Background and Project Overview

The high prevalence of tuberculosis (TB) in Pakistan is a significant burden to the healthcare system and national economy. The main goal of this HEC/Pak US S&T project is to build healthcare capacity in Pakistan by developing new multiplex diagnostic methods for detecting TB. This multiplex immunoassay uses antigens found in *Mycobacterium tuberculosis* (*M. tb.*). In a collaboration with scientists in Pakistan and the US, this project combines recombinant DNA technology and the multiplex immunoassay technology to achieve four objectives as described below.

Importantly, to establish the collaboration between the Khanum and Luciw laboratories, Dr. Imran Khan (PhD, Research Associate at U.C. Davis) had traveled to Islamabad and Rawalpindi. Dr. Khan, originally from Lahore, received his PhD (Albert Einstein School of Medicine, NY) and post-doctoral training (U.C. Davis) in the U.S. Currently, he holds a research faculty position in the Department of Pathology at U.C. Davis and is a member of Dr. Luciw’s laboratory. Dr. Khan initiated the research collaboration with Dr. Khanum’s laboratory in 2005 with a focus on the use of recombinant *M. tb.* antigens for the development of novel multiplex immunoassays built on the multiplex micrbead system from Luminex (Austin, Tx). Several bacterial expression plasmids with cloned *M. tb.* genes were obtained from the NIH TB Resource Center at Colorado State University (Fort Collins, CO). These plasmids included those with cloned *M. tb.* specific genes ESAT-6, CFP-10, and HspX. In the last three years Dr. Khan has made four trips to Dr. Khanum’s laboratory at UAAR, each trip lasting about 2-4 weeks. During these visits he has trained graduate and undergraduate students in her laboratory on state-of-the-art molecular techniques. The Khanum laboratory is currently set up to perform a variety of nucleic acid and protein analyses. Three graduate students in her laboratory are a part of the team working on the expression and purification of recombinant *M. tb.* proteins expressed by these plasmids; these proteins are being used in the HEC/Pak-US S&T project on multiplex detection of TB. Importantly, two of Dr. Khanum’s graduate students, Ms. Irum Nawaz and Mr. Mirza Shahzad, have joined my lab at UC Davis for training on multiplex serodiagnosics for TB (March 2008 to March 2009).

Dr. Imran Khan attended the HEC/Pak-US S&T conference (August 24 – 28, 2008) in Islamabad. He delivered a presentation on the project entitled “Multiplex Immunoassays for the Detection of Tuberculosis”.

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**U.S. Principal Investigator - Paul A. Luciw**, PhD, Professor, University of California, Davis, CA  
**Pakistan Principal Investigator - Azra Khanum**, PhD, Professor and Dean, University of Arid Agriculture (UAAR), Rawalpindi; Clinical collaborator – **Dr. Sabira Tasheen**, National TB Control Program, Islamabad.
A key part of this project is collection of blood samples from TB patients at the National TB Center in (NTBC) in Islamabad. During these previous visits to Islamabad/Rawalpindi, Dr. Khan, in collaboration with Dr. Khanum, established a working relationship with Dr. Sabira Tahseen at the National TB Control Program of Pakistan, Islamabad. Under Dr. Tahseen's guidance, a blood collection protocol and experimental design was established in 2006. Two graduate students in Dr. Khanum’s laboratory, Ms. Irum Nawaz and Mr. Kumail Rizvi, have been duly trained and are actively involved in the collection of blood samples at the NTBC. To date, blood samples from approximately 290 TB patients have been collected. Plasma and dried blood spots are prepared and appropriately stored at UAAR. Half of the patient plasma samples have been received at UC Davis in Nov. 2008. These samples are currently in the process of being analyzed by multiplex immunoassay for antibodies against M. tb., at Dr. Luciw’s laboratory. An optimized multiplex TB serodiagnostic system will be transferred to Dr. Khanum’s laboratory at UAAR in the spring of 2009. This will be coordinated with Dr. Khan’s next two-week visit to UAAR. Dr. Khan will focus on the following activities: (1) supervise multiplex system installation, establish routine operation, train additional students and technical staff in the use of multiplex instrument, and (2) monitor progress on ongoing expression of M. tb. proteins in genetically engineered bacterial vectors by Dr. Khanum’s laboratory personnel. In summary, these efforts of Dr. Khan have been critical for providing the preliminary results and establishing the basis for the HEC/Pak-US S&T grant proposal, which now ties together the Luciw and Khan laboratories with strong involvement of Dr. Tasheen and clinicians at the NTBC.

