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Revealing hidden clonal complexity in *Mycobacterium tuberculosis* infection by qualitative and quantitative improvement of sampling

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Abstract

The analysis of microevolution events, its functional relevance and impact on molecular epidemiology strategies, constitutes one of the most challenging aspects of the study of clonal complexity in infection by *Mycobacterium tuberculosis*. In this study, we retrospectively evaluated whether two improved sampling schemes could provide access to the clonal complexity that is undetected by the current standards (analysis of one isolate from one sputum). We evaluated in 48 patients the analysis by mycobacterial interspersed repetitive unit–variable number tandem repeat of *M. tuberculosis* isolates cultured from bronchial aspirate (BAS) or bronchoalveolar lavage (BAL) and, in another 16 cases, the analysis of a higher number of isolates from independent sputum samples. Analysis of the isolates from BAS/BAL specimens revealed clonal complexity in a very high proportion of cases (5/48); in most of these cases, complexity was not detected when the isolates from sputum samples were analysed. Systematic analysis of isolates from multiple sputum samples also improved the detection of clonal complexity. We found coexisting clonal variants in two of 16 cases that would have gone undetected in the analysis of the isolate from a single sputum specimen. Our results suggest that analysis of isolates from BAS/BAL specimens is highly efficient for recording the true clonal composition of *M. tuberculosis* in the lungs. When these samples are not available, we recommend increasing the number of isolates from independent sputum specimens, because they might not harbour the same pool of bacteria. Our data suggest that the degree of clonal complexity in tuberculosis has been underestimated because of the deficiencies inherent in a simplified procedure.

Keywords: Clonal complexity, hidden complexity, improved detection, sampling schemes, tuberculosis
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Introduction

Infection by *Mycobacterium tuberculosis* is more clonally complex than initially thought. Mixed infection by more than one *M. tuberculosis* strain is the commonest form of clonal complexity; coexistence of clonal variants in the same host is the most unusual [1–3]. The emergence of clonal variants by microevolution from a homogeneous parental bacterial population has been described at both intrapatient and interpatient levels [4–6]. These subtle changes have been considered functionally significant because they can affect the ability of specific variants to infect respiratory/extrarespiratory sites and macrophages, as well as their ability to express genes in the regions where these variations map [6–11].

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Until recently, analysis of microevolution and identification of clonal variants could only be performed using standard genotyping tools; therefore, variants were revealed only when the genetic variations directly affected the regions or markers used in the common fingerprinting methods. The recent introduction of whole genome sequencing has increased our knowledge of the magnitude of the changes occurring in strains as a result of microevolution and how it can impact on the inference of tuberculosis transmission [12]. However, improvements in the strategies applied to analyse microevolution and clonal complexity have not been accompanied by a parallel effort to improve sampling methods so that they can provide a true picture of the composition of the population of bacilli infecting a host. In fact, the standard sampling procedure for fingerprinting—analysis of only one *M. tuberculosis* isolate from a single sputum specimen—has not been extensively evaluated to ascertain whether it is efficient enough to record the true clonal composition of the *M. tuberculosis* population infecting the lung [13]. In this study, we evaluate the efficiency of two improved methods for detecting clonal complexity in *M. tuberculosis* that might have gone undetected using standard sampling procedures. The two methods evaluated were analysis of higher-quality respiratory specimens (bronchial aspirate (BAS) or bronchoalveolar lavage (BAL)), and analysis of a higher number of isolates from sequential sputum specimens taken from the same patient.

Materials and methods

Selection of cases

In all cases, we retrospectively analysed drug-susceptible *M. tuberculosis* strains that had been stored frozen since isolation. For the analysis of higher-quality specimens we selected cases with at least two *M. tuberculosis* isolates: one *M. tuberculosis* isolate from a BAS or BAL specimen and another *M. tuberculosis* isolate from sputum. For the analysis of isolates from multiple sputum specimens, we selected cases with at least four or more isolates from independent sputum specimens taken early after diagnosis (less than 16 days).

We lacked the necessary data to establish the chronology for samples corresponding to sequential extractions from 3 consecutive days that were received on the same day; therefore, we considered these samples as though they were from a single day.

Cultures

Selected isolates were thawed, inoculated in mycobacterial growth indicator tube liquid medium (Becton Dickinson, Sparks, MD, USA), and incubated for 14 days at 37°C.

