TM-REST: a new platform for fast molecular detection of MDR and XDR resistant strains of *M. tuberculosis* and of drug resistant malaria

Duration: 01/01/2008 - 30/06/2011 (42 months) - CONCLUDED
Instrument: Collaborative Project (Small or medium-scale focused research project) - FP7

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Project website: http://www.tm-rest.org/

Executive summary

The increasing threat of infections due to *M. tuberculosis*, including MDR-TB and extensively drug-resistant (XDR) *M. tuberculosis* infections, poses important questions that call for development of integrated tools for rapid diagnosis. In the specific case of TB, an integrated rapid diagnostic approach should be able to permit species identification, drug susceptibility testing (DST) and molecular typing.

Another poverty-related disease, Malaria, caused by the protozoan parasite *Plasmodium falciparum*, kills between 1.5 million and 3 million people each year. Deaths from malaria are increasing, especially in Africa, largely because of antimalarial drug resistance. A rapid diagnostic tool for drug resistance would be affordable within European healthcare systems and advantageous in countries where patients are treated in walk-in clinics with little follow-up.

With the aim to release an highly automated commercial product (diagnostic kit for TB and malaria), with minimum hands-on time and based on the same technology, we have developed, tested and validated a new rapid diagnostic tests for molecular diagnosis and monitoring of TB and its drug-resistant variants, as well as for the detection of malaria using a lab-on-chip (LoC) platform (In-Check™, ST Microelectronics).

The In-check™ technology is based on an integrated PCR and a DNA microarray for the end-point analysis and consists in a single disposable device (biochip) and on associated specific instruments (Figures 1 and 2). Main advancement over existing technology (i.e. Real-time Polymerase Chain Reaction, or RT-PCR) consists in the possibility to perform PCR and hybridization in a single device at competitive costs, using an higher number of genetic probes by integrating multiple PCR

chambers and low-density array (126 probes), with faster and more stable amplification and hybridisation reactions through optimised and controlled thermal ramps and profile.

At present, the developed test can reliably, both from clinical samples and strains, and on the same chip *M. tuberculosis* complex, main non-tuberculous species, and the most frequent mutations leading to the MDR phenotype (resistance to rifampicin and isoniazid) with high sensitivity and specificity.

The malaria assay allows the specific identification of all human *Plasmodium* parasite species. In addition the LoC can reliably detect drug resistant parasites carrying the acknowledged resistance mutations in *Pfcrt* (chloroquine resistance), *Pfdhfr* (pyrimethamine resistance), and *Pfcytb* (atovaquone resistance).

ST Microelectronics is constantly improving the micro fluidic technology with the aim to release an highly automated product with minimum hands-on time and a sample extraction protocol with very low level of biosafety requirements.

The In-Check™ platform is already commercially available for the detection and typing of human strains of Influenza A and B viruses, including the Avian Flu strain H5N1. With the development of the chips for TB and malaria, this technology could be widely adopted, especially in TB- and malaria-endemic countries where it could have significant impact on morbidity and mortality.

Summary description of project context and objectives

Drug-resistant ***Mycobacterium tuberculosis*** strains are a threat to TB control Worldwide, as strains resistant to available anti-TB drugs have emerged. Drug-resistant (DR-TB) and multidrug-resistant tuberculosis (MDR-TB) are man-made problems mainly related to poor case management and quality of drugs, and they require expensive chemotherapy with low cure and increased fatality rates. In Eastern Europe, economic crises and health systems weaknesses lead to an increase of TB cases together with the establishment of hotspots for MDR-TB. Laboratory surveillance of resistance and correct identification of resistant strains are critical, while currently poor laboratory performance still continues to jeopardize the efforts to control the transmission of TB in Europe and globally. Solutions to this increasing problem are therefore urgently needed. Immediate detection of DR cases through rapid identification and drug susceptibility testing (DST) is a key element to ensure that patients receive a quick diagnosis and adequate treatment, and this benefit to the interruption of the transmission of the disease. More advanced, robust, fast and affordable technologies are needed to strengthen laboratory capacity for rapid and unambiguous diagnosis of tuberculosis, DR tuberculosis and other non tubercular mycobacteriosis.

