

ÚSTAV ZEMĚDĚLSKÝCH A POTRAVINÁŘSKÝCH INFORMACÍ

# VETERINÁRNÍ MEDICÍNA

Veterinary Medicine – Czech

ČESKÁ AKADEMIE ZEMĚDĚLSKÝCH VĚD

6

VOLUME 45  
PRAHA  
JUNE 2000  
ISSN 0375-8427

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

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

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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.

**Periodicita:** Časopis vychází měsíčně (12x ročně), ročník 45 vychází v roce 2000.

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**Periodicity:** The journal is published monthly (12 issues per year), Volume 45 appearing in 2000.

**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: [edit@uzpi.cz](mailto:edit@uzpi.cz). Detailed instructions for authors are available in the editorial office and at URL address <http://www.vri.cz> or <http://www.uzpi.cz>

**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 2000 is 159 USD (Europe), 167 USD (overseas).

# THE COLLECTION OF OVIDUCTAL FLUID FROM SHEEP\*

## ODBĚR OVIDUKTÁLNÍ TEKUTINY OD OVCÍ

T. Slavík<sup>1</sup>, R. Doležel<sup>2</sup>, J. Fulka<sup>1</sup>

<sup>1</sup>*Institute of Animal Physiology and Genetics, Academy of Sciences of the Czech Republic, Liběchov, Czech Republic*

<sup>2</sup>*Faculty of Veterinary Medicine, Veterinary and Pharmaceutical University, Brno, Czech Republic*

**ABSTRACT:** Oviduct provides natural environment for gamete transport, fertilization and early embryonic development. The information about composition of milieu in the oviduct at defined stage of estrus cycle is valuable for the improvement of media employed in mammalian embryo production *in vitro* and for the investigation of sperm-egg interaction at fertilization. The intention of these studies was to collect oviductal secretion for biological assays and for chemical analyses using permanent indwelling oviductal cannulae in 14 merino ewes (28 oviducts). In 4 females (8 oviducts) with previously synchronized estrus cycle the harvest of secretion was low (maximal average 0.3 ml/day) and the flow stopped before day 7 after surgical intervention, mostly due to the occlusion by fibrinous plugs. The effect of unilateral ligation localized close to the uterotubal junction was compared in 6 ewes (6 ligated and 6 unligated oviducts). The ligation of the oviducts promoted the function of the canulae for several subsequent estrous cycles, while in unligated counterparts the cannulae became usually occluded with fibrin mostly before third cycle. In remaining 4 females (8 oviducts) in random stage of estrous cycles bilateral ligation was performed. The results show that this treatment was most suitable for collection of oviductal fluid. Despite fluctuation, in 8 from totally ligated 14 oviducts the undisturbed function of cannulae was recorded for more than 2 estrous cycles and in two instances for 150 days. The harvested volumes of secretion were in close relation to the estrous cycle with typical highest amount around the heat (1.2 ml per day) and lowest at the luteal phase (0.3 ml/day). The causes of flow cessation were mostly the formation of fibrinous plugs at the junction with oviduct or evulsion of cannulae by the subject. Nevertheless, the experiments indicate that cannulae may represent effective method for collection of oviductal secretion for biochemical analysis or direct physiological experiments.

oviduct; oviductal fluid; collection; cannulation; sheep

**ABSTRAKT:** Savčí vejcovod vytváří přirozené prostředí pro transport gamet, oplození a vývoj raných stadií embryí. Informace o tomto prostředí mohou přispět ke zlepšení metod užívaných k produkci embryí v podmínkách *in vitro* i k prohloubení teoretických poznatků o vzájemné interakci samčích a samičích gamet. Cílem této práce bylo nashromáždění dostatečného množství oviduktálního sekretu pomocí permanentní kanyly zavedené do ampulární části vejcovodu pro následné biologické experimenty a chemické analýzy. Nejlepších výsledků bylo dosaženo u těch zvířat, jejichž vejcovody byly v místě uterotubálního spoje podvázány. Tento postup zaručuje dlouhodobou funkci kanyly. Objem získaného sekretu kolísal mezi 0,3 ml/den v období luteální fáze a 1,2 ml/den v období říje, zatímco u nepodvázaných vejcovodů byl objem odebrané tekutiny významně nižší. Rovněž spolehlivost a doba odběru byla u podvázaných kanyl podstatně vyšší. Zatímco nepodvázané kanyly fungovaly maximálně 75 dní, některé experimenty s podvázanými vejcovody byly v plné funkci ukončeny 150 den. Výsledky ukazují, že kanylace ovčích vejcovodů je vhodnou metodou k získání dostatečného množství oviduktální tekutiny jak pro biochemické analýzy, tak pro přímé fyziologické experimenty.

vejcovod; oviduktální tekutina; odběr; kanylace; ovce

\* Supported by the Grant Agency of the Czech Republic (Grant No. 524/99/0844) and by the National Agency for Agricultural Research in Prague (Grant No. EP0960006203).

## INTRODUCTION

Oviduct in mammals is essential for gamete transport, fertilization and embryonic development. During estrus cycle the cells lining the oviductal wall produce under the control of circulating gonadal steroid hormones specific molecules that are not present in blood serum. The volume and the composition of oviductal fluid fluctuate in relation to the stage of estrus cycle. The precise role of oviductal fluid in fertilization and embryonic development has not been clearly defined at present. While the fertilization rate of most mammals reaches in artificial media relatively high constant level, the viability of cultured embryos remains mostly far below the counterparts developing in the oviducts. Which components are responsible for the differences waits for elucidation. Unexplained is also the role of specific proteins secreted by oviductal cells around ovulation. From review articles published by Gandolfi (1995) and Nancarrow and Hill (1995) seems evident that they bound to the surface of the oocytes after ovulation and to the spermatozoa after mating. This binding most probably contributes to the interaction between male and female gametes, to the induction of cortical granule reaction and subsequently to the modification of zona pellucida (Kim et al., 1996).

Therefore, it is not surprising that many attempts have been made to study the composition of oviductal secretion and to define the physiological role of individual components. The prerequisite for the analysis is sufficient amount of suitable experimental material. For this aim three basic approaches were chosen. Joshi (1988), Murray (1992) and Abe et al. (1993) used for this purpose simple flushing of bovine and ovine oviducts. Gandolfi et al. (1989, 1991); Buih et al. (1991) and Wegner and Killian (1992) exploited for analysis medium obtained after cultivation of oviductal cell suspensions or tissue explants harvested at defined stages of estrus cycle. From several aspects appears most acceptable the fluid collected by means of well operating permanent cannula inserted to the oviduct. This approach was applied nearly 30 years ago in rabbits and involved the installation of rather complicated devices in abdominal cavity. Later, Sutton et al. (1984, 1986) described the cannulation of sheep oviduct and the collection of the secretion into test-tube situated outside the body. Gradually the technique have been modified and improved by several authors (Kavanaugh and Killian, 1988; Killian et al., 1989; Gerena and Killian, 1990; Kavanaugh et al., 1992) and resulted in the prolongation of undisturbed flow of the fluid. To the progress contributed mainly the exploitation of new and more suitable materials for intubation and collection of fluid. However, irrespective to the improvement of employed technique not all attempts were successful and the flow of fluid stopped for various reasons at different periods after surgical intervention.

For our experiments addressed to the characterisation of surface properties of *in vitro* matured and ovulated

oocytes we required the secretion of physiological composition from sheep oviducts. After evaluation of previously published procedures, considering particularly details provided by Kavanaugh and Killian (1988) and Kavanaugh et al. (1992) we suggested own modification that enabled to collect the fluid through several estrus cycles.

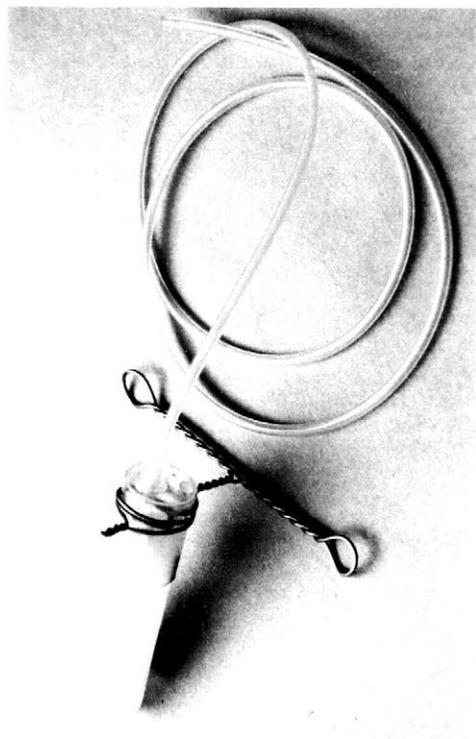
## MATERIAL AND METHODS

In the experiments were included 14 adult ewes of Merino breed 2 to 3 years old. In 4 animals the estrus cycles were synchronised with progesterone sponges for 14 days and these animals were operated at a day of the sponges removal. Preoperative preparation consisted of withholding all food and water. For the immobilisation of the subjects 1.5 ml of Rometar (Spofa Praha) was applied *i.m.* Then the animal was placed on operation table, fixed the legs and elevated back part of body. The abdominal wall was shorn and shaved and after careful scrubbing, 70% ethyl alcohol was used as a final rinse. For regional anesthesia the wall was infiltrated with 2% procain. Finally, the operation field was covered with sterile gauze. The incision, about 15 cm long, was placed in *linea alba* close to the udder.

After the entry to abdomen cavity firstly one uterine horn, oviduct and ovary were exposed by gentle traction. The ostium surrounded by fimbria was identified and cannula (Silastic Medical-Grade Tubing, Dow Corning, Cat. No. 602-205) introduced through ostium into the lumen of ampulla for about 3 cm. Two cuffs localized at the end of cannula were easily palpated through the oviductal wall and allowed the fixation by the ligature that was tied around the oviduct between the cuffs. The ligature was drawn tight enough to hold catheter in place and prevent leakage of oviductal fluid without encroaching on the catheter lumen patency. In most instances one oviduct was tightly ligated near to the tip of uterine horn to prevent the flow of fluid to uterine horn, in 6 others the ligation was not applied for comparison only unilaterally. After completion of the cannulation of one oviduct, the second one was treated by the same way. Subsequently cannulae were situated into abdomen cavity and both ends were ran along the inner wall to the area *tuber coxae*. From outside skin and musculature of abdominal wall were perforated by a trocar. After removing of spike both free ends were pulled through the trocar tube out. The tube was then removed and the length of cannulae adjusted. The incision in *linea alba* was closed in two layers and animal treated with antibiotics.

Already on standing animal the belt with devices for two small collectors was fixed around the body. Free ends were then localized through stopper inside collectors. To reduce contamination all these manipulations were carried out under sterile conditions. Because these belts were not stable, in part of animals, another system

of collectors holder has been used. Small incision (1.5 cm long) was made approx. 2 cm under the site, where cannulae leave the abdomen cavity. Then, T-piece connected with collector (Fig. 1) was inserted under the skin and the incision closed.



I. T-piece connected with collector

T-pieces were prepared from titan rod Osteofix of 0.8 mm in diameter (Beznoska, Ltd., Kladno, Czech Republic). Vertical leg, approx. 1 cm long was connected with ring allowing simple change of collector. Suitable combination, used in our experiment was composed of 5 ml syringe connected to T-piece as holder and 2 ml

NUNC cryotube as removable collector. Immediately after surgery the animals were not restricted in food, water and free movement.

Correct patency of cannulae was evident next day and the volume of accumulated fluid should correspond with the presence of follicles or *corpora lutea* that were recorded on the ovaries at operation. The amount of secretion was monitored daily and each day was also changed both collectors. The fluid was centrifuged at 5 000 g for 20 min, the supernatant inactivated at 56 °C and stored in -80 °C until use. For better orientation, the estrus cycle of experimental ewes was checked by the ram and their behaviour compared with the amount of obtained fluid. If the daily volume of secretion decreased under 0.1 ml per oviduct or when its appearance had changed, the animals were excluded from the experiment.

## RESULTS

The experimental protocol allows to evaluate the results from 3 aspects:

- A. The effect of synchronization of estrous cycle before surgical intervention.
- B. The collection of fluid from unligated oviducts.
- C. the ligation of oviducts at uterotubal junction.

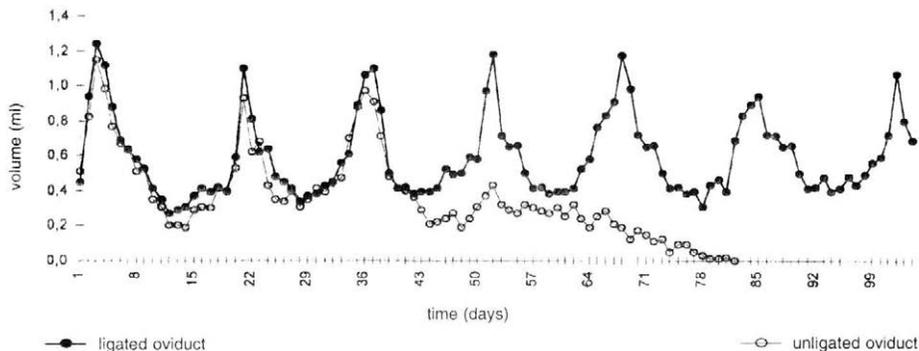
A. From Tab. I it seems obvious that the volume of collected secretion from 4 females (8 oviducts) with previously synchronized estrus cycles was very low. The longevity of flow did not exceed 7 days after surgery and the volume ranged from 0.2–0.5 ml during 24 hrs before it stopped completely. The cause of insufficient function was most frequently the occlusion of cannulae with the fragments of tissue or with fibrin. For that reason the manipulation with estrus cycle was excluded in subsequent experiment.

B. Fig. 2 presents results obtained by direct comparison of the flow in animals with unilaterally ligated oviducts. From originally 6 ewes included in the experiment only in 3 of them both cannulae maintained undisturbed function for longer time interval. In unligated tubes the flow was well conserved for maximally 3 estrous cycles and then followed rapid drop of the harvested volumes without recovery to the original level in subsequent

I. Total number of cannulated oviducts, volumes of harvested secretion and the effect of different treatments

Treatment of animals and oviducts	No. of cannulated oviducts	Flow of secretion in days (min-max)	Collected volume per day in ml (min-max)
Synchronisation of estrus	8	2-7	0.2-0.5
Ligated oviduct	14	3-150*	0.3-1.2
Non-ligated oviduct	6	2-75	0.2-1.0

\* functional cannula has been removed and experiment stopped after 150 days



2. Comparison of secretion activity of ligated and unligated sheep oviducts (data averaged from six ewes, each with one ligated and one unligated oviduct)

period. In contrast, in ligated counterparts, the secretion could be collected for several estrous cycles. Furthermore, the harvested volume were in close relation to the stage of estrous cycle. These results allow to conclude that only during first 2–3 cycles the collected volumes from treated and untreated tubes were similar. Their maximum was in the heat in average 1.0 ml/oviduct per day and minimum at luteal phase 0.2 ml/oviduct/day.

