobúdať rezistenciu voči antibiotikám, ktorú si zvyčajne aj dlhú dobu udrží (Thorasberry a i., 1997). Z viacero prác vyplýva, že v oblasti antimikrobiálnej terapie je potrebné venovať zvýšenú pozornosť sledovaniu a charakterizácii vývoja rezistencie k antibiotikám u ochorenie vyvolávajúceho bakteriálneho pôvodcu, pričom obzvlášť dôležité je to u kmeňov *S. aureus* (McDonald a Anderson, 1981; Schultze, 1983; Kotovski, 1991; Ayliffe, 1997). Ak sa vrátime do histórie už Christie a i. (1974) upozorňujú, že v Anglicku zavedenie širokého používania antibiotík v liečbe mastitíd dojníc viedlo ku skutočnosti, že v priebehu štyroch rokov stúpol výskyt rezistentných kmeňov *S. aureus* zo 6,0 % (v roku 1969) na 45,4 % (v roku 1972). V oblasti humánnej medicíny na Slovensku Krčméry a i. (1975) upozorňujú, že pri vyšetreniach v rokoch 1973 až 1975 bol výskyt rezistentných kmeňov *S. aureus* ku penicilínu

82,4 %, k ampicilínu 40,6 %, k streptomycínu 27,6 %, k tetracyklínu 35,6 % a k erytomycínu 25,7 %, pričom táto situácia s istými obmenami pretrváva dodnes. Pozdnejšie Federič a i. (1980) upozorňujú na nepriaznivú situáciu vo veterinárnej medicíne, keď zo sledovaných 136 kmeňov *S. aureus* nebol ani jeden kmeň citlivý ku všetkým ôsmim testovaným antibiotikám, pričom na pretrvávanie nepriaznivého stavu vo vývoji rezistencie k antibiotikám pri kmeňoch *S. aureus* upozorňujú autori aj v roku 1988 (Federič a i., 1988). Malinowski a i. (1992) upozorňujú na nepriaznivú situáciu v citlivosti k antibiotikám u kmeňov *S. aureus* pri penicilínu (48,6% rezistencia) v Poľsku. Vasiľ (1994) opisuje výskyt rezistencie nielen pri tomto patogénovi za roky 1986 až 1992, pričom upozorňuje na potrebu neprestať v zostavovaní prehľadov citlivosti pôvodcov mastitíd k antibiotikám za väčšie lokality a regióny. Šimko a Bartko (1996) upozorňujú na nepriaznivý vývoj rezistencie k antibiotikám pri kmeňoch *S. aureus* izolovaných z klinických a latentných mastitíd oviec ku penicilínu. Malinowski a i. (1997) upozorňujú na pretrvávanie nepriaznivej situácie vo vývoji rezistencie k penicilínu pri *Staphylococcus* sp. zo začiatku 90. rokov. Vasiľ (1998) popisuje výskyt rezistencie k antibiotikám pri bakteriálnych pôvodcoch izolovaných z mastitíd dojníc v období rokov 1995 až 1997 na východnom Slovensku a udáva, že testované kmene *S. aureus* boli dobre citlivé k oxacilínu v 96,9 %, pričom v 95,3 % boli citlivé k chloramfenikolu, erytromycínu a k oxytetracyklínu, avšak vysoko rezistentné k penicilínu (31,3 %). Myllys a i. (1998) porovnávajú vývoj, tak prevalencie mastitíd dojníc, ako aj rezistencie k antibiotikám pri *S. aureus* a koagulázo-negatívnych staťylokokoch (CNS) na základe výsledkov dvoch reprezentatívnych súborov vo Fínsku. Konštatujú, že prevalencia mastitíd dojníc klesla zo 47,8 % v roku 1988 (4 495 vyšetrených dojníc) na 37,8 % v roku 1995 (2 648 vyšetrených dojníc). Zárodky *Staphylococcus* sp. tvorili väčšiu skupinu izolovaných patogénov, avšak proporcionálne výskyt v roku 1995 pri *S. aureus* poklesol na úkor vzostupu izolácie CNS. Výskyt kmeňov *S. aureus*, ktoré boli rezistentné aspoň k jednému zo sledovaných antibiotík, stúpol z 36,9 % (v roku 1988) na 63,6 % (v roku 1995), pričom podobne tomu bolo aj pri CNS – vzostup z 26,6 % na 49,7 %. Na náraste sa najčastejšie podieľali kmene *S. aureus* produkujúce betalaktamázu. Autori udávajú aj vzostup multirezistentných kmeňov *S. aureus* a CNS, ktorý však bol proporcionálny nárastu celkového počtu kmeňov rezistentných k antibiotikám.

Cieľom práce bolo stanovenie citlivosti voči ôsmim antibiotikám u 320 kmeňov *S. aureus* izolovaných z latentných a klinicky zjavných foriem mastitíd dojníc, mlieka, sterov odobratých z kože vemena, distálnych časti cecky, dojacieho zariadenia a prostredia.

