Influence of Polymerase Brand on Microarray-based Spoligotyping in Low Concentrations of Mycobacterial DNA

Stefan Monecke^{1,2 and 3 *}

Ines Engelmann^{2,3}

Ralf Ehricht^{2,3}

(All authors contributed equally)

1) Institute for Medical Microbiology and Hygiene, Technische Universität Dresden;
Dresden, Germany

2) Alere Technologies GmbH; Jena, Germany

3) Infectognostics Research Campus Jena; Jena, Germany

* Corresponding author

ABSTRACT

Spoligotyping is a widely used typing method for the *Mycobacterium tuberculosis* complex. Protocols and platforms can be adapted for direct use on patient samples. Serial dilutions of genomic DNA from *Mycobacterium bovis* BCG strain DSM45071 were spoligotyped by array hybridisation using 32 different commercial PCR polymerase preparations. In samples with very low concentrations of mycobacterial DNA, commercially available PCR polymerases differed in their performance, and some yielded no, or false, identification. Direct spoligotyping from samples with very low concentrations of mycobacterial DNA thus requires careful selection of polymerase and strict standardisation.

INTRODUCTION

Spoligotyping [1] is a widely used method for typing of *Mycobacterium* (*M.*) *tuberculosis* complex (MTC) organisms such as *M. tuberculosis*, *M. bovis*, *M. caprae*, and *M. africanum*. This assay detects the presence or absence of specific DNA spacer sequences in the direct repeat (DR) genomic region. This region consists of short direct repeat sequences that are interspersed by non-repetitive DNA spacers, of 35 to 41 base pairs each. There are 43 different spacer sequences usually used that, however, not necessarily are all present in a single isolate [2]. The entire region is associated with *cas1/2* genes and thus forms a CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats; [3]) – Cas system, *i.e.*, a system that is assumed to recognize and inactivate exogenous genetic elements such as bacteriophages. Detection and identification is performed by a PCR with primers targeting the conserved direct repeat sequences and is, therefore, essentially a multiplex PCR reaction. This

always generates a multitude of different amplicons that differ in length as well as in the sequences of the spacer regions they span. Subsequently, hybridization to spacer-specific oligonucleotide probes is performed resulting in characteristic patterns that depend on the actual presence of spacer sequences. For visualization, reverse line blot hybridization utilizing a nylon membrane [1], microbeads/Luminex [3, 4] and microarray [5] technologies as well as matrix-assisted laser desorption ionization–time of flight mass spectrometry [6] have been used. Patterns are converted into a numerical code for easier analysis, comparison and data exchange and web-based international databases SpolDB4.0, SITVITWEB [7, 8] and Mbovis.org [9] have been created. These numerical codes can be used for assignment of tested isolates to MTC species and to distinct lineages within one species, as well as for comparison of isolates, *e.g.*, for epidemiological and infection control purposes. One advantage of the newer assay platforms is that they theoretically can be used for spoligotyping directly from sample material without performing time-consuming culture enrichment, facilitating rapid intervention in case of suspected transmissions as well as an assignment, for instance, to known multiresistant “problem strains” and lineages such as the Beijing clone [10]. While this appears to be an interesting approach to provide accelerated guidance to infection control measures as well as to, possibly, therapy, one problem might arise due to the multiplex character of the amplification. Aim of the study was therefore to assess the influence of experimental conditions, especially of the selection of polymerases on the stability of spoligotyping patterns in experiments with low concentrations of mycobacterial DNA.

MATERIALS & METHODS

Microarray based spoligotyping was performed using a previously described system [5] using as template genomic DNA from *Mycobacterium bovis* BCG strain DSM45071 in different

dilutions. DNA was prepared by treatment with lysozyme, proteinase K and lysis buffer AL from the Qiagen DNeasy kit (Qiagen, Hilden, Germany); and subsequently, DNA was purified using the spin columns from that kit and measured using a Nanodrop device (PEQLAB, Fürth, Germany) at 260 nm. For amplification, a total of 32 different commercial polymerase preparations were used according to the respective manufacturer's specifications and – if available – with the respective kit buffers. One polymerase was used with the kit buffer and yielded no useable results. The experiment was repeated with additional magnesium ions (1.5 mM) added.

All polymerases are coded in Table 1, their true designations can be provided on request.

RESULTS & DISCUSSION

Table 1 summarizes the results, *i.e.*, spoligotypes as functions of coded polymerase brand and target dilution. Most polymerases yielded correct identifications down to target DNA dilutions corresponding to 10 or 100 genome copies per sample. Positive amplifications, but incorrect spoligotypes were observed for some polymerases in a range of 1 to 10 genome copies per sample. One brand proved to be completely unusable, even after addition of Mg²⁺.

Adapted protocols of spoligotyping can be performed in samples with very low numbers of DNA target molecules. Therefore, such methods could be used directly on patient samples, rather than on culture material. In some cases, correct results were achieved with as few as 10 copies of genomic DNA per sample. This might prove extremely useful to confirm or rule out a presence of, *e.g.*, a local problematic or outbreak strain or to timely guide infection control measures.

69 However, a problem can be caused by different performances of different brands of
70 polymerases. Because of the different lengths and sequences of the simultaneously generated
71 spoligotyping PCR products, the reaction rather resembles a multiplexed PCR despite its use
72 of only two primers. Therefore, it is likely to encounter problems known from multiplexed
73 PCRs. These include a loss of sensitivity and occurrence of false-negative results due to a
74 competition between simultaneous amplifications for polymerases and nucleotides. Due to the
75 non-linear kinetics of PCR, such effects are hardly predictable. Therefore, it can be expected
76 that the actual outcome of a PCR system with a high degree of multiplexing is strongly
77 influenced by minor variations in the experimental setup and manual handling. The
78 experiments presented in this study show that in spoligotyping reactions, a false result from a
79 compromised or suboptimal run might not necessarily be just a false-negative one. Apparently
80 valid, positive results also might potentially yield a wrong strain assignment.

81 When performing spoligotyping on high amounts of target DNA, *i.e.*, on culture material in
82 the traditional reverse line blot hybridization on nylon membranes, the described effects likely
83 can be neglected. This might be different when using one of the newer technologies directly
84 on patient samples as target DNA concentrations are low. Beside the influence of different
85 polymerases as described above, other technical influences, such as consistency and
86 reproducibility of the temperature regimes and the speed of heating and cooling (“ramp
87 speeds”) in various thermocycling and thermoshaking devices need to be scrutinized. The
88 inhibiting effects of host DNA in patient samples [11] might additionally aggravate these
89 effects.

90 In order to compensate for such effects, conditions need to be strictly standardized.
91 Practically, a use of spoligotyping on patient samples requires confection of assays, optimally

in closed cartridges, as well as assay-specific hardware that minimize any deviations from the recommended protocol.

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123 **AUTHORS CONTRIBUTIONS**

124 RE conceived and planned the experiments, IE performed the experiments, all authors
125 analysed the data, SM wrote the manuscript. All authors read and approved the final
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128 The authors are employees of Alere Technologies. This had no influence on planning and
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