Detecting Active and Latent Tuberculosis with Serology and Multiplex Diagnostics

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Abstract
After HIV/AIDS Tuberculosis is the biggest cause of death worldwide, making a lot of children orphans. Tuberculosis can be either pulmonary or extrapulmonary, the latter site of disease makes it hard for the methods used today to confirm infection of mycobacterium tuberculosis. The methods, especially in developing countries, do far from detecting all those with mycobacterium tuberculosis.

Latent tuberculosis is detected by PPD-skin test, which has many false-positive or false-negative responses.

Detecting tuberculosis in children is even harder due to their inability to produce enough sputum for the test used in developing countries. Tuberculosis in children can be mistaken for a normal child’s disease like pneumonia.

It is important to develop a method that is faster and better than the ones used today. Using serology and multiplex diagnostics could be such a way. Firstly we have to identify proteins or peptides connected to mycobacterium tuberculosis. This was done by testing 35 different synthetic peptides that could be antigens from mycobacterium tuberculosis with tuberculosis sera.

Unfortunately most of the peptides tested showed no significance except for one peptide, Ag85b, that showed some connection to mycobacteria. If expanding the experiments with new peptides or even whole proteins it would be possible to find some epitopes that can be connected to mycobacterium tuberculosis.

Sammanfattning

Teknikerna som används idag, speciellt i U-länder, upptäcker inte alla de med mycobacterium tuberculosis infection.

Latent tuberkulos detekteras med hjälp av ett PPD-hudtest, som kan ge falsk-positiva eller falsk-negativa svar.

Att diagnosticera tuberkulos hos barn är väldigt svårt på grund av deras oförmåga att producera tillräckligt med sputum. Tuberkulos hos barn kan även härma andra mer vanliga sjukdomar, så som lunginfektion.


Tyvärr visade de flesta peptider ingen significans, förutom en peptid, Ag85B, som visade ett samröre med mycobacteria. Om man utökar experimentet med fler peptider eller till och med hela protein är det inte omöjligt att hitta epitope som kan ha ett samband med mycobacterium tuberculosis.
1. Introduction
More than one-third of the world’s population is thought to carry the infectious agent that causes tuberculosis (TB), mycobacterium tuberculosis (mtb) (1).

Year 2010, there was approximately 8.8 million new tuberculosis cases worldwide and about 1.5 million died from active disease (1), which 95% of the deaths occurred in developing countries (2). After HIV/Aids tuberculosis is the leading disease of causing the most deaths in the world (1).

In 90% of the active TB-cases the lungs are affected and the remaining percent are extrapulmonary lesions, which can affect lymph nodes, joints etc (1).

The active TB in developing countries is in first place diagnosed with sputum smear acid-fast bacillus microscopy or by growing cultures, which is the golden standard according to the World Health Organization (WHO) (1).

Growing cultures takes 3-8 weeks (2, 3) and due to the way mtb spreads, via aerosol droplets, the person infected can spread the disease to others before a positive growth culture may be established. In 10-20% of the cases it will be a negative culture even though the person is infected with mycobacterium tuberculosis (2), which also will contribute to the spread of the disease.

The sputum smear acid-fast bacillus microscopy cannot be used if the person has extrapulmonary tuberculosis due to lack of bacilli in the sputum or if the person has paucibacillary tuberculosis, i.e. too few tubercle bacilli (1). This could be one of the problems with detection of early tuberculosis because bacilli are growing slowly.

The sensitivity of the sputum smear acid-fast bacillus examination is only 50-60%. These low numbers depends on that a greater load of bacteria is needed for the detection of mycobacterium tuberculosis (4).

Another way to diagnose tuberculosis, for those that are asymptomatic, is the purified protein derivative (PPD)-skin test. Tuberculin is injected below the skin surface and if you get a reaction you may have tuberculosis or may have had an infection. The reaction from the skin test may also be induced by BCG-vaccination or other atypical mycobacteria, therefore making the test not reliable enough (3, 5). A population whose country has a high burden of tuberculosis cases will have antibodies against mycobacteria and therefore give a positive reaction to the PPD-skin test although they are not infected. This test may only be useful in countries with low rates of tuberculosis (4).

It is hard to detect children with tuberculosis due to 50% of the children being asymptomatic when infected in the early stages (6). One reason is that TB can mimic other childhood diseases like pneumonia, malnutrition or HIV. Their inability to produce enough sputum makes the sputum smear microscopy inefficient (7). Children are often paucibacillary which makes the cultures ineffective as well (6). 20-30% of the children infected have extrapulmonary tuberculosis (6), this might be due to that the immune system is not fully developed in children allowing migration of the bacteria to other locations.

