

Piotr Cieślak¹, Józef Knap², Agata Bielawska-Drózd^{1*}

¹ Biological Threats Identification and Countermeasure Centre of the General Karol Kaczkowski Military
Institute of Hygiene and Epidemiology, Puławy, Poland

² Department of Epidemiology, Warsaw Medical University, Second Faculty of Medicine Warsaw, Poland

Submitted in October, accepted in December 2017

Abstract: In the early twentieth century, *Francisella tularensis* was identified as a pathogenic agent of tularaemia, one of the most dangerous zoonoses. Based on its biochemical properties, infective dose and geographical location, four subspecies have been distinguished within the species *F. tularensis*: the highly infectious *F. tularensis* subsp. *tularensis* (type A) occurring mainly in the United States of America, *F. tularensis* subsp. *holarctica* (type B) mainly in Europe, *F. tularensis* subsp. *mediasiatica* isolated mostly in Asia and *F. tularensis* subsp. *novicida*, non-pathogenic to humans. Due to its ability to infect and variable forms of the disease, the etiological agent of tularaemia is classified by the CDC (Centers for Disease Control and Prevention, USA) as a biological warfare agent with a high danger potential (group A). The majority of data describing incidence of tularaemia in Poland is based on serological tests. However, real-time PCR method and MST analysis of *F. tularensis* highly variable intergenic regions may be also applicable to detection, differentiation and determination of genetic variation among *F. tularensis* strains. In addition, the above methods could be successfully used in molecular characterization of tularaemia strains from humans and animals isolated in screening research, and during epizootic and epidemic outbreaks.

1. Historical overview. 2. Characteristics and taxonomy of *F. tularensis*. 3. Morphology. 4. Culture media and conditions. 5. Biochemical properties. 6. Survivability and persistence of *F. tularensis*. 7. *F. tularensis* as a biological weapon agent. 8. Tularaemia vaccines. 9. Pathogenicity of *F. tularensis*. 10. Tularaemia treatment. 11. Laboratory diagnostics of *F. tularensis*. 12. Summary

Francisella tularensis – przegląd

Streszczenie: *Francisella tularensis* została rozpoznana jako czynnik chorobotwórczy ludzi i zwierząt na początku XX wieku. Analizy biochemiczne i patogenne właściwości czynnika biologicznego pozwoliły na wyróżnienie 4 podgatunków przypisanych do różnych regionów geograficznych: wysoce zakaźny *F. tularensis* subsp. *tularensis* (type A) występujący głównie w Ameryce Północnej, *F. tularensis* subsp. *holarctica* (type B) z obszaru Starego Świata, *F. tularensis* subsp. *mediasiatica* izolowany głównie z Azji oraz *F. tularensis* subsp. *novicida*, niepatogenny dla ludzi. Pałeczka tularemii, ze względu na swoje zdolności infekcyjne oraz zdolność do wywoływania różnych postaci chorobowych, została zakwalifikowana przez CDC (Centrum Kontroli i prewencji Chorób, USA) jako potencjalny wysoce niebezpieczny czynnik broni biologicznej (Grupa A). Większość danych opisujących przypadki zachorowań na tularamię opartych jest na testach serologicznych. Jakkolwiek, technika real-time PCR (Polymerase Chain Reaction) i MST (Multi Sequence Typing) jako analiza wysoce zmiennych obszarów międzygenowych *F. tularensis* może być zastosowana do wykrywania, różnicowania i określania wariantów genetycznych izolatów *F. tularensis*. Techniki te mogą być również wykorzystywane w charakterystyce molekularnej pałeczek tularemii izolowanych od ludzi i zwierząt w badaniach przesiewowych oraz w ogniskach epizootyczno-epidemicznych.

1. Rys historyczny 2. Charakterystyka *F. tularensis* i systematyka taksonomiczna 3. Morfologia 4. Podłoża hodowlane i warunki wzrostu 5. Właściwości biochemiczne 6. Wytrzymałość i żywotność *F. tularensis* 7. *F. tularensis* jako czynnik broni biologicznej 8. Szczepionki przeciwko tularemii 9. Patogenność *F. tularensis* 10. Leczenie tularemii 11. Diagnostyka laboratoryjna *F. tularensis* 12. Podsumowanie

Key words: characteristic of etiological agent, diagnostic methods, *F. tularensis*, treatment, vaccination

Słowa kluczowe: charakterystyka czynnika etiologicznego, metody diagnostyki, *F. tularensis*, leczenie, szczepienia

1. Historical overview

Francisella tularensis was recognised as an agent of human and animals infection at the beginning of the 20th century [82]. It is thought that the tularaemia *F. tularensis* evolved simultaneously in the Old and New Worlds about 11 million years ago at the turn of Miocene and Pliocene [31]. Prior to the disease being recognised by McCoy in 1911, information about an unusual illness (called lemming fever) appeared in 1653 in Norway; in 1818 in Japan it was described as “Yato-byo” – wild hare disease [82]. Cases similar to tularaemia were also described in 1741 in Western Siberia, later in 1825 also

in Astrakhan (Siberia, former Soviet Union) as “pestis minor” (1877–1879) („pestis minor”, 1877–1879 but in 1908 in Utah (USA) it was recognised as “deerfly fever”. In 1921 the disease, “polyadenitis”, was found along the river Irtysh and in 1925 as “lymphadenitis” – an epidemic inflammation of lymph nodes – in the Voronez region (both former Soviet Union) [20, 28, 31, 84].

The first certain tularaemia cases in people were described in 1904 in California and in 1907 in Arizona [31]. Next cases were found in 1908 and 1910 in Utah; Pearse named them “deerfly fever”. The infection was caused by a deerfly bite and symptoms appeared 3–5 days after the bite [84]. In 1911 in Tulare (Califor-

* Autor korespondencyjny: Dr. Agata Bielawska-Drózd, Biological Threats Identification and Countermeasure Centre of the General Karol Kaczkowski Military Institute of Hygiene and Epidemiology, Lubelska 2, 24-100 Puławy Poland; tel. 81 261 519 816; e-mail: abielawska@wihe.pulawy.pl

nia, USA) a pestis-like rodent disease was described; it attacked field rodents and ground squirrels; lesions found in organs during autopsies were similar to those caused by pestis [84]. In 1912 McCoy and Chapin isolated a Gram-negative bacterium and named it *Bacterium tularensis* [6, 20, 65, 84]. Later research classified the tularaemia bacterium as *Bacterium tularensis*, *Bacillus tularensis*, *Brucella tularensis*, *Pasteurella tularensis* [31]. In 1947–1959 the final agreement on the biological agent taxonomy was reached and the genus was named *Francisella* as a tribute to Dr Edward Francis [31].

During intensive research on *F. tularensis*, significant differences were found in symptoms in patients from North America and other parts of the world. The differences were related to the presence of two variable types of *F. tularensis* present in sub-arctic regions, and the second, found in Palaearctic. Further biochemical analyses and the biological agent's pathogenic properties allowed distinguishing 4 subspecies originating from different geographical regions: *F. tularensis nearctica* (mainly North America), *F. tularensis holarctica* (the Old World), in central Asia – *F. tularensis mediasiatica*, in Japan – *F. tularensis japonica*. Including *F. novicida* in a taxonomic group was problematic due to a limited known reservoir [31].

Basing on the research of sensitivity to erythromycin, two biovars were found within *holarctica* species: biovar I Ery^s – sensitive to erythromycin, and biovar II Ery^r – resistant. Moreover, strains originating from Japan were included in the third biovar: *F. tularensis* subsp. *holarctica* biovar *japonica* Radionova. Finally, the descriptors of type A and B were accepted in *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* respectively [60, 82].

