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Proteomic Analysis of Antibody Response in a Case of Laboratory-Acquired Infection with *Francisella tularensis* subsp. *tularensis*

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ABSTRACT. Immunoproteomic analysis was applied to study the immunoreactivity of serum samples collected at different time points from a laboratory assistant accidentally infected with highly virulent strain of *Francisella tularensis* subsp. *tularensis*. Immunoblotting showed that the spectrum of *F. tularensis* antigens recognized specifically by immune sera remained with the exception for 1 antigen stable for up to 16 years after infection. Using immunoproteomics approach 10 immunoreactive antigens were successfully identified. Several new immunogenic *F. tularensis* proteins were described for the first time.

Abbreviations

F.t.	*Francisella tularensis*	2-DE	2-dimensional electrophoresis
F.t.t.	*Francisella tularensis* subsp. *tularensis*	LVS	live vaccine strain
F.t.h.	*Francisella tularensis* subsp. *holarctica*	MS	mass spectrometry

Francisella tularensis is a Gram-negative, facultative intracellular bacterial pathogen causing disease in humans and other mammals. The species include two predominant subspecies, *viz. F.t.t.* (type A) which is prevalent in North America, and *F.t.h.* (type B) occurring mainly in North America and in Eurasia. However, only strains of *F.t.t.* are considered to be the most virulent for humans, with an infectious dose as low as 10 organisms. Because of its extreme infectivity and its ability of dissemination as aerosol, *F.t.* has been classified as a dangerous potential biological weapon (Dennis *et al.* 2001).

Vaccine protection against tularemia infection has been achieved after immunization with *F.t.* LVS which is derived from *F.t.h.* strain and is attenuated in humans (Eigelsbach *et al.* 1961). However, LVS vaccination has been used to immunize only a risk group such as technical personnel and is not licensed for human use. Moreover, incomplete protection against laboratory-acquired infection is afforded with this type of vaccine (Burke *et al.* 1977). Thus, the development of a new generation of *F.t.* vaccines is needed. One of the approaches to generate a safe vaccine would be to identify protective immunogens of *F.t.* and utilize them for the construction of a subunit vaccine (Oyston *et al.* 2004). The effective vaccines should contain such antigens that are able to induce protective immune response against the cell surface and secreted components (Vytvytska *et al.* 2002).

So far immunoreactive proteins of *F.t.* LVS have solely been reported (Havlasová *et al.* 2002, 2005). To identify immunogenic proteins of the highly virulent strain of *F.t.t.* is difficult. This type of analysis is usually hampered by the lack of accessibility of immune sera from patients infected with type A strains due to their exclusive occurrence in North America, on the one hand, and the problems with getting the isolates from patients and their subtyping, on the other hand. So, in the present study we utilized 3 serum samples from the laboratory assistant who was accidentally infected with the virulent strain of *F.t.t.* SCHU S4 originally isolated from a human case of tularemia (Eigelsbach *et al.* 1951). The infection was acquired by the contamination of an eye and resulted in oculoglandular tularemia. To identify *F.t.t.* immunoreactive proteins we exploited the immunoproteomics approach based on the combination of 2-DE, immunoblotting and the MS. The serum samples collected 2, 5 and 16 years after infection were utilized for the analysis and the membrane proteins enriched fraction of *F.t.t.* was used as the source of antigens.

MATERIALS AND METHODS

Clinical data. The laboratory assistant was accidentally infected with the virulent strain of *F.t.t.* in May, 1989. The patient was treated with rifampine for 2 weeks. The drug treatment was discontinued for 1 week and then the patient was treated again with rifampine for another week. Results from serological tests showed a low titer of antibodies against *F.t*. Four months after infection the patient had a high fever (40 °C) that disappeared in a few days after treatment with doxycycline. However, the cause of the high fever was not determined. Seventeen months after acquired infection, the patient was admitted to the hospital with a history of persistent dyspepsia and a higher titer of antibodies against *F.t.* (250; January 1990). Dyspeptic symptoms improved after antacid treatment and a diet. The serological tests revealed decreasing titer (160) in comparison with previous data (titer 250).

Serum samples were obtained 2, 5 and 16 years after the tularemia infection and a positive titer of *Francisella* antibodies was confirmed by a microagglutination test. The informed consent of patient was obtained.

