Ticks and Tick-borne Diseases 11 (2020) 101322

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

Original article

Genetic diversity of *Francisella tularensis* in Poland with comments on MLVA genotyping and a proposition of a novel rapid v4-genotyping

Kamila Formińska[a], Tomasz Wołkowicz[a], Klaudia Brodzik[a], Paweł Stefanoff[a,1], Elżbieta Gołąb[a], Aleksander Masny[a], Beata Osiak[b], Piotr Cieślik[c], Agata Bielawska-Drózd[c], Aleksandra Anna Zasada[a,*]

[a] National Institute of Public Health – National Institute of Hygiene, Warsaw, Poland
[b] Polish Air Force University, Dęblin, Poland
[c] Military Institute of Hygiene and Epidemiology, Puławy, Poland

ARTICLE INFO

Keywords:
Francisella tularensis
MLVA
Ticks
Mosquitoes
v4-genotyping
Schu S4

ABSTRACT

We investigated the genotypes of *Francisella tularensis* (*F. tularensis*) strains isolated in Poland during the period 1953–2013 and studied their genetic relationship to *F. tularensis* strains isolated in other countries using MLVA. We examined the mosquito and tick samples collected in Poland for the presence of *F. tularensis* DNA using PCR. Our results revealed a high genetic diversity among the strains of *F. tularensis* collected from Poland, suggesting that the bacterium is commonly found in the environment. However, we did not detect *F. tularensis* DNA in ticks and mosquitoes, showing that the arthropod bites might not be the main source of infection.

We also propose the application of a practical assay called v4-genotyping that can be directly performed on the clinical and environmental samples.

In addition, we discovered genetic variations among Schu S4 reference strains used in various laboratories and showed that MLVA analysis should not be based on amplicon sizes only because point mutations occurring within the MLVA loci might not always be manifested by a change in the amplicon size.

1. Introduction

Francisella tularensis (*F. tularensis*) is a small, highly virulent, Gram-negative intracellular pathogen that can cause an infectious disease called tularemia (Olsufjew and Meshcheryakova, 1983; Barns et al., 2005). Currently, four subspecies of *F. tularensis* have been identified: *tularensis*, *holarctica*, *mediasiatica*, and *novicida* (Pilo et al., 2009). This bacterium is reported to have been isolated in many countries of the northern hemisphere. *F. tularensis* ssp. *tularensis* is confined to North America, and is the most virulent subspecies infecting humans and animals. *F. tularensis* ssp. *holarctica* is found in Europe and Asia and causes a less virulent form of the disease than ssp. *tularensis*. The subspecies *mediasiatica* and ssp. *novicida* rarely infect human beings (Kingry and Petersen, 2014). Tularemia is commonly observed in wild rodents and its causative agent *F. tularensis* is transmitted to humans through direct contact with the infected animals and ruptured skin; bites of infected ticks, flies, and mosquitoes; and by consuming infected water or meat (Byström et al., 2015). What is worrisome is that tularemia is transmitted through not only arthropods but also wild animals, which can consequently spread the disease to the uninfected regions of the world. Symptoms of tularemia depend on the route of entry of the bacteria into the body and usually are nonspecific, such as fever, chills, headache, diarrhea, muscle and joint pain, nonproductive cough, and weakness. Depending on the nature of exposure, other symptoms including pneumonia, chest pain, ulcers of the skin or mouth, swollen lymph nodes, painful eyes, and sore throat may also be observed (Johansson et al., 2004). In humans, six clinical forms of tularemia are identified: ulceroglandular, glandular, typhoidal, oculoglandular, oropharyngeal, or septic (Pilo et al., 2009). Many cases remain unnoticed, because of the diagnostic challenges associated with this infection.

Outbreaks of tularemia have occurred in most of the European countries. During 2010–2014, a total of 3426 confirmed tularemia cases were recorded in EU/EEA, with the highest number of cases being reported in Sweden (1682 cases), Finland (426), Hungary (346 cases), Norway (337 cases), and the Czech Republic (233 cases) (ECDC, 2018).

* Corresponding author at: National Institute of Public Health – National Institute of Hygiene, Department of Sera and Vaccines Evaluation, Chocimska 24, 00-791 Warsaw, Poland.
E-mail address: azasada@pzh.gov.pl (A.A. Zasada).
[1] Current affiliate: Norwegian Institute of Public Health, Oslo, Norway.

https://doi.org/10.1016/j.ttbdis.2019.101322
Received 13 June 2019; Received in revised form 15 October 2019; Accepted 25 October 2019
Available online 31 October 2019

In Poland, 35 cases were recorded during this period (ECDC, 2018) and 614 cases during 1949–2009. In recent years, the number of tularemia cases has increased in Poland from 9 in 2015, with an incidence of 0.023 per 100,000, to 30 in 2017, with an incidence of 0.078 per 100,000 (Meldunki epidemiologiczne, 2019 NIZP-PZH). Tularemia is a notifiable disease in Poland and other EU countries.

In this research, we studied the genetic characteristics of *F. tularensis* strains isolated in Poland during the period 1953–2013 and assessed their genetic relationship to *F. tularensis* strains isolated in other parts of the world. In addition, we investigated the mosquito and tick samples collected from Poland for the presence of *F. tularensis*. Also, for the rapid genotyping of *F. tularensis* strains, we developed a novel rapid genotyping method which could be directly performed on a clinical sample.

2. Materials and methods

2.1. Bacterial strains and DNA samples

A total of 53 samples were investigated, including 16 *F. tularensis* strains isolated from human and animal samples in Poland during 1953–1962, five DNA samples isolated directly from clinical samples of human tularemia cases diagnosed in 2012–2013 in Poland, and 32 *F. tularensis* strains isolated in other European countries, USA, Japan, and China in 1960s. The reference strains used in this study were *F. tularensis* ssp. *novicida* Ft26, *F. tularensis* ssp. *holarctica* Ft104-15, and *F. tularensis* ssp. *tularensis* Schu S4 (Table 1).

