IMMUNO-REACTIVE PROTEINS OF *FRANCISELLA TULARENSIS*

Jana HAVLASOVÁ, Jiří STULÍK, Lenka HERNYCHOVÁ
Proteome Center for the Study of Intracellular Parasitism of Bacteria, Purkyně Military Medical Academy, Hradec Králové, Czech Republic

Francisella tularensis is the ethiological agent of tularemia, an infection of humans and other mammals. The laboratory diagnosis of human tularemia is based on serological tests. However, there is a lack of information about the *Francisella tularensis* antigens inducing antibody response to date. Except for the complex antigen preparations as sonicated whole bacteria [Viljanen 1983], only the *Francisella* lipopolysaccharide [Carlsson 1979] and the partially purified fraction containing outer membrane antigens [Bevanger 1988] have been described as immunogens suitable for diagnostic purposes. Proteome technology, based on two-dimensional (2D) gel electrophoresis followed by immunoblotting and identification of protein spots by peptide mass fingerprinting (PMF), is the current method of choice for analysis of antigenic repertoire in bacterial pathogens. In the case of *F. tularensis*, 2D-immunoblotting approach has the potential to reveal new *Francisella* specific markers suitable for diagnostic purposes as well as potentially protective antigens useful for the construction of subunit vaccine.

In our study, we have launched the systematic mapping of the immuno-reactive antigens of *F. tularensis* live vaccine strain LVS. First, the proteomic analysis of the antigens recognized by human serum antibodies produced in the course of natural infection has been started. Furthermore, the mapping of the specific antibody response in *F. tularensis* LVS challenged C3H/HeJ mice in comparison with their congenic C3H/HeN counterparts is now underway.

Human sera were collected from 44 patients with tularemia serologically confirmed by positive microaglutination test (titer ≥ 320). Control groups consisted of 20 healthy blood donors and 16 patients with Lyme borreliosis. None of the control sera had a positive microaglutination titer (≥ 20).

In the first step, specific anti-*F. tularensis* response was analyzed by the means of 1-D immunoblotting. Proteins of the *F. tularensis* LVS whole cell lysate were separated by 1-D SDS-PAGE in a separating 12% gel [Laemmli 1970] using Protean II xi Cell (BioRad), with a protein load 0.5 mg/gel. Immunoblotting was performed with human sera diluted 1:100. Peroxidase conjugated goat anti-human immunoglobulin (IgG, IgA, IgM) (Pierce) was used as secondary antibody with enhanced chemilumiscence based visualization (Boehringer Mannheim). Sera collected from tularemia patients revealed typical reactions in two dominant regions encompassing antigens with molecular weights from 60 to 120 kDa and from 8 to 18 kDa. With regard to the control groups, immunoreactive bands occured only rarely in blots and their staining intensity was mostly weak.

For a more detailed 2-D analysis, tularemic sera that altogether covered the whole spectrum of antigens recognized on 1-D immunoblots were selected. Further, control sera displaying some reactivity on 1-D immunoblots were tested. The immunoreactivity of several antigen preparations of *F. tularensis* was analyzed (Table 1). 100 μg of protein were resolved on 18 cm long IPG strips pH 3-10 or pH 6-11 (Amersham Pharmacia Biotech) with 9-16% SDS-PAGE in the second dimension. Immunodetection was performed as in the case of 1-D immunoblotting. For micropreparative 2-DE gels, 0.5 mg of protein was loaded on IPG strips. After in gel digestion [Lamer 2001], the mass spectra of Coomassie Blue R-250 stained proteins were recorded on MALDI Voyager-DE STR (Perseptive Biosystems) or LC-MS/MS Q-TOF Ultima™ API (Micromass) mass spectrometer. Proteins were identified by peptide mass fingerprinting (PMF) using ProFound and PeptIdent programs. In the case of proteins that were not found in the NCBI or SWISSPROT databases, we have exploited the nucleotide sequence database of *F. tularensis* Schu4, kindly provided by Karin Hjalmarsson, the Head of the Dept. of NBC-Analysis, FOI NBC-Defence (Umeĺ, Sweden), partially published [Prior 2001]. ProteinProspector program was used in the search for amino acid theoretical protein sequences listed in the ORF database. The search for proteins with sequence homology of other microbial species listed in NCBI database was done with BLAST program.

