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Detecting rifampin and isoniazid resistance in *Mycobacterium tuberculosis* direct from patient sputum using an automated integrated system

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ABSTRACT

While there has been progress in detection of drug resistant tuberculosis globally, WHO estimates only about half of the patients with bacteriologically confirmed tuberculosis were tested for rifampicin resistance over the past two years. To close this drug resistance diagnostic gap, an expansion of testing for rifampicin and isoniazid resistance is critically needed. The Akonni Biosystem Integrated System combines DNA extraction and a Lab-on-a-Film assembly (LFA) to perform rapid probe and PCR-based detection of resistance associated mutations to first-line anti-tuberculosis drugs. Using raw sputum samples from 25 tuberculosis patients at risk for drug resistance, we conducted a proof-of-concept study of the Integrated System with an MDR-TB assay. Performance of the Integrated System was compared to liquid Mycobacteria Growth Indicator Tube (MGIT) culture reference phenotypes using 2012 WHO endorsed critical concentrations for rifampicin and isoniazid. The overall percent agreement for rifampicin and isoniazid was 91.7% and 100% respectively, with agreement for rifampicin increasing to 95.7% after low-level resistance mutations in *rpoB* were excluded. The Integrated System, combining DNA extraction and LFA amplification, is a promising new tool for detection of both rifampicin and isoniazid using liquefied raw sputum.

1. Introduction

Tuberculosis (TB) has consistently been ranked by the World Health Organization (WHO) as one of the top 10 leading causes of death globally, and prior to COVID-19, TB was the leading cause of death by a single infectious disease agent [1]. Despite the End TB Strategy, endorsed by the World Health Assembly in 2014, [2] an estimated 10 million people continue to be infected with *Mycobacterium tuberculosis* each year [1]. Of growing concern is the increased incidence of drug resistant tuberculosis (DR-TB), specifically multi-drug resistant tuberculosis (MDR-TB) strains, which are resistant to both isoniazid (INH) and rifampicin (RIF) [3]. MDR-TB increases the cost and complexity of treatment and increases mortality, with treatment success rates of less than 60% on average [1]. Identifying individuals with MDR-TB through early drug susceptibility testing (DST) is critical, not only to improving treatment outcomes, but also to reducing transmission. Additionally, rapid identification of both RIF and INH resistance simultaneously allows for early modification of treatment regimes. In 2019, it was estimated that 11% of all incident cases of TB were INH resistant and RIF susceptible, and would not receive the recommended modified treatment if only tested for RIF resistance [1]. Phenotypic drug susceptibility testing (pDST) using cultured clinical isolates has been the gold standard for guiding the selection of appropriate antibiotics for patient treatment. However, global scaling of pDST has been limited by delayed time to result (weeks to months), and the laboratory complexity and cost of implementation in low resource settings [4].

In response to the MDR-TB threat, the WHO-developed guidelines that highlight the importance of expanding access to rapid testing and

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immediate DST as an integral part of the fight against MDR-TB, [1] which has stimulated the development of several new commercial diagnostic solutions [5]. However, currently, the WHO has endorsed only a few benchtop, sample-to-answer molecular platforms for the rapid, automated detection of *M. tuberculosis* and drug resistance, including the GeneXpert MTB/RIF and more recently Molbio Truenat MTB-RIF Dx [6]. Both diagnostics are limited to detection of RIF resistance and do not evaluate INH resistance. The WHO has also recently endorsed BD Max MDR-TB and Abbott *m*2000 RealTi*m*e MTB System for detection of RIF and INH resistance, [7] but these are very large, high-throughput solutions likely only suitable for centralized settings.

There is still a lack of near-patient, sample-to-answer, low-cost solutions capable of simultaneously detecting both INH and RIF resistance for rapid MDR-TB diagnosis. Considering that an estimated 3 million new TB cases went unreported in 2018 – many due to a lack of access to diagnostics – and only ~ 33% of the estimated RIF-resistant cases were diagnosed and treated in the same year, it is clear that more needs to be done to advance the goals of the End TB Strategy [1,8,9].

The Integrated System (Akonni Biosystems, MD) is positioned to address this gap with a scalable, sample-to-answer, benchtop platform that uses patient sputum as the input sample [10]. This system has been previously evaluated using spiked sputum samples, [10] but it has not yet been validated using clinical samples. Successful processing of clinical samples is critical for the evaluation of the system, particularly when considering sputum mucoid encapsulates *M. tuberculosis* in clinical samples, unlike spiked bacilli in contrived samples, which does not undergo active encapsulation [11]. In this study, we challenged this system and MDR-TB assay with 25 prospectively collected clinical sputum samples from patients at risk for DR-TB, and compared assay results to liquid Mycobacteria Growth Indicator Tube (MGIT) culture reference phenotypes at WHO 2012 endorsed critical concentrations.