With the commercial tests of today the specificity and sensitivity are not good enough (8, 9), but with some refinement of the tests the specificity and sensitivity could be high for detection of antigens (10). Due to different antibodies produced in different individuals when infected with mycobacterium tuberculosis at different stages of the disease several antigens are needed.
to be included in a serodiagnostic test (11).

In 2009 almost 14 million children were orphans due to tuberculosis (1). Since tuberculosis is curable with an antibiotic course it could be prevented that people die (1).

If a sensitive, specific, quick and cheap way could be found to analyze patient samples for biomarkers (i.e. antigens or antibodies) connected to latent and active tuberculosis the currency for tuberculosis could decline worldwide. With microsphere-bead multiplex immunoassay (i.e. with serology) there is a hope to quickly and efficiently diagnose individuals with active and latent TB.

The aim of this thesis was to investigate if differences in antibody binding to the antigens could be seen between active and latent TB with the help of synthetic peptides from the proteins mentioned above, which were reported to be diagnostically useful.

Synthetic peptides do not represent all the epitopes on a protein, but they could give a hint where the site of antibody binding could be.

Peptides from the following proteins - antigen 85B (Ag85B), ferritin-like protein (BfrB), hypothetical protein (HypProt), mycobacterium tuberculosis 48 (MTB48), urine protein 1 (U1), culture filtrate protein 10 (CFP-10), early secretory antigenic target protein 6 (ESAT-6) and GroEl - were used in the investigation of detection of active and latent tuberculosis.

In order to investigate if a difference between the two phases could be seen, sera from persons with latent and active tuberculosis were obtained. Blood donors were used for comparison.

Antigen 85B (Ag85B) is an enzyme which participates in the final step of the cell wall assembly of mycobacteria (12). The protein is a major part of the secreted antigens from mtb and can be seen already after 3 days (8).

Iron is an important substance for all cells even for mtb cells. Free iron is toxic to the cells because reactive oxygen radicals will be produced (13). Ferritin-like protein (BfrB), which “collects” iron, is up-regulated if the cell is exposed to oxidative stress, which is created by the macrophage host, and when the mtb infection is trying to adapt to a stationary phase (13, 14). BrfB reduces the free ferrous form. It reduces the production of oxygen radicals and this probably ensures survival of the mycobacterium tuberculosis (13).

A gene whose protein has not been experimentally identified in vivo is called a hypothetical protein. The hypothetical protein in this paper is thought to be a protein involved in the membrane of mycobacterium tuberculosis.

The exact function of MTB48 is not known but it can be seen that the gene is conserved in mycobacterium Tuberculosis but deleted or partially absent in atypical mycobacteria (15). According to Wu et al. (15) it is seen that levels of antibodies against recombinant mycobacterium tuberculosis 48 (MTB48) was significantly elevated for the PPD-positive controls and not as much for the negative PPD-controls. It may be due to either a latent stage of mtb infection or a humoral immune response due to vaccination with BCG or even recovery from undiagnosed TB (15).
The gene for hypothetic thiol peroxidase mtb protein was cloned, which led to a highly purified recombinant protein that was named Urine Protein 1 (U1) (16). Thiol peroxidases protect the bacteria and help them grow under oxidative stress (17). According to Sandeep Murkherjee et al. (16), none of the persons without tuberculosis and HIV did react with the U1 protein while about 60% of those infected with pulmonary tuberculosis reacted with the protein. Unfortunately the persons with extrapulmonary forms of mtb had a lower detection of U1, with only 23.6% detection. Since the murine samples were taken in the first 10 till 14 days, which is thought to be under the replication phase of mycobacterium tuberculosis, it is possible for the protein to be a biomarker for active pulmonary tuberculosis, although not good enough as an alone biomarker (16).

The genes encoding CFP-10 and ESAT-6 are next to each other within the region of difference 1 (RD1) and are being co-transcribed (18). They are thought to form a 1:1 heterodimer in order to be active in vivo (18, 19). CFP-10/ESAT-6 complex is believed to regulate apoptosis of macrophages by inhibiting and/or to induce the modulating TNF-α expression (19, 20). TNF-α is vital for the recruitment of adaptive immune cells and therefore capture of mycobacterium tuberculosis in granulomas (19, 20). ESAT-6 is also thought to inhibit TLR signaling in macrophages, which reduces the response from the innate immune system upon infection (19, 21).