Our results document that besides the generally accepted production of anti-lipopolysaccharide antibodies, patients with tularemia mount highly diversified reactions against antigens of protein origin. More than 70 different antigens of the whole cell bacterial lysate resolved on pH 3-10 IPG strips have been immunorecognized. The bacterial stress

proteins chaperone protein dnaK, 10 kDa and 60 kDa chaperonins belonged to the immunodominant antigens (Table 2). In contrast, only a few of the basic constituents of whole cell lysate (pI 6-11) were immunoreactive. As expected, a lot of integral membrane proteins provided a specific strong reaction with patient sera. One of them, which reacted with all tested tularemic sera, has been identified as 17 kDa antigen denoted TUL4. TUL4 has been described as an immunogenic membrane lipoprotein and is declared to be *Francisella* genus specific antigen [Sjöstedt 1992]. In our study, a group of spots containing TUL4 was immunorecognized by serum from a patient with no history of tularemia but suffering from Lyme disease.

Table 1

The list of antigen preparations analyzed by 2-D immunoblotting using human sera
The number of sera that were selected for 2-D immunoblotting is indicated.
***Integral membrane proteins were prepared by carbonate extraction method [Molloy 2000].**

Bacterial strains	Antigen preparations	Number of tested sera		
		Tularemia	Lyme borreliosis	Blood donors
F. tularensis LVS (subsp. *holarctica*)	**Whole-cell proteins** pI 3-10	9	3	2
	Whole-cell proteins pI 6-11	2	1	1
	Integral membrane proteins* pI 3-10	3	1	1
F. tularensis Schu4 (subsp. *tularensis)* wild clinical isolate	**Whole-cell proteins** pI 3-10	2	1	1

Table 2

The list of identified immunoreactive proteins of F. tularensis LVS - proteins included in NCBI and SWISSPROT databases
Proteins of the *F. tularensis* LVS whole cell lysate were identified using MALDI-TOF MS; LC-MS/MS was applied in the case of integral membrane proteins. The maximum staining intensities of the immunoreactive spot in individual groups of patients are included. The staining intensity was evaluated visually and classified from - to +++.

						2-D immunoblotting reactivity		
Spot No.	Protein Name	Accession No.	*Mr*[kDa]/p*I* Theoretical	*Mr*[kDa]/p*I* Measured	Sequence Coverage	Tularemia	Borreliosis	Controls
Whole-cell antigen *F. tularensis* LVS (IEF pH 3-10)								
579	Chaperone protein dnaK	P48205	69.0/4.9	66.0/4.6	13%	+++	+/-	+
862	60 kDa chaperonin	P94798	57.0/5.0	58.0/4.5	30%	+++	+	-
899	60 kDa chaperonin	P94798	57.0/5.0	58.5/4.6	11%	+++	-	-
904	60 kDa chaperonin	P94798	57.0/5.0	58.5/4.7	30%	++	-	-
2198	Hypothetical 23 kDa protein	CAA70085	22.0/5.7	25.0/5.6	40%	+/-	+	+/-
2725	10 kDa chaperonin	P94797	10.0/5.5	17.0/5.4	32%	++	-	-
2793	10 kDa chaperonin	P94797	10.0/5.5	17.2/5.1	20%	+++	-	-
2797	10 kDa chaperonin	P94797	10.0/5.5	16.6/5.4	38%	+++	-	-
2922	10 kDa chaperonin	P94797	10.0/5.5	16.1/5.4	20%	++	-	-
Integral membrane proteins *F. tularensis* LVS (IEF pH 3-10)								
1351	TUL4, FRATU 17 kDa	P18149	16.0/9.2	19.0/4.7	56%	+++	+	-
223	60 kDa chaperonin	P94798	57.0/5.0	57.0/5.0	14%	+++	-	-

Table 3

The list of identified immunoreactive proteins of *F. tularensis* LVS - protein homologues according to BLAST Proteins were identified using MALDI-TOF MS. The maximum staining intensities of the immunoreactive spot in individual groups of patients are included. The staining intensity of the immunoreactive spot was evaluated visually and classified from - to +++.

