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14. ABSTRACT

TB is considered to be among the most dangerous health threats to military men and women deployed to overseas battlefields and humanitarian missions. Time-consuming culture tests are still commonly used for TB diagnosis and to identify drug-resistant TB cases in much of the world. A rapid, easy-to-use and reliable diagnostic platform is therefore highly needed to allow rapid TB diagnosis and treatment of military forces in harsh environmental conditions, such as war zones. With the support of the CDMRP award, a translational research team, including the engineering lab at Tulane University, the clinician scientists at Baylor College of Medicine and NanoPin Technologies, Inc., aim to develop a nanoparticle-based approach to address the issues associated with detection of Mycobacterium tuberculosis (Mtb)-derived factors in human blood samples. This diagnostic platform can rapidly and sensitively detect and quantitate TB antigen levels directly from blood samples and provides quantitative results to allow prompt diagnosis and rapid evaluation of a patient's response to treatment. Ultimately, this proposal seeks to leverage our recent success in molecular diagnostics and biomedical nanotechnologies to produce a portable point-of-care diagnostic platform that has the potential to markedly improve TB treatment and control by improving the accuracy, speed and cost of TB diagnosis and treatment monitoring. If successful, our strategy should have a significant impact on populations in developing countries as well as military personnel deployed to areas where this disease is prevalent.

15. SUBJECT TERMS

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ANNUAL REPORT FOR W8IXWH-19-1-0926

TITLE: Quantification of Circulating Mtb Antigens for Rapid TB Diagnosis and Treatment Monitoring

PI: Tony Hu PhD, Tulane University School of Medicine

1. INTRODUCTION:

With the support of the CDMRP award, a translational research team that includes scientists at Tulane University, clinician scientists at Baylor College of Medicine and researchers at NanoPin Technologies, Inc. will develop a nanoparticle-based approach to address issues associated with the detection of *Mycobacterium tuberculosis* (*Mtb*)-derived factors in human blood samples. This diagnostic platform will rapidly and sensitively detect and quantify TB antigen levels from blood samples to allow prompt disease diagnosis and evaluation of a patient's response to treatment. In the first award year, we have developed a robust and integrated workflow to identify pathogen-specific peptides that can be utilized as circulating biomarkers to diagnose TB. A monoclonal antibody to an new *Mtb*-derived peptide is now under development, and will be integrated into our current platform to improve its sensitivity and specificity. While we are still evaluating the archived specimens provided from our Eswatini site, we have completed analyzing another archived serum cohort from the Houston Tuberculosis Initiative (HTI), which ran from October 1995 through September 2002. We analyzed serum from 105 children evaluated for TB (55 TB cases and 50 close contacts without TB), identifying TB cases with 85.5% diagnostic sensitivity (95% confidence interval [CI]: 73.3 to 93.5). Similar diagnostic sensitivities were found for culture-positive (87.5%; 95% CI: 67.6 to 97.3) and -negative (83.9%; 95% CI: 66.3 to 94.5) TB cases and for culture-negative pulmonary (77.8%; 95% CI, 40.0 to 97.2) and extrapulmonary (86.4%; 95% CI, 65.1 to 97.1) TB cases. We next evaluated the diagnostic sensitivity of this assay approach in a diagnostically challenging cohort of HIV-positive children aged <10 years from Kenya. These results suggest that serum biomarker analysis holds significant promise for rapid and sensitive diagnosis of pediatric TB, including extrapulmonary and paucibacillary TB. The ability to use frozen samples for this analysis should also permit assays to be performed at central sites, without requiring strict timelines for sample analysis.

2. KEYWORDS:

cGMP, scaled-up, assay kit, nanoparticles, monoclonal antibodies, sample digestion, mass spectrometry, protocols, tuberculosis diagnostics, technology transfer, quality management system

3. ACCOMPLISHMENTS:

- **What were the major goals of the project?**

Cohort Development: Obtain IRB approval for the retrospective cohorts and prospective sample collection (0-3 mon, **100% complete**)

Aim 1) Optimize the sensitivity and specificity of our existing blood-based prototype assay to meet or exceed minimum WHO performance criteria for screening and diagnosing TB.

Subtask 1.1: Organize the retrospective clinical samples (Month 0-12, **50% complete**)

1. Our Eswatini cohort (**Eswatini**) contains >800 subjects who were classified into one of four categories based on their diagnosis: active TB, LTBI, NTM, and close contacts of these three groups without evidence of mycobacterial disease.
2. The TB group (n=>350) spans the spectrum of TB disease severity. Individuals enrolled in this cohort were diagnosed at enrollment using microbial evidence from Xpert MTB/RIF and/or *Mtb* culture (69.9%) or clinically diagnosed (29.7%) using consensus guidelines.
3. This cohort also contains 290 asymptomatic household contacts of these TB cases, who were followed for 12-months after being offered preventative isoniazid therapy, These study participants were classified as close contacts without mycobacterial disease or as TB, LTBI, NTM cases based on their final diagnosis during this 12-month follow-up period.
4. The organization of archived serum samples from these individuals and their transport to the US will be managed by Baylor team members, including Drs. Kay (based in Eswatini) and Mandalakas and Dinardo (based in Houston, TX).

Subtask 1.2: Evaluate Nanoparticle-MS assay diagnostic sensitivity improvements with additional *Mtb*-specific antigen peptides (Month 1-12, **90% complete**; antibody fabrication delayed by the COVID-19 pandemic with final antibody due by the end of April 2021)

Subtask 1.3: Evaluate specificity Improvements by performing logistic analyses to evaluate the relative diagnostic specificity of individual peptide and protein combinations (1-12 months, **90% complete**)

Subtask 1.4: Evaluate the analytical performance of an established peptide biomarker panel. Dr. Hu's lab (Tulane) will generate the assay data, the Baylor team (Baylor) will provide clinical data and patient classifications required to analyze diagnostic performance of the Nanoparticle-MS assay (5-18 months, **20% complete**).

Aim 2) Validate the performance of our optimized assay using blood samples previously collected from well-characterized TB and non-TB patients and determine if assay performance on a prototype mobile miniMS instrument is non-inferior to that observed on a standard MS instrument.

Subtask 1: Establish analysis and validation protocols for the Nanoparticle-MS system with clinical samples (7-12 months, **100% complete**)

Subtask 2: Optimize the direct analysis of nanoparticle-captured biomarkers (Month 13-18, **20% complete**)

Aim 3) Determine the clinical sensitivity and specificity of the final assay on a MiniMS-12 or bench-top MS using >3,000 blood samples prospectively collected from serial patients recruited from intended use settings in high (Swaziland), medium (Tanzania) and low (US) burden TB screening clinics.

Subtask 3.1: Submit documents for IRB approvals (Month 1-3, **100% complete**).

- The IRB for the prospective sample collection in Swaziland was approved and submitted to DOD on Sept. 4, 2019 (**Eswatini**).
- The IRBs for prospective sample collection in **Houston** and **Tanzania** (Site 2 and Site 4) were approved by the relevant institutions in both countries and submitted to DOD on February 17, 2020.

Subtask 3.2: Human sample collection (Month 4-35, **10% compete**, site preparation complete; awaiting HRPO approval to proceed for remaining 90%):

- Enroll individuals being evaluated for TB once IRBs for the prospective sample collection is approved for **Houston, Tanzania and Eswatini** (recruitment will commence after receiving HRPO approval).
- Prospectively evaluate the sensitivity of the miniMS or bench-top MS instrument with serum from a cohort of individuals with microbiologically confirmed TB (**Tulane**).
- Prospectively evaluate the specificity of the miniMS or bench-top MS instrument with serum from asymptomatic controls without TB (**Tulane**).

Subtask 3.3: Perform clinical tests: sputum smear, culture and DST, HIV testing at **Houston** and **Tanzania** (Month 15-18, **not yet initiated**).

Subtask 3.4: Determine the overall diagnostic sensitivity and specificity of Nanoparticle-MS for active TB cases (18-20 months, **not yet initiated**).

Subtask 3.5: Track *Mtb*-derived peptides in sera as a function of TB treatment for patients with documented therapies (21-22 months, **not yet initiated**).