C. For the evaluation of the effect of the ligation, 14 oviducts were available, including those from 6 animals that were treated unilaterally. Despite broad variation in the duration of the flow, it could be seen the tendency indicating the prolongation of the time period for the collection of suitable secretion. In 8 instances the cannulae maintained their function for more than 4 estrous cycles and 2 of them were running for 150 days. In all these cases the collected volumes were in close relation to estrous cycles as shown in Fig 2. The variable results were recorded irrespective to the use of identical experimental protocol, similarly as in articles reported in literature. Also the causes of the cessation of flow were similar, i.e. the occlusion of cannulae by fibrous plug at the junction with the oviduct or the evulsion by experimental subject.

## DISCUSSION

The procedure used for the collection of oviductal fluid in our experiments represents the modification of previous approaches. Last studies addressed to oviduct cannulation in cows were published by Kavanaugh and Killian (1988) and Kavanaugh et al (1992). From their reports was also obvious broad variation of the patency of indwelling cannulae. The failure was caused as in our experiments by closing with fibrous plug at the junction with the oviduct. In some cases they were not able to identify the reason of the cessation reliably. Particular

role in their experiments played the evulsion by experimental subject or by the rest of the herd. In contrast, in our studies the evulsion represented only marginal events.

Despite these limitations more than 50% of cannulated animals provided suitable source of oviductal fluid. In some of them the cannulae maintained their proper function for several estrus cycles and allowed to collect sufficient amount of clearly defined secretion at any day of estrus cycle. Such criteria can not be fully guaranteed after flushing oviducts of slaughtered donors (Murray, 1992, 1993; Abe et al., 1993). More valuable information about the composition of oviductal cell secretion offered the analyses of media after cultivation of cell suspensions or tissue explants. Gandolfi et al. (1989, 1991); Buhi et al. (1991) and Wegner and Killian (1992) determined after using this approach the origin of individual components and the binding of selected estrus specific proteins released to culture medium to surface membranes of spermatozoa and to zona pellucida. They also suggested the significance of some components produced by oviductal cells for sperm-egg recognition and subsequent sperm penetration through zona pellucida. However, not even this system fully reflects physiological situation in which the cells are *in vivo* under permanent hormonal control and their secretion is strictly regulated mainly by rapid changes of circulating gonadal steroid hormones. The fluid obtained by cannulation of the oviducts has all natural parameters typical for each day of estrus cycle. Our recent studies addressed to the determination of zonae pellucidiae properties of *in vitro* and *in vivo* matured oocytes revealed that the components present in oviductal secretion at estrus play critical role in the generation of species specificity and in the prevention of penetration of oocytes by foreign spermatozoa (Slavik and Fulka, 1999).

Moreover, oviductal secretion may be valid tool also in the investigation of the regulation of early embryonic development. It is well known that the media used at pre-

sent for cultivation of preimplantation embryos provide suboptimal environment and adversely affect their development (Czlonkowska et al., 1991; Kelk et al., 1992). In addition, Walker (1992), Holm et al. (1996) observed that *in vitro* culture system resulted in prolonged gestation and increased birth weight in sheep after embryo transfer. Behboodi et al. (1995) and Farin and Farin (1995) described similar phenomenon in cattle. However, these anomalies were even more frequent in cloned bovine embryos as it was clearly documented by Willadsen et al. (1991) and Walker et al. (1996). It was suggested that to the origination of these deviations may contribute not yet defined compounds that are absent in conventional culture media. Their identification in oviductal secretion and introduction to culture system may reduce adverse effect of *in vitro* procedures that are associated with new embryo technologies. The described method of oviductal fluid collection provides feasible approach to the harvesting of sufficient amount of suitable material for analysis and subsequent isolation of substances that may be involved, either alone or in the combination, in the regulation of preimplantation embryonic development. Moreover, the characterisation of fraction (s) present in oviductal secretion at the period of estrus may contribute to better understanding of interaction between gametes.

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Received: 99–12–17

Accepted: 00–02–21

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*Contact Address:*

Ing. Tomáš Slavík, Ústav živočišné fyziologie a genetiky AV ČR, 277 21 Liběchov, Česká republika  
Tel. +420 206 69 71 47, e-mail: slavik@iapg.cas.cz

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# SYSTEMIC DISSEMINATION OF CHLAMYDIAE AFTER GENITAL INFECTION IN RABBIT\*

## SYSTÉMOVÁ DISEMINACE CHLAMYDIÍ PO GENITÁLNÍ INFEKCI KRÁLÍKA

L. Pospíšil, Z. Věžník, Z. Zralý, I. Diblíková

*Veterinary Research Institute, Brno, Czech Republic*

**ABSTRACT:** Findings of chlamydiae in ejaculates of various farm animal species were reported in an earlier paper (Věžník et al., 1998). The objectives of this experiment were to test the hypothesis that such findings are due rather to genital infection than to contamination that may occur during the passage of semen through urethra, and to demonstrate possible dissemination of chlamydiae in the organs of the host. Ten male rabbits were inoculated with 0,5 ml of suspension of *Chlamydia psittaci* (ewe abortion strain PK-5082) divided into both testicles. The clinical state of the animals was monitored before and after the inoculation. The rabbits were successively sacrificed (the last one on p.i. day 122) and samples of testicles, epididymis, seminal vesicles, urethra, kidney, liver, spleen, and lung were examined for the presence of chlamydiae using simultaneously the diagnostic kits Progen for direct immunofluorescence and Welcozyme ELISA Test. Shedding of chlamydiae in ejaculates was demonstrated in two rabbits from p.i. day 63 and in all the remaining from p.i. day 77. Elementary of reticular bodies were demonstrated in testicular, epididymal, renal, hepatic, lung and splenic tissues. The results of both the methods were quite identical. The significance of systemic dissemination of chlamydiae after genital infection is discussed with regard to their role as a cofactor in the development of polyfactorial diseases.

rabbit; chlamydia; genital infection; ejaculate, dissemination

**ABSTRAKT:** Cílem předkládané práce bylo zjištění zda-li k vylučování chlamydií ejakulátem dochází v důsledku genitální infekce nebo zda-li ke kontaminaci semene dochází až při průchodu uretrou osídlenou chlamydiemi. Dalším cílem bylo určit rozsah orgánové diseminace chlamydií po genitální infekci. Králíci samci ( $n=10$ ) byli infikováni suspenzí *Chlamydia psittaci* (ewe abortion strain PK-5082) v množství 0,5 ml rozděleného do obou varlat. Klinický stav pokusných zvířat byl sledován před inokulací a po ní. Ejakulát byl odebírán ve stanovených intervalech do umělé vagíny. Králíci byli postupně sakrifikováni (poslední 122. dne po infekci). Ejakulát a tkáň varlat, nadvarlat, váček semenných, močové trubice, ledvín, jater, sleziny a plic byly vyšetřeny přímou imunofluorescencí (Progen), vzorky tkání současně ELISA reakcí (Welcozyme test). Přítomnost chlamydií v ejakulátu byla potvrzena u dvou infikovaných králíků 63. den po infekci a od 77. dne po infekci u všech jedinců. Elementární a retikulární tělíska byla prokázána ve varlatech, nadvarlatech, v ledvině, v játrech, ve slezině a v plicích. Výsledky dosažené přímou imunofluorescencí a ELISA reakcí byly shodné. Autoři diskutují význam systémové diseminace chlamydií po genitální infekci se zřetelem na kofaktorové působení chlamydií v etiologii polyfaktorálních onemocnění.

králík; chlamydia; genitální infekce; ejakulát; orgánová diseminace

### INTRODUCTION

A rather frequent occurrence of chlamydiae in ejaculates of man and several animal species was demonstrated in our earlier investigations (Věžník et al., 1998). The results of the investigations did not allow the conclusion that the contamination of semen does not occur until the semen reaches urethra, which tends to be colo-

nised by chlamydiae frequently. The objective of the experiments presented here was to test the hypotheses that

a) secretions of the gonadal accessory glands of animals which are reliably free of chlamydiae (incl. urethra) can become the source of chlamydia contamination of ejaculates;

b) genital chlamydiosis can result in a dissemination of the agent into other organs.

\* Presented on the 1st Slovak Dermatological Congress with International Participation, Bratislava, Slovak Republic, October 28–30, 1999.

## MATERIAL AND METHODS

For the experiment 10 male rabbits were used. Each of 10 male Californian rabbits with well developed testicles and a mean live weight of 2.5 kg in light ether anaesthesia was inoculated intratesticularly with of *C. psittaci* – infected (ewe abortion strain PK 50-82) McCoy cell suspension (0.25 ml for each testicle). Before the inoculation, all the rabbits were tested by complement fixation test (CFT) and ELISA for antibodies to chlamydiae and by direct immunofluorescence for the presence of chlamydiae in semen. Only animals negative in all the tests were eligible.

Blood samples for serological tests were collected at pre-set intervals. At the same time, semen samples were collected into artificial vagina to be tested for the presence of chlamydiae by direct immunofluorescence using the kit Progen (Biotechnik, Ltd., BRD). For the latter purpose, the samples fixed in a 1 : 1 alcohol-acetone mixture, were processed according to the manufacturer's instructions.

The rabbits were killed on post-inoculation day 122 and samples of testicular, epididymal, urethral, renal, hepatic, lung tissues and seminal vesicles were collected

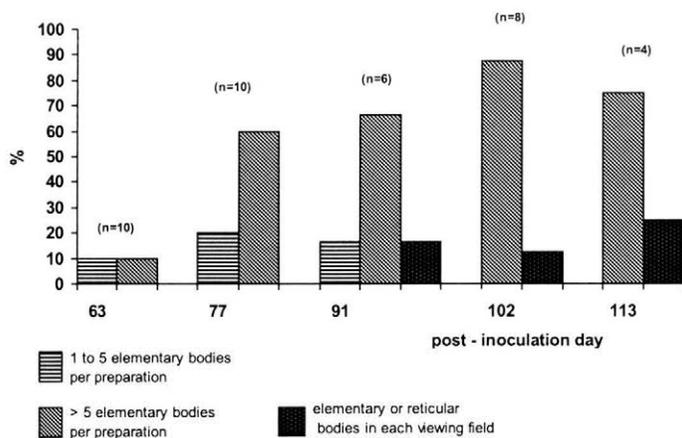
and tested for the presence of chlamydiae by direct immunofluorescence and ELISA (Welcozyme Murex Diagnostic kit).

The microscopic findings were expressed in terms of counts of elementary or reticular bodies and scored as follows: 0 – negative; 1 – solitary elementary bodies (1 to 5 bodies per preparation); 2 – scarce elementary bodies (> 5 elementary bodies per preparation); 3 – positive (elementary and reticular bodies in each viewing field).

## RESULTS

*C. psittaci* was demonstrated in semen in two rabbits from post-inoculation day 63 and in all the other rabbits from the subsequent sampling regularly up to the end of the experiment (Fig. 1).

The data on the systemic dissemination of chlamydiae as found upon necropsy at post-inoculation day 122, including the 62% accordance with the results of direct immunofluorescence and ELISA, as shown in Tab. I.



1. Isolation of chlamydiae from ejaculates after experimental intratesticular infection in rabbits ( $n = 10$ ); in some cases it was not possible to collect the ejaculate

I. Demonstration by IF and ELISA of chlamydiae in various tissues after experimental intratesticular infection of rabbits

IF	ELISA	Number (%) of cases
+	+	12 (19.7 %)
+	-	21 (34.4 %)
-	+	2 (3.3 %)
-	-	26 (42.6 %)
Total		61

Full agreement between IF and ELISA in 38 (62%) cases.

The scoring of microscopic findings of chlamydiae in the individual tissues is presented in Tab. II.

Both elementary and reticular bodies were demonstrated by direct immunofluorescence in tissue sections.

## DISCUSSION

Our experiment showed, that the intratesticular inoculation of chlamydia resulted not only topical propagation, but also shedding of chlamydiae in semen and

II. The of chlamydia findings in tissues after experimental intratesticular infection of rabbits (n = 10)\*

	Number of samples*	Positive total (%)	Score (% of intensity degree)		
			1	2	3
Testicle	18	100	0	55.6	44.4
Epididymis	30	80	52.2	43.5	4.3
Seminal vesicles	8	37.5	67	33	0
Urethra	8	50	100	0	0
Kidney	10	30	33.3	33.3	33.3
Liver	10	40	50	25	25
Spleen	8	12.5	100	0	0
Lung	10	10	100	0	0

\* More than one smear were prepared from some tissue samples

1 = 1 to 5 elementary bodies per preparation

2 = > 5 elementary bodies per preparation

3 = elementary or reticular bodies in each viewing field

dissemination into other organs (epididymis, seminal vesicles, kidney, liver, spleen, and lung). Our findings of various stages of the chlamydial cycle conclude that intratesticularly inoculated chlamydia propagate also in other organs and that genital chlamydiosis can developed into a generalised infection. Although most of the human genital chlamydial infection are caused by *C. trachomatis* (Van den Brule et al., 1993) and the results of experiments in animals cannot be applied without any limitation, we assume that considering the current views of the co-factor role of chlamydiae in the development of other serious disease, our results are not irrelevant to human pathology (Plumer et al., 1991; Paavonen and Lehtinen, 1994; Mlot, 1996; Paavonen, 1996). Possible predisposing effect of preceding chlamydial infection, belonging to the group of sexually transmitted diseases (STD), on the rise of HIV infection was pointed out. Tissues of the urogenital tract damaged due to STD can more easily become the site of entrance of HIV. It is highly probable that chlamydiae, which are currently responsible for most of the diagnosed cases of STD (Plumer et al., 1991), play a significant role in this respect.

It has also been demonstrated that chlamydia-induced pelvic inflammatory diseases (PID) are accompanied by increased levels of ovarian carcinoma markers (Paavonen and Lehtinen, 1999). Cofactor activity of chlamydiae in the rise of ovarian and cervical carcinoma is evident also from results of other analyses (Paavonen,

1996). A significant cofactor effect in the development of atheromatosis, in particular that of coronary arteries, is attributed to *C. pneumoniae* (Mlot, 1996).