2.2. Reidentification of strains and identification of F. tularensis in clinical samples

DNA was extracted from the strains and clinical samples using the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions. The extracted DNA was used directly for the tests or stored at −20 °C for further use. For species and subspecies identification, PCR assay was performed for *tul4*, *pdpD*, *FtC1-C4*, and *RD1* genetic markers (Johansson et al., 2000; Farlow et al., 2001; Broekhuijsen et al., 2003).

2.3. Occurrence of F. tularensis in mosquitoes and ticks

A total of 2995 ticks were collected from the region of central-eastern Poland (Lubelskie Voivodeship, southern part of Podlaskie Voivodeship, eastern part of Mazovian Voivodeship, and eastern part of Świętokrzyski Voivodeship) in 2010. Seventy-seven ticks were collected from southern Poland (neighborhood of Częstochowa), and 2180 mosquitoes were collected from the residential areas of central Poland during 2011–2012. DNA samples were extracted from ticks and mosquitoes using the DNeasy Blood & Tissue Kit according to the manufacturer's instructions. The extracted DNA was directly used for performing the tests. The presence of *F. tularensis* was analyzed by PCR for *tul4* genetic marker.

2.4. MLVA and v4-genotyping

MLVA was performed for six VNTR loci (Ft-V1, Ft-V2, Ft-V3, Ft-V4, Ft-V5, and Ft-V6) according to the method described by Farlow et al. (Farlow et al., 2001). The precise size of amplicons of VNTR markers and exact number of repeats were determined by DNA sequencing. Purified PCR products were sequenced by Genomed Inc. The results were analyzed using Chromas Ver. 1.45 and BioNumerics Ver. 6.6.

The MLVA (and v4-genotyping) diversity (D) was calculated as G/N (where G represented the number of genotypes and N the number of isolates). The discriminatory index (DI) was determined according to Hunter and Gaston (Hunter and Gaston, 1988), and the confidence intervals were calculated according to Grundmann et al. (Grundmann et al., 2001).

3. Results

3.1. Reidentification of the strains and identification of F. tularensis in clinical samples

The PCR results were positive for the presence of *tul4* marker in all the investigated strains and clinical samples and confirmed the presence of *F. tularensis*. However, the results obtained for subspecies identification were difficult to interpret. The repeatability of amplification pattern for the *pdpD* marker was a point of concern. For the FtC1-C4 marker, we obtained amplicons of 150 bp for 51 samples (strains and clinical samples) and amplicons of 180 bp for 5 strains. The size of the amplicons was confirmed by DNA sequencing. However, according to Farlow et al. (2001), the size of amplicons should have been 300 and 330 bp. To verify the results, we conducted a bioinformatics analysis using the available databases. We confirmed the allele size of the genome of *F. tularensis* ssp. *tularensis* Schu S4 strain to be 180 bp (GenBank: NC_006570.2) using CLC Sequence Viewer Ver. 6.9.1. We also performed *in silico* PCR for the described and sequenced reference strains available on http://isilico.ehu.es. For the tested genome of *F. tularensis* ssp. *holarctica,* the size of the amplified product was found to be 150 bp, which was in accordance with our study results. The FtC1-C4 marker is known to differentiate *F. tularensis* ssp. *tularensis* from the other subspecies. However, we obtained identical amplicons for ssp. *tularensis* Schu S4 and ssp. *novicida* Ft-26. Finally, we identified the subspecies based on the RD1 marker. All the strains isolated in Poland and detected in clinical samples belonged to ssp. *holarctica*.

3.2. Occurrence of F. tularensis in mosquitoes and ticks

Francisella tularensis DNA was not detected in any of the 3072 tick and 2180 mosquito samples investigated in this study. But the presence of DNA in the samples after extraction was confirmed using agarose gel electrophoresis.

3.3. MLVA

We successfully amplified almost all the MLVA loci of all the investigated strains and clinical samples, except for locus Ft-V1 in strain 5352 (Poland), locus Ft-V2 in strain 17 (the Czech Republic), locus Ft-V3 in strain 16 (Poland), and locus Ft-V4 in strains 2A (the Czech Republic) and 5246 (Poland) where amplification was unsuccessful. The genetic relationship between the investigated strains is presented in Fig. 1. The number of VNTRs was analyzed based on the nucleotide sequences of the loci and not only by analysis of the final length of the amplified product. Among the 56 *F. tularensis* strains tested, we identified 32 unique MLVA genotypes (Table 1). Among the 21 *F. tularensis* strains isolated in Poland during 1951–2013, we identified 14 unique MLVA genotypes. The variability of the Polish strains was limited to 2 loci, Ft-V1 and Ft-V4, that varied in the number of repeats from 3 to 5 and from 7 to 19, respectively. Only strain 5352 had 5 repeats in the Ft-V5 locus, whereas the rest of the Polish strains had 4 repeats in this locus. The investigated strains collected in the other parts of the world showed variability in loci Ft-V1 (3–5 repeats), Ft-V2 (1–18 repeats), Ft-V3 (1–4 repeats), Ft-V4 (2–21 repeats), and Ft-V5 (4–5 repeats). No variability was detected in the Ft-V6 locus which had 5 repeats in all the strains. The diversity of 21 *F. tularensis* ssp. *holarctica* strains isolated in Poland and counted based on the aforementioned 6 MLVA loci was 0.67. Interestingly, the 5 DNA samples collected from tularemia patients during 2012–2013 in Poland represented 5 distinct genotypes of *F. tularensis,* and only the isolate 7116 corresponded to a genotype identified previously in a strain isolated from a hare. We also observed changes in the genome of *F. tularensis* ssp. *tularensis* Schu S4 reference strain. The locus Ft-V4 (called Ft-M3 by Johansson et al., 2004) contained 21 repeats, whereas it was supposed to contain 25 repeats according to Farlow et al. (2001) and Johansson et al. (2004). The locus

Table 1
MLVA genotypes and v4-genotypes of the investigated *F. tularensis* strains.