Malaria, together with HIV/AIDS and TB, has been identified as one of the major public health challenges undermining development in the poorest countries in the world. Caused by the protozoan parasite ***Plasmodium falciparum***, malaria kills between 1.5 and 3 million people each year the majority of whom are young children living in Africa – malaria kills an African child every 30 seconds. Worldwide there are 300-500 million acute illnesses annually. Deaths from malaria are increasing, especially in Africa, largely because of anti-malarial drug resistance. Within the European region, imported malaria in immigrants and travellers is a growing public health issue for malaria-free European countries. With increasing travel around the world, drug resistant malaria parasites present further challenges in prescribing effective prophylactic treatment for tourists, military personnel and humanitarian aid workers. A rapid diagnostic tool for drug resistance would be very advantageous and affordable within European healthcare systems. It would also be very advantageous in malaria-endemic countries, where frequently patients are treated in walk-in clinics with no follow-up of the efficacy of the treatment. Diagnosis of drug susceptibility of the infecting patients at the same time as diagnosis of infection would allow selection of the most efficacious drug, and could reduce drug costs as prescription of ineffective drugs could be avoided.

Main objectives of the TM-REST project:

- To develop, test and validate a specific diagnostic assay on a lab-on-chip (LoC) based new platform (In-check^{TM}) for the molecular diagnosis and monitoring of tuberculosis and its drug-resistant variants, and for the support and guidance of therapeutic interventions. This tool will allow the identification of DR tuberculosis by the use of selected genomic markers. The integrated PCR and microarray lab-on-chip and the solid-state microarray optical reading tool represents a clear innovation over the conventional readers for its robustness, simplicity of use and low-cost. Non-invasive and quantitative methods will be implemented, including quality assurance (QA) and biosafety aspects.
- To develop, test and validate a specific diagnostic assay able to detect specific markers of drug-resistant variants of malaria, another poverty-related disease using the same platform technology and methodology.

Main S&T results/foregrounds

We have developed, tested and validated a new rapid diagnostic tests for molecular diagnosis and monitoring of TB and its drug-resistant variants as well as for the detection of malaria, using a silicon-based lab-on-chip (LoC) open platform for molecular biology testing (In-check^{TM}, ST Microelectronics).

The In-Check^{TM} technology is based on an integrated PCR and a DNA microarray for the end-point analysis, and consists in a single disposable device (biochip) and on associated specific instruments (Figure 1).

The LoC (biochip) provides an all-in-one device for fast-PCR amplification and detection of targets on a low-density microarray, by integrating all the functions needed to identify multiple oligonucleotide sequences in a sample: an integrated high-speed PCR reactor (Ultrafast PCR Reactor), a low-density microarray, and microfluidic handling. The LoC is composed of two modules:

1) PCR module, thermally driven by the In-Check^{TM} temperature control system (TCS):
   - Microfluidic technology (25µL, two independent chambers of 12.5µl each)
   - Ultra-fast PCR :
     - heating rate: 40 °C/s
     - cooling rate: 10 °C/s
   - Multiple independent control stations
   - 5 different tests simultaneously
   - Possibility to link several TCSs via ethernet
   - Remote control available

2) Microarray module, used to detect the amplified labelled DNA sequences through hybridization on a low-density microarray:
   - 100 available positions for specific probes
   - 26 control probes at fixed positions.

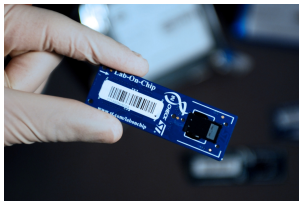
The dedicated, low-cost set of optimized instruments consists of (i) a compact temperature control system (TCS) for amplification and hybridization; (ii) a compact, solid state, user-friendly optical reader able to analyze the microarray automatically and to provide an easy-to-read diagnostic report within few seconds, and (iii) a PC.