**Progress Report**

**Objective 1:** to develop and optimize the Luminex/BioPlex multiplex system for detecting antibodies and cell-based immune responses to M. tb. and for detecting M. tb. antigens.

The basic multiplex immunoassay method for TB detection plasma/serum has been optimized at UC Davis. The first publication reporting the optimization of the multiplex immunoassay for TB serodetection in nonhuman animal model of TB (Rhesus macaque) was published in 2008 (Khan et al., 2008, Clin. Vaccine Immunol. 15: 433-438). Thus, Specific Aim 1 of the proposal has been successfully achieved. Briefly, M. tb. antigens e.g., ESAT-6, CFP-10, HspX, MPT53, Mpt63 and Ag85 complex were included in the basic multiplex serodetection assay. Certain antigens ESAT-6, CFP-10 and HspX were specifically included to enhance specificity of the multiplex immunoassay because these antigens are absent in non-tuberculous Mycobacteria and the vaccine strain BCG (bacillus Clamette-Guerin). This assay enabled simultaneous detection of multiple M. tb. plasma antibodies in several cohorts of macaques representing different stages of infection and/or disease. Antibody profiles were defined in early and latent/chronic experimental infection. These findings demonstrate the potential of multiplex immunoassay for clinical application in detection of TB in humans. About half of the 290 clinical samples collected in Pakistan have arrived at UC Davis. These samples will now be tested using the optimized methodologies to achieve Specific Aim 3 of the proposal.
Objective 2: to set up the Luminex/BioPlex instrument at UAAR and train technical staff in multiplex detection of TB.

A new Luminex/Bioplex instrument has been purchased by UAAR. Dr. Imran Khan will be visiting UAAR in Spring 2009 to help establish and validate the system for routine use in TB serodetection. Importantly, two visiting students (Irum Nawaz and Mirza Imran Shahzad) have trained in my laboratory for the past ten months. In March 2009 they will return to Pakistan ahead of Dr. Khan’s visit and assist him in establishing the multiplex system at UAAR. In addition, they will help train other students and technicians in the use of the system.

In an effort to facilitate capabilities for production of \( M. \text{tb} \) proteins for immunoassays in Pakistan, we have previously provided Dr. Khanum with protocols for expression of recombinant proteins in genetically engineered bacteria (see Background section). In Nov. 2008, two of the purified antigens were shipped to UC Davis for testing in multiplex assays. The tests are currently in progress. These recombinant \( M. \text{tb} \) proteins were produced from the plasmid clones containing \( M. \text{tb} \) antigen genes that were shipped to UAAR in July 2007. The clones were provided by the NIAID (NIH) TB Contract to Colorado State University. These activities have helped establish a key “in-country” capability for production of proteins for infectious disease diagnostics. For additional details see Appendix 1 (Progress report from Dr. Azra Khanum).

In an effort to maintain the ongoing relationship with National TB Center, during his visit to Pakistan in Spring 2009, Dr. Khan will also meet with infectious disease clinicians at the National TB Center (Dr. S. Tasheen and colleagues, Islamabad). Importantly, he will review and update the procedures at the Center for ongoing collection and processing of blood samples from TB patients for routine multiplex analysis at UAAR. Dr. Kathy DeRiemer, co-investigator at U.C. Davis, has been assisting the project with important issues dealing with clinical evaluation of the multiplex assay in TB patients (e.g., criteria for enrollment, design of the clinical study).

Plans for Jan 2009 to May 2009

My laboratory will continue to work with Dr. Khanum’s research group over the next several months to complete the transfer of the multiplex Luminex/Bioplex technology to UAAR. Research funding for Dr. Khanum’s laboratory runs until Summer 2009. At present, we have evaluated over 12 \( M. \text{tb} \) antigens for multiplex serodetection. The plan for the Luciw/Khanum collaboration is to add additional 4 to 6 additional novel antigens to the multiplex immunoassay system. Dr. Luciw’s laboratory at U.C. Davis will optimize microbead conjugation and multiplex assay conditions for these antigens as well. The graduate students, Ms. Nawaz and Mr. Shahzad, will be directly involved in these activities on the multiplex immunoassay over the next few months. These students are currently involved in optimizing antibody detection in plasma samples obtained from TB patients that arrived in my lab at UC Davis in Nov. 2008.