Aim 4) Establish GMP product development processes to produce an optimized assay kit.

Subtask 4.1: Establish a cGMP fabrication process for multilayered stacks of Nanoparticle particles by following internally established and externally validated Standard Operating Procedures (SOPs). The designated research staffs from Tulane University (Tulane) will work closely with Nanopin Technologies Inc. (Nanopin) to optimize the cGMP fabrication of nanoparticles, develop and verify the prototype and establish a quality control system (18-36 months, **15% complete**).

Subtask 4.2: Build a variety of product prototype configurations for the assay nanoparticle, using low-cost injection molding to study plate-to-silicon interfaces, including overmolding (20-24 months, **10% compete**).

Subtask 4.3: Conduct prototype verification (22-28 months, **not yet initiated**).

Subtask 4.4: Develop and implement a thorough ISO13485 quality management system (QMS) (24-30 months, **not yet initiated**).

Subtask 4.5: Identifying monoclonal antibodies for our assay targets (7-18 mon, **85% complete**).

Subtask 4.6: Evaluate the physical and functional stability of new antibody-conjugated nanoparticles by technical staff at Nanopin and Tulane during storage over a range of temperatures, both in solution and as a lyophilized powder (25-34 mon, **30% compete**).

- **What was accomplished under these goals?**

Progression for Aim 1.

Aim 1. Optimize the sensitivity and specificity of our existing blood-based prototype assay to meet or exceed minimum WHO performance criteria for screening and diagnosing TB.

The IRB for using retrospective samples for specific aim 1 has been approved and submitted to DOD on Sept. 4, 2019 (**Baylor**).

Selection of Species-Specific Antigen Peptides

Related pathogens may produce similar symptoms and demonstrate significant genomic and proteomic conservation, making them difficult to distinguish, particularly if they infect sites where invasive procedures are required to obtain isolates. However, pathogen-derived proteins shed by infected cells may be detectable in the circulation even in such cases, and contain short regions of unique sequence that distinguish them from related pathogens. Such proteins should ideally be expressed throughout infection, play essential roles in replication or virulence, and contain pathogen-specific peptide sequences suitable for antibody capture. Structural proteins represent good candidates for biomarker peptides, but virulence factors may permit discrimination of active versus latent infections to influence treatment decisions[1].

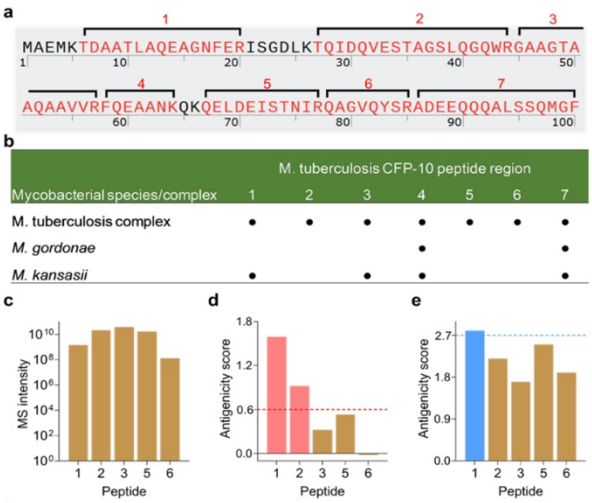


Figure 1. Selection of species-selective peptides for TB diagnosis. **a**, Map of *Mtb* CFP-10 tryptic peptides ≥ 7 amino acids. **b**, Match of *Mtb* CFP-10 peptides with those of species responsible for most (>80%) pulmonary mycobacterial infections. **c**, LC-MS intensity of *Mtb* CFP-10 tryptic peptides. **d-e**, Antigenicity of peptide candidates by predictive algorithms from (d) Genescript or (e) Thermo Scientific.

Mycobacterium tuberculosis (*Mtb*) infections that cause tuberculosis (TB), the leading cause of death from infectious disease prior to the SARS-CoV-2 pandemic, may resemble those caused by nontuberculous mycobacteria (NTM) superfamily members that respond to different drug regimens, and comprise 4–30% of suspected TB[2-4] and 18–27% of chronic, multi-drug resistant TB cases in some settings[5-8]. Specific identification is thus necessary to avoid ineffective treatment with toxic drugs and drug resistance, and normally requires pathogen-rich respiratory or tissue samples. However, peptides derived from circulating *Mtb* proteins can be species-specific, distinguish latent and active disease, and reflect viable pathogen load[1], unlike current methods that lack one or more of these abilities.

In the first project year, we comprehensively studied the specificity of antigen peptides for the *Mtb* virulence factor CFP-10. We developed and validated a robust criteria set to determine the true signal of the target peptide on the mass spectra and will apply this protocol to other antigen peptides of interest (e.g.,ESAT-6, MPT53 and CFP-2) in the next program year as we develop new antibodies to these targets.

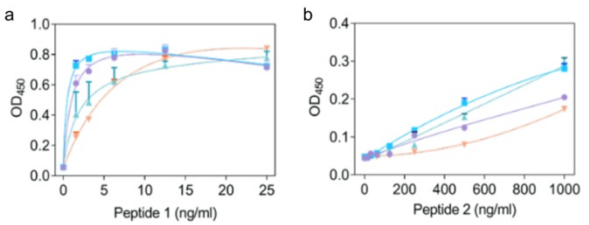


Figure 2. Indirect ELISA results for equal antibody concentrations from rabbits immunized with *Mtb* CFP-10 (a) peptide 1 or (b) peptide 2.

The detection of CFP-10 in serum can diagnose all TB manifestations[9], but some NTM express CFP-10 orthologs[10], including two of the six NTM responsible for >80% of human mycobacterial respiratory disease[11]. Sequence analysis of *Mtb* CFP-10 identified seven tryptic peptides containing ≥ 7 amino acids (**Fig. 1a**) that could serve as enrichment targets and that exhibited variable degrees of sequence identity among CFP-10 orthologs of the two NTM responsible for most respiratory disease (**Fig. 1b**). Five

peptides (peptides 1-3, 5-6) exhibited species specificity or selectivity, and produced similarly strong LC-MS signal intensity (**Fig. 1b-c**), but only peptides 1-2 exceeded the acceptance threshold of one or both antigenicity algorithms (**Fig. 1d-e**), and only peptide 1 (CFP10pep) was recognized by high affinity antibodies (**Fig. 2**).

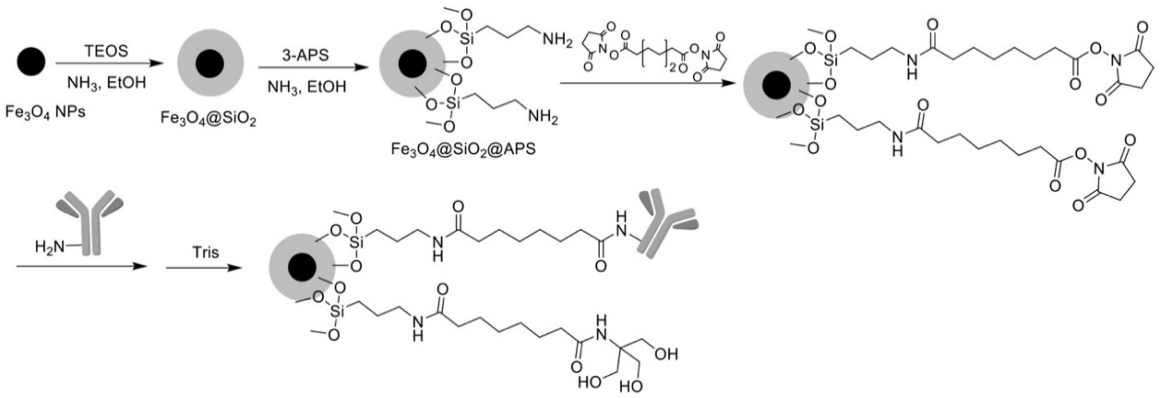
Progression for Aim 2.

Aim 2) Validate the performance of our optimized assay, using blood samples previously collected from well-characterized TB and non-TB patients, and determine if assay performance on a prototype mobile miniMS instrument is non-inferior to that observed on a standard bench-top MS instrument.