				Protein homologues according to BLAST			2-D immunoblotting reactivity		
Spot No.	*Mr*[kDa]/p*I* Theoretical	*Mr*[kDa]/p*I* Measured	Sequence Coverage	***Protein Name*** ***Bacteria***	***Accession No.***	E Value	Tularemia	Borreliosis	Controls
Whole-cell antigen *F. tularensis* LVS (IEF pH 3-10)									
1145	49.5/4.7	49.5/4.5	20%	***Enolase*** **Borrelia burgdorferi**	***O51312***	1e-153	-	+++	-
1232	41.8/5.0	43.7/4.6	30%	***Elongation factor TU*** **Salmonela typhimurium**	***P21694***	1e-168	+++	+++	-
1479	39.6/5.7	38.9/5.8	33%	***Glycine-cleavage system protein T1*** **Pseudomonas aeruginosa**	***G82994***	2e-97	+++	-	-
2139	25.7/7.7	25.2/5.5	23%	***Hypothetical protein*** **Plasmodium falciparum**	***CAB38995***	0.12	+	-	-
2150	26.0/6.1	25.5/6.2	22%	***Oxidoreductase*** **Vibrio cholerae**	***G82383***	2e-84	+	-	-
2531	16.5/5.0	19.5/4.8	42%	***Biotin carboxyl carrier protein*** **Pseudomonas aeruginosa**	***P37799***	6e-27	+++	+/-	-
2575	16.1/5.6	19.2/5.6	45%	***50S ribosomal protein L9*** *Pseudomonas aeruginosa*	***F83029***	2e-30	-	+	+
2681	18.5/5.3	17.7/5.6	40%	***Probable bacterioferritin*** **Pseudomonas aeruginosa**	***B83036***	3e-4	+++	+/-	-
2828	16.3/6.1	17.0/6.0	42%	***3-dehydroquinase*** *Buchnera sp.*	***P57479***	5e-30	++	-	-
2835	12.8/4.6	16.9/4.6	20%	***50S ribosomal protein*** *Campylobacter jejuni*	***H81392***	3e-25	+++	++	+++
Whole-cell antigen *F. tularensis* LVS (IEF pH 6-11)									
354	9.5/9.8	15.0/9.2	35%	***Histone-like protein HU form B*** **Pseudomonas aeruginosa**	AF345628	3e-27	++	+/-	-