The System is composed of :

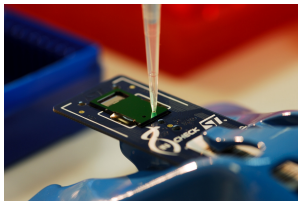
- *Temperature Control System (TCS)*
- *Optical Reader*
- *Control and analysis software*
- *Bar-code reader*
- *Lab-on-Chip*

FIG. 1. In-Check™ platform. Courtesy of ST Microelectronics

The most important phase in building up a customized chip consists on the microarray lay out definition. This phase, performed by means of a "design rule manual" developed by ST Microelectronics, allowed the construction of a microarray on which each position is well known. The microarray is designed in order to have two different sub-microarrays in the same chip with a plane of symmetry, which allows a better control in terms of microarray efficacy. In addition, the probes are printed in duplicate, one in each sub-microarray. This eliminates the risk of surface effects on the chip and/or non uniform washing of the microarray. The outcome of this process is a grid defining all the positions to be used by the software or the operator for the interpretation of the results.



Lab-on-Chip



Sample loading

Chip loading

The In-Check™ platform integrates the advantages provided by a thermal cycler (RT and PCR) with the detection of target sequences on low-density microarray. Main advancement over existing technology (i.e. Real-time Polymerase Chain Reaction, RT-PCR) consists in the possibility to perform PCR and hybridization in a single device at competitive costs, using an higher number of genetic probes by integrating multiple PCR chambers and low-density array (126 probes), with faster and more stable amplification and hybridisation reactions through optimised and controlled thermal ramps and profile.

Additionally, the use of this platform doesn't require advanced molecular biology background. Training of laboratory technicians can be successfully performed in one-two days.

TB and malaria assays rely on the amplification of target genes by PCR performed on DNA extracted from both cultures and clinical samples. An additional chip based on the spoligotype technology was also designed and validated on clinical samples.

Target genes:

Tuberculosis: most relevant mycobacterial species are identified by targeting the 16S rRNA gene and IS*6110* insertion sequence. Species-specific probes were designed. A multiplex PCR was developed for *rpoB*, *katG*, and *inhA* as the most frequently mutated genes involved in the MDR phenotype in *M. tuberculosis* complex (MTBC). Probes were designed targeting the hot-spot region of *rpoB*, the codon 315 of *katG*, and nucleotides -15 and -8 in the promoter region of *inhA*. Probes to target mutations in *rrs* and *gyr A* were also designed.

Malaria*:* detection of the 5 human malaria-causing Plasmodium species (*P. falciparum*, *P. vivax*, *P. ovale*, *P. knowlesi* and *P. malariae*) was performed by PCR of 18S rRNA genes and hybridization with species-specific probes (setting up of amplification conditions and cycling parameters; optimization of hybridization and washing conditions). Detection of *P. falciparum* parasites resistant to the commonly used antimalarial drugs chloroquine, pyrimethamine, proguanil and atovaquone was performed by PCR of the genes *Pfcrt* (chloroquine), *Pfdhfr* (pyrimethamine, proguanil) and *Pfcytb* (atovaquone), with hybridisation to different probes specific to the codons that are known to be mutated in drug-resistant parasites.

Results – Tuberculosis:

The performance of the *In-Check*™ platform was assessed using DNA extracted from both isolates and clinical specimens. Results were analysed by the signal/background ratio calculated by the array software analysis. Provisional thresholds for probe evaluation (ON/OFF) were set up performing a ROC curve based on data from probes "expected ON" and probes "expected OFF". Median signals with interquartile range for each target region were defined and thresholds were set between lowest interquartile range "expected ON" signals, and highest interquartile range "expected OFF" signals.
Selected probes identified MTBC, and 10 clinically relevant non-tubercular mycobacterial species.
Concerning MDR-TB detection, the assay detected mutations D516V, S531L for *rpoB*, S315T for *katG* and c-15t, t-8c, t-8a for *inhA*. Other mutations are identified by a negative signal from wild-type probes. Mycobacterium species and MDR mutations detected are shown in Tables 1 and 2.

- ✓ Overall the assay had a **PPV and NPV of >95%,** and shows a **sensitivity of 95%** and a **specificity of >98%**.
- ✓ The test detection limit is 10^3 genome copies/mL.
- ✓ **The diagnosis (identification of TB species, and drug sensitivity testing to detect drug-resistant and MDR-TB strains) is complete in three hours**, including DNA extraction and master mix preparation.

TABLE 1: Mycobacterial species of clinical interest targeted by the LoC.

