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## REVIEW ARTICLE

# *FRANCISELLA TULARENSIS* - 100 YEARS: TULAREMIA RESEARCH IN FORMER CZECHOSLOVAKIA AND IN THE CZECH REPUBLIC

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**Summary**

The history of national tularemia research started in 1936 when the first outbreak was recognized in south-east Moravia. Since then in average about one hundred cases have been recorded annually. As tularemia was endemic in former Czechoslovakia, three research groups which concentrated on this disease were formed during decades. The first two groups have worked from sixties and were associated with Jiri Libich (Prague) and Darina Gurycova (Bratislava). The third group which concentrated on the research of natural foci started during late seventies in Valtice (Zdenek Hubalek). The experimental research was, and still is, mainly associated with military research, recently with the Proteomic Center (Faculty of Military Health Sciences, University of Defence) in Hradec Kralove. This center opens molecular approaches to the analysis of *Francisella tularensis* microbes on one side and the studies on mutual host-pathogen interaction on the other side. One of the significant aims of the research is searching for the new typing and diagnostic markers of *Francisella tularensis* for the military and medical practice. Thus, scientists from former Czechoslovakia and the Czech Republic contributed significantly to current knowledge on *Francisella* pathogenesis and their results were highly appraised by international scientific community.

**The authors would like to dedicate this review to Jiri Libich, M.D., a leading researcher on tularemia in former Czechoslovakia.**

*Key words: Francisella tularensis; tularemia; former Czechoslovakia; Czech Republic*

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**ABBREVIATIONS**

Δ, deletion;
AAA+, ATPases Associated with diverse cellular Activities;
ATP, adenosine-5´-triphosphate;
dsbA, disulfide bonded protein A;

clpB, caseinolytic peptidase B;
*F. tularensis*, *Francisella tularensis*;
*F. tularensis* strain 15, *Francisella tularensis* subsp. *holarctica* strain 15;
hsp, heat shock protein;
igl, intracellular growth locus;
LVS, live vaccine strain;
MAPK, Mitogen-Activated Protein kinase;
MHC, major histocompatibility complex;
NK, natural killer;
subsp., subspecies

## INTRODUCTION

### Background on *Francisella tularensis*

*Francisella tularensis* (*F. tularensis*) - a highly virulent, nonsporulating, pleomorphic, facultative intracellular, Gram-negative coccobacillus is capable of causing a zoonotic disease called tularemia in a large number of mammals. The first description of this disease was probably made on September 19, 1907 by Ancil Martin, an ophthalmologist of Phoenix, the territory of Arizona, in his letter addressed to F. G. Novy, a professor of bacteriology at the University of Michigan. Martin stated that under observation and treatment he had five cases of an infection caused by the skinning and dressing of wild jack rabbits (reviewed by [1]). The first published description of the disease may be found in the article by R. A. Pearse who reported six atypical cases of fever caused by a deer-fly bite. He called this disease a "deer-fly fever" [2]. However, it is generally accepted that the history of tularemia started in 1911 when this illness was discovered in ground squirrels in Tulare County, California by G. W. McCoy from the US Plague Laboratory in San Francisco [3]. Subsequently, the bacillus, identified as the causative agent of tularemia, was isolated and named *Bacterium tularense* [4]. The first human case of tularemia, which was confirmed bacteriologically, was reported by Wherry and Lamb when they isolated the bacteria from a conjunctival ulcer [5]. The man who contributed most to our knowledge of tularemia as a separate clinical entity was Edward Francis. Francis studied the "deer-fly fever" in Utah and recognized its identical traits with the illness from Tulare County and named the infection tularemia [6]. At the same time, the attention was attracted to the acute febrile disease transmitted to man by wild rabbits of the Abukuma Mountains in the eastern part of Fukushima prefecture in Japan. The illness was known as “yato-byo” (the hare illness) or, according to the discoverer, as Ohara´s illness [7]. Edward Francis and Dunlop Moore finally concluded that Ohara's disease and tularemia were identical on the basis of the exchanged clinical sample analysis in 1926 [8]. *F. tularensis* was recognized as the causative agent of “water rat-trappers’ disease”, an illness acquired by trappers who skinned water-rats for their pelts [9, 10]. Soon after that, tularemia was also reported in Norway (1929), Canada (1930), Sweden (1931) and Austria (1935) [11]. Several other cases of tularemia were then reported from more than 15 countries in North America, Asia, and Europe during the third decade of the twentieth century.

### Taxonomy of *Francisella tularensis*

Tularemia is a zoonotic disease appearing in the entire north hemisphere. *F. tularensis*, as the etiological agent of, tularemia comprised of four subspecies: *tularensis* (Type A), *holarctica* (Type B), *mediasiatica*, and *novicida*. Genotyping methods have demonstrated that both subspecies, Type A and Type B, can be further divided into subpopulations. The subspecies differ in their pathogenicity and in their geographic distribution. *F. tularensis* subsp. *tularensis* Type A (in North America), is further divided into distinct Type A1 (east) and Type A2 (west), resp., differs according to clinical severity [12, 13], *F. tularensis* subsp. *holarctica* Type B (in North America, Europe and Asia), and *F. tularensis* subsp. *mediasiatica* (dominating in Central Asia). The taxonomy of *F. novicida* is still a matter of a debate. An attempt of Huber et al. [14] to classify *F. novicida* as the fourth subspecies of *F. tularensis* was refused by others [15].