Antibody modified magnetic nanoparticles for peptide enrichment.

Peptide enrichment from trypsin-digested serum is critical to detect target peptide biomarkers (e.g. peptide 1593) analyzed by our assay. Our current protocols utilize custom nanoparticles for bench-top MALDI MS and protein A/G-modified magnetic Dynabeads for LC-MS/MS analysis studies. Both these nanoparticles are conjugated with an antibody specific for *Mtb* peptide 1593 to capture this CFP-10-derived peptide (m/z 1593), as well as a spiked-in heavy-isotope-labeled internal standard peptide of identical sequence (m/z 1603), from trypsin-digested serum samples. The optimization of our nanoparticle-MS TB assay requires the refinement and standardization of several components of the nanoparticle, including the sensitivity and specificity of this target antibody, the efficiency and stability of its binding to the nanoparticle, and multiple aspects of the nanoparticle (e.g., lot-to-lot structural and compositional variance, its stability under routine use and storage conditions, and its performance as a MALDI co-matrix). We have focused our effort on defining the best conditions for antibody antibody-mediated enrichment since these conditions are not greatly affected by nanoparticle matrix structure or composition, and have used LC-MS/MS analysis for this evaluation. There are several advantages to this approach. First, LC-MS/MS analyses tend to be more sensitive and specific than MALDI-TOF-MS, albeit at the cost of significantly lower throughput, allowing us to more precisely evaluate antigen enrichment and contamination than would be feasible with MALDI-TOF-MS. Due to this enhanced ability to evaluate sensitivity, LC-MS/MS is generally accepted as the gold-standard for such analyses, and thus this information will be useful for future commercialization efforts. Further, this information is valuable in its own right, since antibody-conjugated nanoparticles used in these analyses could also be directly used for alternate low-throughput LC-MS-based TB assays, since the LC-MS/MS systems required for this assay are present in many well-equipped clinical laboratories.

Covalent binding of the capture antibody to the nanoparticle matrix used for target peptide enrichment is important to avoid antibody losses during enrichment or wash steps and during peptide elution. The former event reduces nanoparticle binding capacity or peptide recovery, and thus peptide enrichment, while the latter results in contamination of the peptide fraction, which can reduce target peptide binding to the LC column and the lifetime of the LC column. Both events can reduce analytical sensitivity for the target peptide. Antibody losses during the wash and elution steps are of particular concern since stringent conditions may be required to efficiently remove high abundance proteins and peptides that may non-specifically interact with these nanoparticles or fully recover target peptide from high-affinity antibodies. We did not, however, achieve high binding efficiency when using commercial nanoparticle preparations and protocols designed to allow covalent antibody conjugation reactions. We therefore prepared (3-glycidyloxypropyl)trimethoxysilane (GLYMO) coated Fe_3O_4 nanoparticles as



Scheme 1. Synthetic route of antibody modified superparamagnetic nanoparticles

an antibody conjugation matrix, which efficiently formed covalent bonds with our target-specific via an epoxy-amine reaction, as shown in **Scheme 1**.

Superparamagnetic Fe_3O_4 nanoparticles used as the matrix in this approach were prepared by solvothermal reaction of $FeCl_3 \cdot 7H_2O$ and sodium acetate dissolved in ethylene glycol in the presence of polyethylene glycol. After washing with diluted HCl, the resulting Fe_3O_4 nanoparticles were coated with varying thickness of SiO_2 and 3-aminopropyltriethoxysilane (APS) generated by the reaction of tetraethyl orthosilicate (TEOS) with APS, to form Fe_3O_4@SiO_2@APS core shell nanoparticles. Disuccinimidyl suberate (DSS) was used as a linker molecule to conjugate peptide antibody to the Fe_3O_4@SiO_2@APS nanoparticles *via* its two terminal succinimidyl ester (NHS) functional groups, and excess free NHS groups remaining after antibody conjugation were quenched by the addition of Tris. These antibody-conjugated nanoparticle variants were then analyzed for their ability to enrich CFP-10 peptide 1593 and the peptide 1603 internal standard from trypsin digests of healthy serum spiked with recombinant CFP-10 and peptide 1603. In this analysis, the nano-HPLC-MS spectra of peptides 1593 and 1603 in enrichment samples generated with each nanoparticle were recorded by a Thermofisher TSQ Altis Triple Quadrupole Mass Spectrometer operating under selected reaction monitoring (SRM) and multiple reaction monitoring (MRM) modes to quantify the relative enrichment efficiency of each nanoparticle particle. Based on the mass spectra intensities measured for peptides 1593 and1603 relative to the nonspecific peptide background, we determined that a Fe_3O_4@SiO_2@APS nanoparticle generated using a 1:3:3 (mg/μL/μL) ratio of Fe_3O_4 to TEOS and APS exhibited the best enrichment efficiency among all the analyzed particle variants. In our early evaluation studies, the enrichment activity of this nanoparticle was found to be similar when employed immediately after synthesis and after 3 months storage at 4 °C to allow. The ability to store this nanoparticle for an extended period under standard laboratory conditions without loss of its enrichment activity is essential for any potential clinical applications. We therefore also intend to employ this antibody conjugation approach to the nanoparticles we are now generating as a MALDI matrix.

Accurate target peptide identification

Pathogen-derived biomarkers may circulate at low levels, particularly during early infection, and biomarker-derived peptide detection may be masked by MS fragment ion overlaps with co-eluting peptides contaminants in immunoprecipitation (IP) samples. While we are still evaluating the archived specimens provided from our Eswatini site, we evaluated archived retrospective samples from two completed studies in Houston (sponsored by **NIH/NIAID N01-AO-02738, PI: Edward A. Graviss**) and

Kenya (sponsored by **NIH/NICHD R01-HD023412 PI: Grace John Stewart**) that were provided by our collaborators on other projects.

Serum samples and clinical data utilized in the Houston study were drawn from a pediatric subset of the Houston Tuberculosis Initiative (HTI) cohort, where all children who were ≤ 18 years of age at HTI enrollment were eligible for inclusion and were excluded only for inconclusive diagnosis, insufficient serum volume, or substantial hemolytic or lipid contamination of their serum samples. Samples and clinical data analyzed in this study were drawn from 206 children with suspected TB cases enrolled between 1995 and 2002, of which 105 met the criteria for serum biomarker analysis. Children were identified as "confirmed TB" cases if they had a positive Mtb culture result or as "unconfirmed TB" cases if they lacked a positive culture result but met two or more of the following criteria: 1) they exhibited a clinical course consistent with TB, 2) they had close TB exposure or a positive tuberculin skin test (TST) result consistent with TB exposure (>5 mm induration), or 3) they demonstrated clinical improvement upon treatment with ≥2 anti-TB drugs. Children who did not meet the criteria for confirmed TB or unconfirmed TB were designated as "non-TB" cohort, since they were not analyzed by Mtb culture and could not be classified as "unlikely TB" cases using the updated 2015 NIH diagnostic criteria. This group contained 24 confirmed TB, 31 unconfirmed TB, and 50 non-TB cases. No differences in age, gender, or ethnicity were detected among the confirmed and unconfirmed TB groups, and these groups did not differ by TB-related criteria except for their history of close TB contact and prevalence of any form of extrapulmonary TB, which were both more common in the unconfirmed TB group. Xpert MTB/RIF data was not available for this cohort, which was enrolled and evaluated well before the development of this test, but the microbiologic reference standard detected only 43.6% of the identified TB cases. Notably, a recent meta-analysis indicates that Xpert has reduced diagnostic sensitivity for culture-positive sputum (62%) and gastric lavage (66%) samples from pediatric pulmonary TB cases than for culture-positive sputum (98%) from adult PTB cases, it appears unlikely that Xpert would outperform the diagnostic performance of culture in this cohort. Serum CFP-10 and/or ESAT-6 levels were detected for most TB cases (85.5%), however, but undetectable in all the non-TB cases, including 15 children with positive TST results who may have had latent TB infections. Further, serum CFP-10 and/or ESAT-6 signal was exhibited similar diagnostic sensitivity for confirmed TB (87·5%) and unconfirmed TB (83·9%) cases, indicating that children with paucibacillary Mtb culture specimens were not diagnosed with reduced efficiency despite the significant CFP-10 and ESAT-6 difference between these groups (**Fig. 3A**). Serum biomarker levels, however, did not differ between PTB and EPTB cases (**Fig. 3B**) or with age (**Fig. 3C**), implying that