MATERIÁL A METÓDA

V práci bolo celkom testovaných 320 kmeňov *S. aureus* izolovaných v priebehu rokov 1993 až 1997 z latentných a klinicky zjavných foriem mastitíd dojníc (subakútnych, akútnych a chronických), nadojeného mlieka, sterov odobratých tak z kože vemena, ako aj distálnych častí cecky, dojacieho zariadenia a prostredia. Odber a spracovanie vzoriek mlieka boli vykonané podľa platných metodík (Bulletin IDF, 1981). Agar-difúzny test citlivosti k ôsmim antibiotikám bol vykonaný podľa Bauer-Kirbyovej metódy (Bauer, 1966) na Miller-Hintonovom (MH) agare (Imuna, Š. Michaľany) v Petriho miskách o priemere 90 až 100 mm a hrúbke agaru 4 mm. Inokulum pre sledovanie citlivosti testovaných kmeňov patogénnych zárodkov k antibiotikám bolo pripravené podľa Urbáškovej a i. (1985) tak, aby výsledný zákal zodpovedal hustote 0,5 McFarlandovej zákalovej stupnice. Inokulácia bola vykonaná rozterom za pomoci upravených sterilných vatových tampónov. Odčítanie výsledkov testov sa vykonalo po 18hodinovej inkubácii platní v termostate pri teplote 37 °C.

Kontrola kvality používaných pôd a testačných diskov bola vykonaná podľa Urbáškovej a i. (1985) referenčným kmeňom *Staphylococcus aureus* ATCC 25923. Hodnotenie inhibičných zón sledovaných antibiotík u jednotlivých kmeňov bolo vykonané podľa Urbaškovej a i. (1985). Sledovanie citlivosti bolo vykonané u osem antibiotík. Boli použité testačné antibiotické disky Lachema Brno (tab. I).

VÝSLEDKY

Súhrnné výsledky sledovania rezistencie voči jednotlivým testovaným antibiotikám u 320 kmeňov *S. aureus* izolovaných z latentných a klinicky zjavných foriem mastitíd dojníc, mlieka, sterov odobratých z mliečnej žľazy dojníc a dojacieho zriadenia sú uvedené v tab. II.

Rezistencia voči penicilínu bola vysoká a predstavovala 22,5 %, pričom medzi výskytom penicilín-rezistentných kmeňov v rámci kmeňov sledovaných v štyroch skupinách nie markantného rozdielu.

Rezistencia voči ampicilínu bola vykazovaná 6,9 % všetkých testovaných kmeňov, pričom medzi výskytom ampicilín-rezistentných kmeňov v rámci sledovaných skupín boli pozorované rozdiely. Najmenej rezistentných kmeňov (3,0 %) bolo u kmeňov izolovaných z latentných mastitíd, pričom u kmeňov pochádzajúcich z klinických foriem mastitíd bol výskyt rezistentných kmeňov viac ako dvojnásobný (6,3 %). Najvyšší počet rezistentných kmeňov bol v skupinách nadojené mlieko (11,4 %) a sterov odobratých z tela dojníc a prostredia (8,6 %).

Rezistencia voči oxacilínu sa v rámci všetkých vyšetrovaných kmeňov vyskytovala v 1,9% frekvencii, keď všetky kmene izolované z latentných a klinických foriem mastitíd boli citlivé, ale kmene izolované zo sterov odobratých z tela dojníc a prostredia vykazovali 5,7% rezistenciu a kmene izolované z nadojeného mlieka boli necitlivé k oxacilínu v 2,5 %.

Rezistencia voči tertracyklínu sa vyskytovala celkove u 7,2 % testovaných kmeňov. Najvyšší podiel tetracyklín-rezistentných kmeňov vykázali kmene izolova-

I. Interpretácia priemerov inhibičných zón – Interpretation of the averages of inhibition zones

Antibiotikum[1]	Skratka[2]	Obsah účinnej látky v disku[3] (μg; IU)	Hodnotenie testu (priemer inhibičnej zóny v mm)[4]	
			rezistentný[5]	citlivý[6]
Penicilín	PNC	10*	<28	>29
Ampicilín	AMP	10	28	29
Oxacilín	OXA	1	12	13
Tetracyklín	TET	30	14	18
Chloramfenikol	CHF	30	12	18
Streptomycin	STR	30	15	20
Neomycin	NEO	30	20	26
Erytromycín	ERY	15	20	26

* = obsah účinnej látky je udávaný v medzinárodných jednotkách – content of active ingredient is given in IU

[1]antibiotic, [2]abbreviation, [3]content of active ingredient in disc, [4]test evaluation (average of inhibition zone in mm), [5]resistant, [6]sensitive

