A Novel Molecular Strategy for Surveillance of Multidrug Resistant Tuberculosis in High Burden Settings

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Abstract

Background

In South Africa and other high prevalence countries, transmission is a significant contributor to rising rates of multidrug resistant tuberculosis (MDR-TB). Thus, there is a need to develop an early detection system for transmission clusters suitable for high burden settings. We have evaluated the discriminatory power and clustering concordance of a novel and simple genotyping approach, combining spoligotyping with \textit{pncA} sequencing (SpoNC), against two well-established methods: IS\textit{6110}-RFLP and 24-loci MIRU-VNTR.

Methods

A total of 216 MDR-TB isolates collected from January to June 2010 from the NHLS Central TB referral laboratory in Braamfontein, Johannesburg, representing a diversity of strains from South Africa, were included. The isolates were submitted for genotyping, \textit{pncA} sequencing and analysis to the Centre for Tuberculosis in South Africa and the Public Health Research Institute Tuberculosis Center at Rutgers University in the United States. Clustering rates, Hunter-Gaston Discriminatory Indexes (HGI) and Wallace coefficients were compared between the methods.

Results

Overall clustering rates were high by both IS\textit{6110}-RFLP (52.8\%) and MIRU-VNTR (45.8\%), indicative of on-going transmission. Both 24-loci MIRU-VNTR and IS\textit{6110}-RFLP had similar HGI (0.972 and 0.973, respectively), with close numbers of unique profiles (87 vs. 70), clustered isolates (129 vs. 146), and cluster sizes (2 to 26 vs. 2 to 25 isolates). Spoligotyping alone was the least discriminatory (80.1\% clustering, HGI 0.903), with 28 unique types. However, the discriminatory power of spoligotyping was improved when combined with
pncA sequencing using the SpoNC approach (61.8% clustering, HGI 0.958). A high proportion of MDR-TB isolates had mutations in pncA (68%, n = 145), and pncA mutations were significantly associated with clustering (p = 0.007 and p = 0.0013 by 24-loci MIRU-VNTR and IS6110-RFLP, respectively), suggesting high rates of resistance to pyrazinamide among all MDR-TB cases and particularly among clustered cases.

Conclusion
We conclude that SpoNC provides good discrimination for MDR-TB surveillance and early identification of outbreaks in South Africa, with 24-loci MIRU-VNTR applied for pncA wild-type strains as needed.

Background
South Africa has the world’s third highest tuberculosis (TB) burden, after India and China and is second highest in terms of absolute numbers of multidrug-resistant (MDR) TB [1]. Moreover, since the 2006 Tugela Ferry outbreak of extensively drug-resistant (XDR) TB in Kwa-Zulu-Natal, XDR-TB has been reported in all provinces in South Africa [2, 3]. Both MDR- and XDR-TB are associated with elevated mortality and high treatment costs, thus contributing a disproportionate burden on health systems [4]. The emergence of MDR-TB has been associated with inappropriate drug regimens, treatment default and HIV co-infection [5, 6]. In addition, evidence from population studies carried out in South Africa and other high prevalence settings suggest that transmission is a significant contributor to globally rising rates of MDR-TB [7–9].

The World Health Organization has set ambitious targets for TB control for the next 20 years [10]. However for these to be achieved, catalysts of transmission will need to be identified for development of targeted interventions, as discussed by Dowdy et al [11]. This would require establishment of an early warning surveillance system to detect genotypic clusters among clinical isolates of Mycobacterium tuberculosis (M. tuberculosis) as an indication of transmission in the population. Although routine genotyping forms an important component of TB control programs in many low prevalence settings, wide-scale genotyping in countries with high TB burden has been hampered by constrained resources and by the prevalence of endemic strains, which complicate the molecular discrimination of epidemiologically linked clusters [12].

The choice of an appropriate genotyping method depends on the extent of diversity of the biomarker among M. tuberculosis isolates in the population. The selected biomarker must be sufficiently polymorphic to distinguish unrelated strains and also stable enough to detect epidemiologically-linked strain clusters [13]. In addition, for optimal application in surveillance, the method should be rapid, reproducible, and easy to interpret in order to facilitate high-throughput analysis to limit cost and ensure timely results for public health action [14].

The use of IS6110-restriction fragment length polymorphism (RFLP) for TB genotyping is internationally accepted as the gold standard and provides excellent discrimination [15]. However, the method requires time for subculture to isolate high quality DNA, has limited discrimination for strains with low IS6110 copy numbers and the results are not readily digitized, making inter-laboratory comparisons difficult [16, 17]. Polymerase chain reaction (PCR)-based mycobacterial interspersed repetitive units-variable number tandem repeats (MIRU-VNTR) typing, using 15- or 24-loci, has high discriminatory power and the results are readily compared as standardized digital codes, but this method is relatively expensive and has
shown lower discrimination in settings where Beijing strains are dominant [18, 19]. Compared to IS6110-RFLP and MIRU-VNTR, spacer oligonucleotide typing (spoligotyping) is the simplest method to perform, yielding rapid results in the form of an internationally standardized binary pattern, making it ideal for high-throughput analysis [20]. However, spoligotyping has relatively low discriminatory power and may distort estimates of recent MDR-TB transmission by clustering endemic strains that are not necessarily epidemiologically linked [21].