2. Methods

2.1. Clinical samples

Raw sputum aliquots were selected from a biobank of existing clinical samples, collected for a prospective cohort study of patients at risk for drug resistant TB and described in detail in Hillery *et al* [12]. Briefly, samples were collected in the Republic of Moldova at several regional TB clinics. All samples were previously characterized by liquid MGIT DST to determine phenotypic drug resistance profiles [12] and bacterial load was quantified by qPCR as described previously [13]. Phenotypic DST was performed using the MGIT 960 following 2012 WHO recommendations for critical concentrations (all units in μg/ml): INH 0.1; RIF 1.0; Ethambutol (ETH) 5.0; Pyranzinamide (PZA) 100; Kanamycin (KAN) 2.5; Amikacin (AMK) 1.0; Capreomycin (CAP) 2.5; Levofloxacin (LEV) 1.5; and Moxifloxacin (MOX) 0.25, 0.5, and 2.0 [12]. Selection criteria for clinical samples to be included in this proof-of-concept study were: a) bacterial load as determined by qPCR (CT value of $\leq$ 28); and b) susceptibility to fluoroquinolones and second-line injectable drugs by pDST. Twenty-five samples met this criteria and were included in this proof of concept study. The integrated system was run in a clinical research laboratory at University of California, San Diego in La Jolla, California.

2.2. Akonni biosystems integrated system

The Integrated System has been previously described in detail [10]. Briefly, the system automatically processes up to six liquefied sputum samples simultaneously, performs nucleic acid amplification and hybridization, and analyzes TruArrays controlled with a graphical user interface on a laptop computer. Raw, liquefied patient sputum were added into lysis tubes that were loaded on a receptacle on the Integrated System along with a pre-filled reagent plate and a TruArray consumable before executing an automated control script [10].

2.3. Study procedure

2.3.1. Study design

The study consisted of seven runs of the Integrated System to evaluate 25 clinical samples. The first six runs contained one control of H37Ra cells-in-sputum, one negative control (sterile water) and four clinical samples. The location of positive and negative controls changed for each run. The seventh run contained only one clinical sample, negative control, and one spiked-sputum control. Cells-in-sputum positive controls, consisted of raw, disease-free purchased sputum (Laboratory Sciences of Arizona, LLC and Sonora Quest Laboratories, LLC; collectively LSA/SQL) which was confirmed TB-negative by PCR prior to testing combined with H37Ra stock cells (Zeptometrix #0801660) spiked in for a final concentration of $4x10^4$ CFU/ml cells.

2.3.2. Liquefaction and heat inactivation

Prior to loading samples onto the Integrated System, 500 μL of thawed sample (for both clinical samples and cell-spiked positive controls) underwent liquefaction as described previously [10]. Liquefied samples were then heat killed at 95 °C for 30 min, vortexed every 10 min. All positive controls and patient samples were liquefied and inactivated at one time and stored at −70 °C prior to the run.

Integrated System setup. All samples and equipment were prepared, loaded and run accordingly as described in Kukhtin et al [10].

2.3.3. Analyses

Analytical drug resistance detection sensitivity and specificity were calculated by comparing the Integrated System drug resistance results to the reference pDST. Ninety five percent confidence intervals were calculated using the Wilson score method [14].

3. Results

The acid-fast bacteria (AFB) smear status of analyzed samples was as follows: 2 (8%) 1+, 3 (12%) 2+, and 20 (80%) 3 + . Two of the 3 + samples were reported by the Integrated System as indeterminate for a single drug. Sample BP2-1217 was indeterminate for RIF and BP2-1168 was indeterminate for INH. Based on the Integrated System results, the patient samples were categorized as: 17 (74%) MDR (RIF + INH resistant), 1 (4%) INH mono-resistant, 2 (9%) RIF mono-resistant, and 3 (13%) susceptible to INH and RIF (Table 1). Sensitivity and specificity for detection of RIF and INH resistance were calculated against MGIT pDST at 2012 WHO critical concentrations as reference (Table 2). Two samples had discordant calls for RIF when compared to pDST.

4. Discussion

The automatic detection of *M. tuberculosis* and simultaneous characterization of resistance/susceptibility to INH and/or RIF directly from sputum has the potential to aid drug resistance detection and guide treatment for TB patients. In this proof-of-concept study, 25 liquefied, inactivated clinical sputum samples were run on the Akonni Biosystems' Integrated System using raw sputum as input. The performance of the

Table 1
Drug resistance classification for Akonni Integrated device, and MGIT DST.

	Susceptible	RIF mono-resistant	INHmono-resistant	MDR	RIF R + INH IND	INH S + RIF IND
Akonni Integrated device (n = 25)	3	2	1	17	1	1
MGIT DST (n = 25)	6	0	1	18	0	0

Table 2
Sensitivity and specificity of RIF and INH resistance detection with 95% CI.