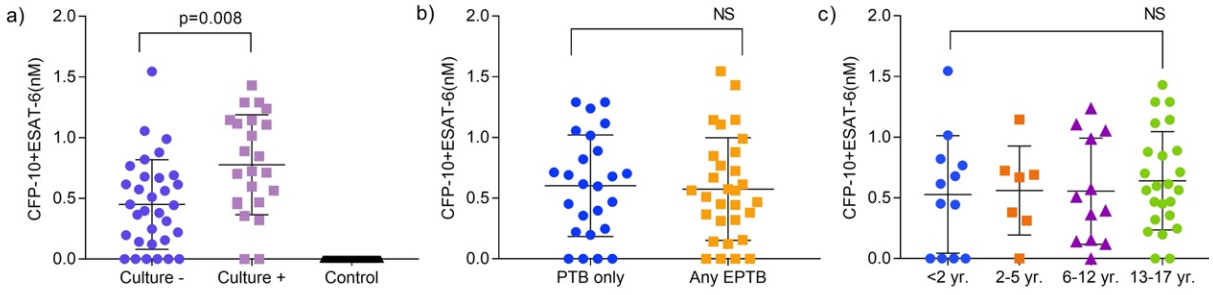


Figure 3. Combined serum CFP-10 and ESAT-6 concentration (CFP-10+ESAT-6) in: A) culture-positive (Confirmed) and culture-negative (Unconfirmed) TB cases and control (non-TB) subjects; B) PTB only and Any EPTB cases; and C) by age in all TB cases. Differences between different groups for were determined using the Kruskal Wallis test. NS = no significant difference.

biomarker levels were not affected by differences in infection site or potential age-associated changes in immune function.

We next evaluated the diagnostic sensitivity of this assay approach in a diagnostically challenging cohort of HIV-positive children aged <10 years from Kenya, who were classified as TB cases by microbiologic evidence from respiratory samples by GeneXpert MTB/RIF or mycobacterial culture (Confirmed TB), or via

	Confirmed TB					Unconfirmed TB						Unlikely TB						
Participants	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
CFP-10 at enrollment[a]	-	+	+	+	+	-	+	+	-	-	+	-	+	-	-	-	-	+	+
Severe immunosuppression[b]	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	-	+	+
TB contact past year	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TST[c]	-	-	+	-	-	/[d]	/	-	-	-	-	-	-	-	-	-	-	-	-
NIH TB symptoms[e]	-	-	+	+	+	+	+	+	+	+	+	-	+	-	-	+	-	-	-
CXR with TB features[f]	+	+	+	+	+	+	+	-	+	+	/	-	-	-	-	-	+	+	+
Respiratory GeneXpert[g]	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Respiratory *Mtb* Culture[g]	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Stool GeneXpert	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Urinary LAM	-	/	-	-	/	-	/	-	-	-	-	-	-	+	-	-	-	-	-
Anti-TB treatment response	-	+	+	+	+	+	+	+	/	/	/	+	/	/	/	/	/	/	/

Figure 4. Combined serum CFP-10 and ESAT-6 concentration (CFP-10+ESAT-6) in: A) culture-positive (Confirmed) and culture-negative (Unconfirmed) TB cases and control (non-TB) subjects; B) PTB only and Any EPTB cases; and C) by age in all TB cases. Differences between different groups for were determined using the Kruskal Wallis test. NS = no significant difference.

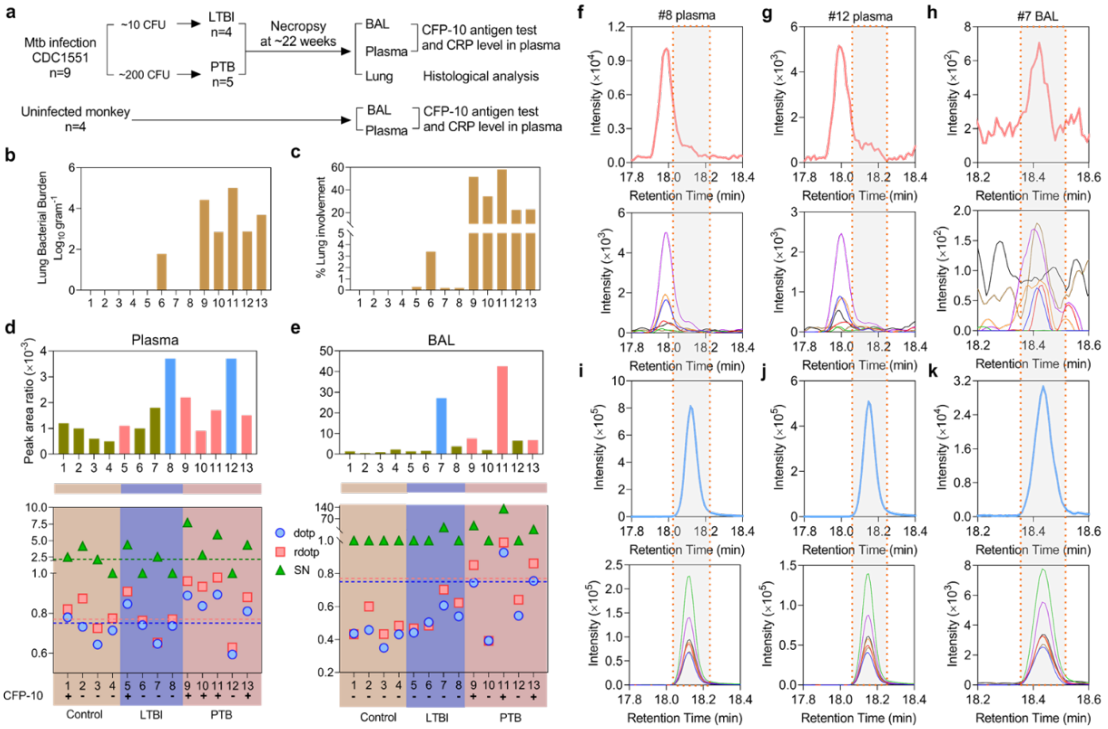


Figure 5. a, Study design employed to evaluate CFP10 plasma signal in a rhesus macaque NHP model of latent TB infection (LTBI) and pulmonary TB (PTB) and in *Mtb*-naïve animals. **b**, *Mtb* burden at necropsy in the lungs of NHPs that were *Mtb* naïve (control) or were diagnosed with LTBI or PTB. **c**, Evaluation of respiratory pathology as indicated by percentage of lung involvement calculated by H&E staining of random sections from each lung lobe. **d-e**, CFP10pep positive rates, as determined by detection by dotp, rdotp, and SNR values, with corresponding peak area ratios (CFP10pep/IS) for (d) serum and (e) bronchoalveolar lavage (BAL) samples collected at necropsy. Peaks were considered negative for CFP100pep (dark green) if any of their corresponding dotp (blue), rdotp (red) or SNR (green) values were below the indicated thresholds, including peaks with high peak area ratios (blue), or positive (red) if all parameter met their thresholds. **f-k**, EICs of the (f-h) CFP10pep and (i-k) IS precursor and their seven fragments (lower) are shown separately for three samples indicated by blue peak in (d) and (e). The gray shadowed box with red dotted frames indicates the RT window of the CFP10pep and IS peptides.

a clinical algorithm based on guidance from an NIH expert panel (Unconfirmed TB), which required each child to meet at least two of the following criteria: have TB-associated symptoms; or a chest radiograph consistent with TB; TB exposure or immunologic evidence of infection (a positive tuberculin skin test or interferon gamma-release assay), or a positive TB treatment response. [14]. Children who did not meet these criteria were classified as Unlikely TB cases. CFP10 signal was detectable in all but one Confirmed TB case, who displayed no TB symptoms (**Fig. 4**; 80% sensitivity), and in 2/5 and 4/9 children classified as Unconfirmed and Unlikely TB cases. However, this changed to 3/7 for both groups as two children were reclassified from unlikely to unconfirmed TB cases based on review of their clinical data. All the children with CFP10-positive serum had severe immunosuppression, with or without TB symptoms or chest radiograph data suggestive of TB. *Mtb* Culture and Xpert data had variable sensitivity in the confirmed TB cases, and the detection of the *Mtb* membrane glycolipid lipoarabinomannan (LAM) in urine samples was not sensitive or specific for confirmed or unconfirmed TB cases. CFP10pep also demonstrated good performance in distinguishing active TB from absent or latent TB in a non-human primate (NHP) model of aerosol-mediated *Mtb* exposure (**Fig. 5**).

