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DIAGNOSTICS

Gold nanoprobes for multi *loci* assessment of multi-drug resistant tuberculosis

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SUMMARY

Tuberculosis, still one of the leading human infectious diseases, reported 8.7 million new cases in 2011 alone. Also, the increasing rate of multidrug-resistant tuberculosis (MDRTB) and its treatment difficulties pose a serious public health threat especially in developing countries. Resistance to isoniazid and rifampicin, first line antibiotics, is commonly associated with point mutations in *katG*, *inhA* and *rpoB* genes of *Mycobacterium tuberculosis* complex (MTBC). Therefore, the development of cheap, fast and simple molecular methods to assess susceptibility profiles would have a huge impact in the capacity of early diagnosis and treatment of MDRTB.

Gold nanoparticles functionalized with thiol-modified oligonucleotides (Au-nanoprobes) have shown the potential to provide a rapid and sensitive detection method for MTBC and single base mutations associated with antibiotic resistance, namely the characterization of the three most relevant codons in *rpoB* gene associated to rifampicin resistance. Here we extend the Au-nanoprobe approach towards discriminating specific mutations within *inhA* and *rpoB* genes in PCR amplified DNA from isolates. Using a multiplex PCR reaction for these two genes, it is possible to assess both loci in parallel, and extend the potential of the Au-nanoprobe method to MDRTB molecular characterization with special application in the most frequent Portuguese genotypes.

1. Introduction

Tuberculosis (TB) is still one of the leading human infectious diseases with reports of 8.7 million new cases in 2011 alone which, together with the increasing rate of multidrug-resistant tuberculosis (MDRTB) and associated difficulties of effective treatment, pose a serious public health problem [1,2]. MDRTB is defined as TB resistance to at least isoniazid (INH) and rifampicin (RIF), two first line antibiotics, and almost 60 000 MDRTB patients were notified worldwide in 2011 that is estimated to represent only 19% of the overall cases [1]. Resistance to rifampicin has been associated to single point alterations within an 81 bp region (codons 507–533) of the *rpoB* gene that encodes for the RNA polymerase beta subunit [3]. Single nucleotide alterations within this region confer resistance to RIF in circa 95% of all examined clinical isolates where more than 35 distinct variations have been described [3]. Among these, two mutations – H526D and S531L – account for two-thirds of RIF resistance and are absent in susceptible isolates, making them an ideal target for molecular characterization [2–5]. About 75–85% of point mutations associated with resistance to INH are related either with *inhA* or *katG* genes [3,6]. *katG* encodes for catalase-peroxidase [7] that activates INH. When activated, INH forms a hypothetical isonicotinic acyl radical that binds to the NAD originating a ternary complex, the INH-NAD adduct [8], which binds tightly to the enoyl-acyl carrier protein reductase (encoded by *inhA*) thereby blocking its action as mycolic acid synthase [8,9]. Synthesis of mycolic acids is essential for *Mycobacterium* sp. survival. Point mutations in these genes confer resistance to INH, either by loss of KatG activity (*katG locus* mutations) [10] or by the *inhA* over expression (mutations in the promoter region of *inhA* are sometimes associated with mutations in *inhA* gene locus) [8,9].

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Furthermore, these latter single nucleotide alterations have also been described as conferring cross-resistance to ethionamide (ETH) [3,9].

The large majority of mutations responsible for isoniazid resistance in clinical isolates of *Mycobacterium tuberculosis* occur in the *katG* gene and the most common mutation is a point mutation in codon 315, where a serine is substituted by a threonine (S315T). This substitution is responsible for about 50–90% of the isoniazid resistant mutants globally but not in Portugal [2,3,8,9]. The incidence rate of tuberculosis in Portugal is high for Western Europe (22.6 cases per 100 000 inhabitants in 2011) with high prevalence of MDRTB and extensively drug-resistant TB in the Lisbon Health Region. More than 90% of these resistant strains show no mutation in *katG*, and INH resistance has been associated only to the C−15T mutation in the promoter of *inhA* coupled with mutations in *inhA* gene locus [9]. The only effective solution to control this situation was the early detection by molecular tools of these resistant strains for the proper implementation of control and treatment measures [2].

Several molecular diagnostic approaches have been used to screen for these mutations, the majority relying on highly trained lab technicians devoted to time consuming assays [2,11,12]. Among these, particular emphasis has been brought upon molecular characterization assays based on PCR amplification and hybridization approaches that may provide for molecular signatures associated with drug resistance profiles. These strategies allow collection of the genetic susceptibility profile results in a few hours, compared with the classical methods based on the growth of bacilli on culture media taking at least 16–20 days. Attaining fast results is of utmost importance for the management of the drug resistant patient in terms of therapeutic and transmission control measures.