RD1 is not expressed in other less harmful mycobacteria, for example mycobacterium BCG, therefore it is thought that the proteins encoded by RD1 are important for the virulence of the mycobacterium tuberculosis (22). This makes them possible candidates for detection of tuberculosis (5, 19).

GroEl is a gene that creates a lot of heat shock protein under stress, for example under hypoxia, transformation and phagocytosis. These proteins are highly conserved and exist also in eukaryotic and prokaryotic cells. They are thought to be important for cellular growth (23). Mycobacterium tuberculosis has two GroEL-like genes, which are referred to as cpn60.1, which is nonessential for the survival of the bacteria, and cpn60.2, which is essential and thought to be a housekeeping gene (23, 24). They have a similarity of 61%. Both of the GroEL-proteins act as virulence factors and cytokine stimulators (23). It is thought that they act like chaperonins, hence they participate in protein folding and to prevent aggregation of proteins in the bacterial cytosol (23, 25). They also are thought to function as signaling molecules and are shown to activate a T-cell response. (23)
2. Material and Methods

2.1 Selection of antigens and control sera

Clinical Samples
From Uppsala Academic Hospital from the blood bank 61 blood donor sera were obtained.

Control sera
30 tuberculosis serum samples (positive or negative) were donated from The Karolinska Institute in Stockholm by Dr. Kristian Ängeby.
9 tuberculosis serum samples were picked out by Kåre Bondeson, clinical virologist and laboratory doctor at the Microbiologic Laboratory at Uppsala Academic Hospital.

Treatment of dangerous agents
The tuberculosis sera obtained from Karolinska Institute could have contained not only mycobacterium tuberculosis but also HIV, Human Immunodeficiency Virus. The sera were heat treated at 60 degrees Celsius for 2 hours to reduce the risk of infectious agents in the samples.
The tuberculosis samples from Kåre Bondeson were treated with 0.4% Triton X-100 made from 100% Triton X-100 (Merck, cas nr 9036-19-5). One part sample and one part 0.4% Triton X-100 was mixed. Later StabilGuard was added to the mixture to yield a 1:10 dilution.

Synthetic peptides
35 different synthesized peptides from eight different proteins were searched for in literature data by Professor Jonas Blomberg. The peptides were provided by Rüdinger Pipkorn, Deutsches Krebsforschungszentrum (DKFZ), Heidelberg, Germany.
These are the peptides used in the experiment:

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>Ag85B 234-263</td>
<td>TQQIPKLVANNTRLWVYCGN GTPNELGGAN</td>
</tr>
<tr>
<td>BfrB 114-143</td>
<td>RDEGDFLGEQFMQWFLQEIQIEEVALMATLV</td>
</tr>
<tr>
<td>HypProt 73-102</td>
<td>KADELQHALQDSGVHDVAVISEAQAATALV</td>
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<td>HypProt 116-145</td>
<td>DETATLSVVGDPDAPPTMVAVAPVAGADAT</td>
</tr>
<tr>
<td>HypProt 391-420</td>
<td>PIPVPIIPFPWQPGMPTIPTAPTPTPV</td>
</tr>
<tr>
<td>MTB48 79-108</td>
<td>KAYGEVDEEAATALNDNDEGTQVAESAGAV</td>
</tr>
<tr>
<td>MTB48 257-286</td>
<td>SEKVLTEYNNKAALEPVNPPKPPPAIKIDP</td>
</tr>
<tr>
<td>MTB48 310-339</td>
<td>TPGTGMPAAPMVPPTGSPGGLPADTAAQL</td>
</tr>
<tr>
<td>MTB48 414-443</td>
<td>MGAAHQQGGAKSKGSQQEDEALYTEDERAW</td>
</tr>
<tr>
<td>MTB48 431-460</td>
<td>QED EALYTEDERAWTEAVIGNRRRDQDSKESK</td>
</tr>
<tr>
<td>U1 15-44</td>
<td>GELPAVGSPAPAFTLTGGDLGVISSDQFRG</td>
</tr>
<tr>
<td>U1 128-157</td>
<td>LARAIVVIGADGNVAYTELVPEIAQEPNYE</td>
</tr>
<tr>
<td>CFP10 21-50</td>
<td>DLKTQIDQVESTAGSLQGWRGAAAGTAQA</td>
</tr>
<tr>
<td>CFP10 46-75</td>
<td>TAAQAAVVRFQEAANKQKQELDEISTNIRQ</td>
</tr>
<tr>
<td>CFP10 64-93</td>
<td>QELDEISTNIRQAGVQYSRADEEEQQALSS</td>
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<td>ESAT6 1-30</td>
<td>MTEQQWNFAGIEAAAASAIQGNVTSHLDD</td>
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<td>ESAT6 21-50</td>
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<td>ESAT6 37-66</td>
<td>TKLAAAWGGSGSEAYQGVQQKWDATETEL</td>
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<td>ESAT6 61-90</td>
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<td>M tuberculosis GroEL 7-36</td>
<td>YDEEARRGLERGLNALADAVKVTLGPKGRN</td>
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<td>M tuberculosis GroEL 24-53</td>
<td>DAVKVTNLGPKGRNVLKKWAGAPTITNDGV</td>
</tr>
<tr>
<td>M tuberculosis GroEL 71-100</td>
<td>ELVKEVAKKTDDVAGDGTATATVLIVALVR</td>
</tr>
<tr>
<td>M tuberculosis GroEL 91-120</td>
<td>M ATVLAQALVREGLRNVAAGANPLGLKRGIE</td>
</tr>
<tr>
<td>M tuberculosis GroEL 168-197</td>
<td>GNEGVTVEESNTFGQLEELECMRFDKGY</td>
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<td>M tuberculosis GroEL 185-214</td>
<td>LELTEGMRFDKGYISGYVFTDPERQEA</td>
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<td>M tuberculosis GroEL 222-251</td>
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</tr>
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<td>M tuberculosis GroEL 249-278</td>
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<td>M tuberculosis GroEL 326-355</td>
<td>DETTIVEGAGTDAIAGRVAQIRQIE</td>
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<td>M tuberculosis GroEL 356-385</td>
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<td>M tuberculosis GroEL 405-434</td>
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</tr>
<tr>
<td>M tuberculosis GroEL 456-485</td>
<td>GLEPGVVAEKVRNLPAHGGLNAQTVG</td>
</tr>
<tr>
<td>M tuberculosis GroEL 495-524</td>
<td>KVTNRSLQNAASIAGLFLTTEAVV</td>
</tr>
</tbody>
</table>

