Lab-on-Chip-Based Platform for Fast Molecular Diagnosis of Multidrug-Resistant Tuberculosis

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A lab-on-chip based platform for fast molecular diagnosis of multi-drug resistant tuberculosis

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Running title: Lab-on-chip for detection of MDR-TB

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Abstract:

We evaluated the performance of the molecular lab-on-chip-based VerePLEX Biosystem for detection of multi-drug resistant tuberculosis, obtaining diagnostic accuracy over 97.8% compared to sequencing and MTBDR*plus* for *M. tuberculosis* complex, rifampicin and isoniazid resistance detection on clinical isolates and smear-positive specimens. The fastness, user-friendly interface and versatility make it suitable for routine laboratory use.

Text:

Multi-drug resistant tuberculosis (MDR-TB) requires long and expensive treatment often resulting in poor clinical outcome in both low- and high-income countries (1, 2). The World Health Organization (WHO) has endorsed specific molecular diagnostics to improve fast diagnosis of MDR-TB (3-5). However, the genotypic diversity and geographical distribution of *Mycobacterium tuberculosis* complex (MTBC), together with the inability to provide appropriate interpretation of silent mutations and the limited versatility represent some of the restraints undermining the effectiveness of the current tools on global scale (6-13).

In the present study we evaluated a lab-on-chip (LoC) device, developed by STMicroelectronics (Geneva, Switzerland) and marketed by Veredus Laboratories (Republic of Singapore) as the VerePLEX Biosystem, for the diagnosis of MDR-TB and detection of common nontuberculous mycobacteria (NTM). The molecular assay was evaluated on both clinical isolates and direct specimens in low and high burden settings.

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We tested 91 MTBC isolates (Table S1) harbouring different patterns of mutations in *rpoB* and *katG/inhA* genes to evaluate the probes on the array listed in Table 1. Eighty respiratory specimens positive for acid-fast bacilli by smear microscopy and MTBC culture-positive were decontaminated according to international guidelines and included in the study (Table S1) (14). Additional 116 MTBC culture negative specimens were included in the analysis. DNA from isolates and specimens was extracted by thermal lysis and sonication as described elsewhere (15). Phenotypic drug susceptibility testing (DST) for rifampicin (RIF) and isoniazid (INH) was performed according to international recommendations (16). Part of the specimens was tested in a representative high-burden setting in Uganda, Nsambya Hospital, Kampala, by trained staff.

DNA samples extracted from both isolates and specimens were tested in parallel and results compared with GenoType® MTBDR*plus* (Hain Lifescience, Nehren, Germany) assay and Sanger sequencing performed as described elsewhere (17).

The VerePLEX consists of a single disposable device comprising microfluidic PCR and microarray modules. The platform includes a temperature control system (TCS), and an optical reader (OR) which allows to automatically analyze the microarray, providing a user-friendly diagnostic report (Figure S2) (18). The protocols for MDR-TB assay are described in Text S3, and Table S4. The assay allows to detect MTBC and other common NTM, together with the most frequent mutations affecting *rpoB*, *katG* and *inhA* genes, involved in phenotypic resistance to RIF and INH in MTBC.

Analysis of the diagnostic performances of LoC assay on clinical isolates

MTBC was detected in all the 91 cases (Table 2). Concerning the *rpoB* and *inhA* targets, 100% concordance was observed between MTBDR*plus* and LoC assays. In one case the LoC revealed both WT and mutated signals from probes targeting positions 523-526 in *rpoB*, not confirmed by

MTBDR*plus*. A 95.74% concordance was observed between MTBDR*plus* and LoC for *katG* target. In two cases probes complementary to the WT sequence of the 315 codon of *katG* were detected slightly over the ON/OFF cut-off but the MTBDR*plus* showed absence of signal from the WT probe. In other two cases a double pattern (MUT+WT) was detected by the LoC but mutation only was identified by MTBDR*plus*.

Other mutations identified by sequencing (*rpoB*: L530M, S531P and Q513; *katG*: S315N, S315R) were correctly detected on the chip by the absence of signal from respective WT probes.

Compared with DST, sensitivity and specificity were 98.53% and 100%, and 82.76% and 100% for RIF and INH, respectively (Table 3).

Analysis of the diagnostic performances of LoC assay on clinical specimens

DST results for RIF and INH were available for 58 and 57 samples, respectively. The chips presenting incomplete results were repeated once and then included in the analysis (Table 4).

