Clonal Complexity in *Mycobacterium tuberculosis* Can Hamper Diagnostic Procedures

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ABSTRACT Clonal complexity is increasingly accepted in *Mycobacterium tuberculosis* infection, including mixed infections by ≥ 2 strains, which usually occur in settings with a high burden of tuberculosis and/or a high risk of overexposure to infected patients. Mixed infections can hamper diagnostic procedures; obtaining an accurate antibiogram is difficult when the susceptibility patterns of the strains differ. Here, we show how mixed infections can also prove challenging for other diagnostic procedures, even outside settings where mixed infections are traditionally expected. We show how an unnoticed mixed infection in an HIV-positive patient diagnosed in Madrid, Spain, with differences in the representativeness of the coinfecting strains in different sputum samples, markedly complicated the resolution of a laboratory cross-contamination false positivity alert.

KEYWORDS tuberculosis, clonal complexity, mixed infection, diagnostic procedures

Clonally complex infections are increasingly accepted in *Mycobacterium tuberculosis* infection. Mixed infection (i.e., the simultaneous infection by ≥ 2 different *M. tuberculosis* strains) is one of the most widely studied types of clonally complex infections (1–4). Mixed tuberculosis (TB) infection requires either simultaneous coinfection by a number of strains or, more likely, overexposure of an infected patient whose initial infection has not resolved to another patient infected by a different strain.

Mixed infections are more likely in prisons, shelters, substandard overcrowded housing, and in other high-burden settings, where the possibility of overexposure is higher (2, 3, 5). The fact that mixed infections are not considered outside these contexts can hamper diagnostic procedures; for example, an unnoticed mixed infection can affect the result of antibiotic susceptibility testing. Rates of resistance are higher in regions where mixed infections are more frequent and in countries hosting immigrants from these regions; therefore, it is not uncommon for mixed infections to involve strains with different susceptibility patterns (6–8). However, although clonally complex infections are now beginning to be taken into account in the above-mentioned settings and for specific diagnostic procedures, equivalent efforts have not been made outside these contexts. Similarly, no efforts have been made to evaluate the impact of mixed infections on diagnostic procedures other than antibiotic susceptibility testing.

We show how an unnoticed mixed *M. tuberculosis* infection in a case diagnosed in Madrid, Spain considerably hampered management of a laboratory cross-contamination alert.

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FIG 1 MLP3 patterns indicating the involvement of 2 strains in the event. In case 1, the 2 patterns overlapped.

RESULTS

Initial management of a false positivity alert. A false positivity alert was set off when only 1 out of 3 respiratory specimens from a patient yielded a positive *M. tuberculosis* culture (pansusceptible). The patient (case 2) was a 46-year-old man who had been diagnosed with HIV infection (C3) in 1998. His history revealed that he was an injection drug user and smoker and that he was homeless. He had been hospitalized in 1998, 2013, and 2014 for bacterial pneumonia and esophageal candidiasis; during the admissions, his respiratory specimens were negative for mycobacteria. His last admission (December 2014) was for worsening of his general status, with weight loss and fever of unknown origin. The patient had not taken antiretroviral treatment for at least 8 months. He presented extensive oropharyngeal candidiasis. The remainder of the physical examination was unremarkable. The chest X-ray was normal. The laboratory work-up revealed the following data: CD4-positive (CD4⁺) T lymphocytes, 2 cells/ μ l (2%); CD8⁺ T lymphocytes, 78 cells/ μ l (53%); CD4⁺/CD8⁺, 0.05; and HIV plasma viral load, 11,400 copies/ml. The *M. tuberculosis*-positive specimen (>50 acid-fast bacilli [AFB]/field, high load) for the current admission was received and processed in the laboratory on the same day as a specimen from another HIV-infected person (case 1) who had accumulated 12 previous positive samples over the previous 53 days (all stain positive, >50 AFB/field, and all isolates pansusceptible). Case 1 was a 48-year-old man who was also an injection drug user and smoker and was homeless. He was hospitalized in December 2014 with a 4-week history of fever, cough, and weight loss. He had a basal partial O₂ pressure (pO₂) of 95%.