Strain/DNA sample	Country	Year of isolation	Source	MLVA genotypes	v4-genotypes
1	France	1962	Nd	3-1-2-4-4-5	4-6-0-0-2
2A	Czechoslovakia	1963	Nd	5-2-2-0-4-5	–
3	Czechoslovakia	1962	Nd	4-2-2-7-4-5	7-4-0-0-2
5	USA	1964	Nd	3-2-2-13-4-5	13-3-0-0-2
6	Czechoslovakia	1962	Nd	5-2-2-8-4-5	8-3-0-0-2
7A	Czechoslovakia	1962	Nd	3-1-2-2-5-5	2-1-0-0-2
10	Poland	1964	Nd	3-2-2-13-4-5	13-3-0-0-2
11	Poland	1959	Nd	5-2-2-7-4-5	7-3-0-0-2
12	Poland	1951	Nd	4-2-2-7-4-5	7-4-0-0-2
16	Poland	1962	Nd	5-2-0-7-4-5	7-3-0-0-2
17	Czechoslovakia	1963	Nd	3-0-2-2-5-5	2-1-0-0-2
19A	Czechoslovakia	1963	Nd	5-2-2-7-4-5	7-3-0-0-2
21	Czechoslovakia	1963	Nd	5-2-2-7-4-5	7-3-0-0-2
23	Czechoslovakia	1963	Nd	4-8-2-2-5-5	2-1-0-0-2
25	USA	1964	Nd	5-2-2-8-4-5	8-3-0-0-2
26	Poland	1961	Nd	3-2-2-14-4-5	14-3-0-0-2
29	Poland	1959	Nd	4-2-2-14-4-5	14-3-0-0-2
31	Czechoslovakia	1963	Nd	3-1-2-6-5-5	6-1-1-0-2
32A	Czechoslovakia	1963	Nd	3-2-2-16-4-5	16-5-0-0-2
32	Czechoslovakia	1963	Nd	3-10-4-18-5-5	18-2-1-0-2
33	Czechoslovakia	1963	Nd	5-2-2-6-4-5	6-3-0-0-2
34	Czechoslovakia	1964	Nd	3-5-2-2-5-5	2-1-0-0-2
35	USA	1964	Nd	3-4-2-2-5-5	2-1-0-0-2
36	USSR	1964	Nd	4-2-2-7-4-5	7-4-0-0-2
38A	USSR	1964	Nd	3-2-1-6-5-5	6-1-1-0-2
40	USSR	1964	Nd	3-18-4-21-5-5	21-1-3-0-2
43	USSR	1965	Nd	3-2-2-15-4-5	15-3-0-0-2
44	China	1964	Nd	4-2-2-15-4-5	15-3-0-0-2
46A	USSR	1965	Nd	3-1-2-2-5-5	2-1-0-0-2
47	USSR	1965	Nd	3-12-2-2-5-5	2-1-0-0-2
49	USA	1961	Nd	4-2-2-10-4-5	10-3-0-0-2
50	USA	1961	Nd	3-2-2-14-4-5	14-3-0-0-2
51	USA	1961	Nd	3-2-2-14-4-5	14-3-0-0-2
58A	Japan	1965	Nd	5-2-2-6-4-5	6-3-0-0-2
60A	USA	1961	Nd	3-2-2-2-5-5	2-1-0-0-2
ACP	Czechoslovakia	1962	Nd	5-4-2-8-4-5	8-3-0-0-2
AC	Sweden	1965	Nd	5-5-2-8-4-5	8-3-0-0-2
NOW	Japan	1961	Nd	3-2-2-2-5-5	2-1-0-0-2
1W	Poland	1953	Hare *Lepus europaeus*	5-2-2-7-4-5	7-3-0-0-2
2W	Poland	1953	Hare *Lepus europaeus*	5-2-2-7-4-5	7-3-0-0-2
3W	Poland	N/A	Hare *Lepus europaeus*	5-2-2-7-4-5	7-3-0-0-2
4W	Poland	N/A	Hare *Lepus europaeus*	5-2-2-8-4-5	8-3-0-0-2
6W	Poland	N/A	Ticks *Ixodes ricinus*	5-2-2-7-4-5	7-3-0-0-2
7W	Poland	1953	Human	3-2-2-15-4-5	15-3-0-0-2
9W	Poland	1953	Ticks *Ixodes ricinus*	3-2-2-15-4-5	15-3-0-0-2
10W	Poland	N/A	Common vole *Microtus arvalis*	3-2-2-15-4-5	15-3-0-0-2
11W	Poland	1954	Hare *Lepus europaeus*	3-2-2-16-4-5	16-3-0-0-2
13W	Poland	1956	Human, lymph node	3-2-2-19-4-5	19-3-0-0-2
7140	Poland	2012	Human, lymph node	3-2-2-9-4-5	9-3-0-0-2
7309	Poland	2012	Human, lymph node	5-2-2-10-4-5	10-3-0-0-2
5246	Poland	2013	Human, lymph node	4-2-2-0-4-5	–
7116	Poland	2013	Human, lymph node	5-2-2-8-4-5	8-3-0-0-2
5352	Poland	2013	Human, lymph node	0-2-2-7-5-5	7-3-0-0-2
Ft-26	–	–	Nd	3-10-2-11-5-5	11-5-3-24-1
Schu S4	–	–	Nd	3-18-4-21-5-5	21-1-3-0-2
Ft-104-15	–	–	Nd	4-2-2-21-5-5	21-1-3-0-2

Ft-V1 (called Ft-M6 by Johansson et al., 2004) contained 3 repeats in our strain, which was consistent with the results of Farlow et al. (2001). Johansson et al. (2004), however, demonstrated the presence of 4 repeats in this locus in the Schu S4 strain. In our study, the fourth repeat in Schu S4 strain contained 2 point mutations, substitution of T to A and deletion of G, and the mutated sequence was found to be TTGGTGAA CTTTCaT-CTCTT (compared to the sequence without mutations: TTG GTGAACTTTCTTGCTCTT).