Progression for Aim 3.

Aim 3) Determine the clinical sensitivity and specificity of the final assay on a MiniMS-12 or MALDI-TOF MS using >3,000 blood samples prospectively collected from serial patients recruited from intended use settings in high (Eswatini), medium (Tanzania) and low (US) burden TB screening clinics.

- The IRB for the prospective sample collection in Eswatini has been approved and was submitted to DOD on Sept. 4, 2019 (**Eswatini**).
- The IRBs for prospective sample collection in Tanzania (**Baylor and Tanzania**) were submitted and approved by both the National Institute for Research (NIR) and the Mbeya Medical Research and Ethics Committee (MMREC), and submitted to DOD on February 17, 2020.
- Non-DOD sources of funding were used to collect and store plasma in the existing Eswatini cohort biorepository. These plasma samples were collected in ACD (acid citrate dextrose) tubes, processed within 4 hours of collection, frozen at -80C freezer, and then rapidly shipped on ice with no more than 2 freeze-thaws occurring before Nanoparticle-MS analysis that were conducted as part of Aim 1. Based on results from Aim 1 analysis on 200 samples, although we are still evaluating wether the specimens stored in ACD tubes are adequate for our prototype blood based assay, we have determined that the prospectively collected specimens will be collected into standard red top tubes and shipped on dry ice once HRPO approval is received.
- Database development for the project was completed over the last year to ensure uniform data collection and storage via RedCap (Research Electronic Data Capture) in both Tanzania and Eswatini.
- Specimen storage capacity has been optimized at all sites through the introduction of freezer works, including related training.
- Training on data collection and prospective enrollment took place over several months in Tanzania and Eswatini.
- All three sites (**Eswatini**, **Baylor and Tanzania**) stopped non-essential research in March 2020 due to the global COVID-19 pandemic and institutional requirements. However, by September 2020, clinical research was allowed to begin at both **Eswatini** and **Tanzania** following national guidelines for implementing clinical research and COVID-19 precautions. All sites are prepared to initiate DOD supported recruitment upon receipt of HRPO approval.

- All team members have completed Good Clinical Practice or Good Laboratory Practice courses conducted *via* CITI training available through Baylor College of Medicine.

Profession for Aim 4.

4.1 Regulatory Feedback

NanoPin believes that early and regular communication with the FDA will support an efficient and effective pathway to regulatory approved medical devices for infectious disease diagnostics. NanoPin has received encouraging feedback directly relevant to the work and goals in this project from the FDA in response to our submission to the FDA's Q-Submission Program, which provides a mechanism for feedback from and meetings with the FDA for medical device submissions. In this response, the FDA has directed our TB diagnostic to the De Novo pathway as a Class II medical device. This means that it will require a 510k submission, which is significantly more straightforward and cost-effective than the Class III premarket approval (PMA) process that would be required for a standard submission.

The FDA highlighted the importance of the TB assay technology and capabilities with specific emphasis on the unique ability to accurately detect extrapulmonary TB and the ability to better address the pediatric population. NanoPin emphasized this feedback to apply for the Breakthrough Devices Program. The FDA agreed that our medical device and its proposed indication for use met the criteria for this program, and designated a Breakthrough Device classification on May 21, 2020. This designation validates the importance of the technology in this project for the field and enables NanoPin to pursue the most efficient pathway to regulatory approval.

4.2 Work Accomplished

In the first year of the three-year project, NanoPin concentrated on Aim 4.1: scaling up the nanoparticle platform production and establishing a robust supply chain for clinical trials and commercialization. Aim 4.2 for monoclonal antibody analytical validation testing will be the focus for Year 2 and Aim 4.3 will be the focus for Year 3.

Aim 4.1 work resulted in several improvements to the original research protocol to develop a scaled-up and high yield cGMP and FDA-compliant assay.

4.2.1 Reagent selection (trypsin): Complete serum digestion by trypsin in the initial research protocol can require >16 hours when employing sequencing grade trypsin. Screening of commercial trypsin reagents with various purity, activity and costs identified an agarose-immobilized trypsin as the best candidate for more rapid digestion, since it: (i) is treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) in order to inhibit chymotrypsin activity but not trypsin activity; (ii) allows efficient removal of trypsin activity by separating the trypsin gel from the digestion solution; (iii) is compatible with automated liquid handling systems to permit process automation; and (iv) is highly cost-effective (<$2/sample vs. $20/sample for sequencing grade trypsin). Comparison of the MS spectra obtained with immobilized and sequencing grade trypsin (**Fig. 7**) revealed that immobilized TPCK trypsin achieved efficient digestion within 3 hours at room temperature, with low non-specific background. Side-by-side comparison of the performance of immobilized and sequencing grade trypsin to digest human plasma spiked with known amounts of recombinant CFP-10 found that both performed similarly during overnight digestions. However, the signal-to-noise ratio and target peak area obtained after 3hrs digestion with sequencing grade trypsin decreased 10× and 60× versus those

obtained after overnight digestion, while the values obtained with immobilized trypsin after 3 hours and 1 6hours digestion did not markedly differ.

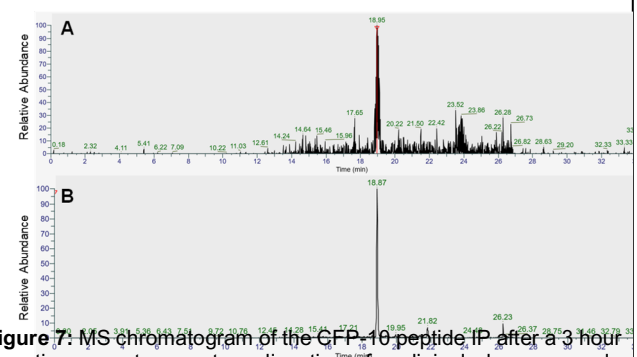


Figure 7: MS chromatogram of the CFP-10 peptide IP after a 3 hour digestion room-temperature digestion of a clinical plasma sample with **A**) sequencing grade and **B**) immobilized TCPK-treated trypsin.

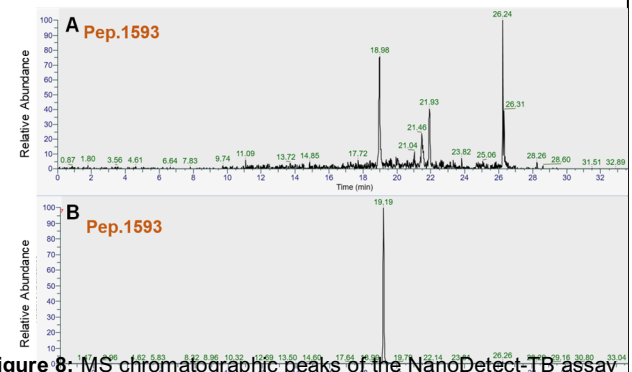


Figure 8: MS chromatographic peaks of the NanoDetect-TB assay with CFP-10-spiked human serum **A**) before and **B**) after protocol optimization.

