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# High-resolution melting PCR assay as a powerful tool for the epidemiological surveillance of tularemia in Western Europe

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## Highlights

- Canonical single nucleotide polymorphisms are used to classify *Francisella tularensis*.
- Discrimination assays were designed for 113 markers identified in Western Europe.
- It enables fast discrimination of *Francisella tularensis* isolates in Western Europe for epidemiological surveillance and outbreak investigation.

## Abstract

In Europe, tularemia is caused by *Francisella tularensis* subsp. *holarctica* and is a sporadic disease affecting mainly wildlife animals and humans. Classification of this species relies on canonical single nucleotide polymorphisms (canSNPs). Four main clades have been described for *F. tularensis* subsp. *holarctica*: B.4, B.6, B.12 and B.16. Phylogeographic studies have shown that clade B.6 is predominant in Western Europe and B.12 in Eastern and Central Europe. Based on this global phylogeny, we aimed to design a molecular typing assay for all genetic subclades of subclade B.11, which is the predominant subclade in clade B.6. We designed high-resolution melting (HRM) primers for the screening of 109 canSNPs divided in seven orders of discrimination for the molecular epidemiology analysis and tracking of *Francisella tularensis* subsp. *holarctica* in Western Europe.

**Key words:** *Francisella tularensis*, High-resolution melting PCR, surveillance, tularemia

### **Short communication**

*Francisella tularensis* is the causative agent of tularemia, a zoonosis that can affect more than 350 animal species (Mörner, 1992). In Europe, the only subspecies known to cause tularemia is *F. tularensis* subsp. *holarctica*. As it is a highly clonal pathogen, classification of closely related subpopulations of *F. tularensis* at a strain level relies on highly resolutive markers (Johansson and Petersen, 2010) and is required for outbreak investigation and long-term surveillance. Selection of polymorphic variable-number tandem repeats (VNTR) for multiple-loci VNTR analysis (MLVA) allowed the differentiation of five clades for subspecies *holarctica* (B.I, B.II, B.III, B.IV and B.V) (Johansson et al., 2004). However, fast evolution and instability of tandem repeats can be responsible for the inherent homoplasy of some VNTRs (Lankester, 1870). Convergent evolution makes VNTRs unsuitable markers to infer phylogenies. Since the decline of the sequencing costs, identification of canonical single nucleotide polymorphisms (canSNP) has become a reference method for high resolution discrimination of bacterial strains (Keim et al., 2004; Girault et al., 2018). Four main clades have been described for *F. tularensis* subsp. *holarctica*: B.4, B.6, B.12 and B.16, corresponding to clades B.II, B.IV, B.I and B.V respectively (Vogler et al., 2009). Since, the number of newly identified canSNPs is constantly increasing over time and across studies (Vogler et al., 2009; Chanturia et al., 2011; Gyuranecz et al., 2012; Karadenizli et al., 2015; Karlsson et al., 2013; Dwibedi et al., 2016; Myrtenäs et al., 2016; Schulze et al., 2016; Wittwer et al., 2018). Studies showed that isolates from Western Europe belonged to numerous subclades of clade B.11, a subclade of B.6, and that these subclades have been able to form spatio-temporal clusters within a country (Allue et al., 2008; Kevin et al., 2020).

High Resolution Melting-PCR (HRM-PCR) analysis is a technique that allows the discrimination of minor genetic variations on amplicons, such as SNPs, through their melting temperature ( $T_m$ ) (Tamburro and Ripabelli, 2017). It enables rapid, reliable and low-cost surveillance of infectious diseases, as well as fast detection of a clonal outbreak. In this study, we developed a genotyping tool based on HRM-PCR to discriminate closely related strains belonging to clade B.11 and its subclades, with the highest possible resolution. A hierarchical HRM-PCR assay was developed using a total of 109 canSNPs retrieved from the CanSNPer database (Lärkeryd et al., 2014) and Kevin et al. (Kevin et al., 2020) (Figure 1). The updated version of the database is available on the following link: <https://github.com/adrlar/CanSNPer>. CanSNPs were classified in “orders of discrimination” (D1-D9) to progressively differentiate the levels of resolution (Figure 1). The SNP-

discriminative typing tool was designed to be used according to the purpose of the study (high level of resolution for outbreak investigation or low level of resolution for long-term surveillance).

