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Project acronym:	TB-DRUG OLIGOCOLOR
Project Title:	Development of a molecular platform for the simultaneous detection of *Mycobacterium tuberculosis* resistance to rifampicin and fluoroquinolones
Instrument:	STREP
Thematic priority:	1

Publishable Final Activity Report

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Project Coordinator organisation name: Institute of Tropical Medicine, Belgium

Revision: Final

Final Activity Report

1. Project Execution

Project objectives:

The Oligocolor project addressed the problem of multidrug resistant tuberculosis (MDRTB) through the development of a versatile and user-friendly molecular platform for the identification of *Mycobacterium tuberculosis* and the simultaneous detection of resistance to key anti-tuberculosis agents directly in clinical specimens and/or liquid cultures.

Most TB cases with treatment failure are due to MDRTB; prolonging the use of first-line drugs would only amplify the epidemiological problem. Under this situation, the spread of MDRTB can only be prevented if it is detected early and treated with a combination of effective antibiotics. For this reason, it would be extremely useful to have a tool able to simultaneously anticipate failure to first-line drugs and determine susceptibility to a major second-line drug.

In this project, the molecular platform proposed was initially developed for the detection of rifampicin resistance because the associated mutations are well defined and their global prevalence is known better. The project focused the development of a modification of the DIAPOPS technique (detection of the immobilized amplified product in one phase system). This system has been successfully applied for the identification of human HLA-A alleles and the detection of bovine leukaemia virus. Unlike other solid support-probe systems used for detection of anti-TB drug resistance, it allows the whole reaction to occur in a single well (capture, amplification and detection) with the hybridisation step being avoided. The visualisation is then accomplished by an enzyme-chromogen colorimetric system. The prototype of this molecular platform was evaluated for reproducibility and proof-of-principle in six laboratories of the project's consortium under local conditions.

The second general objective of the project was the detection of resistance to fluoroquinolones. This was addressed by analysing a collection of *M. tuberculosis* clinical isolates with known phenotypical susceptibility or resistance to these antibiotics. The *gyrA* gene was sequenced to assess new mutations associated with quinolone resistance feasible to be added as primers to the molecular platform previously designed. *M. tuberculosis* specific target segments in the *gyrA* gene also serve for identification purposes.

A third objective of the project was to perform a small pre-clinical evaluation in three laboratories of the consortium to evaluate the combined platform directly on sputum samples and early-positive liquid cultures.

Contractors involved:

Contractor 1: Institute of Tropical Medicine, Antwerp, Belgium (Coordinator)

Contractor 2: National Institute for Public Health and the Environment, Bilthoven, the Netherlands.

Contractor 3: Swedish Institute for Infectious Disease Control, Solna, Sweden.

Contractor 4: Corporación CorpoGen, Bogota, Colombia.

Contractor 5: INEI-ANLIS "Carlos Malbrán", Buenos Aires, Argentina

Contractor 6: Hospital Cetrángolo, Buenos Aires, Argentina.

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Work performed during the project (2005 – 2007)

- A thorough analysis of the prevalence of *rpoB* mutations in rifampicin-resistant *M. tuberculosis* was performed during the project. It is well known that more than 95% of the *M. tuberculosis* rifampicin-resistant strains present mutations within an 81-bp hypervariable region in the *rpoB* gene corresponding to codons 507-533 and between 65-86% of these mutations occur in codons 531 and 526. By searching in GenBank, PubMed and a wide literature review, the most relevant mutations were included in the rifampicin resistance detection platform. All contractors participated in this activity. Based on the analysis performed in the first two years, an improved database was completed and made available to the entire consortium on the project's intranet and will be the basis for a coming publication in a peer-reviewed journal.

- A comparative evaluation of several DNA extraction methods was performed by the participants in order to agree on a common protocol to be used with the oligocolor molecular platform. Due to the initial low reproducibility of the signal obtained after the first evaluation of the oligocolor platform, a new assessment and improvement of the DNA extraction method was performed by two of the participants. A modified protocol was then distributed among all partners.

- Selection of the oligonucleotides to be included in the molecular detection platform for rifampicin resistance was accomplished in the first year of the project. Based on the information obtained of the prevalence of *rpoB* mutations and the use of bioinformatics, the position and frequency of the different point mutations were determined. Specific primers were designed allowing for amplification of wild or mutated sequences. The PCR amplification conditions were then optimised for target detection.