### Tularemia outbreaks in former Czechoslovakia and in the Czech Republic

The first outbreak of tularemia in former Czechoslovakia was recognized in the area of south-east Moravia in 1936-1937. More than 400 cases were diagnosed during this outbreak. All affected people were in contact with hares [16]. After the Second World War, the outbreaks were mainly associated with the campaign in sugar factories. From that time, it was recognized that tularemia is endemic in the territory of Moravia as well as Bohemia. The number of reported cases is about 100 per year with some exceptions (Figure 1). The majority of cases usually appears during a hunting season. The outbreak of water-born tularemia was also registered in the area near the town of Plzen during the onset of the new millennium [17]

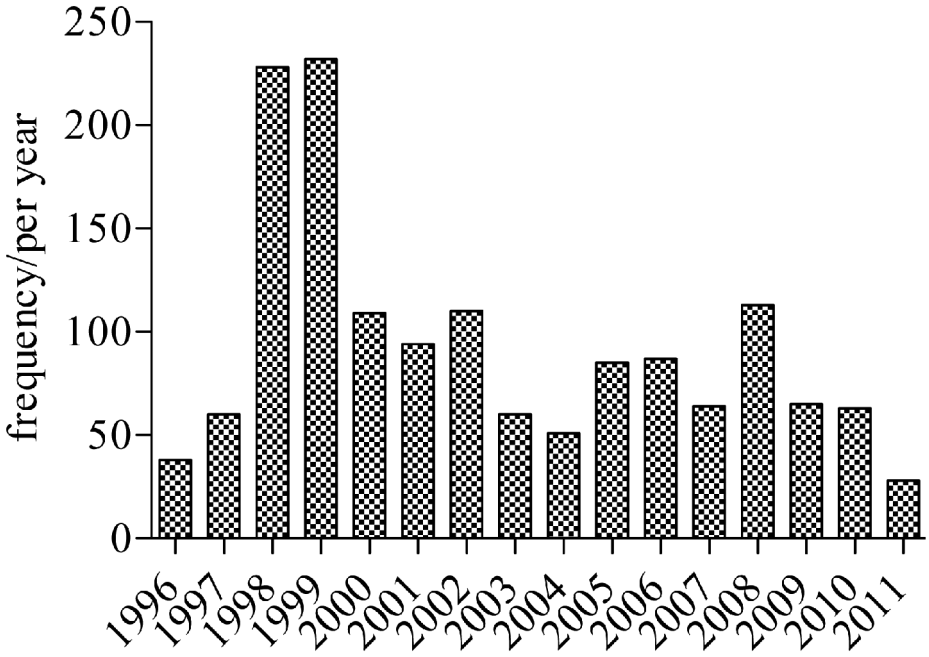


**Figure 1.** The amount of reported cases of tularemia in Czech Republic during the last 16 years.

The existence of tularemia outbreaks became the basis of the intensive research of this disease in the Czech Republic. Altogether three independent research groups started to study this relatively new illness in former Czechoslovakia. One group came from the Medical Faculty of Comenius University in Bratislava. This group is associated with the name of Darina Guricova. She published the first isolation of *F. tularensis* subsp. *tularensis* Type A in Europe, which was a unique report about the presence of Type A in Europe [18]. Second group was established at the branch of the Institute of Landscape Ecology of the Academy of Sciences in the town Valtice. The studies of this group were concentrated on the analyses of vectors in active enzootic foci (floodplain meadow and forest ecosystem) located mainly in South Moravia [19-21]. The third group started their research at the Military Institute of Hygiene, Epidemiology and Microbiology in Prague during the late fifties. This group was engaged solely in the experimental research, which was motivated by the political situation. The dominant person of this group was Jiri Libich, M.D., the head of the Bacteriological Division of the Military Institute of Hygiene, Epidemiology and Microbiology in Techonin. A transformed form of this research group has been working successfully till now and can be currently found at the Faculty of Military Health Sciences, University of Defence, in Hradec Kralove.

**Overview of the former Czechoslovak and Czech tularemia research**

Initially it was important to prepare the culture media for the cultivation of *Francisellae* [22] and to develop a treatment strategy for tularemia based on existing antibiotics [23, 24]. Simultaneously, an experimental model of inhalational tularemia was constructed for the study of pathogenesis and virulence of individual subspecies of *F. tularensis* based on several animal species. The laboratory experiments were enabled by the development of the aerosol technology and exposure procedure named Single Dose Exposure 400 (SINEX 400 – for scheme see Figure 2). A great advantage of this technique was the ability to accurately calculate an inhalation and a deposition dose of microbes in lungs of experimental animals. The calculation was based on several aerosol parameters selected for the experiment (e.g. concentration of dispersed particles, stability of the aerosol, bacterial population density in particles, parameters of the aerosol chamber such as volume of the chamber, air flow in the chamber, relative humidity, temperature or the parameters of an experimental animal used for the experiments). A mathematical model of inhalational tularemia was developed based on theoretical and experimental data, and consequently was confirmed experimentally using

the SINEX 400 technique [25-27]. It was demonstrated that a number of granulomas is strictly dependent on a number of deposited microbes in lungs of infected animals, on a kinetics of microbe dissemination dependent on the infection dose after aerosol challenge, and on generation time of both an attenuated *F. tularensis* subsp. *holarctica* strain 15 (*F. tularensis* strain 15) and a fully virulent *F. tularensis* subsp. *holarctica* strain 130 in murine peritoneal macrophages.

