

**REAL TIME RT-PCR FOR DIRECT DETECTION OF VIABLE
MYCOBACTERIUM AVIUM SUBSPECIES *PARATUBERCULOSIS* IN
CROHN'S DISEASE PATIENTS**

and

**ASSOCIATION OF MAP INFECTION WITH DOWNREGULATION
IN INTERFERON-GAMMA RECEPTOR (*INFG1*) GENE IN
CROHN'S DISEASE PATIENTS**

by

Mounir Chehtane
B.S. University Moulay Ismail Morocco, 1994

A thesis submitted in partial fulfillment of the requirements
for the degree of Master of Science
in the Department of Molecular Biology and Microbiology
in Burnett College of Biomedical science
at the University of Central Florida
Orlando, Florida

Summer Term
2005

© 2005 Mounir Chehtane

ABSTRACT

Association of *Mycobacterium avium subspecies paratuberculosis* (MAP) with Crohn's disease (CD) and not with ulcerative colitis (UC), two forms of inflammatory bowel disease (IBD), has been vigorously debated in recent years. This theory has been strengthened by recent culture of MAP from breast milk, intestinal tissue and Blood from patients with active Crohn's disease. Culture of MAP from clinical samples remained challenging due to the fastidious nature of MAP including its lack of cell wall in infected patients. The advent of real time PCR has proven to be significant in infectious disease diagnostics. In this study, real time reverse transcriptase PCR (RT-PCR) assay based on targeting mRNA of the IS900 gene unique to MAP has been developed. All variables included in RNA isolation, cDNA synthesis and real time PCR amplification have been optimized. Oligonucleotide primers were designed to amplify 165 bp specific to MAP and the assay demonstrated sensitivity of 4 genomes per sample. In hope this real time RT-PCR may aid in the detection of viable MAP cells in Crohn's disease patients, a total of 45 clinical samples were analyzed. Portion of each sample was also subjected to 12 weeks culture followed by standard nested PCR analysis. The samples consisted of 17 cultures (originated from 13 CD, 1 UC and 3 NIBD subjects), 24 buffy coat blood (originated from 7 CD, 2 UC, 11 NIBD and 4 healthy subjects) and 4 intestinal biopsies from 2 CD patients. Real time RT-PCR detected viable MAP in 11/17 (65%) of

suspected cultures compared to 12/17 (70%) by nested PCR including 77% and 84% from CD samples by both methods, respectively. Real time RT-PCR detected MAP RNA directly from 3/7 (42%) CD, 2/2 (100%) UC and 0/4 healthy controls similar to results following long term culture incubation and nested PCR analysis. Interestingly, real time RT-PCR detected viable MAP in 2/11 (13%) compared to 4/11 (26%) by culture and nested PCR in NIBD patients. For tissue samples, real time RT-PCR detected viable MAP in one CD patient with the culture outcome remains pending. This study clearly indicates that a 12-hr real time RT-PCR assay provided data that are similar to those from 12 weeks culture and nested PCR analysis. Consequently, use of real time In our laboratory, we previously demonstrated a possible downregulation in the Interferon-gamma receptor gene (*IFNGR1*) in patients with active Crohn's disease using microarray chip analysis. In this study, measurement of RNA by real time qRT-PCR indicated a possible downregulation in 5/6 CD patients compared to 0/12 controls. The preliminary data suggest that downregulation in *INFGR1* gene, and the detection of viable MAP in CD patients provides yet the strongest evidence toward the linkage between MAP and CD etiology.

I dedicate this work to my beloved parents, my wife Fatima, my daughter Hajar and to the rising star of the family: Ibrahim. To all, I would say many thanks for the help and support. Without you I wouldn't be able to make it this far.

ACKNOWLEDGMENTS

I would like to thank my advisor, Doctor Saleh A. Naser, for his strong support and help to make this work possible. To the members of my dissertation committee, Doctor Antonis Zervos, Doctor Henry Daniell, who have generously given their time and expertise to better my research. To the members of my research laboratory for their constant support. To the National Institutes of Health who funded this work, and finally, to my family and friends, specially my wife Fatima for her endless encouragement and patience

TABLE OF CONTENTS

ABSTRACT iii

ACKNOWLEDGMENTS vi

LIST OF FIGURES x

LIST OF TABLES xii

LIST OF ABBREVIATIONS xiv

CHAPTER ONE: INTRODUCTION 1

CHAPTER TWO: RT-PCR FOR DETECTION OF VIABLE MYCOBACTERIUM
AVIUM SUBSPECIES PARATUBERCULOSIS IN BLOOD AND TISSUE FROM
PATIENTS WITH CROHN'S DISEASE 4

 Summary 4

 Introduction 6

 Materiel and Methods 9

 Bacterial strains: 9

 Patients Populations: 9

 Clinical Samples: 10

 Cultures: 11

 RNA extraction: 11

 Reverse Transcription: 12

 Real time PCR: 13

 Specificity: 14

 Sensitivity: 14

Generating Standards for Real time RT_PCR:	15
Melt Curve analysis:	15
Results.....	15
RNA Extraction:	16
Optimization of the RT-PCR:	17
Annealing temperature:.....	18
Optimization of Magnesium ions:	19
Over All RT-PCR Protocol:.....	19
Sensitivity of RT-PCR:.....	19
Specificity of the RT-PCR:.....	20
Clinical Evaluation of the RT-PCR assay.....	20
MAP cultures:.....	21
Peripheral Blood Buffy coats:.....	21
Tissue samples:.....	22
Discussion.....	23
CHAPTER THREE: IFNGR1 GENE IS DOWNREGULATED IN PATIENTS WITH CROHN'S DISEASE: A MYCOBACTERIAL INFECTION?	27
Summary.....	27
Introduction.....	28
Materials and methods	32
Patient Selection and Specimen Source.....	32
Peripheral Blood Withdrawal and Processing	32