- The first prototype of the oligocolor platform was designed and constructed during the first year of the project. The selected oligonucleotides were synthesised and linked to Nucleolink microtiter plates where the PCR takes place. A modification of the DIAPOPS (Detection of the Immobilized Amplified Product in One Phase System) technique was used for detection. In the second year of the project, a modification was introduced by using newly synthesized primers containing a single-locked nucleic acid (LNA). These primers are modified at the penultimate 3' terminal position, thus, improving the specificity and sensitivity of the annealing step in the PCR reaction. This type of oligonucleotide has been used as probes in real-time PCR applications showing to be more specific that conventional DNA probes.

- A preliminary evaluation of the oligocolor molecular prototype with coded *M. tuberculosis* strains was organized in the second year of the project. For this purpose a panel of strains having different *rpoB* mutations was selected. They were fully characterized to confirm their identity and resistance profile. The strain panel together with the oligocolor prototype kit was distributed to all participants for evaluation. Based on the results obtained in this preliminary evaluation and after some technical modifications, a pre-clinical evaluation of the oligocolor rifampicin resistance detection platform was carried out in the final year of the project with DNA isolated directly from sputum samples. Both smear-positive and smear-negative sputum samples were included in the evaluation. The test was also evaluated in early-positive culture samples.

- A fully-characterized collection of fluoroquinolone-resistant *M. tuberculosis* strains was also completed in the first year of the project. The phenotypic characterization was accomplished by the resazurin microtiter assay, the proportion method in 7H11 agar or the BACTEC TB-460 system. Strains found to be resistant to quinolones were submitted to sequencing of the *gyrA* gene to identify the most common mutations associated with drug resistance. This information was used to incorporate specific oligonucleotides for quinolone resistance detection in another version of the oligocolor molecular platform. The oligocolor *gyrA* platform was re-designed using the conventional DIAPOPS technique for the detection of gene mutations in *gyrA*. Primers and labeled probes were synthesized and the conditions were standardized for the identification of the most prevalent mutations in codons 90 and 94. The oligocolor quinolone resistance detection platform was evaluated in the final year of the project with DNA samples isolated from the panel of fully-characterized *M. tuberculosis* strains.

- Extended sequencing of the whole *rpoB* gene of selected *M. tuberculosis* strains was also accomplished as part of the project.

RESULTS ACHIEVED

Several results were accomplished during the lifetime of the project that are shortly described below organized by workpackage.

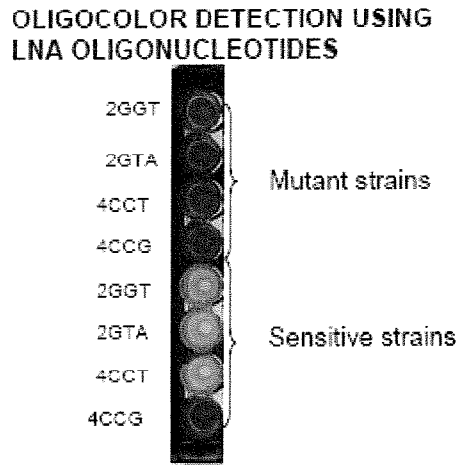
Identification of *rpoB* mutations in rifampicin-resistant *M. tuberculosis*, synthesis of specific oligonucleotides and design of primers (WP1, WP2)

The most relevant *rpoB* mutations included in the first rifampicin-resistance detection platform were selected after a thorough literature review and search on PubMed and other relevant databases. Also, an inquiry into the global distribution of *rpoB* mutations associated with rifampicin-resistant TB was completed including Africa, Asia, Latin America and Europe. All collected data was entered into the database included in the project's intranet, which was continuously updated. All participants in the project contributed to this analysis. This database allows carrying out several analyses and correlations with the stored data, such as determining the most common mutations, and correlating the frequency of these mutations with the geographic origin of the mycobacterial isolate. It will be also possible to analyze mutations outside the hotspot region that have not been previously characterized. This will be important to uncover new and important characteristics of the mechanisms related to the acquisition of rifampicin resistance.