2. Characteristics and taxonomy of *F. tularensis*

F. tularensis belongs to the phylum *Proteobacteria*, class *Gammaproteobacteria*, order *Thiotrichales*, family *Francisellaceae* [33, 64]. Within the genus *Francisella*, 2 species were distinguished: *F. tularensis* and *F. philomiragia* [29, 33, 62, 63]. Taxonomy of *F. tularensis* according to *Bergey's Manual Systematic Bacteriology* [33, 46].

Kingdom: *Bacteria*
 Phylum: *Proteobacteria*
 Class: *Gammaproteobacteria*
 Order: *Thiotrichales*
 Family: *Francisellaceae*
 Genus: *Francisella*
 Species: *Francisella philomiragia*
 Species: *Francisella tularensis*
 Subspecies: *Francisella tularensis* subsp. *tularensis*
Francisella tularensis subsp. *holarctica*

Francisella tularensis subsp. *mediasiatica*

Francisella tularensis subsp. *novicida*

Moreover, it has been suggested that a subspecies *F. japonica* should be distinguished within the type B for the strains isolated in Japan [60, 87, 92] and that 4 species ought to be distinguished within the genus [77].

F. philomiragia previously called *Yersinia philomiragia* was isolated from water samples, muskrats and humans [39]. This species can be found mainly in water environment, i.e. in streams, rivers and lakes. It may cause infections in patients with suppressed immune system [7]. It may also be found in various fish species [44, 45, 52, 61, 63].

Extensive research on the *Francisella* genus allowed describing new, previously unknown species such as *F. noatunesis* [77], *F. hispaniensis* [41], *F. guangzhouensis* [69], *F. haliotica* [8], *F. asiatica* [54] and *F. persica* [51].

3. Morphology

F. tularensis is a small (0.2–0.5 µm × 0.7 – 1.0 µm), non-spore forming single, Gram-negative coccobacillus staining weakly with Gram method [20]. Bilecki (1974) recommends using carbolfuchsin and crystal violet with an extended exposure up to 1.5 hours. For histological slides he recommends Giemsa method, also adopted for staining smears from cultures. Slides prepared in such way may show the bacteria stained pinkish-violet to purplish-blue. Cells from cultures only several days old will stain pink, bacteria from older cultures will show as navy blue. The methylene blue dyeing, Kozłowski method, Burry method may also be adopted for *F. tularensis* microscopy analysis. The Kozłowski technique allows to distinguish *F. tularensis* (stains green) from *Brucella* spp. (stains red). *F. tularensis* is characteristic for its bipolar staining [6].

4. Culturing media and conditions

F. tularensis has high nutritional requirements and needs cysteine-enriched culturing media [20, 64, 65]. One of them is blood agar with glucose and cysteine, another is chocolate agar with cysteine (CHAB) [20]. Moreover, culturing of *F. tularensis* is commonly done using cysteine agar with glucose, thioglycollate fluid medium, modified Theyer-Martin medium, buffered charcoal-yeast extract agar or cysteine agar with heart infusion and 9% sheep blood [20, 65]. On CHAB medium *F. tularensis* grows from 2 to 5 days at the temperature 35°C in the atmosphere of 5% CO₂, they form small (2–4 mm in diameter), smooth, round mucous colonies [18]. *F. tularensis* is highly pleomorphic. In animals highly susceptible to infection (mice) the

bacterium is thin, short and delicate. In organs taken from other animals, for example rabbits, it may be a coccus or a bacillus, but the predominant form is coccobacillus. Pleomorphism may also be observed in artificial media. Bacilli dominate in fresh cultures, while in older ones mainly cocci are present. Liquid media show bipolar or curved forms frequently joined into threads with rounded ends. A theory has been formulated that there exists a connection between the morphology and level of infectiveness of the bacterium. Pleomorphism is absent in old colonies and in non-infectious strains and the bacteria present the form of a coccus. Among the bacteria found in animals susceptible to infection and in highly infectious strains of *F. tularensis* the bacillus is dominated form [6].

Another medium which may be used successfully in culturing samples of *F. tularensis* collected from animals and environment is a cysteine medium with added antibiotics CHAB-A and CHAB-PACCV. The Center for Disease Control and Prevention USA (CDC) recommends CHAB as appropriate for culturing *F. tularensis* from clinical samples, despite other media such as blood agar and Thayer-Martin medium are also being used in the USA, [95]. Morris *et al.* (2017) proposed a novel broth medium (BVFH – BHI supplemented with 2% Vitox, 10% Fildes and 1% histidine). This medium enhanced growth of *F. tularensis* Type A1, A2 and B strains [58].

F. tularensis grows slowly at 37°C and very weakly at 28°C. This feature may be used to distinguish the tularaemia *F. tularensis* from *Y. pestis*, *F. philomiragia* and *F. tularensis* subsp. *novicida*, which show growth at 28°C [20]. Culturing tularaemia in the environment with 5% CO₂ may increase growth but it is not particularly significant. Colonies of *F. tularensis* subsp. *novicida* and *F. philomiragia* are slightly larger than *F. tularensis* [59, 64].

5. Biochemical properties

Biochemical properties of *F. tularensis* subspecies are slightly different. *F. tularensis* subsp. *tularensis* has the ability to ferment glycerol, glucose, maltose and citrulline. It is also very sensitive to erythromycin and other macrolide antibiotics and lincomycin. In comparison to *F. tularensis* subspecies *holarctica*, *F. tularensis* subsp. *tularensis* presents a higher level of fatty acids C24 (12–16%). What is more, its antigen structures contain more hexosamine (6–12%) than *F. tularensis* subsp. *holarctica* (5–7%) [60]. *F. tularensis* subsp. *holarctica* has the ability to ferment glucose and maltose but not citrulline or glycerol (except for biovar *japonica*). These strains contain more fatty acids C14 (9–11%), but less C24 (7–11%) than other subspecies. Immunising laboratory animals with *F. tularensis* subsp. *holarctica* resulted

in a lower level of protection against infections than when strains *F. tularensis* subsp. *tularensis* were used [60]. *F. tularensis* subsp. *mediasiatica* was described by Aikimbaeva; it originates from the centre of the ex-soviet part of Asia. The strains from this subspecies ferment glycerol and citrullin but they show very weak fermentation ability towards glucose and maltose. They are very sensitive to macrolides and lincomycin. The research on fatty acids content showed low levels for C14 (4.2–4.5%) and, similarly to *F. tularensis* subsp. *tularensis*, high levels for C24 (12–16%) [60].

6. Survivability and persistence of *F. tularensis*

F. tularensis may survive many weeks in soil, animal carcasses and their skins, many months in water and silt and for years in frozen rabbit meat. It may survive in watercourses, which may constitute sources of local outbreaks. In North America the main sources of contamination of watercourses are beavers and musk rats, in Scandinavia beavers and lemmings, in the former USSR – rats and voles [38, 84]. Moreover, *F. tularensis* may be found as VBNC (Viable but non culturable state) [20, 38]. VBNC does not guarantee infectivity which was shown during attempts to infect a mouse with a suspension of cells [27]. It was also shown that *F. tularensis* is capable of replication in a free-living protozoan *Acanthamoeba castellanii* [1] similarly to *Legionella pneumophila* [80]. An interesting and not fully explained phenomenon is the existence of FLEs (*Francisella*-like endosymbionts) [20, 22, 42, 50, 78, 86, 94].

