Dubious Role Of Mycobacterium Paratuberculosis In Pathogenesis Of Type I Diabetes

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DUBIOUS ROLE OF *MYCOBACTERIUM PARATUBERCULOSIS* IN PATHOGENESIS OF TYPE I DIABETES

by

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ABSTRACT

***Background**:* Type 1 Diabetes mellitus (TIDM) is a chronic disorder in which the insulin producing beta cells are selectively self-destroyed. Although the etiology of the disease has not been determined, genetic dispositions such as *SLC11A1* polymorphism in suffering patients have been reported. The role of pathogenic microorganisms such as *Mycobacterium avium subspecies paratuberculosis* (MAP) in TIDM has also been recently debated. MAP is already known to cause paratuberculosis in cattle and now it is a strong suspect of causing autoimmune diseases in humans such as Crohn's disease, multiple sclerosis, autoimmune Thyroiditis, rheumatoid arthritis and autoimmune diabetes. We hypothesis that molecular mimicry between MAP Heat shock protein 65K (*Hsp65*) and human Glutamic Acid Decarboxylase 65K (*GAD65*) can be the trigger which leads to the autoimmune destruction of beta cell in patients exposed to MAP .

Method: To test the hypothesis, peptide sequences of MAP *Hsp65* and human *GAD65* were investigated using BLAST and PyMOL bioinformatics tools. Moreover, 18 blood samples from humans with TIDM and controls, and 100 sera samples from cattle with paratuberculosis and controls were evaluated for the presence of MAP, MAP DNA and its antibodies. Glucose, insulin and *GAD65* antibodies were also determined in some of the clinical samples.

Results: Peptide BLAST analysis revealed 44% identity between the two proteins with 75% positive identities in a 16 amino acid region. PyMOL structural analysis identified possible shared epitope regions of the proteins in its 3D conformation. Immunoblot analysis revealed a strong cross reactivity between lysate of *E.coli* recombinant of MAP *Hsp65* and plasma from human subject with TIDM. A weak cross reactivity was also observed between healthy rat pancreatic homogenate and rabbit anti MAP IgG. Nested PCR using IS900-specific

oligonucleotide primers did not detect MAP DNA in peripheral blood from 18 subjects with Type I Diabetes, Type II Diabetes and non-diabetic controls. Long term culture of leukocytes from blood samples from same subjects resulted in the presence of MAP in 3/10 (30%) TIDM and 4/8 (50%) control subjects. However anti MAP IgG were detected in 5/10 (50 %) TIDM samples compared to 3/8 (37.5 %) controls. Insulin level was measured in sera from paratuberculosis cattle and controls. In MAP infected cattle, insulin level ranged from below 0.1ng/ml to 2.456 ng/ml with an average of 0.36 +/- 0.57ng/ml compared to below 0.1ng/ml to 13.47ng/ml with an average of 2.86 +/- 3.00ng/ml in healthy cattle.

Conclusion: Bioinformatics analysis between MAP *Hsp65* and human *GAD65* through BLAST and PyMOL analysis revealed a homology of 16 amino acid motif and possible shared epitope regions; immunohistochemistry analysis revealed a cross reactivity between rabbit anti-MAP IgG and pancreatic cell homogenate from a healthy rat. Moreover, plasma from patient with TIDM (TD8), who was confirmed to be positive for MAP DNA and MAP IgG, reacted strongly with MAP *Hsp65* in MAP protein lysate and MAP *Hsp65* recombinant clone pmptb20. Culture of MAP from human leukocytes is significant despite the lack of correlation between MAP in samples from TIDM and controls. It is worth noting that some of the control subjects have not been evaluated for other autoimmune diseases possible MAP role. Additionally, anti-MAP IgG levels in TIDM subjects compared to controls have raised a suspicion on the involvement of MAP in TIDM. The poor correlation of MAP in blood *versus* either the anti-MAP IgG or the insulin level may be related with the fastidious nature of MAP and *in vitro* cultivation. Since MAP is the sole causative agent of Johne's disease, it is significant that the insulin level is 8 folds less in MAP infected cattle compared to MAP free cattle. Overall, the data

is mixed and suggest that further study is needed to investigate the intriguing question to whether MAP is involved in TIDM or not.

Dedicated to my dear parents and brother who have been very supportive and encouraging in each and every step of my life.

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LIST OF ACRONYMS/ABBREVIATIONS

BB Rat- Bio Breeding Rat

BLAST-Basic Local Alignment Search Tool

CD4-Cluster of Differentiation 4

CD8- Cluster of Differentiation 8

DAB- 3,3'-Diaminobenzidine

dATP-2'-deoxyadenosine triphosphate

dCTP-2'-deoxycytidine triphosphate

dGTP-2'-deoxyguanosine triphosphate

dH20- Distilled Water

DNA- Deoxyribonucleic Acid

dTTP-2'-deoxythymidine triphosphate

EDTA-Ethylenediaminetetraacetic Acid

ELISA- Enzyme Linked Immunosorbent Assay

GABA- Gamma Amino Butyric Acid

GAD67- Glutamic Acid Decarboxylase 67

GAD-Glutamic Acid Decarboxylase

HCl- Hydrochloric Acid

HLA-Human Leukocyte Antigen

HRP- Horse Radish Peroxidase

Hsp-Heat Shock Protein

IA-2- Insulinoma Associated proteins 2

IDDM- Insulin Dependent Diabetes Mellitus

IFN-γ-Interferon γ

IgG- Immunoglobulin G

IL2- Interleukin 2

Kb-Kilobase

KDa- Kilodalton

MAP-*Mycobacterium avium subspecies paratuberculosis*

$MgCl_2$-Magnesium Chloride

MGIT-Mycobacteria Growth Indicator Tube

MHC-Major Histocompatibility Complex

Mtb-*Mycobacterium tuberculosis*

NOD- Non Obese Diabetic

OADC-Oleic Albumin Dextrose Catalase

PBS- Phosphate Buffered Saline

PBST-Phosphate Buffered Saline-Tween 20

PCR- Polymerase Chain Reaction

RPM- Rotation Per Minute

SDS-PAGE-Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SLC11A1- Solute carrier 11A1

TEMED- Tetramethylethylenediamine

Th1- T helper cell

TIDM- Type I Diabetes Mellitus

TIIDM- Type II Diabetes Mellitus

TMB-3,3',5,5'-tetramethylbenzidine

TNF-α- Tumor Necrosis Factor-α

TNF-β- Tumor Necrosis Factor- β

UV-Ultraviolet

VDR- Vitamin D Receptor

CHAPTER ONE: INTRODUCTION

Type I Diabetes Mellitus

Type I diabetes mellitus is a chronic disease in which the insulin producing beta cells of the pancreas are selectively destroyed due to T lymphocyte infiltration[1-3]. The disease is also known as juvenile diabetes as it is most commonly diagnosed in children and young adults. It is the second most chronic disease of the childhood which accounts to only 5 to 10 % of all diabetic cases[1]; however there is a worldwide increase in the incidence of the disease. Frequent urination, excessive thirst, extreme hunger, unusual weight loss, extreme fatigue, itchy skin, poor wound healing, blurred vision and irritability are the most common symptoms of the disease [1].

According to the American Diabetes Association the disease can be broadly classified into two forms: (a) type IA which is due to autoimmune destruction of the beta cell and is usually characterized by the presence of auto-antibodies against host proteins such as insulin, heat shock protein 60, insulinoma associated proteins (IA-2) and glutamic acid decarboxylase (*GAD65*) and (b) type IB which is less frequent and has no known cause yet. Insulin replacement therapy is the only treatment available for the disease [1, 4].

Insulin is a hormone produced by endocrine beta cells of the pancreas. It regulates carbohydrate and fat metabolism by its uptake into liver, muscle and adipose tissues and storing it as glycogen [5]. In TIDM patients, insulin deficiency is created due to autoimmune destruction of the beta cells [6]. This leads to impaired glucose and fat metabolism leading to its accumulation in blood and urine[1]. Persistence of hyperglycemic condition for a prolonged period leads to serious conditions such as diabetic nephropathy, diabetic neuropathy, diabetic retinopathy, cardio vascular ailments and stroke [1].

Pathogenesis of the disease

In the 1980s Eisenbarth postulated the development of immune form of type I diabetes. The hypothesis suggests that every person is born with diverse degrees of susceptibility of acquiring TIDM[1]. These significant susceptibility factors are inherited and it mainly includes the HLA genotypes DR and DQ. IDDM genes are also among the genes responsible for the pathogenesis of the disease but to the least extent [1, 7, 8]. Though predisposition of genetic factors play a major role in pathogenesis of TIDM; concordance study of the monozygotic twins, rapid increase in the incidences of the disease over the last 50 years and the migrant study suggests that there is a promising role of exogenous factors as well [9]. It has been found in past few decades that viruses such as enterovirus and coxsackie and bacteria such as *Mycobacterium avium subspecies paratuberculosis* can act as a possible trigger for generating the autoimmune response [10, 11]. Other environmental factors that may play a possible role as a trigger for the disease includes cow's milk, wheat protein and vitamin D[7, 12].Though etiology of the disease are being extensively studied using animal models such as non-obese diabetic(NOD) mouse and Bio Breeding (BB) rat, but the exact trigger of how the auto immune response is generated during the disease is still not clearly known[13].