RNA extraction:	33
cDNA Analysis:	33
Quantitative Real time RT-PCR:	33
IFN gamma ELISA Test:	34
Results.....	35
Quantitative real time RT-PCR:.....	35
Evaluation of levels of IFN- γ in Clinical Sera:.....	36
Discussion.....	37
CHAPTER FOUR: GENERAL DISCUSSION	39
APPENDIX A: FIGURES	41
APPENDIX B: TABLES.....	66
Table 6: Analysis of fresh tissue samples for MAP detection using real time RT-PCR versus culture and nested PCR.	75
Table7: Genes with Differences in the Expression Levels in each subtype of IBD Disorder. (This table was used from doctor Romero PhD thesis).	76
LIST OF REFERENCES.....	81

LIST OF FIGURES

Figure1: comparison of RNA yield with Qiagen protocol, Modified Qiagen protocol and combination of Mechanical and chemical extraction.....	42
Figure 2: Evaluation of RNA extracts for the presence of DNA using DNase treatment using agarose gel electrophoresis.....	43
Figure3: Evaluation of RNA extracts for the presence of DNA in the presence and absence of DNase using PCR analysis.....	44
Figure 4: Evaluation of the RNA extracts for the presence of MAP DNA in the presence and absence of cDNA synthesis step.....	45
Figure 5: Evaluation of our real-time RT-PCR for specific detection of MAP.....	46
Figure 6: Effect of annealing Temperature on secondary structure formation in IS900 sequence.....	48
Figure 7: Optimization of the concentration of Oligonucleotide primers.....	50
Figure 8: evaluation of the annealing temperature used in this study.....	52
Figure 9: Optimization of the concentration of MgCl ₂	54
Figure 10: Evaluation of our real-time RT-PCR for sensitive detection of MAP RNA...56	
Figure11: Representative figure for evaluation of samples from patients using real time RT-PCR.....	58
Figure 12: Detection of MAP in human samples by Real time RT-PCR (12hours) and	

culture confirmed by nested PCR (12weeks).....	59
Figure13: Evaluation of MAP positive samples from CD, UC and NIBD individuals using real time RT-PCR compared to culture confirmed by nested PCR.....	60
Figure 14: Biological themes predicted by MeV with EASE microarray analysis software.....	61
Figure 15: variation in some of the immune response gene expression patterns including IFNGR1 in human white blood cells.....	62
Figure 16: Representative figure for IFNGR1 gene expression analysis using real time RT-PCR.....	63
Figure17: graph illustrating quantitative real time RT-PCR data.....	65

LIST OF TABLES

Table1: List of microorganisms used in the study.....	67
Table2: Oligonucleotide primers used in this study.....	68
Table 3: Optimized protocols for PCR analysis used in this study.....	69
Table 4A: Analysis of culture samples For MAP by real time RT-PCR versus culture and nested PCR.....	70
Table 4B: Over all summaries of MAP detection in culture samples by direct analysis using real time RT-PCR versus culture and nested PCR.....	71
Table5A: Analysis of peripheral blood buffy coat samples for Map detection by real time RT-PCR versus culture and nested PCR.....	72
Table 5B: Over all summaries of MAP detection in peripheral blood buffy coat samples by direct analysis using real time RT-PCR versus culture and nested PCR.....	74
Table 6: Analysis of fresh tissue samples for MAP detection using real time RT-PCR versus culture and nested PCR.....	75
Table7: Genes with Differences in the Expression Levels in each subtype of IBD Disorder.....	76
Table 8 a: Analysis of Buffy coats from peripheral blood of patients to evaluate IFNGR1	

gene expression using quantitative real time RT-PCR.....	77
Table 8 b: overall Summary of IFNGR1 gene expression in CD patients and Non CD individuals.....	78
Table9: Evaluation of IFNGR1 gene expression in with regard to MAP diagnosis in samples from CD, UC and NIBD subjects.....	79
Table 10: Evaluation of level of IFN- γ in sera from peripheral blood of CD, UC and NIBD subjects using ELISA.....	80

LIST OF ABBREVIATIONS

CARD: Caspase-activation recruitment domain

CD: Crohn's disease

cDNA: complementary Deoxy-ribonucleic acid

DNA: Deoxy-ribonucleic acid

DNase: Deoxy-ribonuclease

ELISA: Enzyme-linked immunosorbent assay

FISH: Fluorescence *in situ* hybridization

GI: Gastrointestinal

IBD: Inflammatory bowel disease

IgG: Immunoglobulin G

IFN- γ : Interferon gamma

JD: Johne's disease

MAP: *Mycobacterium valium* subspecies *paratuberculosis*

MGIT: Mycobacterial growth indicator tube

NIBD: non inflammatory bowel disease

NOD2: Nucleotide oligomerization binding domain 2

OADC: oleic acid-albumin-dextrose-catalase

PCR: Polymerase chain reaction

RNA: Ribonucleic acid

RNase: Ribonuclease

RT-PCR: reverse transcription polymerase chain reaction

TNF-alpha: Tumor necrosis factor alpha

UC: Ulcerative colitis

CHAPTER ONE: INTRODUCTION

Crohn's disease (CD) is known to be an important cause of gastrointestinal disease worldwide but occurring more frequently in the United States, United Kingdom and Scandinavia (19). CD can affect any part of the gastrointestinal tract; inflammation is discontinued in form of patches and transmural resulting in fistula formation, the most common location is the ileum and small bowel (32). CD is present in two distinct forms: perforating and non-perforating in clear analogy to two other mycobacterial diseases, leprosy and tuberculosis (27, 33, 43). The etiology of CD remains unknown; however granuloma and pathologic manifestations resemble aspects found in mycobacteria-caused diseases such as tuberculosis, leprosy and paratuberculosis (16, 27, 53, 8).

