The use of gel element microarrays (GEM) in a lateral flow cell to detect *Mycobacterium tuberculosis* (*Mtb*) and drug susceptibility determinants

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Background

University of California, San Diego; University of Arkansas; and Akonni Biosystems have formed a partnership that leverages prior United States National Institutes of Health (NIH), UCSD, UofAR and Akonni Biosystems' investments in bioinformatics of drug resistance , automated sample preparation, multiplexed amplification microarrays, integrated biodetection systems, and manufacturing *in vitro* diagnostics.

Our unique partnership combines **UCSD's** & **University of Arkansas's** comprehensive bioinformatics experience and knowledge of the role of clinically relevant genetics of DR-TB with Akonni Biosystems' pioneering diagnostic technology to:

1) Test and upgrade the existing MDR-TB prototype Gel Element Microarray (GEM) platform to an XDR-TB platform.
2) Verify that upgrades to the XDR-TB GEM will have a high sensitivity and specificity against 200 well characterized *Mtb* isolates.
3) Clinically evaluate the XDR-TB GEM in a high-throughput clinical laboratory in Chisianu, Moldova.

Here we report on testing of the MDR-TB GEM.

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Introduction

Worldwide, less than 60% of TB cases are confirmed with a laboratory test[1] and regional increases in the prevalence of DR-TB,[2] the broadening distribution of MDR and XDR-TB,[2] and the emergence of what is being called "totally drug-resistant TB"[3-6] all impose a clear threat to global TB control and public health.[7]

The best approach to prevent transmission and cure TB is quick diagnosis and effective treatment.[8] However, conventional laboratory identification by liquid culture of *Mtb* may take 3-8 weeks followed by an additional 2-3 weeks for liquid culture and even longer in laboratories that are forced to rely on solid culture where bacterium grow even slower.

In 2010, WHO endorsed Xpert MTB/RIF[9] (Cepheid, Inc.), an assay which accurately detects *Mtb* and its resistance to the first-line drug rifampicin (RIF^R) in less than two hours.[10,11] Over the last five years, global acceptance has been unprecedented - over 10 million Xpert MTB/RIF tests have been administered.[12] While this real-time method of detecting RIF^R has changed the paradigm of TB diagnosis, it has underscored the need for testing all forms of DR-TB.

There is a clear and critical need for a rapid, integrated, table-top platform with high sensitivity and specificity, for diagnosis of M/XDR-TB from direct patient samples. University of California, San Diego; University of Arkansas; and Akonni Biosystems are working together to produce a rapid XDR-TB detection platform based on detection of resistance conferring mutations. During the initial design and development stage, we are testing an existing prototype Gel Element Microarray (GEM) platform to detect MDR-TB. During Phase 2, we will develop and test a XDR-TB GEM platform.

Citations

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Results

Of the clinical samples obtained, the identification of *M. tuberculosis* was high (94% for culture positive/ smear positive) but for smear negative samples identification was lower, suggesting that identification may be dependent upon bacteria load (Table 1 & 2).

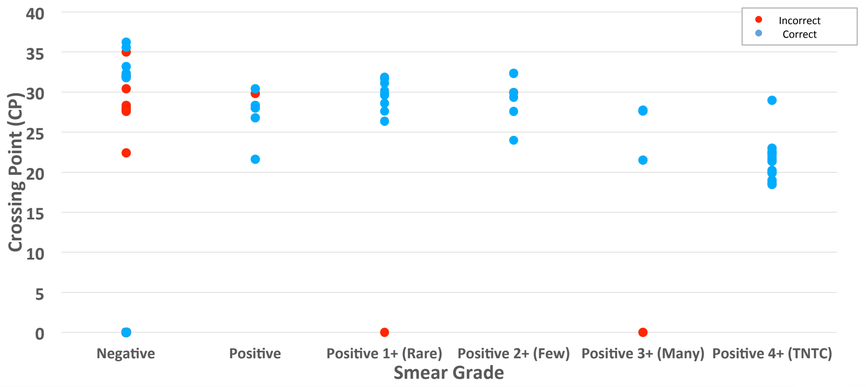
Table 1: Raw Counts		Culture and Smear				Total
		Mtb Culture Negative (-)		*Mtb* Culture Positive (+)		
		Smear -	Smear +	Smear -	Smear +	
LFC Results	*Mtb* Not Identified	11	0	2	1	14
	Mtb Identified	2	2	13	38	55
	Total	13	2	15	39	69