Role of MAP in TIDM

MAP is an occult antigen that acts as a trigger for various autoimmune diseases such as multiple sclerosis, autoimmune Thyroiditis, rheumatoid arthritis and autoimmune diabetes[14]. The role of MAP in pathogenesis of TIDM has always been debatable. There are increasing evidences of shared genetic susceptibility between TIDM and Mycobacterial infection that supports the role of MAP infection as a possible trigger for the disease [6, 15, 16]. The genes

that are found to have common susceptibility to TIDM and Mycobacterial infections are *SLC11A1*, and *VDR* [10, 17]. *SLC11A1* (Solute carrier 11a1) gene encodes an integral membrane protein of the lysosomal part of monocyte and macrophages[17]. The membrane protein functions as a cation transporter which helps in maintaining iron homeostasis[17]. During infection the *SLC11A1* causes acidification of phagosome which helps protect the host against infection. But mutation of *SLC11A1* gene leads to malfunction of the protein leading to creation of suitable environment for bacterial infestation. It has been observed that *SLC11A1* gene polymorphism is coupled with MAP infection and TIDM in Sardinian population[17]. Vitamin D Receptor (VDR) is a protein commonly expressed in immune cells such as lymphocytes and macrophages. It is also widely expressed in β cells of pancreas. Despite its role in regulation of bone and mineral metabolism, Vitamin D is also a potent modulator of immune system [10]. Recently it has been implicated in the pathogenesis of TIDM and susceptibility to Mycobacterial infections[9]. There are speculations of epitope sharing between the host proteins and MAP proteins that might act as a possible trigger for activation of auto immune response [14, 18, 19]. Moreover, elevated immune response against MAP specific proteins such as MAP3733c and MAP3738c has been observed in TIDM population of Sardinia [6, 20].

Mechanism of MAP infection

MAP is an obligate intracellular pathogen that affects a broad range of host. MAP infection leads to Paratuberculosis in ruminants. It is also implicated to cause Crohn's disease in human which is also an inflammatory bowel disease [9]. Crohn's disease has similar clinical symptoms as that of Johne's disease in cattle. MAP is a slow growing, acid fast bacterium that is surrounded by lipid rich cell envelopes. It has been found in pasteurized milk, surface water, soil

and cow manure which is used as a fertilizer in agricultural lands[9]. *Mycobacterium* infests the host organism via the fecal-oral route. Later it invades the mucosa associated lymphoid tissue of the small intestine. Immune responses are elicited in the infected host and the bacteria are endocytosed by M cells of Peyer's patch which is then phagocytosed by intra epithelial macrophages. The macrophage gets activated and results in inflammation of the tissue which is one of the clinical symptoms of the disease [4]. Usually the affected patients suffer from severe abdominal cramps, diarrhea, fever and significant weight loss [21]. MAP infection induces autoimmune reaction in the host system and one of the predicted mechanisms of it is molecular mimicry between the host and bacterial protein. It has been postulated that molecular mimicry between MAP *Hsp65* and human *GAD 65* might play a major role in inducing the autoimmune reaction during pathogenesis of TIDM [10].

Function of Mycobacterial Heat Shock Protein 65

Heat shock proteins are the chaperone protein that helps in protein folding and is constitutively expressed in the cells [9]. The HSPs are synthesized largely during stressful conditions like infection, exposure to heat or ultra violet radiation; thus helps the cells to survive [9]. Besides its protective role in the cell, molecular mimicry between the bacterial HSPs and host proteins leads to autoimmune reactions in the host system eventually resulting in disease state [14]. The role of Mycobacterial *Hsp65* has been implicated in many autoimmune diseases such as rheumatoid arthritis, autoimmune hepatitis, Kawasaki disease, scleroderma, Behcet disease and Takayasu's arteritis [14]. It has been found to activate the macrophages that might lead to production of cytokines such as TNF-α, Il-6 and reactive nitrogen intermediates which might be harmful to the cells [13]. In case of TIDM, it has been proposed that molecular mimicry

between MAP *Hsp65* and human *GAD 65* might trigger a possible autoimmune reaction leading to insulin deficiency [9, 14].

Glutamic Acid Decarboxylase 65 and its significance

Glutamic acid decarboxylase is an enzyme that catalyzes the synthesis of gamma-amino butyric acid (GABA) by α-decarboxylation of L-glutamic acid [22, 23]. There are two isoforms of GAD found – *GAD65* (65 KDa) and *GAD67* (67 KDa). Both the isoforms are synthesized as hydrophilic soluble molecules in the cytoplasm but *GAD65* is post translationally modified and is anchored to the membranes of pancreatic cells[22]. Pyridoxal phosphate acts as the cofactor for the enzyme. Despite central and peripheral nervous system, GAD is also expressed in pancreatic islets, testes, ovaries, thymus and stomach. *GAD65* specifically is expressed in the islets of pancreas and is considered as one of the major auto-antigen involved in the pathogenesis of the disease. Humoral immune response was observed against *GAD65* in TIDM patients[22].

Proposed mechanism of autoimmune destruction of Beta cells

Infection of the host by a pathogen such as bacteria or virus leads to the activation of B cells. The antigens are processed by macrophages and presented to T helper cells in association with MHC class II molecules. These activated macrophages secrete IL-2 which activates Th1 type CD4+ T cells. CD4+ T cells releases a wide range of cytokines like IFN-γ, TNF-α, TNF-β and IL-2. Simultaneously the T precytotoxic cells are recruited to the β cells. These recruited cytotoxic cells are differentiated into CD8+ effector cells by IL2 and the cytokines released by CD4+ T cells. IFN-γ released by CD4+ cells induces the differentiation of macrophages into cytotoxic macrophages. These cytotoxic macrophages releases β cell toxic free radicals and

cytokines that destroy the β cell by Fas mediated apoptosis. The toxic granzyme and cytolysin also mediate destruction of β cells [3, 13, 24].

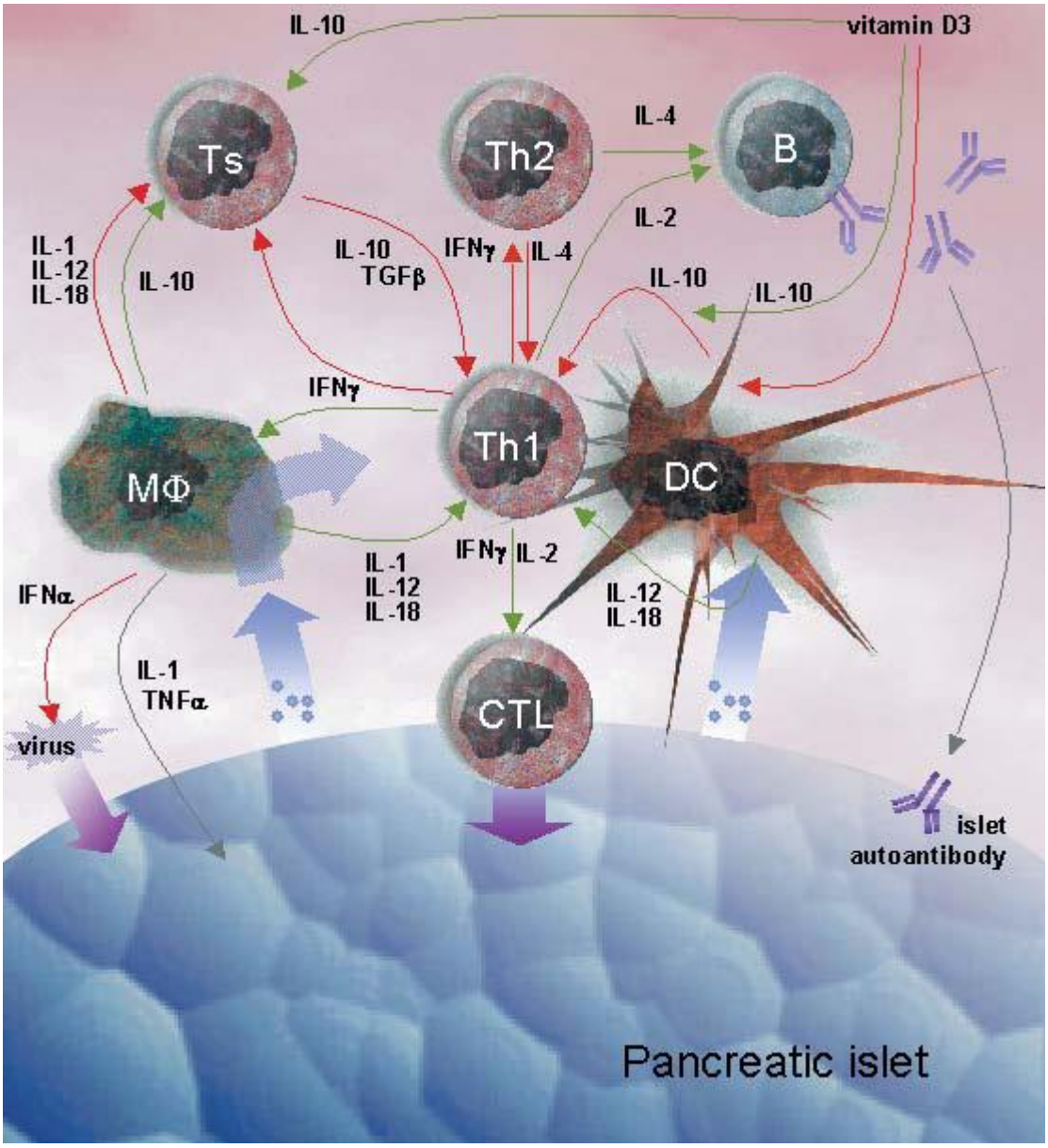

Figure 1: Mechanism of autoimmune destruction of pancreatic β cells.