Mycobacterium avium subspecies paratuberculosis (MAP) has been reported as possible causative agent for CD, due to the very important similarities in symptoms and aspect of the disease with Johne's disease, an inflammatory bowel disease in ruminants caused by MAP; Association of Map with CD was first sighted by Dalziel in 1913 and has got more attention recently when MAP was isolated from blood, gut, lymphoid tissue, and milk of CD patients (7, 46, 47).

MAP is a member of the *Mycobacterium avium* complex (MAC), it is well characterized by its acid fastness, slow growing rate and resistance to acid and alcoholic treatments (14) as well as to regular pasteurization techniques this mainly due to their

cell-wall rich in lipid and long chain peptidoglycans. MAP is considered very fastidious and requires long-term incubation during primary isolation from animal and human sources (47, 48, 52).

The role of MAP in CD has been controversial and was debated vigorously; isolation of MAP from clinical samples of CD patients has been inconsistent adding to the controversy. This is mainly due to the fastidious nature of MAP, its presence in Spheroplastic form in CD patients and to its slow growing nature, culture of MAP from clinical samples may take between 12 and 52 weeks. A new strategy of study is needed to investigate the role of MAP in CD etiology.

It had been reported that mutations in NOD2/ CARD15 gene are associated exclusively with CD (31; 35, 49) identifying the gene as the first susceptibility gene for Crohn's disease NOD2 is expressed as cytoplasmic protein in monocytes, and includes a C-terminus leucine-rich repeat (LRR) domain that senses muramyl dipeptide (MDP), the minimal motif of bacterial cell wall peptidoglycan, suggesting a role for intracellular pathogen-host interactions in the etiology of the disease.

In previous study, An RNA Microarray analysis of the expression profile of genes from CD and UC patients using the buffy coats from peripheral blood had shown that IFNGR1 (interferon-gamma receptor) gene, which encodes the ligand-binding chain of the IFN-gamma receptor, is Downregulated exclusively in CD patients. Early studies had shown that deficiencies in IFNGR1 gene are associated with familial disseminated atypical mycobacterial infection and disseminated BCG infection. Individuals carrying these deficiencies have an immunologic defect predisposing them to infection with

mycobacteria (38, 39, 40).

In this study, we developed a novel real time RT-PCR to detect the presence of viable MAP in blood samples from patients with CD, UC, NIBD and healthy controls as described in Chapter 1. In chapter 2, these clinical samples were subjected to *INFGR1* gene analysis using quantitative real time RT-PCR to evaluate the expression of IFNGR1 gene in an attempt to confirm the Microarray findings. Ultimately the study will provide for the first time weather there is a link between the INFGR gene downregulation and susceptibility to MAP infection in patients with active Crohn's disease. If confirmed our finding will add a strong argument to the role of MAP in CD etiology.

CHAPTER TWO: RT-PCR FOR DETECTION OF VIABLE MYCOBACTERIUM AVIUM SUBSPECIES PARATUBERCULOSIS IN BLOOD AND TISSUE FROM PATIENTS WITH CROHN'S DISEASE

Summary

Association of *Mycobacterium avium subspecies paratuberculosis* (MAP) with Crohn's disease (CD) and not with ulcerative colitis (UC), two forms of inflammatory bowel disease (IBD,) has been vigorously debated in recent years. This theory has been strengthened by recent culture of MAP from breast milk, intestinal tissue and Blood from patients with active Crohn's disease. Culture of MAP from clinical samples remained challenging due to the fastidious nature of MAP including its lack of cell wall in infected patients. The advent of real time PCR has proven to be significant in infectious disease diagnostics. In this study, real time reverse transcriptase PCR (RT-PCR) assay based on targeting mRNA of the IS900 gene unique to MAP has been developed. All variables included in RNA isolation, cDNA synthesis and real time PCR amplification have been optimized. Oligonucleotide primers were designed to amplify 165 bp specific to MAP and the assay demonstrated sensitivity of 4 genomes per sample. In hope this real time

RT-PCR may aid in the detection of viable MAP cells in Crohn's disease patients, a total of 45 clinical samples were analyzed. Portion of each sample was also subjected to 12 weeks culture followed by standard nested PCR analysis. The samples consisted of 17 cultures (originated from 13 CD, 1 UC and 3 NIBD subjects), 24 buffy coat blood (originated from 7 CD, 2 UC, 11 NIBD and 4 healthy subjects) and 4 intestinal biopsies from 2 CD patients. Real time RT-PCR detected viable MAP in 11/17 (65%) of suspected cultures compared to 12/17 (70%) by nested PCR including 77% and 84% from CD samples by both methods, respectively. Real time RT-PCR detected MAP RNA directly from 3/7 (42%) CD, 2/2 (100%) UC and 0/4 healthy controls similar to results following long term culture incubation and nested PCR analysis. Interestingly, real time RT-PCR detected viable MAP in 2/11 (13%) compared to 4/11 (26%) by culture and nested PCR in NIBD patients. For tissue samples, real time RT-PCR detected viable MAP in one CD patient with the culture outcome remains pending. This study clearly indicates that a 12-hr real time RT-PCR assay provided data that are similar to those from 12 weeks culture and nested PCR analysis. Consequently, use of real time RT-PCR may be significant in investigating the controversial role of MAP in CD pathogenesis.

