A novel strategy based on genomics and specific PCR reveals how a multidrug resistant *Mycobacterium tuberculosis* strain became prevalent in Equatorial Guinea 15 years after its emergence

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Summary

Objective: Molecular epidemiology techniques in tuberculosis (TB) can identify high-risk strains that are actively transmitted. We aimed to implement a novel strategy to optimize the identification and control of MDR-TB in a specific population.

Methods: We developed a strain-specific-PCR tailored from whole-genome-sequencing (WGS) data to track a specific multidrug-resistant prevalent strain in Equatorial Guinea (EG-MDR).

Results: The PCR was applied prospectively on remnants of GeneXpert reaction mixtures owing to the lack of culture facilities in EG. In 147/158 cases (93%), we were able to differentiate between infection by the EG-MDR strain or by any other strain and found that 44% of all rifampicin resistant-TB cases were infected by EG-MDR. We also analyzed 93 isolates obtained from EG 15 years ago, before MDR-TB had become the problem it is today. We found that 2 of the scarce historical MDR cases were infected by EG-MDR. WGS revealed low variability—6 SNPs acquired by this strain over 15 years—likely owing to the lack of a national program to treat MDR-TB.

Conclusions: Our novel strategy, which integrated WGS analysis and strain-specific PCRs, represents a low-cost, rapid, and transferable strategy that allowed a prospective efficient survey and fast historical analysis of MDR-TB in a population.

Introduction

Molecular epidemiology studies in tuberculosis (TB) are generally based on the universal application of a fingerprinting tool, mycobacterial interspersed repetitive units-variable number of tandem repeats (MIRU-VNTR), [1] to all the *Mycobacterium tuberculosis* (MTB) isolates in a population. MIRU-VNTR makes it possible to identify clusters of epidemiologically linked isolates and thus alerts to the existence of ongoing transmission chains that should be targeted by control measures.

Once the most relevant ongoing transmission chains have been identified in a population, efforts can be prioritized to survey transmission hotspots and to track the transmission of the strains involved. In some cases, specific molecular tools have been developed to track selected strains [2-7]. We proved the efficiency and flexibility of a new strategy based on strain-specific PCRs targeting specific SNPs identified from whole genome sequencing [8, 9].

We sought a suitable scenario to further evaluate these novel surveillance strategies, specifically, a context where a single high-risk multidrug-resistant (MDR) strain could have a relevant role at the population level. We opted for Equatorial Guinea, a small, densely populated country in central Africa, recently revealed as a MDR hot-spot [10]. Indirect data point to the presence of a prevalent strain leading MDR transmission in this country. We found particularly relevant data [11] showing that all 10 Equatorial Guinean immigrants who arrived in Spain in different years and were residing in 6 different cities shared the same MDR strain, thus suggesting multiple independent importations of a highly prevalent MDR strain from the country of origin.

In the present study, we go one step further in the application of novel strategies based on WGS. Our objectives were as follows: i) to evaluate the efficiency

of the strategy in the most relevant challenge that TB control offers today, namely, monitoring of transmission of multidrug resistance; and ii) to activate a fully comprehensive design that included both the prospective surveillance of transmission of MDR strains in a specific population and to perform a retrospective historical analysis to locate the time-point when the culprit MDR strain emerged in that population.

Methods

Clinical specimens

For the prospective study, we used the remnants of the mixtures of sputa with GeneXpert MTB/RIF Sample Reagent (Cepheid, Sunnyvale, CA, USA) used to perform GeneXpert tests in a study running between February 1st and April 30th 2015 in Equatorial Guinea [10]. The study followed the standard ethical regulations. All smear-postive specimens from consecutive TB cases notified to the 2 main TB units in the country during the study period were collected. Sputa were mixed 1:2 with Sample Reagent and the reaction mixtures were stored at room temperature until delivery to our laboratory in Madrid, Spain. Deliveries were organized to ensure a maximum delay of 20 days between specimen collection in Equatorial Guinea and reception in our laboratory.

On arrival, mixtures were used to perform the GeneXpert assay. Three milliliters of the remaining mixture was used for DNA purification with a column-based purification method (QIAamp DNA Mini Kit; Qiagen, Courtaboeuf, France) and eluted in 75 µl of buffer AE. We selected all the remnants from reaction mixtures in which GeneXpert detected MTB,

For the retrospective analysis, we took a 100-µl microliter aliquot from the isolates stored frozen at –70ºC, boiled it for 13 minutes, and centrifuged it at 13,000 rpm. The supernatant was taken for analysis.

Whole genome sequencing

WGS from 3 representative isolates was performed as indicated elsewhere [12]. We followed the standard library preparation protocol and used HiSeq 2000 (GATC Biotech, Constance, Germany) and MiSeq (FISABIO, Valencia, Spain), which generated 51-300–bp paired-end reads. SNP analysis was performed following a workflow described elsewhere [12].