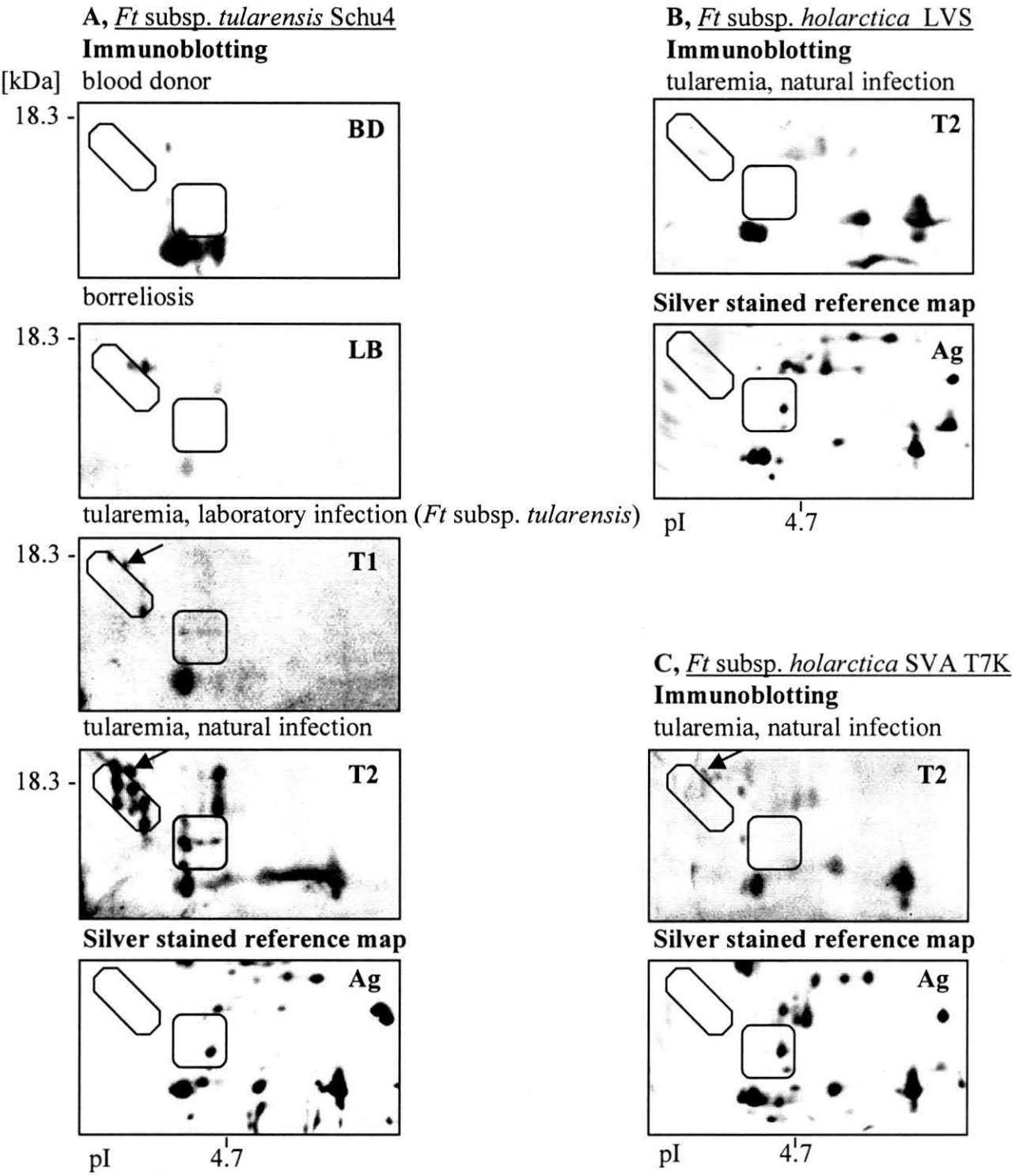
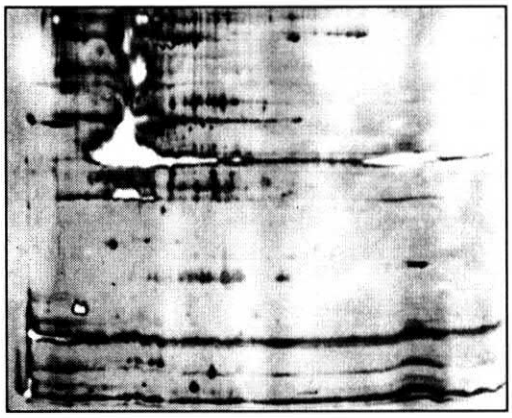


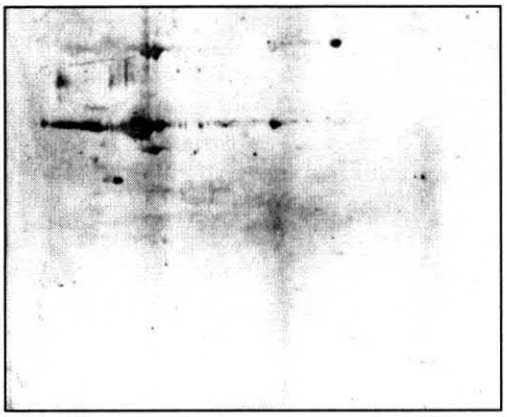
Figure 1. Low molecular weight region on F. tularensis Schu4 immunoblots
F. tularensis subspec. tularensis Schu4 strain, isolated from human ulcer, was used as a source of antigen (A). The whole cell bacterial lysate resolved on IPG 3-10 and 9-16% SDS-PAGE was subjected to immunoblotting with healthy blood donor serum (BD), Lyme disease serum (LB), tularemic serum obtained from a technician accidentally infected with F. tularensis subspec. tularensis strain (T1), and tularemic serum from patient with natural infection (T2). Silver stained 2-DE reference map (Ag) is shown below the immunoblots. For comparison, the reactivity of the same serum from tularemia patient (T2) with proteins of two F. tularensis strains belonging to subspec. holarctica is shown (B, C). First, attenuated live vaccive strain LVS (B) and second, SVA T7K (C) strain, which was isolated from hare and then passaged in animals, were used. The area with F. tularensis Schu4 strain specific immunoreactive antigens is circled. The arrows denote the same immunostained spot that was found on several 2-D immunoblots.