To clarify the false positivity alert, the attending clinicians requested 4 additional specimens from case 2 for staining and culture and ordered genotyping of the *M. tuberculosis* strains from both case 1 and case 2.

Genotyping was performed on the cultured isolate from case 2 and directly using the smear-positive specimen from case 1 (the liquid culture from this specimen was being used for diagnostic laboratory procedures). Our comparison of the mobility of the triplex PCR products obtained (mycobacterial interspersed repetitive-unit-variable-number tandem-repeat [MIRU-VNTR] length polymorphism triplex [MLP3] approach) enabled us to make 2 observations. First, 2 different genotyping patterns were involved in the event (Fig. 1); this finding would usually lead automatically to an interpretation of true positivity for case 1 and case 2. However, our second observation was that there were more than 3 amplicons in the triplex reactions for case 1 and that the mobility of

these additional amplicons was identical to that of the amplicons obtained from case 2. In a standard infection, only 3 amplicons are expected for each triplex reaction. We repeated the assay to rule out contamination in the PCR and when loading the electrophoresis gel, although the same results were obtained. Therefore, our first preliminary interpretation of the 2 observations from the MLP3 analysis was that the 2 cases were true-positive TB cases and that laboratory cross-contamination had occurred. However, we considered that the contamination went from case 2 to case 1, that is, in the direction opposite to that initially suspected, thus leading to the detection of 2 overlapping fingerprinting patterns in case 1.

New data excluding TB in one of the cases. New laboratory findings during the following days were not consistent with our interpretation. A set of 4 additional specimens from case 2 was negative for both staining and culture, and the hypothesis that case 2 did not have TB seemed more robust to the clinician. *M. tuberculosis* analysis had initially been requested in case 2 at admission, following the standard screening procedure applied to all newly diagnosed HIV-positive patients with severe immunosuppression (very low CD4 counts), lack of antiretroviral treatment, and fever of unknown origin at admission, all of which applied to case 2. Fever resolved with the initiation of antiretroviral treatment, and the clinicians responsible for the case excluded TB.

Introducing mixed infection into the reanalysis of the event. Once it was clear from the clinical data that case 2 was a false positive, we retrospectively determined the reasons behind our unusual genotyping data, that is, a mixed pattern in case 1 and a single pattern in case 2 (corresponding to 1 of the 2 strains present in case 1). An alternative hypothesis was proposed, namely, that case 1 was a true-positive TB case coinfecting by 2 susceptible strains (A and B); however, the 2 strains were not equally represented in all of his specimens, and 1 of his specimens, enriched only in strain B, was mislabeled as belonging to case 2.

Given that the marked coincidence of so many unexpected findings was highly unlikely, we were obliged to search for additional evidence supporting the mixed infection and heterogeneous bacterial composition of the sputum samples in case 1.

We first focused on the demonstration of mixed infection for case 1. We plated the culture from the specimen from case 1 that had been used in the initial MLP3 analysis to demonstrate that 2 strains can be detected from single colonies using MIRU-VNTR analysis. However, after analyzing 160 colonies, only the pattern from strain A was detected.

In a second step, we applied MIRU-VNTR typing directly on decontaminated sputum in order to determine the true bacterial composition in case 1, ruling out any potential influence of the culture. At this point, in 7 out of 8 specimens from case 1, we observed more than 3 amplification peaks in the electropherograms for the triplex reactions (Fig. 2A), thus confirming that case 1 was coinfecting by 2 strains (strains A and B). The pattern for one of the coinfecting strains was that observed in the specimen that had been assigned as belonging to case 2. The mixed patterns in case 1 were already present 2 months before he coincided with case 2, thus demonstrating that case 2 played no role in the infection of case 1.

Extending the analysis: heterogeneous representativeness of coinfecting strains. After demonstrating the mixed infection in case 1, we investigated the possibility that not all of the specimens taken from case 1 were representative of the true composition of bacteria in his lung and that some specimens harbored only 1 of the 2 coinfecting strains. Detailed analysis of the patterns of additional specimens from case 1 revealed varying representativeness for each coinfecting strain in the sputum samples (Fig. 2A). In fact, we identified 1 specimen so enriched in strain A that the accompanying coinfecting strain was barely identified; we made the same observation in another specimen, which at this point was enriched in strain B (Fig. 2B). This finding supported our hypothesis that some of the sputum samples from case 1 were enriched in only 1 of the coinfecting strains.