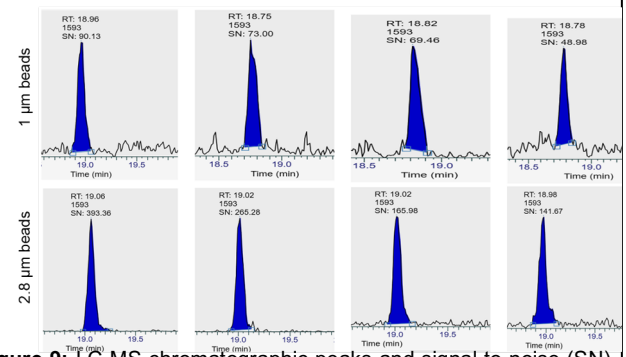


Figure 9: LC-MS chromatographic peaks and signal-to-noise (SN) ratios of plasma spiked with the indicated concentration of the target CFP-10 peptide, IP-enriched with two different magnetic bead sizes, and analyzed using an MS-optimized ICIS Peak detection algorithm.

4.2.2 Reagent selection (magnetic nanoparticles): A second analysis was performed to evaluate the relative performance of protein G-coupled magnetic beads available from different companies as platforms to immunoprecipitate (IP) antibody-bound target peptides from CFP-10 spiked and digested human serum/plasma. No marked differences were observed in peak retention time, peak area, signal-to-noise ratio, or background when protein G-coupled magnetic beads (1µm and 2.8 µm) from three different vendors were used to capture target peptide from human plasma spiked with a 50 pM final concentration of recombinant CFP-10. Non-specific signal reduction: Several modifications were made to the initial research laboratory protocol to reduce non-specific MS spectra peaks in CFP-10-spiked plasma/serum (**Fig. 8A**). A protocol revision to omit a wash step with 10% trifluoroacetic acid, adjust the assay binding and wash buffers, and pre-concentrate the captured IP eluate on C8 or C18 spin tips prior to the LC-MS analysis markedly reduced the relative intensity of non-specific peaks in the MS chromatographs of these samples (**Fig. 8B**). Selection of MS analysis approach: Nanoparticle-MS utilizes LC-MS for target detection, whereas the research laboratory assay utilized matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) MS for peak detection. MALDI-TOF MS offers rapid through-put at the expense of sensitivity and specificity that can be obtained with most LC-MS systems now in use in many clinical laboratories for other diagnostic assays. NanoPin's decision to proceed with LC-MS instead of MALDI-TOF for the final NanoDetect-TB assay was based on the advanced regulatory status of LC-MS instruments, their ability to accurately and reproducibly identify and quantify low abundant peptides in complex biological matrixes, and their successful use in other assays on the market. NanoPin has employed a ThermoFisher Altis TSQ system for most of our initial efforts to integrate, validate and optimize the utilization of LC-MS in as the readout for the

NanoDetect-TB assay, demonstrating that this system was able to consistently detect our target peptide in human serum/plasma spiked with low concentrations the target peptide and its matching heavy-isotope-labeled internal standard peptide.

4.2.3 Limit of detection (LoD): To establish the LoD of this assay approach, plasma samples spiked with serial dilutions of CFP-10 were trypsin digested and IP-enriched using different size protein G-coupled nanoparticles (1µm and 2.8 µm) obtained from different vendors after pre-incubation with our target-specific mAb. This analysis revealed that assays performed with magnetic beads of both sizes both consistently produced high signal-to-noise peaks corresponding to our target peptide (m/z 1593) when analyzing 100 µL healthy plasma samples spiked with low- to sub-pM concentrations of this peptide (**Fig. 9**). We selected the ICIS algorithm for peak detection due to its robust ability to detect peaks at the sub-pM range, which should be more than sufficient to detect active TB cases. LC-MS instrument selection: NanoPin has also analyzed several FDA-compliant LC-MS hardware and software systems as candidates for a phase II studies. This analysis focused on triple quadrupole-based MS instruments that allow ultrasensitive peptide detection and high-throughput LC-MS sample analyses. Candidate systems analyzed include the Thermo-Fisher Altis MD coupled with Vanquish MD LC system, the Waters Xevo TQXS ESI coupled with an UPLC I-Class LC system, and the AB SCIEX 6500+ Series coupled with a M5 microflow UPLC system. Through collaboration agreements with these three leading LC-MS manufacturers, we have conducted preliminary studies to evaluate the ability of these systems to detect our target peptide (e.g., sensitivity, dynamic range, reproducibility, and robustness), and their suitability as a readout system for clinical and/or analytical validation studies of the NanoDetect-TB diagnostic assay kit. These studies detected roughly similar pre-optimization LoDs for the ThermoFisher, Waters, and AB SCIEX systems (0.2, 0.5, and 1 nM), with fast elution times (1.6, 5.5, and 4.25 min) that should permit high-sample through-put. Results for the Thermofisher system (**Fig. 10**) are shown as an example of the level of initial performance achieved with this system.

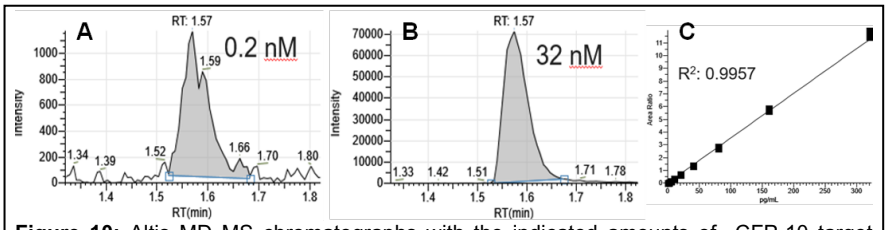


Figure 10: Altis MD MS chromatographs with the indicated amounts of CFP-10 target peptide (**A-B**) and (**C**) the reproducibility of the target to internal standard ratio obtained over a wide dynamic range of input peptide (0.16 – 320 pg/mL; 0.1 nM – 200 nM).

- **What opportunities for training and professional development has the project provided?**

  Nothing to Report.

- **How were the results disseminated to communities of interest?**

  Nothing to Report.

- **What do you plan to do during the next reporting period to accomplish the goals?**

In the Year 2, the research lab at Tulane aims to complete the development of a multi-factor blood-based TB assay and diagnostic algorithm that meets or exceeds the minimum WHO

performance criteria for TB screening and diagnosis. After 7 months delay due to the COVID-19 pandemic, the antibody production company has confirmed the antibody fabrication procedure would be completed by April. 2021 and the product would be delivered to us without delay. We will continue to apply the protocol of peptide selection criteria for the *Mtb*-specific CFP-10 peptide to other *Mtb*-derived factors identified by our research group (e.g., Ag85B, MPT53 and CFP-2). We anticipate that inclusion of one or more peptides from these factors, or other abundantly secreted *Mtb* factors, will yield an assay with >90% diagnostic sensitivity without negatively affecting diagnostic specificity.

We will determine the theoretical maximum sensitivity and specificity of our nanoparticle-MS assay under ideal laboratory conditions (both MALDI-TOF MS and LC-MS/MS) with blood samples from well-characterized retrospective samples from the Baylor Swaziland cohort biorepository, with the goal of achieving 90% sensitivity and specificity.

We will start evaluating the analytical parameters of the integrated nanopartiple-miniMS assay, including linearity, precision, LOD and LLMI, accuracy, and matrix effects with the goal of achieving similar performance to that established for the current assays.

During reporting year 2, the clinical team at Baylor College of Medicine plans to commence enrollment at all three sites, depending on pandemic conditions at each site. Enrollment began in September and October 2020 at both the Mbabane and Mbeya sites. Pending hospital approval, which currently depends on the nature of the COVID pandemic, approval should rapidly start in Houston. Pending the nature of the pandemic, annual enrollment from these three sites should be similar to that as previously described.

In Year 2, NanoPin will focus on Aim 4.2, which will identify monoclonal antibodies for assay targets and perform thorough a thorough analytical validation of these antibodies. These validated monoclonal antibodies will replace the previous polyclonal antibodies, which were could not be employed in a commercial product as they lack the capacity to allow reproducible and scalable production of a defined material. The work in this term will analyze the performance of a panel of monoclonal antibodies with specificity for assay targets, utilizing a thorough analytical validation study approach to select the candidates with the best affinity and specificity for the chosen assay targets. The antibodies will be tested for linearity, precision, limit of detection (LoD) and limit of blank (LoB), accuracy, and matrix effects.