One of the main factors influencing *F. tularensis* development is temperature. The pathogen may survive in natural waters for up to 14 weeks at the temperature of 7°C and even up to 4 months at 4°C to 6°C. The temperatures of 20–21°C allow its survival for about 3 weeks. Mud is another environment conducive to the bacterium's survival (62 days); in humid soil it may live for up to 30 days and in mud kept at the temperature of 7°C even up to 14 weeks [31]. According to Skrodzki [84], bacteria suspended in isotonic saline solution at 58°C die after 10 minutes [31, 84], at the temperature of 56°C – after 20 minutes, at 54°C after 1 hour. However, heating a suspension of bacterial cells to the temperature 50°C – 52°C does not deactivate the biological agent [84]. Other authors state that the bacteria die after 10 minutes in 56°C – 58°C [6, 31]. From the epidemiological and epizootic viewpoint, the crucial factor is the bacteria's survival in living organisms and dead tissues, where they can live for up to 93 days, and in liver for 53 days. The bacteria were also found after 90 days in mice kept at 0°C; in mice kept at room temperature they survived for up to 15 days. In carcasses of dead hares kept in cold stores (–16°C to –20°C) the microorganism

survived for up to 120 days in solid organs and up to 75 days in muscles [84]. In ticks it may live for up to 700 days and in skins of animals kept at 15°C – 20°C it may survive for at least 20 days [31]. Moreover in vole skins dried at the temperature of 8°C – 12°C *F. tularensis* was found for up to 40 days, at 32°C the bacteria die after 5–6 days [84]. Research concerning survivability of *F. tularensis* was conducted, among others, by Bilecki [6], who discovered that *F. tularensis* found in vole skins exposed to sunlight at the temperatures of 24°C – 29.5°C survived for only 3 hours. Where the skins were dried in diffused light at the temperatures 17.5°C – 20°C, the bacteria were active for 30 hours and at 17°C – 18°C when dried indoors, the skins contained living bacteria for up to 20 days [6].

7. *F. tularensis* as an agent of biological weapon

Tularaemia was already known in 14th century B.C. in Central Anatolia (present day Turkey) during the region of Hittites. It is thought that the disease was brought to Anatolia from the Middle East when, as a result of wars and voyages, with infected rodents and ticks. The tularaemia outbreaks with many victims from varied social groups were observed in Syria, Egypt and Lebanon. Military use of *F. tularensis* was known as long ago as 1320–1318 B.C. when, during a war between Hittites and Arzawa in the Middle East, herds of infected sheep were driven by the Hittites behind the enemy's defence lines and, as a result, the Arzawan forces were greatly weakened by the disease [36].

In 1932 Japanese scientists initiated research on the possibility of using *F. tularensis* as an agent of biological weapon; this research was tested in paramedical experiments conducted by Unit 731 in Manchuria during the Second Sino-Japanese War (1937–1945) of World War II [38, 82]. In the mid-20th century both the USA and the USSR conducted extensive research on *F. tularensis* strains resistant to streptomycin. Moreover, a Soviet scientist Ken Alibek suggested a hypothesis of intentional *F. tularensis* use as an agent of biological weapon on the eastern front during WW II (the Battle of Kursk). He also disclosed that the Soviets continued the research on biological warfare until the 1990s, as a result of which antibiotic-resistant and vaccine-resistant strains may have been created [2, 20, 38, 80, 82].

Extensive research on humans and animals was also conducted by USAMRIID (United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland). For the research purposes aerosol chamber was used; there the effectiveness of preventive therapies and vaccines was tested on infected volunteers.

In 1972 Convention on the Prohibition of the Development, Production, and Stockpiling of Bacteriological

and Toxin Weapons was implemented [54]. The convention was accepted by 140 countries; in spite of this, however, the number of countries possessing hazardous biological agents has been steadily increasing. This increase constitutes a threat demanding continuous research on the potential results of releasing biological agents, on symptoms, pathomechanisms, human immunological responses and appropriate treatments, vaccinations and other possible defensive actions [49].

8. Tularaemia vaccine

Extensive studies of *F. tularensis*, particularly in the USA and former USSR, resulted in the commencement of research on an effective tularaemia vaccine. Observations of initial infections with *F. tularensis* showed that the following infections were much milder [82].

First vaccines used cellular extracts of *F. tularensis*. They proved to be ineffective, however, and caused strong reactions in the vaccinated organisms. The induced immunoresponse allowed to reduce the symptoms of ulceroglandular and typhoidal tularaemia in volunteers, but many presented symptoms of the acute form of the disease. Similar trials done on animals did not result in noticeable therapeutic effects. Infections with low virulence strains can be defeated by antibodies and immunity against highly virulent strains of *F. tularensis* is provided by T cells. One of the target units potentially useful in therapy is LPS, which may induce immunoresponse against low virulence strains and extend the survival of immunised mice [64].

In the 1930s and 1940s there were extensive studies aimed at finding a strain suitable to produce a vaccine. As a result, in the USSR a Live Vaccine Strain (LSV) was produced; it caused a satisfactory immunoresponse in organisms [38]. LVS was tested on humans during Operation Whitecoat. Two routes of administration of the vaccine are known: via aerosol or scarification. Aerosol application caused more effective immunity, however in most cases scarification was used [64]. First human testing was done in 1942. Six months after the vaccination volunteers were infected with *F. tularensis* virulent strains. The experiments confirmed usefulness of this strain for the purpose of immunisation against tularaemia [82]. In 1946 in the USSR a programme of preventive vaccinations against tularaemia was initiated; an attenuated vaccine was used. At the end of the 1950s tens of millions of inhabitants of endemic areas were inoculated. The Russian vaccine resulted in immunity for the period of at least 5 years, in some cases from 10–15 years [23]. Among the vaccinated only 0.36% showed tularaemia symptoms, in the non-vaccinated population the disease incidence was 4.3% [38].

Presently LVS of *F. tularensis* is not officially approved for use. One of the reasons may be variable cell morphology and immunogenicity. Moreover, there is not much information regarding the mechanisms of the strain attenuation. Despite these unknowns however, attenuated LVS may induce immunoresponse resulting in immunity to tularaemia. This phenomenon proves that a strain selected for its particular genetic features and a rationally prepared vaccination strain may be useful in research on a potential vaccine. The strain used ought to be non-virulent and to be capable of replication *in vivo* with a limited ability to survive, so no disease symptoms would result [57].

ISCOMS – Immune Stimulating Complexes and CpG as a new adjuvants are very hopeful [64]. Currently used vaccines are available on a limited scale only, live attenuated vaccines are available in Russia (LVS) and the USA. Apart from that several others are being tested: attenuated, killed and subunit vaccines [53, 64].

9. Clinical forms of tularaemia

Pathomechanism virulence factors and interactions between the host and the pathogen were widely discussed. Clinical forms of tularaemia and its course depend on many factors: virulence potential of the bacterium, the path of contact with organism the infectious dose, the disease course in the human body and the immunological status of the host. The effect may be a nearly symptomless form of tularaemia, but in some cases sepsis and sudden death may result. The incubation period of disease is between 3 to 5 days (sometimes up to 21 days) [20, 65, 70, 88] or from 1 to 14 days [88]. The onset of tularaemia is usually sudden, with high fever, shivers, headache, apathy, general fatigue, weakness, vomiting and conjunctivitis [65, 70, 71, 88]. Other symptoms may also be present, such as cough, chest discomfort, stomachache and diarrhoea. There exist several clinical forms of tularaemia: ulceroglandular, glandular, oculoglandular, oropharyngeal, pneumonic, intestinal and typhoidal [6, 31, 47, 48, 57, 65, 70, 71, 84, 88].