Figure adapted from [25].

CHAPTER TWO: MATERIALS AND METHODS

Bioinformatics Analysis

Protein BLAST analysis was initialized to determine the sequence homology between peptide sequences of MAP *Hsp65* and human *GAD 65* (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The peptide sequences of the proteins for the analysis were retrieved from Uniprot protein database (http://www.uniprot.org/). PyMOL visualization tool was then used to localize the homologous sequences of the proteins in their 3D conformation. Predicted protein structures for PyMOL analysis were retrieved from the protein data bank (http://www.rcsb.org/pdb/home/home.do).

Evaluation of cross reactivity between MAP *Hsp65* and *GAD 65*

The antigenic cross reactivity between the proteins *Hsp65* and *GAD65* was evaluated by immunoblot method. The lysate from *Hsp65 E. coli*-recombinant clone and pancreatic homogenates from diabetic and control rat were diluted in 2X sample loading buffer(3.55 ml dH20, 1.25 ml 0.5M Tris-HCl pH 6.8, 2.5 ml glycerol, 2 ml 10% SDS, 0.2 ml 0.5% bromophenol blue). The samples were boiled and then loaded in appropriate wells of 12% SDS-PAGE gel. The gel was run for 50 minutes at 110 V for the resolution of proteins in the sample according to the standard molecular weight. The resolved proteins were transferred to the nitrocellulose membrane using a Bio-Rad electro blotting apparatus at 85 V for 45 minutes. Membranes were then blocked by incubating them with PTM (PBST with 3% dry milk) at room temperature for 1 hr with gentle shaking. They were then incubated with goat polyclonal antibody to human *GAD65* (1:500), rabbit anti-MAP polyclonal antibodies (1:250), human sera containing anti-*GAD65* (1:50) and bovine sera containing anti MAP antibodies (1:50) in different

containers throughout night at 4°C with gentle shaking. On the following day the primary antibody was discarded and the membranes were washed with PBS-T for 5 min at room temperature. Mouse anti human IgG conjugated with horse radish peroxidase (HRP) (1:100) and goat anti rabbit with conjugated HRP (1:500) diluted in PTM was added respectively and incubated at room temperature for 1.5 hours with gentle shaking. The membranes were then washed thrice with PBS-T for 10 min and once with PBS for 10 min. It was then developed by using a developing solution prepared by dissolving a tablet of diaminobenzidine in 15 ml of 1X tris buffered saline and 120 µl of 3% hydrogen peroxide filtered through 0.2µm filter. The membranes were incubated in the developing solution for 30 minutes and later documented.

Clinical Samples

EDTA blood was collected from a total of 29 subjects. The source, diagnosis and demographic information for the samples are listed in Table 1. Out of 18 subjects under study, 10 were TIDM and 8 were control subjects (with 2 T2DM). All the subjects considered for the study were non inflammatory bowel disease patients. Informed consent was obtained from subjects in accordance with the Institutional Review Board (IRB) regulations. Whole blood sample was drawn into sterile K2 EDTA vacutainer tube. The samples were coded to mask the identity and diagnosis of the subject. Bovine sera were kindly received from Dr.Mike Collin. One hundred sera samples were out of which 50 samples were MAP positive and 50 samples were control.

Table 1: Demographic characteristics of clinical samples from human participants

Code	Sex	Diagnosis	Source	Specimen
TD 3	F	TIDM	UF	Whole Blood
TD 4	M	TIDM	UF	Whole Blood
TD 5	F	TIDM	UF	Whole Blood
TD 6	M	TIDM	UF	Whole Blood
TD 7	M	TIDM	UF	Whole Blood
TD 8	F	TIDM	UF	Whole Blood
TD 9	M	TIDM	UF	Whole Blood
TD 14	F	TIDM	UF	Whole Blood
TD 15	M	TIDM	UF	Whole Blood
TD 18	F	TIDM	UF	Whole Blood
TD 1	M	T2DM	UF	Whole Blood
TD 2	M	T2DM	UF	Whole Blood
TD 10	F	CONTROL	UF	Whole Blood
TD 11	F	CONTROL	UF	Whole Blood
TD 12	F	CONTROL	UF	Whole Blood
TD 13	F	CONTROL	UF	Whole Blood
TD 16	F	CONTROL	UF	Whole Blood
TD 17	M	CONTROL	UF	Whole Blood

Table 2: Summary of human samples used in the study

Diagnosis	TIDM	T2DM	Control
Total	10	2	6
Male	5	2	1
Female	5	-	5

Table 3: Demographic characteristic of samples from cattle used in the study

Sample code	Specimen	Sample code	Specimen
P1	Serum	N1	Serum
P2	Serum	N2	Serum
P3	Serum	N3	Serum
P4	Serum	N4	Serum
P5	serum	N5	serum
P6	serum	N6	serum
P7	serum	N7	serum
P8	serum	N8	serum
P9	serum	N9	serum
P10	serum	N10	serum
P11	serum	N11	serum
P12	serum	N12	serum
P13	serum	N13	serum

Sample code	Specimen	Sample code	Specimen
P14	Serum	N14	Serum
P15	Serum	N15	Serum
P16	Serum	N16	Serum
P17	Serum	N17	Serum
P18	Serum	N18	Serum
P19	Serum	N19	Serum
P20	Serum	N20	Serum
P21	Serum	N21	Serum
P22	Serum	N22	Serum
P23	Serum	N23	Serum
P24	serum	N24	serum
P25	Serum	N25	Serum
P26	Serum	N26	Serum
P27	Serum	N27	Serum
P28	Serum	N28	Serum
P29	Serum	N29	Serum
P30	Serum	N30	Serum
P31	Serum	N31	Serum
P32	Serum	N32	Serum

Sample code	Specimen	Sample code	Specimen
P33	Serum	N33	Serum
P34	Serum	N34	Serum
P35	Serum	N35	Serum
P36	Serum	N36	Serum
P37	Serum	N37	Serum
P38	Serum	N38	Serum
P39	Serum	N39	Serum
P40	Serum	N40	Serum
P41	Serum	N41	Serum
P42	Serum	N42	Serum
P43	Serum	N43	Serum
P44	Serum	N44	Serum
P45	Serum	N45	Serum
P46	Serum	N46	Serum
P47	Serum	N47	Serum
P48	Serum	N48	Serum
P49	Serum	N49	Serum
P50	Serum	N50	Serum

Isolation of peripheral leukocytes

The EDTA blood tubes were received cold within 24 hours since the blood was withdrawn from the subjects. The blood was processed by centrifugation at 3000 rpm for 10 minutes at room temperature. Tri layered separation was formed. A volume of 1mL of plasma was transferred to a new sterile tube and stored at -70̊ C for future testing for anti-MAP/anti-GAD, glucose and insulin. The leukocyte containing buffy coat middle layer was carefully transferred to a new sterile tube. The leukocytes were then mixed with two volumes of red blood cell lysis buffer purchased from Roche Applied Sciences, IN, USA. The tubes were mixed thoroughly and then incubated at room temperature for 10 minutes on an automatic inverter. The hemolyzed samples were then centrifuged at 2500 rpm for 5 minutes at room temperature. The supernatant was discarded and the leukocyte pellet was either inoculated into culture media or stored at -20̊ C for further use[26].

Culture of Peripheral leukocytes

The purified buffy coat sample was inoculated into Mycobacterial Growth Indicator Tubes (MGIT) para tubes purchased from Becton Dickinson Diagnostic systems, MD, USA. MGIT is a MAP selective media containing 4mL of modified Middlebrook 7H9 broth base. Approximately 1L of the medium contains 5.9g of Modified Middlebrook 7H9 Broth base and 1.25g of casein peptone. In addition, the media was supplemented with 800µl of OADC supplement purchased from Becton Dickinson Diagnostic systems, MD, USA and 500µl of egg yolk enrichment (Becton Dickinson Diagnostic systems, MD, US). All the inoculated tubes were incubated at 37̊ C incubator. . Following 12 weeks incubation, aliquot were subjected to DNA

extraction and PCR analysis for MAP DNA detection was performed. The process was repeated after further culture incubation for a total of six months.

