IOCD 2025 Conference

The 1st International Online Conference on Diseases

09–11 September 2025 | Online

MDPI

Robust detection of *Mycobacterium tuberculosis* Haarlem genotype by real-time PCR assay

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INTRODUCTION & AIM

The Haarlem genotype of *Mycobacterium tuberculosis* belongs to its Euro-American lineage. It is spread across many regions of the world. This genotype was named after the city of Haarlem in the Netherlands where it was first isolated. Its strains exhibited interesting pathogenic properties in mouse and macrophage models (Reiling et al., 2013).

Spoligotyping remains a classical method for Haarlem detection, but fails to reliably classify strains with abridged spoligoprofiles. According to SITVIT2, spoligotyping-based criteria for Haarlem genotype require the absence of signals 31, 33-36 along, and presence of signal 32.

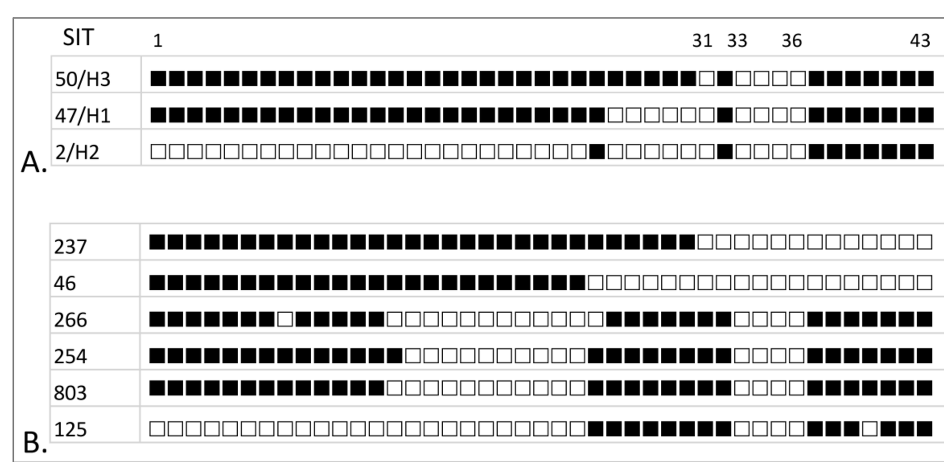


Figure 1. Schematic spoligotyping profiles based on hybridization analysis of 43 spacers in the CRISPR locus.
A. The main ancestral spoligotypes of the Haarlem genotype.
B. Undetectable profiles with long blocks of deleted signals ("unknown genotype", according to the SITVIT2 resource).