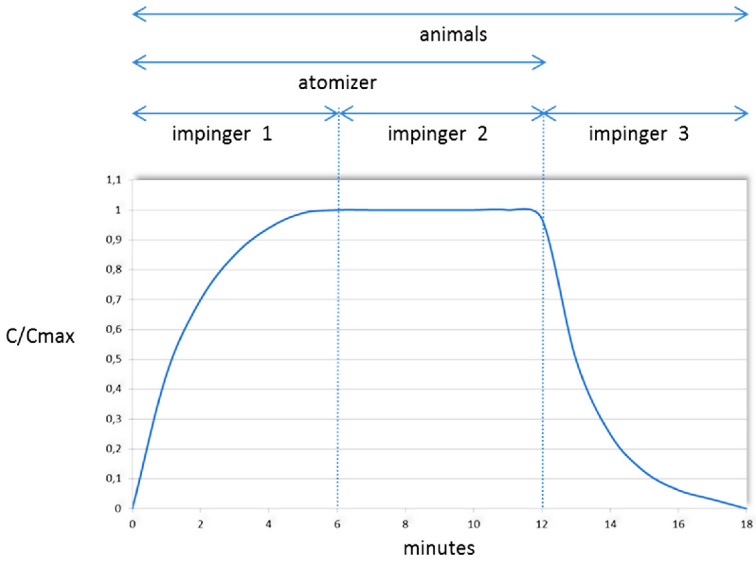


**Figure 2**. The scheme of an aerosol technique constructed and named by Jiri Libich as Single Dose Exposure 400 (SINEX 400). An aerosol chamber of 400 liter volume and a pneumatic nebulizer constructed for creation of monodisperse aerosol with a mean diameter of particles 3.5 um were used for an inhalation model of infection. Three impingers were put into the aerosol chamber during nebulization of 1ml bacterial suspension per minute. One impinger contains a simple cultivation medium for aerosolized bacteria. The constant flow of air was drowned through the impinger for an indicated time (axis x). A number of microbes in the aerosol was determined by plating the impinger liquid on a solid thioglycolate-glucose-blood-agar plate. An amount of bacteria in the aerosol was expressed as C/Cmax (axis y) calculated from the impinger liquid.

After this initial period, tularemia research had moved on to a study of a host immune response to experimental *F. tularensis* infection. The conditions for the best effectiveness of immunization against virulent strains of *F. tularensis* were studied during the eighties of the last century. The challenging of immunized animals with the virulent strains of subsp. *holarctica* Type B and *tularensis* Type A led to the observation that a protective effect of inhalational immunization is significantly better than a subcutaneous one. Furthermore, it was also demonstrated that the induction of protective immunity is not an exclusive property of a live vaccine. Some degree of protection was also obtained when a proliferation of a live vaccine was limited by an administration of antibiotics (streptomycin, kanamycin) in immunized animals. Similar protective effect was observed after an immunization with attenuated *F. tularensis* strain 15 autolysate. It was also documented that an intensity of immune reaction and a spectrum of activated immune mechanisms were dependent on a genetic background of mice, on a route of infection, and partially on a dose of infection. A production of specific antibodies, an activation of macrophages, a blast transformation of lymphocytes, and a production of several cytokines were used for monitoring an immune response [28-30]. It was also proved that an infection with *F. tularensis* leads to an activation of "Natural" killer (NK) cells during early stages of infection briefly after its discovery [31]. The NK cell activation was accompanied by a production of regulatory interferon gamma cytokine [32]. Transfer experiments also confirmed an MHC class II restriction of a protective immune response induction [33]. A phenomenon of an early protective response to virulent *F. tularensis* Type A occurring between 24 and 48 h after immunization was published by Karen Elkins and co-workers later in 1997 [34].

**Table 1**. A survival of C3H/Cbi/Bom mice irradiated by $^{60}$Co in a dose of 4 Gy. The resistance to *F. tularensis* infection is totally abrogated using gamma irradiation ($^{60}$Co) higher than 3 Gy. A significant rapid depression in a specific T lymphocyte count is observed after irradiation (3 – 4 Gy), and their nadir is reached 36 to 48 h after an irradiation event. The duration of this decrease correlates with a radiation dose, while the recovery begins 10 to 15 days after a dose of 3 – 4 Gy.

| Irradiation | Infection* | Survival | % | MTD (days) |
|---|---|---|---|---|
| - | + | 45/49 | 91.8 | 8.5 |
| 1 Gy | + | 18/20 | 90.0 | 9.0 |
| 2 Gy | + | 8/20 | 40.0 | 9.8 |
| 3 Gy | + | 0/20 | 0.0 | 8.8 |
| 4 Gy | + | 0/20 | 0.0 | 7.8 |
| 5Gy | + | 0/20 | 0.0 | 6.4 |
| 5 Gy | - | 20/20 | 100.0 | - |

* Mice were *s.c.* infected with *F. tularensis 15* infection in a dose of 1.05 x $10^2$ live microbes, 72h after irradiation.