The ulceroglandular form constitutes from 45% to 85% of all tularaemia cases [70, 71]. Most commonly it results from an infected bite. Symptoms commonly appear between 3 to 6 days after infection, later an ulcer forms. During illness the patient may suffer from flu-like symptoms, including fever (38°C – 40°C), headache, chills and general fatigue [57]. Skin lesions may appear before, during or from one to a few days following adenopathy as red, painful pustules in the area of lymph nodes. There may also appear blisters resembling a herpes simplex varicella-zoster infection. Skin lesions may necrotise, forming ulcers with raised

edges. If no treatment is undertaken, the ulcers may be present for weeks and result in scarring. The site of ulceration depends on the route of infection in case of animal contact, palms and forearms are affected; after a tick bite ulceration is present on the torso, crotch, legs, head and neck. Lymphadenopathy location also depends on pathogenesis. Neck and nape adenopathy is most common in children; adults usually suffer from groin adenopathy. Skin lesions appearing over adenopathies, observed in 19.1% cases in Sweden, suggest suppuration. Lymphadenopathy is uncommon, but may accompany this disease form especially in cases of secondary infections of the initial ulceration with other bacteria [63]. Skin lesions may also present as black pustule surrounded by erythema and swelling. Infected lymph nodes may be the source of pathogen penetrating other organs and tissues, such as liver, spleen, intestines, kidneys, central nervous system and skeletal muscles. Often hyperbilirubinemia can be observed (including elevated levels of liver transaminases) and leukocytosis [70]. Unless antibiotics are given, lymph nodes may swell within 7–10 days and suppuration may follow. This may occur in 30–40% of cases and is one of the most serious complications of infections by *F. tularensis* subsp. *holarctica*. The ulceroglandular is the dominant form of the disease and constitutes more than 90% of tularaemia cases in Europe. Other subspecies of *F. tularensis* may also cause this form of the disease [82]. Frequently observed large swelling of lymph nodes may resemble bubonic plague. Glandular form does not present ulceration or suppuration; the form with fever but showing no lymphadenopathy is described as typhoidal or septic [57].

Oculoglandular form is a localised form of glandular tularaemia (Gałęzowski-Parinaud syndrome), initially described by a Polish ophthalmologist Gałęzowski working in France and a Frenchman Parinaud. It was also described in Poland.

Glandular form occurs in patients with a local lymphadenopathy, but lacking visible skin lesions. Such form occurs in the USA in fewer than 20% of cases. The glandular form develops similarly to the ulceroglandular type except for the development of skin lesions which may not occur at all or be minimal, atypical or unnoticed. Both glandular and ulceroglandular forms may present suppuration in lymph nodes. Suppuration is present in more than 20% of cases if no therapy with antibiotics is undertaken or if antibiotics are administered after the first two weeks of illness. The form of tularaemia with suppurating lymph nodes should be distinguished from: infections by *Streptococcus pyogenes*, cat scratch disease, syphilis, chancroid, lymphogranuloma venereum, tuberculosis, mycobacterial infections, toxoplasmosis, sporotrichosis, rat-bite fever, anthrax, plague and herpes simplex infections [65].

The oropharyngeal form constitutes only several percent of cases and is associated mainly with intestinal infections by contaminated food or water [64, 65]. It may be a variant of the ulceroglandular form [65]. A typical symptom is ulcerating and exudative stomatitis with or without tonsillitis. In the mouth and throat area ulceration may form causing pustules. Large cervical lymphadenopathy is usually one-sided, rarely symmetrical. Late commencement of treatment may result in suppuration, as it was observed in Turkey in 40% of oropharyngeal form of cases [65, 88]. In the pharyngeal form the main symptoms are high fever and acute throat ache. Some patients may show lesions on mucous membranes of the larynx, resembling diphtheria. In differential diagnosis streptococcal pharyngitis, mononucleosis, diphtheria and adenovirus infections should be considered. The suspicion of pharyngeal tularaemia should be taken under consideration in endemic regions and in patients with acute throat ache in the cases where penicillin treatment was unsuccessful and routine diagnostic tests showed no satisfactory results [65].

Pulmonary and septic forms of tularaemia, caused by *F. tularensis* subsp. *tularensis*, are the most dangerous disease forms with average mortality rates on 30–60%. Both forms may develop from ulceroglandular or glandular tularaemia, but the pulmonary form mainly follows infection by inhalation [57]. This form may manifest as typical pneumonia with cough, chest pains and elevated breathing frequency. High fever, nausea and vomiting may also be present as additional symptoms [88].

Significant differences in symptoms caused by A and B types of *F. tularensis* are well illustrated by the pulmonary type. Infections with the A type show fast decline in health with sudden chills, high fever, shortness of breath, dry or wet cough, chest pains, sweating, sleepiness and general fatigue [88]. Research from 1950s conducted on volunteers exposed to aerosol infection showed that the pulmonary form develops 3 to 5 days following the exposure [18]. High fever, up to 40°C, is a characteristic symptom. Other symptoms resemble pneumonia: bradycardia, chills, shortness of breath and dry cough. Headache, throat ache, muscular pains and nausea are also present. Frequently observed haemorrhagic airway inflammation may lead to bronchopneumonia [57]. The pulmonary form should be distinguished from pneumonia caused by *Mycoplasma* spp., *Legionella* spp., *Chlamydophila pneumoniae*, *Coxiella burnetii*, *Chlamydia psittaci*, *Mycobacterium tuberculosis*, pulmonary plague and others [66]. Intestinal tularaemia follows ingestion of contaminated food or water and may lead to gastritis and inflammation of intestines. This form may show symptoms from mild diarrhoea up to acute form with numerous ulcerations of the intestines [70, 71]. Typhoidal tularaemia is uncommon [70]. It may develop following an

infection via any of the possible paths of contact with microorganisms and it is the hardest to diagnose. In many patients it has a very serious form, from extreme exhaustion to sudden death. The main symptoms are fever with chills, headache, muscular pains, throat ache, nausea, vomiting, diarrhoea, stomachache, cough. Patient examination will show dehydration, lowered blood pressure, mild pharyngitis, cervical adenopathy, meningismus, increased muscle tone of stomach muscles. Hepatomegaly and splenomegaly are also encountered in acute cases of the disease and the risk of these increases with the progress of the disease [65]. The onset of the disease can be sudden (fever 38°C – 40°C). One of the symptoms is breakdown of skeletal muscles (rhabdomyolysis), morbidity in such cases reaches 50% [70, 71]. Typhoidal tularaemia should be distinguished from: typhoid fever (infection with *Salmonella typhi*), typhus fever (*Rickettsia provazekii*), infections with *Brucella* spp., *Legionella* spp., *C. burnetii*, mycobacteria, fungi, rickettsiae, *Plasmodium* spp. [65].

Among the diseases which should be differentiated from the ulceroglandular form, the following should be mentioned: skin lesions caused by *Staphylococcus* and *Streptococcus* infections, anthrax, pasteurellosis, tuberculosis, lymph node tuberculosis, infections with mycobacteria and rickettsiae, toxoplasmosis, cat scratch disease, plague, and viral infections with HIV (*Human Immunodeficiency Virus*), CMV (*Cytomegalovirus*) and EBV (*Epstein-Barr Virus*). Pulmonary tularaemia may present similarly to acute brucellosis, *Hantavirus* infections, leptospirosis, flu, *M. Pneumoniae* infection, Legionnaires' disease, TWAR (*Chlamydia trachomatis pneumoniae*), psittacosis, anthrax, Q fever, plague, SARS (*Severe Acute Respiratory Syndrome*) [88] and MERS-CoV (*Middle East Respiratory Syndrome Coronavirus*) and some deep mycoses. In tularaemia transmission the arthropods bite may play important role [25, 30, 47, 72, 91, 94, 96].