Table 2: Percentages		Culture and Smear				
		Mtb Culture Negative (-)		*Mtb* Culture Positive (+)		
		Smear -	Smear +	Smear -	Smear +	
LFC Results	*Mtb* Not Identified	85%	0%	13%	3%	
	Mtb Identified	15%	100%	87%	97%	
	Total	100%	100%	100%	100%	

Sensitivity and specificity for isoniazid resistant was high (90.9% and 100% respectively, pink cells Table 3) and high for rifampin (100% for both pink cells, Table 4).

Table 3: Isoniazid Results		DST results		Total
		Susceptible	Resistant	
LFC Results	Susceptible	34	1	35
	Resistant	-	10	10
	Mtb pos, DST Indeterminate	6	-	6
	Mtb negative	1	1	2
	Total	41	12	53*
*16 samples had no DST - 13 were culture negative and 3 DSTs were never done				

Table 4 Rifampin Results		DST results		Total
		Susceptible	Resistant	
LFC Results	Susceptible	39	-	39
	Resistant	-	6	6
	Mtb pos, DST Indeterminate	6	-	6
	Mtb negative	1	1	2
	Total	46	7	53*
*16 samples had no DST - 13 were culture negative and 3 DSTs were never done				



Conclusions

The MDR-TB prototype Gel Element Microarray (GEM) platform has very good performance characteristics against a set of clinical samples collected in San Diego and Imperial Counties.

Test failure rate for the drug susceptibility of isoniazid and rifampin (number of tests performed that did not produce actionable results) was low – only 6 of 69 tests (8.7%).

Test sensitivity and was high (90.9% for isoniazid and 100% for rifampin) as was specificity (100% for both isoniazid and rifampin).

Incorrect drug susceptibility results seem to be concentrated within smear negative samples indicating that low bacterial loads may confound results. The amount of DNA extracted from samples (indicated by the crossing point) does not appear to be related to smear grade and whether samples were incorrectly or correctly classified.

Future Work

The results of the MDR-TB prototype Gel Element Microarray (GEM) platform are promising and we plan to continue testing and development.

Over the next three years we plan on

1. Add a set of markers to determine XDR-TB (Table 5).
2. Verify that the XDR-TB GEM has a high sensitivity and specificity against 200 well characterized *Mtb* isolates.
3. Clinically evaluate the XDR-TB GEM in a high-throughput clinical laboratory in Chisianu, Moldova.

Methods

We obtained three types of clinical specimens from San Diego County Public Health Laboratory (SDPHL) Imperial County Public Health Laboratory (IMPHL). Specimens included retained sediment and excess sputa from routine tuberculosis testing.

Retained Sediments clinical samples from suspected MDR patients no longer needed for public health purposes will be identified by county laboratory staff. Retained sediments with a minimum volume of 550 microliters were transported to UCSD for further processing. Demographic and laboratory data corresponding to the selected sediments was entered into an online collection form.

Real Time Sputa and Real Time Sediment clinical samples for tuberculosis positive patients returning for repeat sputa collection were sub-sampled prior to processing and both an aliquot of a minimum of 0.50 mL of sputa and a paired aliquot of a minimum of 0.55 mL of sediment was collected. Demographic and laboratory data corresponding to the selected samples was entered into an online collection form.

Specimens received from either from San Diego County or Imperial County were divided into aliquots 0.55 ml of sediment or 0.50 ml of raw sputum. Aliquots were heat killed in a 90°C waterbath for five minutes. Excess aliquots were stored for confirmatory or replicate testing.