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Received: 99–12–30

Accepted after corrections: 00–04–18

### Contact Address:

Prof. MUDr. et MVDr. Leopold Pospišil, DrSc., Výzkumný ústav veterinárního lékařství, Hudcova 70, 621 32 Brno, Česká republika  
Tel. +420 5 41 32 12 41, fax +420 5 41 21 12 29, e-mail: pospisil@vri.cz

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# PRODUCTION OF ANTI-SPERM ANTIBODIES ASSOCIATED WITH GENITAL *CHLAMYDIA* INFECTION IN RABBITS\*

## VZNIK PROTILÁTEK PROTI SPERMIÍM PŘI CHLAMYDIÁLNÍ GENITÁLNÍ INFEKCI KRÁLÍKŮ

L. Pospíšil<sup>1</sup>, I. Diblíková<sup>1</sup>, Z. Věžník<sup>1</sup>, Z. Zralý<sup>1</sup>, I. Horová<sup>2</sup>, M. Budíková<sup>2</sup>

<sup>1</sup>*Veterinary Research Institute, Brno, Czech Republic*

<sup>2</sup>*Masaryk University, Faculty of Sciences, Institute of Applied Mathematics, Brno, Czech Republic*

**ABSTRACT:** Male Californian rabbits ( $n = 10$ ) with well developed testicles were inoculated intratesticularly with 0.5 ml suspension of *Chlamydia psittaci* (ewe abortion strain PK-5082) divided in both testicles. Control rabbits ( $n = 5$ ) received the same volume of physiological saline. Blood samples for serological examinations were collected from the ear vein one week before and weekly intervals after the inoculation. Antibodies to chlamydiae and anti-sperm antibodies were determined by ELISA. The results of ELISA for each of the rabbits were plotted against time and mean data for the group were processed by the kernel smoothing (Härdle, 1990). It is evident from the results that the intratesticular inoculation of chlamydiae induced the formation of specific anti-chlamydial antibodies. At the same time formation of anti-sperm antibodies was observed in most of the inoculated rabbits. The dynamic of titres of two types were similar.

rabbit; intratesticular infection; *Chlamydia psittaci*; anti-chlamydial and anti-sperm antibodies

**ABSTRAKT:** Králíčím samečům kalifornského plemene ( $n = 10$ ) s dobře vyvinutými varlaty byla inokulována suspence *Chlamydia psittaci* (ewe abortion strain PK-5082) intratestikulárně v objemu 0,5 ml rozdělené do obou varlat. Kontrolním králíkům ( $n = 5$ ) byl aplikován stejný objem fyziologického roztoku. Vzorokry krve byly odebírány pro sérologické vyšetření z vény ušního boltee před inokulací a v týdenních intervalech po ní. Protílátky proti chlamydiím a proti spermii byly prokazovány ELISA metodou. Výsledky jednotlivých měření u každého králíka byly statisticky vyhodnoceny metodou vyhlazování jader (Härdle, 1990). Z výsledků pokusů je zřejmé, že intratestikulární infekce chlamydií indukuje tvorbu specifických antichlamydiálních protilátek. Současně byla pozorována tvorba antispermatických protilátek u většiny králíků. Křivky dynamiky tvorby obou druhů protilátek byly podobné.

králík; intratestikulární infekce; *Chlamydia psittaci*; antichlamydiální a antispermatické protílátky

## INTRODUCTION

Anti-sperm antibodies (ASA) can occur in blood serum and genital secretion collected from male and female humans and animals. Findings indicative of an association between a previous infection of male gonads and the production of anti-sperm antibodies in the affected patient were described in several papers Eggert-Kruse et al. (1996). A considerable importance among such infections is attributed to genital chlamydiae (Soffer et al., 1990; Witkin et al., 1993, 1995; Daya, 1994 Munoz

and Witkin, 1995; Munoz et al., 1995). A significant correlation between the humoral immune response to *C. trachomatis* and ASA response in men was demonstrated, among other authors, by Witkin et al., (1995). A similar significant relation between the presence of anti-chlamydial antibodies (ACA) and ASA in blood serum of bulls was also demonstrated (Zralý et al., 1998). The experiment described in this paper was encouraged by finding of anti-sperm antibodies within our studies of experimental chlamydial orchitis in rabbit.

\* Presented on the 1st Slovak Dermatological Congress with International Participation, Bratislava, Slovak Republic, October 28–30, 1999.

## MATERIAL AND METHODS

### Rabbits and inoculation

The experiment was carried out in ten experimental and five control male Californian rabbits with a mean live weight of 2.5 kg. Before the experiment, all the rabbits were tested for the presence of anti-chlamydial antibodies by CFT (complement fixation test) and ELISA and anti-sperm antibodies by ELISA. Only animals free from the antibodies and with well developed testicles were eligible. Under light ether anaesthesia, the selected rabbits were inoculated bilaterally intratesticularly with Mc Coy cell-line culture of *Chlamydia psittaci* (ewe abortion strain PK 50-82). The dose size was 0.25 ml for each testicle. The second inoculation of the same dose was applied on day 63. The control group received the same volume of physiological saline. Blood samples were collected after the inoculation at pre-set intervals.

### Anti-chlamydia antibodies

Blood serum were determined by ELISA using the commercial *Chlamydia psittaci* antigen for CFT (Bioveta, Ivanovice na Hané, Czech Republic) as the coating antigen. All active components of the assay were used in 0.1 ml volumes and separate microtitre plates P for ELISA (Gama, Czech Republic) were used for individual rabbits. The coating antigen, diluted 1 : 100 with the bicarbonate buffer, was pipetted into the wells and the plates were incubated at room temperature for 24 h. The incubation was followed by treble washing with PBS supplement with 0.05% of Tween 20. Blood serum of rabbit inoculated intratesticularly with concentrated suspension of *Chlamydia psittaci* and showing clinically apparent orchitis and CFT titre 1:40 was used as positive control serum. The donor of negative control serum was a clinically normal rabbit with zero CFT titre. All control and tested sera were diluted 1:100 in dilution medium (0.5% lactalbumin hydrolysate in wash solution) before assay. In each microtitre plate the positive control serum (eight values), the negative control serum (eight values), the tested serum samples (four values per sample) were assayed. The remaining four wells were allocated for blank (dilution medium alone). The plates with tested samples and control sera were incubated at 37 °C for 60 min. After incubation, the plates were washed and, after the addition of conjugate (SwAR x Px, 1 : 15 000 in dilution medium), the plates were incubated at 37 °C for 60 min. The second incubation was followed by sucking off the conjugate, quadruple washing and addition of substrate (with tetra-methylbenzidine, TMB). The developing colour reaction was stopped after 15 min at room temperature with 0.05 ml 2N sulphuric acid and absorbances was measured with the iEMS READER MF (Labsystems, Finland) at 450 nm. The results were processed using the GENESIS soft-

ware. Samples showing absorbances higher than the treble of mean absorbance of the negative control serum were scored as positive.

### Anti-sperm antibodies

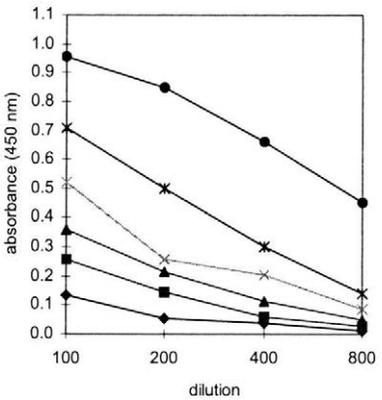
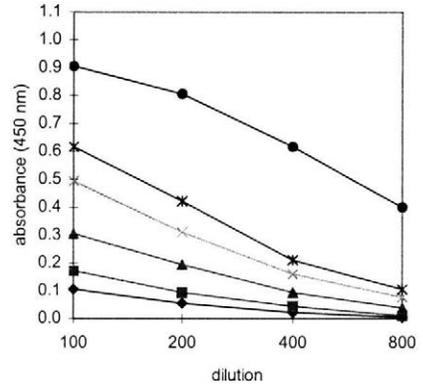
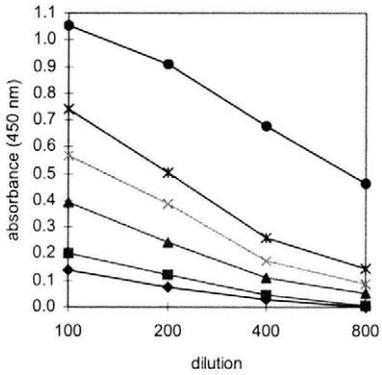
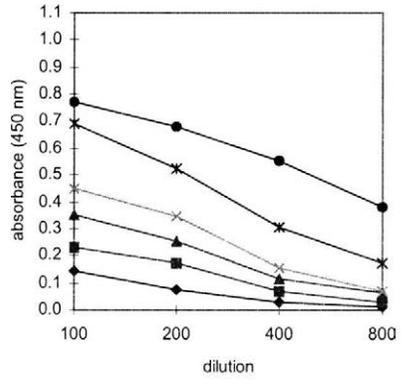
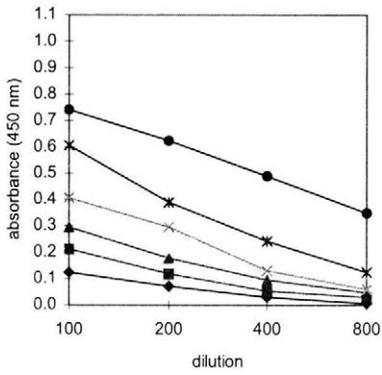
Blood serum ASA were determined by ELISA as described earlier (Zralý et al., 1998). Rabbit sperms were separated by centrifugation, sonicated, and diluted with the coating buffer. Wells of microtitre plates P for ELISA (Gama, Czech Republic) were coated with the antigen by incubation at room temperature for 24 h. The plates with the tested samples and control sera were incubated at 37 °C for 90 min. After the incubation, the plates were washed, and conjugate (SwAR x Px) was added into each well, and the plates were incubated at room temperature for another 60 min. The second incubation was followed by treble washing and addition of substrate (TBM). The developing colour reaction was stopped after 15 min at room temperature with 0.05 ml 4N sulphuric acid and absorbance was measured with the iEMS READER MF (Labsystems, Finland) at 450 nm. The results were processed using the GENESIS software. Samples showing absorbances higher than the mean absorbance of the negative control serum + 3 SD were scored as positive.

### Stabilisation and testing of ELISA for the determination of antibodies to chlamydiae

The following procedure was used to test the repeatability of the results of ELISA. Blood serum of a rabbit infected intratesticularly with a concentrated suspension of *Chlamydia psittaci* and showing apparent clinical signs of orchitis and CFT titre 40 was used as the positive control. Blood serum of a clinically normal rabbit showing CFT titre 0 was used as the negative control. Six samples containing 0%, 2%, 12.5%, 25%, 50% and 100% of the positive serum were prepared by mixing appropriate parts of the positive and the negative control serum. This series of samples was repeatedly tested at dilutions 100, 200, 400, and 800. The positions of the samples on the microtitre plate were randomly changed before each repetition of the tests. The results are presented in Fig. 1. Inter-assay variation coefficients and reproducibility (100% - variation coefficient) were calculated for each dilution of the positive and the negative control sera (Fig. 2, Tab. I).

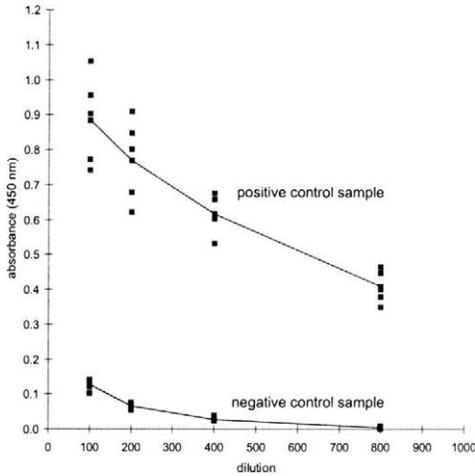
### Cross-reactivity between ELISA-ASA and ELISA-ACA

Two so far non-inseminated female rabbits with zero levels of ASA antibodies were used for the induction of formation of antibodies to chlamydiae without interfer-



- ◆ serum 1 - negative
- serum 2 - 2% of positive
- ▲ serum 3 - 12,5% of positive
- × serum 4 - 25% of positive
- ✱ serum 5 - 50% of positive
- serum 6 - positive

1. Repeatability of the results of ELISA determination of antibodies to chlamydiae



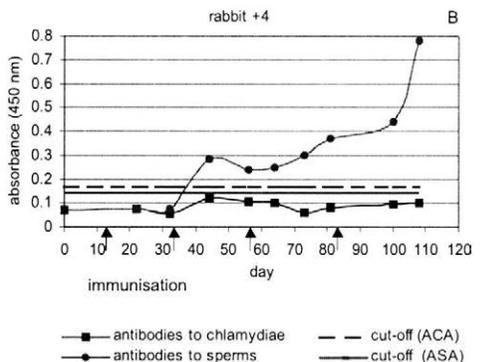
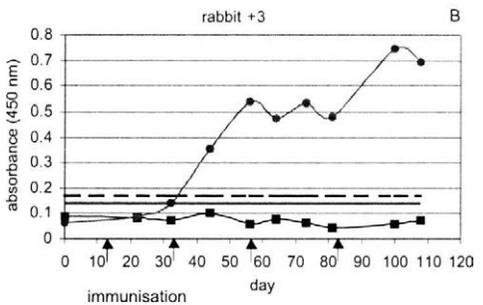
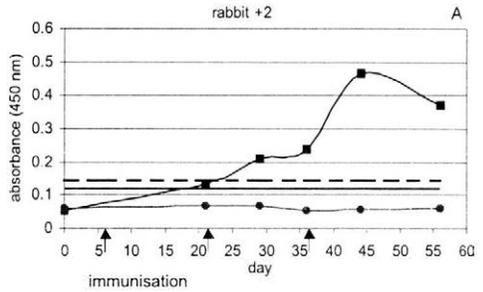
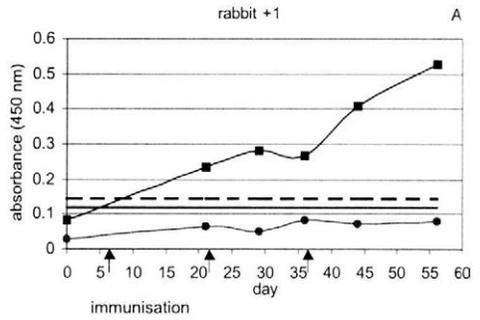
## 2. Repeated determination of antibodies to chlamydiae in rabbit by ELISA method

### 1. Results of the repeated determination of ACA by ELISA method

Dilution of serum	Positive serum		Negative serum	
	Variation coefficient (%)	Reproducibility (%)	Variation coefficient (%)	Reproducibility (%)
100	14.57	85.43	15.65	84.35
200	15.42	84.58	4.20	95.80
400	9.11	90.89	8.08	91.92
800	11.74	88.26	-	-

ence of formation of ASA antibodies. The two rabbits received killed antigen *Chlamydia psittaci* for CFT (Bioveta, Ivanovice na Hané, Czech Republic) emulgated in the All-Span-Oil adjuvant (Sevac, Prague, Czech Republic) according to the instructions of the supplier. The immunization dose of 1 ml was divided into 10 equal portions administered intradermally in 10 sites. Three immunization doses were administered within 60 days. Blood samples were collected currently from the ear vein and the separated blood sera were tested for antibodies to chlamydiae and ASA antibodies. No cross-reactions of the anti-chlamydia antibodies with the antigen used for ASA testing were observed.

