Pyrosequencing for the rapid detection of rifampicin resistance in *Mycobacterium tuberculosis*: a meta-analysis

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**BACKGROUND:** Multidrug-resistant tuberculosis is a major threat to the control of tuberculosis and to public health. Whereas most conventional methods of drug susceptibility testing (DST) are precise but time consuming, pyrosequencing is a rapid, high-throughput technique.

**OBJECTIVE:** To conduct a meta-analysis to evaluate the overall accuracy of pyrosequencing for the detection of rifampicin (RMP) resistance.

**METHODS:** We searched PubMed, Web of Science, Elsevier and BIOSIS databases according to a written protocol and explicit study selection criteria. A summary receiver operating characteristic curve (SROC) and Cochrane (Q*) index were calculated to perform this meta-analysis using Meta-Disc software.

**RESULTS:** Twelve studies involving 594 specimens with RMP resistance and 793 RMP-susceptible specimens met the inclusion criteria. Of these, 11 were based on *Mycobacterium tuberculosis* clinical isolates. The overall sensitivity and specificity were estimated at respectively 0.94 (95% CI 0.92–0.96) and 0.98 (95% CI 0.97–0.99). The area under the SROC curve was 0.99 and the Cochrane (Q*) index was 0.96. For clinical specimens, the overall sensitivity and specificity estimates were respectively 0.89 (range 0.52–1.00) and 0.99 (range 0.95–1.00).

**CONCLUSIONS:** This meta-analysis shows that pyrosequencing is a highly sensitive and specific tool for the detection of RMP resistance in *M. tuberculosis*. The pyrosequencing assay is conducted in a high-throughput format, with a turnaround time of <2 h, making it substantially faster than conventional DST methods. We propose that pyrosequencing applied directly to clinical specimens instead of *M. tuberculosis* isolates could be of greater clinical value.

**KEY WORDS:** Mycobacterium tuberculosis; pyrosequencing; RMP; resistance; meta-analysis

TUBERCULOSIS (TB), particularly multidrug-resistant tuberculosis (MDR-TB), defined as resistance to at least isoniazid and rifampicin (RMP), is a major threat to TB control and to public health in general. According to the World Health Organization global tuberculosis control 2011 report, there were 8.8 million incident cases of TB (range 8.5–9.2 million) and an estimated 650 000 cases of MDR-TB among the world’s 12.0 million prevalent cases of TB in 2010. Rapid methods of diagnosis are needed to ensure that patients can seek effective treatment as soon as possible.

Conventional culture methods using egg- or agar-based media are still the most commonly used approaches in many countries, as they are inexpensive and easily accessible. The standard methods using Löwenstein-Jensen medium include the proportion method, the absolute concentration method and the resistance ratio method to test for drug resistance; however, these procedures take several weeks to months to yield results. In recent years, molecular detection has been widely used in diagnosing *Mycobacterium tuberculosis*, as it is rapid and specific.

Since it was first reported in 1998 by Ronaghi et al., pyrosequencing has become an important tool for the detection of drug resistance.² This is a semi-automated sequencing method based on a chemical light-producing reaction triggered by the enzymatic incorporation of a complementary nucleotide in a growing chain. The method begins with polymerase chain reaction (PCR) amplification of the *rpoB* gene performed with biotin-labelled primers. The purified PCR amplification product is subsequently fixed on the streptavidin-coated sepharose beads to obtain single-stranded templates. Finally, pyrosequencing and data analysis are performed as recommended by the PSQ96MA (Biotage, Uppsala, Sweden) and SQA software manufacturer.

**SUMMARY**

QG and RJZ contributed equally to this work.
Pyrosequencing has recently been used in some studies for the rapid detection of *M. tuberculosis* RMP resistance; however, these trials found that the sensitivity and specificity were statistically inconsistent. In the present study, we aimed to perform a meta-analysis to assess pyrosequencing for the rapid detection of *M. tuberculosis* RMP resistance.

**MATERIALS AND METHODS**

**Search strategy**

We searched PubMed, Web of Science, Elsevier and BIOSIS databases, compiled up to June 2012, to retrieve articles and abstracts using the keywords ‘tuberculosis’, ‘*Mycobacterium tuberculosis*’, and ‘pyrosequencing’. We also searched the reference literature for primary studies and reviews. Where necessary, we contacted the authors to obtain unpublished data.

**Study selection**

Studies were included in the review if they met the following criteria in written protocols: 1) pyrosequencing was used for rapid drug susceptibility testing (DST) of *M. tuberculosis*, 2) rapid detection of *rpoB* gene of RMP-resistant TB was performed in clinical specimens or isolates, 3) the accuracy (sensitivity and specificity) of pyrosequencing was evaluated, and 4) a reference standard was used including the proportion method, absolute concentration method or radiometric BACTEC™ MGIT™ 960/460 TB systems (BD, Sparks, MD, USA). Two reviewers (QG and RJZ) independently screened the study titles and abstracts to identify eligible studies. Any disagreement between the reviews was resolved by discussion until a consensus was reached.