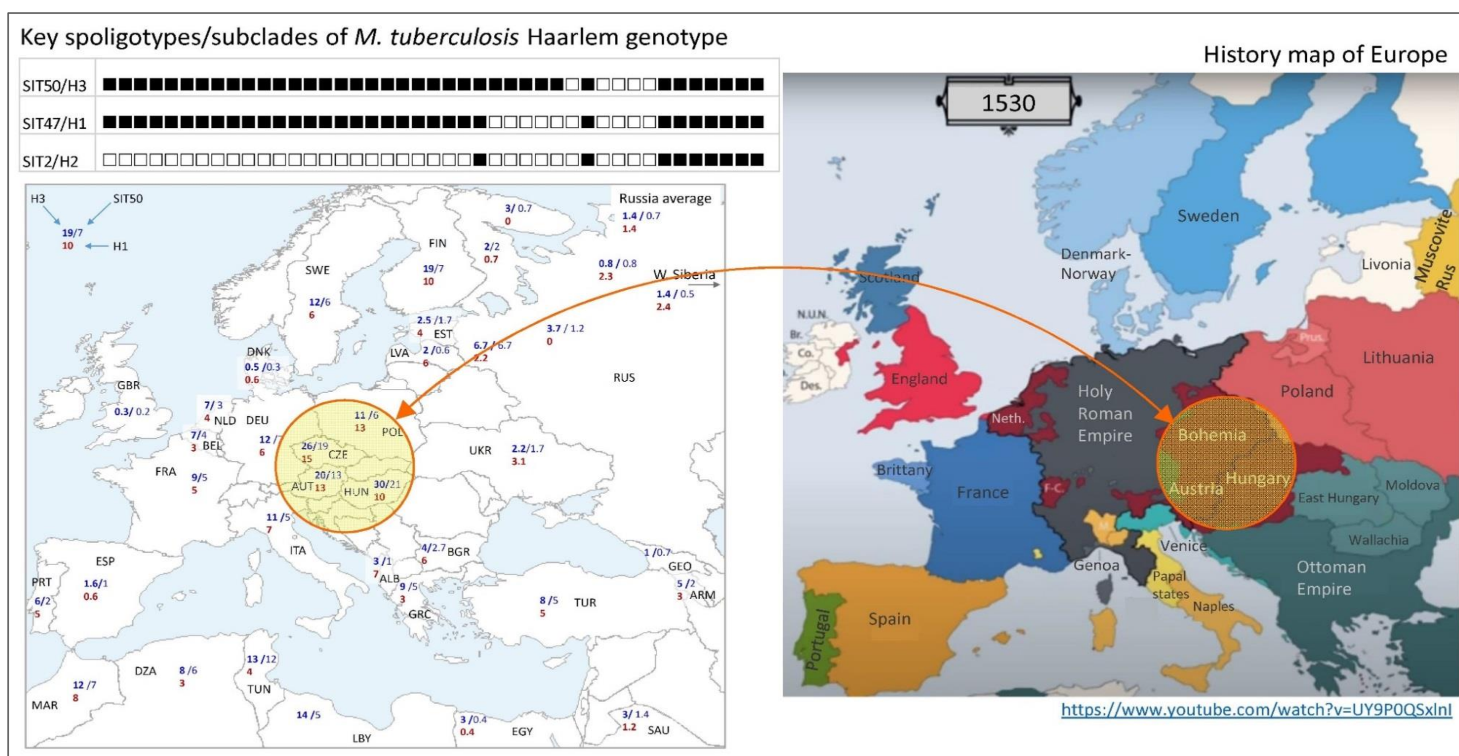


Figure 2. Hypothesis on origin and primary dispersal of the Haarlem genotype in the 12th-19th centuries in Central Europe (Mokrousov, 2024)

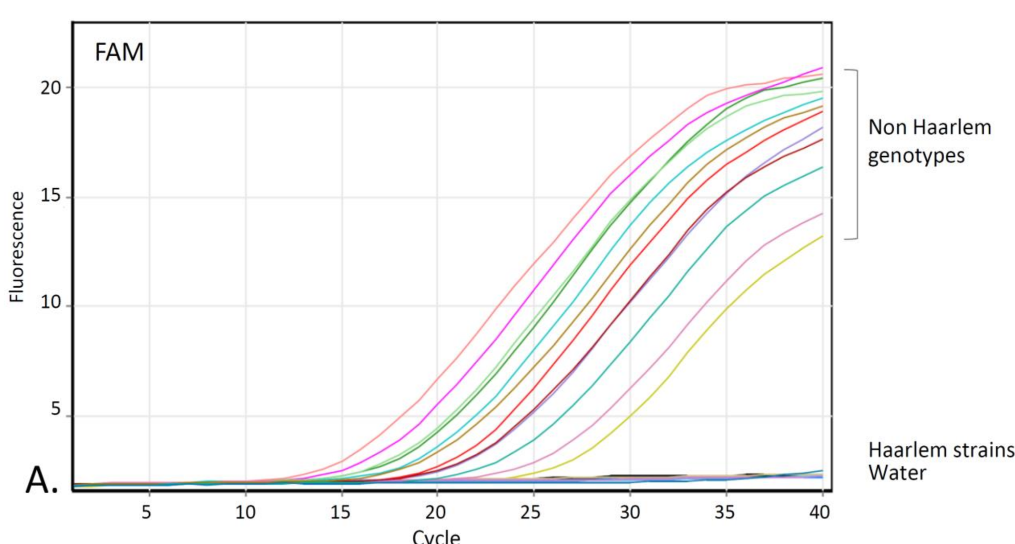
Aim: to develop and validate a real-time PCR (RT-PCR) assay for robust identification of the Haarlem genotype.

METHODS

M. tuberculosis genotyping was performed using spoligotyping with subsequent comparison against the international SITVIT2 database.

Whole-genome sequencing was performed on HiSeq4000.

For RT-PCR, we designed LNA probes to detect previously reported Haarlem-specific mutation in *Rv0282* (Shitikov, Bespiatykh 2023) and used RotorGene thermal cycler.



