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

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Instrument: Collaborative Project (Small or medium-scale focused research project) - FP7

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

Project summary:

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. 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. More advanced, robust, fast and affordable technologies for immediate detection of DR cases through rapid identification and drug susceptibility testing (DST) are needed to ensure a quick diagnosis and adequate treatment, and this benefit to the interruption of the transmission of the disease.

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. Malaria, caused by the protozoan parasite ***Plasmodium falciparum***, kills between 1.5 and 3 million people each year, the majority of whom are young children living in Africa. Worldwide there are 300-500 million acute illnesses annually. Deaths from malaria are increasing, 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. A rapid diagnostic tool for drug resistance would be very advantageous and affordable within European healthcare systems as well as in malaria-endemic countries, where frequently there is 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 drugs.

Main objectives of 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 achievements:

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 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.

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 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.

In parallel to the development and validation of the diagnostic platform for TB, a feasibility study on its applicability to other poverty related diseases, such as malaria was successfully carried out. The malaria assay we developed allows the specific identification of all human *Plasmodium* parasite species. In addition, the LoC can reliably detect parasites resistant to the commonly used anti-malarial drugs: chloroquine (resistance mutations in *Pfcrt* gene), pyrimethamine (*Pfdhfr*), and atovaquone (*Pfcytb*).

Progress to date:

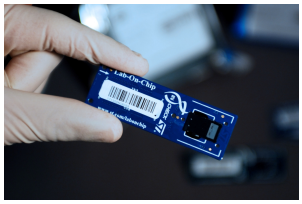
In the second period of the project we have achieved the main goals of the project:

1. Tuberculosis primers and probes design: we have evaluated several combinations of primers and probes for the detection of *M. tuberculosis* complex and other clinically relevant mycobacterial species based on 16S ribosomal gene. In addition, we have worked on assays for the detection of MDR-TB and XDR-TB phenotypes. Based on experimental results and computer analysis for compatibility, we have selected the best combination of primers and probes to be included in the In-check™ devices. For MDR-TB detection we targeted, with wild-type and mutated probes, *rpoB* hot spot (codon 516, 531, 526), *inhA* (-8, -15 mut) and *katG* (codon 315), with one triplex PCR and as a first layout we designed 50 probes in duplicates. For XDR-TB detection we targeted *rrs* and *gyrA* genes in a duplex PCR and designed XDR probes. Experimental conditions for combining the multiplex amplifications in the In-check™ device were established.
2. Malaria primers and probes design: PCR primers and probes were optimised for diagnosis of the five species of *Plasmodium* causing malaria in man on the In-check™ devices. The device was tested using parasite material of known species obtained from experimental infections and from *in vitro* culture, to establish sensitivity and specificity of detection. The device was also tested on samples from individuals diagnosed with malaria, and the results compared with those obtained by microscopy and by standard diagnostic PCR. The device successfully diagnosed the five species of human malaria with 100% accuracy. It was able to detect species in patient samples that were missed by microscopy (but detected by standard PCR). The parasitaemia that can be detected by the device is 20-fold lower than that generally accepted as the limit of detection for microscopy. Diagnosis is complete in one hour. For diagnosis of drug resistant malaria (*Plasmodium falciparum* only) PCR primers and probes were designed and tested for chloroquine resistance (mutation at codon 76 of *Pfcrt* gene), for resistance to antifolate drugs such as pyrimethamine (mutations at codons 51, 59, 108 and 164 of *Pfdhfr*, and for malarone(R) resistance (mutations in *Pfdhfr* codon 16 and *Pfcytb* codon 268).
3. Quality assured production, and distribution to partners of "TB-4.0" chip (MDR-TB) and "Mal-2.0" chip.

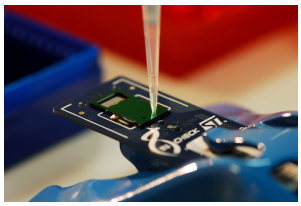
FIG. 1. In-Check™ platform. Courtesy of ST Microelectronics.

The System is composed of :

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



Lab-on-Chip



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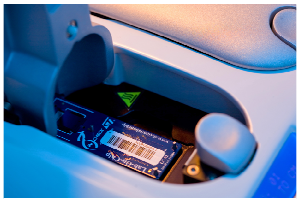

Chip loading

Final results and their potential impact and use:

We have developed 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-CheckTM, ST Microelectronics). 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

Target genes – 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 Table 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 done 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.

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

- ✓ 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 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)	X
	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

CONCLUSIONS:

This integrated PCR-microarray LoC and the solid-state microarray optical reading tool represent a clear innovation over the conventional readers for its robustness, 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).

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

With the development of the chips for TB and malaria, 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.

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

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