***M. tuberculosis* species**	
16S rDNA and IS6110	**Detected by**
M. tuberculosis complex	*specific probe*
M. avium	*specific probe*
M. intracellulare	*specific probe*
M. simiae, M. kansasii, M. scrofulaceum	*specific probe*
M. abscessus, M. chelonae	*specific probe*
All Mycobateria	*specific probe*
M. xenopi	*specific probe*
M. haemophilum	*specific probe*
M. fortuitum (var. IV only)	*specific probe*
M. tuberculosis	*specific probe*

TABLE 2: Target sequences (mutated and wild-type) relevant for multi-drug resistance in *M. tuberculosis* complex by the LoC.

***M. tuberculosis* Multi-Drug Resistance**		
GENES	**MUTATIONS (AA)**	**Detected by**
rpoB (hot-spot region)	gac/gtc (D516V)	*specific probe*
	ctg/ccg (L533P)	*absence of wt*
	cac/gac (H526D)	*absence of wt*
	cac/tac (H526Y)	*specific probe*
	tcg/ttg (S531L)	*specific probe*
katG (coding region)	agc/acc (S315T1)	*specific probe*
	agc/aca (S315T2)	*specific probe*
inhA (promoter region)	c-15t	*specific probe*
	t-8c	*specific probe*
	t8-a	*specific probe*

Results – Malaria:

The LoC was tested with (i) *in vitro* grown *P. falciparum,* (ii) single species infections of the other non-culturable parasites from experimental infections in monkeys or naturally occurring single-species infections in humans, and (iii) patient samples containing mixtures of parasite species. In all cases, it allowed correct identification of all human malaria parasite species (Table 3).
Results were analysed by the signal/background ratio calculated by the array software analysis. The signal to background ratio for probes "expected ON" was always very significantly higher than for probes "expected OFF", although the difference varied depending on the parasitaemia in the sample. To overcome this difficulty, ratios were expressed relative to the PCR control probe, and analyses performed using this methodology.

- ✓ The **accuracy of the test is 100% for the identification of all five *Plasmodium* species** and shows a **sensitivity and specificity equivalent to microscopy and standard PCR assays**.
- ✓ Tests on serial dilutions demonstrated that LoC can specifically detect *P. falciparum* in aliquots containing as few as 1 to 10 parasites (below the accepted threshold for microscopy, and equivalent to standard PCR assays).
- ✓ **The diagnosis is complete in less than one hour.**
- ✓ The test is specific for malaria parasites and there is no cross-reactivity with the other blood-borne infections tested (e.g. *Trypanosoma brucei, Trypanosoma cruzi, Leishmania mexicana, Toxoplasma gondii)*

For drug resistance, the assay was able to detect the key mutation in *Pfcrt* (K76T) necessary for resistance to chloroquine, as well as the initial key mutation (*Pfhdhfr*S108N) responsible for resistance to antifolate drugs such as pyrimethamine), the additional mutation conferring increased resistance (*Pfdhfr*C59R), and the mutation conferring very high level resistance to these drugs (*Pfdhfr*I164L). The assay gave variable results for the detection of a key mutation for resistance to proguanil (*Pfdhfr*A16S/V), and for detection of mutations at another codon responsible for intermediate resistance to pyrimethamine (*Pfdhfr*I51N). Finally the LoC assay was able to detect the wild-type allele of *Pfcytb*268Y, where mutations *(Pfcytb*268S/C) cause resistance to atovaquone.

TABLE 3: Human malaria species detected by the LoC.

Plasmodium species detected	
18S rDNA	**Detection**
P. falciparum	V
P. vivax	V
P. ovale	V
P. knowlesi	V
P. malariae	V

TABLE 4. Target sequences (mutated and wild-type) relevant for drug resistance in *P. falciparum* detected (V) and not detected (X) by the LoC. (*) results ambiguous for one clone. nd= not tested.

P. falciparum multiple drug resistance		
Genes	**Sequence (AA)**	**Detection**
Pfcrt	aaa (76K)	V
	aca (76T)	V
Pfdhfr	gca (16A)	V*
	tca (16S)	nd
	gta (16V)	nd
	tgt (50C)	V
	cgt (50R)	nd
	aac (51N)	nd
	aat (51N)	nd
	att (51I)	X
	tgt (59C)	nd
	cgt (59R)	V
	tcg (108S)	nd
	aac (108N)	V
	acc (S108T)	nd
	atc (164I)	V
	ctc (164L)	V
Pfcytb	tat (268Y)	V
	tct (268S)	nd
	tgt (268C)	nd

Potential impact of the project.