Introduction

Mycobacterium Avium subsp *paratuberculosis* (MAP), a member of the *Mycobacterium avium* complex (MAC) belongs to the genus of *Mycobacteria* which is a large group of high %G+C gram positive microorganisms including more than a 100 species. The main characteristics of this *bacterium* is its acid fastness, slow growing rate and resistance to acid and alcoholic treatments (14) as well as to regular pasteurization techniques due to the complexity of their cell-wall rich in lipid and long chain peptidoglycans. Unlike other *Mycobacteria*, MAP is the only *Mycobacterium* subspecies that depend on the siderophore mycobactin for *in vitro* growth (9). MAP is considered very fastidious and requires long-term incubation during primary isolation from animal and human sources (47, 48, 52).

MAP is an opportunistic intracellular chronic pathogen, it was identified over 100 years ago as the causative agent of Paratuberculosis in ruminants (21), known also as Johne's disease (JD), an inflammatory disease that affect the gastrointestinal (GI) tract and manifest as a severe weight loss, diarrhea, decrease in milk production and ultimately may lead to the death of infected hosts (30). MAP infection in farm animals has great economic impact with an estimated three billions dollars loss per year world wide (6, 25). The resistance of MAP to standard pasteurization and its detection in drinking water have

alarmed Scientists of possible zoonotic potential and impact on humans.

The association of MAP with Crohn's disease, a chronic inflammatory bowel disease with significant histological and pathological similarities with JD (18, 21) tuberculosis and sarcoidosis, has been suggested initially by Dalziel (16) but ruled out by Crohn's et al (15) and reemerged again following the culture of Spheroplastic (cell wall deficient) form of MAP from mesenteric tissue from patients with Crohn's disease following several months of culture incubation (7). The association of MAP with Crohn's disease became more controversial due to the conflicting outcomes in all reported studies. However, Schwartz et al (54) reported the development of modified 7H9 culture media, known as Mycobacterial Growth Indicator Tube (MGIT) and the isolation of MAP from intestinal tissue from Crohn's disease patients following 10-12 weeks of incubation. This was followed by the isolation of MAP from human milk (46) adding more evidence to the similarity between MAP infection in animals and human hosts. The use of MGIT culture media for rapid isolation and detection of MAP from human sources has been recently confirmed by investigators in the United Kingdom (3) and most recently in the united state. World-wide Interest in investigating MAP association in Crohn's disease pathogenesis has been recently stirred following the report of isolation of MAP from human peripheral blood suggesting for the first time a bacteremic phase in Crohn's disease pathogenesis and possible systemic infection in patients with MAP infection (47).

Numerous studies have been done in an attempt to determine the role of MAP in Crohn's disease etiology, but the results were inconsistent which added to the controversy of the association of MAP with the disease.

Due to the challenges in consistent detection of MAP in human clinical samples, some investigators relied on serologic detection of anti-MAP IgG antibodies in sera from Crohn's disease patients. However, using Serological tests such as IDEXX which is based on cytoplasmic proteins from MAP cultured from JD-animal resulted in detection of anti-MAP IgG antibodies in sera from Crohn's disease patients similar to sera from patients with ulcerative colitis and normal controls (11). However, when recombinant MAP proteins such as p35 and p36 proteins were used in an ELISA-based assay, MAP antibodies were detected in the majority of sera from patients with Crohn's disease and significantly less in sera from patients with ulcerative colitis and normal subjects (45). Most recently, MAP DNA has been detected directly in tissue samples from the majority of Crohn's disease patients by FISH (51) and nested PCR (1).

Despite the success in detection of MAP, MAP DNA, or MAP antibodies in tissue or blood samples which added significant evidence toward MAP association in Crohn's disease pathogenesis, many in the gastroenterology community remained skeptics and posted vigorous negative commentary (41, 43). The need for new methodology that can associate viable MAP with Crohn's disease may be the key to providing key evidence to the link. In this study a reverse transcription (RT) PCR based on Oligonucleotides primers derived from the IS900 specific to MAP has been developed using real time PCR. This new and improved technology provides real time detection of MAP RNA (indicating viability) in clinical samples from humans infected with MAP. Ultimately, MAP can be detected, if present, in clinical samples with in 12 hours instead of 12 weeks of culture incubation.

Materiel and Methods

All materials used in this study were prepared and processed aseptically inside Biosafety cabinets dedicated for specific purpose.

Bacterial strains:

Clinical MAP strain Ben obtained and purified from CD tissue was used in this study. Other microorganisms were also included such as *Mycobacterium tuberculosis* strain H37Ra (ATCC 25177), *Mycobacterium avium* subspecies *avium*, *Mycobacterium smegmatis* strain MC2155. All mycobacterial isolates were cultured in Bactec or the MGIT culture media. Supplements including OADC (oleic acid-albumin-dextrose-catalase) and Mycobactin J (0.5 mg/mL) were added to media when MAP was cultured.

Patients Populations:

A total of 24 subjects were included in the study: 7 with Crohn's disease, 2 with ulcerative colitis, and 15 with non-inflammatory bowel disease (of which three were healthy controls). Informed consent was obtained in accordance with institutional review board regulations at the University of Florida, Gainesville Veterans Affairs Medical

Center, and the University of Central Florida. The diagnosis of Crohn's disease or ulcerative colitis was established on standard clinical, endoscopic, histological, and radiographic criteria.