Access to point-of-care diagnostic for these MDRTB cases is the greatest obstacle for reporting these patients since standard methods of diagnostic are either protracted or expensive, creating a huge gap between clinical needs and laboratories. Therefore, the development of cheap, fast and simple molecular methods to assess susceptibility profiles at point-of-care would have a huge impact in the capacity of early diagnosis and treatment of MDRTB patients. Nanotechnology has brought great advances in molecular diagnostics in the last few years. In particular, systems based on gold nanoparticles (AuNPs) functionalized with thiol-modified DNA (Au-nanoprobes) have been extensively used for the detection and characterization of pathogens [13], including *M. tuberculosis* [14]. We have previously reported on a detection strategy for *M. tuberculosis* and members of the *M. tuberculosis* complex (MTBC) based on the observable colorimetric alteration of a solution encompassing Au-nanoprobes. The colorimetric alteration in this non-cross-linking method results from the differential aggregation profiles of Au-nanoprobes induced by increased ionic strength in the presence or absence of the specific target sequence: presence of the complementary target sequence to that of the probe prevents aggregation and the solution remains red (localized surface plasmon resonance (LSPR) band at 526 nm), whereas absence of a specific target sequence leads to extensive aggregation after salt addition and the solution turns blue (red-shift of the LSPR peak) [15–17]. This system is extremely sensitive allowing for single point mismatches characterization that was applied to identification of the 3 most relevant point mutations within the *rpoB* gene associated to rifampicin resistance in MTBC [18].

Here, we extend the Au-nanoprobe approach towards effective application at point-of-care by demonstrating for the first time the simultaneous PCR amplification of the two main loci of interest followed by characterization of the molecular alterations involved in MDRTB via an Au-nanoprobe strategy. Following a multiplex PCR of the two *loci*, *rpoB* 531 and *inhA* C(−)15T, we use a set of Au-nanoprobes to evaluate in a single array the presence/absence of some of the relevant mutations associated with MDRTB, focusing on those present within the cases reported in the Lisbon Health Region (see above). This approach brings new possibilities for MDRTB diagnostics as the Au-nanoprobe methodology may become an useful tool for MDRTB molecular characterization at a point-of-need.

2. Materials and methods

All reagents were purchased from Sigma Aldrich and were of analytical grade. HPLC purified labeled oligonucleotides were purchased from STAB Vida (Portugal) and used without further purification. Thiolated oligonucleotides were used to synthesize the Au-nanoprobes and non-modified oligonucleotides were used as specific controls for calibration of the assay.

2.1. Biological samples

Twenty-five clinical isolates obtained from respiratory samples positive for acid fast bacilli (BAAR) from patients of the Lisbon Health Region including 11 strains susceptible to INH and RIF, 10 MDRTB, and 4 INH mono-resistant strains were used. *M. tuberculosis* H37Rv (ATCC27294^T) was used as positive control and a non-MTBC strain (*Mycobacterium kansasii*) as negative control. The BACTEC™ MGIT™ 960 (BACTEC 960) system was used for primary isolation and standard susceptibility testing for the first line drugs (Streptomycin, isoniazid, rifampicin and ethambutol) according to the manufacturer's instructions (Becton Dickinson Diagnostic Systems, Sparks, MD, USA). MGIT tubes were inoculated with 0.8 ml of SIRE supplement (Becton Dickinson), 0.1 ml of antibiotic (Becton Dickinson) at the desired concentration and 0.5 ml of the strain suspension [9]. For the preparation of the drug-free growth control tube (proportional control), the strain suspension were diluted 1:100 with a sterile saline solution and 0.5 ml inoculated into the MGIT tube. For standard susceptibility testing strains were considered resistant when growth at critical concentration was obtained (0.1 mg/L for isoniazid, 1 mg/L for rifampicin, 1 mg/L for streptomycin, 5 mg/L for ethambutol, 100 mg/L for pyrazinamide). Identification of MTBC and mutations in the *rpo*B gene associated to RIF resistance was performed by INNO-LiPA Rif. TB assay (Innogenetics, Belgium). Mutations associated with INH resistance were identified with Genotype MTBDRplus (Hain Lifescience, Nehren, Germany). DNA was extracted from cultures with the QIAamp DNA Mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

2.2. Multiplex PCR amplification

The two most relevant regions of genes associated to MTBC resistance were amplified in a single PCR multiplex reaction using two sets of primer pairs (Supplementary Data Table S1) designed to obtain two different fragments: 395 bp *rpoB* and 248 bp *inhA*. PCR amplifications were performed on a Biometra®TGradient Thermocycler (Göttingen, Germany) in 50 μL final volume with 1 × DreamTaq Buffer, 0.1 mM of each DNTPs, 2uM of each primer and 0.1 U\μL of DreamTaq DNA polymerase (Amersham Biosciences, GE Healthcare, Europe) and ~1 μg/mL of template DNA with the following thermal cycling conditions: initial 5 min denaturation at 94 °C, followed by 35 amplification cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, elongation at 72 °C for 45 s, and a final elongation at 72 °C for 5 min. PCR products were analyzed in a 3% agarose gel electrophoresis (Supplemental Data Figure S2) and samples were sequenced using the Big Dye V3.1 technology in an Applied Biosystems 3730XL DNA Analyzer (Applied Biosystems, USA) by STAB Vida (Portugal).

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