**BCG-vaccine**

The BCG vaccine SSI (The State Serum Institute, Copenhagen, Denmark), the only vaccine used against tuberculosis, is made from attenuated Mycobacterium Bovis BCG, Bacillus
Calmette-Guerin, Danish strain 1331. 1ml vaccine contains between 2-8 million Mycobacterium Bovis.

**Membrane dialysis of the BCG vaccine**
The lyophilized BCG vaccine was delivered as a powder and with a dilution fluid. The dilution fluid contained dipotassium phosphate which interferes in the coupling to the beads, therefore the dilution fluid was not used and instead the powder was dissolved in 200μl PBS. Unfortunately, the powder contained sodium glutamate which also interferes with the bead coupling. To extract the interfering component the dissolved vaccine was dialyzed in a 1ml dialysis tube, Spectra/Por® Float-A-Lyzer® G2 with an Ultra-pure Biotech Cellulose Ester Membrane, 3.5-5kD, according to:
First the filter membrane was pre-wetted for approximately 30 minutes in PBS. Then the dissolved BCG vaccine (200 μl) was transferred to the dialysis tube. The lid was screwed on well to avoid leakage. A flotation ring was threaded over the dialysis tube, which then was submerged in a glass vessel containing 2L of PBS. A magnet was put in the vessel and the vessel was put on a magnetic stirrer to form a gentle rotating current. The dialysis was performed over night in a cold room.
After dialyzing the sample, 150μl vaccine was obtained and used in the bead coupling.