Finally, our hypothesis led us to assume that 1 specimen from case 1 had been misassigned to case 2. The only specimen with a positive *M. tuberculosis* culture out of the 5 from case 2 was stain positive (high load), thus indirectly supporting the possibility that it may have belonged to case 1, whose 12 specimens were all stain positive (high load).

DISCUSSION

Laboratory cross-contamination is responsible for false-positive TB in 0.1% to 3% of cases (9, 10). The final resolution of an alert requires genotyping the isolates involved. Based on the fingerprints obtained, the case under analysis is finally considered a true positive when the patterns of the isolates involved are different or false positive if the genotypes are identical and no epidemiological links can be identified between the patients.

Although high-resolution genotyping of *M. tuberculosis* requires the combination of as many fingerprinting tools as possible (restriction fragment length polymorphism [RFLP] analysis, spoligotyping, and sequencing) for maximum resolution, other more limited strategies are commonly used for specific purposes. The standard fingerprinting procedure is based on multiplex 24-locus MIRU-VNTR fingerprinting followed by capillary electrophoresis to determine whether or not the numerical code for the 2 isolates involved in the alert is identical (11). In our institution, however, we developed an adapted strategy (MLP3) to simplify and accelerate resolution of the alert (12). MLP3 is aimed exclusively at determining the identity of or differences between isolates. It involves only 5 triplex MIRU-VNTR reactions and compares the mobility of the 3-band patterns in standard electrophoresis for assessment of qualitative identity or differences. This procedure has proven to be highly efficient for rapid resolution of cross-contaminations, determining whether recurrences are due to reactivations or reinfections, and resolving suspected microepidemics and outbreaks (12).

The application of MLP3-based genotyping led us to shift our initial suspicion of laboratory cross-contamination from case 1 (true positive) to case 2 (false positive) to an alternative hypothesis that tried to account for the finding of several alleles for specific loci in case 1 and the finding that case 2 did not have TB. Our novel hypothesis was that case 1 was a true-positive TB case with the coincidence of 2 unexpected features: a mixed infection by 2 *M. tuberculosis* strains whose representativeness differed over several samples and that one case 1 sample, enriched in only 1 of the coinfecting strains, was wrongly assigned to case 2. Given that the coincidence of these findings was highly unlikely, we were obliged to search for additional microbiological, clinical, and epidemiological evidence to support the new hypothesis.

Epidemiological data from case 1 accounted for the mixed infection detected. The patient was an active injection drug user who was outside the public health system, temporarily homeless, and living in an area of slum housing that is well known for its illicit drug trade and high incidence of TB. Consequently, the mixed infection was most likely due to overexposure to different infectious cases.

Mixed infection would have gone undetected in case 1 if the initial fingerprinting analysis had been performed according to the standard procedure, that is, on a cultured isolate instead of directly on the respiratory specimen. It has been reported that culture can affect the true clonal composition of the *M. tuberculosis* population in sputum by modifying the representativeness of some of the coinfecting strains or even eliminating some of them (13, 14). In our study, mixed infection did not have an impact on the outcome of the patient, as the antibiogram from the culture revealed pansusceptibility, thus indicating that neither of the 2 coinfecting strains was resistant.

In addition to the identification of epidemiological features supporting mixed infection in case 1, we needed to demonstrate circumstances that explained the heterogeneous presence of the coinfecting strains in the different sputum samples analyzed. Recent studies in macaques (15) infected with various strains simultaneously have demonstrated that infected sites in the lung can harbor different strains and that not all sites drain equally to each respiratory sample, thus accounting for heteroge-

A Electropherograms of Mix 3

B Electropherograms of mix 6

neous identification of strains in different specimens from cases with complex infections. We also reported cases infected by more than 1 clonal variant of *M. tuberculosis* in which not all of the sequential sputum samples from the same patient harbored the same composition of bacteria (16). In case 1, we reviewed the clinical charts to establish a consistent explanation for the heterogeneous distribution of the coinfecting strains. The patient showed an X-ray pattern with extensive multiple bilateral cavitated lesions in both upper lobes that may have facilitated unusual specific compartmentalization of the 2 strains and may justify potential differential shedding of each coinfecting strain in independent sputum samples.