DNA purification

DNA was extracted from 1 ml of culture using a column-based purification method and eluted in 100 µl of buffer AE (QIAamp DNA minikit protocol, Qiagen, Courtaboeuf, France).

Screening of cases with clonally complex infections

As clonally complex infections, in this study we only considered infections involving coexistence of clonal variants (those in which we detected variants differing by one or two repetitions in only one mycobacterial interspersed repetitive unit (MIRU) locus) and not mixed infections.

Multiplex MIRU-VNTR analysis. Clonal variability was investigated using mycobacterial interspersed repetitive unit–variable number tandem repeat (MIRU-VNTR) (MIRU-24 multiplex PCR format [14,15], as described in ref. [16]). PCR products were analysed by capillary electrophoresis (3130xl Genetic Analyzer, POP-7 polymer, 1200 LIZ ISS standard; Applied Biosystems, Foster City, CA, USA). The PCR fragments were sized using GeneMapper v4.0 [16].

We visually analysed the electropherograms generated by GeneMapper for each multiplex PCR to find minority peaks indicating the presence of a second allelic variant for each of the loci studied. Residual peaks corresponding to fluorescence intensity below 200 in height were not included.

Simplex MIRU-VNTR analysis. All cases of clonal complexity for a locus in the multiplex MIRU-VNTR analysis in capillary electrophoresis were confirmed by repeating the analysis of the locus using simplex MIRU-VNTR analysis and capillary electrophoresis following procedures published elsewhere [14,15].

The cases in which we detected and confirmed two variants in the isolates after screening with MIRU-VNTR were reconfirmed by identifying each of the variants independently in the analysis of multiple single colonies after sub-culturing the original isolate on agar plates. This was performed by means of MIRU-VNTR analysis of the relevant locus by simplex PCR and detection in agarose gel by standard electrophoresis (2% MS-8; Pronadisa, Madrid, Spain) at 45 V for 18.5 h.

Results

Analysis of clonal complexity in *M. tuberculosis* isolates from BAL/BAS specimens

Our first objective was to search for clonal complexity in cases where *M. tuberculosis* was isolated from both BAL/BAS and sputum samples to evaluate whether the deeper and more homogeneous sampling expected with BAL/BAS (considered for this reason as a high-quality respiratory specimen) could provide a more representative picture of clonal complexity than sputum.

We selected 48 patients from two different institutions for whom *M. tuberculosis* had been isolated from a BAL or BAS sample and a sputum sample in the same tuberculosis episode. MIRU-VNTR analysis of the BAS/BAL isolates revealed five (10.4%) cases in which two variants were observed according to the double peaks detected in the electropherograms for the loci MIRU42, MIRU26, 1995, MIRU40, and Q26 (Fig. 1a). All the variations were single-locus variations; in three of these cases the variants differed in one repetition, and in the two remaining cases, the co-infecting variants differed in two repretitions. In all five cases, the double peaks were confirmed by simplex PCR of the relevant locus; confirmation was verified using analysis of single colonies by MIRU-VNTR to reveal the presence of the two variants. This analysis could be performed in four of the five cases and enabled us to determine the relative proportion between the two coexisting variants. The relative ratios for the variants in patients 1, 3, 4 and 5 were 8 : 1, 50 : 1, 12 : 1 and 11 : 1, and, as expected, the variant with the highest allelic peak in the electropherogram was the most abundant in the single-colony analysis.

After detecting the five clonally complex cases, we performed an equivalent analysis on the isolates recovered from the sputum samples from the same five cases. In only one case, the two variants detected in the isolate from the BAL specimen were also detected in the isolate from sputum (case 5, Fig. 1b). In the remaining four cases, only one of the variants detected in the isolate from BAS/BAL was detected in sputum (Fig. 1b). Surprisingly, in two of these cases (1 and 4), the variant detected in sputum was the least frequent in BAS/BAL (Fig. 1a, b).