3.4. v4-genotyping

An in-depth analysis of the nucleotide sequences of MLVA loci revealed that the most variable locus Ft-V4 contained up to 5 different 9-nucleotide length repeats. The nucleotide sequences of the repeats were as follows: AATAAGGAT (the repeat described by Farlow et al., 2001), AACAAAGAC, AATAAAGAC, AATAAAGAT, and CAAAATGAG, referred to as loci Ft-V4a, Ft-V4b, Ft-V4c, Ft-V4d, and Ft-V4e, respectively, in this study. Based only on the 5 Ft-V4 loci (a–e), we created a minimum spanning tree for the investigated strains (Fig. 2). Among the 54 strains, we identified 18 Ft-V4 genotypes, and the calculated diversity was 0.34. The calculated DI was 0–921 and the confidence interval was 89–95.2%. Two strains that did not give positive PCR reactions with Ft-V4 primers were excluded from the analysis. The diversity at each individual locus varied remarkably. Ft-V4a demonstrated greater diversity (D = 0.278) with 15 alleles identified, while Ft-V4d and Ft-V4e showed the least amount of diversity (D = 0.037) with only 2 alleles identified in each of these loci. Among the 20 strains isolated in Poland (1 strain was PCR-negative for Ft-V4), we identified 10 v4-genotypes

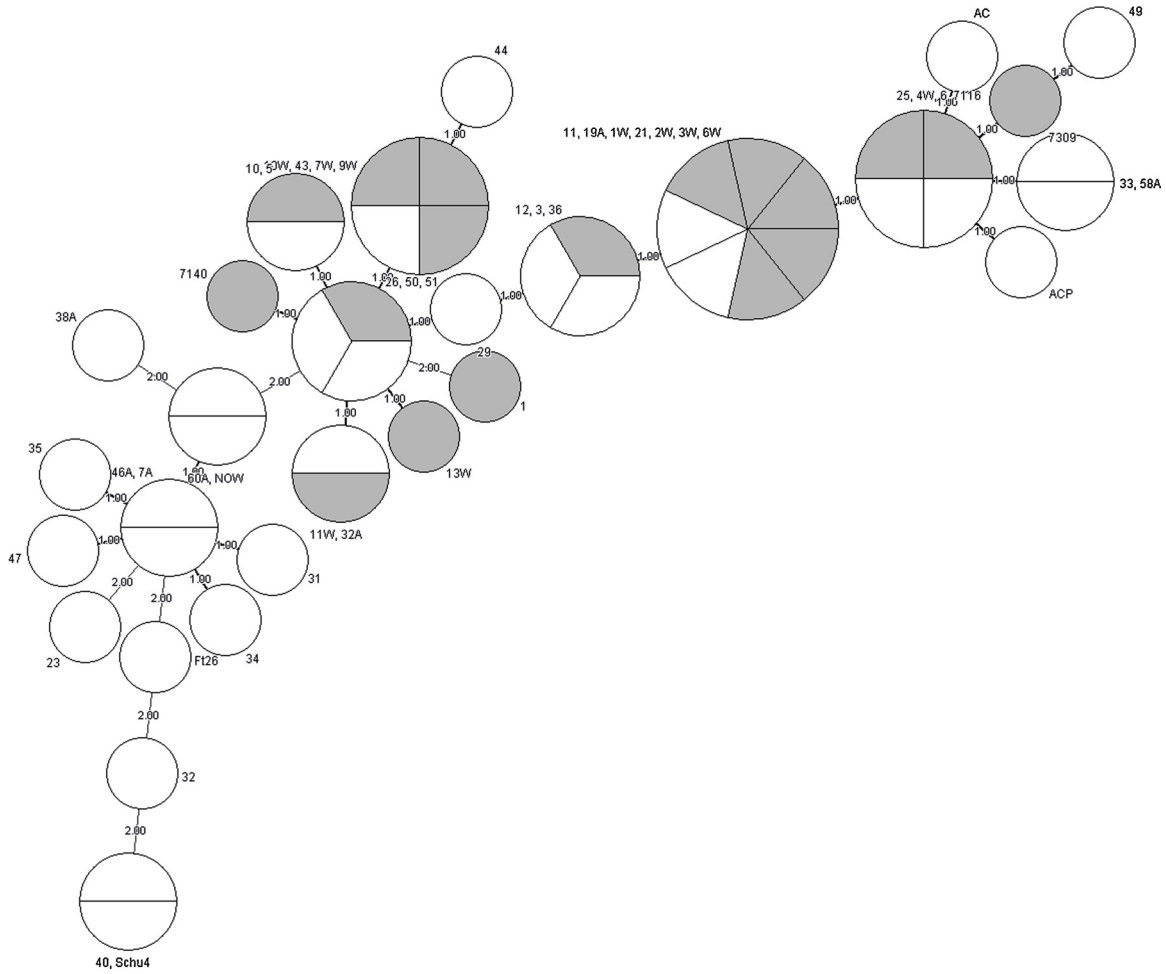

Fig. 1. A minimum spanning tree created based on the MLVA results for the investigated *F.tularensis* strains. Strains isolated in Poland are marked in gray.

and the calculated diversity was 0.5. Among all the investigated strains, 2 Ft-V4 genotypes showed the highest frequency of 16.67% (9/54): 2-1-0-0-2 and 7-3-0-0-2. Seven v4-genotypes were represented by single strain (Table 1).