**Table 2**. A survival of C3H/Cbi/Bom mice irradiated by $^{60}$Co in a dose of 4 Gy. A natural recovery of resistance to infection started during a second week after irradiation. A significant rapid depression in a specific T lymphocyte count is observed after irradiation (3 – 4 Gy), and their nadir is reached 36 to 48 h after an irradiation event. The duration of this decrease correlates with a radiation dose, while the recovery begins 10 to 15 days after a dose of 3 – 4 Gy.

| Infection* after irradiation** (days) | Survival | % | MTD (days) |
|---|---|---|---|
| 1 | 0/8 | 0.0 | 8.0 |
| 3 | 0/8 | 0.0 | 7.1 |
| 7 | 0/8 | 0.0 | 7.0 |
| 14 | 7/8 | 87.5 | 7.0 |
| 21 | 8/8 | 100.0 | - |
| 28 | 7/8 | 87.5 | 7.0 |
| 42 | 8/8 | 100.0 | - |
| 56 | 8/8 | 100.0 | - |
| Un-irradiated | 15/16 | 93.7 | 8.0 |
| Un-infected | 16/16 | 100.0 | - |

* Mice were *s.c.* infected with *F. tularensis 15* infection in a dose of 1.05 x $10^2$ live microbes, 72h after irradiation.
** Mice were infected at the indicated day after irradiation using gamma irradiation ($^{60}$Co).

A separate set of experiments was performed using $^{60}$Co gamma irradiation of mice as a model of immunocompromised animals. It was demonstrated that gamma irradiation of mice greater than 3 Gy totally abrogated resistance to an infection induced by *F. tularensis* strain 15 (Table 1). Minimum two weeks were required to reach full natural recovery from this deep decline of resistance (Table 2). It means that a live vaccine immunization of generally immunocompromised individuals is practically impossible. An immunization with killed microbes or microbial protein extracts lack a sufficient protective effect. Thus, one of the possibilities how to protect irradiated individuals is a passive transfer of immunity. Both immune cells and antibodies are effective in a passive transfer of protective immunity to naive recipient (Table 3). This knowledge can conclude that specific antibodies provide some degree of protection probably mediated by an antibody dependent cell mediated bactericidal activity, originally published by Lovell at al. 1979 [35, 36]. Later, the same conclusion was published by Stephan Stenmark in 2003 [37].

**Table 3**. A survival of C3H/Cbi/Bom mice irradiated by $^{60}$Co in a dose of 4 Gy, passively protected by transfer of cells, sera against *F. tularensis 15* or Tularin.

| Irradiation | Infection* | Treatment** | Survival | % | MTD |
|---|---|---|---|---|---|
| 4 Gy | - | - | 8/8 | 100.0 | - |
| 4 Gy | + | Tularin | 0/8 | 0.0 | 8.2 |
| 4 Gy | + | Naïve spleen cells | 2/22 | 9.1 | 7.9 |
| 4 Gy | + | Naïve thymus cells | 1/10 | 10.0 | 7.8 |
| 4 Gy | + | Immune spleen cells | 22/22 | 100.0 | - |
| 4 Gy | + | Immune spleen cells + NMS + C | 10/10 | 100.0 | - |
| 4 Gy | + | Immune spleen cells + anti Thy1.2 Ab + C | 10/10 | 100.0 | - |
| 4 Gy | + | Ultrasound destroyed immune spleen cells | 2/10 | 20.0 | 8.9 |
| 4 Gy | + | Mouse serum naïve | 0/10 | 0.0 | 8.6 |
| 4 Gy | + | Immune serum (3$^{rd}$ day - live vaccine) | 3/7 | 42.8 | 8.7 |
| 4 Gy | + | Immune serum (7$^{th}$ day - live vaccine) | 4/5 | 80.0 | 7.0 |
| 4 Gy | + | Immune serum (11$^{th}$ day - live vaccine) | 10/10 | 100.0 | - |
| 4 Gy | + | Immune serum (21$^{st}$ day - live vaccine) | 10/10 | 100.0 | - |
| 4 Gy | + | Immune serum (56$^{th}$ day - live vaccine) | 7/7 | 100.0 | - |
| 4 Gy | + | Immune serum (21$^{st}$ day - heat inactivated vaccine) | 7/7 | 100.0 | - |
| - | + | - | 20/22 | 90.9 | 7.5 |

* Mice were *s.c.* infected with *F. tularensis 15* infection in a dose of 1.05 x $10^2$ live microbes, 72h after irradiation.
** Passive transfer of cells or sera was realized 2 h before infecting irradiated mice. A treatment of irradiated mice by a commercial USSR preparation Tularin (antigenic material for skin tests used frequently in the past as a prototype of dead vaccine) was realized 2 h after irradiation.