DNA extraction from peripheral leukocytes

The isolated leukocytes were re-suspended in Tris EDTA (TE) buffer and then incubated in a heating block at 100°C for 30 minutes. The samples were then cooled by placing atop ice bath for 15 min. The tubes were then centrifuged at 12,000 RPM for 10 minutes. The supernatant was then transferred to 2 ml Phase lock gel tubes (PLG, Eppendorf, Westbury, N.Y., USA). 200µl of phenol/chloroform/isoamyl- alcohol (1:1:124 v/v; Acros Organic Morris Plains, N.J., USA) was added to each tube and then centrifuged at 12,000 RPM for 5 minutes at 4°C. The supernatant was carefully extracted to a fresh sterile 1.5 ml micro centrifuge tube.400µl of 100% chilled ethanol was added and the tubes were stored overnight at -20° C. On the following day, the tubes were centrifuged at 14,000 RPM for 20 minutes. The supernatant was extracted and discarded without disturbing the pellets. The pellets were washed with 400µl of 80% chilled ethanol by re-centrifuging at 14000 RPM for 20 minutes. The supernatant is again discarded and the pellets are vacuum dried for 15 minutes. Finally the DNA is re-suspended in 50µl sterile Millipore filtered water and stored overnight at 4° C before further usage.

Nested PCR for MAP DNA detection

DNA extracted from the above procedure was subjected to nested PCR. The first round of PCR reaction mixture consists of 25µl of Master Mix (50units/ml- *Taq*DNA polymerase, 400µM-each dATP,dGTP,dCTP,dTTP and 3mM $MgCl_2$; Promega , Madison, WI, USA), 1µl of each primer (Forward primer: P90-5'-GTTCGGGGCCGTCGCTTAGG-3', Reverse primer: P91-5'-GAGGTCGATCGCCCACGTGA-3') and 23µl of DNA template. In the second round of

PCR reaction there was two sets of reactions which differed by the volume of the DNA template used. First set of the reaction mixture consisted of 25 µl of Master Mix, 1µl of each primer (Forward primer: AV1-5'ATGTGGTTGCTGTGTTGGATGG'3, Reverse primer: AV2-5'CCGCCGCAATCAACTCCAG 3') and 23 µl of DNA template which is the PCR product of the first round of reaction. The second set of the reaction mixture consists of the same except that 1µl of PCR product from the first reaction was used as a template and the total volume was made to 50µl by addition of nuclease free water. The cycle conditions of the primary reaction were: 95℃ for 5 min, 35 cycles of 95℃ for 1 min, 58℃ for 1.5 min, 72℃ for 1.5 min and final extension of 10 min at 72℃. The cycle conditions of the secondary reaction were: 95℃ for 5 min, 35 cycles of 95℃ for 0.5 min, 60℃ for 0.5 min, 72℃ for 1min and final extension of 10 min at 72℃.The PCR product was analyzed on 2% agarose gel. Agarose gel results were analyzed using ultraviolet light and were documented using Biosens SC 645 gel documentation system (Andromeda, FL, USA) and pictured using PS Remote software installed in the computer.

Detection of MAP IgG antibodies

The IDEXX MAP antibody test kit (IDEXX Laboratories, Westbrook, ME, USA) for cattle was modified for determining the MAP antibody levels in human samples[27, 28]. All the reagents were brought to room temperature before starting with the assay. The samples and controls were diluted fifty (50) fold with the sample diluents. A volume of 100µl of each sample and controls were dispensed in the respective wells. The plate was then covered and incubated at room temperature for 45 min. Mean while required volume of wash buffer was prepared by diluting appropriate quantity of wash concentrate to twenty (20) fold with distilled water. Also secondary antibody was prepared by diluting the anti human IgG in conjugate diluents to

hundred (100) fold. All the liquid in the wells were then aspirated into appropriate waste reservoir and the wells were washed with diluted wash solution for 5 times. Caution was taken to avoid plate drying. All the liquids were then aspirated into appropriate waste reservoir.100µl of diluted anti human IgG was dispensed into each well and then incubated for 30 min at room temperature. A volume of 100 µl of TMB substrate was added to each well after all the wells were washed 7-8 times with wash buffer. The plate was then incubated for 10 min at room temperature. A volume of 100µl of stop solution was added to each well to stop the reaction .Absorbance was recorded at 450nm using a microplate reader (Model680, Bio-Rad laboratories Inc, CA, USA) within 30 min of stopping the reaction.

Figure 2: Reagents and components of IDEXX MAP detection kit.

Figure adapted from website of IDEXX laboratories Inc.

Measurement of Insulin

The concentration of insulin in the plasma samples were determined by using the ultrasensitive mouse insulin ELISA kit (Crystal Chem Inc., IL, USA) following the manufacturers protocol with necessary adaptations. A volume of 95µl of sample diluents was added to the antibody coated plate. Following which 5µl of insulin standards and the samples

were added and the plate was incubated for 2 hours at 4℃. The plate was then washed 5times with wash buffer. 100µl of 1:100 diluted anti human IgG was dispensed into each well. The microplate was then covered and incubated at room temperature for 30 min. The plate was then washed for 7-8 times with wash solution and any remaining solution was removed by inverting and tapping the plate but at the same time precautions were taken to avoid plate drying. 100µl of enzyme substrate was added to each well and then incubated in dark at room temperature for 10 min. The reaction was stopped by addition of 100µl stop solution. Absorbance was measured at 450nm within 30 min of stopping the reaction using a microplate reader (Model680, Bio-Rad laboratories Inc, CA, USA). The mouse insulin stock solution (64ng/ml) was used to establish the standards for calculation of concentration of insulin in plasma samples.

Measurement of Glucose

Glucose concentration in the plasma samples were measured using liquid Glucose (Hexo) kit (Pointe Scientific Inc., MI, USA). A volume of 10µl of plasma was added to 1ml of glucose reagent in a micro centrifuge tube. The tube was vortex and then incubated in heating block at 30℃ for 3 min. Absorbance value was measured at 340nm using a spectrophotometer (Smart spec 3000, Bio-Rad, CA, USA). Glucose standard of known concentration (100mg/dl) was used as a standard to calculate the concentration of glucose in plasma samples.

CHAPTER THREE: RESULTS

Bioinformatical analysis of sequence homology between MAP *Hsp65* and *GAD65*

Hsp65 is among the most widely studied *Mycobacterial* proteins and is suggested to be the basis for autoimmune reaction in inflammatory bowel diseases [29]. It has been suggested as a strong cytokine stimulator thus a strong stimulator of immune system [30]. The function of *Hsp65* is to promote proper folding of cellular proteins in an ATP dependent cycle[30]. The molecular weight of the *Hsp65* protein in MAP and other *Mycobacterial* species is about 65 KDa [29]. Specifically MAP *Hsp65* encodes 541 amino acids and Mtb *Hsp65* encodes for 540 amino acids with both expressing an estimated 65KDa protein (http://www.uniprot.org/). Although Mtb *Hsp65* is well characterized including the determination of its 3D conformational structure, MAP Hsp65 characterization including 3D conformational structure is yet to be determined [29]. In this study, Mtb Hsp65 peptide sequence was first blasted with MAP *Hsp65*.BLAST analysis revealed a homology of 96% between the two proteins as shown in Figure 6. BLAST analysis between Mtb Hsp65 and human GAD65 was also done to find the homology between the two peptide sequences. The BLAST analysis between the peptide sequences revealed a 44 % identity between the two proteins with 75% positive identity in a 16 amino acid region as in case between MAP *Hsp65* and human *GAD65* shown in Table 5. Furthermore, proteins' sequences of MAP *Hsp65* and human *GAD65* were retrieved from Uniprot protein database (http://www.uniprot.org/) in FASTA format as shown in Figure 3 and Figure 4, respectively in order to perform BLAST analysis between the two peptide sequences. The BLAST analysis revealed a 44 % identity between the MAP Hsp65 and human GAD65 with 75% positive identity in a 16 amino acid region as shown in Table 3. Interestingly, BLAST analysis revealed a 96%

homology between MAP Hsp65 and Mtb Hsp65 (Figure 6) thus correctly justifying the use of Mtb Hsp65 for further PyMOL analysis. Predicted protein structure was retrieved from Protein Data Bank (http://www.rcsb.org/pdb/home/home.do) and used further in this study.

PyMOL visualization tool was used to localize and identify the identical peptide region between the protein sequences of Mtb *Hsp65* and human *GAD65* .As shown in Figure 7 the PyMOL analysis revealed the localization of 16 amino acid epitope of human *GAD65*.The latter has been reported to be a known potential antigenic site and implicated in production of autoantibodies in TIDM [31].