**2.2 Multiplex detection of antibodies based on the Luminex system**

**Coupling of antigens to beads (Luminex xMAP Technology Microspheres)**
Before the beads could be coupled the peptides were dissolved in PBS (pH 7.5, made by Department of Clinical Microbiology at Uppsala Academic Hospital) to yield a concentration of 2mg peptides/ml PBS.
The first step in the bead coupling was for the beads, stored in 4°C, to warm up in room temperature for approximately 30 minutes. The bottles containing the beads were then vortexed for two minutes, followed by sonication for two minutes. An appropriate amount of beads were transferred to Eppendorf tubes that were put to centrifuge for 3 minutes at 13000 rpm. If a pellet could be seen after centrifugation the supernatant was carefully removed and 100μl of sterile water was added, followed by vortexing and sonication for approximately 20 seconds each. The samples where yet again centrifuged (3 minutes at 13000 rpm).
Supernatants were removed and the beads were resuspended in 80μl of 100mM MSP, monobasic sodium phosphate, NAH$_2$PO$_4$, pH6.2, by vortexing and sonicating (20 seconds each). 10μl of 50mg/ml Sulfo-NHS solution was added. The samples were vortexed carefully.
After the addition of Sulfo-NHS 10μl of 50mg/ml EDC was added to the samples and they were carefully vortexed. The tubes containing the beads were put to incubate for 20 minutes, with a gently vortexing after 10 minutes. The samples were then centrifuged for 3 minutes at 13000 rpm. Appropriate amount of 50mM MES, morpholineethanesulfonic acid, pH5, was added twice to wash the beads after the supernatants were removed, with vortexing, sonicating and centrifugation between the two washes.
After the washing and removal of the supernatant an appropriate amount of 50mM MES, pH 5, was added and the vortexed and sonicated for about 20 seconds each. 100μg of the prepared peptides were added to the tubes containing the beads and were carefully vortexed. The tubes with the peptides and the beads were incubated at room temperature for 2 hours.
After a centrifugation (three minutes at 13000 rpm) the supernatants were removed and substituted with 250μl blocking agent, StabilGuard. Thereafter the samples were vortexed, sonicated and centrifuged. Twice the supernatant was exchanged with an appropriate amount of StabilGuard followed by vortexing, sonication and centrifugation in-between. The supernatants were removed and an appropriate amount StabilGuard was added to yield concentration of 2500 beads/μl. The samples were vortexed and sonicated for 20 seconds each and were the put in 4°C for later use.

Validity control
Over time, with thawing and re-freezing, the sera seem to follow a trend of getting weaker reactions, although the antibodies in the sera are supposed to not get affected. To avoid misleading results from sera that does not work in an optimal way a bead with Haemophilus Influenza vaccine coupled to it was added to the bead mixture. Haemophilus Influenza vaccine was selected as a validity control, because most human sera have antibodies against H. Influenza. The vaccine used was HIB® from Sanofi Pasteur MSD, which contained 10μg of H. Influenza type b polysaccharide conjugated to a tetanus protein.

Naked beads
In the bead mix a naked bead, which means a bead without any antigens coupled to it, was always included. This was performed to set a background from the reaction levels of the naked beads. The reaction to the naked bead is due to the reactivity of antibodies to react to the beads without an antigen. The MFI value from the naked bead was subtracted from all the signal levels from the antigen/antibody complexes.

The process of making naked beads started like the coupling process. The beads were centrifuged and FRO-water (sterile water) was added which after the solution was centrifuged for 3 minutes at 13000rpm to get a pellet. Then 250μl StabilGuard was added, then again centrifuged for three minutes. Last steps were to resuspended the bead in 500μl StabilGuard two times before the appropriate amount of StabilGuard was added to yield the final concentration of 2500 beads/μl.

NTC, Non Template Control
The blocking agent, StabilGuard, used in the bead coupling process was also used as the non template control during each Luminex-run.
There should not be any reactions with this solution, because the solution does not contain any antibodies. When you run the Luminex system you will get a MFI value for the well with the StabilGuard. This is due to reactions with the antigens on the beads that take place in the solution. Therefore the MFI value for NTC must be subtracted from the different antigen/antibody signal values.

Prior to loading the Luminex 96 well filter plate
The blood donors as well as the TB-sera were diluted 1:10 with StabilGuard. Before loading the plates with samples and beads a master bead mix was created from all the coupled beads. There were 35 beads coupled with tuberculosis antigens, one bead coupled with the BCG-vaccine, one bead that was “naked” and one bead with Haemophilus Influenza (validity control), in total it was 38 beads that were added in the mixture. The aim was to have 0.5μl of
every bead diluted to 2500 beads/µl in each well. The total amount that should be taken from
the bead mixture to each well was 50µl, this due to previous optimization that is not described
here. 0.5µl times 38 beads yields 19µl. Since it should be 50µl in each well from the bead
mixture 31µl
StabilGuard was added for each well used in the mix.