Valid results were obtained in 99.00%, 95.80%, and 95.50% of the cases for MTBC, *rpoB* and *katG-inhA* targets, respectively. MTBC was detected with 100% sensitivity and specificity on the LoC, as well as resistance to RIF (Table 3). One discrepant result was detected in *katG*/*inhA* genes leading to a sensitivity of 93.75%, and 90.91% compared to MTBDR*plus*, respectively. Overall, sensitivity and specificity of *katG*/*inhA* targets was 73.33% and 100% compared to DST. Three specimens were invalid on LoC. One sample gave an invalid result for PCR controls possibly due to inhibitors affecting the reaction in the microfluidic environment. The remaining two specimens resulted invalid also by MTBDR*plus*. All 116 MTBC culture negative specimens were classified correctly.

In the current study we developed and evaluated a LoC-based assay for the diagnosis of MDR-TB. LoC devices represent promising tools to fill the diagnostic gap in low-income countries: they integrate many of the laboratory components on a small chip, thus reducing infrastructure and technical

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requirements but preserving analytical capabilities. In addition, operating speed, ease of modification (addition/removal of probes), the ability to perform multiplex tests and to scale-down costs represent other relevant features of LoCs (19, 20).

Our results showed high specificity and sensitivity of the semi-automated VerePLEX for the MDR-TB targets, thus suggesting an usefulness of the platform for fast and simple diagnosis of MDR cases in centralized laboratories. Sensitivity and specificity of NTM probes on the same platform were evaluated by Lazzeri *et al* (21). The assay allowed to identify correctly MTBC in 100% of the smear positive samples tested independently to the smear microscopy score, with a small number of indeterminate results due most likely to a low quality of DNA extracted. Resistance to RIF and INH was detected by the chip with high sensitivity and specificity in agreement with the minimal requirements established by the WHO for molecular tools, comparable to MTBDR*plus* (12). The limit of detection of the assay was observed in the range of 10^1 genome copies/reaction, as reported in Supplementary Table S5.

A separate array layout for spoligotyping of MTBC was also developed within the TM-REST Project (data not shown). The possibility to integrate the probes for spoligotyping, MDR- and extensively DR-TB in one medium-density microarray layout by using separate multiplex-PCR enhances the benefits of the micro-array assays, and would enable the reduction of time-to-results compared to other available tests (22-24).

The ease of customization of the array design makes the LoC a versatile tool for easy integration of relevant targets for local genetic variants, new genes and/or mutations, and novel key-drugs included in new therapeutic regimens. In addition, the LoC can be adapted for other diagnostic or research needs, thus providing a multi-purpose platform suitable for other relevant diseases (e.g. influenza, malaria, tropical diseases) (25, 26).

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213 chip technology. Arch Virol **156:**1371-1378.

214

215 **Table 1**. List of targeted mycobacterial species and MDR-TB targets included in the assay. Legend: the
216 name of probes spotted on the array is placed in brackets.

MICROARRAY LAYOUT PROBES SPECIES IDENTIFICATION AND MDR-TB
M. avium (MYC4a)
M. intracellulare (MYC5a)
M. simiae, M. kansasii, M. scrofulaceum (MYC6a)
M. abscessus, M. chelonae (MYC8a)
M. xenopi (MYC17a)
M. haemophylum (MYC19a)
M. fortuitum (MYC31a)
M. tuberculosis complex (MYC15a-MYC16a)
rpoB WT codons 510-513 (L511_w3a)
rpoB L511P (L511P_m3)
rpoB WT codons 515-518 (D516_w5)
rpoB D516V (D516V_m1)
rpoB WT codons 523-526 (H526_w14)
rpoB H526D (H526D_m2)
rpoB H526Y (H526Y_m5)
rpoB WT codons 530-533 (S531L_w1)
rpoB S531L (S531L_m2)
katG WT codons 313-317 (S315_w2)
katG S315T1 (S315T1_m2)
katG S315T2 (S315T2_m1)
inhA WT nucleotides -21 to -7 (inhA_w3)
inhA t-8a (InhA – 8T>A_m2)
inhA t-8c (InhA – 8T>C_m2)
inhA c-15t (InhA – 15C>T_m3)

217

218 **Table 2**. Phenotypic DST, MTBDR*plus* and VereMTB results for the 91 MTBC clinical isolates
219 included in the study. *: probe signal ON at the cut-off. Δ: no WT signal.

Phenotypic DST		MTBDRplus/ sequencing				VereMTB			
RIF	INH	*rpoB*	*katG*	*inhA*	No.	*rpoB*	*katG*	*inhA*	No.