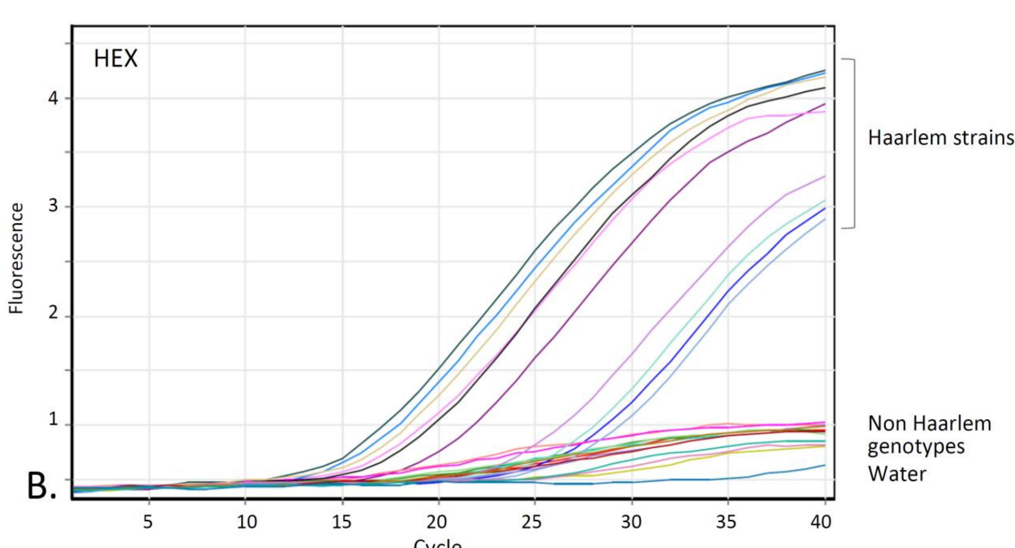


Figure 3. Real time PCR of *Rv0282* gene for detection of Haarlem specific SNP at position 211C>T.
A. FAM channel, non-Haarlem detection signal.
B. HEX channel, Haarlem detection signal.

RESULTS & DISCUSSION

Primers and LNA probes were designed to target a previously reported Haarlem-specific mutation in Rv0282 (Shitikov, Bespiatykh, 2023). The method was optimized using DNA from Haarlem and non-Haarlem strains with available whole-genome sequences.

RT-PCR assay was tested on 293 isolates from the European and Asian parts of Russia, Belarus, Bulgaria, and Vietnam, previously characterized by spoligotyping.

Among Russian isolates, spoligotyping identified 39 as Haarlem and 35 as "unknown family". The RT-PCR assay reclassified 10 of these unknown strains with truncated spoligoprofiles (SIT46, SIT237, SIT1177) as Haarlem and this was confirmed by WGS. For Belarusian isolates, spoligotyping detected only one Haarlem strain alongside six "unknown family" strains, whereas RT-PCR assigned all seven to Haarlem. All Haarlem isolates from Bulgaria (n=8; SIT47, SIT50) and Vietnam (n=1; SIT50) were concordantly confirmed by both methods.

SIT	Spoligoprofile (43 signals)	No of isolates
237	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□□□□□□□□□□	8
46	■■■■■■■■■■■■■■■■■■■■■■■■□□□□□□□□□□□□□□□□□□□	8
1177	■■■■■■■■■■■■■■■■■■■■■■■□■■■■■■□□□□□□□□□□□□□	1
786	■■■■■■■■■■■■■■■■■■■■■■■□□□□□□□□□□□□□□□□□□□□	1
56	■■■■■■■■■■■■□■■■■■■■■■■■□□□□□□□□□□□□□□□□□□□	1
4134	■■■■■□□□□□□□□□□□□□□□□□□□■□□□□□□■□□□□■■■■■■■	1
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Table 1. "Unknown genotype" isolates redefined as Haarlem