II. Rezistencia voči jednotlivým antibiotikám a chemoterapeutikám 320 kmeňov *Staphylococcus aureus* – Resistance to particular antibiotics of 320 *Staphylococcus aureus* strains

Kmene izolované z[1]	Celkový počet kmeňov[2]	Počet kmeňov (v %) rezistentných voči[3]							
		PNC	AMP	OXAC	TET	CHF	STR	NEO	ERY
Klinických mastitíd[4]	80	22,6	6,3	0,0	8,8	3,8	16,3	1,3	0
Latentných infekcií[5]	100	21,0	3,0	0,0	6,0	0,0	13,0	2,0	1,0
Mlieka[6]	70	24,3	11,4	2,5	6,3	2,9	11,3	1,4	0,0
Sterov z tela dojníc a prostredia[7]	70	22,9	8,6	5,7	6,3	2,9	17,1	1,4	1,4
Spolu[8]	320	22,5	6,9	1,9	7,2	2,2	14,7	1,6	0,6

[1]strains isolated from, [2]total number of strains, [3]number of strains (in %) resistant to, [4]clinical mastitis, [5]latent infections, [6]milk, [7]smears from daily cow body and environment, [8]total

III. Typovosť rezistencie voči antibiotikám u 320 kmeňov *Staphylococcus aureus* – Type of resistance to antibiotics in 320 *Staphylococcus aureus* strains

Kmene izolované z[1]	Celkový počet kmeňov citlivých voči testovaným antibiotikám[2]	Počet kmeňov rezistentných voči[3]					
		jednému antibiotiku[4]	2	3	4	5	6
			antibiotikám[5]				
Klinických mastitíd[6]	47	23	7	2	1	0	0
Latentných infekcií[7]	63	31	3	3	0	0	0
Mlieka[8]	46	14	2	5	3	0	0
Sterov z tela dojníc a prostredia[9]	47	11	6	3	1	1	1
Spolu[10]	203	79	18	13	5	1	1

[1]strains isolated from, [2]total number of strains sensitive to tested antibiotics, [3]number strains resistant to, [4]one antibiotic, [5]antibiotics, [6]clinical mastitis, [7]latent infections, [8]milk, [9]smears from dairy co[illegible]body and environment, [10]total

né z klinických foriem mastitíd (8,8 %), pričom v ostatných skupinách bol výskyt rezistentných kmeňov v rozpätí 6,0 až 6,3 %.

Rezistencia voči streptomycínu sa v rámci všetkých testovaných antibiotík vyskytovala ako druhá najčastejšia. Celkove 14,7 % testovaných kmeňov bolo rezistentných ku streptomycínu. Najvyššie percento zastúpenia rezistentných kmeňov ku streptomycínu bolo u kmeňov izolovaných zo sterov odobratých z tela dojníc a prostredia (17,1 %), potom u kmeňov pochádzajúcich z klinických foriem mastitíd (16,3 %), ďalej u kmeňov izolovaných z latentných mastitíd (13,0 %), pričom 17,1 % rezistentných kmeňov pochádzalo z nadojeného mlieka.

V rámci 320 testovaných kmeňov bolo ku chloramfenikolu rezistentných 2,2 %, ku neomycínu 1,6 % a k erytromycínu len 0,6 %, čo svedčí o dobrej citlivosti *S. aureus* k týmto antibiotikám.

Typovosť rezistencie voči antibiotikám u testovaných kmeňov *S. aureus* je uvedená v tab. III.

IV. Výskyt jednotlivých vzorcov antibiotiko-rezistencie u 320 kmeňov *Staphylococcus aureus* – The occurrence of particular formulae of antibiotic resistance in 320 *Staphylococcus aureus* strains

Kmene izolované z[1]	Počet antibiotiko-rezistentných kmeňov[2]	Typovosť antibiotiko-rezistence u sledovaných kmeňov[3]					
		mono-	bi-	tri-	tetra-	penta-	hexa-
Klinicky zjavných mastitíd dojníc[4]	33	P 9 S 8 CH 3 T 3	PA 3 PS 3 TS 1	PAT 1 PTS 1	PATN 1	0	0
Latentných infekcií[5]	37	P 15 S 10 T 6	PA 2 PS 1	PNS 2 PAE 1	0	0	0
Mlieka[6]	24	P 9 S 3 CH 2	PO 1 PS 1	PAT 2 PAS 2 PAO 1	PAST 2 ATSN 1	0	0
Sterov z tela dojníc a prostredia[7]	23	P 6 S 3 CH 2	PA 2 PS 2 TS 2	PAS 1 POS 2	PATS 1	PATON 1	PAOTSE 1

Legend: P – penicillin, A – ampicillin, O – oxacillin, T – tetracycline, CH – chloramphenicol, S – streptomycin, N – neomycin, E – erythromycin

[1]strains isolated from, [2]number of antibiotic resistant strains, [3]type of antibiotic resistances in strains observed, [4]clinically apparent dairy cow mastitis, [5]latent infections, [6]milk, [7]smears from dairy cow body and environment

Z celkového počtu 320 kmeňov sa citlivosťou voči všetkým ôsmi antibiotikám vyznačovalo 203 kmeňov (čo činí 63,44 %).