Our findings indicated that in most of the cases shown to be clonally complex after analysis of BAL/BAS, analysis of a single sputum sample did not reveal complexity. We therefore evaluated whether this deficiency could be compensated by increasing the number of sputum specimens analysed. Unfortunately, we only had additional isolates from independent specimens for one of the five cases (two additional sputum specimens for case 1, collected 3 and 6 days before the BAL isolate). In one of the additional sputum specimens we again detected the same single variant as in the previous specimen (Fig. 1c); however, in the other specimen, we detected the second variant, namely, the one that had been detected in the isolate from BAL. For case 1, the clonal complexity detected in a single isolate from BAL was also detected when analysing the isolates from sputum; however, it was necessary to analyse the isolates from three specimens, because none of them simultaneously harboured the two variants.

Analysis of clonal complexity in multiple sputum samples

The findings reported above suggest that increasing the number of isolates from sputum samples could compensate for the limitations of analysing the isolate from a single sputum specimen to reveal the clonal complexity detected in BAL/BAS. Therefore, to find an alternative improved procedure for the cases in which BAS/BAL specimens were not accessible, we evaluated whether more systematic screening of specimens from a new selection of patients could also reveal clonal complexity that may have gone undetected using the standard one-sputum-sampling procedures.

We selected a population of patients with four or more *M. tuberculosis* isolates from independent sputum samples collected early after diagnosis (within the first 16 days). We analysed 79 samples from 16 patients. For each case, we used MIRU-VNTR for screening, starting with the first diagnostic sample and continuing until clonal complexity was detected. Clonal complexity was detected in two cases (12.5%; patients M4 and M15) (Fig. 2a). In patient M4, we analysed six *M. tuberculosis* isolates from sputum sampled on days 0 and 7. Five isolates showed the same allelic value in the ETRA locus (three repetitions), whereas the remaining sample showed a two-repetition variant. For patient M15, after analysing the isolates from three samples, all from day 0, we found one showing clonal complexity. In this case, 1 isolate simultaneously harboured two clonal variants, differing in one single-locus variant (three and four repetitions) in the MIRU16 locus, whereas the remaining two harboured only the four-repetition variant (Fig. 2b). In both cases (patients M4 and M15), the presence of the two variants was confirmed by simplex PCR of the relevant locus. In patient M15, the original isolate was sub-cultured on agar to analyse multiple independent colonies, showing the allele 3 variant to be a minority variant (1:5).

To confirm the presence of the clonal variants identified in patients M4 and M15 during the remainder of the infection, we analysed the subsequent available samples from both patients using the same MIRU-VNTR analysis, which was now restricted to the relevant loci. For patient M4, we collected four additional isolates on days 17 and 30. We found that one of the isolates from day 30 showed the two previously detected variants in ETRA and a third additional variant (allelic value 4, Fig. 2b); all three were detected simultaneously in the same isolate. The original isolate was sub-cultured on agar; variants corresponding to alleles 2 and 3 were confirmed (1 : 1). The allele 4 variant was not detected after analysing 40 single colonies, suggesting that it was under-represented. Patient M15 had two isolates sampled on day 16 available for analysis. In one isolate, the coexistence of the allele 3 and 4 variants observed in MIRU16 on day 0 was also confirmed, whereas the other isolate from the same day only showed the allele 4 variant (Fig. 2b).