A number of drug resistance mutations have been used for fine resolution of genotyping clusters [13]. The \textit{M. tuberculosis} \textit{pncA} gene encodes an amidase that is required for modifying the anti-TB drug pyrazinamide (PZA) to an active state. Mutations in \textit{pncA} account for the majority of resistance to PZA in both clinical and laboratory isolates and show high correlation with in vitro assays of phenotypic resistance to PZA [22]. Due to the technical challenges of growth-based PZA susceptibility testing, resistance testing is not routinely performed in many high burden countries, including South Africa [23]. However, two surveys conducted in Gauteng and Western Cape between 2000 and 2002 showed that clinical isolates from over 50% of MDR-TB patients harbored mutations in the \textit{pncA} locus [24, 25]. This finding is evidence of a large burden of undiagnosed PZA resistance, particularly among MDR-TB cases. Importantly for its application in molecular typing, mutations in the \textit{pncA} locus are highly diverse, spanning a region of ~600 nt covering the entire gene and putative promoter region [26]. A recently published meta-analysis of global PZA resistance identified 608 unique polymorphisms in 397 positions throughout the \textit{pncA} locus, with a pooled estimated prevalence of 16.2% among all TB cases and 60.5% among MDR cases [23]. Thus, the use of \textit{pncA} sequencing as a secondary genetic marker to improve the resolution of spoligotyping clusters offers a novel strategy to provide a high volume and faster approach for genotyping in endemic settings, with the added benefit of providing resistance information for programmatic purposes.

The present study was designed to evaluate \textit{pncA} sequencing as a secondary marker and to examine the association between \textit{pncA} mutations and clustering in an MDR-TB population in South Africa. In addition, we sought to determine the discriminatory ability and cluster concordance of spoligotyping combined with \textit{pncA} sequence profiling (SpoNC) compared with IS6110-RFLP and 24-loci MIRU genotyping methods.

**Methods**

The wording of the manuscript is suitable for publication.

**Ethics**

This study was approved by the Human Research Ethics Committee of the University of the Witwatersrand and the Institutional Review Board of Rutgers University. The institutional review board committee waived the need for consent.

**Clinical Samples**

A total of 216 clinical isolates of \textit{M. tuberculosis} from culture positive confirmed MDR-TB cases was used in this study. These isolates were collected over the first 6 months of 2010 from the NHLS Central TB referral laboratory in Braamfontein, Johannesburg. The NHLS laboratory is a high-throughput routine diagnostic laboratory that receives specimens for culture and drug susceptibility testing (DST) from over 100 health facilities in Johannesburg and surrounding areas. All isolates were submitted for genotyping and analysis to the Centre for Tuberculosis (CTB) at the National Institute for Communicable Diseases in South Africa and the Public Health Research Institute (PHRI) Tuberculosis Center at Rutgers University in the United States.
Genotyping

Spoligotyping was done using a commercial kit (Ocimum Biosolutions, India) according to the manufacturer's instructions [20]; spoligotypes were uploaded as binary codes in the SpolDB4 database for SIT (Spoligotype International Type) assignment. The MIRU-VNTR typing was performed using an automated 24-loci MIRU-VNTR method, as described [27], and validated using the MIRU-VNTR Calibration Kit (GenoScreen, France). The PCR products were subjected to electrophoresis using the ABI 3130XL genetic analyser (Applied Biosystems, CA, USA) with the LizMarker (Bioventures, TN, USA) as a size standard. Sizing of fragments and MIRU-VNTR allele assignment were performed using GeneMapper software 4.0 (Applied Biosystems). IS6110-RFLP typing was performed using the standardized methods, as described previously [15].

Amplification and sequencing of the pncA locus

Genomic DNA was prepared from all isolates and subjected to PCR amplification (primers: 5’-TGGCCGCCGCAGTCAGCTGGTCATG-3’; 5’-CCACCGCCGCCAACAGTTCAT-3’) followed by Sanger sequencing of pncA promoter and coding DNA sequence (CDS). Mutations were identified by alignment of nucleotide sequences to MTB H37Rv reference strain (NCBI Accession number AL123456) [28] using ClustalW2 [29].

Cluster definition

A cluster was defined for each individual method as two or more M. tuberculosis isolates with an identical genotyping pattern from different patients, while SpoNC clusters were defined as two or more isolates with an identical spoligotyping pattern and pncA mutation. The clustering rate was defined as \(\frac{n_c-c}{n}\), where \(n\) is the total number of cases in the sample, \(c\) is the number of genotypes represented by at least two cases and \(n_c\) is the total number of cases in clusters of two or more patients [30].

Statistical analysis

A chi-square test was used to determine associations between the presence of pncA mutations and clustering defined by IS6110-RFLP or 24-loci MIRU-VNTR. The Hunter-Gaston index (HGI) was calculated as described previously [31] and used to evaluate the level of discriminatory power of the genotyping methods, alone and in combination. The HGI varies between 0.00 (where all strains in the sample are indistinguishable) and 1.00 (all strains are differentiated). Wallace coefficients are determined by calculating the ability to predict clustering generated by one typing method, given the results analysed by a second typing method and were used to measure agreement between methods, as described previously [32]. Potential values range from 0 (no ability to predict) to 100% (perfect predictive ability).