4. **IMPACT:**
    - **What was the impact on the development of the principal discipline(s) of the project?**

      Nothing to report.

    - **What was the impact on other disciplines?**

      Nothing to report.

    - **What was the impact on technology transfer?**

Nothing to report.

- **What was the impact on society beyond science and technology?**

The technology developed in this proposal is a necessary advancement in infectious disease diagnostics. Improved methods for tuberculosis (TB) detection in all patient groups will reduce the global burden of TB on countries and economies throughout the world. TB kills over 1.5 million persons with approximately 10 million new active TB cases annually.

5. **CHANGES/PROBLEMS:**
   - **Changes in approach and reasons for change**

     Nothing to report.

   - **Actual or anticipated problems or delays and actions or plans to resolve them**

     For our Tanzania site, the only "change" was delay of study launch due to COVID-19 and initial clinical research halting/restrictions in early/mid 2020. Dr. Jason Bacha (Site PI) has been working closely with the local organizations to resume project planning and site preparation. The PI has also explored the capacity of other nearby sites to provide additional study entry points. These pro-active explorations were completed to ensure that DOD recruitment targets will be met in light of declining TB case notification well-recognized globally due to COVID-19.

     Based on our preliminary results, we have determined that specimens stored in ACD tubes are inadequate for our prototype blood based assay. Hence, prospectively collected specimens will be collected into standard red top tubes once HRPO approval is received.

     The Tulane lab has experienced delayed delivery of some key reagents and supplies since April, 2020, including the monoclonal peptide antibodies we specially designed for the awarded project. Now that restrictions affecting product fabrication and shipment have been lifted we have received, and will continue to receive, orders placed before the COVID-19 pandemic delayed their production. We are working on completing all sub-aims proposed in Aim 1 and 2 by September 2021.

   - **Changes that had a significant impact on expenditures**

     Nothing to Report

   - **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

     Nothing to Report

   - **Significant changes in use or care of human subjects**

     Nothing to Report

- **Significant changes in use or care of vertebrate animals.**

  Nothing to Report

- **Significant changes in use of biohazards and/or select agents**

  Nothing to Report

6. **PRODUCTS:**
   - **Publications, conference papers, and presentations**
     - **Journal publications.**

Shu, Q., Rajagopal, M., Fan, J., Zhan, L., Kong, X., He, Y. Rotcheewaphan, S., Lyon, C. J., Sha, W., Zelazny, A.M., **Hu, T.** IP-MS Analysis of ESX-5 and ESX-1 Substrates Enables Mycobacterial Species Identification. *bioRxiv.* **2020**, published.
Acknowledgement of federal support? Yes

Mao, L., Lacourse, S., Kim, S., Ning, B., Bao, D., Fan, J., Sun, Z., Nackman, S., Mitchelle, C., **Hu, T.** Evaluation of a blood-based antigen test for tuberculosis in HIV-exposed children younger than 5 years. *BMC Medicine*. **2020**, Revision submitted.
Acknowledgement of federal support? Yes

He, Y., Lyon, C. J., Nguyen D. T., Liu, C., Sha, W. Graviss, E., **Hu, T.** Serum-based diagnosis of pediatric tuberculosis by assay of *Mycobacterium tuberculosis* factors: a retrospective cohort study. *J. Clin. Micro*. **2020**, Univer revision.
Acknowledgement of federal support? Yes

     - **Books or other non-periodical, one-time publications.**

       None.

     - **Other publications, conference papers, and presentations.**

a. Evaluation of a blood-based antigen test for tuberculosis in HIV-exposed children younger than 5 years. *The 50th Union Conference -TB Science 2020 Virtual conference*. Oct. 20-24, **2020**.
b. Blood-based antigen test for rapid TB diagnosis. *CTVD 6th Annual Virtual Meeting*. June 16-17, **2020**.
c. Mycobacterium antigen peptidome enables a rapid differentiation of species and sub-strains. *ASMS Virtual conference*. June 1, **2020**.
d. Rapid differentiation of Mycobacterium using antigen peptidome. *2019 NTM Research Consortium*. Nov. 14-16, **2019**. Portland, OR.

e. Blood-based antigen test for rapid TB diagnosis. *The 50th Union World Conference*. Oct. 27-Nov. 1, **2019**. Hyderabad, India.

- **Website(s) or other Internet site(s)**

  None.

- **Technologies or techniques**

Several advantages were realized in the Aim 4.1 efforts.
1. The trypsin-based enzyme digestion method was translated to a commercially viable process in terms of both cost and time. The previous method was clinically non-viable due to cost (>$20 per test for the trypsin component itself) and time (overnight digestion required). The new method utilizes an agarose resin, TPCK treated immobilized trypsin that enables 1-3 hour digestion times with a >10x cost reduction (<$2 per test for the new trypsin component).
2. Commercial protocols for the entire assay procedure from sample preparation to LC-MS analysis were developed. A validated bill of materials (BOM) for the tuberculosis assay was confirmed and supply chain with commercial terms & conditions was established.
3. These advances were integrated in the NanoPin quality management system (QMS). The QMS and NanoPin's Quality Control incorporated these advances from the determination of user needs to design inputs and outputs, reagent validation and testing, supply chain management, and product labeling.

These advances were shared with the members of this grant and especially with the research group of Professor Tony Hu at Tulane University School of Medicine. Professor Hu's research group directly benefits from the validation of the assay reagents, extreme cost savings for the trypsin digestion step, and the time savings from the refined commercial protocols. The LC-MS maintenance, QC protocols, and information on other application changes has been shared with the entire team.

- **Inventions, patent applications, and/or licenses**

  Nothing to report.

- **Other Products**
  - *data or databases;*

1. The dataset "mycobacterial culture filtrate proteins identified by LC-MS/MS" has been successfully submitted to ProteomeXchange via the PRIDE database.
   Project Name: mycobacterial culture filtrate proteins identified by LC-MS/MS
   Project accession: PXD019069
   Project DOI: 10.6019/PXD019069

2. The dataset "Classification of Mycobacterium down to subspecies and strain level by Proteomic Analysis of Clinical MGIT Sample" has been successfully submitted to ProteomeXchange via the PRIDE database.
   Project Name: Classification of Mycobacterium down to subspecies and strain level by Proteomic Analysis of Clinical MGIT Sample
   Project accession: PXD022102
   Project DOI: 10.6019/PXD022102

ii. biospecimen collections;

As described in Aim 3, a prospective pediatric cohort has been under development specially for the award.

iii. audio or video products;

None

iv. software;

None

v. models;

None

- *educational aids or curricula;*

  None

- *instruments or equipment;*

  None

- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*

  None.

- *clinical interventions;*

  None.

- *new business creation; and*
- *other.*

  None.

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

- **What individuals have worked on the project**

Name:	Tony Y. Hu
Project Role	PI
Researcher Identifier (e.g. ORCID ID):	0000-0001-7255-5409
Nearest person month worked:	3
Contribution to Project:	Dr. Hu has been providing scientific direction, overall project management and coordination, and financial management for the team. He has been also serving as the information conduit between the DOD and the scientific team, while ensuring that the shared resources and infrastructure at all participating institutions are leveraged to their fullest. Dr. Hu evaluates program activities such as discussions of project milestones and data presentation and dissemination.
Funding Support:	W8IXWH1910926, R01AI113725, R01HD090927

Name:	Christopher Lyon
Project Role:	Tulane Site co-investigator
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	6
Contribution to Project:	Dr. Lyon has been participating the analysis of clinical and preclinical biomarkers of multiple different human disease conditions, which he will employ to supervise the scientific rigor of all aspects of assay design, authentication, methodology and reproducibility.
Funding Support:	W8IXWH1910926, R21EB026347

Name:	Qingbo Shu,
Project Role:	Tulane site Postdoctoral Associate

Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	12
Contribution to Project:	Dr. Shu has been focusing on developing mass spectrometry based detection method for *Mtb*-specific antigens in mycobacterium infected patient blood samples.
Funding Support:	W8IXWH1910926