Using Primer 3+ software (Untergasser et al., 2012), HRM-PCR primers were designed to target canSNPs specific of subspecies *holarctica*: major clade B.6 (D1) and subclade B.11 (D2) to confirm the belonging of Western European strains to these clades; canSNPs B.82-B.86 (D3-D7) and B.44 (D3); canSNPs B.228 (D4), B.229 (D5) and B.230 (D6), specific of a newly discovered branch of B.44 (Kevin et al., 2020), and canSNPs specific of B.45 (D4) and its subclades (including B.48-B.62 and B.87-B.92). Genomic positions of these SNPs and their primers sequences are listed in Table SI.

For canSNPs B.57, B.58 and B.88, synthetic DNA oligonucleotides (Eurofins, Germany) carrying the Ancestral and the Derived state of the variant were included as controls. Amplification was performed on the ViiA7™ Real-Time PCR system (Life Technologies) using the LightCycler® 480 High Resolution Melting (HRM) Master Mix (Roche Diagnostics). The reaction mixture consisted in 0.2µM of each primer, 1x LightCycler® HRM Master Mix and 2.5mM MgCl<sub>2</sub> in a 10µl final volume. The following parameters were used: 10 min at 95°C followed by 40 cycles consisting of 10 s at 95°C, 10 s at 58°C and 20 s at 72°C. Samples were next heated to 95°C for 30 s, cooled down to 58°C for 1 min and heated from 58°C to 95°C at a rate of 1°C/s with 25 fluorescence acquisitions/°C. HRM data were analyzed with the QuantStudio™ Real-Time PCR Software. HRM-PCR assays were successfully validated on 317 strains of human or animal origins that are listed in Table SII (figure 2 HRM). However, A-T and G-C transversions could not be deciphered through HRM-PCR because of their low T<sub>m</sub> difference and therefore, these canSNPs were not included in the typing tool. We also propose that these canSNPs should not be retained for the canSNP nomenclature. Average melting temperatures calculated for each bi-allelic assay are reported in Table SI. HRM-PCR primers for canSNPs B.82-B.86 still need to be tested *in vitro*.

As a tool for routine epidemiological surveillance, clusters such as B.44 (D3), B.45 (D4), B.49, B.50, B.55, B.87, B.250 and B.251 (D5), that were found predominant in our previous study (>10 strains)(Kevin et al., 2020), should be typed in priority during HRM-PCR assays. Such clades also present more subclades than other clades, which reflects the evolution of these clades and therefore, their persistence in Europe. Because of PCR inhibitors present in

biological samples, HRM-PCR is more adapted to bacterial strains. HRM-PCR was tested on highly positive biological samples from a marmoset (*Callithrix pygmaea*, Ct ~20) and gave satisfying results (Figure 2).

Contact with hares is one of the most important risk factors for humans in Western Europe as several outbreaks have been associated with hunting or handling hares in these geographical areas (Allue et al., 2008; Mailles and Vaillant, 2013; Otto et al., 2015; Hestvik et al., 2017; Koene et al., 2019). Thus, wildlife surveillance is of great importance not only for animal health, but also for human health, especially because human cases might be under-declared. Indeed, as symptoms are not specific and often of mild severity, patients might be under-diagnosed or might not even request medical consultation. Moreover, most genetic clusters were identified with strains isolated from hares (Kevin et al., 2020) and it has been previously demonstrated that the European Brown hare could be useful as sentinels of environmental risk (Moinet et al., 2016). It is fair to consider that these systems of surveillance (in humans and in wildlife), along with environment surveillance, should be complementary for a One-Health surveillance. As there seems to be no host specialization concerning *F. tularensis* in Western Europe (Dwibedi et al., 2016; Kevin et al., 2020) it is expected that strains isolated in vectors or in the environment would share identical genotypes as humans and other animals. Therefore, our SNP-discriminative HRM-PCR assay would greatly help epidemiological surveillance of tularemia in Western Europe. First, this tool will enable highly resolutive discrimination of *F. tularensis* subsp. *holarctica* isolates to enhance knowledge about tularemia in Western Europe and to detect abnormal incidence of the disease due to a particular genotype. Secondly, this tool can be used for a biodefense purpose as it will allow to identify a clonal outbreak.

In conclusion, we developed a rapid, cost-effective and robust molecular typing tool for the discrimination of *F. tularensis* subsp. *holarctica* from bacterial strains or highly positive samples, with a high resolution in order to introduce a One-Health epidemiological surveillance of tularemia in Western Europe.