Results

The number of IS6110-RFLP bands ranged from 1 to 23 per strain. Of the 216 M. tuberculosis isolates, a total of 32 clusters (146 isolates, 68%) were identified by IS6110-RFLP, the largest containing 25 isolates, with 70 (32%) unique isolates, giving a strain-clustering rate of 52.8% (Table 1). Of these 32 clusters, four (35 isolates, 16%) had less than six IS6110 elements. The 24-loci MIRU-VNTR typing identified 30 clusters (129 isolates, 60%), of which the largest included 26 isolates, and 87 unique isolates (40%), giving a strain-clustering rate of 45.8%.
Spoligotyping resulted in 43 distinct patterns, including 28 (13%) unique and 188 (87%) clustered isolates, yielding a clustering rate of 80.1%. None of the isolates were identified as *M. bovis*. Among the clustered isolates, 14 strain families were identified by spoligotyping, with Beijing (48/188, 25.5%) constituting the largest, followed by LAM4 (31, 16%), H3 (26, 14%), S (18, 9.6%), EAI1_SOM, X2 and X3 (12, 6.4%), T1 (ST 53) (8, 4.3%), T1 (ST 926) (7, 3.7%), T3 (4, 2.1%), T1 (ST719), LAM3 (ST 33) and LAM3 (ST 130) (3, 1.6%), and LAM9 (2, 1.1%).

Based on HGI, the discriminatory power of 24-loci MIRU-VNTR and IS6110-RFLP typing were similar, while spoligotyping was the least discriminatory of the three methods (Table 1). Among the 48 Beijing strains, IS6110-RFLP and 24-loci MIRU-VNTR typing split the group into 8 and 9 clusters with 17 and 20 unique isolates (HGI 0.997 and 0.998), respectively. The level of concordance between typing methods was evaluated by calculation of Wallace coefficients (Table 2). Considering IS6110-RFLP as the reference standard, the Wallace coefficient was 78% for MIRU-VNTR and 83% for spoligotyping. When 24-loci MIRU-VNTR was used as the reference, the Wallace coefficients were somewhat higher: 88% and 87% for IS6110-RFLP and spoligotyping, respectively. Sixteen of the 32 clusters identified by IS6110-RFLP (115 isolates, 53.2%) shared fully identical genotypes by 24-loci MIRU-VNTR typing. Three IS6110-RFLP clusters were split partially by 24-loci MIRU-VNTR typing, while the remaining 7 clusters were fully differentiated. Conversely, 9 clusters defined by 24-loci MIRU-VNTR typing were partially split by IS6110-RFLP typing and 5 clusters were fully differentiated. Taken together, the similarities in discriminatory power and clustering rates and the high concordance between the two typing methods support the validity of 24-loci MIRU-VNTR genotyping, despite the high proportion of Beijing strains in the population.

Table 1. Discriminatory power of different genotyping methods for MDR-TB isolates.

| Method                      | N  | No. of types | No. of unique isolates | No. of clustered isolates | No of clusters | Range of cluster size | HGI   | Clustering rate |
|-----------------------------|----|--------------|------------------------|---------------------------|----------------|-----------------------|-------|-----------------|
| IS6110-RFLP                 | 216| 102          | 70                     | 146                       | 32             | 2 to 25               | 0.973 | 52.8%           |
| 24-loci MIRU-VNTR           | 216| 117          | 87                     | 129                       | 30             | 2 to 26               | 0.972 | 45.8%           |
| Spoligotyping               | 216| 43           | 28                     | 188                       | 15             | 2 to 48               | 0.903 | 80.1%           |
| Spoligotyping + pncA sequence* | 212| 81           | 53                     | 159                       | 24             | 2 to 29               | 0.958 | 63.7%           |

*excluding 4 isolates without pncA sequence data

Table 2. Concordance between the typing methods in total population (N = 216).

| Reference typing method     | Complementary method   | Wallace coefficient |
|-----------------------------|------------------------|---------------------|
| IS6110-RFLP                 | 24-loci MIRU-VNTR      | 78.2                |
| IS6110-RFLP                 | Spoligotyping          | 82.8                |
| 24-loci MIRU-VNTR           | IS6110-RFLP            | 87.8                |
| 24-loci MIRU-VNTR           | Spoligotyping          | 87.4                |
| Spoligotyping               | IS6110-RFLP            | 70.6                |
| Spoligotyping               | 24-loci MIRU-VNTR      | 60.6                |

doi:10.1371/journal.pone.0146106.t001

doi:10.1371/journal.pone.0146106.t002
Use of *pnc*A sequence as a secondary marker and association with clustering

In our study population, 145 isolates (68%) had mutations in the *pnc*A locus, while 67 isolates had wild type *pnc*A sequence. For four isolates, *pnc*A sequencing results were not available. Diversity at the *pnc*A locus was high, with 47 unique mutations identified, including 5 insertions, 6 deletions, 35 non-synonymous single nucleotide polymorphisms (SNPs) and one synonymous SNP. The frequency of *pnc*A mutations was higher among isolates clustered by both IS6110-RFLP (102/133, 77%) and MIRU-VNTR (98/126, 78%) versus non-clustered isolates (43/79, 54% and 47/86, 54.7%; respectively), demonstrating a significant association between *pnc*A mutations and clustering (*p* = 0.0013, *p* = 0.0007; respectively).