One of the critical points in the development of a molecular detection platform is the design of the target oligonucleotides. Taking into account the identified *rpoB* mutations, the reported sequences were aligned selecting the codons carrying the most frequent mutations. As the upper primers had to be specific, mutation frequency and position within the codon were taken into account in their design so that the relevant mutations were located toward the 3' end. The lower primer was common to all the PCR reactions so a segment downstream of the hot-spot region was selected. Oligonucleotides were designed to anneal at the same temperature and synthesized. To control that the DNA being tested belongs to *M. tuberculosis*, a primer was also designed as a species specific positive control. Performance of the designed oligonucleotides was assayed under different testing conditions. When using DNA from rifampicin-susceptible *M. tuberculosis*, only the wild sequences showed amplification while DNA from rifampicin-resistant strain amplified only the mutated segment.

In the second part of the project and in order to improve the specificity and sensitivity of the annealing step in the PCR reaction, new primers were synthesized containing a single locked nucleic acid (LNA) modification at the penultimate 3' terminal position. This is due to the fact that the difference in melting temperature (Tm) for a perfect match and a single-nucleotide mismatch is higher for LNA oligomers than it is for DNA oligomers. This kind of oligonucleotides has been used as probes in real-time PCR experiments and has been shown to be more specific than conventional DNA probes.

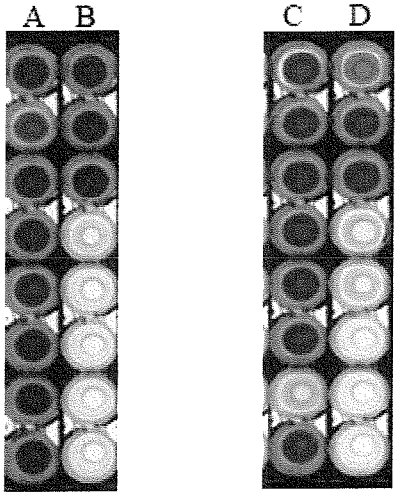
As can be seen in the figure below, the LNA primers exhibited a good specificity for detecting rifampicin-associated *rpoB* mutations.



Concerning fluoroquinolone resistance and the mutations in the gyrA gene, two codons exhibiting the four most frequent mutations were selected. The codon 90 (Ala-GCG) and its mutation 90 (Val-GTG), and the codon 94 (Asp-GCG) with its mutations 94 (Ala-GCC; Gly-GGC and Tyr-TAC) were included. Both codon 90 and 94 contain more than 75% of the reported mutations in the gyrA gene conferring resistance to ofloxacin.

Standardization of the oligocolor molecular platform for detection of rifampicin and quinolone resistance (WP4)

The solid phase PCR (DIAPOPS) technology is based on a specific amplification carried out directly on a solid support and subsequent detection of the amplified product by hybridisation using a labelled specific probe. A modification of the original technique was used with amplification and detection together in the same step. The lower primer was labelled with biotin and the amplified product was detected with the amplifier system developed by Invitrogen.



Strip 1	Strip 2
0W (511)	6W (531)
1.1W (513)	7W (533)
1.2W (515)	M. tb Positive control
2W (516)	M. tb Negative control
2.1W (518)	-
3W (522)	-
4W (526)	-
5W (529)	-

The figure above shows the amplification and detection of a susceptible strain (A and B) and a mutant strain of *M. tuberculosis* (C and D) with a mutation in codon 526.

The initial platform was designed to identify a mutation in the hot-spot region of the *rpoB* gene but not to identify the specific mutated nucleotide. As can be seen in figure 1C above, when there was a mutation in codon 526 of the hot spot region it was visualized as a clear well. Also, in order to make a more friendly system, reducing manipulation and taking into account the preparation complexity of the PCR mix in each well, an innovation was introduced to the procedure. All PCR reagents were added in the ELISA wells in advance with the exception of the magnesium chloride. The PCR mix was coated with wax, which solidifies below 10°C and liquefies above this temperature. The coated plates were frozen at -20°C until used. Their stability was also tested during a 9-week period and no difference was found between the experiments.

Considering that the more frequent mutations reported worldwide are: Asp516Tyr, Asp516Val, His526Asp, His526Tyr, Ser531Trp and Ser531Leu, specific primers were included in the platform (Figure below).