Another two female rabbits were immunised with a sonicated antigen of washed spermatozoa and emulgated in the adjuvant. Similar immunization and blood sampling schemes were used. Four immunization doses were administered within 110 days. The detected ASA antibodies did not show any cross-reactivity with the *Chlamydia psittaci* antigen (Fig. 3).



3. Cross-reactivity between ELISA-ASA and ELISA-ACA: A – rabbits immunised with antigen *Chlamydia psittaci* intradermally (without infection, without detection of anti-sperm antibodies); B – rabbits immunised intradermally with sperms, producing ASA (without reaction with antigen *Chlamydia psittaci*)

Means and variation coefficients were calculated for the individual serum samples and the results were plotted using the kernel smoothing method of Härdle (1990). Whereas this method was used for the first time in the field of experimental biology, it is described in detail (Fig. 4).

Production of ACA was observed already in the 1st week after the experimental genital infection. The titres culminated between the post-infection days 60 and 80 (Fig. 5). Although 90% of the infected rabbits developed positive responses, their intensity differed among indi-

## KERNEL SMOOTHING

Nonparametric approaches to the smoothing of noisy data sets have been demonstrated to be effective for many applications. Especially kernel methods provide a simple way of finding a structure in data sets without the imposition of a parametric model. One of the most fundamental settings where kernel methods can be applied is the single regression model where observations for each of two variables are available and one is interested in determining an appropriate functional relationship between these two variables. This fitting procedure is commonly called "smoothing". Smoothing of the dataset  $\{(X_i, Y_i)\}_{i=1}^n$  involves the approximation of the mean response curve  $m$  in the regression relationship

$$Y_i = m(X_i) + \varepsilon_i, \quad i = 1, \dots, n,$$

where  $\varepsilon_i$  are measurement errors. Let  $\hat{m}$  denote an estimate of  $m$ .

The well-known kernel method is the Nadaraya-Watson estimator. This estimator is defined by the following formula

$$\hat{m}(x) = \frac{\sum_{i=1}^n K_h(x - X_i) Y_i}{\sum_{j=1}^n K_h(x - X_j)},$$

Here  $K$  is a symmetric continuous function satisfying conditions:

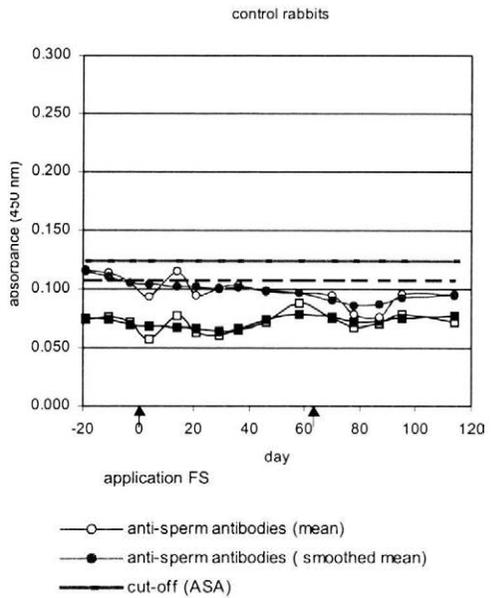
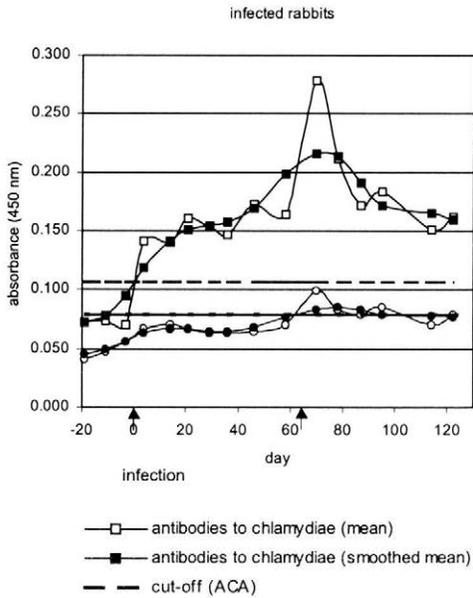
$$\int K(x) dx = 1, \quad K(x) = 0 \text{ for } |x| > 1.$$

The function satisfying these conditions is called a kernel. We denote

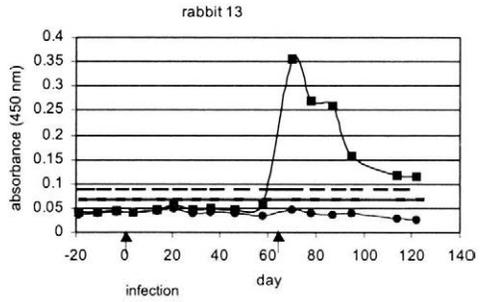
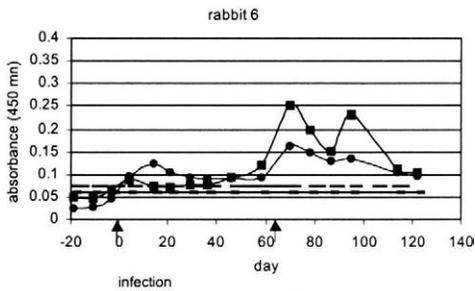
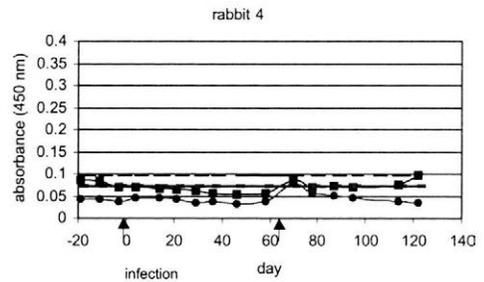
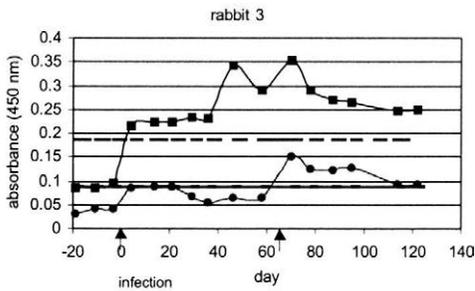
$$K_h(x) = \frac{1}{h} \cdot K\left(\frac{x}{h}\right).$$

Further,  $h$  is a positive constant called a bandwidth. The choice of  $h$  plays an important role since it controls the smoothness of the estimate. It is supposed  $h \rightarrow 0$ ,  $nh \rightarrow \infty$  for  $n \rightarrow \infty$ . In our case the Epanechnikov kernel  $K(x) = \frac{3}{4} \cdot (1 - x^2)$ ,  $|x| < 1$  has been used and  $h = 0,15$ .

The confidence intervals have been constructed by an algorithm given in Härdle W., 1990.



5. Anti-chlamydia and anti-sperm ELISA antibodies in rabbits infected intratesticularly with *Chlamydia psittaci*



6. Individual antibody response of rabbits infected with *Chlamydia psittaci*

vidual animals. Graphic presentation of the data has resulted from statistical processing described in detail in the part Material and Methods.

The production of ASA was demonstrated in 80% of the infected rabbits, but, compared with ACA, the onset of antibody responses was delayed. Positive titres were found between post-infection days 60 and 70, i.e. at a time when the titres of ACA already culminated. The ASA curve is the resultant of the statistical calculation as described above. Positive and negative antibody responses to antigenic stimulation are shown in Fig. 6.

## DISCUSSION

We assume that the results of this experimental study are a significant contribution to the knowledge of the formation of anti-sperm antibodies as a consequence of a previous testicular chlamydia infection. Demonstrable amounts of anti-sperm antibodies were found in 80% of the rabbits inoculated intratesticularly with a strain of *C. psittaci*. The formation of anti-sperm antibodies is often associated with a previous or concurrent inflammation of the male genital tract caused by various agents. However all such hypotheses are derived from clinical and epidemiological observations (Cimino et al., 1993; De Placido et al., 1986; Eggert-Kruse et al., 1990; Eggert-Kruse et al., 1996; Micic et al., 1990; Munoz and Witkin, 1995; Munoz et al., 1995; Soffer et al., 1990; Witkin, 1996; Witkin et al., 1993, 1995). Our attempts to create within this study experimental conditions supporting this hypothesis were successful and the effect of chlamydia orchitis in rabbits on the formation of both anti-chlamydia and anti-sperm antibodies has been demonstrated (Fig 5). Statistical data processing and graphic presentation show a close similarity of the dynamics of anti-chlamydia and anti-sperm antibody. The specificity of the ASA and ACA has been confirmed by demonstration of absence of their cross-reactivity (Fig.3).

Anti-sperm antibodies rank with significant immunological factors that can affect male fertility. Several authors reported increased occurrence of anti-sperm antibodies in infertile individuals (Cohen and Hendry, 1978; Wakefield and Penny, 1983; Toth et al., 1988; Witkin, 1996).

Although the effects of anti-sperm antibodies on the fertilisation capacity of spermatozoa were not investigated within this study, the fact that their formation was associated with a previous chlamydia infection offers a new insight into the fascinating and complex problem of reproductive immunology. The above mentioned findings may have practical implications for the research of the role of chlamydiae in impairment of sperm quality and male infertility. The demonstration of the absence of the anti-sperm and anti-chlamydial antibody cross-reactivities is considered very important.

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Received: 99–12–30

Accepted after corrections: 00–04–18

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*Contact Address:*

Prof. MUDr. et MVDr. Leopold P o s p í š i l, DrSc., Výzkumný ústav veterinárního lékařství, Hudcova 70, 621 32 Brno,  
Česká republika  
Tel. +420 5 41 32 12 41, fax +420 5 41 21 12 29, e-mail: [pospisl@vri.cz](mailto:pospisl@vri.cz)

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# BACTERIA OF THE TRIBE PROTEEEAE – OCCURRENCE IN RAW MATERIALS AND FOOD, AND RESISTANCE TO ANTIBIOTICS

## ZÁSTUPCI TŘÍDY PROTEEEAE V SUROVINÁCH A POTRAVINÁCH ŽIVOČIŠNÉHO PŮVODU A JEJICH REZISTENCE K ANTIBIOTIKŮM

E. Urbanová<sup>1</sup>, K. Manová<sup>2</sup>, Z. Páčová<sup>1</sup>

<sup>1</sup>Masaryk University, Faculty of Science, Czech Collection of Microorganisms, Brno, Czech Republic

<sup>2</sup>Private Microbiology Laboratory, Brno, Czech Republic

**ABSTRACT:** A total 54 strains of *Proteeeae* was isolated and identified from raw materials and foods. The most frequently present species: *Proteus mirabilis* (37%), *Morganella morganii* (25.9%), *Proteus vulgaris* (18.5%). Members of the genus *Providencia* were found less frequent (from waste water and sludge only): *P. rustigianii* (9.3%), *P. rettgeri* (5.6%) and *P. alcalifaciens* (3.7%). All strains were included in the genus using commercial diagnostic kit ENTEROtest 16 and identified by numerical identification program TNW. Correct species identification – excellent (EI) or very good (VGI) – was obtained in eighty-five per cent. Two *Morganella morganii* subsp. *morganii* biogroup C strains were designated as intermediate strains (IS) on the basis of both lysine and ornithine decarboxylases negative results. The antibiotic susceptibility of 50 isolates of *Proteeeae* species was examined according to standard method. Thirty of tested strains (60%) were found resistant to one and more antibiotics. *Proteus vulgaris* (60% of strains), *Morganella morganii* (53.8% of strains), *Proteus mirabilis* (47.4% of strains) were the most resistant members of the tribe *Proteeeae*.

*Proteus; Morganella; Providencia*; identification; resistance to antibiotics

**ABSTRAKT:** Ze vzorků surovin, potravin a prostředí živočišné výroby bylo izolováno a identifikováno 54 kmenů třídy *Proteeeae*. Nejčtenější byly zastoupeny kmeny: *Proteus mirabilis* (37 %), *Morganella morganii* (25,9 %), *Proteus vulgaris* (18,5 %); méně často (převážně z odpadních vod a kalů) zástupci rodu *Providencia*: *P. rustigianii* (9,3 %), *P. rettgeri* (5,6 %) a *P. alcalifaciens* (3,7 %). Na základě výsledků diferenceiace ENTEROtestem 16 bylo numerickým identifikačním programem TNW výborně (VI), resp. velmi dobře (VDI) identifikováno 85 % všech izolátů. Dva izolované kmeny *M. morganii* byly programem hodnoceny jako intermediární kmen (IK). Jde o kmeny *M. morganii* subsp. *morganii* bioskupina C, vyznačující se negativní dekarboxylací lysinu a ornitinu. U 50 izolovaných kmenů třídy *Proteeeae* byla testována citlivost k vybraným antibiotikům. Celkem u 30 kmenů (60 %) byla prokázána rezistence k jednomu nebo více antibiotikům. V našem souboru bylo rezistentních 60 % kmenů *P. vulgaris*, 53,8 % kmenů *M. morganii* a 47,4 % kmenů *P. mirabilis*.