The ability of *pnc*A sequencing to further resolve clusters defined by each of the primary typing methods was examined among the 145 isolates with *pnc*A mutations. Nine IS6110-RFLP-defined clusters (51 isolates) and 12 MIRU-VNTR-defined clusters (54 isolates) harbored identical mutations in the *pnc*A locus unique to each cluster. Fifteen IS6110-RFLP and 11 MIRU-VNTR clusters were subdivided by *pnc*A sequence, increasing the HGI of IS6110-RFLP and MIRU-VNTR to 0.979 and 0.984, respectively (Table 1).

Performance of the SpoNC strategy

Spoligotyping in combination with *pnc*A sequence profiles defined 24 clusters, ranging in size from 2 to 29 members, with 53 unique isolates. With the exception of one cluster, *pnc*A sequence patterns subdivided all spoligotyping clusters, reducing the clustering rate from 80.1% to 63.7% and increasing the discriminatory power from 0.903 to 0.958 (Table 1). The ability of *pnc*A sequencing to improve the discriminatory power of spoligotyping was even more striking when the analysis was limited to those isolates with *pnc*A mutations, resulting in an HGI increased to 0.976 and a clustering rate of 62.8% (Table 1). Furthermore, the concordance was similar when assessing the new strategy with either IS6110-RFLP or 24-loci MIRU-VNTR as the reference standard and was consistently within the same range when the reference standards were compared against each other (Tables 2 and 3). Interestingly, while diverse *pnc*A mutations were able to further resolve clusters defined by each of the three primary typing methods, there were no instances where identical *pnc*A sequences were found to be present in more than one cluster defined by IS6110-RFLP. There was only one instance where the same *pnc*A mutation was present in two isolates with different spoligotypes (LAM3 and LAM4). Four distinct *pnc*A mutations were present in more than one 24-loci MIRU-VNTR cluster, but in all cases, the clusters differed by only a single MIRU-VNTR locus and were thus closely related. Table 4 shows the 16 clusters defined by SpoNC. Each of these clusters is defined by a unique non-synonymous SNP in the *pnc*A locus, and 11 of these specific combinations of spoligotype and *pnc*A mutation have been described in previous reports from South Africa (Table 4).

Discussion

In our study population, 68% of all MDR-TB strains were clustered by IS6110-RFLP typing and 60% were clustered by 24-loci MIRU-VNTR. Similarly high levels of clustering among MDR-TB cases have been described in previous studies from South Africa [33–35], consistent with transmission as a significant contributor to the growing MDR burden. These findings highlight the need for an early warning surveillance system based on genotyping of clinical strains from MDR-TB patients. The relative distribution of spoligotypes in our study population was similar to that reported in a previous study of clinical isolates collected from 434
MDR-TB cases in Gauteng from March 2004 to December 2007 [34]. However, our data suggest that there were increases in the relative proportions of Beijing and H3 (ST 50) MDR strains in the Gauteng MDR population between 2007 and 2010. Routine genotyping of MDR-TB strains will help in tracking such shifts in the population, which may reflect transmission and/or migration of MDR-TB within and between different regions in South Africa.

A large proportion of isolates (68%) in the MDR population carried mutations in the \( pncA \) locus, consistent with other reports from South Africa [24, 25]. While not all \( pncA \) polymorphisms confer drug resistance, a strong association between \( pncA \) mutations and phenotypic PZA resistance has been found in clinical isolates from diverse settings [36, 37]. Of the 16 \( pncA \) mutations found among the clustered isolates shown in Table 4, ten have been reported to confer PZA resistance [23]. The strong association between PZA resistance and \( pncA \) mutations supports an additional benefit of routine \( pncA \) sequencing, which can provide a rough estimate of PZA resistance in the MDR population and may be of value in designing protocols for improved clinical management.

As expected, a high degree of diversity among \( pncA \) mutations was observed. Moreover, we found a significant association between mutations in \( pncA \) and clustering by both IS6110-RFLP and 24-loci MIRU-VNTR, consistent with a recent report on PZA resistance among TB cases in New York City [38]. Although only 60% of clinical MDR isolates in our study population carried mutations in \( pncA \), the cluster analysis indicates that our SpoNC typing approach will enable us to follow the largest clusters of MDR-TB cases. Thus, while problematic from a

| Reference typing method        | Complementary method         | Wallace coefficient |
|--------------------------------|------------------------------|---------------------|
| IS6110-RFLP                    | Spoligotyping + pncA         | 88.1                |
| Spoligotyping + pncA           | IS6110-RFLP                  | 87.2                |
| 24-loci MIRU-VNTR              | Spoligotyping + pncA         | 87.3                |
| Spoligotyping + pncA           | 24-loci MIRU-VNTR            | 83.8                |