Clinical Samples:

One 4-mL whole blood sample drawn into sterile K2-EDTA Vacutainer tube was obtained from every patient participating in the study. All samples were coded to conceal the patient's identity and the diagnosis. The samples were immediately processed in a class II biosafety cabinet. The buffy coat layer from each tube was transferred into a separate sterile RNase free tube containing 750ul of RNA later solution (Ambion, California) and was used immediately for extraction of RNA. Surgically removed tissue specimens were also recruited and used in this study. The tissue samples were stored in empty sterile tubes and coded as described earlier. All clinical samples were stored on ice and transferred to the laboratory within 24 hours of collection. Blood and tissue samples were processed immediately either for culture in Bactec and MGIT media (Becton Dickinson) with supplements or used for direct isolation of RNA.

Cultures:

Blood and tissue samples were cultured as described previously (47), a total of 14 cultured samples suspected of mycobacterial growth were analyzed by extraction of RNA and real time RT-PCR analysis as described later. The concentration of microorganism cells used in the specificity experiments was estimated using spectrophotometric measurement of 500ul of each bacterial culture at O.D. 680 nm in Smartspec (BioRad, CA)

RNA extraction:

Clinical MAP strain Ben was used in all experiments related to extraction of RNA, cDNA and PCR analysis. Extraction of RNA was performed using an RNA isolation kit from Qiagen (Qiagen, Valencia, CA) with some modifications as follows. A 500uL of MAP culture (0,58CFU/mL) was centrifuged at 13,000 rpm (17400g) at 4°C for 3min. Cell pellet was resuspended in 1 ml of lysis buffer. Cells were physically disrupted using Fast-prep system and pro blue cap tubes at speed of 6.5 m/s for two cycles with each 45 Seconds (Qbiogene, CA). Cell lysate was then centrifuged at 13000 rpm (17400g) in 4 °C for 5 min Supernatant was removed to a sterile 2-ml RNase free tube followed by the addition of 1mL elution buffer. The mixture was poured into the column as described by the manufacturer. RNA in eluted buffer was then mixed with two volumes of ethanol,

stored at -20 °C for 2 hours. Tubes were then centrifuged at 13000rpm (17400g) at 4 °C for 15min. Nucleic acid pellet was then washed with 70% ethanol, dried in a speed vacuum (BioRad, Hercules, CA) and finally dissolved in 50uL Of DEPC (Diethylpyrocarbonate) sterile water. DNA was removed by the addition of DNase (2units/ul) and incubation at 37 °C for 30min. DNA removal was confirmed by agarose gel electrophoresis. The concentrations of RNA solutions were measured at an O.D. ₂₆₀ nm.

Reverse Transcription:

RNA extracted from MAP strain Ben was used as template during reverse transcription and cDNA analysis. Superscript III reduced RNase H activity (envitrogen, CA) was used and we followed the protocol described in the kit with some modifications, we added 2pmole of oligonucleotide PrimersF1/R1, 200g of RNA and 10mM of dNTP mix into a sterile PCR tube, the mixture was heated at 65°C for 5min and incubated on ice for 3min then we added the buffer, RNase inhibitor and the enzyme (200units), we incubated the reaction mixture at 60°C for 45min then at 70 °C for 15 min to inactivate the reaction.

Real time PCR:

cDNA obtained by reverse transcription was subject to real time PCR using Syber green and MY IQ system (BioRad, Hercules, CA).12.5ul of 2x SYBR Green (100mM KCl, 40 mM Tris-HCl, pH8.4, 0.4mM of each dNTP, enzyme(50units/ml, 6mM Mgcl2, SYBR Green I, fluorescein, and stabilizers) were added to sterile PCR tube as well as Betaine, oligonucleotide primers (6pM), 2.5ul of cDNA and sterile water for a final volume of 25ul.the PCR tubes were then subject to the following protocol: hot start step of 5min at 95°C followed by 20 sec at 95°C then 30 sec at 60°C for annealing, the fluorescence reading was done at this step. Two set of primers were used, the first set(Av1/Av2) amplify 280 base pair of MAP specific sequence IS900 and was used to generate the standards while the second set(F1/R1) amplify 165 base pair within the 280 amplified by Av1/Av2

- Av1: 5' ATGTGGTTGCTGTGTTGGATGG 3'
- Av2: 5'CCGCCGCAATCAACTCCAG 3'
- F1: 5' CACGTCGGCGTGGTCGTCT 3'
- R1: 5' CGTCACCGCCGCAATCAACT 3'

Optimization of our Real time PCR was done by determining the optimal concentration of primers, the optimal annealing temperature as well as by determining the MgCl2 concentration.

Specificity:

Specificity was tested for F1/R1 Oligonucleotide primers using template from known bacteria; strains including *Mycobacterium avium* subspecies *avium*, *Mycobacterium tuberculosis* (strain H37 Ra, non virulent) and *Mycobacterium smegmatis* (strain MC²155).

Sensitivity:

MAP RNA was subjected to reverse transcription as described earlier. The standard PCR was performed using Taq DNA polymerase Kit from eppendorf (Brinkman instruments, Westbury, NY) as follows: PCR reactions consisted of PCR buffer (20mM Tris-HCl pH8.0, 100mMKCl, 0.1 EDTA was added to a final concentration of 1X, 1mM DTT, 50% Glycerol, 0.5% tween 20, Betaine (0.5mM), MgCl₂ (5mM), forward and reverse primers (F1/R1), 5ul cDNA and sterile distilled water for a final volume of 50ul into sterile PCR tube. Tubes were incubated in iCycler system (BioRad, CA) for 5 min at 95°C as hot start step, followed by 40 cycles composed each of denaturation phase at 95°C for 30seconds, annealing phase at 60°C for 45 seconds, and extension phase at 72°C for 1min, followed by an extension cycle for 10 min. Product was then purified using PCR purification kit (Qiagen, CA). Concentration was determined by absorbance reading at 260nm in nanodrop system (Nanodrop technologies, Wilmington, DE) and subjected to a series of dilutions (1:1, 1:10, 1:100, 1:1000, 1:10000, 1:100000and 1:1000000) with

an initial quantity of 33ng . Real time PCR was done using F1/R1 set of primers as described in Table 3 after optimization.