Akonni Biosystems has developed a prototype MDR-TB PCR TruArray analysis system which conducts DNA extraction and purification, PCR amplifiaction, and image analysis for full sample-in-answer-out system (Figure 1). The Prototype TruArray® MTB Lateral Flow Cell Test for RIF and INH Drug Susceptibility is a gel element, microarray-based test for detecting and genotyping mycobacteria in the M. tuberculosis complex (MTB). If MTB DNA is detected, then the test will also report on the detection of mutations known to confer resistance to Isoniazid and Rifampicin (Table X).

DNA extraction was accomplished using 0.5ml of sputum or sediment and batched in the Sample Preparation sub-system (Inset (C), Figure 1 and Figure 2) with six samples plus a negative and a positive control. The amount of DNA in a sample was later quantified using qPCR.

The TruArray system consists of a 3-dimensional co-polymer gel elements immoblized on the LFC glass slide (Figure 3). Oligonucleotide probes are covalently coupled to the polymer backbone, and immobilized throughout the three-dimensional volume. Microarray probes target common mutations in *rpoB*, *katG* genes and *inhA* promoter and insertion elements IS6110 (Table X). The PCR mix is introduced directly into the LFC, sealed, and placed in the TruCycler (Figure 1 (F)) for thermal cycling and hybridization. After hybridization, microarrays are washed and dried, and imaged on the TruDx Imager (Figure 1 (E)). The analysis software automatically analyzes the fluorescence image and reports on the detection or non-detection of MTB, resistance-conferring mutations, and internal controls.

Drug	Gene	MDR-TB GEM Codons (number of mutations)		Proposed XDR-TB GEM Codons (number of mutations)	
***Mtb*/NTM**		IS6110		IS6110, MPB64, hsp65	
Isoniazid	*katG*	315 (1)		315 (3)	
	inhA promoter	-15 (1)		-8 (1) -15 (1) -17 (1)	
	ahpC promoter			-6 (1) -10 (1)	
Rifampicin	*rpoB*	511 (1) 513 (1) 516 (3)	526 (4) 531 (2) 533 (1)	513 (2) 516 (3) 522 (1)	526 (6) 531 (2) 533(1)
Fluoroquinolones	*gyrA*			88 (3) 90 (1)	91(1) 94 (3)
Injectables	*rrs*			1401 (1) 1402 (1) 1484 (1)	
	eis promoter			-10 (1) -12 (1) -14 (1)	-15 (1) -37 (1)

Table 5: Mutations detected by the MDR-TB TruArray GEM and proposed XDR-TB GEM

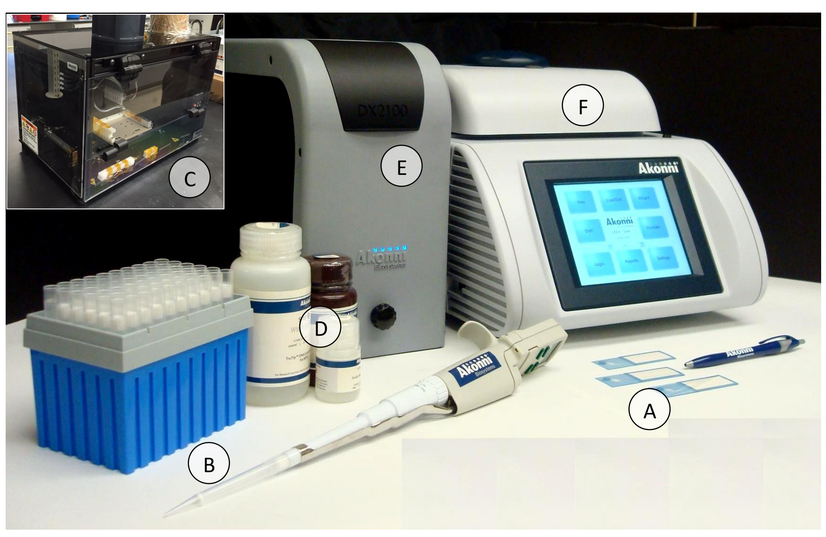


Figure 1: Akonni MDR-TB PCR TruArray System Sputum . (A) Lateral flow cell with microarray probes. (B) TruTip s for single or 8-channel pipettor used for DNA extraction and purification. (C) Inset shows the stand-alone, automated sample preparation sub-system (D) Primer and hybridization reagents. (E) Portable imager (F) thermal cycler.