The most challenging issue in our assessment was the demonstration of the possible misassignment of 1 specimen from case 1 to case 2. We found that the 2 cases coincided in the same ward and that their samples were taken on the same day by the same nurse. The suspicion of mislabeling led us to thoroughly review the sample labeling procedure and to improve monitoring of the steps involved to ensure that they are carried out correctly.

In summary, not considering mixed infection with heterogeneous representativeness in various sputum samples hampered the relatively straightforward resolution of a routine occurrence—cross-contamination—thus forcing us to consider several possible interpretations. Fortunately, the integration of microbiological, clinical, and epidemiological data with a refined molecular analysis performed on both cultured isolates and, very importantly, directly on respiratory specimens revealed a false-positive case that would have gone undetected using standard analysis. Consequently, we were able to avoid the personal, therapeutic, and epidemiological impact of a misdiagnosis of TB. Although ours is a case report and additional studies with a larger number of samples should be carried out, we can conclude that clonal complexity in *M. tuberculosis* must be borne in mind in clinical practice, even in areas of moderate incidence, to ensure that challenging situations are appropriately assessed.

MATERIALS AND METHODS

Microbiological procedures. Clinical specimens (sputum samples) were processed according to standard methods (17) and inoculated on Lowenstein-Jensen slants and in mycobacterial growth indicator tube (MGIT) liquid medium (Becton Dickinson, Sparks, MD, USA). Susceptibility testing for isoniazid, rifampin, streptomycin, and ethambutol was performed using the mycobacterial growth indicator tube SIRE system (Becton Dickinson, Sparks, MD, USA) according to standard methods.

MIRU-VNTR length polymorphism triplex. MLP3 (12) is based on nonfluorescent triplex PCR and separation of the amplified products using conventional agarose gel electrophoresis. This approach uses the electrophoretic mobility of the 3-band pattern as a fingerprint, without the need to assign numerical values to alleles. The analysis therefore enables a qualitative comparison between the patterns from different strains in order to determine whether they are identical or different. We applied the procedure as determined by Sisilema-Egas et al. (12), although in this case, without having to relocate loci in the multiplex PCR to rule out potential overlap between patterns.

MIRU-VNTR analysis. The 24-locus MIRU-VNTR multiplex analysis was performed either directly from respiratory specimens (18) or from cultured isolates. DNA was purified using the Qiagen DNA minikit (Qiagen, Hilden, Germany).

For the analysis of single colonies, we performed serial dilutions of a concentrated inoculum (0.5 to 0.8 McFarland standard) and plated serial dilutions (10^2 to 10^6) from the stock on 7H11 agar plates. We selected plates that allowed us to select around 100 independent colonies for analysis.

The final reaction mixture (50 μ l) included 25 μ l of PCR master mix (Qiagen multiplex PCR kit), 5 μ l of Q solution, and 0.25 μ M each of labeled and unlabeled oligonucleotide (3.9 μ M for loci 4156 and 2059). The primers used for PCR amplification were previously described by Supply et al. (11) (Table 1). Amplification profiles were as described elsewhere (19), except for the number of cycles (20 or 35 cycles when using DNA from culture or from clinical specimens, respectively). PCR products were analyzed using capillary electrophoresis (20) in an ABI Prism 3100 genetic analyzer (Applied Biosystems, NLLab Centraal B.V., Haarlem, The Netherlands).

Ethical features. Informed consent was not required because all of the explorations and procedures followed were part of the standard clinical management of the case.