4. Discussion

Tularemia is endemic in many regions of Europe, including countries bordering Poland like Germany, the Czech Republic, Slovakia, and Ukraine (Maurin and Gyuranecz, 2016). The genetic diversity of *F. tularensis* isolates has been investigated in particular regions of Europe (Johansson et al., 2004; Gehringer et al., 2013; Borde et al., 2017). However, in the case of Poland, no data on *F. tularensis* genotypes have been published, except for a recent paper which presented the results of Multispacer Typing (MST) for 15 strains isolated during the period 1953–1966 (Cieślik et al., 2018). Despite the fact that the number of isolates available for investigation was limited, we focused our study on Poland. The strains of *F. tularensis* isolated in Poland revealed significant genetic variability. Changes in the number of repeats were detected not only in the hypervariable locus Ft-V4 but also in the less variable Ft-V1 and the stable Ft-V5 loci. The variability is reflected in historical strains isolated mainly from animals and humans as well as in DNA samples isolated from patients with tularemia in recent years (2012–2013). Each of these 5 DNA samples isolated from patients with tularemia represented a different genotype of *F. tularensis*, although they were obtained from 3 regions of Poland (2 cases from Szczecin, 2 from Warsaw, and 1 from Chorzów) as described previously (Formińska et al., 2015). These findings suggest that *F. tularensis* ssp. *holarctica* occurred and dispersed in Poland long time ago and that the present genetic structure of the species exhibits high variability. In contrast to these results, Ariza-Miguel et al. (2014) revealed limited diversity of *F. tularensis* strains isolated from tularemia outbreaks in Spain. The 98 isolates obtained from Spain were classified into 8 MLVA types, which were essentially grouped into 2 closely related clonal complexes that differed only at 1 of the 16 MLVA markers used for genotyping. Less diverse *F. tularensis* strains were observed in France, where 8 MLVA genotypes were identified among 103 strains and the majority of the strains belonged to 2 predominating genotypes (Vogler et al., 2011).

In general, some of the MLVA loci showed extremely high variability, whereas others were more conserved. To study *F. tularensis* evolution comprehensively, highly mutable loci and loci with low mutation rates should be included in the analysis. Goethert et al. (2009) excluded Ft-V1 (Ft-M6), among others, in their analysis because they did not notice any differences in this locus among the investigated strains. In our study, this locus was represented by 4 alleles.

We also identified 5 distinct tandem repeat sequences at the Ft-V4 locus, also referred to as Ft-M3 locus according to the nomenclature proposed by Johansson et al. (2004). Three of the 5 tandem repeat sequences were present in all the investigated strains. Identification of additional repeats within the Ft-V4 locus enables extending the MLVA typing scheme and increasing the discriminatory power of the method. We used the newly identified repeats, referred to as loci Ft-V4a, Ft-V4b, Ft-V4c, Ft-V4d, and Ft-V4e, for v4-genotyping which has been found to be a rapid and cost-effective method for the investigation of initial outbreaks. The PCR reaction with only a pair of primers and a sequencing reaction can give preliminary information regarding

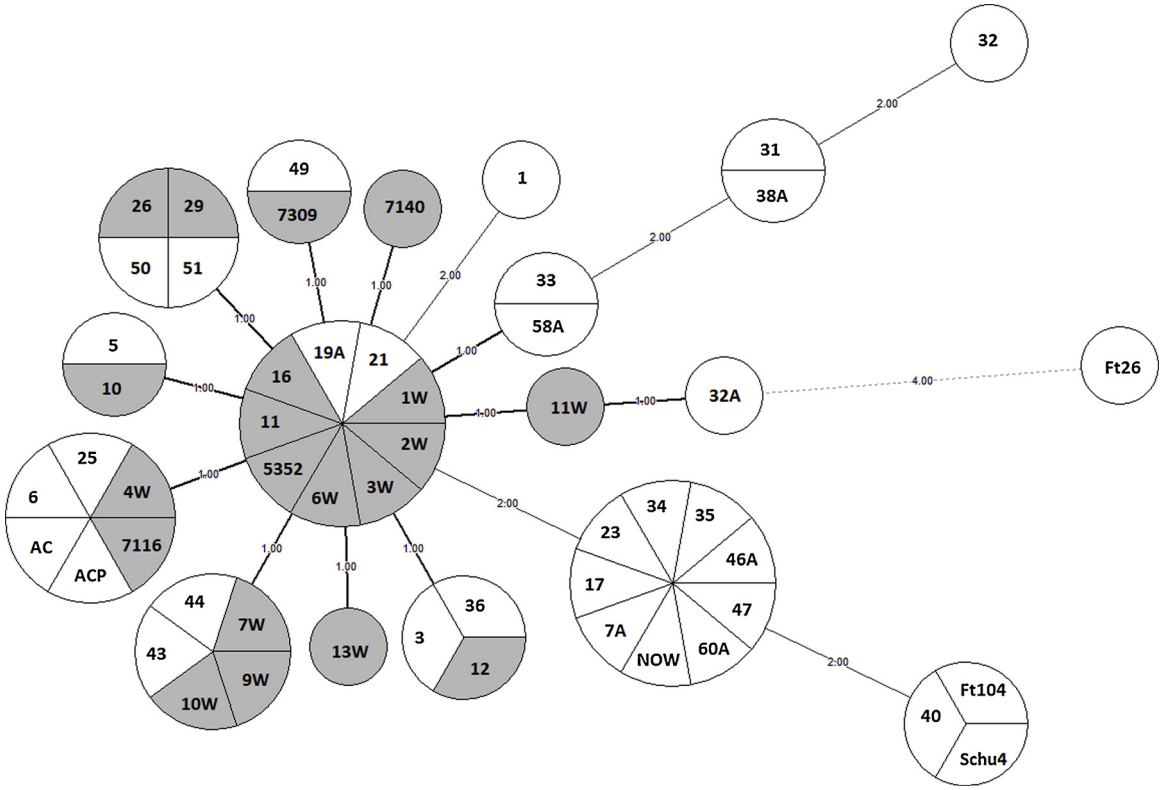

Fig. 2. A minimum spanning tree created based on the v4-genotyping results for the investigated *F. tularensis* strains. Strains isolated in Poland are marked in gray.