A B

	A	B
1	0W (511)	1.1W (513)
2	2W (516)	2GTA + 2GGT* (516)
3	2.1W (518)	1.2W (515)
4	3W (522)	5W (529)
5	4W (526)	4CCT + 4CCG* (526)
6	6W (531)	6GTT + 6GTG* (531)
7	7W (533)	M. tb positive control
8	W (146)	M. tb negative control

Evaluation of the oligocolor platform under different laboratory settings (WP6)

This evaluation was performed by all participants in the project. The oligocolor strips and coded strains of *M. tuberculosis* were sent to all laboratories. The tests were performed in triplicate and the results were evaluated by the coordinating partner. The evaluation generated a large amount of raw data with almost 7000 PCR tests performed on the strips. A summary of the most relevant findings and subsequent analysis is shown below.

Certain criteria were established for the interpretation and analysis of results. First, for a result to be taken into account at least two out of three replicate tests had to show consistent results; otherwise, the result was considered as "unclear". Some cut-off values were also defined to determine the value or colour intensity from which it was considered a positive or negative result. It was also determined that

any sample where the positive or negative control did not work properly was considered as invalid and was not taken into account for further analysis.

The figure below shows a global analysis of the results obtained with the first oligocolor platform for rifampicin resistance detection.

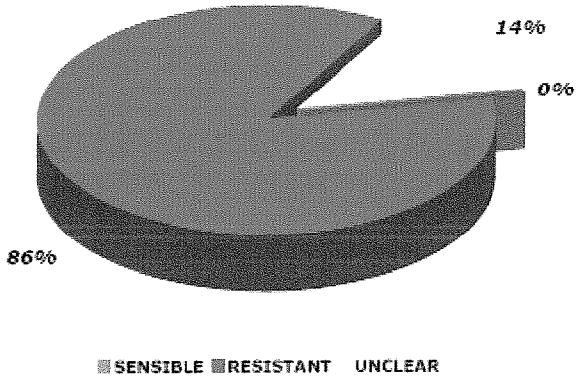


Figure Global distribution of results obtained in the evaluation.

Eighty-six percent of the coded samples were detected as resistant and most importantly, no false negative results were obtained; this means that no sample was determined as susceptible being resistant, which is known as a very major error; however, 14% of the samples gave unclear results.

Based on the results obtained in the previous evaluation, in the second part of the project the performance of the oligocolor platform was assessed for rifampicin resistance detection directly on sputum specimens. The design of the study involved a small-scale demonstration activity comprising sputum samples and early-positive liquid cultures in two clinical settings in Argentina with moderate to high prevalence of multidrug resistant TB.

The oligocolor rifampicin strips were produced at CorpoGen (Colombia) and the evaluation was performed at the Malbran Institute and Hospital Cetrángolo in Buenos Aires, Argentina. The study design at Malbran involved consecutively detected highly-positive sputum specimens and their early positive cultures in liquid medium, obtained from new patients at risk of multidrug-resistant tuberculosis and collected in a three-month period.

A total of 27 sputum specimens were selected in collaboration with the Hospital Muñiz that complied with the following inclusion criteria:

- AFB smear positive (++) or (+++)

- originated from new consecutive patients with respiratory symptoms suggestive of tuberculosis

- originated form patients at risk of multidrug resistant tuberculosis

The specimens were coded and decontaminated with the NALC-NaOH method. One third of the decontaminated pellet was stored at -20°C for DNA extraction. The remaining pellet was inoculated into the BACTEC MGIT960 system. At the earliest alert of a positive culture, an aliquot of 1.0-1.5 ml was transferred into an Eppendorf microtube and centrifuged at 12,000 rpm for 15 min. The supernatant was discarded and the pellet was conserved frozen for testing with the oligocolor rifampicin strips. Drug susceptibility testing was performed with the proportion method on Löwenstein-Jensen slants and the BACTEC TB-460 system.