>sp|P42384|CH602_MYCPA 60 kDa chaperonin 2 OS=Mycobacterium paratuberculosis (strain ATCC BAA-968 / K-10) GN=groL2 PE=3 SV=4
MAKTIAYDEEARRGLERGLNALADAVKVTLGPKGRNVVLEKKWGAPTITNDGVSIAKEIE
LEDPYEKIGAELVKEVAKKTDDVAGDGTTTATVLAQALVREGLRNVAAGANPLGLKRGIE
KAVEKVTETLLKSAKEVETKDQIAATAAISAGDQSIGDLIAEAMDKVGNEGVITVEESNT
FGLQLELTEGMRFDKGYISGYFVTDAERQEAVLEDPFILLVSSKVSTVKDLLPLLEKVIQ
AGKPLLIIAEDVEGEALSTLVVNKIRGTFKSVAVKAPGFGDRRKAMLQDMAILTGGQVIS
EEVGLSLESADISLLGKARKVVVTKDETTIVEGAGDSDAIAGRVAQIRTEIENSDSDYDR
EKLQERLAKLAGGVAVIKAGAATEVELKERKHRIEDAVRNAKAAVEEGIVAGGGVALLHA
IPALDELKLEGEEATGANIVRVALEAPLKQIAFNGGLEPGVVAEKVRNSPAGTGLNAATG
EYEDLLKAGIADPVKVTRSALQNAASIAGLFLTTEAVVADKPEKAAAPAGDPTGGMGGMD
F

Figure 3 : Peptide sequence of MAP *Hsp65* in FASTA format

Source: http://www.uniprot.org/[32]

>sp|Q05329|DCE2_HUMAN Glutamate decarboxylase 2 OS=Homo sapiens GN=GAD2 PE=1 SV=1
MASPGSGFWSFGSEDGSGDSENPGTARAWCQVAQKFTGGIGNKLCALLYGDAEKPAESGG
SQPPRAAARKAACACDQKPCSCSKVDVNYAFLHATDLLPACDGERPTLAFLQDVMNILLQ
YVVKSFDRSTKVIDFHYPNELLQEYNWELADQPQNLEEILMHCQTTLKYAIKTGHPRYFN
QLSTGLDMVGLAADWLTSTANTNMFTYEIAPVFVLLEYVTLKKMREIIGWPGGSGDGIFS
PGGAISNMYAMMIARFKMFPEVKEKGMAALPRLIAFTSEHSHFSLKKGAAALGIGTDSVI
LIKCDERGKMIPSDLERRILEAKQKGFVPFLVSATAGTTVYGAFDPLLAVADICKKYKIW
MHVDAAWGGGLLMSRKHKWKLSGVERANSVTWNPHKMMGVPLQCSALLVREEGLMQNCNQ
MHASYLFQQDKHYDLSYDTGDKALQCGRHVDVFKLWLMWRAKGTTGFEAHVDKCLELAEY
LYNIIKNREGYEMVFDGKPQHTNVCFWYIPPSLRTLEDNEERMSRLSKVAPVIKARMMEY
GTTMVSYQPLGDKVNFFRMVISNPAATHQDIDFLIEEIERLGQDL

Figure 4: Peptide sequence of human *GAD65* in FASTA format

Source: http://www.uniprot.org/[32]

>sp|P0A520|CH602_MYCTU 60 kDa chaperonin 2 OS=Mycobacterium tuberculosis GN=groL2 PE=1 SV=2
MAKTIAYDEEARRGLERGLNALADAVKVTLGPKGRNVVLEKKWGAPTITNDGVSIAKEIE
LEDPYEKIGAELVKEVAKKTDDVAGDGTTTATVLAQALVREGLRNVAAGANPLGLKRGIE
KAVEKVTETLLKGAKEVETKEQIAATAAISAGDQSIGDLIAEAMDKVGNEGVITVEESNT
FGLQLELTEGMRFDKGYISGYFVTDPERQEAVLEDPYILLVSSKVSTVKDLLPLLEKVIG
AGKPLLIIAEDVEGEALSTLVVNKIRGTFKSVAVKAPGFGDRRKAMLQDMAILTGGQVIS
EEVGLTLENADLSLLGKARKVVVTKDETTIVEGAGDTDAIAGRVAQIRQEIENSDSDYDR
EKLQERLAKLAGGVAVIKAGAATEVELKERKHRIEDAVRNAKAAVEEGIVAGGGVTLLQA
APTLDELKLEGDEATGANIVKVALEAPLKQIAFNSGLEPGVVAEKVRNLPAGHGLNAQTG
VYEDLLAAGVADPVKVTRSALQNAASIAGLFLTTEAVVADKPEKEKASVPGGGDMGGMDF

Figure 5: Peptide sequence of Mtb Hsp65 in FASTA format

Source: http://www.uniprot.org/[32]

Table 4: BLAST analysis between MAP *Hsp65* and *GAD65* peptide sequences

Protein	Amino acid region	Amino acid Sequence
GAD65	520-535	EERMSRLSKVAPVIKA
		+ER+++L+ VIKA
MAP *Hsp65*	364-379	QERLAKLAGGVAVIKA

Table 4: Result of BLAST analysis between MAP *Hsp65* and human *GAD65* revealing a 44% sequence identity in 16 aa antigenic motif.

Table 5: BLAST analysis between Mtb *Hsp65* and *GAD65* peptide sequences

Protein	Amino acid region	Amino acid Sequence
GAD65	520-535	EERMSRLSKVAPVIKA
		+ER+++L+ VIKA
Mtb *Hsp65*	364-379	QERLAKLAGGVAVIKA

Table 5: Result of BLAST analysis between Mtb *Hsp65* and human *GAD65* revealing a 44% sequence identity in 16 aa antigenic motif.

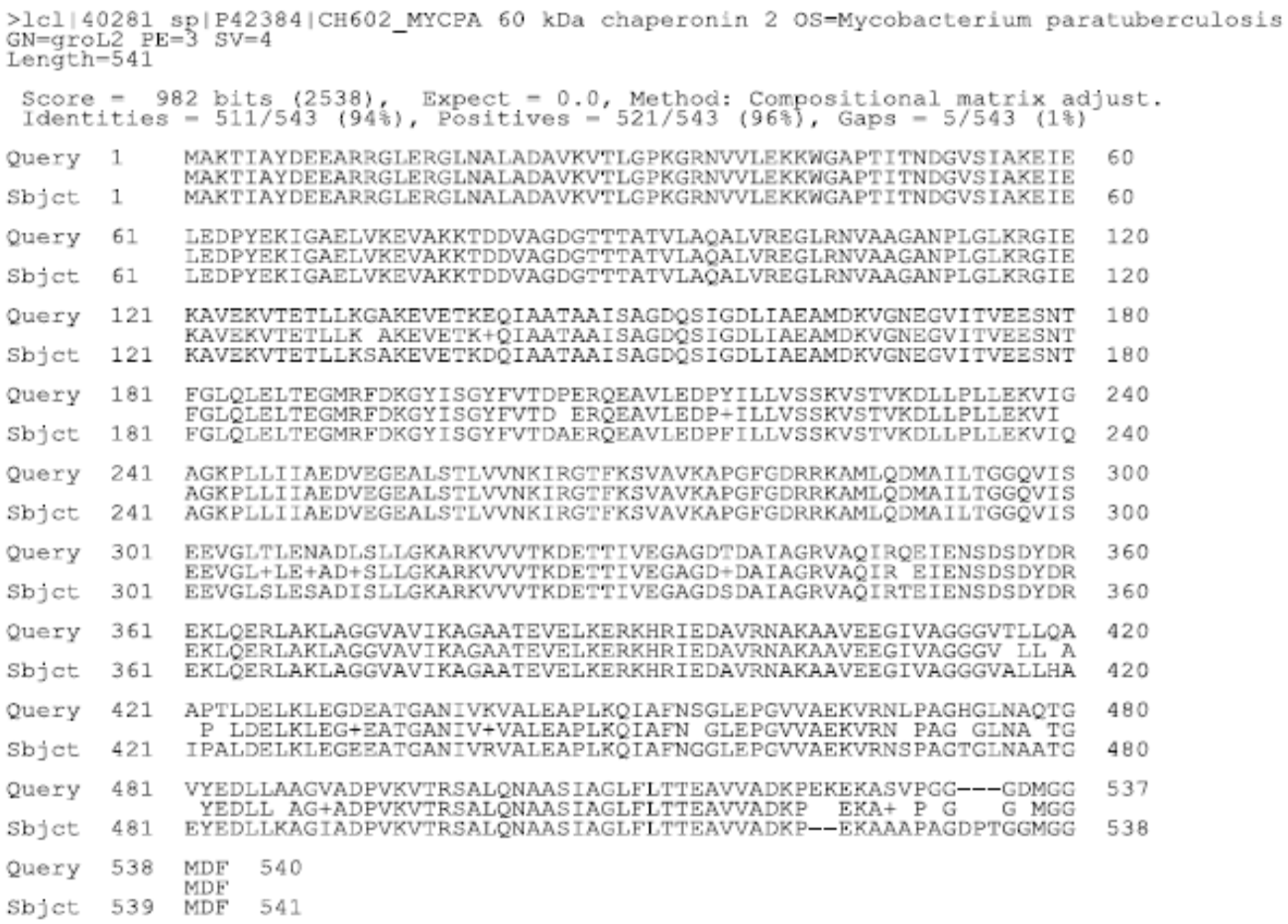

Figure 6: BLAST analysis between Mtb *Hsp65* and MAP *Hsp65* peptide sequences

Query: Mtb *Hsp6*5, Subject: MAP *Hsp65*

Table 6: Summary of BLAST analysis between Mtb *Hsp65* and *GAD65* peptide sequences