*Proteus; Morganella; Providencia*; identifikace; rezistence k antibiotikům

### INTRODUCTION

Three bacterial genera *Proteus*, *Providencia* and *Morganella* belong to the tribe *Proteeeae*. These bacteria are important human pathogens causing a variety of nosocomial infections. They also occur as contaminants in food and the food can be a transfer vector of alimentary and nosocomial diseases. The *Proteeeae* may also influence the quality of raw materials and food because they produce hydrogen sulfide, indole etc. with typical bad odour. In poor hygienic conditions the quality of chilled and frozen poultry (McMeekin and Patterson, 1975; Pipová et al., 1997), refrigerated ground and packed meat and fish (Klausen and Huss, 1987), and

cheese (Tornadijo et al., 1993) can be affected. The increasing supplementation of feed with antibiotics leads to a selection of resistant microorganisms in animals and in the food industry (Manic et al., 1998) as well as in fishing industry (De Paola et al., 1995). A transfer of bacterial resistance to antibiotics by food to the human population, for example by eggs, is discussed (Coghlan, 1996; Papadopoulou et al., 1997). Present taxonomy of the tribe *Proteeeae* includes the genus *Proteus* with four species (*P. mirabilis*, *P. vulgaris*, *P. penneri*, *P. myxofaciens*) while *P. vulgaris* consists of two biogroups: 2, 3 (biogroup 2 strains are indole, esculin and salicin positive and sensitive to chloramphenicol, while biogroup 3 strains are indole positive, esculin and salicin negative

and sensitive or resistant to chloramphenicol). The use of DNA hybridisation techniques revealed considerable heterogeneity in the biogroup 3 strains (Costas et al., 1993). From biogroup 1 is the separate species *Proteus penneri* (Hickman et al., 1982) that is indole, esculin and salicin negative and chloramphenicol resistant. The genus *Morganella* contains the species *M. morganii* only which was divided into two subspecies *M. morganii* subsp. *morganii* and *M. morganii* subsp. *sibonii* by means of DNA hybridisation (Jensen et al., 1992). The difference between them underlies trehalose fermentation. These subspecies consist of recognized 7 biogroups on the basis of lysine and ornithine decarboxylase activities: *M. morganii* subsp. *morganii* biogroups A, B, C, D; *M. morganii* subsp. *sibonii* biogroups E, F, G (Jensen et al., 1992). Finally the genus *Providencia* includes five species: *P. stuartii*, *P. alcalifaciens*, *P. rettgeri* (previously *Proteus rettgeri*), *P. rustigianii* (previously *P. alcalifaciens* biogroup 3) (Hickman-Brenner et al., 1983) and *P. heimbachae* (Müller et al., 1986) have been isolated from animals so far.

The intention of this work was to describe the occurrence of members of the tribe *Proteeae* in raw materials, food, environment and to determine their susceptibility to antimicrobials. The *Proteeae* grow very well on common culture media but it is very useful to use a selective medium for their isolation (Thaller et al., 1992; Mazoyer et al., 1995; Pompei et al., 1996). In this work the selective medium employed for the phosphatase activity of the *Proteeae* was used for the isolation of most strains (Urbanová, 2000). Isolated strains were determined and identified by the commercial diagnostic system and the numerical identification program.

## MATERIAL AND METHODS

### Isolation of bacteria

Selective phosphate agar (Urbanová, 2000) was used as a primary medium for isolation and detection of strains of the tribe *Proteeae*. The selectivity of a "Selective *Proteeae* Medium" (SPM) is based on the phosphatase activity of *Proteeae* and on their resistance to the polymyxin B. The medium contains bile salts as inhibitors for Gram-positive bacteria. Fifty-four strains of the genera *Proteus*, *Providencia* and *Morganella* were isolated from samples of raw milk, cheese, ham foam, raw and frozen chicken, raw sausage, waste water and sludge.

### Determination of bacteria

The *Proteeae* strains were identified by the commercial diagnostic kit ENTEROtest 16 which contains the following biochemical tests: hydrogen sulfide production, lysine decarboxylase, ornithine decarboxylase, indole production, urea hydrolysis, phenylalanine

deaminase, esculin hydrolysis, citrate (Simmons') and malonate utilisation, myo-inositol, ribitol, cellobiose, sucrose, glucitol, trehalose and mannitol fermentation and inclusive of OXItest and ONPtest (Lachema Co., Brno, Czech Republic). For correct determination some conventional tests were used (production of hydrogen sulfide, Simmons' citrate utilisation, lysine and ornithine decarboxylases, ribitol, galactose, salicin, trehalose, maltose and mannose fermentation) (Hugh and Liefson, 1953; Brooks and Sodeman, 1974; Barrow and Feltham, 1993).

### Identification of bacteria

The numeric identification program TNW 5.1 (Czech Collection of Microorganisms, Brno, Czech Republic) was used for the identification of isolated bacterial strains.

### Susceptibility to antibiotics

The antibiotics susceptibility was tested by the standard disk method (Urbášková, 1991; Urbášková and Lochman, 1997). The following antibiotics (Sanofi Diagnostic Pasteur) were used: amikacin 30 µg (AMI), ampicillin 10 µg (AMP), amoxicillin/clavulanic acid 30 µg (20 µg/10 µg = AMC), cephalothin 30 µg (CLT), cefoxitin 30 µg (CXT), cefuroxime 30 µg (CRX), co-trimoxazole 25 µg (trimethoprim 1.25 µg + sulfonamide 23.75 µg = COT), gentamycin 10 µg (GEN), chloramphenicol 30 µg (CMP), netilmycin 30 µg (NET), ofloxacin 5 µg (OFL), tetracycline 30 UI (TET).

## RESULTS AND DISCUSSION

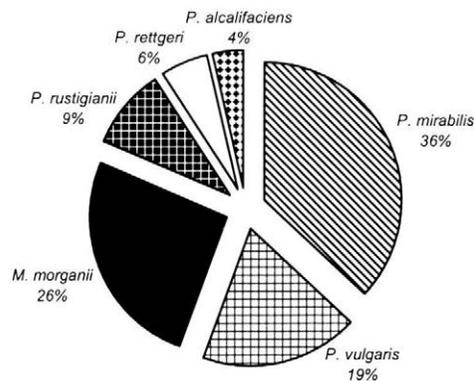
Isolated strains and their sources are listed in Tab. 1 and shown at Fig. 1. Twenty *Proteus mirabilis* strains (37%) were isolated. *P. mirabilis* is a widespread ubiquitous species. Fourteen strains of *Morganella morganii* (25.9%) were found. In some literature sources *M. morganii* have been described as a producer of histidine causing hygienic problems in the fishing industry (Klausen and Huss, 1987). *Morganella morganii* subsp. *sibonii* was isolated only from waste water and sludge in agriculture. Ten strains (18.5%) of *Proteus vulgaris* (three strains of biogroup 3 and seven strains of biogroup 2) were isolated. *P. vulgaris* occur as an undesirable contaminant in food production. Majority of biogroup 2 (70%) corresponds with Costas et al. (1993), but the share of biogroup 2 and/or 3 in hygienic problems in food technology as well as in important nosocomial infections or diarrhoea in human medicine is unknown. Species from the genus *Providencia* were isolated in small amounts from the environment mainly (waste water, sludge in agriculture) – 5 strains of *P. rusti-*

I. Proteaceae species isolated from raw materials, food and environment

Source	No.	Species	No.	%	(No.)
Raw milk and milking	17	<i>P. mirabilis</i>	7	41.2	
		<i>P. vulgaris</i>	4	23.5	biogroup 3 (2)
		<i>M. morgani</i>	6	35.3	ssp. <i>morgani</i>
Meat and poultry industry	25	<i>P. mirabilis</i>	13	52.0	
		<i>P. vulgaris</i>	5	20.0	biogroup 3 (1)
					biogroup 2 (4)
		<i>M. morgani</i>	4	16.0	ssp. <i>morgani</i>
		<i>P. alcalifaciens</i>	1	4.0	
Food	1	<i>P. vulgaris</i>	1	100.0	biogroup 2
		<i>P. rustigianii</i>	2	8.0	
Environment (waste water, sludge)	11	<i>M. morgani</i>	4	36.3	ssp. <i>sibonii</i>
		<i>P. rettgeri</i>	3	27.3	
		<i>P. rustigianii</i>	3	27.3	
		<i>P. alcalifaciens</i>	1	9.1	

*gani* (9.3%), 3 strains of *P. rettgeri* (5.6%) and 2 strains of *P. alcalifaciens* (3.7%). These results agree with natural appearance of genus *Providencia*: *P. rustigianii* – animals and human intestinal tract; *P. rettgeri* – poultry, reptiles' and amphibians' excrements, surface water; *P. alcalifaciens* – invasive enteropathogen (Stock and Wiedemann, 1998).

Eighty-five per cent of all *Proteaceae* isolates were identified excellent (EI) or very good (VGI) by the numerical system TNW on the basis of results from the ENTEROtest 16 (Tab. II). Only three false results were obtained due to false negative tests of the trehalosa fermentation in the ENTEROtest 16. Good identification (GI) of two *P. mirabilis* strains has been done of negative hydrogen sulfide reaction, which was also confirmed by the conventional test as well (2% H<sub>2</sub>S negative strains of *P. mirabilis* are referred to in literature). Four isolates of *M. morgani* subsp. *sibonii* were identified excellent (EI).



I. Bacteria isolated from raw materials, food and environment (%)

All of them belong to biogroups A (lysine negative, ornithine positive). Identification of *M. morgani* subsp. *morgani* was very good (biogroup A – lysine negative, ornithine positive like a type strain) except for two strains of biogroup C (lysine negative, ornithine negative) that were evaluated as intermediate strains (IS). These two strains have been isolated from raw milk and frozen chicken. If these isolates belong to the species *M. morgani* was validated in maltose (MLT) and mannose (MNE) fermentation tests: *M. morgani* MLT negative, MNE positive; *Proteus* spp. mostly MLT positive, MNE negative. The identification to the genus (GnI) due to a positive Simmons' citrate test and a negative sucrose fermentation test was obtained for four strains of *P. rustigianii* isolated from waste water, sludge and frozen chicken (15% SCI positive *P. rustigianii* is reported). To confirm the identification of *P. rustigianii* a conventional galactose fermentation test has been done. Together with

II. ENTEROtest 16 and TNW system ability to identify isolated species

Species	Identification by TNW	Number of isolates	%
<i>P. mirabilis</i>	EI;VGI	18	90.0
	GI	2	10.0
<i>P. vulgaris</i>	EI;VGI	10	100.0
	EI	4	28.6
<i>M. morgani</i>	VGI	8	57.1
	IS	2	14.3
<i>P. rettgeri</i>	EI;VGI	3	100.0
<i>P. rustigianii</i>	EI	1	20.0
	GnI	4	80.0
<i>P. alcalifaciens</i>	EI	2	100.0

EI = excellent identification; VGI = very good identification  
GnI = identification to genus; IS = intermediate strain

a negative ribitol, inositol and trehalose fermentation validated correct identification to the species. To advantage the ENTEROtest II can be used.

From a human medical point of view the appearance of bacteria resistant to antibiotics in food is a relevant problem now (Coghlan, 1996; Manie et al., 1998), so that antibiotic susceptibilities of 50 isolated strains were tested (Tab. III). The composition of the used antibiotics was different for individual species (Urbášková, 1998) according to the natural resistance of members of the tribe *Proteeae* (for example, to polymyxin, older cephalosporins, tetracycline, colistin, ampicillin and nitrofurantoin) without respect to an epidemical situation. Most resistance mechanisms to antimicrobial agents are known such as  $\beta$ -lactamases production by indol positive

Two strains *M. morganii* subsp. *morganii* biogroup A were intermediate to tetracycline (Tab. III). The resistance to tetracycline in biogroup A has been reported too (Janda et al., 1996; Stock and Wiedemann, 1998). Further increase in of the occurrence of resistant bacteria is going to be probable in tetracycline which is used commonly as a supplement of feed for farm animals (De Paola et al., 1995; Manie et al., 1998).

*Providencia* spp. were susceptible to all agents with the exception of one strain *P. rettgeri* (resistant to CPM) and one strain *P. rustigianii* (resistant to COT) – Fig. 2. It has been shown that it is not correct to use the diffuse method because strains of *Providencia* can show a false sensitivity (Biedenbach and Jones, 1994). *P. stuartii* is the most resistant species of the genus (naturally resis-

### III. Antibiotics resistance of isolated strains

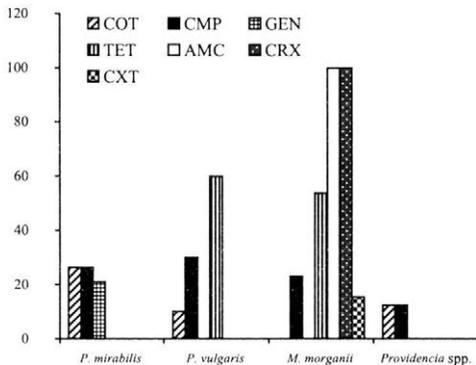
Species	Number of strains	Set of antibiotics	RS			IS		RS + IS	
			ATB	No.	%	No.	%	No.	%
<i>P. mirabilis</i>	19	AMP, CLT, CMP, COT, AMC, CRX, CXT, OFL, GEN, NET, AMI	COT	4	21.1	1	5.3	5	26.3
			CMP	3	15.8	2	10.5	5	26.3
			GEN	3	15.8	1	5.3	4	21.1
			total RS					9	47.4
<i>P. vulgaris</i>	10	CXT, OFL, GEN, NET, AMI, CMP, TET, COT	TET	5	50.0	1	10.0	6	60.0
			CMP	3	30.0	–	–	3	30.0
			COT	1	10.0	–	–	1	10.0
			total RS					6	60.0
<i>M. morganii</i>	13	CXT, OFL, GEN, NET, AMI, CMP, TET, COT, AMC, CRX,	AMC	13	100.0	–	–	13	100.0
			CRX	7	53.8	6	46.2	13	100.0
			TET	5	38.4	2	15.4	7	53.8
			CMP	1	7.7	2	15.4	3	23.1
			CXT	–	–	2	15.4	2	15.4
			total RS					13	100.0
<i>Providencia</i> spp.	8	CXT, OFL, GEN, NET, AMI, CMP, CRX, COT,	CMP	1	12.5	–	–	1	12.5
			COT	1	12.5	–	–	1	12.5
			total RS					2	25.0

ATB = antibiotics; RS = resistant strains; IS = intermediate strains; AMI = amikacin; AMP = ampicillin; AMC = amoxicillin/clavulanic acid; CLT = cephalothin; CXT = cefoxitin; CRX = cefuroxime; COT = co-trimoxazole; Gen = gentamycin; CMP = chloramphenicol; NET = netilmycin; OFL = ofloxacin; TET = tetracycline

*Proteeae* (von Graevenitz and Nourbakhsh, 1972). Thirty strains (60%) were found to be resistant to one or more antibiotics (Tab. III). *M. morganii* showed a high-level of resistance to amoxicillin/clavulanic acid and cefuroxim (nearly 100%) – Fig. 2. Mechanisms of resistance to these antibiotics on *M. morganii* are akin to mechanisms of natural resistance to ampicillin or first-generation cephalosporins. Recently the occurrence of false results in susceptibility of *M. morganii* to cephalosporins in a disk method has been confirmed (Biedenbach and Jones, 1994). The higher resistance to tetracycline (38.4%) is shown by two *M. morganii* subsp. *sibonii* strains (100% resistance) and three *M. morganii* subsp. *morganii* biogroup C (90% resistance).

tant to tetracyclines, penicillines and older cephalosporins) and is generally considered to be an antibiotic multiresistant bacterium. *P. rettgeri*, *P. alcalifaciens* and *P. rustigianii* are more and/or less sensitive (Stock and Wiedemann, 1998), but multiresistant *P. rettgeri* strains have been described.