Strain-specific SNPs were identified after comparing the SNPs extracted from WGS data with those from an in-house database of 219 strains representing the geographic and phylogenetic diversity of MTB complex [13]. We finally selected synonymous SNPs mapping in essential genes to be targeted in the ASO-PCR analysis to ensure their stability as genetic markers [14].

Strain-specific ASO-PCR design

We designed a multiplex ASO-PCR to target 4 SNPs (Table)specific to the Equatorial Guinea MDR strain (EG-MDR strain), in order to rule out false negatives, as recommended elsewhere [15]. We designed 2 selective primers (SNP2 and SNP4) to target the alleles found in the surveyed strain, whereas the remaining 2 primers (SNP1 and SNP3) targeted the alleles from the non-surveyed strains (Table). Our design generated 2 different patterns depending on whether the strain tested was EG-MDR or another strain.

The assay conditions were 1.6 mM $MgCl_2$, 1%DMSO, 0.8 µM of SNP1 primers, 0.3 µM of SNP2 primers, 0.09 µM of SNP3 primers, 0.11 µM of SNP4 primers, 200 µM dNTPs, and 0.4 µL AmpliTaq Gold (Applied Biosystems, Foster

City, California, USA). The PCR conditions were 95°C for 10 minutes followed by 30 cycles (95°C for 1 minute, 65°C for 1 minute, and 72°C for 1 minute) and 72°C for 10 minutes.

MIRU-VNTR analysis

The isolates were genotyped using the MIRU-15 multiplex PCR [1] as described in Alonso *et al.* [16], but with 30 amplification cycles. The MIRU-VNTR loci order was as described elsewhere [11].

Susceptibility testing

Susceptibility to isoniazid, rifampin, and second-line anti-TB drugs was assessed using Anyplex II MTB/MDR/XDR Detection (Seegene Inc, Seoul, Korea).

Results

Optimization of a strain-specific PCR for a prevalent Equatorial Guinean MDR strain

An immigrant who had recently arrived to Spain from Equatorial Guinea was diagnosed with MDR-TB (resistant to rifampicin, isoniazid, ethambutol, and pyrazinamide) in October 2014 in our institution. Her MTB isolate was analyzed using MIRU-VNTR, and the pattern (244214232324115153522722) was identical to the one, belonging to Lineage 4, described as prevalent in EG immigrants in Spain [11].

The isolate was analyzed by whole genome sequencing, and the comparison with the reference strain (the most recent common ancestor of MTB [13, 17]) revealed 868 SNPs. These SNPs were compared with those found in a global database of representative MTB strains circulating worldwide, and, after filtering out shared SNPs, we kept 85 SNPs as potentially strain-specific for EG-MDR strain. Four of these SNPs (Table) were finally selected to be targeted in a newly designed ASO-PCR (Figure 1).

The specificity of our EG-MDR strain-specific ASO-PCR was tested on a selection of strains, including those from the newly diagnosed immigrant and a selection of 15 unrelated strains randomly selected from our collection (one lineage 2, one lineage 6 and the remaining lineage 4). The expected ASO-PCR patterns were obtained in all cases.

Prospective application of the ASO-PCR

When the optimized EG-MDR strain-specific PCR was about to be prospectively applied on new cases diagnosed in Equatorial Guinea, we found ourselves faced with a challenging situation, namely, the absence of cultured MTB isolates resulting from the lack of capacity in laboratories in Equatorial Guinea. The only material available was the remnants from the reaction mixtures from an assessment of resistance based on GeneXpert that was simultaneously running in the country.

The ASO-PCR was applied to 158 purified remnants from the GeneXpert reaction mixtures from consecutive respiratory specimens. The evaluators were blind to the GeneXpert resistance data. An interpretable result was obtained in 147 cases (93.6%) (Figure 1b). The EG-MDR strain was identified in 12 cases (8%) of all those with an ASO-PCR result. All specimens with an EG-MDR pattern corresponded to strains shown to be rifampicin resistant by GeneXpert, and 44.4% of all cases with resistance in Equatorial Guinea corresponded to infections with the EG-MDR strain.

A mixed pattern including the EG-MDR and non-EG-MDR profiles was found in 1 case. When the ASO-PCR results were compared with those from GeneXpert, the 11 specimens without an interpretable ASO-PCR result corresponded to specimens with low (8 specimens) or medium (3 specimens) bacterial load.