doi:10.1371/journal.pone.0146106.t003

| Spoligotype | ST | pncA SNP | pncA nucleotide position | pncA SNP result | Cluster size | References |
|-------------|----|----------|--------------------------|-----------------|-------------|------------|
| BEIJING     | 1  | T>C      | 40                       | C14R            | 10          | [6], [25]  |
|             |    | G>C      | 518                      | frame shift     | 2           | [6]        |
|             |    | C>T      | 200                      | S67W            | 2           | [6]        |
|             |    | ins C    | 456                      | frame shift     | 10          | [39]       |
|             | 60 | del T    | 389                      | frame shift     | 8           |            |
|             |    | G>C      | 395                      | G132A           | 3           | [2]        |
|             |    | T>G      | 416                      | V139G           | 5           |            |
| H3          | 50 | C>T      | 211                      | H71Y            | 24          | [25], [24] |
|             |    | C>T      | 305                      | A102V           | 3           | [25]       |
|             |    | C>T      | 100                      | Y34D            | 2           |            |
|             | 92 | A>G      | 460                      | R154G           | 10          | [25]       |
|             | 18 | A>G      | 289                      | G97C            | 9           | [25], [43] |
|             | 18 | G>T      | 289                      | G97D            | 7           | [25]       |
| X3          | 48 | G>A      | 289                      | G97D            | 7           | [25]       |
| X2          | 92 | T>C      | 452                      | L151S           | 6           | [25]       |
| T1          | 37 | T>C      | 175                      | S59P            | 4           | [25]       |

doi:10.1371/journal.pone.0146106.t004
public health perspective, the elevated frequency of \textit{pnc}A mutations among clustered isolates may enhance our ability to identify possible chains of transmission and conduct outbreak investigations.

The use of \textit{pnc}A sequencing as a secondary marker improved the discriminatory power of each of the three genotyping methods. In particular, \textit{pnc}A sequence profiles reduced the clustering rate of spoligotyping from 80\% to 62\%, and yielded the highest Wallace coefficient when either IS\textit{6110}-RFLP or MIRU-VNTR was used as a reference. Among the 16 clusters defined by spoligotyping and \textit{pnc}A sequencing, eleven carried distinct non-synonymous SNPs that were identical to \textit{pnc}A mutations previously seen in MDR-TB isolates from South Africa (Table 4). Two of these SNPs (T\textgreater{}C in position 40 and insertion G in position 518) were identified in association with a large cluster of Beijing strains predominantly from Eastern Cape [6]. Two other mutations (insertion C in position 456 and G\textgreater{}C in position 395) have been observed among a group of closely related LAM4/KZN strains that were originally associated with the 2006 Tugela Ferry outbreak and appear to be spreading in South Africa [2, 39]. The presence of identical \textit{pnc}A sequences in clustered strains may be indicative of clonal strains of MDR \textit{M. tuberculosis} circulating in the population of Gauteng, through transmission and/or migration of people into the region.

The discriminatory power of the SpoNC strategy was similar to IS\textit{6110}-RFLP and 24-loci MIRU-VNTR typing, and was able to detect important clonal strains that are relevant to the South African context. This strategy is a simple high-throughput method and results can easily be interpreted and readily be compared between different laboratories. Although genomic DNA was amplified for \textit{pnc}A sequencing in the present study, a recent report describes a method for sequencing of \textit{pnc}A directly from clinical specimens that shows high sensitivity and specificity [40]. Thus, unlike IS\textit{6110}-RFLP, SpoNC can be done directly from clinical samples without the need for prior culture, as well as from Ziehl-Neelsen smear slides. Importantly, the turnaround time for both SpoNC (1–10) and MIRU-VNTR typing (2–10 days) are greatly reduced compared to IS\textit{6110}-RFLP (20–40 days). Moreover, SpoNC is less costly in terms of consumables and labor as compared to either IS\textit{6110}-RFLP or 24-loci MIRU-VNTR typing. Taken together, the results of this study support the value of SpoNC strategy for MDR-TB surveillance in a high burden setting. Based on our findings, we propose to employ a tiered approach for MDR-TB transmission surveillance, involving SpoNC strategy as a first-line method, followed where relevant by 24-loci MIRU-VNTR typing of isolates that carry wild type \textit{pnc}A sequence and are not uniquely differentiated by spoligotyping alone. MIRU-VNTR typing has a technical advantage over IS\textit{6110}-RFLP and, despite the relatively high proportion of Beijing isolates in our population, we found 24-loci MIRU-VNTR as discriminatory as IS\textit{6110}-RFLP for genotyping. These findings support the use of 24-loci MIRU-VNTR for genotyping isolates with wild type \textit{pnc}A sequences and non-unique spoligotypes. However the follow on method would be applied on risk or need basis, since within this group that are \textit{pnc}A wild type the cluster sizes were small between 2–4 and these small clusters may not be an early priority in high burden settings where emphasis would be on targeting the large clusters with greater risk potential. This would simplify the approach and ensure resources are directed where the need is greatest.

A limitation of the present study is the lack of epidemiological and contact tracing data to validate and interpret clusters identified by the three typing methods. However, we have used both IS\textit{6110}-RFLP and 24-loci MIRU-VNTR as reference standards, which have been widely used in contact tracing and epidemiological investigations. In addition, the collection of isolates may not be fully representative of the MDR-TB population in South Africa, where regional diversity in \textit{M. tuberculosis} strain populations has been noted [41, 42]. Further work is planned to evaluate the performance of this new strategy in other regions of South Africa to address this...
short coming. It should also be noted that, although the strains in this study were only from one region in South Africa, this collection identified clusters representing all the major clones present in the country.