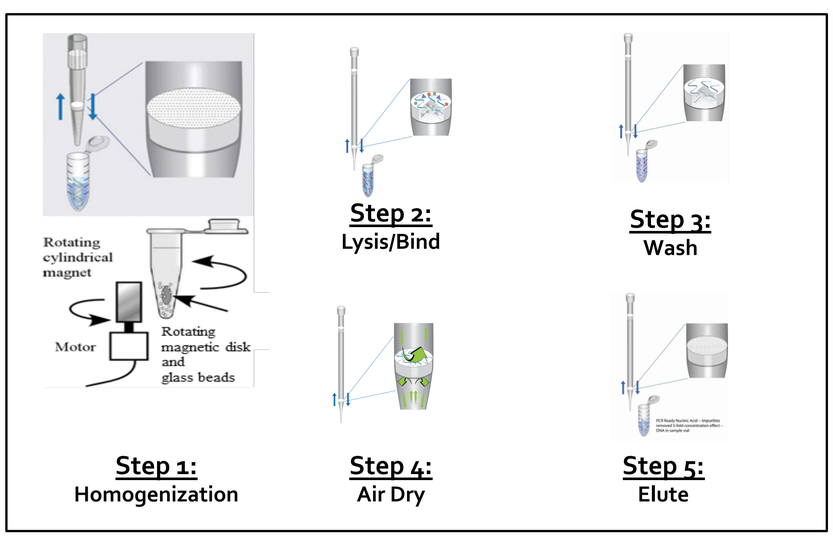


Figure 2: Sputum sample preparation steps using TruTip, a porous, monolithic binding matrix is inserted into a pipette tip. Step 1: Homogenizing viscous samples and lysing microorganisms is accomplished using an exterior cylindrical magnet which rotates and causes the magnetic disk and glass beads in the TruTip to physically break up the sample. Step 2: Lysis buffer formula releases and extracts DNA. Step 3: The wash step removes lysis buffer and other impurities. Step 4: Drying step removes residual solvents. Step 5: The elution step releases extracted and purified DNA.

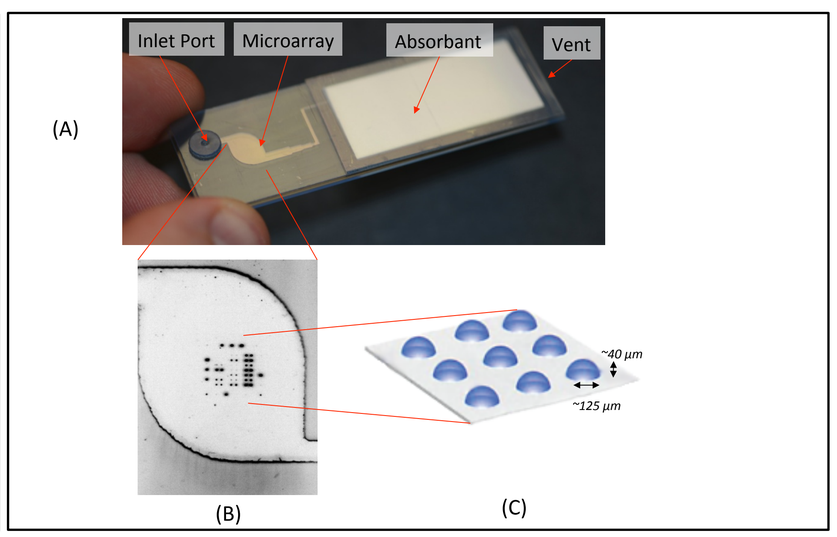


Figure 3: Aspects of Lateral Flow Cell (LFC) which is approximately the size of a slide . (A) The entire LFC incorporates four components: Inlet port where extracted DNA is deposited, microarray, absorbant material that will receive waste materials, and vent; (B) close up view of microarray showing Gel Element Microarray (GEM) as well as fiduciary marks and ; (C) close up view of probes immobilized within discreet 3D gel elements.