similarity of the investigated isolates. The v4-genotyping scheme includes highly variable locus Ft-V4a, variable locus Ft-V4b, and less variable loci Ft-V4c, Ft-V4d, and Ft-V4e. A combination of loci with different variabilities enables risk minimization for erroneous estimates of relationships among strains. Genotyping using only highly variable loci may show extremely diverse results among closely related strains, whereas the use of only low variable loci may not discriminate between unrelated strains. Moreover, in the v4-genotyping assay, PCR can be performed on the clinical and environmental samples directly, without requiring any bacterial culture that is especially important in the case of *F. tularensis* as it is a fastidious and slow-growing bacterium. Genotyping involving only one PCR reaction (single tube) is also crucial when the amount of sample is very limited. This feature of v4-genotyping technique provides an advantage over the MLVA genotyping methods which can also be conducted on a clinical sample directly (e.g., Pailhories et al., 2015). However, it is important to note that DNA quality, concentration, and presence of inhibiting factors may exert an influence on PCR, and therefore selection of an appropriate method for DNA extraction is essential for genotyping of the clinical samples directly.

Comparing the results of our study with the published results of other authors, we noticed changes in the genome of *F. tularensis* ssp. *tularensis* Schu S4 reference strain. The observed genetic differences among Schu S4 strains obtained from various laboratories might be related to laboratory-induced mutations. Our finding showed that the use of a particular strain, such as Schu S4, as a reference strain for genotyping methods should be preceded by careful maintenance of its genome stability. Special attention should be paid to hypervariable markers like Ft-V4 (Ft-M3). Moreover, the MLVA analysis should not be based on amplicon size only, as has been suggested by other studies (Johansson et al., 2004; Vogler et al., 2009), and the amino acid sequence of each MLVA locus should also be analyzed. Point mutation within a locus would not be reflected in differences in amplicon size but would rather affect the number of tandem repeats.

An increasing role of ticks and mosquitoes in tularemia transmission has been observed in many countries (Rojko et al., 2016; Borde et al., 2017). *F. tularensis* DNA was detected in 8.4% of *Ixodes ricinus* ticks and in 57% of the ticks of the genus *Dermacentor* (nymphs and adults life stage were investigated) from south-western Germany (Gehringer et al., 2013). In Sweden, mosquitoes are considered to be major vectors of the bacterium and the presence of *F. tularensis* ssp. *holarctica* was confirmed in 11 out of the 14 mosquito species sampled (Thelaus et al., 2014). In South Moravia, the Czech Republic, *F. tularensis* was detected in 2.6% of *Dermacentor reticulatus* and 0.2% of *I. ricinus* ticks but was not detected in mosquitoes (Hubálek et al., 1996). In Poland, tularemia cases related to arthropod bites were also described recently (Switaj et al., 2009; Moniuszko et al., 2011; Formińska et al., 2015). However, the presence of *F. tularensis* in ticks and mosquitoes is far from common. Wójcik-Fatla et al. (2015) identified a *F. tularensis*-positive tick sample from 1391 ticks collected in eastern Poland during 2011–2012, and Bielawska-Drózd et al. (2018) identified 0.49% tularensis-positive ticks among 1551 ticks collected in Drawsko County (north-western Poland in 2017). In contrast, we did not detect *F. tularensis* DNA in any of the tick and mosquito samples collected in southern Poland (the neighborhood of Częstochowa), central-eastern Poland, and in residential areas of central Poland. This finding might suggest that a relatively small number of arthropods are infected with *F. tularensis* in Poland, since the investigated arthropods were collected in the period preceding and corresponding to the period when the arthropod bite-related tularemia cases were recorded. Nevertheless, the situation is subjected to changes and should be monitored continuously because arthropods and wild animals do not respect borders. Moreover, the role of arthropods as vectors of animal and human diseases is gaining importance due to climatic changes. The range of occurrence of arthropod species is growing. For example, ticks have been found recently in the polar circle (Soleng et al., 2018) and in Iceland (Alfredsson et al., 2017), and mosquito species related to dengue, Zika, West Nile fever, and chikungunya have been detected in more and more regions of Europe (Ebi and Nealon, 2016; Conduto et al., 2018).

All the *F. tularensis* strains isolated in Poland and investigated in this

study belong to the ssp. *holarctica* that is found to be broadly distributed in the northern hemisphere. The ssp. *tularensis* has not been identified in Poland yet, although there are some reports of isolation of this species in Europe (Gurycova, 1998; Maurin and Gyuranecz, 2016).

5. Conclusions

The present study revealed a high genetic diversity among the strains of *F. tularensis* in Poland. Arthropods are not the main source of infection, although arthropod bite-related tularemia cases have been described in Poland recently (Switaj et al., 2009; Moniuszko et al., 2011; Formińska et al., 2015).

Our study showed that MLVA analysis should not be based on amplicon size only but should also be accompanied by nucleotide sequence analysis, because point mutations occurring within the MLVA loci might not be reflected in the amplicon size.

V4-genotyping, proposed in this study, is a practical assay that enables rapid genotyping of *F. tularensis* strains and is also a cost-effective technique. It incorporates the advantages of MLVA, such as numeric characters of the obtained data, which are well-suited for easy transfer among laboratories, and lack of necessity to culture the bacterium. It also overcomes the disadvantages of MLVA such as requiring several PCRs and sequencing reactions, which require larger amount of sample.