Rezistenciu len voči jednému antibiotiku vykazovalo 79 kmeňov (24,69 %);

Rezistenciu súčasne voči dvom antibiotikám vykazovalo 18 kmeňov (5,63 %);

Rezistenciu súčasne voči trom antibiotikám vykazovalo 13 kmeňov (4,06 %).

Výskyt rezistencie súčasne voči štyrom antibiotikám možno označiť za vzácny – touto charakteristikou sa vyznačovali štyri kmene (1,56 %).

Výskyt kmeňov rezistentných voči piatim a šiestim antibiotikám súčasne treba označiť za výnimočný – v oboch prípadoch sa jednalo len o jediný kmeň (0,31 %). Oba kmene vyznačujúce sa týmito širokými vzorcami antibiotikorezistencie boli izolované zo sterov odobratých z dojacieho zariadenia.

Konkrétny rozpis jednotlivých vzorcov antibiotiko-rezistencie u 320 kmeňov *S. aureus* je uvedený v tab. IV.

V rámci kmeňov rezistentných len k jednému antibiotiku dominujú kmene rezistentné ku penicilínu (celkom 39), menej často sa vyskytujú kmene rezistentné voči streptomycínu (24 kmeňov), ďalej voči tetracyklínu (deväť kmeňov) a voči chloramfenikolu (sedem kmeňov).

V rámci kmeňov rezistentných súčasne voči dvom antibiotikám sa najčastejšie vyskytujú kmene rezistentné voči penicilínu a streptomycínu (sedem kmeňov), ale aj penicilínu a ampicilínu (sedem kmeňov), menej často súčasne voči tetracyklínu a streptomycínu (tri kmene) a penicilínu a oxacilínu (jeden kmeň).

Medzi kmeňmi rezistentnými súčasne voči trom antibiotikám sa najčastejšie vyskytovali kmene rezistentné súčasne voči penicilínu, ampicilínu a tetracyklínu (tri kmene) a penicilínu, ampicilínu a streptomycínu (tri kmene), menej kmene rezistentné súčasne voči penicilínu, oxacilínu a streptomycínu (dva kmene), kým výskyt rezistencie súčasne voči penicilínu, ampicilínu a oxacilínu (jeden kmeň) a rezistencie súčasne voči penicilínu, ampicilínu a erytromycínu (jeden kmeň), ako aj rezistencie súčasne voči penicilínu, streptomycínu a tetracyklínu (jeden kmeň) bol ojedinelý.

Z celkového počtu piatich kmeňov vykazujúcich rezistenciu súčasne voči štyrom antibiotikám tri kmene boli rezistentné súčasne voči penicilínu, ampicilínu, streptomycínu a tertracyklínu, jeden kmeň bol rezistentný súčasne voči penicilínu, ampicilínu, tetracyklínu a neomycínu a tak isto jeden kmeň vykazoval rezistenciu súčasne voči ampicilínu, tetracyklínu, streptomycínu a neomycínu.

Kmeň vykazujúci rezistenciu súčasne voči piatim antibiotikám mal vo svojom vzorci antibiotiko-rezistencie penicilín, ampicilín, oxacilín, tetracyklín, neomycín a kmeň vykazujúci rezistenciu voči šiestim antibiotikám mal nasledujúci vzorec antibiotiko-rezistencie – penicilín, ampicilín, oxacilín, tetracyklín, streptomycín a erytromycín.

DISKUSIA

Ako vyplýva z výsledkov 22,5% výskyt rezistencie u kmeňov *S. aureus* k penicilínu i napriek poklesu oproti predchádzajúcim rokom (Vasiľ, 1994) je vysoký, podobne ako aj 14.7% výskyt rezistentných kmeňov tohoto patogéna pri streptomycíne. Druhou prezentovanou skutočnosťou je pomerne častý výskyt kmeňov re-

zistentných k viacerým antibiotikám súčasne, ktorá je sprievodným javom nerešpektovania takých zásad pri terapii mastitíd, ako je voľba antibiotického intramamárneho prípravku v súlade s dlhodobými prehľadmi citlivosti *S. aureus* k antibiotikám, priestupnosť požitého antibiotika do ložiska zápalu, dodržanie požadovanej dĺžky liečby pri udržaní požadovaných hladín použitého antibiotika v mieste zápalu atď. Používanie kombinácie antibiotík v jednom intramammárnom preparáte je do istej miery odrazom typovosti vzorcov antibiotiko-rezistencie pri jednotlivých sledovaných kmeňoch *S. aureus*. Nepoužívanie chloramfenikolu v posledných rokoch v liečbe mastitíd sa zjavne odrazilo v poklese výskytu rezistentných kmeňov k tomuto antibiotiku. Výsledky dokladajú, že jedným z najdôležitejších faktorov pri nadobúdaní rezistencie u kmeňov *Staphylococcus* sp. je s najväčšou pravdepodobnosťou nedosiahnutie požadovaných terapeutických hladín požívaného antibiotika v mieste liečeného zápalu počas nevyhnutne nutného času pre liečbu.