How to load the Luminex 96 well filter plate
The 96 well filter plate (Millipore MX-plate) was loaded accordingly:
First 100µl PBS was added to each well, followed by aspiration by a vacuum manifold. This
procedure was done twice.
After the pre-wetting of the wells 50µl of the serum (in a suitable dilution with StabilGuard
buffer) was added to each well. From the bead mix 50µl was taken to each well. The filter
plate with the bead mix and sera were incubated for 30 minutes in room temperature on a
shaker at the lowest speed. The wells were then aspirated (approximately 2 seconds so that the
beads would not pass through the filter) and washed two times with 100µl of PBS per well.
50µl of StabilGuard was added before 50µl of a 4µg/ml biotinylated protein G solution
(Pierce, cat nr. 29988, diluted in StabilGuard) was added to each well. While adding Protein
G the beads were resuspended by pipetting the solutions up and down five times. A further
incubation on a shaker for 30 minutes in room temperature at low speed was conducted. After
aspiration with the vacuum manifold the beads were washed 2 more times in 100µl PBS per
well, followed by addition of 50µl StabilGuard to each well. 50µl of a Streptavidin R-PE
conjugate solution (SA-PE, Invitrogen, cat nr. SA1004-4, diluted in StabilGuard) was added
to the wells. The beads were resuspended by pipetting the solutions up and down five times.
The filter plate was then incubated for 15 minutes on a shaker at lowest speed in room
temperature. After incubation the plate was aspirated and washed twice with 100µl PBS per
well. The last step before the plate was ready to be run in the Luminex machine, the beads
were resuspended with 150µl PBS per well by pipetting the solutions up and down.

Luminex computer settings
The following settings were used for the Luminex Analyzer when creating a protocol in the
computer:
The analysis time for each well was set to 100 seconds and the number of beads needed for
time-out was set to 100beads/type. The volume to analyze from each well was set to 100µl.
The number needed to get a signal was 1, although bead count under 10 was excluded as Not-
a-Number, NaN.

The bead numbers and the names of the analytes were typed into the analyzer protocol, as
well as the location of the wells and names for the different samples and controls. After these
decisions the machine was ready to analyze the plate.

Interpreting results
In each Luminex run there was a non template control, NTC, well. The highest value from
this well was set as the background and subtracted from the other MFI values.
A negative value after subtracting the background was set as 0.
3. Results
BCG vaccine in blood donors was used to possibly discover similar patterns in the tuberculosis sera and therefore exclude those Tb-sera that were heat treated from being active tuberculosis.

In the data from the first Luminex run (Figure 1) it can be seen that blood donors have higher levels of antibodies against the vaccine, although two Tb-sera reacted as the blood donors with high antibody levels.

In the second Luminex run with tuberculosis sera (Figure 2), treated with 0.4% Triton X-100, and with more blood donors included it can be seen that also here two of the tuberculosis sera got high MFI values.

Figure 1. Antibody reaction levels against the BCG vaccine in heat treated tuberculosis sera and in blood donors.

Figure 2. Antibody reaction levels against the BCG vaccine in tuberculosis sera, treated with 0.4% Triton X-100, and in blood donors.

When testing the 35 antigens, possibly being mycobacterium tuberculosis antigens, one interesting antigen was revealed.
In the first run (Figure 3) antibody reaction in tuberculosis sera against Ag85B is higher than in blood donors, except in three cases where the reactivity in blood donors were equivalent to the reactivity of the Tb-sera.

The reactivity against Ag85B is higher in blood donors than in the tuberculosis sera in the second run (Figure 4) with the tuberculosis sera obtained from Uppsala Academic Hospital.

Figure 3. Antibody reaction levels against Antigen 85 B in heat treated tuberculosis sera and in blood donors.

Figure 4. Antibody reaction levels against Antigen 85 B in tuberculosis sera, treated with 0.4% Triton X-100 and in blood donors.

If looking at figure 5, with data from the first run, at the first glance BfrB could be coupled to mycobacterium tuberculosis, but the MFI levels and therefore the reactivity is not high enough to be significant.

The tuberculosis sera treated with Triton X-100 showed even lower MFI values (Figure 6) for BfrB than the tuberculosis sera that were heat treated.
Figure 5. Antibody reaction levels against BfrB in heat treated tuberculosis sera and in blood donors.

Figure 6. Antibody reaction levels against BfrB in tuberculosis sera, treated with 0.4% Triton X-100, and in blood donors.

Heat treated tuberculosis sera does not show any high levels of antibody reactivity against the three hypothetical protein (HypProt) peptides. In Tb-sera 12 the peptide HypProt 116-145 is slightly elevated but not significantly (Figure 7). The tuberculosis sera treated with Triton X-100 the reactivity was even lower than with the heat treated sera (Figure 9).

Blood donor 407 (Figure 8 and Figure 9) showed high levels of one of the hypothetical peptides, HypProt 391-420. The other blood donors gave no reaction to the HypProt peptides.
Figure 7. Antibody reaction levels against three Hypothetical Protein peptides in heat treated tuberculosis sera.