MIRU-VNTR for the specimens with an ASO-PCR pattern corresponding to the EG-MDR strain was performed from the scarce amount of purified DNA that was available after applying the ASO-PCR. Given the limited material available, a complete 15-loci MIRU-VNTR pattern was obtained from 5 of the 12 specimens with a result that was consistent with the EG-MDR strain; only incomplete patterns (9-12

loci) were obtained for the remaining 7 specimens. In all cases, the allelic values coincided with those of the EG-MDR strain. As for the specimen with a mixed EG-MDR/non–EG-MDR pattern by ASO-PCR, the mixed infection was confirmed as indicated by double alleles at 5 loci, with one of the mixed MIRU-VNTR profiles identical to that of the EG-MDR strain.

In addition to the consecutive Equatorial Guinean specimens, the ASO-PCR was also applied to a selection of 6 Equatorial Guinean patients who had travelled to Cameroon to receive treatment for resistant TB. In these cases, the ASO-PCR was performed on the remnants received from the purified DNA used for the Genotype (Hain) test performed to confirm resistance in Cameroon. The ASO-PCR revealed that all 6 cases were infected by the EG-MDR strain, thus confirming its high prevalence among resistant cases.

Second-line susceptibility patterns were obtained in a selection of 10 cases infected by the EG-MDR strain. In 1 case, we detected resistance to fluoroquinolones in addition to resistance to rifampicin and isoniazid.

Retrospective application of the ASO-PCR

Our objective was to evaluate whether the EG-MDR strain, which is highly prevalent in Equatorial Guinea today, or alternatively the parental strain preceding the acquisition of resistance was also present 15 years previously, when multidrug resistance was not yet a major problem in the country.

The same ASO-PCR used to prospectively survey the EG-MDR in Equatorial Guinea was now applied to a collection of MTB isolates obtained 15 years ago from a population-based survey to assess resistance in Equatorial Guinea between March 1999 and February 2001. We selected 93 isolates, which included 1 representative

isolate of 18 of the 21 clusters detected in that study using IS6110-RFLP [18], and the remaining orphan strains. These included the viable resistant strains from that study (9 isoniazid-resistant, 2 MDR, and 2 polyresistant).

An interpretable pattern was obtained from a crude boiled extract in all 93 isolates (Figure 2). The EG-MDR pattern was identified in 2 cases, which corresponded to 2 of the 8 MDR cases in that study (1 new isoniazid+rifampicin–resistant case and a previously treated isoniazid+rifampicin+streptomycin–resistant case). MIRU-VNTR confirmed that the pattern from the 2 cases corresponded to the EG-MDR strain.

The 2 representative isolates for the EG-MDR strain identified 15 years ago were analyzed by WGS to compare data with those from the representative strain circulating today. All 3 shared the mutations conferring resistance to rifampicin (position 761155CT; *rpo*B S450L) and isoniazid (position 1673425CT; *inh*A promoter mutation –15). One of the two historical isolates showed 2 SNPs not shared by the others (position 1472358CT, which confers resistance to streptomycin, and position 2155198GA [*kat*G G305D]). The 2014 isolate yielded 6 SNPs not found in the historical representatives: *emb*B (M306I), which is involved in ethambutol resistance, *pcn*A (X187R), which is involved in pyrazinamide resistance, and 4 additional SNPs in *hem*C, *gid*B, *ack*A, and Rv2090.

DISCUSSION

The implementation of molecular epidemiology strategies in various countries has demonstrated the usefulness of this approach for identifying recent transmission chains and the extent to which they occur in contexts where they have been missed by standard contact-tracing analysis. TB control programs could benefit from being able to prioritize the often limited resources they assign to transmission control activities by applying them to the transmission events responsible for the highest number of secondary cases in their population. Strain-specific PCRs have been developed to track the most efficiently transmitted strains in a population [2-5]. However, the design of strain-specific PCRs requires in-depth knowledge of the genetic composition of the strains targeted for surveillance in order to identify specific genetic features or genotypic rearrangements to be targeted. Today, WGS enables us to identify specific genetic features, namely, SNPs, in any strain, thus expanding our capacity to design strain-specific PCRs to track the most efficiently transmitted strains in a population.

We recently proved the effectiveness of these novel strategies for specifically tracking strains transmitted efficiently in a population with a high proportion of immigrants [8] and for rapid interrogation of a retrospective collection to determine the presence of an outbreak strain [9]. Another example of the application of specific PCRs in refining the definition of an outbreak has been published elsewhere [15].

In the present study, we go one step further, by integrating these 2 challenges (prospective efficient survey and fast historical analysis) into a single problem, which is representative of the highest alert in TB from a clinical/epidemiological point of view, namely, the transmission of multidrug resistance. The application of our

strategy in this scenario enabled us to illustrate the predominance of a single MDR strain in Equatorial Guinea 15 years after its emergence.

Our simple and fast test was able to identify cases infected by the prevalent EG-MDR strain, even in a challenging analytical situation, because cultured isolates were not available. Our test performed well with the remnants of GeneXpert reaction mixtures, and we observed a high percentage of interpretable results (93%), considering that remnants were stored at room temperature for several weeks before being sent to our laboratory for analysis.