Watts a i. (1995) sledovali MIC používaných a novozavádzaných antibiotík určených k liečbe mastitíd. Zo 135 kmeňov *S. aureus* izolovaných zo sekrétu mliečnej žľazy 90,0 % kmeňov vykazovalo nasledovné MIC: penicilín 13,0 μg/ml; kloxacilín 0,5 μg/ml; cephapirín 1,0 μg/ml; ceftiofur 0,5 μg/ml; novobiocín 0,5 μg/ml; enrofloxacin 0,5 μg/ml; erytromycín 0,5 μg/ml; pirlimycín 0,5 μg/ml. V rámci 1 225 kmeňov *S. aureus* sp. boli MIC nasledovné: penicilín 1,0 μg/ml; kloxacilín 1,0 μg/ml; cephapirín 0,5 μg/ml; ceftiofur 1,0 μg/ml; novobiocín 0.5 μg/ml; enrofloxacin 0,5 μg/ml; erytromycín 1,0 μg/ml; pirlimycín 0,5 μg/ml. Pereira a Siquire (1995) testovali 46 kmeňov *S. aureus* na výskyt rezistencie k 21 antimikrobiálnym činidlám. Najčastjše bola rezistencia zaznamenaná ku streptomycínu, tetracyklínu, erytomycínu, kanamycínu, neomycínu, ale aj ku kádmiu a arzénu. Všetky testované kmene boli citlivé k chloramfenikolu, doxycyklínu, gentamycínu, methicilínu, minocyklínu, novobiocínu, rifamycínu, tylozínu, vankomycínu, ale aj k etidium bromidu, cetrimidínu, benzalkonim chloridu. Iba šesť testovaných kmeňov bolo citlivých k všetkým testovaným činidlám. Autori udávajú, že po ošetrení rezistenciu vykazujúcich kmeňov etidium bromidom bola eliminovaná rezistencia k penicilínu, tetracyklínu, streptomycínu, erytomycínu a kadmiu, ktorá s najväčšou pravdepodobnosťou bola viazaná plazmidicky. Ayliffe (1997) popisuje 16 skupín fagotypov meticilín-rezistentných kmeňov *S. aureus*, pričom fagotyp 84 vykázal súčasne rezistenciu k penicilínu, tetracyklínu, erytromycínu, kanamycínu, gentamycínu a bacitracínu. Fagotyp *S. aureus* 28/83C/a32 bol rezistentný súčasne ku penicilínu, tetracyklínu, fusidic acidu, kanamycínu, gentamicínu, bacitracínu a fagotyp *S. aureus* 29/52/75/83A/83C vykazoval resistenciu voči penicilínu, erytromycínu, kanamycínu, gentamycínu, ciprofloxacínu. Zároveň upozorňuje, že v Anglicku v roku 1970 bol 9% výskyt methicilín rezistentných kmeňov *S. aureus*, ktorý v roku 1980 poklesol až na 1 %, ale nepriaznivý vývoj v antibiotickej politike znamenal, že v roku 1993 výskyt presahoval už 14% úroveň.

Šimko a Bartko (1996) sledovali difúznou diskovou metódou rezistenciu ku 14 antibiotikám a k jednému chemoterapeutiku u kmeňov *S. aureus* izolovaných z klinických a latentných mastitíd oviec, ovčieho mlieka a výrobkov z neho. Najvyšší stupeň rezistencie bol zaznamenaný pri kmeňoch izolovaných z klinických foriem mastitíd, ktorý sa pohyboval v rozpätí od 10 % (pri kanamycíne) do 68 % (pri penicilíne) až 69 % (pri tetracyklíne). U kmeňov *S. aureus* izolovaných z latentných mastitíd bol stupeň výskytu rezistentných kmeňov k antibiotikám v porovnaní s kmeňmi izolovanými z klinických foriem nižší u 13 sledovaných antibiotík a pohyboval sa v rozpätí od 3 % (pri spiramycíne) do 30 % (pri penicilíne), pričom výskyt rezistentných kmeňov ku kanamycínu stúpol na 13 %.