FIG 2 Electropherograms obtained from the analysis of MIRU-VNTR triplex products in capillary electrophoresis. Each peak corresponds to 1 amplicon (the respective loci are indicated). (A) selection of specimens in which the mixed infection by 2 strains can be observed for a representative MIRU-VNTR triplex reaction (mix 3); (B) selection of specimens in which enrichment in only 1 of the 2 coinfecting strains can be observed for a representative MIRU-VNTR triplex reaction (mix 6). A/B, since the sizes of the amplicons corresponding to locus MIRU 39 were identical for the two strains, the respective peaks overlapped.

TABLE 1 Primers used for multiplex fluorescent MIRU-VNTR analysis

Primer	Sequence
4-1	GCG CGA GAG CCC GAA CTG C (FAM)
4-2	GCG CAG CAG AAA CGT CAG C
26-1	TAG GTC TAC CGT CGA AAT CTG TGA C
26-2	CAT AGG CGA CCA GGC GAA TAG (VIC)
40-1	GGG TTG CTG GAT GAC AAC GTG T (NED)
40-2	GGG TGA TCT CGG CGA AAT CAG ATA
10-1	GTT CTT GAC CAA CTG CAG TCG TCC (FAM)
10-2	GCC ACC TTG GTG ATC AGC TAC CT
16-1	TCG GTG ATC GGG TCC AGT CCA AGT A
16-2	CCC GTC GTG CAG CCC TGG TAC (VIC)
31-1	ACT GAT TGG CTT CAT ACG GCT TTA
31-2	GTG CCG ACG TGG TCT TGA T (NED)
2-1	TGG ACT TGC AGC AAT GGA CCA ACT
2-2	TAC TCG GAC GCC GGC TCA AAA T (FAM)
23-1	CTG TCG ATG GCC GCA ACA AAA CG (VIC)
23-2	AGC TCA ACG GGT TCG CCC TTT TGT C
39-1	CGC ATC GAC AAA CTG GAG CCA AAC
39-2	CGG AAA CGT CTA CGC CCC ACA CAT (NED)
20-1	TCG GAG AGA TGC CCT TCG AGT TAG (FAM)
20-2	GGA GAC CGC GAC CAG GTA CTT GTA
24-1	CGA CCA AGA TGT GCA GGA ATA CAT
24-2	GGG CGA GTT GAG CTC ACA GAA (VIC)
27-1	TCG AAA GCC TCT GCG TGC CAG TAA
27-2	GCG ATG TGA GCG TGC CAC TCA A (NED)
42-1	CTT GGC CGG CAT CAA GCG CAT TAT T
42-2	GGC AGC AGA GCC CGG GAT TCT TC (FAM)
43-1	CGA GAG TGG CAG TGG CGG TTA TCT (VIC)
43-2	AAT GAC TTG AAC GCG CAA ATT GTG A
ETRA-1	AAA TCG GTC CCA TCA CCT TCT TAT (NED)
ETRA-2	CGA AGC CTG GGG TGC CCG CGA TTT
47-1	CTT GAA GCC CCG GTC TCA TCT GT (FAM)
47-2	ACT TGA ACC CCC ACG CCC ATT AGT A
52-1	CGG TGG AGG CGA TGA ACG TCT TC (VIC)
52-2	TAG AGC GGC ACG GGG GAA AGC TTA G
53-1	TGA CCA CGG ATT GCT CTA GT
53-2	GCC GGC GTC CAT GTT (NED)
QUB-11b-1	CGT AAG GGG GAT GCG GGA AAT AGG
QUB-11b-2	CGA AGT GAA TGG TGG CAT (FAM)
1955-1	AGA TCC CAG TTG TCG TCG TC (VIC)
1955-2	CAA CAT CGC CTG GTT CTG TA
QUB-26-1	AAC GCT CAG CTG TCG GAT (NED)
QUB-26-2	CGG CCG TGC CGG CCA GGT CCT TCC CGA T
46-1	GCCAGCCGCCGTGCATAAACCT (FAM)
46-2	AGCCACCCGGTGTGCCTTGTATGAC
48-1	ATGGCCACCCGATACCGCTTCAGT (VIC)
48-2	CGACGGGCCATCTTGGATCAGCTAC
49-1	GGTGCGCACCTGCTCAGATAA (NED)
49-2	GGCTCTCATTGCTGGAGGGTGTAC

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We declare that we have no conflicts of interest.

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