R	R	S531L	S315T1	WT	15	S531L	S315T1	WT	15
R	R	WT	WT	WT	1	WT	WT	WT	1
S	R	WT	WT	WT	5	WT	WT	WT	5
R	R	S531L	WT	C-15T	16	S531L	WT	C-15T	16
R	R	S531L	WT	WT	7	S531L	WT	WT	7
R	S	S531L	WT	WT	2	S531L	WT	WT	2
R	R	H526D	S315T1	WT	1	H526D	WT*+S315T1	WT	1
R	R	H526D	S315T1	WT	1	WT+H526D	WT+S315T1	WT	1
R	R	L511P	S315N	WT	1	L511P	WT*	WT	1
R	R	H526D	S315R	WT	1	H526D	Δ 313-317 WT	WT	1
R	R	H526Y	S315N	WT	1	H526Y	WT*	WT	1
R	S	D516V	WT	WT	1	D516V	WT	WT	1
R	R	S531L	S315T1	T-8A	2	S531L	S315T1	T-8A	2
R	R	L530M+S531P	S315T1	T-8C	1	Δ 530-533 WT	S315T1	T-8C	1
R	R	S531L	S315T2	WT	2	S531L	S315T2	WT	2
R	R	D516V	S315T1	T-8A	3	D516V	S315T1	T-8A	3
R	R	D516V	S315T1	T-8C	1	D516V	S315T1	T-8C	1
S	R	WT	WT	C-15T	11	WT	WT	C-15T	11
R	R	D516V	S315T1	WT	5	D516V	S315T1	WT	5
S	R	WT	S315T1	WT	5	WT	S315T1	WT	5
R	R	H526D	S315T1	WT	1	H526D	S315T1	WT	1
R	R	S531L	S315T1	C-15T	3	S531L	S315T1	C-15T	3
R	R	Q513P	S315T1	WT	1	Δ 510-513 WT	S315T1	WT	1
S	R	WT	S315N	WT	1	WT	Δ 313-317 WT	WT	1
R	R	H526Y	S315T1	C-15T	2	H526Y	S315T1	C-15T	2
S	S	WT	WT	WT	1	WT	WT	WT	1

Table 3. Diagnostic performance of VereMTB on clinical isolates and specimens. Legend: Percentages of sensitivity, specificity, positive (PPV) and negative (NPV) predictive values, diagnostic accuracy were calculated according to the Wilson score (www.OpenEpi.com), as well as positive and negative Likelihood ratios (with lower-upper 95% CIs). The effective number of samples considered for the analysis is reported for each target. The positive likelihood ratio cannot be computed since specificity is always 100%.