In conclusion, spoligotyping in combination with pncA sequencing is an appropriate method for first-line approach for the detection of MDR-TB genotypic clusters in South Africa and similar settings, with complementary genotyping by 24-loci MIRU-VNTR, as needed. The results of this study further support the need for implementation of prospective MDR-TB transmission surveillance for early identification of outbreaks. Lastly, molecular surveillance at a national level will provide insights into MDR-TB transmission that can inform targeted interventions to control spread and reduce incidence of MDR-TB in South Africa.

S1 File. Typing methods used and gen sequenced for each patient.

"IS6110-RFLP" = Insertion sequence 6110 restriction fragment length polymorphism; "pncA" = gene sequenced for Pyrazinamide resistance; "ST" = shared type; "MIRU-VNTR" = Mycobacterial Interspersed Repetitive Unit-Variable Number tandem repeats

Supporting Information

S1 File. (XLSX)

Acknowledgments

The authors would like to thank the staff members of NHLS Central TB laboratory in Braamfontein for their assistance during specimen collection and Drs. Natalia Kurepina and Barun Mathema for valuable comments on the manuscript.

Author Contributions

Conceived and designed the experiments: HS NI DF SV. Performed the experiments: HS NK YG IR. Analyzed the data: HS NI DF. Contributed reagents/materials/analysis tools: NI DF NB AD. Wrote the paper: HS NI DF SV AD HK LE YG IR ES GK NK YG IR.

References

1. World Health Organization. Global Tuberculosis Report 2013. Geneva: WHO, 2013. http://www.who.int/tb/publications/global_report/en/index.html
2. Ioerger TR, Koo S, No EG, Chen X, Larsen MH, Jacobs WR Jr, et al. Genome analysis of multi- and extensively-drug-resistant tuberculosis from KwaZulu-Natal, South Africa. PLoS One. 2009; 4(11): e7778. Epub 2009/11/06. doi: 10.1371/journal.pone.0007778 PMID: 19890396; PubMed Central PMCID: PMC2767505.
3. Moodley P, Shah NS, Tayob N, Connolly C, Zetola N, Gandhi N, et al. Spread of extensively drug-resistant tuberculosis in KwaZulu-Natal province, South Africa. PLoS One. 2011; 6(5): e17513. doi: 10.1371/journal.pone.0017513 PMID: 21655324; PubMed Central PMCID: PMC3104985.
4. Gandhi NR, Nunn P, Dheda K, Schaaf HS, Zignol M, van Soolingen D, et al. Multidrug-resistant and extensively drug-resistant tuberculosis: a threat to global control of tuberculosis. Lancet. 2010; 375 (9728): 1830–43. doi: 10.1016/S0140-6736(10)60410-2 PMID: 20486523.
5. Sanchez-Padilla E, Dlaminii T, Ascorsa A, Rusch-Gerdes S, Tefera ZD, Calain P, et al. High prevalence of multidrug-resistant tuberculosis, Swaziland, 2009–2010. Emerging infectious diseases. 2012; 18 (1): 29–37. doi: 10.3201/eid1801.110850 PMID: 22260950; PubMed Central PMCID: PMC3310109.
6. Muller B, Chihota VN, Pillay M, Klopper M, Streicher EM, Coetzee G, et al. Programmatically selected multidrug-resistant strains drive the emergence of extensively drug-resistant tuberculosis in South Africa. PloS one. 2013; 8(8): e70919. doi: 10.1371/journal.pone.0070919 PMID: 24058399; PubMed Central PMCID: PMC3751934.
7. Strauss OJ, Warren RM, Jordaan A, Streicher EM, Hanekom M, Falmer AA, et al. Spread of a low-fit-ness drug-resistant Mycobacterium tuberculosis strain in a setting of high human immunodeficiency...
virus prevalence. Journal of clinical microbiology. 2008; 46(4):1514–6. doi: 10.1128/JCM.01938-07 PMID: 18272712; PubMed Central PMCID: PMC2292903.

8. Johnson R, Warren RM, van der Spuy GD, Gey van Pittius NC, Theron D, Streicher EM, et al. Drug-resistant tuberculosis epidemic in the Western Cape driven by a virulent Beijing genotype strain. Int J Tuberc Lung Dis. 2010; 14(1):119–21. PMID: 20003705.

9. Moss AR, Alland D, Telzak E, Hewlett D Jr., Sharp V, Chilade P, et al. A city-wide outbreak of a multiple-drug-resistant strain of Mycobacterium tuberculosis in New York. The international journal of tuberculosis and lung disease: the official journal of the International Union against Tuberculosis and Lung Disease. 1997; 1(2):115–21. PMID: 9441074.

10. World Health Organization. Global strategy and targets for tuberculosis prevention, care and control after 2015. Geneva: WHO, 2015. http://www.who.int/tb/publications/global_report/en/.

11. Dowdy DW, Azman AS, Kendall EA, Mathema B. Transforming the fight against tuberculosis: targeting catalysts of transmission. Clinical infectious diseases: an official publication of the Infectious Diseases Society of America. 2014; 59(8):1123–9. doi: 10.1093/cid/ciu506 PMID: 24982034; PubMed Central PMCID: PMC481585.

12. Cardoso Oelemann M, Gomes HM, Willery E, Possuelo L, Batista Lima KV, Allix-Beguec C, et al. The forest behind the tree: phylogenetic exploration of a dominant Mycobacterium tuberculosis strain lineage from a high tuberculosis burden country. PLoS One. 2011; 6(3):e18256. doi: 10.1371/journal.pone.0018256 PMID: 21464915; PubMed Central PMCID: PMC3064675.