Generating Standards for Real time RT_PCR:

These dilutions used in the sensitivity assay were later used as standards when applied this method to clinical samples and cultures.

Melt Curve analysis:

Melt curve analysis was applied to all samples in order to exclude contamination in our study where the nature of the amplified sequence can be confirmed.

The reaction was carried out as follow: the PCR tubes containing real time RT-PCR products were incubated at 95°C for 1 minute followed by another minute at 60°C then 80 cycles with incubation at variable temperature for 10 seconds each, temperatures varied between 65°C and 94°C with an increment of 0.5 °C.

Results

Real time RT-PCR targets and amplifies RNA templates signaling viability of the

template source. In this study we used RT-PCR based on oligonucleotide primers derived from the IS900 sequence which is unique to MAP. All parameters and variables involved in the isolation of RNA, synthesis of cDNA and PCR analysis were evaluated and finally optimized to provide sensitive and maximum specificity outcome. Initially, stock MAP culture was used to prepare MAP cultures in order to be used for RNA extraction which in turn was used in the evaluations of all parameters.

RNA Extraction:

Due to the complexity of the cell wall of MAP and its absence in the MAP cell in human clinical samples, different RNA isolation methods were evaluated. Initially, an RNA isolation kit; RNA/DNA mini kit (Qiagen, CA); was used, the outcome was disappointing due to the lower yield of isolated RNA (Figure 1, lane 1). However, increasing the incubation period with lysosyme from 10 to 30 minutes and at 37 °C instead of room temperature resulted in significant and quality increase in the isolated RNA (Figure 1, lane 2). Further optimization of RNA isolation protocol had resulted in the development of a modified procedure based on the combination of chemical lysis and physical disruption of the MAP cells (Figure 1, lane 3). Physical disruption was achieved using Fast-Prep Ribolyser (Qbiogene, CA). As shown in Figure 1, maximum RNA yield was obtained when the latter RNA isolation protocol (combined chemical and physical disruption treatment) was used (lane 4) compared to the protocol from Qiagen (Lane 1).

Treatment with DNase for 30 min at 37°C resulted in complete DNA removal from the RNA extracts. This was confirmed following direct analysis of the RNA solution by agarose gel electrophoresis which resulted in absence of any DNA band (Figure 2). The purity of the RNA extracts was also confirmed by performing PCR analysis on the RNA extract without synthesis of cDNA template (Figure 3, lanes 1). The MAP target was amplified when the cDNA step was included but not when the cDNA synthesis was eliminated confirms the complete hydrolysis and removal of any DNA traces from the RNA extracts (figure 4).

RNA was quantified using spectrophotometric measurement and then stored at -80 °C until use.

Optimization of the RT-PCR:

Primer Design: MAP DNA consists of high percentage of G+C which is estimated to be 69%. This characteristic favors formation of secondary structures and may lead to false positive and lack of amplification. We used DNA folding software, available at <http://www.bioinfo.rpi.edu/~zukerm/>, to determine the right annealing temperature to minimize secondary structures of the template in order to design and evaluate Oligonucleotide primers specific to MAP. The AV1/AV2 Oligonucleotide primers (Table 2) have been used successfully in our laboratory (3, 47, 51). Figure6 illustrates the structure of IS900 under different temperatures. We designed new Oligonucleotide

primers termed F1/R1 using the Primer 3 software available at <http://frodo.wi.mit.edu/cgi-bin/primer3>. Blast analysis of the F1/R1 primers confirmed the theoretical amplification of MAP template only and provided poor identity with other genomes targets.

The optimal concentration of F1/R1 primers was determined by testing several dilutions of each primer. In this study, primer concentrations ranged from 1 pmole/ul to 30pmole/ul were evaluated. As shown in Figure 7. Concentration of 6pmole/ul has been identified to be the optimal and provided best amplification. Additionally, 6 pmole/ ul resulted in significant reduction of primer dimerization even in negative controls (those tubes lacking any templates).

Annealing temperature:

Following primers design and the estimation of appropriate annealing temperature, we performed a temperature gradient assay on the MAP RNA template. As shown in Figure 8, the annealing temperature ranged from 54 °C to 64 °C with an increment of 1.2 °C. Optimum amplification was detected at 58 °C and 60 °C. Due to the nature of high %G+C content of the MAP genome, we favored to use the 60 °C annealing temperature.

Optimization of Magnesium ions:

The concentration of MgCl₂ in PCR mixtures is known to play a major role in the level of the assay sensitivity. In this study, MgCl₂ concentrations ranged from 0 uM to 30 uM were evaluated. As shown in Figure 9, MgCl₂ at 10 uM has been identified to provide the optimal amplification signal in a 50 ul PCR reaction.

Over All RT-PCR Protocol:

Following the evaluation of all variables included in this RT-PCR assay, we have developed and optimized protocol which is described in Table 3

Sensitivity of RT-PCR:

The optimized protocol described in Table 3 was employed on serial dilutions of MAP cDNA ranged from 33ng to 0.000033ng in order to determine the lowest concentration of RNA template needed for this assay. Figure 10 shows that MAP template was detected in PCR mixtures containing 33ng/ul, 3.3ng/ul, 330pg and 33pg. The lowest concentration of MAP template detected by this assay (33pg) corresponds to 61 copy of the IS900 gene. MAP contains between 14 to 18 copies of the IS900 indicating that this RT-PCR assay may be able to detect as low as 4 MAP cells per sample.