Higher resistance rates were found in *Proteus vulgaris* (60%) and *P. mirabilis* (47.4%) – Tab. III, Fig. 2. It is important for their part in the human urine tract disease. The high resistance to cephalosporins (for example, cefuroxime > 50% and/or ceftrozil > 93%) has been reported (Biedenbach and Jones, 1994). Our strains of *P. vulgaris* from animal production were sensitive to cefoxitin (CXT). Resistance to tetracycline (50–60% of



2. The resistance to antibiotics for tested species of the tribe Proteaceae (%)

strains) in *P. vulgaris* agrees with what has been reported (von Graevenitz and Nourbakhsh, 1972; Manic et al., 1998). Siboni (1980) referred that strains which fermented trehalose had nontransmissible resistance to tetracycline, too. However, *P. vulgaris* strains tested in this work have not been trehalose positive. *P. vulgaris* biogroup 2 has been designated to be selective to chloramphenicol (Hickman et al., 1982) but one of the seven tested strains was resistant to CMP.

About one half of *Proteus mirabilis* strains (47.4%) was resistant to one and/or more antibiotics. 26.3 per cent of strains resistant to co-trimoxazole (the antibiotic very often used in treatment of urinary diseases) was found. It is a question whether strains from raw material or food origin participate in the growth of resistance to COT of nosocomial strains of *P. mirabilis* as have been reported by Perrin et al. (1998). The increasing resistance to antibiotics in *P. mirabilis* can be shown on chloramphenicol (26.3%) and gentamycin (21.1%) – Fig. 2 – in contrast with results of von Graevenitz and Nourbakhsh (1972). In their study out of the 864 isolates of *P. mirabilis* 5.5% were resistant to CMP only and none to GEN. The increase in numbers of resistant strains to gentamycin from food production can be partially due to the fact that in the last ten years gentamycin has been used in veterinary medicine. Since 1992 several gentamycin preparations for treatment of endometritis and mastitis in cows, diarrhoea in sucking – pigs and calves, etc., have been registered in the Czech Republic. With respect to the increasing numbers of resistant bacterial strains we suppose with the other authors (Costas et al., 1993; Stock and Wiedemann, 1998) that the resistance of bacteria to antibiotics can be only a limited taxonomic sign.

#### Acknowledgement

We thank RNDr. L. Hrubá, OHS Jihlava, MVDr. J. Vyhnáková, VFÚ Brno, and RNDr. M. Laštůvková, MHS Brno, for their help with isolation of strains from raw materials, food and environment.

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Received: 99-09-09

Accepted after corrections: 00-02-03

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Contact Address:

RNDr. Eva Urbášková, CSc., Čaderský-Envitek Ltd., Bednářova 21, 619 00 Brno, Česká republika  
Tel. +420 5 43 25 07 64, fax +420 5 43 25 07 60, e-mail: cadersky@brn.pvtnet.cz

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## FUNCTIONAL ACTIVITY OF BLOOD LYMPHOCYTES AND NEUTROPHILS IN PERSISTENT BOVINE PAPILLOMATOSIS

### FUNKČNÁ AKTIVITA KRVNÝCH LYMFOCYTOV A NEUTROFILOV PRO PERZISTENTNEJ BOVINNEJ PAPILOMATÓZE

Š. Paulík, J. Mojžišová, Š. Švrček, Z. Beníšek, R. Ondrejka, F. Lešník

*University of Veterinary Medicine, Košice, Slovak Republic*

**ABSTRACT:** The functional activity of blood lymphocytes and neutrophils in bulls with persistent cutaneous papillomatosis was evaluated. During clinical manifestation of papillomatosis, a blastogenic response of lymphocytes to T-cell mitogens significantly decreased. In the regression period of papillomas gradual elevation of lymphocytic activity was found out and, at the end of evaluation it was comparable with activity of lymphocytes in control animals. The phagocytic activity of neutrophils in affected animals was not significantly altered in comparison with that in control animals. We demonstrated similar findings by evaluation of leukocyte, neutrophil and lymphocyte counts.

cattle; papillomatosis; blastogenesis of lymphocytes; phagocytic activity of neutrophils

**ABSTRAKT:** Hodnotili sme funkčnú aktivitu krvných lymfocytov a neutrofilov u býčkov s perzistentnou kutánnou papilomatózou. Počas klinickej manifestácie papilomatózy bola preukázaná signifikantne znížená blastogénna odpoveď lymfocytov k T bunkovým mitogénom. V dobe regresie kožných papilómov bolo zaznamenané postupné zvyšovanie tejto aktivity lymfocytov, ktorá na záver sledovania bola porovnateľná s aktivitou kontrolných zvierat. Fagocytárna aktivita neutrofilov nebola u postihnutých býčkov v porovnaní s kontrolnými zvieratami signifikantne alterovaná. Podobné zistenie sme zaznamenali aj pri hodnotení počtu leukocytov, neutrofilov a lymfocytov.

hovádzí dobytok; papilomatóza; blastogenéza lymfocytov; fagocytárna aktivita neutrofilov

#### INTRODUCTION

Bovine papillomatosis manifested by skin fibropapillomas is caused by three types of bovine papillomaviruses (BPV), including BPV-2 (Radostits et al., 1994). Papillomatosis caused by BPV-2 affects primarily animals at the age under 2 years. Lesions usually regress spontaneously in any stage of their development (Lee and Olson, 1969). In some cases persistent papillomatosis can develop. This is probably associated with the suppression of cell-mediated immunity. We examined an alteration of the functional activity of blood lymphocytes and neutrophils in progressive and regressive stages of bovine persistent skin papillomatosis.

#### MATERIAL AND METHODS

##### Animals

9 bulls (Pinzgau breed) at the age of about 2 years were examined. Experimental group (E) consists of 4 animals with naturally developed generalized and persistent (> 1 year) cutaneous papillomatosis, caused probably by BPV-2 (Levkut et al., 1997). Blood samples were taken in the progressive (sampling A – 14 days before surgery) and the regressive (sampling B – day 105; sampling C – day 139 after surgery) stage of papillomatosis (Levkut et al., 1997). To induce regression, surgery (excision of bigger papillomas) and immunization with autologous tis-

sue vaccine were used. Results of investigation were compared with those found in control animals ( $n = 5$ , clinically healthy, without clinical manifestation of papillomatosis).

### Blastogenic response of blood lymphocytes to mitogens

Lymphocytes were separated from venous blood on the Ficoll density gradient. PHA (15 µg/ml) and Con A (5 µg/ml) were used for lymphocyte stimulation. The level of blastogenic response was estimated using ethidium bromide fluorescent test and expressed as fluorescence intensity of stimulated (FISC) and unstimulated cells (FIUC) and stimulation index (SI) (Nagahata et al., 1986).

### Phagocytic activity of neutrophils (PA)

Phagocytic activity of blood neutrophils was examined as described by Větvička et al. (1982) using the phagocytosis of 2-hydroxyethylmetacrylate particles. Phagocytic activity was expressed as the percentage of phagocytizing neutrophils (% PNe) and the phagocytic index (PI).

### Absolute leukocyte, neutrophil and lymphocyte counts

Total leukocyte count was determined with blood cells analyser (SERONO, 150, Plus, USA). Neutrophil and lymphocyte counts were determined by calculation from their percentage rate in differential blood smear.

### Statistical analysis

Results were expressed as mean  $\pm$  standard deviation (SD) and analysed by Student's *t*-test. Only values  $P < 0.05$  were considered to be statistically significant.

## RESULTS

In the experimental (E) group of bulls in the progressive stage of papillomatosis (sampling A) a significantly decreased blastogenic response of blood lymphocytes to both mitogens was found. In the regressive stage (sampling B), a gradual increase of blastogenic response of blood lymphocytes was found and at the end of observation (sampling C) was comparable with values found in control (C) group of animals (Tab. I). The parameters of PA of neutrophils were not significantly altered in E group when compared with C group of animals in either of the terms of sampling. Total leukocyte, neutrophil and lymphocyte counts in both groups of animals were within the range of the reference values (Radostits et al., 1994).

## DISCUSSION

In bovine papillomatosis spontaneous recovery is common. Under some circumstances papillomas can persist for 12–18 months (Olson, 1990; Radostits et al., 1994). Such animals have probably inadequate immunological responses (Scott and Anderson, 1992). In bulls under our investigation fibropapillomas persisted more than 1 year. This progressive stage of infection was manifested by conspicuous alteration of the blastogenic response of blood lymphocytes. Duncan et al. (1975) have also reported cellular immunity suppression (evaluated by skin test) in bovine persistent papillomatosis. Cellular immunity depression in fibropapillomatosis was also documented by histological examination of skin lesions (Lee and Olson, 1969; Levkut et al., 1997).

The regressive stage of papillomatosis is accompanied by a positive change in the immunity. This is manifested by papilloma infiltration with mononuclear cells (Lee and Olson, 1969; Levkut et al., 1997) as well as by an increased blastogenesis of blood lymphocytes in humans (Morison, 1975). In the regressive stage of remain-

I. Blastogenic response of blood lymphocytes after mitogen induction

Mitogens	Group	Sampling	FISC	FIUC	SI
PHA	E	A	8.15 $\pm$ 1.79 <sup>a</sup>	7.00 $\pm$ 1.28 <sup>b</sup>	1.17 $\pm$ 0.17 <sup>c</sup>
		B	18.71 $\pm$ 5.81	7.63 $\pm$ 1.50 <sup>d</sup>	2.43 $\pm$ 0.56
		C	26.50 $\pm$ 7.28	10.60 $\pm$ 1.32	2.50 $\pm$ 0.49
		–	29.59 $\pm$ 9.36	11.45 $\pm$ 1.34	2.54 $\pm$ 0.53
ConA	E	A	12.10 $\pm$ 2.23 <sup>e</sup>	7.00 $\pm$ 1.28 <sup>f</sup>	1.74 $\pm$ 0.23 <sup>f</sup>
		B	21.45 $\pm$ 8.54	7.63 $\pm$ 1.50 <sup>b</sup>	2.82 $\pm$ 1.14
		C	31.26 $\pm$ 8.42	10.60 $\pm$ 1.32	2.92 $\pm$ 0.32
		–	34.74 $\pm$ 8.38	11.45 $\pm$ 1.34	3.05 $\pm$ 0.79

E = experimental group

C = control group

A = before surgery

B = day 105 after surgery

C = day 139 after surgery

FISC = fluorescence intensity of stimulated cells

FIUC = FI of unstimulated cells

SI = stimulation index

g =  $P < 0.05$

d; h =  $P < 0.025$

a =  $P < 0.01$

b;c;e;f =  $P < 0.005$  (vs. control)

ing papillomas, a significant increase of blastogenic response of blood lymphocytes was found. Surgical excision of some papillomas is accompanied by the regression of the papillomas which were not removed (Radostits et al., 1994). Tissue extracts from papillomas have also therapeutical effects (Makay, 1989; Beníšek et al., 1996). Despite this, we cannot answer the question if the increase of lymphocytic activity was the cause or the consequence of papilloma regression.

In the case of neutrophil dysfunction, increased sensitivity to infection can occur (Moriarty, 1984). Persistent cutaneous papillomatosis can be complicated by the secondary bacterial infection if papillomas were traumatised (Radostits et al., 1994). Necrotic changes of fibropapilloma lesions on the ventral side of abdomen in bulls under our observation prove the fact mentioned above.

Although in the progressive stage of the disease we did not find any depression of the phagocytic activity of neutrophils, we cannot exclude if the inflammatory changes were the consequence of an alteration of other activities of these cells. By this time we have no data at our disposal about the dysfunction of neutrophils in bovine persistent cutaneous papillomatosis. The clarification of the relationship papillomatosis – alteration of functional activities of neutrophils require further observation.