10. Tularaemia treatment

In acute tularaemia cases in adults where hospital admission is deemed necessary, parenteral administration of aminoglycosides is recommended gentamicin dosed 5 mg/kg/day is preferred; it should be divided into two doses and the concentration of the antibiotic in the serum should be monitored. Alternatively, streptomycin may be administered as an intramuscular injection given twice daily at 2 g/day for 10 days. In extremely severe cases the period of treatment will depend on the organism's response and it may extend much further than 10 days. In less extreme cases or when mass attack is suspected, oral administration of ciprofloxacin is preferred at 800–1000 mg/day,

intravenously in two doses; the period of treatment should be between 10–14 days. Doxycycline is also possible, given orally in two doses at 200 mg/day for no fewer than 15 days [86].

In the studies carried out by Grossman *et al.* (2017) the synthetic fluorocycline TP-271 was tested in outbreak events of aerolized exposure to *F. tularensis* for complicated inhalational infections [34].

In children requiring hospital treatment, parenteral administration of aminoglycosides is preferred, with gentamicin being the drug of choice at 5–6 mg/kg/day divided into two or three doses; the concentration of the antibiotic in the serum should be monitored. Alternatively, streptomycin can be administered at 15 mg/kg/day in two doses (maximally 2 g of the antibiotic per day). In milder cases caused by *F. tularensis*, type B which may occur in endemic areas, ciprofloxacin may also be used at the dosage of 15 mg/kg/day (maximally 1 g/day) [95].

In pregnant women it is necessary to consider possible side effects of treating severe infections. Despite gentamicin and ciprofloxacin not being admitted by the FDA (Food and Drug Administration, USA) to use in tularaemia infections treatment in pregnant women, these antibiotics are recommended by Johns Hopkins Working Group on Civilian Biodefense [18]. The dosage is identical with the dosage recommended for non-pregnant women and the treatment period should be decided on an individual basis [95].

Not much information is available for the patients with compromised immunological systems. Aminoglycosides should be the drug of choice, alternatively ciprofloxacin can be given. The dosage is similar to the patients with stable immunological systems and the treatment period may be up to 14 days. In cases requiring extended treatment, initial administration of aminoglycosides may be supplemented by oral or parenteral administration of ciprofloxacin [95]. Significant categorisation of tularaemia treatments in various population groups was presented by Dennis *et al.* [18].

11. Laboratory diagnostics of *F. tularensis*

F. tularensis as a facultative intracellular pathogen may be cultured on artificial media. One of them is CHAB (cysteine heart agar with blood), containing cysteine [55, 66, 67, 93], another is BCYE (buffered charcoal yeast extract) [56, 73, 74], also used as a selective medium in *L. pneumophila* culturing [14, 15]. Other media successfully used in culturing of *F. tularensis* are as follows: McCoy and Chapin, Francis, Cystine Heart Agar and fluid thioglycollate medium [84]. Recently two new selective media have been developed on the base of CHAB [59, 66]; they may be used to obtain and

multiply *F. tularensis* from contaminated solid tissues. The CHAB – A medium contains colistin, amphotericin, lincomycin, trimethoprim and ampicillin [66]. CHAB – PACCV, also successful in culturing *F. tularensis* from environmental samples, contains polymyxin B, amphotericin B, cycloheximide, cefepime and vancomycin [59]. Serological methods are most commonly used in laboratories confirming tularaemia diagnoses, with ELISA being the most popular method [11, 12, 13, 68, 76, 79]. Among others there are microagglutination tests [15, 68], immunochromatographic tests [12], Western blot tests [12, 68, 76], a micromatrix method [11] and an indirect immunofluorescence method [68].

Using a PCR (Polymerase Chain Reaction) method enables detection of *F. tularensis* genetic material in both clinical and environmental samples. The method is an essential tool in detection of microorganisms difficult to culture. For identification of species with a real time PCR technique, the most convenient nucleotide sequences are the genes *fopA* and *tul4*, coding the proteins of the external membrane of the *F. tularensis* cells [4, 5, 13, 21, 32, 37, 50, 81, 95]. In order to distinguish the type A from type B and other subspecies, the recommended PCR techniques should be aimed to detect the following genes: *pdpD* (present in type A *F. tularensis*, and missing in type B and *novicida*), *ISFtu2*, absent in type A and *novicida* [50], the gene coding 23 kDa protein [19, 83, 92, 93], *lpnA* (*tul4*) coding 17 kDa protein [10, 89]. For further detailed analyses within the species *F. tularensis* a real-time PCR technique has been developed which allows differentiation of subtypes A.I. and A.II. The goal sequences are RDs (Regions of differences) [87]. Another method allowing for differentiation within the species and subspecies is detection and sequencing of the genes 16S rRNA [17, 28, 50, 81].

12. Summary

Due to low infectious dose, high virulence, invasion and variable forms of the disease, the etiological agent of tularaemia is classified by the CDC (Centers for Disease Control and Prevention, USA) as a potentially highly dangerous agent of biological weapons (group A). Moreover, a broad distribution of the pathogen's reservoir makes *F. tularensis* a serious threat to human life and health. Clinical presentation of tularaemia varies largely and therefore poses diagnosis problems; in effect the number of tularaemia cases may go underreported. Diagnostics of tularaemia in Poland is mainly based on serological analyses and therefore not fully satisfactory due to the low specificity of serological methods resulting from an extremely high diversity among the *F. tularensis* species. As an alternative to these methods, genetic techniques may be used successfully in molecu-

lar characterisation of tularemia strains isolated from humans and animals in screening research and during epizootic and epidemic outbreaks.