Výskyt rezistencie u kmeňov *S. aureus* aj v budúcnosti bude závisieť od mnohých faktorov, pričom ale zväčša je sprevádzaný nesprávnymi návykmi pri liečbe mastitíd dojníc. Úspešné zvládnutie intramamárnych infekcií spôsobených *S. aureus* bude podmienené racionálnym používaním antibiotík zohľadňujúc pritom požadovanú MIC antibiotika pre vyvolávajúceho pôvodcu, pričom voľba antibiotika pre liečbu hlavne akútnych mastitíd, kde sa vyžaduje rýchlosť zásahu, musí vychádzať z dlhodobých prehľadov citlivosti jednotlivých kmeňov za danú lokalitu, čo určite obmedzí aj častý výskyt rezistentných kmeňov.

LITERATÚRA

Ayliffe G. A. J. (1997): The progressive intercontinental spread of methicillin-resistant *Staphylococcus aureus*. Clin. Inf. Dis., *24*, (Suppl 1), 74–79.

Bauer A. W., Kirby W. M., Sherris J. C., Turck M. (1966): Antibiotic susceptibility by a standardized single disc. Am. J. Clin. Pathol., *45*, 493–496.

Benda P., Vyletělová M. (1995): Výskyt *Staphylococcus aureus* v bazénových vzorcích mléka. Vet. Med. – Czech, *40*, 221–226.

Christie G. J., Keffe T. J., Strom P. W. (1974): Cloxacillin and the dry cow. Vet. Med. Small Anim. Clin., *69*, 1403–1408.

Federič F., Jordánová V., Petrík P. (1980): Rezistencia voči antibiotikám u grampozitívnych kokovitých baktérií izolovaných pri prvovýrobe mlieka. [Záverečná správa.] Košice, ÚEVM. 47 s.

Federič F., Elečko J., Vasiľ M., Petrík P., Jordánová V., Fotta M., Rosocha J., Naďová P. (1988): Staťylokokové mastitídy prežúvavcov. [Záverečná správa.] Košice, ÚEVM. 73 s.

IDF (1981): Laboratory Methods for Use in Mastitis Work. IDF Bull., Document No. 132, 27 s.

Kotowski K. (1992): Częstotliwosc występowania klinicznych przypahków mastitis u krów chowie wielkostadnym. Med. Vet., *47*, 12, 555–556.

Krčmery V., Grunt J., Rosival L., Výmola F. (1975): Nation-wide survey of antibiotic resistance by means of

a computer analysis of 200 000 strains of problem bacteria isolated in 1973–1975. In: Mitsuhashi S., Rosival L., Krčmery V. (eds.): Drug-inactivating Enzymes and Antibiotic Resistance. Praha, Avicenum. 17–23.

Malinowski E., Kłossowska A., Kuźma K., Krukovski H. (1992): Wrazliwosc na antybiotyki bakterii wyosobnionych z wydzieliny zapalnej gruczolu lekowego krow. Med. Wet., *48*, 366–367.

Malinowski E., Pilaszek J., Kłossowska A., Sobolewsk S., Sobolewski J. (1997): Zamiany wrazliwosci na antybiotyki bakterii wyosobnionych z klinicznych postaci mastitis u krow w latach 1987–1996. Med. Wet., *53*, 12, 722–725.

McDonald J. S., Anderson A. J. (1981): Antibiotic sensitivity of *Staphylococcus aureus* and coagulase negative staphylococci isolated from infected bovine mammary glands. Cornell Vet., *71*, 4, 391–396.

Myllys V., Asplund K., Hirvela-Koski V., Honkanen-Buzalski T., Juttila J., Kulkas L., Myllykangas O., Niskanen M., Saloniemi H., Sandholm M., Saranpaa T. (1998): Bovine mastitis in Finland in 1988 and 1995 – changes in prevalence and antimicrobial resistance. Acta. Vet. Scand., *39*, 1, 119–126.

Pereira M. S. V., Siquiera J. P. Jun. (1996): Antimicrobial drug resistance in *Staphylococcus aureus* isolated from cattle in Brazil. Lett. Appl. Microbiol., *20*, 6, 391–395.

Schultze W. D. (1983): Effets of selective regimen of dry cow therapy on intramammary infection and antibiotic sensitivity of surviving pathogens. J. Dairy Sci., *66*, 4, 892–903.

Šimko Š., Bartko P. (1996): Rezistencia na antibiotiká u *Staphylococus aureus* pri mastitídach oviec, ovčom mlieku a výrobkov z neho. Vet. Med. – Czech, *41*, 241–244.

Thornsberry C., Burton P. J., Yee Y. E., Watts J. L., Yancey J. R. (1997): The activity of a combination of penicillin and novobiocin against bovine mastitis pathogens: development of a disk diffusion test. J. Dairy Sci., *80*, 413–421.

Urbašková P., Hausnerová S., Chaloupecký V., Lochmanová J., Výmola F., Zahradnický J. (1985): Vyšetření pro antimikrobiálni terapii. In: Schindler J. a i.: Mikrobiologické vyšetřovací metody. 2. sv. Praha, Avicenum. 152 s.