Bacterial strain and antigen preparation. F.t.t. was obtained from the *Collection of Animal Pathogenic Microorganisms* and prepared in the *Veterinary Research Institute* (Brno, Czechia). The strain was cultured on McLeod agar (36.6 °C, 1 d). The colonies were harvested into cold PBS, centrifuged and the pellets were resuspended in 50 mmol/L Tris-HCl (pH 8). The bacterial cells were subjected to several cycles of freeze-thawing in liquid nitrogen and undisrupted bacteria were removed by centrifugation. The membrane proteins enriched fraction was prepared using carbonate extraction (pH 11) followed by ultracentrifugation (115 000 ***g***, 1 h, 4 °C) in a *Beckman* Optima MAX ultracentrifuge (USA) (Molloy *et al.* 2000).

Two-dimensional electrophoresis. The pellet was solubilized in 1.5 mL rehydration buffer containing 7 mol/L urea, 2 mol/L thiourea, 1 % ASB-14, 1 % Triton X-100, 40 mmol/L Tris, 2 mmol/L tributylphosphine, 1 % Pharmalyte pH 3–10 and 0.5 % Pharmalyte pH 8–10.5. The sample was loaded onto commercial strips with nonlinear immobilized pH 3–10 gradient. Isoelectric focusing was performed using Multiphor II (*Amersham Biosciences*, Sweden). In the 2nd dimension, gradient 9–16 % SDS-PAGE was used.

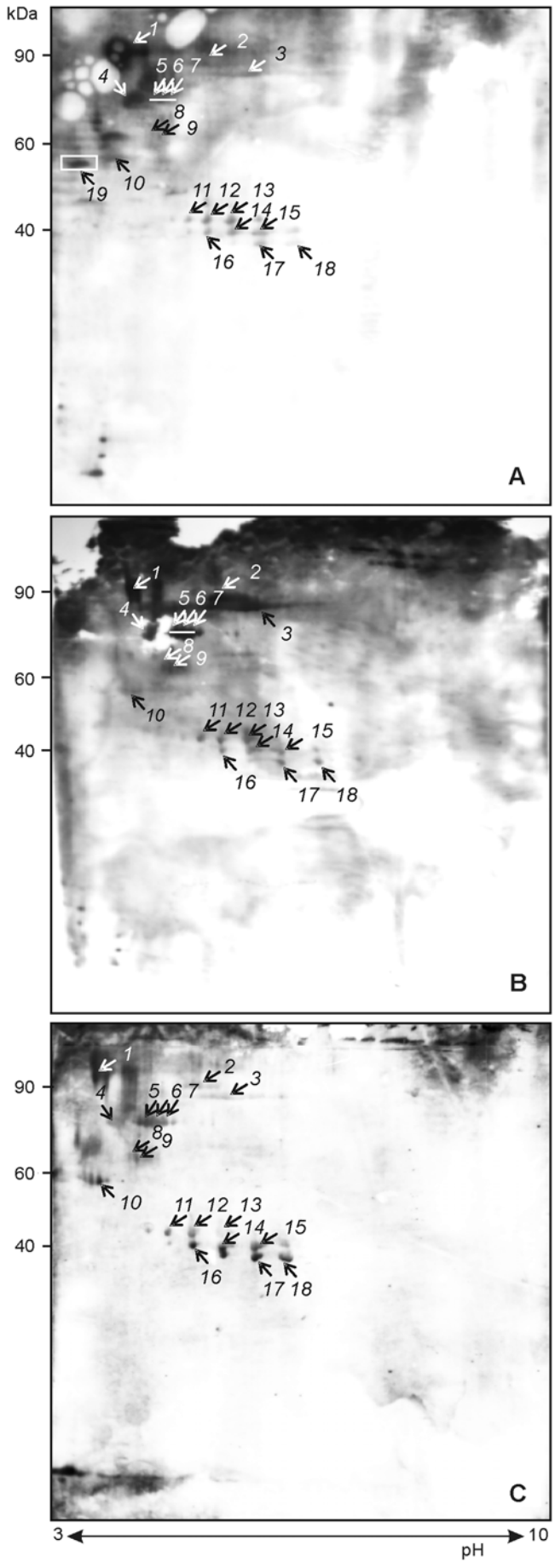
Immunoblotting. The separated proteins were electroblotted onto polyvinylidene difluoride membranes, which were then probed with tularemic sera, diluted 1 : 100 in the blocking buffer (4 % non-fat milk, 0.1 % Tween in TBS). After the incubation (overnight at 4 °C), the membranes were washed and peroxidase-conjugated goat antibody directed against human IgM, IgG, IgA (*Pierce*, USA) was used for probing. The incubation was for 45 min at room temperature. After washing, the immunostained spots were visualized with a chemiluminescence detection kit (*Boehringer Mannheim*, Germany). Immunoblotting experiments were performed 2× for each serum sample.