References

1. Abd H., Johansson T., Golovliov I., Sandström G., Forsman M. Survival and growth of *Francisella tularensis* in *Acanthamoeba castellanii*. *Appl. Environ. Microb.* **69**, 600–606 (2003)
2. Alibek K.: The Soviet Union's Anti-Agricultural Biological Weapons. *Ann. N.Y. Acad. Sci.* **894**, 18–19 (1999)
3. Barns S.M., Grow C.C., Okinaka R.T., Keim P., Kuske C.R.: Detection of diverse new *Francisella*-like bacteria in environmental samples. *Appl. Environ. Microb.* **71**, 5494–5500 (2005)
4. Bielawska-Drózd A., Gawel J., Bartoszcze M., Kołodziej M., Graniak G., Joniec J., Michalski A.: Identification of *Francisella tularensis* by real-time PCR with TaqMan probes. *Lek. Wojsk.* **2**, 98–102 (2011)
5. Bielawska-Drózd A., Niemcewicz M., Gawel J., Bartoszcze M., Graniak G., Joniec J., Kołodziej M.: Identyfikacja *Francisella tularensis* techniką real-time PCR z wykorzystaniem sond hybrydujących zaprojektowanych dla fragmentów sekwencji genów *fopA* i *tul4*. *Med. Dosw. Mikrobiol.* **62**, 351–360 (2010)
6. Bilecki Ś.: Tularemia. Państwowe Wydawnictwo Rolnicze i Leśne. Warszawa, 1974
7. Birkbeck T.H., Bordevik M., Frøystad M.K., Baklien Å.: Identification of *Francisella* sp. from Atlantic salmon, *Salmo salar* L., in Chile. *J. Fish Dis.* **30**, 505–507 (2007)
8. Brevik O.J., Ottem K.F., Kamaishi T., Watanabe K., Nylund A.: *Francisella haliotica* sp. nov., a pathogen of farmed giant abalone (*Haliotis gigantea*) in Japan. *J. Appl. Microbiol.* **5**, 1044–1056 (2011)
9. Broman T., Thelaus J., Andersson A.C., Bäckman S., Wikström P., Larsson E., Forsman M.: Molecular detection of persistent *Francisella tularensis* subspecies *holarctica* in natural waters. *Int. J. Microbiol.* DOI: 10.1155/2011/851946 (2010)
10. Byström M., Böcher S., Magnusson A., Prag J., Johansson A.: Tularemia in Denmark: identification of a *Francisella tularensis* subsp. *holarctica* strain by real-time PCR and high-resolution typing by multiple-locus variable-number tandem repeat analysis. *J. Clin. Microbiol.* **43**, 5355–5358 (2005)
11. Carlsson H.E., Lindberg A.A., Lindberg G., Hederstedt B., Karlsson K.A., Agell B.O.: Enzyme-linked immunosorbent assay for immunological diagnosis of human tularemia. *J. Clin. Microbiol.* **10**, 615–621 (1979)
12. Chaignat V., Djordjevic-Spasic M., Ruettger A., Otto P., Klimpel D., Müller W., Tomaso H.: Performance of seven serological assays for diagnosing tularemia. *BMC Infect. Dis.* **14**, 234 (2014)
13. Chróst A., Gielarowiec K., Kałużewski S., Rastawicki W. Występowanie zakażeń wywołanych przez *Francisella tularensis* u ludzi w Polsce oraz laboratoryjna diagnostyka tularemii. *Med. Dośw. Mikrobiol.* **69**, 55–63 (2017)
14. Cianciotto N.P., Fields B.S.: *Legionella pneumophila* mip gene potentiates intracellular infection of protozoa and human macrophages. *Proc. Nat. Acad. Sci. USA*, **89**, 5188–5191 (1992)
15. Cirillo J.D., Falkow S., Tompkins L.S.: Growth of *Legionella pneumophila* in *Acanthamoeba castellanii* enhances invasion. *Infect. Immun.* **62**, 3254–3261 (1994)
16. Croddy E., Krcalova S.: Tularemia, biological warfare, and the battle for Stalingrad (1942–1943). *Mil. Med.* **166**, 837–838 (2001)
17. Del Blanco N.G., Dobson M.E., Vela A.I., De La Puente V.A., Gutierrez C.B., Hadfield T.L., Ferri E.R.: Genotyping of *Francisella tularensis* strains by pulsed-field gel electrophoresis, amplified fragment length polymorphism fingerprinting, and 16S rRNA gene sequencing. *J. Clin. Microbiol.* **40**, 2964–2972 (2002)
18. Dennis D.T., Inglesby T.V., Henderson D.A., Bartlett J.G., Ascher M.S., Eitzen E., Tonat K.: Tularemia as a biological weapon: medical and public health management. *JAMA*. **285**, 2763–2773 (2001)
19. Dentan C., Pavese P., Pelloux I., Boisset S., Brion J.P., Stahl J.P., Maurin M.: Treatment of tularemia in pregnant woman, France. *Emerg. Infect. Dis.* **19**, 996–998 (2013)
20. Ellis J., Oyston P.C.F., Green M., Titball R.W.: Tularemia. *Clin. Microbiol. Rev.* **15**, 631–646 (2002)
21. Emanuel P.A., Bell R., Dang J.L., McClanahan R., David J.C., Burgess R.J., Hadfield T.: Detection of *Francisella tularensis* within infected mouse tissues by using a hand-held PCR thermocycler. *J. Clin. Microbiol.* **41**, 689–693 (2003)
22. Escudero R., Toledo A., Gil H., Kováčsová K., Rodríguez-Vargas M., Jado I., Anda P. Molecular method for discrimination between *Francisella tularensis* and *Francisella*-like endosymbionts. *J. Clin. Microbiol.* **46**, 3139–3143 (2008)
23. Fedorova V.A., Sayapina I.V., Corbel M.J., Motin V.L.: Russian vaccines against especially dangerous bacterial pathogens. *Emerg. Microbes Infect.* **3**, DOI:10.1038/emi.2014.82 (2014)
24. Fong I.W., Alibek K.: Bioterrorism and infectious agents: a new dilemma for the 21st century (Vol. 4) (w) Tularemia and Bioterrorism, Chapter 3, red. L. Hodges, R.L. Penn, Springer Science & Business Media, Berlin, 2005, s. 71–98
25. Formińska K., Zasada A.A., Rastawicki W., Śmietanińska K., Bander D., Wawrzynowicz-Szczewska M., Yanushevych M., Niściogórska-Olsen J., Wawszczak M.: Increasing role of arthropod bites in tularemia transmission in Poland – case of reports and diagnostic methods. *Ann. Agric. Environ. Med.* **22**, 443–446 (2015)
26. Formińska K., Zasada A.A.: *Francisella tularensis* – podstępny patogen. *Post. Mikrobiol.* **56**, 187–195 (2017)
27. Forsman M., Henningson E.W., Larsson E., Johansson T., Sandström G. *Francisella tularensis* does not manifest virulence in viable but non-culturable state. *FEMS Microbiol. Ecol.* **31**, 217–224 (2000)
28. Forsman M., Sandström G., Jaurin B.: Identification of *Francisella* species and discrimination of type A and type B strains of *F. tularensis* by 16S rRNA analysis. *Appl. Environ. Microb.* **56**, 949–955 (1990)
29. Forsman M., Sandstrom G., Sjöstedt A.: Analysis of 16S ribosomal DNA sequences of *Francisella* strains and utilization for determination of the phylogeny of the genus and for identification of strains by PCR. *Int J Syst Microbiol.* **44**, 38–46 (1994)
30. Franke J., Fritsch J., Tomaso H., Straube E., Dorn W., Hildebrandt A. Coexistence of Pathogens in Host-Seeking and Feeding Ticks within a Single Natural Habitat in Central Germany. *Appl. Environ. Microbiol.* **76**, 6829–6836 (2010)
31. Friend M.: Tularemia. U.S. Geological Survey, Reston, 2006, s. 1–68
32. Fujita O., Tatsumi M., Tanabayashi K., Jamada A.: Development of a real-time PCR assay for detection and quantification of *Francisella tularensis*. *Jpn. J. Infect. Dis.* **59**, 46–51 (2006)
33. Garrity G.M., Bell J.A., Lilburn T.G.: Taxonomic Outline of the Prokaryotes Release 5.0. Bergey's Manual of and Systematic Bacteriology, 2nd ed, Springer, 2004
34. Grossman T.H., Anderson M.S., Christ D., Gooldy M., Henning L.N., Heine H.S., Kindt M.V., Lin W., Siefkas-Patterson K., Radcliff A.K., Tam, V.H. Sutcliffe J.H. The fluorocycline TP-271 is efficacious in models of aerosolized *Francisella tularensis* SCHU S4 infection in BALB/c mice and cynomolgus macaques. *Antimicrob. Agents and Ch.* doi:10.1128/AAC.00448-17 (2017)