Vasiľ M. (1994): Výskyt rezistencie k antibiotikám pri bakteriálnych pôvodcoch mastitíd dojníc. Vet. Med. – Czech, *39*, 9, 503–509.

Vasiľ M., (1998): Rezistencia k antibiotikám pri bakteriálnych pôvodcoch izolovaných z mastitíd dojníc. Vet. Med. – Czech, *43*, 201–205.

Watts J. L. a i. (1995): Antimicrobial susceptibility of microorganisms isolated from the mammary glands of dairy heifers. J. Dairy Sci., *78*, 1637–1648.

Received: 98–06–08
Accepted after corrections: 98–11–24

Kontaktná adresa:

MVDr. Milan Vasiľ, CSc., Výskumný ústav veterinárnej medicíny, Hlinkova 1/A, 040 01 Košice, Slovenská republika
Tel. +421 95 633 20 11–12, fax +421 95 633 18 53

POKYNY PRO AUTORY

Časopis uveřejňuje původní vědecké práce, krátká sdělení a výběrově i přehledné referáty, tzn. práce, jejichž podkladem je studium literatury a které shrnují nejnovější poznatky v dané oblasti. Práce jsou uveřejňovány v češtině, slovenštině nebo angličtině. Rukopisy musí být doplněny krátkým a rozšířeným souhrnem. Časopis zveřejňuje i názory, postřehy a připomínky čtenářů ve formě kurzívy, glosy, dopisu redakci, diskusního příspěvku, kritiky zásadního článku apod., ale i zkušenosti z cest do zahraničí, z porad a konferencí.

Autoři jsou plně odpovědni za původnost práce a za její věcnou i formální správnost. K práci musí být přiloženo prohlášení o tom, že práce nebyla publikována jinde.

O uveřejnění práce rozhoduje redakční rada časopisu, a to se zřetelem k lektorským posudkům, vědeckému významu a přínosu a kvalitě práce. Redakce přijímá práce imprimované vedoucím pracoviště nebo práce s prohlášením všech autorů, že se zveřejněním souhlasí.

Rozsah původních prací nemá přesáhnout 10 stran psaných na stroji včetně tabulek, obrázků a grafů. V práci je nutné používat jednotky odpovídající soustavě měrových jednotek SI.

Rukopis má být napsán na papíře formátu A4 (30 řádek na stránku, 60 úhozů na řádku, mezi řádky dvojité mezery). K rukopisu je vhodné přiložit disketu s textem práce, popř. s grafickou dokumentací pořízenou na PC s uvedením použitého programu. Tabulky, grafy a fotografie se dodávají zvlášť, nepodlepují se. Na všechny přílohy musí být odkazy v textu.

Pokud autor používá v práci zkratek jakéhokoliv druhu, je nutné, aby byly alespoň jednou vysvětleny (vypsány), aby se předešlo omylům. V názvu práce a v souhrnu je vhodné zkratek nepoužívat.

Název práce (titul) nemá přesáhnout 85 úhozů a musí dát přesnou představu o obsahu práce. Jsou vyloučeny podtitulky článků.

Krátký souhrn (Abstrakt) musí vyjádřit všechno podstatné, co je obsaženo v práci, a má obsahovat základní číselné údaje včetně statistických hodnot. Nemá překročit rozsah 170 slov. Je třeba, aby byl napsán celými větami, nikoliv heslovitě.

Rozšířený souhrn prací v češtině nebo slovenštině je uveřejňován v angličtině, měly by v něm být v rozsahu cca 1–2 strojopisných stran komentovány výsledky práce a uvedeny odkazy na tabulky a obrázky, popř. na nejdůležitější literární citace. Je vhodné jej (včetně názvu práce a klíčových slov) dodat v angličtině, popř. v češtině či slovenštině jako podklad pro překlad do angličtiny.

Literární přehled má být krátký, je třeba uvádět pouze citace mající úzký vztah k problému. Tato úvodní část přináší také informaci, proč byla práce provedena.

Metoda se popisuje pouze tehdy, je-li původní, jinak postačuje citovat autora metody a uvádět jen případné odchylky. Ve stejné kapitole se popisuje také pokusný materiál a způsob hodnocení výsledků.

Výsledky tvoří hlavní část práce a při jejich popisu se k vyjádření kvantitativních hodnot dává přednost grafům před tabulkami. V tabulkách je třeba shrnout statistické hodnocení naměřených hodnot. Tato část by neměla obsahovat teoretické závěry ani dedukce, ale pouze faktické nálezy.

Diskuse obsahuje zhodnocení práce, diskutuje se o možných nedostatcích a výsledky se konfrontují s údaji publikovanými (požaduje se citovat jen ty autory, jejichž práce mají k publikované práci bližší vztah). Je přípustné spojení v jednu kapitolu spolu s výsledky.