At the turn of the millennium, brand-new tularemia research stimuli were discovered on a molecular level due to the development of advanced proteomic analyses of both pathogen and infected host cells. A proteomic technology based on combining various gel electrophoresis procedures, a Western blot technique, and mass spectrometry identification approaches were used for analyzing *F. tularensis* immunoreactive proteins [38-42], for identifying unique typing markers of *Francisella* subspecies [43-45], and for studying host-pathogen interaction at a molecular level. These studies were ranging from a subcellular proteome of bacterial membranes [46-48] to bacterial secreted proteins important for early stages of a host-pathogen interaction [49]. Highly sophisticated approaches of quantitative shotgun proteomics were then applied to a protein profiling study of bacteria exposed to stress conditions *in vitro*. Such conditions were able to immitate prevailing conditions inside host cells *in vivo* [51-54] alongside with the identification of several proteins whose expression was changed. Among them there were proteins encoded by an *igl* operon, a Hsp100 chaperone ClpB with its assumed function in reactivation of aggregated proteins under *in vivo* stress conditions, and an ORF FTL_0200 encoding a protein of putative AAA+ ATPase of a MoxR subfamily. All these proteins seem to be indispensable for the resistance to stress conditions and are substantial factors controlling a virulence and a pathogenicity of *F. tularensis*. Further, the comparative shotgun proteome analyses of *F. tularensis* subtypes revealed several promising candidate proteins for constructing a new type of attenuated live vaccines. Deletion mutants were prepared for some of the identified proteins and two of them, FSC200Δ*dsbA* and FSC200Δ*iglH*, were successfully tested for their attenuation and their immunogenicity. Regarding the DsbA deletion mutant, a molecular mechanism of its attenuation was studied comparing protein patterns of the original wild and deletion mutant strains. Several proteins accumulating in a membrane of the mutant strain were found and some of them were later identified as important factors of *F. tularensis*

virulence [55, 56]. Current proteomic experiments are focused on structural characterization of *F. tularensis* proteins, especially on identifying bacterial membrane glycoproteins [50].

The above-mentioned proteome technologies were also used in a study of a host response to an ongoing infection. These studies clarified that a mutual interaction is stressful for both organisms. Various production of a highly stress-inducible hsp72 protein, a member of the hsp70 family, has been demonstrated in macrophages of three different inbred strains of mice exhibiting either a resistance or a susceptibility to an *F. tularensis* LVS infection. The hsp72 was observed to be preferentially produced and accumulated in intracellular space of a murine peritoneal adherent cell [57]. *F. tularensis* LVS induces apoptosis in a macrophage after infecting these cells. This process requires activation of a p42/p44 MAPK pathway and is associated with a reduced p38 MAPK activity, indicating that infection-induced cell death can be caused by perturbation of these two signalling pathways [58]. A mapping of *Francisella* intracellular trafficking inside macrophages discovered a complicated fate of intracellularly localized bacteria. Bacteria of *F. tularensis* strain LVS disintegrate a phagosome early after its entry to a host cell followed by an escape to a cytosol where it intensively proliferates. A significant part of bacterial population merges into an autophagosome expressing MHC class II molecules. Subsequently, formed autophagosome can be a source of immunogenic signal for CD4+ T cells [59].

Macrophages are considered to be primary host cells for *F. tularensis*, but several other cell types in the immune system also serve as host cells for tularemia infection. *F. tularensis* is able to infect, "disturb", and to activate not only cells of a mononuclear phagocytic system but also dendritic cells [60], epithelial cells [61] and hepatocytes [62]. Moreover, Krocova and co-workers demonstrated that *F. tularensis* also infects mouse (A20) or human (Ramos RA-1) B cell line cells as well as murine primary spleen B cells in series of experiments [63]. Within an infection of B cells, it has been observed that *F. tularensis* FSC200 activates several caspases such as caspase 8, 9 and 3. An activation of Bid, cytochrome c, apoptosis-inducing mitochondria factor and proapoptic Bcl-2 family member has also been determined. This causes depolarization of a mitochondrial membrane potential in a Ramos cell line, thus leading these cells to apoptosis. Unlike live bacteria, killed *F. tularensis* FSC200 is capable of activating caspase 3 only and does not cause apoptosis of Ramos cells. Killed bacteria also cause accumulation of anti-apoptotic protein $Bclx_L$ in mitochondrial membranes. Therefore, live *F. tularensis* activates both caspase-dependent pathways (receptor-mediated and intrinsic) and caspase-independent mitochondrial death in B cells [64].

## CONCLUSION

In conclusion, former Czechoslovak and consequently Czech scientists contributed significantly to current knowledge on tularemia pathogenesis and their results are highly appraised by international scientific community. Former Czechoslovak and Czech scientists underwent a long way from identifying the illness at the beginning to recent molecular analyses of a bacterial virulence during the last 50 years. Therefore, the authors would like to dedicate this article to a successful, long-time work of Jiri Libich (Figure 3), which attracted many of us to become a part of tularemia research community.

**Figure 3**. One of the very rare photos of Jiri Libich who was the leading researcher on tularemia in former Czechoslovakia during the sixties and the seventies during the last century.