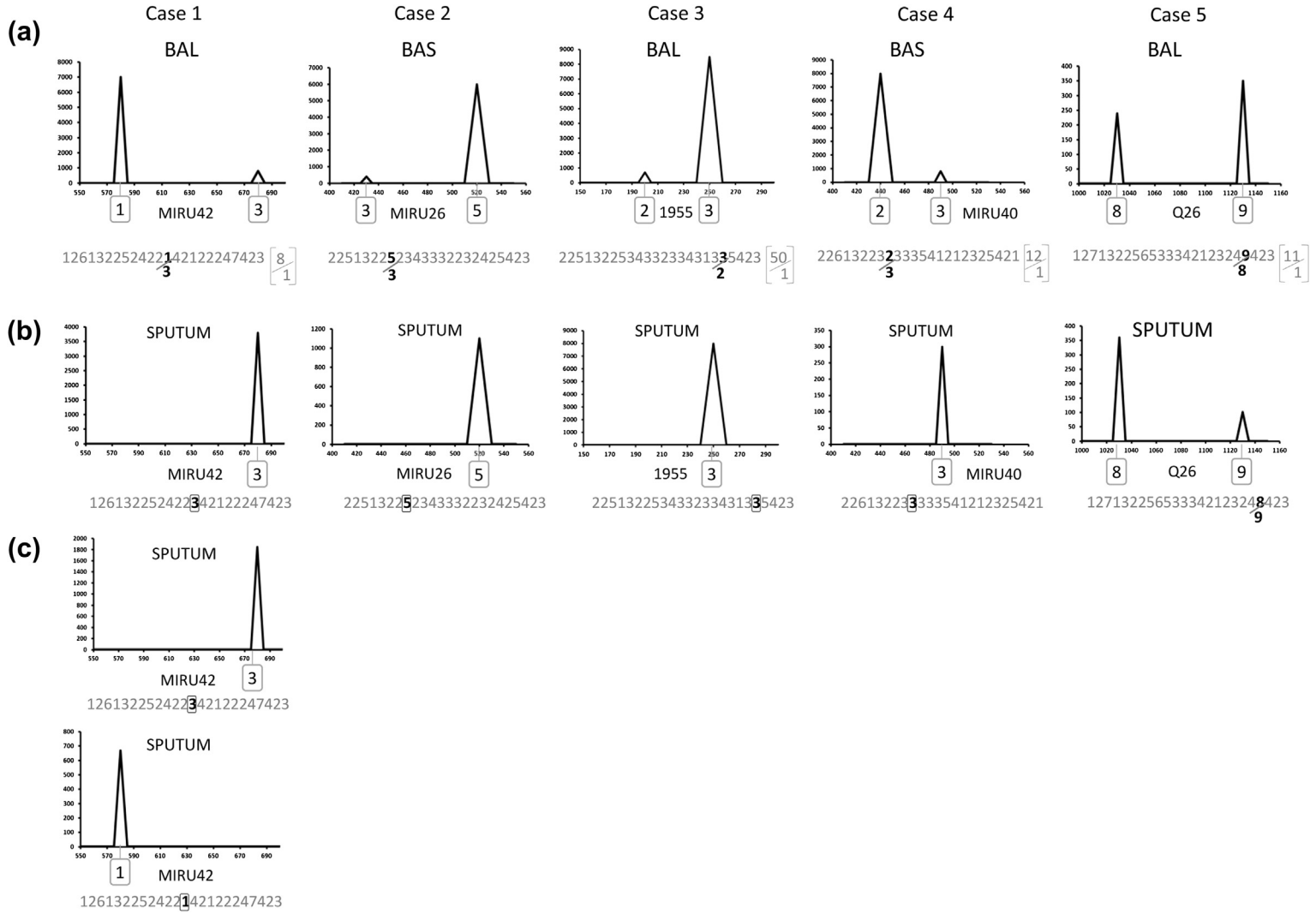

FIG. 1. Representation of the electropherograms (each peak corresponds to a mycobacterial interspersed repetitive unit–variable number tandem repeat (MIRU-VNTR) amplification product, its height corresponds to fluorescence intensity and its position in the *x*-axis indicates its size in bp) corresponding to the MIRU-VNTR loci showing clonal complexity in patients 1 to 5. The result for the MIRU loci involved in clonal heterogeneity is the only one shown (MIRU locus indicated under each graph). The results are shown according to origin: (a) isolates from bronchial aspirate (BAS) or bronchoalveolar lavage (BAL); (b) isolates from a sputum specimen from the cases in (a); and (c) isolates from two additional sputum specimens for patient 1. The allelic values for the peaks are indicated in boxes. The MIRU-VNTR pattern is shown under each graph with the allelic differences between the co-infecting variants highlighted in bold. The relative proportion between the two variants is indicated by the ratio at the right side of the MIRU-VNTR pattern.