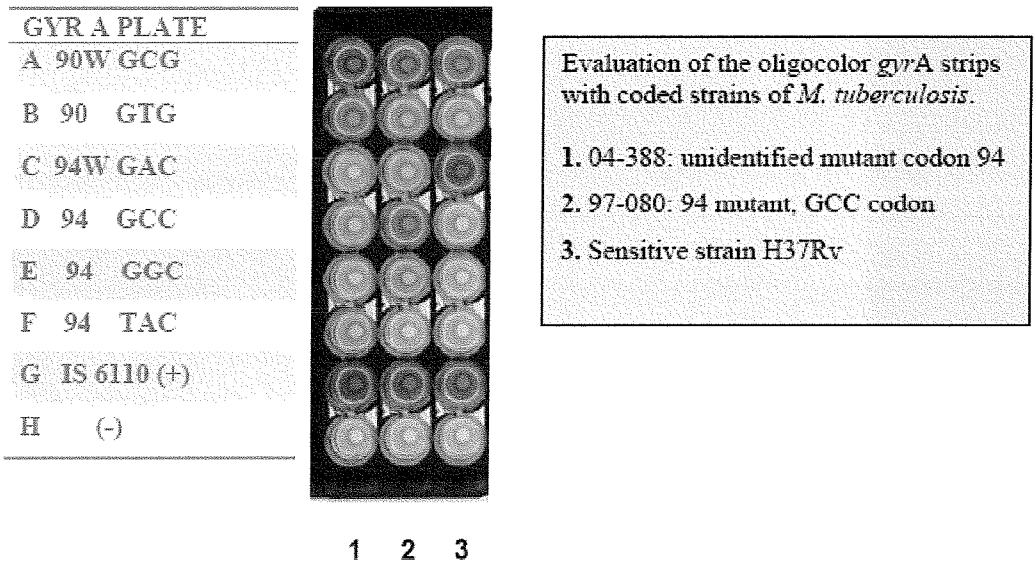
At the Cetrángolo Hospital the evaluation involved eleven rifampicin-resistant *M. tuberculosis* clinical isolates, 29 decontaminated sputa and 22 early-positive cultures (10 to 13 days) obtained from the BACTEC MGIT960 system. Sputum specimens were selected based on their bacillary load and decontaminated by the NALC-NaOH method. *M. tuberculosis* H37Rv was used as the reference strain. The isolates obtained from liquid medium were fully drug susceptible according to results from drug susceptibility testing obtained afterwards.

The oligocolor molecular platform was also evaluated for their capacity to detect *gyrA* mutations associated with resistance to quinolones in *M. tuberculosis*. The evaluation was performed by the ITM in Belgium and the SMI in Sweden. As for the detection of rifampicin resistance, the quinolone-resistance detection strips were prepared and distributed by CorpoGen in Colombia. For this evaluation the order of the oligonucleotides was maintained in the new version of the plate. Enough plates to test 12 DNA samples in triplicate were prepared and sent to the participants.

Due to logistical problems related to the international distribution of the testing kits, additional tests were performed at CorpoGen with selected strains sent by SMI, which showed encouraging results. All coded samples evaluated were correctly identified by the oligocolor gyrA strips and the test was highly reproducible. All drug-susceptible strains were also correctly identified. Mutant at codon 90 GTG, as well as mutants at codon 94 GGC and GCC were also correctly genotyped. Additionally, three of the mutants at codon 94 were identified as mutants by the gyrA plate, but could not be assigned a specific genotype; all of them presenting a TAC mutation.

The table and figure below summarizes the results for this evaluation

Strain code	*gyrA* Oligocolor	Characterized strain (SMI)
- 03-143	Susceptible	Susceptible
- 05-597	94 mutant GGC	94 mutant GGC
G 00-324	Susceptible	Susceptible
O 04-388	94 mutant *	94 mutant TAC
O 05-656	Susceptible	Susceptible
O 97-077	94 mutant GGC	94 mutant GGC
O 97-080	94 mutant GCC	94 mutant GCC
V 02-113	Susceptible	Susceptible
V 05-582	90 mutant GTG	90 mutant GTG
V 06-050	94 mutant *	94 mutant TAC
V 97-156	94 mutant GGC	94 mutant GGC
V 99-509	94 mutant *	94 mutant TAC



The impact of this project could be summarized in the availability of newer and rapid molecular techniques for the detection of new forms of drug resistance in TB. The oligocolor molecular platform as developed in the current project still needs refinement and improvement to be used directly with sputum samples but it represents advancement to the currently available tools for the molecular detection of drug resistance in *M. tuberculosis*.