Objectives 3: to apply these novel multiplex assays for cross-sectional and longitudinal studies of TB patients at NTBC in Rawalpindi:

Plasma samples from 290 TB patients collected in Pakistan have been stored in
multiple aliquots at -80°C at UAAR (see Appendix 1: Project Report by Dr. Khanum from the Pakistani side). One aliquot per patient from 150 patients has been received at UC Davis. Multiplex analysis to detect antibodies against *M. tb.* in these samples from Pakistan is in progress at UC Davis. It is anticipated that in the coming months all of the received samples will have been analyzed. Arrangements are under way to receive aliquots at UC Davis from the rest of the patient samples stored at UAAR. Upon completion of the multiplex analysis of patient samples, results will be published in an appropriate peer reviewed journal. For additional details see Appendix 1.

**Objective 4:** to produce a detailed laboratory manual and training materials that can be implemented in Pakistan for detection of *M. tb.* infection by multiplex immunoassay:

See Project Report by Dr. Khanum from the Pakistani side (Appendix 1).

**Training of Visiting Students (Ms. Irum Nawaz and Mr. Mirza Shahzad):**

Two visiting students from Dr. Azra Khanum’s laboratory at UAAR joined my research group in March 2008. These students were closely supervised by Dr. Khan and myself on the use of TB antigens in developing multiplex serodiagnostic tests. The two students have learned a variety of methodologies necessary for the successful completion of the project including gene expression, protein purification, Western blot, ELISA, and microbead coating for multiplex assay development. Upon their return to UAAR in the spring of 2009, they will help establish the multiplex system for continual serosurveillance of *M. tb.* infection in patient samples collected in Pakistan. Dr. Khan will visit UAAR a few weeks after the return of the two students to Pakistan. During his two week stay at UAAR, Ms. Nawaz, and Mr. Ahahzad will assist Dr. Khan in the completion of the transfer of technology to UAAR which will include testing and validation of the new BioPlex instrument and finalizing the standard multiplex assay protocols.

**Summary:**

At the end of the current activities described above (Summer 2009), the multiplex TB detection data will be used to make a comparison of this novel immunoassay diagnostic method with conventional methods for TB diagnosis. Drs. Khanum and Luciw will work with their students and post-doctoral associates to generate manuscripts for publication in peer-review journals. The multiplex data will be used to make a determination of cost-effectiveness by comparing with conventional TB detection methods. If these comparisons prove favorable for the multiplex technology, then a future study can be done for a full clinical validation. Such a study will determine the utility of the multiplex immunoassay system for improving TB diagnosis in Pakistan.

**Summary of capacity-building through this 2-year collaborative project:**

1. Development and optimization of the basic multiplex microbead immunoassay for detection of anti-*M. tb.* antibodies has been successfully achieved and published.

2. Training of graduate students from UAAR is in progress and will complete by Spring 2009. This training includes preparation of the microbeads and operations of the Luminex/BioPlex instrument, including use of software for data analysis.
3. Technology transfer to UAAR will be completed by late Spring 2009 to enable Luminex/BioPlex instrumentation at UAAR for in-country surveillance of TB in individuals monitored by the NTBC.

Notes:
1. Two of Dr. Khan’s trips to Pakistan were sponsored by HEC’s foreign faculty program: July 2005 and Dec. 2006. The USAID supported trips were in Oct. 2007 and Aug. 2008 (Annual conference in Islamabad plus trip to UAAR/Khanum Laboratory).
2. The USAID project has been leveraged by funds to Dr. Luciw’s research program from the Department of pathology, School of Medicine, UC Davis.
Reporting Indicators for the Pakistan-US Science and Technology Cooperative Program and the Pakistan-US Cooperative Program