Protein	Amino acid region	Amino acid Sequence
GAD65	520-535	EERMSRLSKVAPVIKA
		+ER+++L+ VIKA
MAP *Hsp65*	364-379	QERLAKLAGGVAVIKA
Mtb *Hsp65*	364-379	QERLAKLAGGVAVIKA

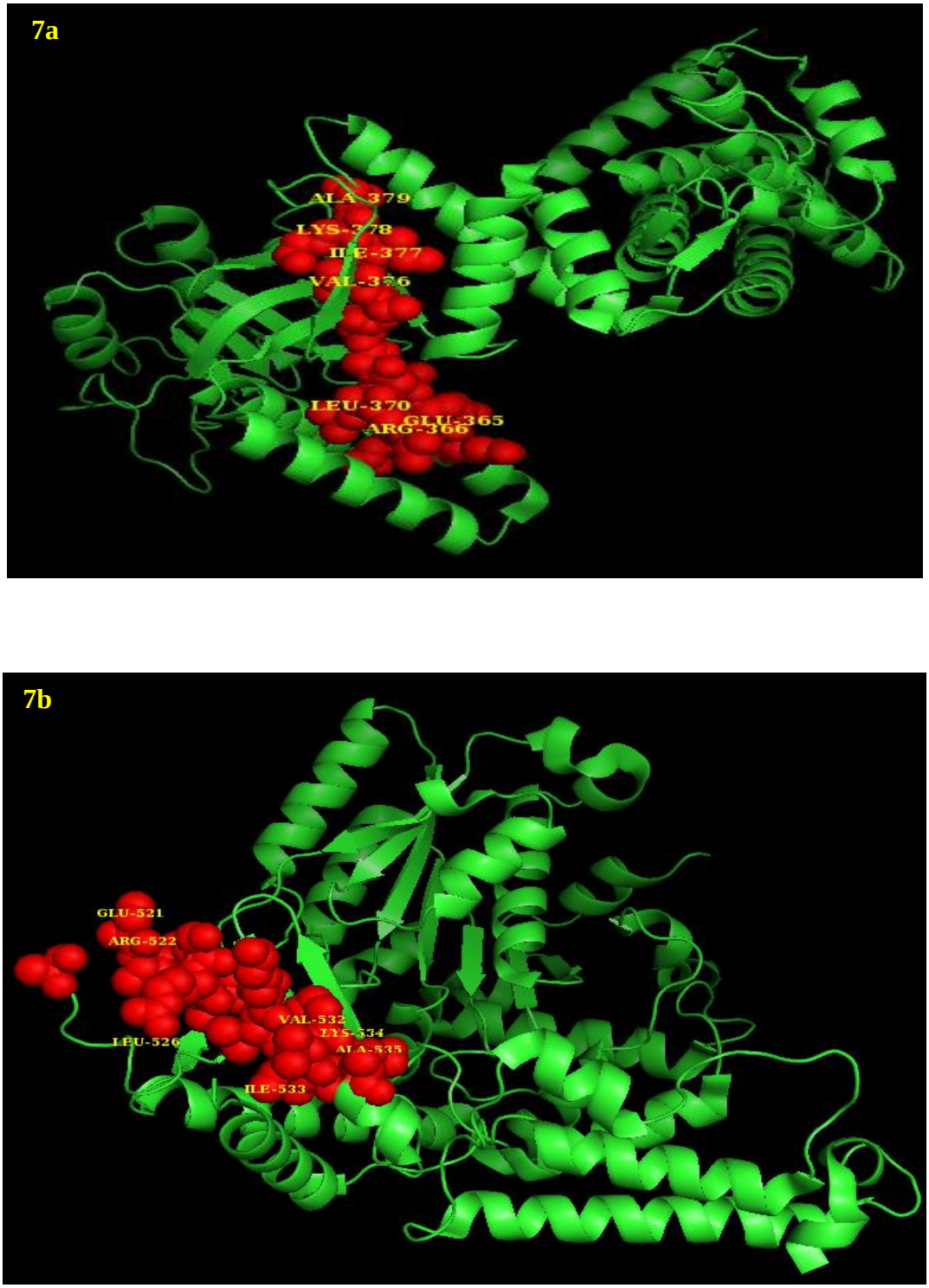

Figure 7 : PyMOL structural analysis of Mtb *Hsp65* and human *GAD65*

(7a) Protein structure of Mtb *Hsp65* PDB ID:3RTK[33]

(7b) Protein structure of human *GAD65* PDB ID: 20KK[34]

Highlighted are the sequences that were identical between MAP *Hsp65* and *GAD65* by BLAST analysis.

Immunoblot analysis to evaluate antigenic cross reactivity

Immunoblot analysis was used to evaluate the cross reactivity between the proteins MAP *Hsp65* and human *GAD65*. Antigens used for the immunoblot analysis were pancreatic tissue homogenate from TIDM-induced rat and healthy control, cell lysate from pmptb20 which is an *E. coli*-recombinant clone of MAP *Hsp65* (Dr.Naser's lab) [29], cell lysate of *E.coli* recombinant clone with vector (Negative control, Invitrogen, NY, USA) and lysate from Mtb strain, MAP strain and M.avium strain The latter's were used as positive controls. The primary antibodies used in the analysis were rabbit anti MAP serum [35] and human plasma isolated from TD8, a subject with TIDM. TD8 has been confirmed to be positive for MAP by culture and nested PCR (lane 8 Figure 9 and Table 7) and for MAP IgG (Table 8). As shown in Figure 8A, rabbit anti-MAP IgG reacted strongly with Hsp65 from Mtb (lane 2), M. avium (lane 1), MAP (lane 3) and pmptb20 (lane 4). Interestingly rabbit anti MAP IgG also reacted with pancreatic tissue lysate from healthy rat (lane 5, Figure 8A). There was no reactivity with pancreatic tissue lysate from diabetic rat (lane 6). Furthermore, plasma from TD8 (subject with TIDM) reacted strongly with protein lysate from MAP (lane 1) and pmptb20 (lane 2) as shown in Figure 8B. There was no reactivity by immunoblot between diabetic and healthy rat pancreatic tissue homogenate and plasma from TD8 (lanes 4 and 5 respectively).

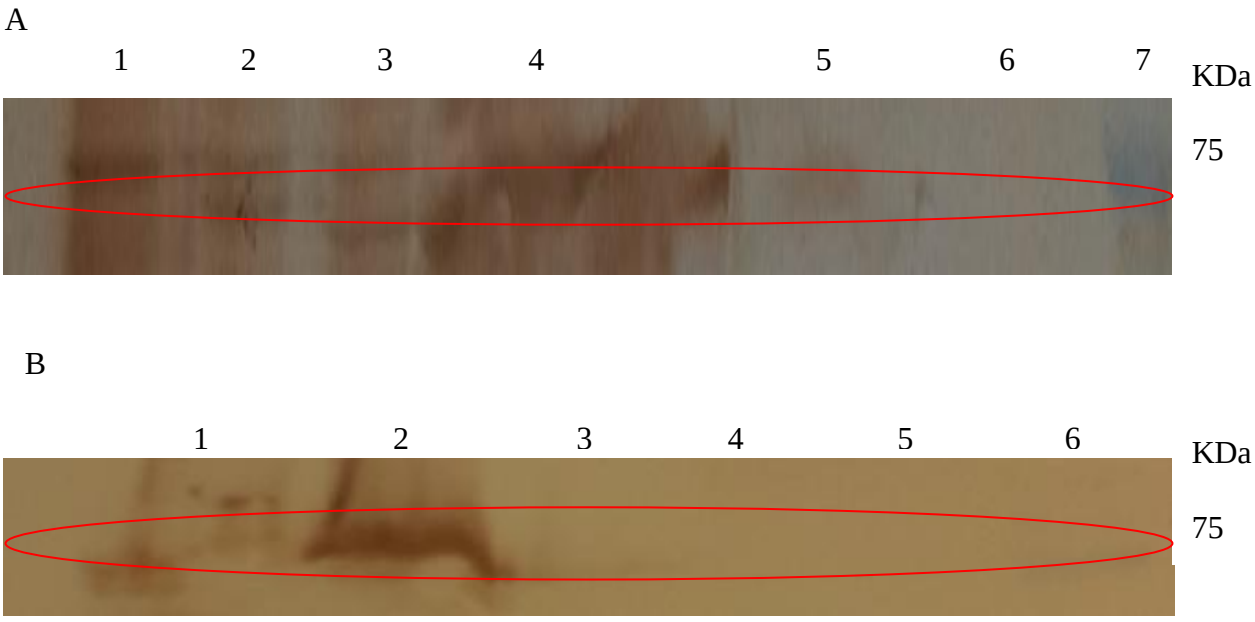

Figure 8: Immunoblot analysis to evaluate antigenic cross reactivity between MAP *Hsp65* and *GAD65*.