Declaration of Competing Interest

None.

Acknowledgments

This work was supported by National Science Center (grant no. UMO-2013/11/N/NZ6/01247).

References

Alfredsson, M., Olafsson, E., Eydal, M., Rut Unnsteinsdottir, E., Hansford, K., Wint, W., Alexander, N., Medlock, J.M., 2017. Surveillance of *Ixodes ricinus* ticks (Acari: ixodidae) in Iceland. Parasit. Vectors 10, 466. https://doi.org/10.1186/s13071-017-2375-2.

Ariza-Miguel, J., Johansson, A., Fernández-Natal, M.I., Martínez-Nistal, C., Orduña, A., Rodríguez-Ferri, E.F., Hernández, M., Rodríguez-Lázaro, D., 2014. Molecular investigation of tularemia outbreaks, Spain, 1997–2008. Emerg. Infect. Dis. 20, 754–761. https://doi.org/10.3201/eid2005.130654.

Barns, S.M., Grow, C.C., Okinaka, R.T., Keim, P., Kuske, C.R., 2005. Detection of diverse new *Francisella*-like bacteria in environmental samples. Appl. Environ. Microbiol. 71, 5494–5500. https://doi.org/10.1128/AEM.71.9.5494-5500.2005.

Bielawska-Drózd, A., Cieślik, P., Żakowska, D., Głowacka, P., Wlizło-Skowronek, B., Zięba, P., Zdun, A., 2018. Detection of *Coxiella burnetii* and *Francisella tularensis* in tissues of wild-living animals and in ticks of north-west Poland. Pol. J. Microbiol. 67, 529–534. https://doi.org/10.21307/pjm-2018-059.

Borde, J.P., Zange, S., Antwerpen, M.H., Georgi, E., von Buttlar, H., Kern, W.V., Rieg, S., 2017. Five cases of vector-borne *Francisella tularensis holarctica* infections in southwestern Germany and genetic diversity. Ticks Tick-borne Dis. 8, 808–812. https://doi.org/10.1016/j.ttbdis.2017.06.009.

Broekhuijsen, M., Larsson, P., Johansson, A., Byström, M., Eriksson, U., Larsson, E., Prior, R.G., Sjöstedt, A., Titball, R.W., Forsman, M., 2003. Genome-wide DNA microarray analysis of *Francisella tularensis* strains demonstrates extensive genetic conservation within the species but identifies regions that are unique to the highly virulent F. tularensis subsp. tularensis. J. Clin. Microbiol. 41, 2924–2931. https://doi.org/10.1128/JCM.41.7.2924-2931.2003.

Byström, M., Böcher, S., Magnusson, A., Prag, J., Johansson, A., 2015. Tularemia in Denmark: identification of a *Francisella tularensis subsp. holarctica* by real-time PCR and high-resolution typing by multiple-locus variable-number tandem repeat analysis. J. Clin. Microbiol. 10, 5355–5358. https://doi.org/10.1128/JCM.43.10.5355-5358.2005.

Cieślik, P., Knap, J.P., Piechowicz, L., Bielawska-Drózd, A., 2018. Subspecies differentiation and genotyping of *Francisella tularensis* strains isolated from clinical and environmental samples. Lett. Appl. Microbiol. 67, 550–556. https://doi.org/10.1111/lam.13063.

Conduto, A.D., Sanseverino, I., Pozzoli, L., Lettieri, T., 2018. Toward Climate Change Impact: Vectors Carrying Viral Infection: What We Should Know. IRC Technical Reports. EUR 29001 EN. Publications Office of the European Union, Luxembourg ISBN 978-92-79-80856-2, doi:10.2760/067022, JRC107421.

Ebi, K.L., Nealon, J., 2016. Dengue in a changing climate. Environ. Res. 151, 115–123. https://doi.org/10.1016/j.envres.2016.07.026.

European Centre for Disease Prevention and Control, 2018. Annual Epidemiological Report 2015. ECDC, Tularaemia. Stockholm (Accessed 13 June 2019). https://ecdc.europa.eu/sites/portal/files/documents/AER_for_2015-tularaemia.pdf.

Farlow, J., Smith, K.L., Wong, J., Abrams, M., Lytle, M., Keim, P., 2001. *Francisella tularensis* strain typing using multiple-locus variable – number tandem repeat analysis. J. Clin. Microbiol. 9, 3186–3192. https://doi.org/10.1128/JCM.39.9.3186-3192.2001.

Formińska, K., Zasada, A.A., Rastawicki, W., Śmietańska, K., Bander, D., Wawrzynowicz-Syczewska, M., Yanushevych, M., Niścigórska-Olsen, J., Wawszczak, M., 2015. Increasing role of arthropod bites in tularaemia transmission in Poland – case reports and diagnostic methods. Ann. Agric. Environ. Med. 22, 443–446. https://doi.org/10.5604/12321966.1167711.

Gehringer, H., Schacht, E., Maylaender, N., Zemna, E., Kayser, P., Oehme, R., Pluta, S., Splettstoesser, W.D., 2013. Presence of an emerging subclone of *Francisella tularensis holarctica* in *Ixodes ricinus* ticks from south-western Germany. Ticks Tick-borne Dis. 4, 93–100. https://doi.org/10.1016/j.ttbdis.2012.09.001.

Goethert, H.K., Saviet, B., Telford III, S.R., 2009. Metapopulation structure for perpetuation of Francisella tularensis tularensis. BMC Microbiol. 9, 147. https://doi.org/10.1186/1471-2180-9-147.

Grundmann, H., Hori, S., Tanner, G., 2001. Determining confidence intervals when measuring genetic diversity and the discriminatory abilities of typing methods for microorganisms. J. Clin. Microbiol. 39, 4190–4192. https://doi.org/10.1128/JCM.39.11.4190-4192.2001.