Name of US Principal Investigator:  Paul A. Luciw

Project Title: Multiplex Immunoassays for the Detection of Tuberculosis

<table>
<thead>
<tr>
<th>Indicators/Targets: For each Program Element selected, one of the following three entries is required for the indicator targets -</th>
<th>Reporting Period</th>
</tr>
</thead>
</table>
| • Zero (0) – if there is work specific to that indicator being done but results were not reportable in this timeframe;  
• A number – reflecting what the implementing mechanism accomplished in FY2007; or  
• N/A – if the indicator does not apply to the work being done. | 10/1/07 - 9/30/08 |

1. Number of higher education partnerships between US and host country higher education institutions that address regional, national, and/or local development needs

2. Total number of Pakistanis completing exchange visits on your project during the reporting period

   Number of women

   Number of men

3. Total number of Pakistanis trained in research as part of your project during the reporting period

4. Total number of Pakistanis trained as a result of participation in your project during the reporting period (this figure may be higher than the number reported in item 3, as this item will include not only people trained in research but also trained in other aspects, such as public health measures, criminal investigative procedures, legal processes, or technical lab procedures that do not involve actual research.)

   Number of women

   Number of men
Appendix 1
Progress Report by Dr. Azra Khanum:
Pak-US Joint Academic & Research Program

Title: Multiplex Immunoassays for the Detection of Tuberculosis

Period: October 2007 to December 2008

Pakistan Principal Investigator - Azra Khanum, PhD, Professor of Biochemistry and Dean, Sciences, University of Arid Agriculture Rawalpindi (UAAR), Rawalpindi, Pakistan

US Principal Investigator - Paul A. Luciw, PhD, Professor, Center for Comparative Medicine, University of California, Davis (UC Davis), CA, USA

Background

Tuberculosis (TB) is a global disease and Pakistan ranked 8th in high burden countries. The main objective of this project is to develop a novel, accurate and specific technique as multiplex immunoassays for the diagnosis of TB. Different Mycobacterium tuberculosis (M. tb) specific antigenic genes will be used in this project. This project includes recombinant genes, their expression and purification and multiplex immunoassay. This project involves collaboration between UC Davis and the Biotechnology Laboratory, Department of Biochemistry, UAAR. Blood sample collection for TB patients will be accomplished at the National TB Center, Rawalpindi.

Progress from October 2007 to December 2008

Objective 1: To develop and optimize the Luminex/Bioplex Multiplex System for detecting antibodies and cell- based immune responses to M tb and for detecting M tb Antigens.

The multiplex immunoassay methods for TB detection are being developed and optimized at UC Davis.

Blood Sampling

At the National TB Centre, Rawalpindi, Doctors refer patients for sputum microscopy to the Centre Laboratory. Two different sputum samples are collected by staff at the TB Centre. One sample is taken on the same day and the other on the next day. A report is provided to the patient on the second day. If the patient is declared as sputum positive i.e. active TB disease, then the patient is interviewed and history is taken on a clinical information form who gives his/her consent. Thereafter, 3.5 ml blood sample is drawn by syringe. The blood is collected in EDTA coated tubes and then taken to the laboratory at UAAR.

A total of 290 blood samples have been collected from patients in different TB disease groups from the TB Centre. Plasma has been separated from these blood samples,
 aliquots 0.2 ml each in 5 vials have been made and stored at -80 °C. One aliquot of plasma of each sample has been sent to UC Davis (Dr. Paul Luciw’s laboratory) for optimization of in the multiplex Luminex immunoassay. These samples are mailed through renowned courier service at the required temperature (frozen on dry ice). The details of different disease groups are shown in Table 1. Blood spots of TB patients are also prepared on standard blood stain cards; these dried blood spot samples were sent earlier to UC Davis (September 2008) to test this method for anti M.tb. antibody detection.

The optimization of expression and purification of five, out of seven, new recombinant genes from *M. tb* i.e. groES, bfrB, acg/Rv2032, RV2626C, MPT51, has been completed. Protein products of recombinant genes bfrB and groES have been sent to Dr. Luciw’s laboratory at UC Davis for further processing and purification. The expression of the remaining two antigens has already been optimized and purification is under process in Dr. Khanum’s laboratory.