227

	Clinical isolates (No. 91)			Clinical specimens (No. 80 MTB+ smear pos / 116 MTB-)			
	MTBDR*plus* / seq		DST	MTBDR*plus* / seq /Xpert MTB-RIF		DST	Indeterminate
RIFAMPICIN (*rpoB*)				**tot N° 71**		**tot N° 58**	Molecular 3/71 (4.23%) Phenotypic 2/58 (3.45%)
Sensitivity	100.00 (94.58, 100.00)		98.53 (92.13, 99.74)	100.00 (77.19, 100.00)		100.00 (75.75, 100.00)	
Specificity	100.00 (86.2, 100.00)		100.00 (85.69, 100.00)	100.00 (93.47, 100.00)		100.00 (91.97, 100.00)	
PPV	100.00 (94.58, 100.00)		100.00 (94.58, 100.00)	100.00 (77.19, 100.00)		100.00 (75.75, 100.00)	
NPV	100.00 (86.2, 100.00)		95.83 (79.76, 99.26)	100.00 (93.47, 100.00)		100.00 (91.97, 100.00)	
Likelihood ratio pos	Undefined		Undefined	Undefined		Undefined	
Likelihood ratio neg	0.00 (0.00, ?)		0.01 (0.00, 0.10)	0.00 (0.00, ?)		0.00 (0.00, ?)	
Diagnostic accuracy	100.00 (95.95, 100.00)		98.90 (94.03, 99.81)	100.00 (95.95, 100.00)		100.00 (93.58, 100.00)	
ISONIAZID (*katG*. *inhA*)	***katG***	***inhA***		***katG* (tot No. 67)**	***inhA* (tot No. 67)**	**tot No. 57**	Molecular 3/67 (4.48%) Phenotypic 2/57 (3.5%)
Sensitivity	95.74 (87.75, 98.83)	100.00 (91.03, 100)	82.76 (73.48, 89.26)	93.75 (71.67, 98.89)	90.91 (62.26, 98.38)	73.33 (55.55, 85.82)	
Specificity	100.00 (91.97, 100.00)	100.00 (93.12, 100.00)	100.00 (51.01, 100.00)	100.00 (92.59, 100.00)	100.00 (93.24, 100.00)	100.00 (86.68, 100.00)	
PPV	100.00 (92.13, 100.00)	100.00 (91.03, 100.00)	100.00 (94.93, 100.00)	100.00 (79.61, 100.00)	100.00 (72.25, 100.00)	100.00 (85.13, 100.00)	
NPV	95.65 (85.47, 98.90)	100.00 (93.12, 100.00)	21.05 (8.51, 43.33)	97.96 (89.31, 99.64)	100.00 (90.23, 99.67)	75.76 (58.98, 87.17)	
Likelihood ratio pos	Undefined	Undefined	Undefined	Undefined	Undefined	Undefined	
Likelihood ratio neg	0.04 (0.02, 0.11)	0.00 (0.00, ?)	0.17 (0.15, 0.20)	0.07 (0.009, 0.44)	0.09 (0.01, 0.65)	0.26 (0.21, 0.34)	
Diagnostic accuracy	97.8 (92.34, 99.4)	100.00 (95.95, 100.00)	83.52 (74.57, 89.75)	98.44 (91.67, 99.72)	98.44 (91.67, 99.72)	85.45 (73.84, 92.44)	
MTB				**tot No. 196**			
Sensitivity	100.00 (95.95, 100.00)		100.00 (95.95, 100.00)	100.00 (95.31, 100.00)			2/196 (1.02%)
Specificity	Undefined		Undefined	100.00 (96.79, 100.00)			
PPV	100.00 (95.95, 100.00)		100.00 (95.95, 100.00)	100.00 (95.31, 100.00)			
NPV	Undefined		Undefined	100.00 (96.79, 100.00)			
Likelihood ratio pos	Undefined		Undefined	Undefined			
Likelihood ratio neg	Undefined		Undefined	0.00			
Diagnostic accuracy	Undefined		Undefined	100.00 (98.06, 100.00)			

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229 **Table 4**. Phenotypic DST, MTBDR*plus*, Xpert MTB-RIF and VereMTB results for the 80 smear-
230 positive MTBC culture positive clinical specimens included in the study. Legend: ND: not detected. Δ:
231 no WT signal.

	DST		MTBDRplus/sequencing			Xpert MTB-RIF		VereMTB			
N°	**RIF**	**INH**	***rpoB***	***katG***	***inhA***	**MTB**	**RIF**	***rpoB***	***katG***	***inhA***	**N°**
9	S	R	WT	WT	C-15T	-	-	WT	WT	C-15T	9
2	R	R	S531L	S315T1	WT	-	-	S531L	S315T1	WT	2
1	R	R	S531L	WT+S315T1	WT	-	-	S531L	WT+S315T1	WT	1
6	S	R	WT	WT	WT	-	-	WT	WT	WT	6
2	R	R	D516V	S315T1	WT	-	-	D516V	S315T1	WT	2
2	R	R	S531L	WT	WT	-	-	S531L	WT	WT	2
4	S	R	WT	S315T1	WT	-	-	WT	S315T1	WT	4
1	R	R	S531L	S315T1/T2	WT	-	-	S531L	S315T1/T2	WT	1
1	R	R	Q513P	S315T1	WT	-	-	Δ 510-513 WT	S315T1	WT	1
1	S	R	WT	S315N	WT	-	-	WT	Δ 313-317 WT	WT	1
1	R	S	S531L	WT	WT	-	-	S531L	WT	WT	1
1	R	R	S531L	WT	C-15T	-	-	S531L	Δ 313-317 WT	WT	1
15	S	S	WT	WT	WT	-	-	WT	WT	WT	15
1	R	R	Δ 518-525 WT, Δ 530-533 WT	S315T1	WT	-	-	Δ 523-526 WT, S531L	S315T1	WT	1
1	-	-	D516V	S315T1	T-8C	-	-	D516V	S315T1	T-8C	1
15	-	-	WT	WT	WT	-	-	WT	WT	WT	15
1	-	-	WT	S315T1	WT	-	-	WT	S315T1	WT	1
9	S	S	-	-	-	-	-	WT	WT	WT	9
4	-	-	-	-	-	pos	WT	WT	WT	WT	4
1	-	-	WT	WT	WT	-	-	PCR controls not valid			1
1	S	S	ND	S315T1	WT	-	-	MTB ND			1
1	S	S	ND	WT	WT	-	-	ND	ND	ND	1

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