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Received: 99–04–13

Accepted after corrections: 00–02–03

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### Contact Address:

Doc. MVDr. Štefan Paulík, CSc., Univerzita veterinárskeho lekárstva, Komenského 73, 041 81 Košice, Slovenská republika  
Tel. +421 95 633 21 11, fax +421 95 76 76 75, e-mail: paulik@uvm.sk

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**BACTERIAL COMMUNICATION AND POSSIBLE APPLICATION  
IN CONTROL AND THERAPY OF BACTERIAL DISEASES\*****BAKTERIÁLNÍ KOMUNIKACE A MOŽNOSTI VYUŽITÍ V PREVENCI  
A TERAPII BAKTERIÁLNÍCH INFEKČÍ****I. Rychlík, J. Volf, M. Ševčík***Veterinary Research Institute, Brno, Czech Republic*

**ABSTRACT:** Biological processes such as control of bioluminescence in *Vibrio fischerii* and *Vibrio harveyi*, expression of virulence factors in *Pseudomonas aeruginosa* and *Erwinia carotovora* or conjugative transfer of the Ti plasmid in *Agrobacterium tumefaciens* are dependent on the production and sensing of specific secondary metabolites derived from the structure of N-acyl homoserine lactone. These metabolites are released and sensed by bacterial populations which in turn modify their metabolism accordingly. In certain bacterial species, virulence is under the control of these metabolites, in genomes of *Salmonella typhimurium* and *E.coli* homologous systems with so far unknown function have been identified. This shows that communication by secondary metabolites is widespread in bacteria. Furthermore, host organism have been shown to respond to these metabolites by non-specific immune reactions. Understanding the biological role of this communication could enable specific modification of behaviour of bacterial populations and thus open a way to new therapeutic measures. All of this becomes even more important in a time when antibiotic resistance in bacteria increases and new ways of prevention and control of infectious diseases are sought.

quorum sensing; *Vibrio*; *Salmonella*; *E. coli*; autoinducer; homoserine lactone

**ABSTRAKT:** Biologické procesy podílející se na kontrole bioluminiscence *Vibrio fischerii* a *Vibrio harveyi*, produkci faktorů virulence u *Pseudomonas aeruginosa* a *Erwinia carotovora* nebo na konjugativním přenosu Ti plazmidu u *Agrobacterium tumefaciens* jsou u těchto bakteriálních druhů řízeny produkcí a vnímáním specifických sekundárních metabolitů odvozených od struktury N-acyl homoserin laktonu. Tyto látky jsou bakteriálními populacemi aktivně vylučovány do prostředí, zpětně vnímány a podle jejich koncentrace bakteriální populace ovlivňuje svůj metabolismus. Skutečnost, že u některých druhů je těmito metabolity kontrolována virulence, a skutečnost, že homologní systémy bez doposud určené funkce byly identifikovány v genomu *Salmonella typhimurium* nebo *E. coli* naznačuje, že vnímání specifických sekundárních metabolitů je mezi bakteriemi velmi rozšířeno. Navíc se jeví, že i hostitelský organismus je schopen vnímat bakteriální metabolity a reagovat na ně nespecifickými imunitními reakcemi. Poznáním smyslu takové komunikace by mohlo umožnit cíleně ovlivnit chování bakteriální populace a tím připravit nové možnosti pro terapii. To vše nabývá dále na významu v době, kdy u bakterií dochází k nárůstu rezistencí na antibiotika a hledají se nové, alternativní směry prevence a kontroly šíření infekčních onemocnění.

quorum sensing; *Vibrio*; *Salmonella*; *E. coli*; autoinduktor; homoserin lakton

\* Supported by the Grant Agency of the Czech Republic (Grant No. 524/98/1089) and National Agency for Agricultural Research in Prague (Grant No. EP6076).

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## 1. ÚVOD

Z posledních výsledků výzkumu chování bakteriálních populací vyplývá, že jednotlivé bakteriální buňky v průběhu růstu mezi sebou komunikují, produkují specifické sekundární metabolity, vnímají je a podle toho zpětně upravují metabolismus a chování celé populace. Přitom způsob komunikace je různý u rozdílných bakteriálních rodů a druhů. Grampozitivní bakterie mohou komunikovat pomocí oligopeptidů, myxobakterie uvolňují tzv. A faktor, *Ralstonia solanaceae* reguluje virulenci pomocí metyl esterů mastných kyselin (Dunny a Leonard, 1997; Dworkin, 1996; Flavier aj., 1997a, b; Horinouchi a Bepu 1992; Kleerebezem aj., 1997). U gramnegativních bakterií je nejvíce rozšířen systém komunikace pomocí metabolitů na bázi N-acyl homoserin laktónů (AHL). A protože mezi gramnegativní bakterie patří i mnoho patogenních mikroorganismů, jim a jejich komunikaci pomocí AHL bude věnována pozornost v tomto přehledném článku.

## 2. BIOLUMINISCENCE U *VIBRIO FISHERI*

Výzkum bakteriální luminescence se začal intenzivně rozvíjet počátkem 70. let detailním studiem bioluminiscence u *Vibrio fischeri* (syn. *Photobacterium fischeri*) (Eberhard, 1972; Hastings a Nealson, 1977; Nealson a Hastings, 1979). Tato bakterie žije buď volně v mořské vodě, nebo se vyskytuje jako symbiont ve světelných orgánech hlubinných ryb. Díky příznivým podmínkám uvnitř těchto orgánů v nich dorůstá do vysokých koncentrací (až  $10^{10}$  bakterií v 1 ml) a je zde zodpovědná za produkci světla. Příležitostně se však *V. fischeri* uvolní ze světelných orgánů do volného moře a za takových okolností je produkce světla pro bakteriální buňku nepotřebná. Protože koncentrace *V. fischeri* ve vol-

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ném moři nikdy nedosahuje takových koncentrací jako ve světelných orgánech ryb, postupně se u tohoto bakteriálního druhu vyvinul systém kontroly bioluminiscence v závislosti na koncentraci bakteriální populace – při vysokých bakteriálních koncentracích je bioluminiscence zapnutá, při nízkých je vypnutá.

Podobné výsledky byly dosaženy i v podmínkách *in vitro*. Na začátku *in vitro* kultivace je luminescence *V. fischeri* téměř nulová. Po dosažení kritické koncentrace bakteriální populace však dochází k velmi prudkému nárůstu luminescence. Tyto charakteristiky byly studovány počátkem 70. let a již tehdy bylo zjištěno, že za prudký nárůst bioluminiscence jsou zodpovědné metabolity, které jsou uvolňovány kulturou *V. fischeri* do prostředí (Eberhard, 1972). Trvalo dalších 10 let než byly tyto metabolity identifikovány. V roce 1981 Eberhard aj. popsali N-(3-oxo)-hexanoyl-L-homoserin laktón jako první komunikační molekulu *V. fischeri*, která se podílí na kontrole bioluminiscence (Eberhard aj., 1981). Pro označení jevů spojených s vnímáním koncentrace/hustoty bakteriální kultury a produkci specifických sekundárních metabolitů se ujal označení quorum sensing (Fuqua aj., 1994; Hastings a Greenberg, 1999), volně přeloženo vnímání kritické koncentrace. Specifické sekundární metabolity stimulující bioluminiscenci případně jiné metabolické dráhy bakterií se nejčastěji označují jako autoinduktory (autoinducer) (Eberhard aj., 1981; Surette a Bassler, 1999), objevuje se však i označení bakteriální feromony (Hardman aj., 1998).

U *V. fischeri* se na bioluminiscenci podílí geny *lux* regulonu. Celý *lux* regulon se u *V. fischeri* sestává ze dvou operonů, *luxR* a *luxICDAB* (Egland a Greenberg, 2000; Shadel a Baldwin, 1991; Shadel a Baldwin, 1992). Protein LuxR reguluje expresi všech ostatních genů *lux* operonu. Modelování sekundární struktury LuxR

odhalilo helix-turn-helix motiv typický pro proteiny regulující transkripci. Dále se prokázalo, že AHL reaguje s LuxR a mění jej z neaktivní formy na aktivní. Aktivovaný LuxR se váže na promotor genů *luxICDAB*, zvyšuje jejich transkripci a tím i celkovou expresi (Shadel a Baldwin, 1991; Shadel a Baldwin, 1992). Expresie genů *luxICDAB* je rovněž závislá na produkci cAMP a přítomnosti železa v prostředí (Dunlap, 1992; Dunlap a Kuo, 1992). Protein LuxI katalyzuje syntézu AHL. Za samotnou bioluminiscenci jsou pak zodpovědné proteiny LuxA a LuxB, proteiny LuxC a LuxD se podílejí na syntéze aldehydu, který je substrátem pro luciferázu (proteiny LuxA a LuxB). Nejvhodnějším substrátem pro luciferázu jsou aldehydy s 8 až 12 uhlíky, jejichž oxidací na příslušné karboxylové kyseliny se uvolní kvantum světla. Protože se při oxidaci aldehydu spotřebovává přímo molekulární kyslík, stejně jako při respiraci, někteří autoři považují bioluminiscenci u *V. fischeri* za alternativu k respiračnímu řetězci (Nealson a Hastings, 1979). Chemická rovnice definující bioluminiscenci může být vyjádřena takto:



FMNH<sub>2</sub> získává *V. fischeri* oxidací NADH<sub>2</sub>, vznikající karboxylová kyselina je recyklována zpětnou redukcí na aldehyd za spotřeby další molekuly NADH<sub>2</sub>. Na jedno světelné kvantum se tak spotřebují dvě molekuly NADH<sub>2</sub>. Bioluminiscence je tedy energeticky poměrně nákladná a proto je u *V. fischeri* tak přísně regulována.

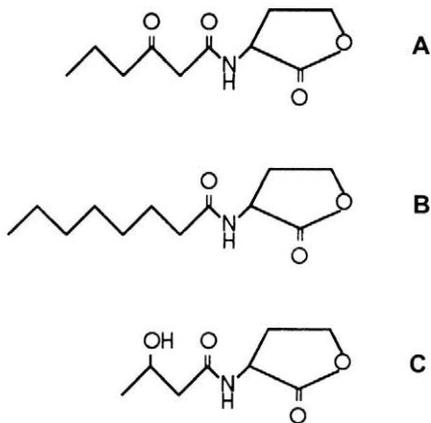
### 3. DALŠÍ QUORUM SENSING BAKTERIE

Quorum sensing systémy založené na produkci AHL byly prokázány i u jiných bakterií, jako například *Vibrio harveyi* (syn. *Beneckea harveyi*) (Bassler aj., 1993; Bassler aj., 1994a, b), *Pseudomonas aeruginosa* (Latifi aj., 1996; Winson aj., 1995), *Agrobacterium tumefaciens* (Piper a Farrand, 2000; Zhu aj., 1998), *Yersinia pseudotuberculosis* (Atkinson aj., 1999), *Rhizobium leguminosarum* (Thorne a Williams, 1999), *Aeromonas hydrophilla*, *A. salmonicida* (Swift aj., 1997) nebo *Erwinia carotovora* (Cui aj., 1995; Jones aj., 1993). U všech těchto bakteriálních druhů bylo možno vyzorovat některé obecné rysy:

- všechny komunikační molekuly vycházely ze struktury N-acyl homoserin laktону,
- na biosyntéze a vnímání AHL se podílejí homologní enzymy/geny,
- koncentrace N-acyl homoserin laktónů v prostředí je kritická pro spuštění specifických odpovědí (Swift aj., 1996).

#### 3.1. N-acyl homoserin laktony

Přestože je základní struktura autoinduktorů velmi podobná, je zároveň i druhově specifická a každý bakteriální druh reaguje jen na svůj specifický autoinduktor.



1. Různé autoinduktory odvozené od struktury N-acyl homoserin laktónu

A = N-(3-oxo)-hexanoyl-L-homoserin laktón, autoinduktor 1 *V. fischeri*;

B = N-oktanoyl-L-homoserin laktón, autoinduktor 2 *V. fischeri*;

C = N-butanoyl-L-homoserin laktón, autoinduktor 1 *V. harveyi*

Different autoinducers derived from the structure of N-acyl homoserine lactone

A = N-(3-oxo)-hexanoyl-L-homoserine lactone, autoinducer 1 of *V. fischeri*;

B = N-octanoyl-L-homoserine lactone, autoinducer 2 of *V. fischeri*;

C = N-butanoyl-L-homoserine lactone, autoinducer 1 of *V. harveyi*

Kultura *V. harveyi* nebo *P. aeruginosa* proto nereaguje například na autoinduktory produkované *V. fischeri*. Toho je dosaženo různou délkou a různými modifikacemi uhlíkového řetězce (acylu) navázaného na homoserin laktónové (HSL) jádro. Délka uhlíkového řetězce je vždy sudé číslo a pohybuje se od 4 (butanoyl HSL u *V. harveyi*) až do 14 atomů uhlíku (tetradecanoyl HSL u *R. leguminosarum*). Uhlíkové řetězce bývají modifikovány navázáním hydroxylové nebo keto skupiny (obr. 1). Není výjimkou, produkuje-li jedna bakterie dva odlišné autoinduktory (*V. fischeri*, *P. aeruginosa*) o různé délce a postranní modifikaci acylového řetězce (Eberhard aj., 1981; Hanzelka aj., 1999; Latifi aj., 1996). Každý z těchto autoinduktorů může mít odlišnou funkci.

#### 3.2. Biosyntéza a vnímání AHL

Doposud byly popsány dva zcela nezávislé mechanismy syntézy AHL. První známý mechanismus byl charakterizován u *V. fischeri*. Identifikovaný enzym hexanoyl HSL syntetáza byl pojmenován LuxI, jehož exprese je pozitivně regulována proteinem LuxR. Systémy homologní s LuxR/LuxI u *V. fischeri* byly později objeveny u *P. aeruginosa* (LasR/LasI), *E. carotovora* (CarR/CarI) atd. (Swift aj., 1996).

U *V. harveyi* byl později popsán enzym LuxM, který rovněž katalyzuje syntézu AHL. Překvapivě však nebyla zjištěna žádná homologie mezi proteiny LuxM *V. harveyi* a LuxI *V. fisheri*. Později byly AHL syntetázy homologní k LuxM popsány u dalších bakteriálních druhů, jako například AinS u *V. fisheri* (Hanzelka aj., 1999). Biochemie syntézy AHL však není ani v případě LuxI ani v případě LuxM plně objasněna. Přesto je zřejmé, že acyl pochází z biosyntézy mastných kyselin, HSL jádro z metabolismu aminokyseliny metioninu (More aj., 1996; Swift aj., 1996).

Autoinduktory jsou produkovány bakteriemi průběžně a postupně se s růstem populace hromadí v prostředí. Autoinduktor uvolněný do prostředí reaguje se svým receptorem (například LuxR v případě *V. fisheri*) a ten zpětně pozitivně reguluje jeho produkci. Jedná se tedy o autokatalytický proces, autoindukci. Vlastností vyplývající z takového způsobu regulace je exponenciální chování celého systému. Autoinduktor se nejprve velmi pomalu hromadí v prostředí. Protože množení bakterií je exponenciální funkcí, dochází i k exponenciálnímu růstu produkce AHL. V době prudkého nárůstu koncentrace autoinduktoru je rychle překročena jeho kritická koncentrace; v případě *V. fisheri* dojde k prudkému nárůstu bioluminiscence, v případě *P. aeruginosa* se začnou produkovat enzymy elastáza a chitináza a v případě *E. carotovora* enzymy pektináza, celulóza a polygalakturonáza. Skutečnost, že pro spuštění quorum sensing regulovaných procesů je důležitá spíše koncentrace autoinducerů než koncentrace bakteriální populace, byla prokázána pokusy, při nichž se bakterie kultivovaly s přísadkou použitého, filtrací sterilizovaného média. Například *V. fisheri* nebo *V. harveyi* začne produkovat světlo při mnohem nižších koncentracích bakteriální populace, pokud je kultivováno v půdě, která byla již jednou pro kultivaci *V. fisheri* (resp. *V. harveyi*) použita (culture conditioned medium – kultivaci podmíněná půda). Příčina tohoto jevu je zřejmá – v takovéto půdě je z předchozí kultivace přítomný autoinduktor.