13. Mathema B, Kurepina NE, Bifani PJ, Kreiswirth BN. Molecular epidemiology of tuberculosis: current insights. Clin Microbiol Rev. 2006; 19(4):658–85. PMID: 17041139.

14. Schurch AC, van Soolingen D. DNA fingerprinting of Mycobacterium tuberculosis: from phage typing to whole-genome sequencing. Infection, genetics and evolution: journal of molecular epidemiology and evolutionary genetics in infectious diseases. 2012; 12(4):602–6. doi: 10.1016/j.meegid.2011.08.032 PMID: 22067515.

15. van Embden JD, Cave MD, Crawford JT, Dale JW, Eisenach KD, Gicquel B, et al. Strain identification of Mycobacterium tuberculosis by DNA fingerprinting: recommendations for a standardized methodology [see comments]. J Clin Microbiol. 1993; 31(2):406–9. PMID: 8381814

16. Goyal M, Saunders NA, van Embden JD, Young DB, Shaw RJ. Differentiation of Mycobacterium tuberculosis isolates by spoligotyping and IS6110 restriction fragment length polymorphism. J Clin Microbiol. 1997; 35(3):647–51. PMID: 9041405

17. van Soolingen D, Arbet RD. Dealing with variation in molecular typing of Mycobacterium tuberculosis: low-intensity bands and other challenges. J Med Microbiol. 2001; 50(9):749–51. doi: 10.1099/0022-1317-50-9-749 PMID: 11549175.

18. Shamputa IC, Lee J, Allix-Beguec C, Cho EJ, Lee JI, Rajan V, et al. Genetic diversity of Mycobacterium tuberculosis isolates from a tertiary care tuberculosis hospital in South Korea. J Clin Microbiol. 2010; 48(2):387–94. doi: 10.1128/JCM.02167-09 PMID: 20018816; PubMed Central PMCID: PMCPMC2815581.

19. Hanekom M, van der Spuy GD, Gey van Pittius NC, McEvoY CR, Hoek KG, Ndabambi SL, et al. Discordance between mycobacterial interspersed repetitive-unit-variable-number tandem-repeat typing and IS6110 restriction fragment length polymorphism genotyping for analysis of Mycobacterium tuberculosis Beijing strains in a setting of high incidence of tuberculosis. J Clin Microbiol. 2008; 46(10):3338–45. doi: 10.1128/JCM.00770-08 PMID: 18716230; PubMed Central PMCID: PMCPMC2566133.

20. Kamerbeek J, Schols L, Kolk A, van Agterveld M, van Soolingen D, Kuipper S, et al. Simultaneous detection and strain differentiation of Mycobacterium tuberculosis for diagnosis and epidemiology. J Clin Microbiol. 1997; 35(4):907–11. PMID: 9157152

21. Kremer K, van Soolingen D, Frothingham R, Haas WH, Hermans PW, Martin C, et al. Comparison of methods based on different molecular epidemiological markers for typing of Mycobacterium tuberculosis complex strains: interlaboratory study of discriminatory power and reproducibility. J Clin Microbiol. 1999; 37(8):2607–18. PMID: 10405410

22. Scorpio A, Zhang Y. Mutations in pncA, a gene encoding pyrazinamidase/nicotinamidase, cause resistance to the antituberculous drug pyrazinamide in tubercle bacillus [see comments]. Nat Med. 1996; 2(6):662–7. PMID: 8640557

23. Whitfield MG, Soeters HM, Warren RM, York T, Sampson SL, Streicher EM, et al. A Global Perspective on Pyrazinamide Resistance: Systematic Review and Meta-Analysis. PloS one. 2015; 10(7):e0133869. doi: 10.1371/journal.pone.0133869 PMID: 26218737; PubMed Central PMCID: PMC4517823.

24. Louw GE, Warren RM, Donald PR, Murray MB, Bosman M, Van Helden PD, et al. Frequency and implications of pyrazinamide resistance in managing previously treated tuberculosis patients. The
international journal of tuberculosis and lung disease: the official journal of the International Union against Tuberculosis and Lung Disease. 2006; 10(7):802–7. PMID: 16848344.

25. Mphahlele M, Syre H, Valvatne H, Stavrum R, Mannsaker T, Muthivi T, et al. Pyrazinamide resistance among South African multi-drug resistant Mycobacterium tuberculosis isolates. J Clin Microbiol. 2008. Epub 2008/08/30. JCM.00973-08 [pii] doi: 10.1128/JCM.00973-08 PMID: 18753350.

26. Cheng SJ, Thiibert L, Sanchez T, Helfets L, Zhang Y. pncA mutations as a major mechanism of pyrazinamide resistance in Mycobacterium tuberculosis: spread of a mono-resistant strain in Quebec, Canada. Antimicrobial agents and chemotherapy. 2000; 44(3):528–32. PMID: 10681313; PubMed Central PMCID: PMC89721.

27. Supply P, Lesjean S, Savine E, Kremer K, van Soolingen D, Locht C. Automated high-throughput genotyping for study of global epidemiology of Mycobacterium tuberculosis based on mycobacterial interspersed repetitive units. J Clin Microbiol. 2001; 39(10):3563–71. PMID: 11574573; PubMed Central PMCID: PMC88389.