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**REFERENCES**

1. Martin, A: Tularemia Infection of Conjunctiva (Case Report). *Southwest Med*. **1925**.
2. Pears, R. A. Insect Bites. *Northwest Medicine*. **1911**, 3, 81-82.
3. McCoy, G. W. Plague-like Disease of Rodents. *Bull. Hyg. Lab., U. S. Pub. Health Service*. **1911**.
4. McCoy, G. W.; Chapin, C. W. Bacterium Tularense, Cause of a Plague-like Disease of Rodents. *Bull. Hyg. Lab., U. S. Pub. Health Service*. **1912**.
5. Wherry, W.B.; Lamb, B.H. Infection of Man with *Bacterium Tularense. J. Infect. Dis*. **1914**, 15, 331-340.
6. Francis, E. A new disease of man. *J Am Med Assoc*. **1922**, 78, 1015-1018.
7. Ohara, S. Studies on Yato-Byo (Ohara´s disease, tularemia in Japan), Report I. *Japan J Exp Med*. **1954**. 24, 69–79.
8. Francis, E.; Moore, D. Identity of Ohara´s disease and tularemia. *JAMA*. **1926**, 86, 1329-1332.
9. Suvarov, S.; Volfertz, V.; Voronkova, M.M. Plague like lymphadentitis of the Astrikhansky Region. *Vestnik Mikrobiol. Epidemiol. Parazitol. 1928*, 7, 293.
10. Zarkai, G.I. Tularemia among water rats; methods of studying them, *Bull. Hyg*. **1930**, 5, 875.
11. Francis, E. Sources of infection and seasonal incidence of tularaemia in man, *Public Health Rep*. **1937**, 52, 103–113.
12. Kugeler, K.J.; Mead, P.S.; Janusz, A.M.; Staples, J.E.; Kubota, K.A.; Chalcraft, L.G.; Petersen, J.M. Molecular Epidemiology of *Francisella tularensis* in the the United States. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 2*. **2009**, 48, 863-870.
13. Staples, J.E.; Kubota, K.A.; Chalcraft, L.G.; Mead, P.S.; Petersen, J.M. Epidemiologic and molecular analysis of human tularemia, United States, 1964–2004. *Emerg Infect Dis*. **2006**, 12, 1113–8.
14. Huber, B.E.; Escudero, R.; Busse, H. J.; Seibold, E.; Scholz, H. C.; Anda, P.; Kämpfer, P.; Splettstoesser, W. D. Description of *Francisella hispaniensis* sp. *nov.*, isolated from human blood, reclassification of *Francisella novicida* (Larson et al. 1955) Olsufiev et al. 1959 as *Francisella tularensis* subsp. *novicida* comb. nov., and emended description of the genus *Francisella. Int J Syst Evol Microbiol*. **2010**, 60, 1887–1896.
15. Johansson, A.; Celli, J.; Conlan, W.; Elkins, K.L.; Forsman, M.; Keim, P.S. Larsson, P.; Manoil, C.; Nano, F.E.; Petersen, J.M.; Sjöstedt, A. Objections to the transfer of *Francisella novicida* to the subspecies rank of *Francisella tularensis. Int J Syst Evol Microbiol*. **2010**, 60, 1717-8.
16. http://ekolist.cz/cz/zpravodajstvi/zpravy/v-okrese-plzen-jih-propukla-epidemie-tularemie
17. Tomanek, E. Tularemia in Czechoslovakia and Austria During 1936 and 1937. *Am J Public Health Nations Health*. **1937**, 27, 443.
18. Gurycová, D. First isolation of *Francisella tularensis* subsp. *tularensis* in Europe. *Eur J Epidemiol*. **1998**, 14, 797-802.
19. Hubalek, Z.; Treml, F.; Halouzka, J.; Juricová, Z.; Hunady, M.; Janik, V. Frequent isolation of *Francisella tularensis* from *Dermacentor reticulatus* ticks in an enzootic focus of tularaemia. *Med Vet Entomol*. **1996**, 10, 241-6.
20. Hubalek, Z.; Sixl, W.; Halouzka, J.; Mikulasková, M. Prevalence of *Francisella tularensis* in *Dermacentor reticulatus* ticks collected in adjacent areas of the Czech and Austrian Republics. *Cent Eur J Public Health*. **1997**, 5, 199-201.
21. Hubalek, Z.; Sixl, W.; Halouzka, J. *Francisella tularensis* in *Dermacentor reticulatus* ticks from the Czech Republic and Austria. *Wien Klin Wochenschr*. **1998**, 110, 909-10.
22. Lukas, B.; Libich, J. Modification of a simple blood agar plate for the culture of *Pasteurella tularensis*. *Cesk Epidemiol Mikrobiol Imunol*. **1962**, 11, 290-7. 23.
23. Libich, J. Effect of the administration of streptomycin in the incubation and manifest phase on the course of inhalation tularaemia in guinea pigs. *Folia Microbiol (Praha)*. **1962**, 7, 320-5.
24. Lukas, B.; Hejzlar, M.; Libich, J. Effect of streptomycin and kanamycin on the course of experimental tularaemia in guinea-pigs. *Folia Microbiol (Praha)*. **1963**, 8, 80-8.
25. Kliment, V.; Libich, J.; Edl, J. A mathematical model of in vitro survival of guinea pig alveolar macrophages in correlation to time. *Folia Morphol (Praha)*. **1970**, 18, 330-4.
26. Kliment, V.; Libich, J. Experimental inhalation tularaemia: statistical distribution of infectious particles deposited in the lung tissue in guinea