A, C3H/HeN
control serum

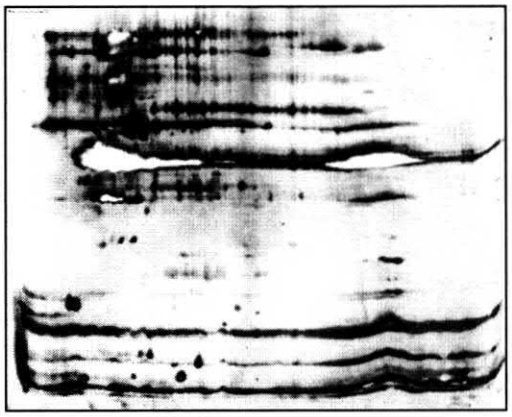
A, C3H/HeN
immune serum, day 28



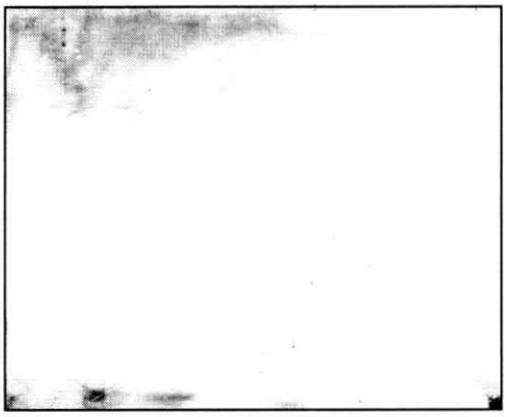
B, C3H/HeJ
control serum



B, C3H/HeJ
immune serum, day 28



C,
healthy blood donor



C,
patient with tularemia

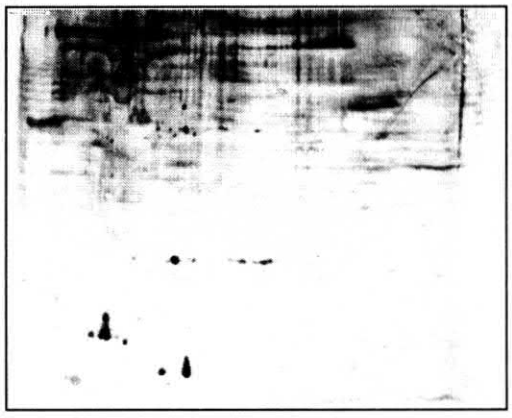


Figure 2. The representative 2-D immunoblotting pattern of specific antibody response to F. tularensis infection
The immune sera were collected from C3H/HeN (Lpsn) mice (A) and C3H/HeJ (Lpsd) mice (B), 28 days after the F. tularensis LVS challenge. Non-immune control sera were obtained from mice treated only with saline. For comparison, the immunoreactivity pattern found in human serum obtained from a patient with natural infection as well as the minor immunoreactivity in the serum from healthy blood donor (C) are shown. The whole cell bacterial lysate of F. tularensis LVS separated on pH 3-10 IPG strips was used for 2-D immunoblotting. All the sera were diluted 1:100.

In total, 22 immunogenic spots were identified by PMF. 11 of them provided specific reactions only with sera from tularemia patients in our study, therefore represent promising candidates for *Francisella* specific markers (Table 2 and 3). An example of both the specific as well as immunogenic antigen is 10 kDa chaperonin (Table 2). Immune reactions with at least one charge or mass variant of the 10 kDa chaperonin were found in 4/9 patients with tularemia and no reaction was disclosed in control sera.