35. Gunnell M.K., Robinson R.A., Adams B.J.: Natural selection in virulence genes of *Francisella tularensis*. *J. Mol. Evol.* **82**, 264–278 (2016)
36. Gürçan Ş.: Epidemiology of Tularemia. *Balk. Med. J.* **31**, 3–10 (2014)
37. Higgins J.A., Hubalek Z., Halouzka J., Elkins K.L., Sjöstedt A., Shipley M., Ibrahim M.S.: Detection of *Francisella tularensis* in infected mammals and vectors using a probe-based polymerase chain reaction. *Am. J. Trop. Med. Hyg.* **62**, 310–318 (2000)
38. Hodges L., Penn R. L.: Tularemia and bioterrorism (w) Bioterrorism and infectious agents: A new dilemma for the 21st century, red. I.W. Fong, Kenneth Alibek, Springer, New York, 2005, s. 71–98
39. Hollis D.G., Weaver R.E., Steigerwalt A.G., Wenger J.D., Moss C.W., Brenner D.J.: *Francisella philomiragia* comb. nov. (formerly *Yersinia philomiragia*) and *Francisella tularensis* biogroup *novicida* (formerly *Francisella novicida*) associated with human disease. *J. Clin. Microbiol.* **27**, 1601–1608 (1989)
40. Hubalek Z., Rudolf I.: *Francisella tularensis* prevalence and load in Dermacentor reticulatus ticks in an endemic area in Central Europe. *Med. Vet. Entomol.* **31**, 234–239 (2017)
41. Huber B., Escudero R., Busse H.J., Seibold E., Scholz H.C., Anda P., Spletstoesse W.D.: Description of *Francisella hispaniensis* sp. nov., isolated from human blood, reclassification of *Francisella novicida* (Larson et al. 1955) Olsufjev et al. 1959 as *Francisella tularensis* subsp. *novicida* comb. nov. and emended description of the genus *Francisella*. *Int. J. Syst. Evol. Micr.* **60**, 1887–1896 (2010)
42. Ivanov I.N., Mitkova N., Reye A.L., Hübschen J.M., Vatcheva-Dobrevska R.S., Dobрева E.G., Muller C.P.: Detection of new *Francisella*-like tick endosymbionts in *Hyalomma* spp. and *Rhipicephalus* spp. (Acari: Ixodidae) from Bulgaria. *Appl. Environ. Microb.* **77**, 5562–5565 (2011)
43. Jones C.L., Napier B.A., Sampson T.R., Llewellyn A.C., Schroeder M.R., Weiss D.S.: Subversion of host recognition and defense systems by *Francisella* spp. *Microbiol. Mol. Biol. Rev.* **76**, 383–404 (2012)
44. Kamaishi T., Fukuda Y., Nishijama M., Kawakami H., Matsujama T., Yoshinaga T., Oseko N.: Identification and pathogenicity of intracellular *Francisella* bacterium in three-line grunt *Parapristipoma trilineatum*. *Fish Pathology.* **40**, 67–72 (2005)
45. Kay W., Petersen B.O., Duus J.Ø., Perry M.B., Vinogradov E.: Characterization of the lipopolysaccharide and β -glucan of the fish pathogen *Francisella victoria*. *FEBS J.* **273**, 3002–3013 (2006)
46. Keim P., Johansson A., Wagner D.M.: Molecular epidemiology, evolution, and ecology of *Francisella*. *Ann. NY. Acad. Sci.* **1105**, 30–66 (2007)
47. Kłapeć T., Cholewa A.: Tularemia – wciąż groźna choroba. *Med. Og. Nauk Zdr.* **17**, 155–160 (2011)
48. Knap J. Tularemia (Tularaemia) (w) Choroby zakaźne i pasożytnicze – epidemiologia i profilaktyka. 6th ed., red. W. Magdzik D. Naruszewicz-Lesiuk, A. Zieliński, α-medica press, Bielsko-Biała, 2007 s. 312–317
49. Kocik J. Tularemia (w) Bioterroryzm. Zasady postępowania lekarskiego, red. K. Chomiczewski, J. Kocik, M.T. Szkoda, Państwowy Zakład Wydawnictw Lekarskich, Warszawa, 2002, s. 142–152
50. Kugeler K.J., Gurfield N., Creek J.G., Mahoney K.S., Versage J.L., Petersen J.M.: Discrimination between *Francisella tularensis* and *Francisella*-like endosymbionts when screening ticks by PCR. *Appl. Environ. Microb.* **71**, 7594–7597 (2005)
51. Larson M.A., Nalbantoglu U., Dayood K., Zentz E.B., Cer R.Z., Iwen P.C., Francesconi S.C., Bishop-Lilly K.A., Mokashi V.P., Sjöstedt A., Hinrichs S.H.: Reclassification of *Wolbachia persica* as *Francisella persica* comb. nov. and emended description of the family Francisellaceae. *Int. J. Syst. Evol. Microbiol.* **66**, 1200–1205 (2016)
52. Manuel M.J., Soto E., Moralis J.A., Hawke J.: A piscirickettsiosis-like syndrome in cultured Nile tilapia in Latin America with *Francisella* spp. as the pathogenic agent. *J. Aquat. Anim. Health.* **19**, 27–34 (2007)
53. Maron M.E., Barry E.M.: Live attenuated tularemia vaccines: recent development and future goal. *Vaccine* **31**, 3485–3491 (2013)
54. Mikalsen J., Colquhoun D.J.: *Francisella asiatica* sp. nov. isolated from farmed tilapia (*Oreochromis* sp.) and elevation of *Francisella philomiragia* subsp. *noatunensis* to species rank as *Francisella noatunensis* comb. nov., sp. nov. *Int. J. Syst. Evol. Micr.* DOI:10.1099/ijs.0.002139-0 (2009)
55. Mikalsen J., Olsen A.B., Tengs T., Colquhoun D.J.: *Francisella philomiragia* subsp. *noatunensis* subsp. nov., isolated from farmed Atlantic cod (*Gadus morhua* L.). *Int. J. Syst. Evol. Micr.* **57**, 1960–1965 (2007)
56. Mitchell J.L., Chatwell N., Christensen D., Diaper H., Minogue T.D., Parsons T.M., Weller S.A.: Development of real-time PCR assays for the specific detection of *Francisella tularensis* ssp. *tularensis*, *holarctica* and *mediaasiatica*. *Mol. Cell. Probes.* **24**, 72–76 (2010)
57. Mitchell S.L., Griffin K.F., Titball R.W.: Tularemia pathogenesis and immunity (w) Microorganisms and Bioterrorism, Springer, New York, 2006 s. 121–137
58. Morris B.J., Buse H.Y., Adcock N.J., Rice E.W. A novel broth medium for enhanced growth of *Francisella tularensis*. *Lett. Appl. Microbiol.* **64**, 394–400 (2017)
59. Nano F., Elkins K.: The Genus *Francisella*. Chapter 3.3.44, *Prokaryotes*. **6**, 1119–1132 (2003)
60. Olsufjev N.G., Meshcheryakova I.S.: Subspecific taxonomy of *Francisella tularensis* McCoy and Chapin 1912. *Int. J. Syst. Evol. Micr.* **13**, 872–874 (1983)
61. Ostland V.E., Stannard J.A., Creek J.J., Hedrick R.P., Ferguson H.W., Carlberg J.M., Westerman M.E.: Aquatic *Francisella*-like bacterium associated with mortality of intensively cultured hybrid striped bass *Morone chrysops* × *M. saxatilis*. *Dis. Aquat. Organ.* **72**, 135–145 (2006)
62. Ottem K.F., Nylund A., Karlsbakk E., Friis-Møller A., Krossøy B., Knappskog D.: New species in the genus *Francisella* (Gammaproteobacteria; Francisellaceae); *Francisella piscicida* sp. nov. isolated from cod (*Gadus morhua*). *Arch. Microbiol.* **188**, 547–550 (2007)
63. Ottem K.F., Nylund A., Karlsbakk E., Friis-Møller A., Kamaishi T.: Elevation of *Francisella philomiragia* subsp. *noatunensis* to *Francisella noatunensis* comb. nov. [syn. *Francisella piscicida* Ottem et al. (2008) syn. nov.] and characterization of *Francisella noatunensis* subsp. *orientalis* subsp. nov., two important fish pathogens. *J. Appl. Microbiol.* **106**, 1231–1243 (2009)
64. Oyston P.C.: *Francisella tularensis*: unravelling the secrets of an intracellular pathogen. *J. Med. Microbiol.* **57**, 921–930 (2008)
65. Penn R.L.: *Francisella tularensis* (Tularemia) (w) Principles and Practice of Infectious Diseases, red. L. Gerald, J.E. Mandell, R.D. Bennett, D. Mendel, Bennett, 7th ed. Churchill Livingstone, Elsevier, 2010, volume 2, s. 2927–2950
66. Petersen J.M., Schrieffer M.E., Carter L.G., Zhou Y., Sealy T., Bawiec D., Chu M.C.: Laboratory analysis of tularemia in wild-trapped, commercially traded prairie dogs, Texas, 2002. *Emerg. Infect. Dis.* **10**, 419–425 (2004)
67. Petersen J.M., Schrieffer M.E., Gage K.L., Monteneri J.A., Carter L.G., Stanley M., Chu M.C.: Methods for enhanced culture recovery of *Francisella tularensis*. *Appl. Environ. Microb.* **70**, 3733–3735 (2004)
68. Porsch-Özcürümez M., Kischel N., Priebe H., Spletstösser W., Finke E.J., Grunow R.: Comparison of enzyme-linked immu-