pigs. *J Hyg Epidemiol Microbiol Immunol*. **1971**, 15, 316-23.
27. Kliment, V.; Libich, J.; Kaudersova, V. Geometry of guinea pig respiratory tract and application of Landahl's model of deposition of aerosol particles. *J Hyg Epidemiol Microbiol Immunol*. **1972**, 16, 107-14.
28. Kovarova, H.; Macela, A.; Stulik, J. The activation of macrophages in the immune response against the intracellular bacterial pathogen *Francisella tularensis. Sb Ved Pr Lek Fak Karlovy Univerzity Hradci Kralove*. **1990**, 33, 565-77.
29. Kovarova, H.; Macela, A.; Stulik, J. The production of oxygen metabolites and their possible regulatory role in the course of tularemia infection. *Folia Microbiol (Praha)*. **1990**, 35, 413-22.
30. Kovarova, H.; Macela, A.; Stulik, J. Macrophage activating factors produced in the course of murine tularemia: effect on multiplication of microbes. *Arch Immunol Ther Exp (Warsz)*. **1992**, 40, 183-90.
31. Kiessling, R.; Klein, E.; Wigzell, H. "Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. *Eur J Immunol*. **1975**, 5, 112-7.
32. Macela, A.; Stulik, J.; Hernychova, L.; Kroca, M.; Krocova, Z.; Kovarova, H. The immune response against *Francisella tularensis* live vaccine strain in Lps(n) and Lps(d) mice. *FEMS Immunol Med Microbiol*. **1996**, 13, 235-8.
33. Anthony, L.S.; Kongshavn, P.A. H-2 restriction in acquired cell-mediated immunity to infection with *Francisella tularensis* LVS. *Infect Immun*. **1988**, 56, 452-6.
34. Culkin, S.J.; Rhinehart-Jones, T.; Elkins, K.L. A novel role for B cells in early protective immunity to an intracellular pathogen, *Francisella tularensis* strain LVS. *J Immunol*. **1997**, 158, 3277-84.
35. Lowell, G.H.; Smith, L.F.; Artenstein, M.S.; Nash, G.S.; MacDermott, R.P. Jr. Antibody-dependent cell-mediated antibacterial activity of human mononuclear cells. I. K lymphocytes and monocytes are effective against meningococci in cooperation with human imune sera. *J Exp Med*. **1979**, 150, 127-37.
36. Lowell, G.H.; Smith, L.F.; Griffiss, J.M.; Brandt, B.L.; MacDermott, R.P. Antibody-dependent mononuclear cell-mediated antimeningococcal activity. Comparison of the effects of convalescent and postimmunization immunoglobulins G, M, and A. *J Clin Invest*. **1980**, 66, 260-7.
37. Stenmark, S.; Lindgren, H.; Tärnvik, A.; Sjöstedt, A. Specific antibodies contribute to the host protection against strains of *Francisella tularensis* subspecies *holarctica. Microb Pathog*. **2003**, 35, 73-80.
38. Stulik, J.; Cerna, J.; Kovarova, H.; Macela, A. Protein heterogeneity of *Francisella tularensis*: detection of proteins with antigenic determinants. *Folia Microbiol (Praha)*. **1989**, 34, 316-23.
39. Havlasova, J.; Hernychova, L.; Halada, P.; Pellantova, V.; Krejsek, J.; Stulik, J.; Macela, A.; Jungblut, P.R.; Larsson, P.; Forsman, M. Mapping of immunoreactive antigens of *Francisella tularensis* live vaccine strain. *Proteomics*. **2002**, 2, 857-67.
40. Havlasova, J.; Hernychova, L.; Brychta, M.; Hubalek, M.; Lenco, J.; Larsson, P.; Lundqvist, M.; Forsman, M.; Krocova, Z.; Stulik, J.; Macela, A. Proteomic analysis of anti-*Francisella tularensis* LVS antibody response in murine model of tularemia. *Proteomics*. **2005**, 5, 2090-103.
41. Janovska, S.; Pavkova, I.; Hubalek, M.; Lenco, J.; Macela, A.; Stulik, J. Identification of immunoreactive antigens in membrane proteins enriched fraction from *Francisella tularensis* LVS. *Immunol Lett*. **2007**, 108, 151-9.
42. Janovska, S.; Pavkova, I.; Reichelova, M.; Hubalek, M.; Stulik, J.; Macela, A. Proteomic analysis of antibody response in a case of laboratory-acquired infection with *Francisella tularensis* subsp. *tularensis. Folia Microbiol (Praha)*. **2007**, 52, 194-8.
43. Hernychova, L.; Stulik, J.; Halada, P.; Macela, A.; Kroca, M.; Johansson, T.; Malina, M. Construction of a *Francisella tularensis* two-dimensional electrophoresis protein database. *Proteomics*. **2001**, 1, 508-15.
44. Hubalek, M.; Hernychova, L.; Havlasova, J.; Kasalova, I.; Neubauerova, V.; Stulik, J.; Macela, A.; Lundqvist, M.; Larsson, P. Towards proteome database of *Francisella tularensis. J Chromatogr B Analyt Technol Biomed Life Sci*. **2003**, 787, 149-77.
45. Hubalek, M.; Hernychova, L.; Brychta, M.; Lenco, J.; Zechovska, J.; Stulik, J. Comparative proteome analysis of cellular proteins extracted from highly virulent *Francisella tularensis* ssp. *tularensis* and less virulent *F. tularensis* ssp. *holarctica* and *F. tularensis* ssp. *mediaasiatica. Proteomics*. **2004**, 10, 3048-60.