Figure 8. Antibody reaction levels against three Hypothetical Protein peptides in blood donors.
Figure 9. Antibody reaction levels against three Hypothetical Protein peptides in tuberculosis sera treated with 0.4% Triton X-100.

Figure 10. Antibody reaction levels against three Hypothetical Protein peptides in blood donors.

Figure 11 shows low reactivity levels against the five peptides derived from MTB48 protein in heat treated tuberculosis sera (Figure 11).

In blood donor 412 the peptide MTB 257-286 gave a high signal (Figure 12).

The antibody reaction levels for the five MTB48 peptides are lower in the tuberculosis sera treated with Triton X-100 (Figure 13) than in those that were heat treated (Figure 11).
Blood donor 412 showed the same tendency for peptide MTB48 257-286 in the second run (Figure 14).

Figure 11. Antibody reaction levels against five MTB48 peptides in heat treated tuberculosis sera.

Figure 12. Antibody reaction levels against five MTB48 peptides in blood donors.
Figure 13. Antibody reaction levels against five MTB48 peptides in tuberculosis sera treated with 0.4% Triton X-100.

Figure 14. Antibody reaction levels against five MTB48 peptides in blood donors.

Peptide U1 128-157 shows higher reactivity than U1 15-44 in the heat treated tuberculosis sera, although the reaction in all was not high enough to be significant (Figure 15). The blood donors showed even lower reactivity to the two peptides from U1 (Figure 16). Tuberculosis sera treated with Triton X-100 shows even lower reactivity to the two peptides from U1 (Figure 17). The antibody reaction for blood donors in figure 18 shows similarities to the reaction for blood donors in figure 16.
Figure 15. Antibody reaction levels against two U1 peptides in heat treated tuberculosis sera.

Figure 16. Antibody reaction levels against two U1 peptides in blood donors.
Figure 17. Antibody reaction levels against two U1 peptides in tuberculosis sera treated with 0.4% Triton X-100.

Figure 18. Antibody reaction levels against two U1 peptides in blood donors.

As seen in figure 19 there is not much reactivity in the heat treated tuberculosis sera against the three CFP10 peptides. The reactivity levels are lower for those tuberculosis sera treated with Triton X-100 (Figure 21). Both of the runs for blood donors show low levels of antibody reaction against the three peptides from CFP10 (Figure 20 and Figure 22).
Figure 19. Antibody reaction levels against three CFP10 peptides in heat treated tuberculosis sera.

Figure 20. Antibody reaction levels against three CFP10 peptides in blood donors.
Figure 21. Antibody reaction levels against three CFP10 peptides in tuberculosis sera treated with 0.4% Triton X-100.

Figure 22. Antibody reaction levels against three CFP10 peptides in blood donors.

ESAT6 peptides shows no high reactivity in the heat treated tuberculosis sera (Figure 23). In figure 25 it can be seen that the tuberculosis sera treated with Triton X-100 gives lower reactivity levels than the heat treated Tb-sera in figure 23.

The peptides derived from ESAT6 shows low reactivity in blood donors (Figure 24 and Figure 26).
Figure 23. Antibody reaction levels against five ESAT6 peptides in heat treated tuberculosis sera.

Figure 24. Antibody reaction levels against five ESAT6 peptides in blood donors.
Figure 25. Antibody reaction levels against five ESAT6 peptides in tuberculosis sera treated with 0.4% Triton X-100.

Figure 26. Antibody reaction levels against five ESAT6 peptides in blood donors.

The heat treated tuberculosis sera does not show any significant reactivity to the fifteen MTB GroEL peptides used in the experiments (Figure 27). Triton treated tuberculosis sera show low reactivity (Figure 29).

One blood donor, BD 407, shows significant higher reactivity against peptide MTB GroEL 356-385 (Figure 28 and Figure 30). The other blood donors did not react notably to the peptides.
Figure 27. Antibody reaction levels against fifteen MTB GroEL peptides in heat treated tuberculosis sera.

Figure 28. Antibody reaction levels against fifteen MTB GroEL peptides in blood donors.
Figure 29. Antibody reaction levels against fifteen MTB GroEL peptides in tuberculosis sera treated with 0.4% Triton X-100.