(A) Lane 1, M.avium lysate. Lane2, Mtb lysate. Lane 3, MAP lysate. Lane 4, pmptb20 (*E.coli* recombinant clone of MAP *Hsp65*), Lane 5, Healthy rat pancreas homogenate. Lane 6, Diabetic rat pancreas homogenate. Lane 7, Protein marker. (B) Lane 1, MAP lysate. Lane 2, pmptb20 (*E.coli* recombinant clone of MAP *Hsp65*), Lane 3, pcDNAII (*E.coli* recombinant clone containing vector), Lane 4, Healthy rat pancreas homogenate. Lane 5, Diabetic rat pancreas homogenate. Lane 6, Protein marker.

Detection of MAP DNA

Human blood samples were all negative for MAP DNA in uncultured buffy coat from all subjects using direct nested PCR analysis (Table 7)Following a total of 6 months incubation, culture aliquots subjected to DNA extraction and nested PCR revealed that MAP DNA was detected in 3/10 (30%) TIDM, 1/2 (50%) T2DM and 3/6 (50%) of control. As shown in Figure 9, MAP DNA was detected in lanes 7, 8 and 18.(TIDM), lanes 2 (T2DM) and lanes 10,13 and 17 (controls)(Table 7). Table 7summarizes the PCR results in all samples.

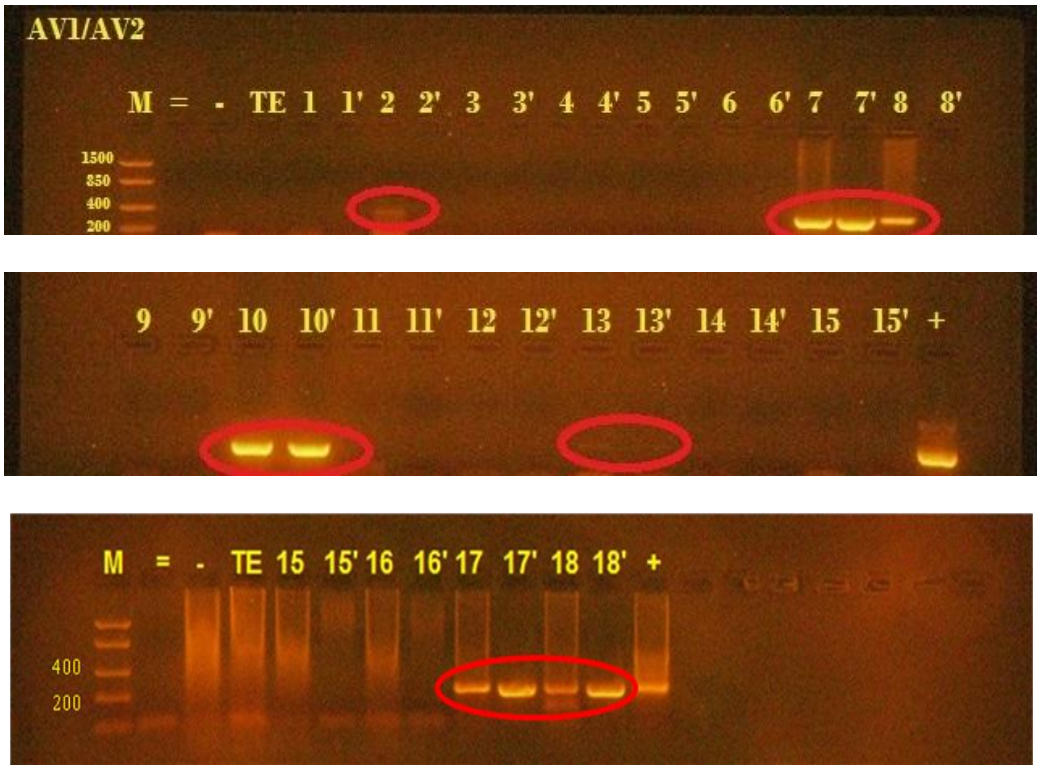

Figure 9: Detection of MAP in blood by culture PCR

Table 7 : Results of Direct PCR and culture PCR analysis of human samples

Code	Diagnosis	Direct PCR	Culture PCR
TD 03	TIDM	-	-
TD 04	TIDM	-	-
TD 05	TIDM	-	-
TD 06	TIDM	-	-
TD 07	TIDM	-	+
TD 08	TIDM	-	+
TD 09	TIDM	-	-
TD 14	TIDM	-	-
TD 15	TIDM	-	-
TD 18	TIDM	-	+
TD 01	T2DM	-	-
TD 02	T2DM	-	+
TD 10	CONTROL	-	+
TD 11	CONTROL	-	-
TD 12	CONTROL	-	-
TD 13	CONTROL	-	+
TD 16	CONTROL	-	-
TD 17	CONTROL	-	+

Detection of MAP IgG Antibodies

The IDEXX ELISA kit was used to evaluate the level of anti-MAP IgG antibodies, if present, in all samples. The titer level was calculated following manufacturer protocol where:

S/P={sampleA(450)-negative control mean}/{ positive control mean- negative control mean}

by using the absorbance values obtained. A correlation was observed between the MAP ELISA results and the culture results.

Table 8 summarizes the titer level for each sample used in this study. Over all MAP antibodies were detected in 3/8 (37.5 %) controls whereas 5/10 (50 %) TIDM samples were positive for MAP. The analysis was repeated twice and the average of all attempts is summarized in Table 8.

Table 8 : MAP ELISA results

Code	DIAGNOSIS	ELISA S/P ratio	ELISA interpretation
TD 01	T2DM	0.13	Negative
TD 02	T2DM	0.69	Low Positive
TD 03	TIDM	0.73	Low Positive
TD 04	TIDM	0.79	Low Positive
TD 05	TIDM	0.83	Positive
TD 06	TIDM	0.99	Positive
TD 07	TIDM	0.22	Negative
TD 08	TIDM	2.11	Strong Positive
TD 09	TIDM	0.49	Negative
TD 10	CONTROL	0.28	Negative
TD 11	CONTROL	0.5	Negative
TD 12	CONTROL	0.09	Negative
TD 13	CONTROL	2.12	Strong Positive
TD 14	TIDM	0	Negative
TD 15	TIDM	0.25	Negative
TD 16	CONTROL	0.53	Negative
TD 17	CONTROL	2.09	Strong positive
TD 18	TIDM	0.36	Negative

Table 9 : Summary of results of clinical analysis of human samples

	MAP Positive by culture PCR	MAP Antibody Positive
TIDM	3/10 (30%)	5/10(50%)
T2DM	1/2 (50%)	1/2 (50%)
CONTROL	3/6 (50%)	2/6 (33.33%)

Clinical Investigation in Cattle

The bovine sera were analyzed for MAP IgG levels using the IDEXX. The cutoff threshold of 0.6 was provided by the manufacturer. Fifty sera from cattle were positive for MAP with an S/P ratio ranging from 1.525 to 3.018 and were declared positive following IDEXX protocol. Another 50 cattle serum revealed a 0.027 to 0.104 anti-MAP IgG S/P ratio and was declared negative following IDEXX protocol. The insulin levels were determined in each sample. Overall, the insulin level in MAP infected cattle ranged from below 0.1ng/ml to 2.456ng/ml with an average of 0.36 +/- 0.57ng/ml compared to below 0.1ng/ml to 13.47ng/ml with an average of 2.86 +/- 3.00ng/ml in MAP negative cattle (Table 10). Insulin level determination revealed that MAP negative cattle produced 2.86ng/ml which is 8 fold higher when compared to MAP positive cattle that produce 0.36ng/ml Glucose levels were also measured in each sera sample and the results are listed in Table 10(Figure 10). Glucose levels in MAP positive cattle ranged from 68mg/dl to125.96 mg/dl and 50mg/dl to 106.09mg/dl (Table 10). Insulin and glucose levels in all samples were measured in duplicates.