46. Pavkova, I.; Hubalek, M.; Zechovska, J.; Lenco, J,; Stulik, J. *Francisella tularensis* live vaccine strain: proteomic analysis of membrane proteins enriched fraction. *Proteomics*. **2005**, 5, 2460-7.
47. Pavkova, I.; Reichelova, M.; Larsson, P.; Hubalek, M.; Vackova, J.; Forsberg, A.; Stulik, J. Comparative proteome analysis of fractions enriched for membrane-associated proteins from *Francisella tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* strains. *J Proteome Res*. **2006**, 5, 3125-34.
48. Dresler, J.; Klimentova, J.; Stulik, J. *Francisella tularensis* membrane complexome by blue native/SDS-PAGE. *J Proteomics*. **2011**, 75, 257-69.
49. Konecna, K.; Hernychova, L.; Reichelova, M.; Lenco, J.; Klimentova, J.; Stulik, J.; Macela, A.; Alefantis, T.; Delvecchio, V.G. Comparative proteomic profiling of culture filtrate proteins of less and highly virulent *Francisella tularensis* strains. *Proteomics*. **2010**, 10, 4501-11.
50. Balonova, L.; Hernychova, L.; Mann, B.F.; Link, M.; Bilkova, Z.; Novotny, M.V.; Stulik, J. Multimethodological approach to identification of glycoproteins from the proteome of *Francisella tularensis*, an intracellular microorganism. *J Proteome Res*. **2010**, 9, 1995-2005.
51. Lenco, J.; Pavkova, I.; Hubalek, M.; Stulik, J. Insights into the oxidative stress response in *Francisella tularensis* LVS and its mutant DeltaiglC1+2 by proteomics analysis. *FEMS Microbiol Lett*. **2005**, 246, 47-54.
52. Lenco, J.; Hubalek, M.; Larsson, P.; Fuciková, A.; Brychta, M.; Macela, A.; Stulik, J. Proteomics analysis of the *Francisella tularensis* LVS response to iron restriction: induction of the *F. tularensis* pathogenicity island proteins IglABC. *FEMS Microbiol Lett*. **2007**, 269, 11-21.
53. Meibom, K.L.; Dubail, I.; Dupuis, M.; Barel, M.; Lenco, J.; Stulik, J.; Golovliov, I.; Sjöstedt, A.; Charbit, A. The heat-shock protein ClpB of *Francisella tularensis* is involved in stress tolerance and is required for multiplication in target organs of infected mice. *Mol Microbiol*. **2008**, 67, 1384-401.
54. Dieppedale, J.; Sobral, D.; Dupuis, M.; Dubail, I.; Klimentova, J.; Stulik, J.; Postic, G.; Frapy, E.; Meibom, K.L.; Barel, M.; Charbit, A. Identification of a putative chaperone involved in stress resistance and virulence in *Francisella tularensis*. *Infect Immun*. **2011**, 79, 1428-39.
55. Straskova, A.; Pavkova, I.; Link, M.; Forslund, A.L.; Kuoppa, K.; Noppa, L.; Kroca, M.; Fucikova, A.; Klimentova, J.; Krocova, Z.; Forsberg, A.; Stulik, J. Proteome analysis of an attenuated *Francisella tularensis* dsbA mutant: identification of potential DsbA substrate proteins. *J Proteome Res*. **2009**, 8, 5336-46.
56. Straskova, A.; Cerveny, L.; Spidlova, P.; Dankova, V.; Belcic, D.; Santic, M.; Stulik, J. Deletion of IglH in virulent *Francisella tularensis* subsp. *holarctica* FSC200 strain results in attenuation and provides protection against the challenge with the parental strain. *Microbes Infect*. 2012, 14, 177-87.
57. Stulik, J.; Hernychova, L.; Macela, A.; Krocova, Z.; Kroca, M. Production of stress-inducible form of heat-shock protein 70 in mouse peritoneal adherent cells after in vivo infection by *Francisella tularensis. Folia Microbiol (Praha)*. **1999**, 44, 306-10.
58. Hrstka, R.; Stulik, J.; Vojtesek, B. The role of MAPK signal pathways during *Francisella tularensis* LVS infection-induced apoptosis in murine macrophages. *Microbes Infect*. **2005**, 7, 619-25.
59. Hrstka, R.; Krocova, Z.; Cerny, J.; Vojtesek, B.; Macela, A.; Stulik, J. *Francisella tularensis* strain LVS resides in MHC II-positive autophagic vacuoles in macrophages. *Folia Microbiol (Praha)*. **2007**, 52, 631-6.
60. Bosio, C.M.; Dow, S.W. *Francisella tularensis* induces aberrant activation of pulmonary dendritic cells. *J Immunol*. **2005**, 175, 6792-801.
61. Craven, R.R.; Hall, J.D.; Fuller, J.R.; Taft-Benz, S.; Kawula, T.H. *Francisella tularensis* invasion of lung epithelial cells. *Infect Immun*. **2008**, 76, 2833-42.
62. Conlan, J.W.; North, R.J. Early pathogenesis of infection in the liver with the facultative intracellular bacteria *Listeria monocytogenes, Francisella tularensis*, and *Salmonella typhimurium* involves lysis of infected hepatocytes by leukocytes. *Infect Immun*. **1992**, 60, 5164-71.
63. Krocova, Z.; Härtlova, A.; Souckova, D.; Zivna, L.; Kroca, M.; Rudolf, E.; Macela, A.; Stulik, J. Interaction of B cells with intracellular pathogen *Francisella tularensis. Microb Pathog*. **2008**, 45, 79-85.
64. Zivna, L.; Krocova, Z.; Härtlova, A.; Kubelkova, K.; Zakova, J.; Rudolf, E.; Hrstka, R.; Macela, A.; Stulik, J. Activation of B cell apoptotic pathways in the course of *Francisella tularensis* infection. *Microb Pathog*. **2010**, 49, 226-36.