- nosorbent assay, Western blotting, microagglutination, indirect immunofluorescence assay, and flow cytometry for serological diagnosis of tularemia. *Clin. Diagn. Lab. Immunol.* **11**, 1008–1015 (2004)
69. Qu P.H., Chen S.Y., Scholz H.C., Busse H.J., Gu Q., Kämpfer P., Yang Z.C.: *Francisella guangzhouensis* sp. nov., isolated from air-conditioning systems. *Int. J. Syst. Evol. Microb.* **63**, 3628–3635 (2013)
 70. Rastawicki W., Jagielski M.: Tularemia. *Post Mikrobiol.* **44**, 265–273 (2005)
 71. Rastawicki W.: Tularemia (Tularaemia) (w) Choroby zakaźne i pasożytnicze – epidemiologia i profilaktyka, 7th ed., red. A. Bauman-Popczyk, M. Sadkowska-Todys, A. Zieliński, α-medica press, Bielsko-Biała, 2014, s. 423–433
 72. Reis C., Cote M., Paul R.E., Bonnet S. Questing ticks in suburban forest are infected by at least six tick-borne pathogens. *Vector Borne Zoonotic Dis.* **11**, 907–916 (2011)
 73. Rydzewski K., Schulz T., Brzuszkiewicz E., Holland G., Lück C., Fleischer J., Heuner K.: Genome sequence and phenotypic analysis of a first German *Francisella* sp. isolate (W12-1067) not belonging to the species *Francisella tularensis*. *BMC Microb.* **14**, 169 (2014)
 74. Santic M., Akimana C., Asare R., Kouokam J.C., Atay S., Kwaik Y.A.: Intracellular fate of *Francisella tularensis* within arthropod-derived cells. *Environ. Microb.* **11**, 1473–1481 (2009)
 75. Sato T., Fujita H., Ohara Y., Homma M.: Microagglutination test for early and specific serodiagnosis of tularemia. *J. Clin. Microbiol.* **28**, 2372–2374 (1990)
 76. Schmitt P., Splettstoesser W., Porsch-Özcürümez M., Finke E.J., Grunow R.A.: Novel screening ELISA and a confirmatory Western blot useful for diagnosis and epidemiological studies of tularemia. *Epidemiol. Infect.* **133**, 759–766 (2005)
 77. Schrallhammer M., Schweikert M., Vallesi A., Verni F., Petroni G.: Detection of a novel subspecies of *Francisella noatunensis* as endosymbiont of the ciliate *Euplotes raikovi*. *Microbial Ecology.* **61**, 455–464 (2011)
 78. Scoles G.A. Phylogenetic analysis of the *Francisella*-like endosymbionts of *Dermacentor* ticks. *J. Med. Entomol.* **41**, 277–286 (2004)
 79. Sharma N., Hotta A., Jamamoto Y., Fujita O., Uda A., Morikawa S., Tanabayashi K.: Detection of *Francisella tularensis*-specific antibodies in patients with tularemia by a novel competitive enzyme-linked immunosorbent assay. *Clin. Vaccine Immunol.* **20**, 9–16 (2013)
 80. Sinclair R., Boone S.A., Greenberg D., Keim P., Gerba C.P.: Persistence of category A select agents in the environment. *Appl. Environ. Microb.* **74**, 555–563 (2005)
 81. Sjöstedt A., Eriksson U., Berglund L., Tärnvik A.: Detection of *Francisella tularensis* in ulcers of patients with tularemia by PCR. *J. Clin. Microbiol.* **35**, 1045–1048 (1997)
 82. Sjöstedt A.: Tularemia: history, epidemiology, pathogen physiology, and clinical manifestations. *Ann. NY Acad.* **1105**, 1–29 (2007)
 83. Skottman T., Piiparinen H., Hyytiäinen H., Myllys V., Skurnik M., Nikkari S.: Simultaneous real-time PCR detection of *Bacillus anthracis*, *Francisella tularensis* and *Yersinia pestis*. *Eur. J. Clin. Microbiol. Infect. Dis.* **26**, 207–211 (2007)
 84. Skrodzki E.: Tularemia. Państwowy Zakład Wydawnictw Lekarskich. Warszawa, 1978
 85. Steiner D.J., Furuya Y., Jordan M.B., Metzger D.W.: Protective role for macrophages in respiratory *Francisella tularensis* infection. *Infect. Immun.* **85**, e00064-17 (2017)
 86. Sun L.V., Scoles G.A., Fish D., O'Neill S.L. *Francisella*-like endosymbionts of ticks. *J. Invertebr. Pathol.* **76**(4), 301–303 (2000)
 87. Svensson K., Larsson P., Johansson D., Byström M., Forsman M., Johansson A.: Evolution of subspecies of *Francisella tularensis*. *J. Bacteriol.* **187**, 3903–3908 (2005)
 88. Tärnvik A., Sandström G., Sjöstedt A. Epidemiological analysis of tularemia in Sweden 1931–1993. *FEMS Immunol. Med. Mic.* **13**, 201–204 (1996)
 89. Thelaus J., Andersson A., Broman T., Bäckman S., Granberg M., Karlsson L., Forsman M.: *Francisella tularensis* subspecies *holarctica* occurs in Swedish mosquitoes, persists through the developmental stages of laboratory-infected mosquitoes and is transmissible during blood feeding. *Micro. Ecol.* **67**, 96–107 (2014)
 90. Titball R.W., Petrosino J.F.: *Francisella tularensis* genomics and proteomics. *Ann. NY. Acad. Sci.* **1105**, 98–121 (2007)
 91. Tomanović S., Radulović Z., Masuzawa T., Milutinović M. Coexistence of emerging bacterial pathogens in *Ixodes ricinus* ticks in Serbia. *Parasitic Zoonoses in Europe.* **17**, 211–217 (2010)
 92. Tomaso H., Scholz H.C., Neubauer H., Al Dahouk S., Seibold E., Landt O., Splettstoesser W.D.: Real-time PCR using hybridization probes for the rapid and specific identification of *Francisella tularensis* subspecies *tularensis*. *Mol. Cell Probe.* **21**, 12–16 (2007)
 93. Versage J.L., Severin D.D., Chu M.C., Petersen J.M.: Development of a multitarget real-time TaqMan PCR assay for enhanced detection of *Francisella tularensis* in complex specimens. *J. Clin. Microbiol.* **41**, 5492–5499 (2003)
 94. Wójcik-Fatla A., Zajac V., Sawczyn A., Cisak E., Sroka J., Dutkiewicz J. Occurrence of *Francisella* spp. in *Dermacentor reticulatus* and *Ixodes ricinus* ticks collected in eastern Poland. *Ticks Tick-Born Dis.* **6**, 253–257 (2005)
 95. World Health Organization: Tularemia, <https://www.cdc.gov/tularemia/diagnosistreatment/index.html>. (26.09.2017)
 96. Zhang F., Liu W., Wu X.M., Xin Z.T., Zhao Q.M., Yang H., Cao W. C. Detection of *Francisella tularensis* in ticks and identification of their genotypes using multiple-locus variable-number tandem repeat analysis. *BMC Microbiology*, **8**, 152 (2008)