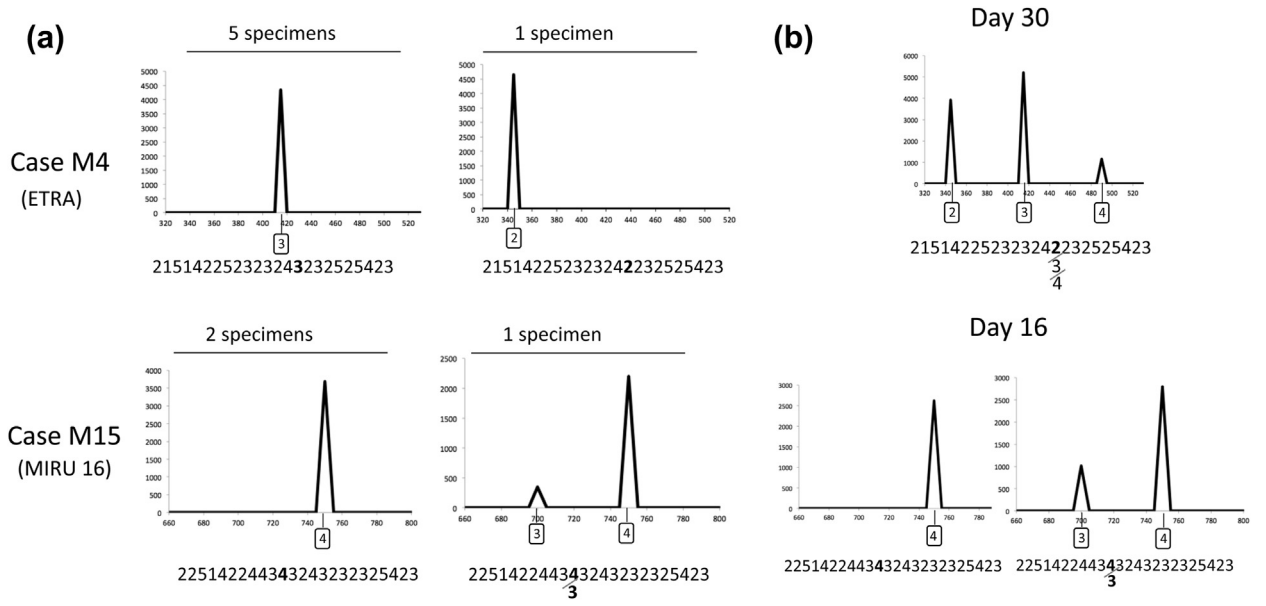

FIG. 2. Representation of the electropherograms (each peak correspond to a mycobacterial interspersed repetitive unit–variable number tandem repeat (MIRU-VNTR) amplification product, its height corresponds to fluorescence intensity and its position in the *x*-axis indicates its size in bp) corresponding to the MIRU-VNTR loci showing clonal complexity in patients M4 and M15. The result for the MIRU loci involved in clonal heterogeneity is the only one shown (the cases and the MIRU loci involved are indicated at the left side of the figure). (a) Patterns supporting the identification of clonal complexity close to the time of diagnosis. The number of specimens in which each pattern was detected is indicated above each graph. (b) Analysis of additional specimens to confirm the permanence of the clonal complexity detected in (a). The allelic values are indicated in boxes. The MIRU pattern is shown under each graph with the allelic differences between the co-infecting variants highlighted in bold.

Discussion

Major advances have been made in the study of clonal complexity in *M. tuberculosis* infection. The findings of several studies have highlighted the extent of this phenomenon in a variety of settings. Microevolution leading to the emergence of clonal variants has been described at both intrapatient and interpatient levels [3–6], and cut-offs for tolerance of microevolution within clusters have been proposed [17].

However, the advances made in this area overshadow, to a certain extent, the evaluation of whether standard diagnostic sampling procedures are also valid for determining the true clonal composition of the bacterial population infecting the lungs of patients with tuberculosis. In most studies that address clonal complexity and microevolution, fingerprinting is performed using an *M. tuberculosis* isolate cultured from a single sputum specimen; therefore, we may be underestimating the clonal complexity of tuberculosis because our sampling procedures are too simple. Moreover, it may be naive to assume that a single isolate from one sample could provide a true picture of clonal composition in the respiratory compartment. Some studies have found that different sputum samples from the same patient lead to the isolation of different variants [3,13]. Since autopsy-based evidence has shown that different clones could be located at different lung sites [18], the composition of bacteria in sputum specimens from the same patient could differ. In addition, microevolution leading to the emergence of clonal variants seems to be a highly dynamic process with interactions, even competition, between the variants, before one or more are evolutionarily fixed [12]. Consequently, the snapshot provided by a single sputum specimen may not be a reliable representation of true clonal complexity.