Specificity of the RT-PCR:

In order to confirm the result of the Blast analysis of the Oligonucleotides primers used in this study, several bacterial isolates (Table1) have been cultured and prepared for extraction of RNA. RNA templates were then used for synthesis of cDNA and PCR analysis. As expected, only MAP template was detected. There was no amplification signal from all other microorganisms including the closely related *M. avium* subspecies *avium* (Figures 5).

Clinical Evaluation of the RT-PCR assay

Since the ultimate application of this newly developed RT-PCR assay is to detect viable MAP in clinical samples in hope it will aid in rapid identification of viable MAP in clinical samples, we evaluated a total of 45 samples originated from subjects diagnosed with Crohn's disease, ulcerative colitis and NIBD including healthy controls. All samples were blindly coded in order to conceal the identity and diagnosis of the sample. The diagnosis was revealed following the conclusion of all experiments. Of the 45 samples, 17 were MAP cultures previously identified by acid-fast stain and nested PCR, 24 buffy coat preparations from peripheral blood samples and 4 tissue homogenates from fresh surgically removed tissue specimens. Tables 4 and 5 describe the clinical code of each sample, diagnosis and the nature of the sample. The 17 MAP cultures originated from 13 patients with Crohn's disease, 1 with Ulcerative colitis and 3 from NIBD subjects. The 24

buffy coats preparations consisted of 7 from Crohn's disease patients, 2 from Ulcerative colitis patients and 15 from NIBD individuals. All 4 tissue samples were removed from two Crohn's disease patients with each patient providing inflamed and normal tissue samples. Interestingly, patients who provided tissue samples have also provided peripheral blood samples.

MAP cultures:

Of the 17 cultures used in this study which originated from human sources, 12 (70%) were confirmed to have MAP by conventional nested PCR. All 17 cultures were also evaluated using real time RT-PCR and 11 (65%) were also MAP positive. Interestingly, the 11 MAP cultures confirmed by real time RT-PCR were also MAP positive by conventional nested PCR (Table 4 a and b). Over all, 11/12 (92%) of the MAP confirmed culture using nested PCR were positive by our real time RT-PCR.

Peripheral Blood Buffy coats:

A total of 24 Buffy coats preparations originated from 7 CD, 2 UC and 15 NIBD subjects were evaluated using standard nested PCR and real time RT-PCR assay. By Real time PCR, a total of 7/24 (29%) were confirmed to have viable MAP. Of the 7 MAP

positive samples, 3/7 (43%) from CD, 2/2 (100%) from UC and 2/15 (13%) were from NIBD patients. Using culture and nested PCR assay, a total of 9/24 (37%) was MAP positive, specifically, 3/7 (43%) from CD patients, 2/2(100%) from UC patients, and 4/15 (26%) from NIBD subjects (Table 5a and b). It is important to note that both methods confirmed viable MAP in the same three samples from CD patients. Similar outcome were observed from samples originated from UC patients. However, of the 4 samples from NIBD subjects that were MAP positive by culture and nested PCR, only one was positive by our real time RT-PCR. The real time RT-PCR detected MAP in one NIBD sample that was negative by culture and nested PCR.

Tissue samples:

A total of 4 tissue samples from 2 CD patients (1 inflamed and 1 non-inflamed specimen from each patient) were evaluated by culture and nested PCR and real time RT-PCR. Real time RT-PCR analysis detected MAP in 1/4 (25%) tissue samples. Results from the culture and conventional nested PCR are still pending (table 6). The detection of MAP in tissue by real time RT-PCR originated from inflamed lesion of CD patient. The non-inflamed tissue from this CD patient was negative for MAP using real time RT-PCR. Interestingly, blood sample from the same patient was also negative for MAP using real time RT-PCR indicating a non-systemic state of MAP infection in this CD patient.

Discussion

MAP has been confirmed to be the etiologic agent of JD. Its thermal tolerance, resistance to alcohol and acid treatment and survival in pasteurized milk add more characteristics to its pathogenicity and its virulence potential. As described earlier, MAP role in Crohn's disease pathogenesis has been suggested and vigorously debated. Development of new methodology that facilitates rapid and consistent detection of MAP in clinical samples from humans with Crohn's disease may assist in the elucidation of MAP role in Crohn's disease. The development of RT-PCR assay is challenging due to several reasons. Of which, working with RNA is extremely tedious and when the number of bacterial cells such as MAP in clinical samples is naturally low, it adds to the limitations of the assay. In this study, the RNA yield has been maximized and all precautions were enforced in order to maximize cell lysis and minimize RNA degradation. As shown earlier, a combination between chemical lysis and physical disruption of MAP cells resulted in maximum recovery of MAP RNA. The use of oligonucleotide primers derived from the IS900 of MAP added quality characteristic to this RT-PCR assay. This is due to the presence of about 17 copies of the IS900 genes in a single MAP cell which improves the sensitivity level by several folds.

The potential application of this RT-PCR assay in clinical diagnosis has been validated in this study following the analysis of a total of 45 samples originated from patients with Crohn's disease, Ulcerative colitis and non-IBD subjects. In addition the

coding system enforced here, the majority of these samples were also analyzed in our laboratory for the presence of MAP using other methodology. Ultimately, this technology will aid other conventional techniques such as culture media, despite the need for long term incubation, in rapid detection of viable MAP. Most importantly, the application of this technology will significantly aid the elucidation of MAP role in Crohn's disease pathogenesis. Regardless of the outcome, this test may aid in resolving the MAP debate in Crohn's disease etiology.