Table 10: Clinical investigation of Insulin and Glucose concentration levels in cattle sera

Sample code	MAP IgG +/-	Average Insulin concentration in ng/ml	Average Glucose concentration in(mg/dl)
P1	+	0.1	124.04
P2	+	1.335	62.50
P3	+	1.489	53.21
P4	+	0.013	70.19
P5	+	0.413	53.85
P6	+	0.419	67.63
P7	+	0.1	60.90
P8	+	1.573	49.68
P9	+	0.1	65.71
P10	+	2.456	70.19
P11	+	0.555	82.05
P12	+	0.1	70.19
P13	+	0.1	81.41
P14	+	2.482	57.37
P15	+	0.1	67.95
P16	+	0.1	66.67
P17	+	0.851	118.91
P18	+	0.1	106.09
P19	+	0.1	125.96
P20	+	0.001	81.09
P21	+	0.697	94.23
P22	+	0.1	55.13
P23	+	0.02	72.76
P24	+	0.1	58.65
P25	+	0.1	51.60
P26	+	0.1	99.36
P27	+	0.1	77.24
P28	+	0.1	70.51
P29	+	0.1	121.47
P30	+	0.1	58.01
P31	+	0.1	117.31
P32	+	0.1	96.47

Sample code	MAP IgG +/-	Average Insulin concentration in ng/ml	Average Glucose concentration in(mg/dl)
P33	+	0.1	55.13
P34	+	0.052	75
P35	+	0.1	79.17
P36	+	0.1	76.60
P37	+	0.078	82.69
P38	+	0.1	58.33
P39	+	0.1	59.94
P40	+	0.677	62.82
P41	+	0.117	69.55
P42	+	0.336	77.56
P43	+	0.091	63.46
P44	+	0.1	55.45
P45	+	0.1	60.9
P46	+	0.1	81.09
P47	+	0.6	60.26
P48	+	0.1	110.58
P49	+	0.071	55.45
P50	+	0.626	61.22
N1	-	6.561	82.69
N2	-	1.090	50.32
N3	-	7.502	79.17
N4	-	6.986	81.73
N5	-	0.1	73.4
N6	-	4.84	106.09
N7	-	2.849	63.14
N8	-	3.97	58.65
N9	-	2.495	67.31
N10	-	4.647	71.79
N11	-	1.985	83.33
N12	-	13.47	66.67
N13	-	1.605	101.28
N14	-	0.1	75.64
N15	-	2.108	98.08
N16	-	5.304	75.32
N17	-	0.1	71.79

Sample code	MAP IgG +/-	Average Insulin concentration in ng/ml	Average Glucose concentration in(mg/dl)
N18	-	2.727	54.49
N19	-	2.752	71.47
N20	-	2.121	95.19
N21	-	2.101	79.81
N22	-	0.1	82.37
N23	-	NA	75.64
N24	-	NA	80.77
N25	-	NA	82.05
N26	-	0.271	100
N27	-	NA	81.09
N28	-	NA	81.09
N29	-	NA	69.23
N30	-	NA	62.18
N31	-	NA	74.36
N32	-	NA	76.60
N33	-	1.438	54.49
N34	-	0.258	105.77
N35	-	NA	60.90
N36	-	NA	80.45
N37	-	NA	72.12
N38	-	NA	76.6
N39	-	NA	65.06
N40	-	NA	66.67
N41	-	NA	79.49
N42	-	NA	74.36
N43	-	NA	72.12
N44	-	NA	66.03
N45	-	0.716	91.99
N46	-	NA	75.64
N47	-	NA	63.46
N48	-	NA	68.91
N49	-	0.065	95.83
N50	-	1.264	94.87

Table 11: Summary of glucose and insulin levels in cattle serum

Diagnosis	Number of Samples	Average Glucose Levels in mg/dl	Average Insulin Levels in ng/ml
MAP Positive	50	75.07 +/- 20.92	0.36 +/- 0.57
MAP Negative	50	76.75 +/- 13.14	2.86 +/- 3.00

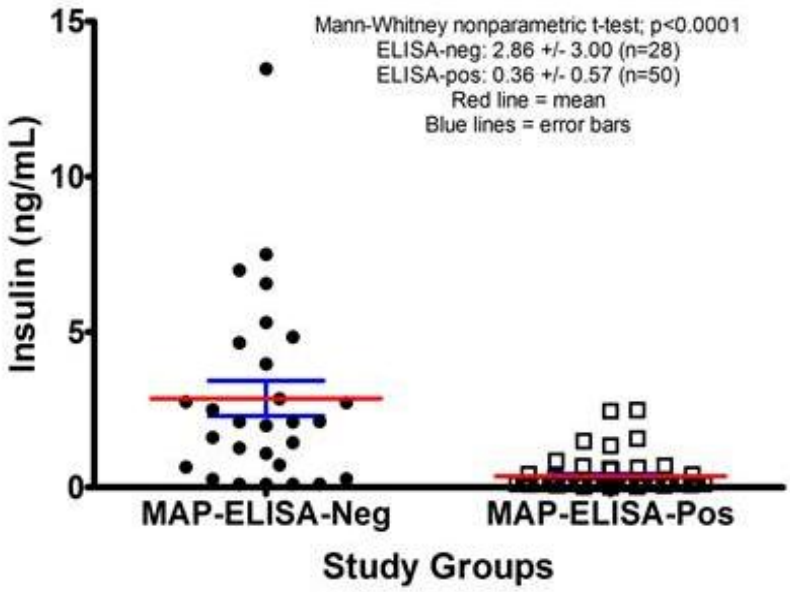

Figure 10: Analysis of Insulin levels versus MAP positive and negative cattle groups

CHAPTER FOUR: DISCUSSION

TIDM association with MAP has been reported exclusively in Sardinia, Italy and by Sechi group only [6, 15-17, 20, 36]. In this study, we investigated the possibility of MAP *Hsp65* as a possible trigger for auto-destruction of beta cell through recognition of *GAD65* autoantigen. BLAST analysis between MAP *Hsp65* and human *GAD65* revealed the identification of 16 amino acid regions with 75% positive identity. Interestingly the 16 amino acid region in *GAD65* has been suggested earlier to be a potential antigenic binding site (Figure 7) [31]. Since the protein structure of MAP *Hsp65* is not available in protein structure database, alternative protein structure of a closely related peptide sequence of Mtb *Hsp65* was used. This was facilitated by the fact that MAP *Hsp65* and Mtb *Hsp65* had a homology of 96% with perfect match within the 16 amino acid region identified earlier (Figure 6). Consequently, Figure 7 suggests that the 16 amino acid site localized on the 3D structure of MAP *Hsp65* may play an antigenic role in provoking the host immune response to produce antibodies following MAP infection. The anti-MAP *Hsp65* antibodies may then bind to the *GAD65* epitope on the surface of Beta cell leading to auto destruction of pancreatic Beta cell. Immunohistochemistry analysis initially revealed a cross reactivity between rabbit anti-MAP IgG and pancreatic cell homogenate from healthy rat (Figure 8). Moreover, plasma from patient with TIDM (TD8), who was confirmed to be positive for MAP DNA and MAP IgG, (lane 8 Figure 9 and Table 7) reacted strongly with MAP *Hsp65* in MAP protein lysate and MAP *Hsp65* recombinant clone pmtb20 (Figure 8). Despite these preliminary data, TD8 plasma failed to react with pancreatic tissue homogenate from rat. One possible reason for this result is the fact that 1) the level of *GAD65* in rat pancreatic tissue is diluted since whole pancreatic tissue were used instead of beta cell homogenate, 2)*GAD65* is a

cell membrane protein and homogenate from whole pancreatic tissue may contain only small amount of *GAD65* to be recognized by anti-MAP IgG from TD8 plasma, 3) sensitivity of immunoblot may not be efficient enough to detect low level of *GAD65* protein present in rat pancreatic tissue homogenate, and 4) the 75% positivity in 16 amino acid epitope region may not be antigenic enough to be recognized by anti-MAP IgG.

Therefore, ELISA measurement was necessary in order to evaluate any cross reactivity between MAP *Hsp65* and *GAD 65*. The ELISA results confirmed the cross reactivity when anti-MAP IgG was detected in 5/10 TIDM subjects (Table 8). The detection of MAP DNA in leukocytes from TIDM is first in this field despite the low positivity rate in the samples tested. MAP is fastidious and many declared it unculturable from human [37, 38]. The detection of MAP in 1/2 TIIDM could be related to potential misdiagnosis as many clinicians agree to. The detection of MAP DNA in controls samples is pending investigation. MAP has been suggested to play a role in many autoimmune disease including inflammatory bowel disease and Crohn's disease. The lack of history information on the control samples provides challenges to us to correctly explain the detection of MAP DNA in these samples. The glucose and insulin data were discarded in this study since the subjects did not comply with fasting and insulin therapy. In short, the patients were not fasting and some were on insulin therapy which alters the glucose and insulin levels.

The cattle sera were added late to the study. MAP infection in cattle is also known as Johne's disease or Paratuberculosis [39]. A total of 100 sera were a gift from Dr. Collins of University of Wisconsin where 50 samples were identified to be MAP IgG positive and an equal 50 samples were identified to be MAP IgG negative (Table 10). Glucose and insulin levels were

measured. The glucose level values were not beneficial to the study since the cattle were continuously fed. However the insulin values were a great indicator of possible hyperglycemia in those with MAP infection. In fact, Figure 10 illustrates just that where MAP infected cattle revealed a lower level of insulin up to 8 fold when compared to healthy cattle with normal or elevated insulin levels (Table 10).

Over all, the data suggests that further study is needed. The molecular mimicry and the antigenic cross reactivity between MAP *Hsp65* and human *GAD65* may play some role in the auto-destruction of pancreatic beta cell and development of TIDM. However, other factors may also play equal or more roles in disease pathogenesis.

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