28. Cole ST. Comparative and functional genomics of the Mycobacterium tuberculosis complex. Microbiology. 2002; 148(Pt 10):2919–28. PMID: 12368425.

29. Li W, Cowley A, Uludag M, Gur T, McWilliam H, Squizzato S, et al. The EMBL-EBI bioinformatics web and programmatic tools framework. Nucleic acids research. 2015; 43(W1):W580–4. doi: 10.1093/nar/gkv279 PMID: 25845596; PubMed Central PMCID: PMC4489272.

30. Supply P, Allix C, Lesjean S, Cardoso-Oelemann M, Rusch-Gerdes S, Willery E, et al. Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of Mycobacterium tuberculosis. Journal of clinical microbiology. 2006; 44(12):4498–510. doi: 10.1128/JCM.01392-06 PMID: 17005759; PubMed Central PMCID: PMC1698431.

31. Hunter PR, Gaston MA. Numerical index of the discriminatory ability of typing systems: an application of Simpson’s index of diversity. Journal of clinical microbiology. 1988; 26(11):2465–6. PMID: 3069867; PubMed Central PMCID: PMC266921.

32. Wallace DL. A method for comparing two hierarchical clusterings. J Am Stat Assoc. 1983; 78:559–76.

33. Calver AD, Falmer AA, Murray M, Strauss OJ, Streicher EM, Hanekom M, et al. Emergence of multidrug-resistant tuberculosis in Johannesburg, South Africa. Emerg Infect Dis. 2010; 16(2):264–71. doi: 10.3202/eid1602.090968 PMID: 20113557; PubMed Central PMCID: PMC2958014.

34. Marais BJ, Mlambo CK, Rastogi N, Zozo T, Duse AG, Victor TC, et al. Epidemic spread of multidrug-resistant tuberculosis in Johannesburg, South Africa. Journal of clinical microbiology. 2013; 51(6):1818–25. doi: 10.1128/JCM.00200-13 PMID: 23554196; PubMed Central PMCID: PMC3716102.

35. Klopper M, Warren RM, Hayes C, Gey van Pittius NC, Streicher EM, Muller B, et al. Emergence and spread of extensively and totally drug-resistant tuberculosis, South Africa. Emerg Infect Dis. 2013; 19(3):449–55. doi: 10.3202/eid1903.120246 PMID: 23622714; PubMed Central PMCID: PMC3647643.

36. Hirano K, Takahashi M, Kazumi Y, Fukasawa Y, Abe C. Mutation in pncA is a major mechanism of pyrazinamide resistance in Mycobacterium tuberculosis. Tubercle and lung disease: the official journal of the International Union against Tuberculosis and Lung Disease. 1997; 78(2):117–22. PMID: 9692180.

37. Morlock GP, Crawford JT, Butler WR, Brim SE, Sikes D, Mazurek GH, et al. Phenotypic characterization of pncA mutants of Mycobacterium tuberculosis. Antimicrobial agents and chemotherapy. 2000; 44(9):2291–5. PMID: 10952570; PubMed Central PMCID: PMC30060.

38. Verdugo D, Fallows D, Ahuja S, Schluger N, Kreiswirth B, Mathema B. Epidemiologic Correlates of Pyrazinamide-Resistant Mycobacterium tuberculosis in New York City. Antimicrob Agents Chemother. 2015; 59(10):6140–50. doi: 10.1128/AAC.00784-15 PMID: 26195530; PubMed Central PMCID: PMCPMC4576106.

39. Gandhi NR, Weissman D, Moodley P, Ramathal M, Elson I, Kreiswirth B, et al. Nosocomial transmission of extensively drug-resistant tuberculosis in a rural hospital in South Africa. J Infect Dis. 2013; 207(1):9–17. doi: 10.1093/infdis/jis631 PMID: 23166374; PubMed Central PMCID: PMC3523793.

40. Streicher EM, Maharaj K, York T, Van Heerden C, Barnard M, Diacon A, et al. Rapid sequencing of the Mycobacterium tuberculosis pncA gene for detection of pyrazinamide susceptibility. J Clin Microbiol. 2014; 52(11):4056–7. doi: 10.1128/JCM.02438-14 PMID: 25165061; PubMed Central PMCID: PMCPMC4313251.

41. Said HM, Kock MM, Ismail NA, Mphahlele M, Baba K, Omar SV, et al. Molecular characterization and second-line antituberculosis drug resistance patterns of multidrug-resistant Mycobacterium tuberculosis isolates from the northern region of South Africa. J Clin Microbiol. 2012; 50(9):2857–62. doi: 10.1128/JCM.00358-12 PMID: 22649019; PubMed Central PMCID: PMC3421812.

42. Chiho VN, Muller B, Mlambo CK, Pillay M, Tait M, Streicher EM, et al. Population structure of multidrug-resistant Mycobacterium tuberculosis strains in South Africa. J Clin Microbiol.
43. Bishop KS, Blumberg L, Trollip AP, Smith AN, Roux L, York DF, et al. Characterisation of the pncA gene in Mycobacterium tuberculosis isolates from Gauteng, South Africa. Int J Tuberc Lung Dis. 2001; 5(10):952–7. PMID: 11605890.