**Data extraction and assessment of study quality**

Data from study reports were extracted twice and included the first author, year of publication, country where the study was conducted, sample size (number of resistant/number of susceptible samples), reference standard test, whether consecutive or random, specimen type and true-positive (TP), false-positive (FP), false-negative (FN) and true-negative (TN) values. Dates were extracted to fill the four cells of a $2 \times 2$ table from the study report.

The quality of the diagnostic studies was assessed using the criteria based on the quality assessment of studies of diagnostic accuracy included in systematic reviews (QUADAS, University of Bristol, Bristol, Wales, UK).4

**Statistical analysis**

The random effects model was used in the meta-analysis.5 Forest plots of sensitivity, specificity, positive likelihood ratio (PLR), negative likelihood ratio (NLR), and diagnostic odds ratio (DOR) were analysed using Meta-Disc software, version 1.4 (Hospital Ramon y Cajal and Universidad Complutense de Madrid, Madrid, Spain). Heterogeneity was assessed using meta-regression.

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**Figure 1** Flow chart of study inclusion. TB = tuberculosis; RMP = rifampicin.
RESULTS

Study selection and characteristics of studies included
Of 115 citations identified, 12 eligible articles were included in the study.7–18 The flow chart for study selection is shown in Figure 1. Among the articles, the average sample size of the included reports was 116 (range 42–280). Table 1 shows the characteristics of the studies.

Diagnostic accuracy for \textit{M. tuberculosis} clinical isolates
The overall sensitivity and specificity estimates were respectively 0.94 (range 0.91–0.95) and 0.98 (range 0.97–0.99). For clinical isolates, the sensitivity and specificity estimates were respectively 0.94 (range 0.92–0.96) and 0.98 (range: 0.97–0.99). Figure 2 shows a forest plot of the accuracy measures. The area under the summary receiver operating characteristic curve (SROC) was 0.99 and the Cochrane (Q*) index was 0.96 (Figure 3).

Diagnostic accuracy for clinical specimens of \textit{M. tuberculosis}
For clinical specimens, the sensitivity and specificity estimates were respectively 0.89 (range 0.52–1.00) and 0.99 (range 0.95–1.00; Figure 4).

Figure 5 shows the workflow of testing \textit{M. tuberculosis} in clinical isolates and specimens. Pyrosequencing allows the detection of RMP resistance directly from sputum specimens, if combined with molecular diagnostic methods.

Heterogeneity in accuracy estimates
The Spearman correlation coefficient was 0.546, with \(P = 0.066\). Heterogeneity derived from variation in study characteristics can be evaluated by relative diagnostic odds ratios (RDOR). Meta-regression was used to show RDOR estimates using the restricted maximum likelihood method to measure between-study variance. RDOR = 1 indicates that the particular covariate does not affect the overall DOR, while RDOR > 1 means that studies, study centres or patient subgroups with a particular characteristic have higher DOR than studies without this characteristic.19 As shown in Table 2, the RDORs were all >1, and there was no statistical significance in RDOR values between high- and low-quality studies. There was no significant difference between studies with or without cross-sectional and blinded designs.

DISCUSSION

Earlier detection of drug-resistant TB would reduce the incidence of infection and transmission of MDR-TB. The methods currently available for DST of \textit{M. tuberculosis} typically delay diagnosis by at least 1–2 months. Drug resistance in TB is growing, and...
there is an urgent demand for rapid and accurate diagnostic methods to detect resistance. Pyrosequencing was introduced as a new, highly specific tool for DST, and the literature published on its application provides the opportunity to use meta-analysis to explore the overall accuracy of the method for the detection of RMP resistance.

In this meta-analysis, 12 articles reporting pyrosequencing for the detection of RMP resistance were considered to be eligible for inclusion. Of these, 11 were aimed at *M. tuberculosis* clinical isolates. Point estimates of sensitivity and specificity from each study are shown as solid circles; solid lines represent 95% CIs; circle sizes are proportional to study size. The pooled estimate is represented by a diamond at the bottom of the Figure. CI = confidence interval; df = degree of freedom.