Gurycova, D., 1998. First isolation of *Francisella tularensis subsp. tularensis* in Europe. Eur. J. Epidemiol. 14, 797–802.

Hubálek, Z., Treml, F., Halouzka, J., Juřicová, Z., Huňady, M., Janīk, V., 1996. Frequent isolation of *Francisella tularensis* from *Dermacentor reticulatus* ticks in an enzootic focus of tularaemia. Med. Vet. Entomol. 10, 241–246.

Hunter, P.R., Gaston, M.A., 1988. Numerical index of the discriminatory ability of typing systems: an application of Simpson's Index of Diversity. J. Clin. Microbiol. 26, 2465–2466.

Johansson, A., Farlow, J., Larsson, P., Dukerich, M., Chambers, E., Byström, M., Fox, J., Chu, M., Forsman, M., Sjöstedt, A., Keim, P., 2004. Worldwide genetic relationships among *Francisella tularensis* isolates determined by Multiple-locus Variable-number Tandem Repeat Analysis. J. Bacteriol. 186, 5808–5818. https://doi.org/10.1128/JB.186.17.5808-5818.2004.

Johansson, A., Ibrahim, A., Göransson, I., Eriksson, U., Gurycova, D., Clarridge, J.E., Sjöstedt, A., 2000. Evaluation of PCR-based methods for discrimination of *Francisella* species and subspecies and development of a specific PCR that distinguishes the two major subspecies of Francisella tularensis. J. Clin. Microbiol. 38, 4180–4185.

Kingry, L.C., Petersen, J.M., 2014. Comparative review of *Francisella tularensis* and *Francisella novicida*. Front. Cell. Infect. Microbiol. 4, 35. https://doi.org/10.3389/fcimb.2014.00035.

Maurin, M., Gyuranecz, M., 2016. Tularaemia: clinical aspects in Europe. Lancet Infect. Dis. 16, 113–124. https://doi.org/10.1016/S1473-3099(15)00355-2.

Meldunki epidemiologiczne http://wwwold.pzh.gov.pl/oldpage/epimeld/index_p.html#01 (Accessed 13 June 2019).

Moniuszko, A., Zajkowska, J., Pancewicz, S., Kondrusik, M., Grygorczuk, S., Czupryna, P., 2011. Arthropod-borne tularemia in Poland: a case report. Vector Borne Zoonotic Dis. 10, 1399–1401. https://doi.org/10.1089/vbz.2010.0227.

Olsufjew, N., Meshcheryakova, I.S., 1983. Subspecific taxonomy of *Francisella tularensis* McCoy and Chapin 1912. Int. J. Syst. Bacteriol. 33, 872–874. https://doi.org/10.1099/00207713-33-4-872.

Pilo, P., Johansson, A., Frey, J., 2009. Identification of *Francisella tularensis* cluster in central and Western Europe. Emerg. Infect. Dis. 15, 2049–2051. https://doi.org/10.3201/eid1512.080805.

Rojko, T., Korva, M., Lotrič-Furlan, S., Strle, F., Avšič-Županc, T., 2016. Cluster of ulceroglandular tularemia cases in Slovenia. Ticks Tick-borne Dis. 7, 1193–1197. https://doi.org/10.1016/j.ttbdis.2016.07.016.

Soleng, A., Edgar, K.S., Paulsen, K.M., Pedersen, B.N., Okbaldet, Y.B., Skjetne, E.B., Gur, D., Vikse, R., Andreassen, A.K., 2018. Distribution of *Ixodes ricinus* ticks and prevalence of tick-borne encephalitis virus among questing ticks in the Arctic Circle region of northern Norway. Ticks Tick. Dis. 9, 97–103. https://doi.org/10.1016/j.ttbdis.2017.10.002.

Switaj, K., Olszynska-Krowicka, M., Zarnowska-Prymek, H., Zaborowski, P., 2009. Tularaemia after tick exposure – typical presentation of rare disease misdiagnosed as atypical presentation of common diseases: a case report. Cases J. 2, 7954. https://doi.org/10.4076/1757-1626-2-7954.

Thelaus, J., Andersson, A., Broman, T., Bäckman, S., Granberg, M., Karlsson, L., Kuoppa, K., Larsson, E., Lundmark, E., Lundström, J.O., Mathisen, P., Näslund, J., Schäfer, M., Wahab, T., Forsman, M., 2014. *Francisella tularensis subspecies holarctica* occurs in Swedish mosquitoes, persists through the developmental stages of laboratory-infected mosquitoes and is transmissible during blood feeding. Microb. Ecol. 67, 96–107. https://doi.org/10.1007/s00248-013-0285-1.

Vogler, A.J., Birdsell, D., Wagner, D.M., Keim, P., 2009. An optimized, multiplexed multilocus variable-number tandem repeat analysis system for genotyping Francisella tularensis. Lett. Appl. Microbiol. 48, 140–144 10.1111.j.1472-765X.2008.02484.x.

Vogler, A.J., Birdsell, D., Lee, J., Vaissaire, J., Doujet, C.L., Lapalus, M., Wagner, D.M., Keim, P., 2011. Phylogeography of *Francisella tularensis* ssp. *holarctica* in France. Lett. Appl. Microbiol. 52, 177–180. https://doi.org/10.1111/j.1472-765X.2010.02977.x.

Wójcik-Fatla, A., Zając, V., Sawczyn, A., Cisak, E., Sroka, J., Dutkiewicz, J., 2015. Occurence of *Francisella spp*. in *Dermacentor reticulatus* and *Ixodes ricinus* ticks collected in eastern Poland. Ticks Tick-borne Dis. 6, 253–257. https://doi.org/10.1016/j.ttbdis.2015.01.005.