The potential of GeneXpert remnants as templates for molecular epidemiology analysis had not been previously tested. Our results highlight the value of the combination of cheap and easy-to-implement surveillance tests with the stabilized extracted material offered by GeneXpert, which is used worldwide, even in most low-resource countries. Replicating our strategy could improve our limited knowledge of the molecular epidemiology of TB in many low-resource settings, even in those where culture facilities are not available.

One interesting additional finding was the identification of a mixed infection involving an EG-MDR strain and another strain. Although data available were not sufficient to rule out the involvement of cross-transmission it illustrates the ability of the technique to identify simultaneous infections by more than one strain.

One added value of our strategy was that it enabled us to integrate the prospective survey of the prevalent EG-MDR strain with parallel historical tracking of its emergence in the country. The simplicity of our design facilitated transfer of the reagents to the laboratory in Barcelona, where the isolates obtained in the first molecular epidemiology study run in Equatorial Guinea 15 years ago were stored [18]. The sensitivity of the test made it possible to analyze the stored isolates directly

298 from a crude boiled extract, without the need to subculture or purify DNA, thus
299 making the test fast and inexpensive.

300 Two cases were infected by the EG-MDR strain in the retrospective collection.
301 During those years most cases were monoresistant to isoniazid, and only 8 cases
302 (3.4%) were MDR [19]. One of the 2 cases was newly diagnosed and the other
303 previously treated.

304 Our findings provide a snapshot of 2 relevant moments in the history of
305 multidrug resistance in Equatorial Guinea. The first was the emergence of an MDR
306 strain, most likely in a treated case 15 years ago. In the second, we see the dramatic
307 consequences of this emergence because today, the same strain is responsible for half
308 of all resistant cases in the country, most likely because of transmission, as indicated
309 by the fact that the EG-MDR strain is mainly isolated from new cases (data not
310 shown).

311 The identification of representatives of the EG-MDR strain 15 years apart
312 offers an extraordinary opportunity to analyze the variability acquired by an MDR
313 strain over a long period. Surprisingly, for an MDR strain that was actively
314 transmitted over such a long period (when compensatory mutations and additional
315 variability are likely to be expected) [20, 21], we observed low variability based on
316 SNPs within the same range as that expected in a standard recent transmission chain
317 occurring over a limited period [22]. This finding is consistent with one of the
318 peculiarities of Equatorial Guinea: the lack of a national program to treat MDR-TB
319 means that the EG-MDR strain has not been exposed to efficient therapeutic
320 regimens, thus obviating the selection of variability that had likely occurred under
321 standard anti-TB selective pressure.

It is particularly worrying that, compared with its historical ancestor, the EG-MDR strain developed additional resistance within this prolonged transmission dynamic (ethambutol and pyrazinamide, 1 isolate; fluoroquinolones, 1 isolate). The acquisition of resistance to fluoroquinolones is probably due to the frequent use of ciprofloxacin in many African countries, as recently stated in a report alerting to the cross-border migration of MDR cases from Equatorial Guinea to Cameroon to receive treatment [23]. In a sample of patients who migrated to Cameroon, all members were infected by the EG-MDR strain, thus highlighting the importance of tracking the potential transmission of the EG-MDR strain in the neighboring country. The final objective of our strategy, which was based on tailoring a strain-specific PCR to address local transmission challenges, is to transfer the tests to be implemented locally. We are collaborating with Bamenda hospital in Cameroon to implement the EG-MDR–specific PCR locally and optimize rapid analysis *in situ*.

Our study enabled us to analyze the dramatic consequences of the emergence of a MDR strain 15 years ago that is responsible for half of the MDR cases in Equatorial Guinea today. Transmission was likely enhanced by the dramatic socioeconomic transformation of the country and the lack of a national program to treat MDR-TB. This worrying situation must be appropriately managed to minimize the impact on both neighboring countries and on host countries receiving immigrants from this area. Our novel strategy, which integrated WGS analysis and strain-specific PCRs, could facilitate surveillance and represent a model for new control programs based on low-cost, rapid, and transferable tests tailored to the challenges of various populations.

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452 Figures

453 1. a) Amplification patterns obtained from EG-MDR strain-specific multiplex ASO-
454 PCR results for control EG-MDR and non–EG-MDR strains. The amplicons
455 corresponding to each of the 4 targeted SNPs are indicated. b) A selection of EG-
456 MDR and non-EG-MDR strains. MW, 100-bp ladder.

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458 2. Results obtained after applying the EG-MDR strain-specific multiplex ASO-PCR
459 to a selection of historical isolates. The isolates identified as EG-MDR or non-EG-
460 MDR are indicated. MW, 50-bp ladder.

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