### **Abbreviations**

canSNP: canonical single nucleotide polymorphism; DNA: Deoxyribonucleic acid; HRM: High resolution melting; MLVA: Multilocus variable number tandem repeats analysis; PCR: polymerase chain reaction; SNP: single nucleotide polymorphism; VNTR: Variable number tandem repeat

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### **Figure legends**

**Figure 1. CanSNP nomenclature generated by CanSNPer** (Lärkeryd et al., 2014). Clades for which a HRM-PCR assay was designed are indicated with green circles; clades for which HRM-PCR primers need to be validated on samples are indicated with green circles filled with a black star; countries (Austria, Belgium, France, Germany, Italy, Netherlands, Spain, Switzerland) in which each clade (D5) has been identified are indicated with flags. Data have been retrieved from the NCBI database (<https://www.ncbi.nlm.nih.gov/genome/genomes/511>) as well as from Kevin et al (Kevin et al., 2020).

**Figure 2. Normalized melting curve obtained by HRM-PCR assay (canSNP B.304) on spleen samples from a marmoset (*Callithrix pygmaea*) diluted to the 1/2, 1/4, 1/8 and 1/16 (red).** Positive control for B.304 was included (blue). Expected melting temperatures (T<sub>m</sub>) are listed in supplementary table I.

### **Supplementary data**

**Supplementary table I.** List of primers used for this study and melting temperature (T<sub>m</sub>) values determined for the respective controls.

**Supplementary table II.** List of strains used for *in vitro* validation of HRM-PCR primers.

### **Conflict of Interest**

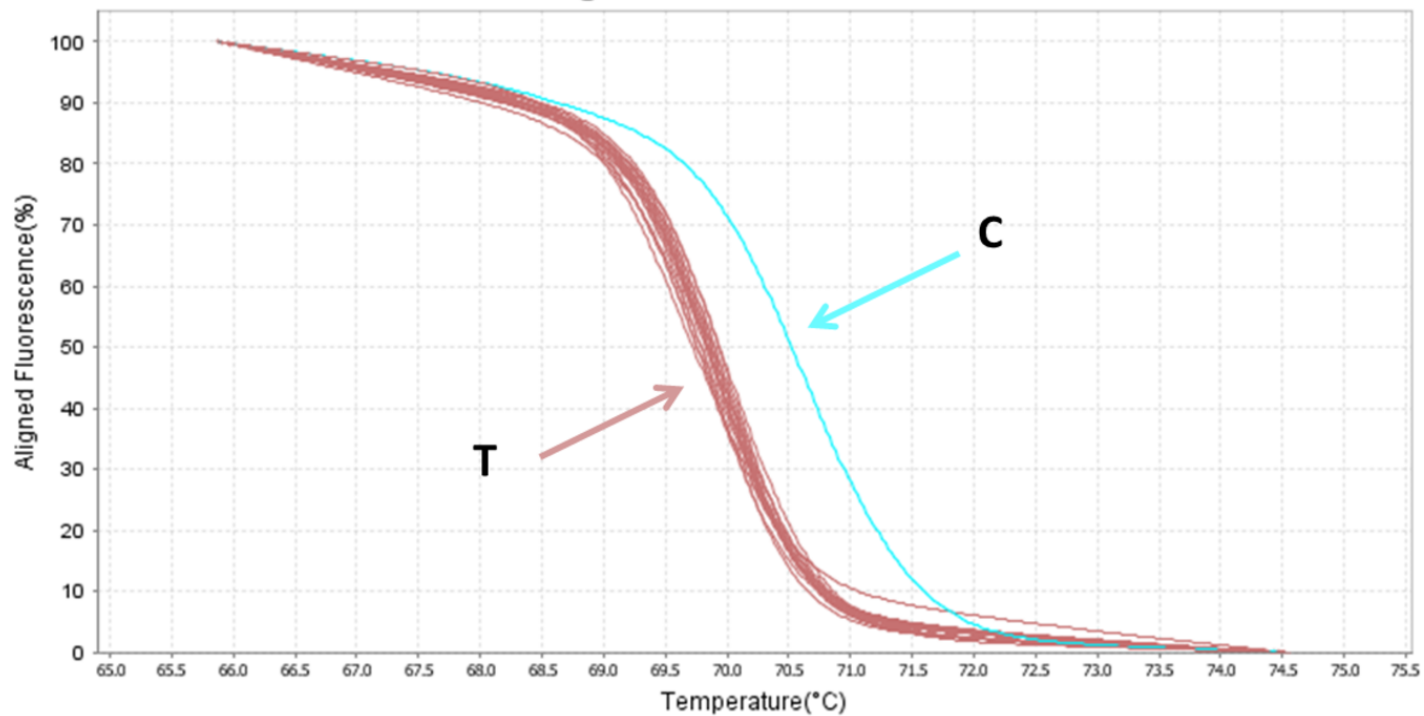
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Aligned Melt Curves



Legend

variant1 variant2