DNA sequencing is considered the gold standard among molecular methods. Compared with DNA sequencing, the accordance rate of pyrosequencing was 100%,7,10 while the overall concordance between pyrosequencing and Geno Type® MTBDRplus (Hain Lifescience GmbH, Nehren, Germany) in clinical strains was 99.1%;17 pyrosequencing for detecting *M. tuberculosis* RMP resistance is therefore reliable. In particular, the cost of a pyrosequencing reaction is also acceptable, at about US$2.30, which is less than that of a conventional DNA sequencing reaction.7 Compared with other methods, such as PCR-single strand conformational polymorphism assay20 and LiPA (line-probe assay),21 pyrosequencing is less labour-intensive and time-consuming. For sequencing analysis, 96 samples can be tested within 1–2 h after PCR amplification,7,8,11,13,22 and 96 single nucleotide polymorphisms can be analysed in 10 min.22 Multiple targets can be assayed in the same 96-well plate, which gives the laboratory tremendous flexibility in designing assays for...
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multiple infectious agents or multiple targets in the same microorganism. In comparison with pyrosequencing and direct microscopy, the assay showed high sensitivity for 97.8% of the acid-fast bacilli (AFB) positive specimens and 83.3% of the AFB-negative specimens tested; pyrosequencing can therefore be considered a high-throughput and rapid sequencing methodology.

Although the turnaround time is significantly lower for pyrosequencing compared to conventional DST, in this analysis we noticed that most of the studies used only culture isolates as detection subjects. As M. tuberculosis culture isolates generally require 4–8 weeks to grow, direct detection in clinical specimens could significantly shorten the turnaround time. There were two studies that used clinical specimens, with sensitivities of respectively 66.7% and 85.7%, and specificities of 100% and 99%. Only three specimens were detected as harbouring drug-resistant M. tuberculosis in one of these studies, which may account both for the statistical bias, and for the considerable difference in sensitivity between the two studies. For the clinical specimens, the sensitivity and specificity estimates were respectively 0.89 (range 0.52–1.00) and 0.99 (range 0.95–1.00) according to the forest plot.

These data show that pyrosequencing could be used to detect M. tuberculosis RMP resistance in clinical specimens. However, there are also several limitations for rapid diagnosis of drug-resistant M. tuberculosis in clinical specimens. Because of the high degree of similarity in rpoB DNA sequences between M. tuberculosis and mycobacteria other than TB, M. tuberculosis-specific PCR should be performed to confirm the presence of M. tuberculosis DNA prior to detection of drug resistance. Furthermore, as DNA extraction from clinical specimens is the key step for pyrosequencing, low DNA copies of M. tuberculosis in specimens could result in poor pyrosequencing signals and failure of the assays.

Heterogeneity is of common concern in diagnostic meta-analysis. In the present analysis, QUADAS scores do not affect the overall DOR and there were no significant differences between studies with or without cross-sectional, case-control and blinded designs. Studies using a cross-sectional design have 2.78 times higher DOR than those that do not; however, in one review, several factors, including variations in study population (e.g., severity of disease and comorbidities), index test (different laboratories use different primers, difference in technology, assays and operator, etc.), reference standard and design methods resulted in variations in accuracy estimates.

This meta-analysis has several limitations. First, despite searching several sources, some eligible studies may not have been identified. Second, a relatively small number of eligible studies were included. In addition, even with meta-regression methods, there are considerable heterogeneities that remain unexplained. Geographic and genetic variations in the distribution of drug-resistant strains of M. tuberculosis may partially explain the present findings.

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References


CONTEXTE : La tuberculose (TB) à germes multirésistants constitue une menace majeure pour la lutte contre la TB et pour la santé publique. Alors que la plupart des méthodes conventionnelles pour tester la sensibilité aux médicaments (DST) sont précises mais exigent beaucoup de temps, le pyrosequençage est une technique rapide et à débit élevé.

OBJECTIF : Mener une méta-analyse pour évaluer la précision globale du pyrosequençage pour la détection de la résistance à la rifampicine (RMP).


RÉSULTATS : Douze études, impliquant 594 échantillons résistants et 793 échantillons sensibles à la RMP, ont répondu aux critères d’inclusion. Parmi celles-ci, 11 études ont concerné des isolats cliniques de Mycobacterium tuberculosis. On a estimé la sensibilité globale à 0,94 (IC95% 0,92–0,96) et la spécificité globale à 0,98 (IC95% 0,97–0,99). L’aire sous la courbe SROC a été de 0,99 et l’indice de Cochrane (Q*) a été de 0,96. Pour les échantillons cliniques, les estimations de la sensibilité globale ont été de 0,89 (extrêmes 0,52–1,00) et pour la spécificité de 0,99 (extrêmes 0,95–1,00). 

CONCLUSIONS : Cette méta-analyse révèle que le pyrosequençage est un outil hautement sensible et spécifique pour la détection de la résistance de M. tuberculosis à la RMP. Le test de pyrosequençage est à débit élevé et la durée d’exécution est <2 h, ce qui en fait une technique substantiellement plus rapide que les méthodes conventionnelles de DST. Nous proposons que l’application directe du pyrosequençage aux échantillons cliniques plutôt qu’aux isolats de M. tuberculosis pourrait avoir une meilleure valeur clinique.