Mass spectrometry. The immunoreactive spots were identified by the MS. 2-DE gel was stained by Coomasie G-250 and selected spots were excised. The in-gel tryptic digestion and protein identification using either MALDI-TOF mass spectrometer Voyager-DE STR (*PerSeptive Biosystems*, USA) or LC-nanoESI-MS/MS system (CapLC Q-TOF Ultima^{TM} API; *Waters*, UK) were done according to Pávková *et al.* (2005).

RESULTS AND DISCUSSION

The results from immunoblot experiments showed that all serum samples displayed almost no variation in spot immunoreactivity, thus *Francisella*-specific antibodies were found positive even 16 years after infection. Such persistence of *Francisella* antibodies is desirable since effective vaccines could provide long-term solutions to many infectious diseases (Bumann *et al.* 2004). The immunoreactivity profile of tularemic sera is shown in Fig. 1. One protein spot for hypothetical protein FTT0918 was recognized solely by the patient serum collected 2 years after laboratory-acquired infection (Fig. 1A).

Application of the MS analysis resulted in successful identification of 19 immunogenic protein spots, corresponding to 10 different proteins, as some of them occurred in multiple charge variants. Identified immunoreactive protein spots of *F.t.t.* are listed in Table I and their position is indicated on 2-D immunoblots in Fig. 1A–C. Seven proteins, *viz*. the hypothetical membrane protein FTT1676, aconitate hydratase, intracellular growth locus, subunit B (IglB), succinyl-CoA synthetase β chain, hypothetical protein FTT0918 and two components of pyruvate degydrogenase, were described as *F.t.* immunorelevant proteins for the first time. Dihydrolipoamide succinyltransferase (another identified immunoreactive protein) has recently been reported to be recognized by antisera from mice immunized with *F.t.* LVS (Twine *et al.* 2006). The remaining two proteins, peroxidase and DnaK, were found to be immunogenic in the whole cell lysate of *F.t.* LVS (Havlasová *et al.* 2005). As negative control, a serum sample from a healthy blood donor was examined. The results revealed a weak cross-reaction with chaperone protein DnaK and IglB (*data not shown*).



Six identified immunodominant protein spots (5 protein spots differing in charge and molar mass for hypothetical membrane protein FTT1676 and 1 spot for pyruvate dehydrogenase E2) (Fig. 1, Table I), were previously found to be uniquely expressed in highly virulent strain of *F.t.t.* compared to less virulent *F.t.h.* strains (Pávková *et al.* 2006). A vaccine for tularemia should be effective particularly against highly virulent *F.t.t.* strains, thus proteins specific for these strains might be suitable candidates for further testing.

Protein FTT1676 recognized by all tularemic sera was characterized as lipoprotein using LipoP server which is accessible at **`http://www.cbs.dtu.dk/services/LipoP/`**. This algorithm is able to distinguish between lipoproteins, secreted, cytoplasmic and transmembrane proteins (Juncker *et al.* 2003). Lipoproteins and other surface-exposed proteins from pathogenic bacteria are attractive candidates for vaccine development as well as for the generation of novel diagnostic tests. Furthermore, the E2 component of pyruvate dehydrogenase (occurring in mass variant; Table I) is associated with intracellular growth and virulence in another intracytosolic pathogen *Listeria monocytogenes* (O'Riordan *et al.* 2003). An additional antigenic protein, IglB, has some homology to a *Rhizobium leguminosarum* protein that is thought to be needed for protein secretion (Nano *et al.* 2004). The gene encoding IglB is part of the *Francisella* pathogenicity island (Nano *et al.* 2004) and its mutation results in reduced intramacrophage growth (Gray *et al.* 2002).