Figure 30. Antibody reaction levels against fifteen MTB GroEL peptides in blood donors.
**4. Discussion**

Since mycobacterium tuberculosis is thought to have infected about 1/3 of the world’s population and nearly 1.5 million people die from it every year, especially in developing countries, and being the cause of nearly 14 million children becoming orphans (1), it is important to develop good diagnostic tools in detection of mycobacterium tuberculosis infections. The methods used today does not detect all those infected with active mycobacterium tuberculosis (1, 4), especially in children it is hard to detect tuberculosis due to the test often being based on patient sputum samples and children being unable to produce enough sputum (6). Latent tuberculosis is even harder to detect due to the test that exists today, the PPD-test, has so many flaws by falsely confirming those with a BCG vaccination to be tuberculosis-positive and by negatively identifying those with an immunosuppression of not having tuberculosis (3, 5).

Our bodies react to all that is not native in our bodies therefore it is likely that the body makes antibodies against the bacteria that infect us.

A faster and better way of identifying mycobacterium tuberculosis antibodies may be accomplished by using antibodies and multiplex diagnostics, but first potential proteins must be found that the immune system respond to.

Since the responses against mycobacterium tuberculosis is thought to be heterogeneous (11) several proteins are needed to be included in a serodiagnostic test to get as high specificity and sensitivity as possible.

It is said that Ag85B is one of the proteins that are abundantly secreted if infection with mycobacterium tuberculosis is established (12). According to the results obtained, the peptide Ag85B 234-263 is interesting. If testing more peptides from the protein an epitope which the antibodies bind strongly to may be found. It could be more efficient to use the whole protein than just a peptide, due to the 3D structure the protein forms which makes the immune system respond in a stronger way than just with a part of the protein.

The rest of the peptides did not give any significant results with anti-IgG. Since the whole protein sequences were not tested it could be that the sequence which the immune system responds to was not among the tested peptides.

It also could be because the antibodies the immune system makes bind to a folded conformation of the protein and not a linear peptide sequence. Therefore it is important to test the whole proteins with the tuberculosis sera since it is established that the proteins which the peptides are made from have been found in other researches (2, 3, 8, 11, 15, 16).

One thing that could be seen with the different treated tuberculosis sera is that the sera treated with 0.4% Triton X-100 solution gave much lower signals than the heat treated sera. This may be due to aggregation of the antibodies upon heat treatment and therefore giving higher MFI values than it should. These data are more likely to be misleading than those from the Triton treated sera.

Maybe the tuberculosis sera should, if handled very carefully, not be treated before adding them to the wells in order to prevent falsely data.

With the results obtained from the experiments the aim of this thesis could not be answered.
There were no significant differences between tuberculosis sera or blood donor sera, but that does not mean that the proteins used, which the peptides were derived from, are not part of the immune response in human bodies. As mentioned earlier it might be due to that the conformation of the whole protein is the important factor in detection of the pathogen by our B-cells.

Since this experiment is just a tentative attempt of finding peptides that can be linked to active and latent mycobacterium tuberculosis phase and our production of antibodies against the bacteria it is important to repeat and refine the experiment. Recombinant proteins could be a better choice of antigens and therefore it would be interesting to investigate whole proteins to see if the antibody reaction would be greater than the antibody reaction against the peptides.

It also would be interesting to see if IgM antibodies give higher antibody signals than the IgG antibodies. Since IgM is known to be the antibodies that first appear upon infection it may be that there is more IgM than IgG antibodies in the sera, therefore a higher antibody level may point to some of the peptides/proteins being more virulent than other peptides/proteins.

According to yet unpublished data from Borbala Katona (candidate thesis project 2012, Department of Clinical Virology) IgM can give a stronger reaction for auto-antigens. The results with mycobacterium tuberculosis peptides and IgM, conducted by Amal Elfaitouri (Department of Clinical Virology), are similar to those results obtained by Katona. It is possible mycobacterium tuberculosis uses mechanisms to fool the immune system that its antigens are “self”, thereby avoiding a potent humoral immune response.

A facilitation of the project would be to begin with defined patient sera, and not a set of sera with controls, in order to find the peptides or proteins that contributes to our immune response and later on try those peptides/proteins on sera that have a hidden status to evaluate if the peptides/proteins could be used in specific detection of sera with mycobacterium tuberculosis.

According to Luciw et al.’s (10) work it is seen that with some refinements and adjustments the method used with detection of antibodies by coupling the proteins to microsphere beads could be useful if identifying the correct antigens that the human immune response react to. Therefore we should not give up the hope of finding the peptides/proteins that our bodies would react to when infected with mycobacterium tuberculosis. Just because the peptides could not be substantiated in this experiment it does not mean that they do not have something to do with the virulence of mycobacterium tuberculosis or the human immune response against the bacteria.
References