	Sensitivity % (95% CI)	Specificity % (95% CI)	Percent Agreement
RIF	100	66.7	91.7%
n = 24	(78.1–100)	(24.1–94)	
INH	100	100	100%
n = 24	(78.1–100)	(51.7–100)	

instrument in comparison to phenotypic MIGT DST resulted in a sensitivity and specificity of 100% for INH, and 100% and 66.7% respectively for RIF (Table 2). The reduced RIF resistance was caused by discordance with pDST in two samples. For sample BP2-1190, the Integrated System detected an *rpoB* L511P mutation, which is a well-documented resistance associated mutation that most often appears RIF susceptible in liquid DST (e.g. MGIT) but resistant on solid DST and has been well documented to be a low minimum inhibitory concentration (MIC) mutation [15,16]. Excluding this sample with a low MIC mutation from the specificity calculation results in RIF specificity estimate of 80% (CI:29.9–98.9%). For the other RIF discordant sample (BP2-1116), the Integrated System detected a *rpoB* S512T mutation, which is associated with phenotypic resistance and was thus reported as "resistance detected", whereas the pDST result was susceptible. In this case, the median of three replicate wild-type hybridization spots on the LFA was lower than the median of three replicate mutant (S512T) spots, resulting in a mutant call. The algorithm uses the median (as opposed to the mean) only when the variance across replicates exceeds a threshold. However, the mean and not the median of the replicates in this instance resulted in the correct call (susceptible), indicating further testing and characterization of the system with clinical samples with diverse genotypes is needed to inform and improve the analysis algorithm.

All clinical samples produced at least partial data, with only 2 samples resulting in partial drug resistance profiles. Two samples resulted in indeterminate calls, one for each drug examined (BP2-1217 RIF and BP2-1168 INH). For BP2-1217 there was poor amplification of the *rpoB* target and thus no mutations could be examined. For BP2-1168 there was adequate amplification on the *inhA* promoter region, but low signals for the *katG* target resulted in an indeterminate call for INH resistance. Probes on the array include universal markers that target conserved regions in the gene as an indicator of amplification efficiency. While the universal markers for the other gene targets (rpoB, inhA, and IS6110) gave acceptable signals, the katG signals were lower than the threshold for acceptable amplification. In this case, the result was correctly reported as indeterminate. One possible explanation for this result is that this sample may have had a mutation in what has been deemed to be a conserved region, resulting in poor amplification.

Rapid MDR detection, with separate and simultaneous identification of INH and RIF resistance will allow for the diagnosis of both INH and RIF mono-resistance and MDR-TB, which is critical as each have different treatment recommendations [16]. The WHO currently recommends the modification of patient treatment regimens specifically for INH-resistant/RIF-susceptible infections [1,17] but such clinical decisions require further testing than is currently possible with GeneXpert MTB/RIF alone, which only tests for RIF resistance. In this diagnostic environment, the Integrated System could provide added diagnostic value as molecular diagnostic solutions that include INH resistance testing are not widely available yet, leaving critical diagnostic gaps for this important group of TB patients, and resulting in amplification of resistance when not appropriately treated [1]. While only one of the 25 patients (Table 1) examined in this proof-of-concept study fits this category, the global prevalence of INH mono resistance is over 11% [1].

There are two important limitations of the study that will be addressed in future studies. The first limitation is the sample selection, which include, by design, only samples that did not have mutations associated with second-line resistance. And there were a limited number of drug susceptible clinical samples (n = 6 pDST RIF and INH susceptible), due to the parent study being focused on drug resistant patients. Future analytical studies will include a larger sample size with more *M. tuberculosis* genetic diversity including pan susceptible samples to more accurately evaluate assay specificity and sensitivity estimates. Follow-on studies of performance across a larger and more diverse patient sample set are needed to improve precision of our findings and generalizability of the assay performance across a broader range of bacterial loads and patient characteristics.

5. Conclusion

This proof of concept study demonstrates the potential of the Integrated System to be used to detect INH and RIF resistance directly from raw sputum. The ability to report on both INH and RIF resistance from raw sputum samples in an integrated, automated solution could have a major impact on the rapid diagnosis and treatment of both MDR and INH mono-resistant TB.

Ethics statement

Raw sputum aliquots were selected from a biobank of existing clinical samples, collected for a prospective cohort study of patients at risk for drug resistant TB and described in detail in Hillery *et al.*

Hillery N, Seifert M, Catanzaro DG, McKinnon S, Colman RE, Chiles PG, et al. Rapid Detection of Extensively Drug-Resistant Tuberculosis in Clinical Samples Using a Novel Tabletop Platform: Protocol for a Prospective Clinical Study. Journal of Medical Internet Research (JMIR) Research Protocols. 2021.

Excerpt from parent study publication listed above: "The study was approved by the UCSD Human Research Protections Program (Project #161864), and the Ethics Committee of the PPI "Chiril Draganiuc." The consent document translation was completed by the study staff in Moldova fluent in Romanian. The participants were compensated with an equivalent of US$10 per patient per visit for time and travel; the compensation was customary and allowable by local norms, as well as the UCSD and Moldova IRB requirements. All participants were assigned a unique study identifier; no personally identifiable data were documented on the study questionnaires. A document linking the patient's name to the study ID was stored securely in locked study files for follow-up purposes only. These records were destroyed upon completion of data collection. The questionnaires were stored in a separate secure location at the PPI and were archived for at least 5 years. No identifiable data were shared outside of the research team. The results from the XDR-LFC were used for research purposes only, and because they were not run in real time, it was not possible to use these experimental results for clinical decision-making. This protected the participants from any potential misuse of the experimental results."

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