This integrated PCR-microarray LoC represents an innovation for its ease of use and cost-effectiveness. The In-Check™ is the first semi-automated platform able to perform the diagnosis of the two major-poverty related diseases, and is easily adaptable for additional diagnostics purposes. The main features of platform (rapidity, contained cost, easy-to-use, presence of independently operated modules) make it suitable for the use in Reference/regional laboratories also in low/middle-income countries with high burden of tuberculosis and malaria.

At present, the developed test can reliably identify, both from clinical samples and strains, and on the same chip *M. tuberculosis* complex, main non-tuberculous species, and the most frequent mutations leading to the MDR phenotype (resistance to rifampicin and isoniazid) with high sensitivity and specificity. A chip for the XDR phenotype was also developed but not fully validated.. Less frequent mutations (L533P, H526D, S315T var. 2) are identified by the absence of hybridization with the wild-type probes.

The malaria assay allows the specific identification of all human *Plasmodium* parasite species. In addition the LoC can reliably detect drug resistant parasites carrying the acknowledged resistance mutations in *Pfcrt* (chloroquine resistance), *Pfdhfr* (pyrimethamine resistance), and *Pfcytb* (atovaquone resistance).

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This technology can be widely adopted, especially in TB- and malaria-endemic countries where it could have significant impact on morbidity and mortality of these diseases.

Main dissemination activities:

The scientific work conducted under this project lead to several publications in international scientific journals, such as “Antimicrobial Agents and Chemotherapy”, and the “Journal of Clinical Microbiology” (American Society for Microbiology, ASM), the “Journal of Antimicrobial Chemotherapy” (Oxford University Press), “BMC Infectious Diseases”, and the” International Journal of Antimicrobial Agents”. During project implementation period, results of the scientific work were also presented during scientific congress at national, European and international level in particular during National conferences and meetings and the annual congresses of the “American Society for Microbiology” and of the “European Society of Mycobacteriology”, that were held in several countries in Europe and in the USA. Project website was also used and constantly updated to disseminate main findings and project achievements.

Project website:

A project website (http://www.tm-rest.org/) has been developed at the beginning of the project and is composed of a public section and a restricted area, accessible by project Partners only for reporting relevant project information. The website is regularly maintained and updated with most relevant progress on research activities for dissemination to the public.
A database of mutations conferring resistance to antimycobacterial first and second line drugs developed by P2-FZB with contribution from project Partners is posted in the website.

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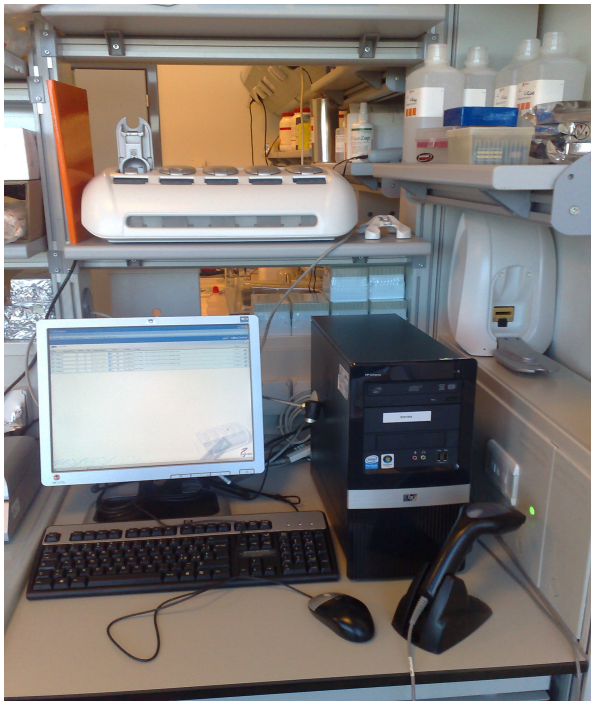

FIG. 2. In-check™ platform (ST Microelectronics) installed at San Raffaele Scientific Institute, Milan (Courtesy of ST Microelectronics)

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