Literatura citovaná v textu práce se uvádí jménem autora a rokem vydání. Do seznamu se zařadí jen publikace citované v textu. Citace se řadí abecedně podle jména prvních autorů.

Klíčová slova mají umožnit vyhledání práce podle sledovaných druhů zvířat, charakteristik jejich zdravotního stavu, podmínek jejich chovu, látek použitých k jejich ovlivnění apod. Jako klíčová slova není vhodné používat termíny uvedené v nadpisu práce.

Na zvláštním listě uvádí autor plné jméno (i spoluautorů), akademické, vědecké a pedagogické tituly a podrobnou adresu pracoviště s PSČ, číslo telefonu a faxu, popř. e-mail.

Úplné znění pokynů pro autory s dodatky najdete na URL adrese
http://www.clark.cz/vri/Pokyny.htm

For full text of instruction for authors see
http://www.clark.cz/vri/Pokynya.htm

INSTRUCTIONS FOR AUTHORS

Original scientific papers, short communications, and selectively reviews, that means papers based on the study of technical literature and reviewing recent knowledge in the given field, are published in this journal. Published papers are in Czech, Slovak or English. Each manuscript must contain a short or a longer summary. The journal also publishes readers' views, remarks and comments in form of a text in italics, gloss, letter to the editor, short contribution, review of a major article, etc., and also experience of stays in foreign countries, meetings and conferences.

The authors are fully responsible for the originality of their papers, for its subject and formal correctness. The authors shall make a written declaration that their papers have not been published in any other information source.

The board of editors of this journal will decide on paper publication, with respect to expert opinions, scientific importance, contribution and quality of the paper. The editors accept papers approved to print by the head of the workplace or papers with all the authors' statement they approve it to print.

The extent of original papers shall not exceed ten typescript pages, including tables, figures and graphs.

Manuscript should be typed on standard paper (quarto, 30 lines per page, 60 strokes per line, double-spaced typescript). A PC diskette with the paper text or graphical documentation should be provided with the paper manuscript, indicating the used editor program. Tables, figures and photos shall be enclosed separately. The text must contain references to all these annexes.

The **title** of the paper shall not exceed 85 strokes and it should provide a clear-cut idea of the paper subject. Subtitles of the papers are not allowed either.

Abstract. It must present information selection of the contents and conclusions of the paper, it is not a mere description of the paper. It must present all substantial information contained in the paper. It shall not exceed 170 words. It shall be written in full sentences, not in form of keynotes and comprise base numerical data including statistical data.

Introduction has to present the main reasons why the study was conducted, and the circumstances of the studied problems should be described in a very brief form. This introductory section also provides information why the study has been undertaken.

Review of literature should be a short section, containing only literary citations with close relation to the treated problem.

Only original method shall be described, in other cases it is sufficient enough to cite the author of the used method and to mention modifications of this method. This section shall also contain a description of experimental material and the method of result evaluation.

In the section **Results**, which is the core of the paper, figures and graphs should be used rather than tables for presentation of quantitative values. A statistical analysis of recorded values should be summarized in tables. This section should not contain either theoretical conclusions or deductions, but only factual data should be presented here.

Discussion contains an evaluation of the study, potential shortcomings are discussed, and the results of the study are confronted with previously published results (only those authors whose studies are in closer relation with the published paper should be cited). The sections Results and Discussion may be presented as one section only.

References in the manuscript are given in form of citations of the author's name and year of publication. A list of references should contain publications cited in the manuscript only. References are listed alphabetically by the first author's name.

Key words should make it possible to retrieve the paper on the basis of the animal species investigated, characteristics of their health, husbandry conditions, applied substances, etc. The terms used in the paper title should not be used as keywords.

If any abbreviation is used in the paper, it is necessary to mention its full form at least once to avoid misunderstanding. The abbreviations should not be used in the title of the paper nor in the summary.

The author shall give his full name (and the names of other collaborators), academic, scientific and pedagogic titles, full address of his workplace and postal code, telephone and fax number, or e-mail.

VETERINARY MEDICINE – CZECH

Volume 44, No. 4, April 1999

CONTENTS

VETERINÁRNÍ MEDICÍNA

Ročník 44, č. 4, Duben 1999

OBSAH

Vědecký časopis VETERINÁRNÍ MEDICÍNA ● Vydává Ústav zemědělských a potravinářských informací ● Redakce: Slezská 7, 120 56 Praha 2, tel.: 02/24 25 79 39, fax: 02/24 25 39 38 ● Sazba: Studio DOMINO – ing. Jakub Černý, Bří. Nejedlých 245, 266 01 Beroun, tel.: 0311/229 59 ● Tisk: ÚZPI Praha ● © Ústav zemědělských a potravinářských informací, Praha 1999

Rozšiřuje Ústav zemědělských a potravinářských informací, referát odbytu, Slezská 7, 120 56 Praha 2