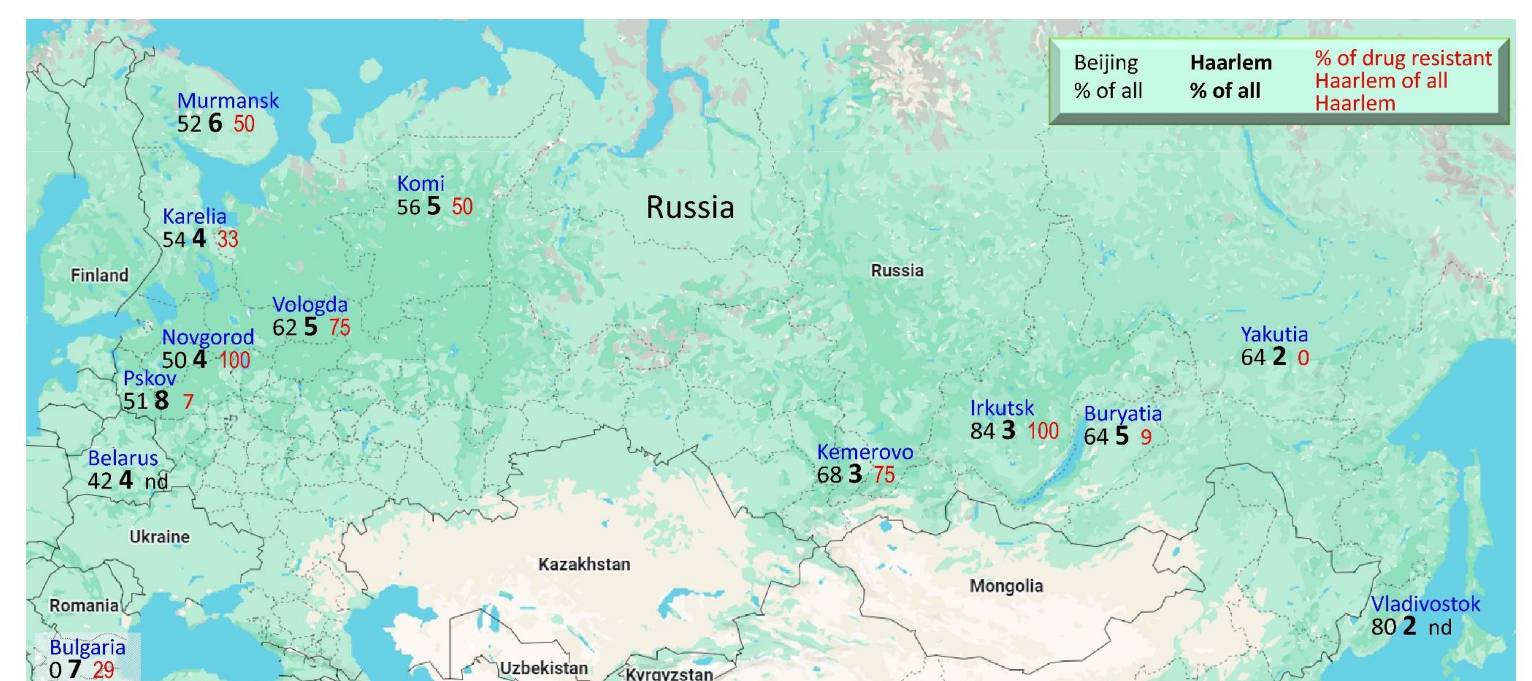

Figure 4. Map with studied locations and prevalence of Haarlem strains (along with major Russian Beijing genotype).

Regarding drug resistance, the Haarlem genotype is generally considered as drug susceptible. Yet, in some regions with epidemic spread of MDR *M. tuberculosis* strains, the situation is more nuanced and is influenced by the general situation with MDR-TB control in given country. In case of large collections, the susceptible Haarlem isolates were dominant (13/14 isolates in Pskov). However, in small subsets, MDR isolates were more visible e.g. all 4 Haarlem isolates in Novgorod, 2 of 4 in Vologda, 3 of 4 in Kemerovo were MDR. The underlying reasons may be different and be related to both global phylogeography, past historical human exchange and migration, and local implementation of TB control programs. A relatively higher prevalence of 7-8% of Haarlem in Pskov is accompanied with low level of drug resistance (7%). Haarlem strains in Europe are commonly drug susceptible and, in this sense, Pskov resembles European situation. Interestingly, it had close historical contacts with Europe dating back to the distant past, until recently.

CONCLUSION

In this study, we focused on the globally spread *M. tuberculosis* Haarlem genotype. The correct assignment of *M. tuberculosis* isolates at genotype level is important for both evolutionary and epidemiological studies and understanding clinical significance of genotypes. Use of the developed method for screening of the large collection of clinical isolates demonstrated that real proportion of the Haarlem genotype in the *M. tuberculosis* populations in some world regions is higher than previously thought.

The developed RT-PCR assay ensures reliable detection of the *M. tuberculosis* Haarlem genotype in retrospective collections and under prospective surveillance of the circulating strains. Abridged spoligoprofiles with large deleted blocks of spacers require caution.

Future research will evaluate this method with more strains from more world regions.

Conflict of interests: The authors declare no conflicts of interest.

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