Name:	Pouya Amrollani
Project Role:	Tulane site graduate student
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	12
Contribution to Project:	Pouya Amrollani has been assisting Dr. Hu to process sample and nanopore operation. He focuses on the metallization of nanoparticles and surface modification for isolating, detecting and validating TB biomarker signatures from patient blood samples.
Funding Support:	W8IXWH1910926

Name:	Bo Ning
Project Role:	Tulane site faculty
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	6
Contribution to Project:	Dr. Ning has been working on sample processing, data collection and analysis under the supervision of Both Dr. Hu and Dr. Lyon.
Funding Support:	W8IXWH1910926

Name:	Andrew DiNardo
Project Role:	Houston site co-investigator
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	3
Contribution to Project:	Dr. DiNardo has overseen work for the project as per the previously described scope of work. He has coordinated previously collected specimen contributing to the existing biorepository and coordinated with Drs Hu and Tombler the initial evaluation of the study assay as well as clinical metadata for the study. He has prepared for prospective enrollment in Houston with the Houston study coordinator, Ms Spieler.
Funding Support:	W8IXWH1910926

Name:	Zoe Spieler
Project Role:	Houston Study coordinator
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	6
Contribution to Project:	Ms. Spieler has organized the regulatory approval documents for Harris Health approval and begun the coordination for enrollment at Harris Health. She has organized RedCap database for clinical epidemiologic data capture.
Funding Support:	W8IXWH1910926

Name:	Jason Bacha
Project Role:	Mbeya, Tanzania site co--investigator
Researcher Identifier (e.g. ORCID ID):	ORCID ID: 0000-0002-1093-5227

Nearest person month worked:	2
Contribution to Project:	Dr. Bacha has acted as the study site PI for Mbeya, Tanzania. He has assisted on pre-study preparation for Tanzania, orientation/training of Tanzanian team, recruitment strategies for Tanzania, procurement of study supplies for Tanzania, obtaining IRBs and ethical clearances for the study in Tanzania, regularly study team meetings participation, and assistance with preparation for data/specimen collection and data entry for Tanzania study participants which will commence upon receipt of HRPO approval.
Funding Support:	W8IXWH1910926

Name:	Lwijisyo Minga
Project Role:	Tanzania study nurse
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	9
Contribution to Project:	Mrs. Minga is a TB study nurse dedicated to implementing the TB study. She has been involved in all aspects of study preparation, and pre-study orientation/training. She has been trained to be responsible for participant enrolment, sample collection, participant data collection/entry, participant follow-up and communications, and assisting with all clinical implementation of the study in Mbeya, Tanzania.
Funding Support:	W8IXWH1910926

Name:	Alexander Kay, MD
Project Role:	Site co-investigator
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1
Contribution to Project:	Dr. Kay has acted as the study site PI for Eswatini. He has assisted on pre-study preparation for Eswatini, orientation/training of Swati team, recruitment strategies for Eswatini, procurement of study supplies for Eswatini, obtaining IRBs and

	ethical clearances for the study in Eswatini, regularly study team meetings participation, and assistance with preparation for data/specimen collection and data entry for Eswatini study participants which will commence upon receipt of HRPO approval.
Funding Support:	W8IXWH1910926

Name:	Jose Euberto Mendez Reyes
Project Role:	Biostatistician
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1
Contribution to Project:	Supported database and analysis plan development
Funding Support:	W8IXWH1910926

Name:	Anna Mandalakas
Project Role:	Senior TB Expert
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1
Contribution to Project:	Dr. Mandalakas has supervised and supported the development of all aspects of study implementation including the development of data collection tools within red cap, standard operating procedures, and training among other activities. She has led weekly Baylor team meeting to ensure seamless communication across all study sites and smooth integration of DOD study activities within existing infrastructure. She has also led communications with stake holders to ensure robust support for planned study activities.
Funding Support:	W8IXWH1910926

Name:	Godwin Mtetwa
Project Role:	Research Nurse
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	4.2

Contribution to Project:	Coordinates the clinical research team in Eswatini and has led preparation for initiation of clinical cohort recruitment across 4 project sites.
Funding Support:	W8IXWH1910926

Name:	Nomthandazo Dlamini
Project Role:	Research Assistant
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	6
Contribution to Project:	Ms. Dlamini has supported site preparation for participant recruitment at one of three Eswatini sites
Funding Support:	W8IXWH1910926

Name:	Qiniso Dlamini
Project Role:	Laboratory Technician
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	4.2
Contribution to Project:	Mr. Dlamini has supported development of lab tools and introduction of freezer works to ensure complete specimens capture in accordance with study SOPs once prospective recruitment commences
Funding Support:	W8IXWH1910926

Name:	Jaquiline Kahari
Project Role:	Laboratory Technician
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	4.2
Contribution to Project:	Ms. Kahari has supported development of lab tools and introduction of freezer works to ensure complete specimens capture in accordance with study SOPs once prospective recruitment commences

Funding Support:	W8IXWH1910926

Name:	Nomathemba Dlamini
Project Role:	Study coordinator
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	3
Contribution to Project:	Ms. Dlamini coordinates procurement and human resources for clinical studies
Funding Support:	W8IXWH1910926

Name:	Nkulungwane Mthethwa
Project Role:	Data Manager
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	3
Contribution to Project:	Has participated in the develop of data capture tools and RedCap for the project
Funding Support:	W8IXWH1910926

Name:	Faith Dlamini
Project Role:	Screening Officer
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	6
Contribution to Project:	Ms. Dlamini has supported site preparation for participant recruitment at one of three Eswatini sites
Funding Support:	W8IXWH1910926

Name:	Sibonisiwe Lokotfwakohas
Project Role:	Screening Officer
Researcher Identifier (e.g. ORCID ID):	N/A

Nearest person month worked:	6
Contribution to Project:	Ms. Lokotfwakohas has supported site preparation for participant recruitment at one of three Eswatini sites
Funding Support:	W8IXWH1910926
Name:	Mduduzi Mbatha
Project Role:	Driver
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	6
Contribution to Project:	Specimen transport
Funding Support:	W8IXWH1910926

Name:	Thomas Tombler
Project Role:	Nanopin site co-investigator
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	4
Contribution to Project:	Dr. Tombler has overseen the project for the sub-award scope of work. He coordinated all the personnel involved in the project and ensured all objectives are being met. He has been responsible for supervision, document management, procurement, and development of supply contracts and licensing requirements for key components.
Funding Support:	W8IXWH1910926

Name:	Wael Abdelgaliel, Ph.D.
Project Role:	Site Co-Investigator
Researcher Identifier (e.g. ORCID ID):	N/A

Nearest person month worked:	6
Contribution to Project:	Dr. Abdelgaliel ran the experiments for the particle standardization, trypsin digestion reagent validation, and limit of detection testing. He supported QMS integration and implementation. He has been responsible for leading sample preparation, nanoparticle testing, and running the mass spectrometry equipment.
Funding Support:	W8IXWH1910926

Name:	Rahul Rao, P.E.
Project Role:	Medical Device Engineer
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	2
Contribution to Project:	Mr. Rao has provided engineering support in setting up the initial quality system which meets requirements of ISO 13485 and 21 CFR 820. He assisted in the design control framework and review process of user needs and design inputs initially and supported the full development, review, and verification of the Quality Management System (QMS).
Funding Support:	

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

  Nothing to Report.

- **What other organizations were involved as partners?**

  Nothing to Report.

8. **SPECIAL REPORTING REQUIREMENTS**
   - **COLLABORATIVE AWARDS:** Not applicable.
   - **QUAD CHARTS:** Attached.
9. **APPENDICES:**

Appendix I. FDA letter granting the Nanodetect-TB (the product name of our assay) as a “breakthrough technology”.

Appendix II. The copy of the manuscript entitled “Evaluation of a blood-based antigen test for tuberculosis in HIV-exposed children younger than 5 years”.

Appendix III. The copy of the manuscript entitled “Serum-based diagnosis of pediatric tuberculosis by assay of Mycobacterium tuberculosis factors: a retrospective cohort study”.

Appendix IV. The copy of the manuscript entitled “IP-MS Analysis of ESX-5 and ESX-1 Substrates Enables Mycobacterial Species Identification”.