We attempted to complete this area of research by evaluating the procedure we use to analyse clonal complexity in *M. tuberculosis*. Our data indicated that standard approaches actually underestimate the true proportion of clonal complexity in tuberculosis; therefore, we suggest alternative procedures to improve the efficiency of our detection strategies. Sampling isolates from BAS/BAL revealed a much higher percentage of clonally complex cases than in previous studies based on sampling from sputum [3,6,13]. In addition, the clonal complexity we found in BAS/BAL could also be detected using the standard sampling from sputum in only one of the five cases. Of note, in the only case with a higher number of sputum samples available, none had revealed the coexistence of two variants, although they were identified in independent isolates from independent sputum specimens. Alternatively, increasing the number of

isolates from different sputum specimens led to a much higher percentage of clonally complex cases than the standard approach. In studies by our group based on standard sampling approaches, a systematic survey showed that the percentage of cases with coexistence of clonal variants (1.6%) was clearly below current figures [3].

It seems clear, therefore, that the true proportion of clonal complexity in *M. tuberculosis* infection will remain underestimated until sampling procedures improve. Nevertheless, our secondary findings emphasise the limitations and unpredictability of sputum sampling for determining the true composition of the expected bacterial population in the respiratory compartment. For example, when the variants in BAL/BAS were compared with the variant detected in sputum in a patient with clonally complex *M. tuberculosis* infection, the variant in sputum was not always over-represented in BAL/BAS and may even be the minority variant.

The expected variability in a single sputum sample is also illustrated by the finding that in the two clonally complex cases, most specimens had a single genotype. The second co-infecting variant was only detected in two of ten specimens from patient M4 and in two of five from patient M15. It is noteworthy that heterogeneity in these infections is not a short and transient finding, because it was detected in chronologically separated specimens. The fact that variants are observed at different points during the chronology of infection confirmed the presence of co-infecting variants and suggested that if they are not detected, it is because of the inability of a single sputum specimen to represent the whole bacterial population. Therefore, the observation window should be extended to minimise the risk of overlooking clonal complexity in *M. tuberculosis* infection. The presence of co-infecting clonal variants at different points during heterogeneous infection is consistent with the functional role underlying this variability. Several authors suggest that the variations generated by microevolution can affect expression of neighbouring genes [6–10], the ability of the variants to replicate in macrophages [11], and the infective behaviour of the variants in the clinical setting [19,20]. The MIRU loci involved in the acquisition of variability detected in this study are located in genomic contexts where the variation in the number of repetitions could have a potential functional effect. In some loci (MIRU42, MIRU26, 1955, MIRU40, Q26 and MIRU16), this variation can affect the expression of adjacent genes, and in others (ETRA, located in the Rv1917 (PPE34) gene), it could affect the antigenic properties of the coded protein [21]. The detection of these variants indicates that they were fixed after their emergence and that the subtle changes they underwent must therefore be advantageous for the strains. In the perspective of patient management, it is relevant to assure a high efficiency to detect the true composition of clonal variants co-infecting the same patient because infectivity, virulence or ability to acquire mutations and therefore resistances are all features that could be modulated by the emergence of certain subtle genetic changes from a parental strain.

Improving the detection of microevolution events it is also essential to assure precision in the identification of recent transmission events. The definition of a cluster lies on the detection of identical fingerprints between two or more cases in a population. The high genetic intra-patient variability in the *M. tuberculosis* population in the lung, recorded by BAS/BAL samples (10.4%), and the different clonal composition observed between the different isolates from different sputum specimens from the same patient, could impact on the final fingerprint assigned to a case and, therefore to the correct identification of recent transmission events. Only the precise identification of the genotypic variability in the *M. tuberculosis* isolate from a patient can assure precision in tuberculosis control programmes supported by molecular epidemiology strategies.

In summary, detection of clonal variants of *M. tuberculosis* co-infecting a single host seems to be more challenging than initially assumed. The highly dynamic events involved in microevolution, the interactions and potential competition between variants, and the differences in representativeness between variants indicate that standard sampling based on fingerprinting of a single isolate cultured from one sputum sample is more likely underestimate the true proportion of clonal complexity in tuberculosis. Our data suggest that more clonally complex events can be detected using higher-quality respiratory specimens, such as BAL or BAS. When these specimens are not accessible, we recommend increasing the number of sputum samples. Every effort should be made to improve sampling procedures to gain access to the as yet undiscovered pool of clonal complexity in tuberculosis and so better understand its significance and its impact on the interpretation of clusters and therefore on the identification of recent transmission events.

Transparency declaration

There is no conflict of interests for any author.

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