## 4. BIOLOGICKÉ A EKOLOGICKÉ VÝHODY QUORUM SENSING

### 4.1. Bioluminiscentní bakterie

Biologie *V. fisheri* byla popsána v úvodu. Rod *Vibrio* zahrnuje i již zmíněný další bioluminiscentní druh *V. harveyi*. Biologie *V. harveyi* je odlišná od *V. fisheri*. *V. harveyi* nebylo nikdy izolováno ze světelných orgánů ryb. Mnohem častěji se vyskytuje ve volném mořském prostředí a zároveň je jako komenzál přítomný ve střevním traktu mořských ryb. Tyto bakterie osídlují i povrch těl koryšů. Na povrchu koryšů přitom mohou dosáhnout koncentrací již dostatečně vysokých pro spuštění bio-

luminescence. Světélkující koryši jsou nápadní a proto jsou snadnější kořisti pro další mořské živočichy, včetně ryb. Tímto způsobem se *V. harveyi* dostane do střevního traktu ryb, který kolonizuje a přežívá v něm (Hastings a Nealson, 1977; Nealson a Hastings, 1979). Pro *V. harveyi* je tento životní styl s pravidelným přísunem živin výhodnější než přežívání ve volné mořské vodě. To odpovídá i zjištění, že bioluminiscence *V. harveyi* je vyšší v minimálních půdách než v plných živných bujonech (Greenberg aj., 1979; Hastings a Nealson, 1977; Makemson a Hastings, 1979; Nealson a Hastings, 1979). Pochopitelně na povrchu koryšů je produkce světla pro *V. harveyi* mnohem důležitější (situace podobná kultivaci v minimální půdě) než produkce světla ve střevním traktu ryb bohatším na živiny (situace analogická kultivaci v plném živném médiu).

Nedávno se objevila i práce naznačující další možnou funkci bioluminiscence u *V. harveyi*. Mutantní kmeny *V. harveyi* defektní v produkci světla jsou mnohem citlivější na ozáření UV světlem. Dále bylo zjištěno, že tato citlivost přímo souvisí s produkcí světla a endogenní produkce světla by tak mohla přispívat k fotoreaktivaci reparačních systémů nukleových kyselin po ozáření UV světlem (Czyz aj., 2000).

### 4.2. Ostatní bakterie

U *P. aeruginosa* a *E. carotovora* se quorum sensing podílí na regulaci virulence. Předpokládá se, že exprimovat geny související s virulencí při nízkých koncentracích bakteriální populace by bylo zbytečné. Malé množství bakterií nemá šanci v souboji s imunitním systémem hostitelského organismu nebo jeho obrannými mechanismy. Až po dosažení určité mezní koncentrace je pravděpodobnost úspěchu vyšší, a to je i vhodný okamžik k plné expresi všech faktorů virulence. U *P. aeruginosa* je tak přítomnosti autoinduktorů v prostředí stimulována exprese elastázy, chitinázy, kyanidu a pyoverdinu (Jones aj., 1993; Stintzi aj., 1998; Winson aj., 1995), u *E. carotovora* se v přítomnosti autoinduktoru otkanoyl HSL zvyšuje exprese pektinázy a celulózy (Jones aj., 1993; McGowan aj., 1995; Pirhonen aj., 1993). V souladu s těmito poznatky byla pozorována u *P. aeruginosa* kmenů defektních v quorum sensing systémech snížená virulence (Rumbaugh aj., 1999; Tang aj., 1996)

## 5. NESPECIFICKÉ KOMUNIKAČNÍ SYSTÉMY

U *V. harveyi* byl popsán, mimo komunikaci pomocí butanoyl HSL, i systém komunikace pomocí doposud neznámého autoinduktoru A12 (Bassler aj., 1993; Bassler aj., 1994a, b). Tento autoinduktor u *V. harveyi*, stejně jako specifický systém, kontroluje bioluminiscenci. Bylo však zjištěno, že podobný autoinduktor,

kteřý stimuluje bioluminiscenci u *V. harveyi*, produkují i jiné bakterie, včetně *E. coli* a *S. typhimurium* (Bassler aj., 1997; Greenberg aj., 1979; Surette a Bassler, 1998). To poukazuje na skutečnost, že mimo přísne druhově specifické systémy komunikace existují mezi bakteriemi i systémy s mnohem širší specifičností (Bassler, 1999).

### 5.1. *Vibrio harveyi*

U *V. harveyi* existují dva systémy mezibuněčné komunikace. V prvním případě se jedná o komunikaci pomocí butanoyl HSL. Proteiny zodpovědné za syntézu autoinduceru byl označen jako LuxM a LuxL, jako sensor pro vnímání tohoto autoinduktoru funguje protein LuxN. Protein LuxO, který je pozitivně regulován proteiny LuxL a LuxN, pak u *V. harveyi* přímo aktivuje celý operon luciferázy, tedy geny *luxCDAB*.

Protein LuxO u *V. harveyi* však může být aktivován i druhým systémem regulace bioluminiscence a autoinduktorem AI2. U druhého systému komunikace byly nejprve identifikovány receptory AI2, proteiny LuxP a LuxQ (Bassler aj., 1994a). Později bylo zjištěno, že luminescence *V. harveyi* může být stimulována širším spektrem bakterií včetně *E. coli* nebo *S. typhimurium*. Nedávno pak byl charakterizován gen *luxS*, který je zodpovědný za produkci AI2 (Surette aj., 1999). Chemická povaha samotného autoinduktoru AI2 však doposud není známa. Předpokládá se, že široká specifita AI2 souvisí s ekologií *V. harveyi*, které ve srovnání s *V. fisheri* musí být adaptováno na život ve volné mořské vodě, na povrchu těl vodních korýšů, nebo ve střevním traktu ryb. Zejména ve střevním traktu ryb je *V. harveyi* ve styku s ostatní mikroflórou a přítomnost komunikačního systému se širokou specifičností pro něj může být výhodná.

### 5.2. Ostatní bakterie

V souvislosti s identifikací genů nezbytných pro bioluminiscenci *V. harveyi* bylo zjištěno, že geny zodpovědné za syntézu a vnímání autoinduktoru AI2 mají své homologы v genomu *E. coli* nebo *S. typhimurium*. Homolog LuxP *V. harveyi* byl u *E. coli* a *S. typhimurium* identifikován jako RbsB, LuxQ *V. harveyi* je podobný proteinům ArcA a ArcB u *E. coli* (Bassler aj., 1994a), homolog LuxS ve vyskytuje v genomu *E. coli* pod označením YgaA a byl rovněž prokázán v genomu *S. typhimurium* (Surette aj., 1999; Surette a Bassler, 1998, 1999). Dále byly *luxS* homologní geny nalezeny v genomech *Haemophilus influenzae*, *Bacillus subtilis*, *Helicobacter pylori*, *Campylobacter jejuni*, *Vibrio cholerae* a dalších (Surette aj., 1999). Největší pozornost budí přítomnost quorum sensing genů u *E. coli* a *S. typhimurium*.

## 6. QUORUM SENSING U *E. COLI* A *S. TYPHIMURIUM*

### 6.1. Autoinduktor AI2

Jak u *E. coli*, tak u *S. typhimurium* bylo prokázáno, že produkují autoinduktor AI2, který stimuluje bioluminiscenci u *V. harveyi*. U *E. coli* a *S. typhimurium* však dochází k produkci AI2 pouze pokud je v živném médiu glukóza (Surette a Bassler, 1999). Proto se předpokládá, že by mohlo jít o modifikovaný meziprodukt fermentace. Navíc, i když je v médiu přítomna glukóza, AI2 se objevuje v médiu jen dočasně a při přechodu do stacionární fáze růstu je kulturou *E. coli* nebo *S. typhimurium* AI2 aktivně metabolizován a odbourán. Osmotický tlak podporuje produkci AI2 nepřímo snížením jeho odbourávání. Ne všechny kmeny *E. coli* však produkují stejné množství autoinduktoru AI2. Dokonce bylo prokázáno, že u laboratorního kmene *E. coli* DH5 $\alpha$  došlo k mutaci v genu *ygaA* a tento kmen vůbec autoinduktor AI2 neprodukuje (Surette aj., 1999; Surette a Bassler, 1999). Je tedy zřejmé, že AI2 není nezbytně nutný pro metabolismus *E. coli*. Rovněž u *E. coli* se objevují první informace o vztahu quorum sensing a virulenci. Nedávno bylo zjištěno, že exprese LEE operonu (locus of enterocyte effacement) u enterohemoragických i enteropatogenních kmenů je pod kontrolou *luxS* (*ygaA*) a quorum sensing (Sperandio aj., 1999).

### 6.2. Další autoinduktor produkováné *S. typhimurium*

Několik prací z poslední doby naznačuje, že *S. typhimurium* produkuje a reaguje i na jiné metabolity než autoinduktor AI2. Nejčastěji je možnost jiného quorum sensing systému u salmonel spojována s genem *sdiA*, který je homologní genu *luxR* u *V. fisheri*. Bylo zjištěno, že SdiA se podílí na kontrole buněčného dělení a že ovlivňuje i virulenci salmonel (Ahmer aj., 1998; Garcia-Lara aj., 1996; Wang aj., 1991). Gen *sdiA* byl částečně stimulován přítomností dekanoyl HSL a autoinduktor produkovánými *V. fisheri* a *V. harveyi*, tato stimulace však nebyla nikterak výrazná (Sitnikov aj., 1998). Regulace exprese *sdiA* se nezdá být spojena s produkcí AI2 (Surette a Bassler, 1999). Není známo, zda se SdiA u *S. typhimurium* spolupodílí na expresi *luxS* a produkci autoinduktoru AI2 (Fuqua a Greenberg, 1998).

Mimo studie zaměřené na funkci *sdiA* a *luxS* u *S. typhimurium* byly popsány i jiné skupiny genů, které jsou aktivovány metabolity hromadícími se ve stacionárních kulturách (Baca-DeLancey aj., 1999). Proto je možno do budoucna očekávat, že mezibuněčná komunikace u kultur *E. coli* a *S. typhimurium* může mít komplexní charakter a výzkum v této oblasti může přinést neočekávané výsledky.

## 7. QUORUM SENSING A HOSTITELSKÝ ORGANISMUS

Bakteriální autoinduktory slouží primárně k modifikaci metabolismu bakteriální populace, která je produkuje. Jsou však v případě infekce tyto látky vnímány i hostitelským organismem?

Vzhledem k molekulové hmotnosti AHL je specifická imunita proti jednotlivým autoinduktorům poměrně nepravděpodobná. Přesto však bakteriální autoinduktory mohou být vnímány hostitelským organismem a mohou stimulovat nespecifické imunitní reakce. Purifikovaný dodecanoyl HSL vykázal imunomodulační účinky, naproti tomu hexanoyl HSL neměl na imunitní systém žádný vliv. Dodecanoyl HSL například snižoval produkci IL12, při vyšších dávkách snižoval i produkci protilátek. Při nižších dávkách naopak stimuloval produkci IgG1 a IgE (Telford aj., 1998).

Fray aj. (1999) vnesli gen pro produkci hexanoyl HSL (*yenI* z *Yersinia enterocolitica*) do chloroplastové DNA tabáku. Taková transgenická rostlina syntetizovala hexanoyl HSL a byla schopna komplementovat defekt v syntéze hexanoyl HSL mutantních kmenů *E. carotovora* anebo *Pseudomonas aureofaciens*. To vedlo u *E. carotovora* k navození plné virulence a v případě *P. aureofaciens* k produkci antibiotika phenazinu s protiplísňovou aktivitou (Fray aj., 1999).

Je tedy možné, že další výzkum povede k přípravě chemických analogů AHL a k jejich terapeutickému využití (Finch aj., 1998; Hardman aj., 1998). Modifikovaný autoinduktor by mohl být podáván terapeuticky s cílem pozastavit růst a množení patogenních organismů a zároveň aktivovat nespecifické imunitní odpovědi hostitele.

## 8. PERSPEKTIVY

Pravděpodobně jsme na počátku nových objevů v oblasti komunikace jednotlivých bakterií v rámci celé bakteriální populace a kontroly růstu bakteriálních populací. Doposud bylo jako jediný cíl růstu bakteriální populace chápáno maximální možné množení až do úplného vyčerpání živin z prostředí. V extrémním případě zůstává toto tvrzení v platnosti – při dlouhodobém skladování/hladovění v živném médiu nakonec bakteriální populace vyčerpá všechny alternativní zdroje organického uhlíku a akceptory elektronů pro respiraci a odumře. Avšak doba odumírání může být produkcí autoinduktorů aktivně prodlužována tím, že bakteriální populace začne utlumovat aktivně svůj metabolismus ještě před tím, než jsou všechny živiny vyčerpány. Takovéto chování dává bakteriální populaci větší šanci na dlouhodobé přežití a tak i na nalezení nové ekologické niky. Poznání těchto procesů v případě patogenních mikroorganismů umožní hledat nové způsoby využití mikrobiální kompetice v ochraně hostitelského

organismu před infekcí, umožní návrh nových atenuovaných vakcinačních kmenů, například s nadprodukcí autoinduktorů, případně povede k terapeutické aplikaci analogů autoinduktorů. To je důležité zejména v době, kdy u řady patogenních mikroorganismů narůstá rezistence na antibiotika a hledají se nové způsoby prevence a léčby infekčních onemocnění.

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Received: 00–03–23

Accepted: 00–04–06

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*Kontaktní adresa:*

RNDr. Ivan Rychlík, Výzkumný ústav veterinárního lékařství, Hudcova 70, 621 32 Brno, Česká republika  
Tel. +420 5 41 32 12 41, fax +420 5 41 21 12 29, e-mail: rychlik@vri.cz

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Vědecký časopis VETERINÁRNÍ MEDICÍNA ● Vydává Česká akademie zemědělských věd – Ústav zemědělských a potravinářských informací ● Redakce: Slezká 7, 120 56 Praha 2, tel.: +420 2 24 25 79 39, fax: +420 2 24 25 39 38, e-mail: edit@uzpi.cz ● Sazba a tisk: ÚZPI Praha ● © Ústav zemědělských a potravinářských informací, Praha 2000

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

Podávání novinových zásilek povoleno Českou poštou, s. p., Odštěpný závod Střední Čechy, č. j. NOV-6588/00-P/1 dne 9. 5. 2000