Recently, the antigenic repertoire of *F. tularensis* subsp. *tularensis* (strain Schu4) has been analyzed with a few first sera. According to the preliminary results, the whole cell lysate of this highly virulent strain possesses strain specific immunogenic components localized in the acidic, low molecular mass region on 2-DE map (Fig. 1). These antigens have not been identified yet, because we were not able to stain them neither by Coomassie blue R-250 nor colloidal Coomassie G-250 nor by silver staining. Work is currently underway to concentrate these antigens on narrow pH gradient IPG strips.

To assess the contribution of genetic background to the pattern of specific antibody response in mice, normal lipopolysacccharide–sensitive (Lps^n) C3H/HeN mice are nowadays studied in comparison with their congenic (Lps^d) C3H/HeJ counterparts. The LPS hyporesponsiveness of the C3H/HeJ mouse strain is caused by a point mutation in Toll–like receptor (TLR4) gene [Poltorak 1998]. As a consequence, C3H/HeJ mice are extremely susceptible to infection with Gram-negative bacteria [O'Brien 1980], including *F. tularensis*.

In our study, mice were inoculated s.c. with the dose of 100 *F. tularensis* LVS microbes. Immune mouse sera were collected at different time intervals after LVS challenge (days 7, 10, 14, 21, 28) to analyze the progress of antibody response in the course of infection. Non-immune control sera were obtained from mice treated with saline. The whole cell bacterial lysate of *F. tularensis* LVS was used as the source of antigens. 100 μg of protein were separated on pH 3-10 IPG strips. Mouse sera were diluted 1:100 for immunoblotting. Peroxidase conjugated goat anti-mouse immunoglobulin, which reacts with immunoglobulin G, IgA and IgM together, (DAKO) was used as secondary antibody.

Fig. 2 shows the 2-D immunoblotting pattern of specific antibody response found in immune mouse sera 28 days after the *F. tularensis* LVS challenge. As expected, both mouse strains mounted strong antibody response to *Francisella* lipopolysaccharide as well as to the antigens of protein origin. The characteristic ladder-like reactivity with lipopolysaccharide, especially in the low molecular mass region, is more expressed in immune mouse sera compared with human tularemia (Fig. 2). The preliminary results also indicate that the antibody response of the more susceptible LPS-defective C3H/HeJ mice covers the wider spectrum of protein antigens in comparison with C3H/HeN (Lps^n) mice. The reaction to LPS is more expressed in C3H/HeJ mice as well. The process of identification of immunogenic proteins is ongoing and the 2--DE database of LVS proteins reactive with murine antibodies is being formed.

Acknowledgement: The presented study was fully supported by Ministry of Education, Youth and Sport, grant No. LN00A033.

References

BEVANGER, L. – MAFLAND, KA. – NAESS, AI. *J. Clin. Microbiol.*, 1988, vol. 26, p. 433–437.
CARLSSOn, HE., et al. *J. Clin. Microbiol.*, 1979, vol. 10, p. 615–621.
LAEMMLI, UK. *Nature*, 1970, vol. 227, p. 680–685.
LAMER, S. – JUNGBLUT, PR., *J. Chromatogr.*, 2001, vol. 52, p. 311–322.
MOLLOY, MP., et al. *Eur. J. Biochem.*, 2000, vol. 267, p. 871–2881.
POLTORAK, A., et al. *Science*, 1998, vol. 282, p. 2085–2088.
O'BRIEN, AD., et al. *J. Immunol.*, 1980, vol. 124, p. 20–24.
PRIOR, RG., et al. *J. Appl. Microbiol.*, 2001, vol. 91, p. 614–620.
SJÖSTEDT, A., et al. *Microb. Pathog.*, 1992, vol. 13, p. 243–249.
VILJANEN, MK. – NURMI, T. – SALMINEN, A. *J. Infect. Dis.*, 1983, vol. 148, p. 715–720.

Correspondence: Jana Havlasová
Proteome Center for the Study of Intracellular Parasitism of Bacteria
Purkyně Military Medical Academy
Třebešská 1575
500 01 Hradec Králové
Czech Republic

Received 16. 9. 2002