Fig. 1. 2-D immunoblotting pattern of *F. tularensis* immunoreactive antigens. The membrane proteins enriched fraction of *F. tularensis* subsp. *tularensis* was probed with serum samples from the laboratory assistant accidentally infected with highly virulent type A strain. Serum samples were obtained 2 (**A**), 5 (**B**) and 16 years (**C**) after infection. A protein spot recognized only with serum collected 2 years after laboratory-acquired infection is boxed. All identified immunogenic protein spots are designated by numbers that refer to Table I.

Table I. Identified immunoreactive proteins of *F. tularensis* subsp. *tularensis*

[a]	Spot no.[b]	Protein[c]	Accession no.[d]	Theoretical		Measured	
				molar mass, kDa	p*I*	molar mass, kDa	p*I*
**	*1*	pyruvate dehydrogenase[e]	YP 170419	67.25	4.74	91.53	4.81
*	*2*	aconitate hydratase	YP 169161	102.61	5.44	87.98	5.31
*	*3*	pyruvate dehydrogenase[f]	YP 170420	100.23	5.56	80.29	5.51
	4	chaperone protein dnaK	YP 170225	69.25	4.86	78.03	4.86
	5	peroxidase/catalase	YP 169735	83.50	5.32	73.65	5.11
	6	*ditto*	*ditto*	*ditto*	*ditto*	73.00	5.15
	7	*ditto*	*ditto*	*ditto*	*ditto*	73.16	5.22
	8	dihydrolipoamide succinyltransferase[g]	YP 169152	52.75	5.09	62.63	4.96
	9	*ditto*	*ditto*	*ditto*	*ditto*	61.94	5.00
*	*10*	intracellular growth locus[h]	YP 170618	58.87	4.66	54.11	4.68
*	*11*	succinyl-CoA synthetase[i]	YP 169539	41.54	5.17	43.61	5.18
*	*12*	HMP FTT1676	YP 170582	37.47	6.31	43.90	5.29
*	*13*	*ditto*	*ditto*	*ditto*	*ditto*	43.70	5.36
**	*14*	*ditto*	*ditto*	*ditto*	*ditto*	40.45	5.24
**	*15*	*ditto*	*ditto*	*ditto*	*ditto*	40.32	5.38
**	*16*	*ditto*	*ditto*	*ditto*	*ditto*	39.99	5.60
**	*17*	*ditto*	*ditto*	*ditto*	*ditto*	38.00	5.61
**	*18*	*ditto*	*ditto*	*ditto*	*ditto*	37.93	5.83
*	*19*	hypothetical protein FTT0918	YP 169915	58.75	4.68	56.11	4.47

[a]*Novel immunogens of *F. tularensis*; **new *Francisella* antigens, specific for subsp. *tularensis* (Pávková *et al*. 2006) as well.
[b]The numbers of spots indicated on 2-D immunoblot in Fig. 1.
[c]HMP – hypothetical membrane protein.
[d]Accession numbers according to NCBI nr database.
[e]E2 component.
[f]E1 component.
[g]Component of 2-oxoglutarate dehydrogenase complex.
[h]Subunit B.
[i]Subunit β.

The hypothetical protein FTT0918 (other identified immunogenic protein) has no homology to other protein. FTT0918 is absent in LVS (Pávková *et al.* 2006) and in the less virulent spontaneous mutant strain of *F.t.t.* (Twine *et al.* 2005). Moreover, the FTT0918-deficient mutant showed reduced virulence in mice, thus FTT0918 contributes to the virulence of the highly virulent strain of *F.t.t.* SCHU S4 (Twine *et al.* 2005).

We demonstrated here a long-term antibody response to accidentally acquired infection with highly virulent strain of *F.t.t.* Moreover, by an immunoproteomics approach, a number of *F.t.t.* immunoreactive proteins have been identified. Among them, several new antigenic *Francisella* proteins were reported. Some of the immunogenic protein spots were specifically recognized only by type A immune sera. The specificity of this reaction is verified by the lack of these antigens on 2-DE protein patterns of membrane proteins extracted from less virulent type B, *F.t.h.* strains (Pávková *et al.* 2006).

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