**Table 1: Disease Groups for TB Patients and their Sampling Details:**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Description</th>
<th>No. of Samples to be taken</th>
<th>No. of Samples taken</th>
<th>No. of Samples sent to UC. Davis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>Non BCG vaccinated, healthy and skin test negative</td>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Group 2</td>
<td>Non BCG vaccinated, healthy and skin test positive (latent TB without symptoms)</td>
<td>15</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>Group 3</td>
<td>with active TB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 3-1</td>
<td>1st time smear positive, newly diagnosed: have never taken TB treatment</td>
<td>250</td>
<td>256</td>
<td></td>
</tr>
<tr>
<td>Group 3-11</td>
<td>TB relapse/recurrence. Previously cured or treated but disease reactivated later</td>
<td>15</td>
<td>18</td>
<td>15</td>
</tr>
<tr>
<td>Group 4</td>
<td>Patients responding to therapy in DOTS program. Declared smear negative after 2 months. These will get same drugs for a total time period of 6 months. These patients will be taken from Group 3-1.</td>
<td>15</td>
<td>26</td>
<td>15x6 (90)</td>
</tr>
<tr>
<td>Group 5</td>
<td>Patients not responding to therapy in DOTS program. Declared smear positive after 2 months. These patients will also be taken from Group 3-1</td>
<td>15</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Objective 2: To set up the Luminex/Bioplex Instrument at UAAR and train technical staff in Multiplex detection of TB.

The multiplex immunoassay is being performed at UC Davis. Patient plasma samples as well as dried blood spots have been sent for detection of antibodies against M. tb. One of our graduate students, Ms. Irum Nawaz, has joined Laboratory of Dr. Luciw at UC, Davis in March, 2008. She has also successfully completed the expression and purification of six different recombinant genes from M. tb., i.e. hspx, esat-6, cfp 10, ag85a, ag85b, and ag85c. She is also working on optimization of the multiplex immunoassay for detection of TB from patient plasma.

Dr. Imran Khan (Research Associate at the Center for Comparative Medicine, UC Davis) has visited UAAR, Rawalpindi, Pakistan and stayed from August 22 to 30th, 2008. He also provided the basic and advanced knowledge to graduate students and technical staff related to novel methods of infectious disease diagnostics. He further conducted detailed discussions with graduate students involved in this project regarding expression studies of various M. tb antigens etc. Dr. Khan also met with clinicians at the National TB Centre in Rawalpindi.

The multiplex Bioplex instrument has been purchased from BioRad (Richmond, CA, USA) for installation at PMAS-AAUR. Dr. Khan looked after the installation process and gave detailed orientation of the instrument operations to the graduate students in DR. Khanum’s laboratory.

Plans from January 2009 to May 2009

Objective 1: (Stated above)

At present, work is in progress for large scale purification of different M. tb specific recombinant proteins with affinity chromatography.

Objective 2: (Stated above)

The multiple/Bioplex instrument was purchased from BioRad (USA) and installed. Dr. Khan will spend two weeks at UAAR in spring 2009 to provide additional training of multiplex immunoassays; during this visit, conditions for detecting antibodies in TB patient plasma will be optimized. He will also monitor the activities undergoing for the full transfer of the multiplex technology, including development of training manuals, from UC Davis to UAAR.

Objective 3: To apply these novel multiplex serodiagnostic assays for cross-sectional and longitudinal studies of TB patients at NTBC in Rawalpindi.
To achieve this objective, blood collection for most patient groups at National TB Center, Rawalpindi has been completed (Table 1). A total 290 blood plasma samples have been collected from patients after obtaining their informed consent and taking their medical histories. Blood spots have also been prepared on standard blood stain cards, which were provided from the laboratory of Dr. Luciw. These blood stain card samples (dried blood spots) were sent to the laboratory of Dr. Luciw in September 2008; subsequently the frozen plasma samples were sent to his laboratory (November 2008). The details of different disease groups are given in Table 1

**Objective 4:** To produce a detailed laboratory manual and training materials that can be implemented in Pakistan for detection of M. tb. infection by multiplex immunoassay.

For this objective, the project staff at UAAR will work in direct collaboration with DRs. Luciw, DeRiemer, and Khan at UC Davis on the development of a detailed laboratory manual and training materials that can be implemented in Pakistan for multiplex detection of antibodies to M. tb.

At the end of project, the serodiagnostic data will be used to compare multiplex immunoassay diagnosis with conventional methods for TB diagnosis. The cost effectiveness, sensitivity and specificity of the multiplex system, if prove favorable, will be followed by a full clinical validation study.