Culture can be delicate, as *M. paratuberculosis* is a slow growing and has extremely long incubation times which can raise a contamination problem (2). Other diagnosis methods had shown specificity and viability issues (11), a conventional RT-PCR is hard to establish for detection of MAP in blood or tissue samples due to the small number of bacterium recovered from these samples (14), development of new Real time RT- PCR for detection of MAP in blood and tissue samples from Crohn's disease patients may open a new horizons in establishing a link between MAP and this debilitating disease as well as in the diagnosis of the disease.

In this study, we have been able to extract MAP RNA from blood and tissue samples using a mechanical disruption of the cells combined with chemical extraction, which goes well with systemic nature of the disease (34, 47). The half life of RNA is markedly short and the presence of RNase in the cell extract render the extraction even more delicate (42), the use of RNase inhibitors in the extraction process and working at temperature relatively low helped increasing the RNA recovery. To avoid DNA contamination and ensure clean RNA, we treated the samples with DNase; RNA samples were used as

negative control for the Real time PCR to check for DNA contamination.

We used Real time RT-PCR for it is very sensitive, does not require as much time and to minimize the risk of contamination. Real-time RT-PCR has been recognized as an accurate and sensitive method of quantifying mRNA transcripts (17, 50). This method allowed us to detect accumulation of cDNA template in real time manner using SYBR Green as an intercalating dye, rather than by conventional end-point analysis. Also there is no need for post amplification procedures such as gel electrophoresis, analysis can then be completed rapidly.

Our study was based on amplification of region of the IS900 sequence unique for MAP using specific primers that amplify 165 base pair within the sequence as shown by the specificity assay. The concentration of primers was determined with regard to dimers formation and optimal amplification of the template, in fact, primer dimers are a very common issue for real time RT-PCR (4, 5, 50). The annealing temperature was determined with computational method that takes in consideration the percentage of base pairs, the thermodynamic of the sequence and the possible secondary structures that could be formed at each specific temperature, this had enable us to prevent any nonspecific annealing of primers and helped reduce the generating of secondary structures.

The sensitivity assay showed that our real time RT-PCR is able to detect as low as 61 copy of the IS900 sequence corresponding to 4 genome copies of MAP, which can be of significance knowing that we are using cDNA as template and the number of bacterium per sample is usually low, in addition to the short period of time that it takes to

analyze the samples (less than 12 hours from the blood processing to real time RT-PCR analysis). This can make the real time RT-PCR an alternative way for detection of MAP in clinical samples from CD patients. Other studies using DNA based real time PCR were able to detect lower copies of the genomes that were interested in, for example Danbing Ke et al were able to detect 1 copy of the GBS genome (17). Our method is an RNA based method so we can go in harmony with the goal of our study which is the detection of viable MAP from clinical samples.

The application of our real time RT-PCR to buffy coat from inflammatory bowel disease patients and individuals with non-inflammatory bowel disease (NIBD) and its comparison with cultures of MAP from buffy coat and tissue samples from same patients had shown a concordance of the results and therefore gave more incredibility to our newly developed method. 54.5 % of IBD patients participating in this study are positive for MAP but only 12.6 % of the NIBD were positive, the 12.6% of NIBD could be exposed to MAP. In fact, MAP was isolated from water (41) and from pasteurized milk (24), other studies had found Map in NIBD control with hyperthyroid and diarrhea (3). Overall, this study show that real time RT-PCR could be an alternative way to detect MAP in Crohn's disease patients, this method has an answer for the viability, specificity issues as well as for the long term incubation raised by other methods used to detect MAP in clinical samples. The 54.5% MAP positive patients is a significant percentage, but view the population studied, we still need to diagnose more samples in order to have a confirmed association of MAP with Crohn's disease.

CHAPTER THREE: IFNGR1 GENE IS DOWNREGULATED IN PATIENTS WITH CROHN'S DISEASE: A MYCOBACTERIAL INFECTION?

Summary

In our laboratory, we previously demonstrated a possible downregulation in the Interferon-gamma receptor gene (*IFNGR1*) in patients with active Crohn's disease using microarray chip analysis. In this study, Measurement of RNA by real time qRT-PCR indicated a possible downregulation in 5/6 CD patients compared to 0/12 controls. Overall, a total of 5/6 (83%) CD patients have MAP infection and downregulation in the *IFNGR1* gene. The preliminary data suggest that downregulation in *IFNGR1* gene and the detection of viable MAP together in CD patients provide yet the strongest evidence toward the linkage between MAP and CD etiology.

Introduction

In normal immune response to microbial infection, cascades of signaling pathways are initiated leading to activation of variety of types of immunity cells such as dendritic cells and macrophages (60, 63). In the presence of an initiating antigenic stimulus, antigen presenting cells (monocytes/ macrophages) produce IL-12 that stimulates TH-1 lymphocytes to increase their IFN-gamma production that will then acts on infected macrophages to induce the production of proinflammatory cytokines, such as TNFalpha, IL-6 and IL-1beta, which are the direct cause of inflammation in CD (1,2). This signaling cascade leads macrophages to enhanced phagocytosis and intra-cellular killing of the antigen producing infectious agent and so to the elimination of the antigenic stimulus. However, if the immune system can not clear the antigen by this mechanism, either because of the quantity of the antigen or genetically determined dysfunction in production of cytokines and/or their respective receptors, a misdirected inflammatory situation is seen such is the case of atypical tuberculosis (48), leprosy or any other chronic infection with persisting antigenic burden. CD falls in the same category, in fact, patients with this disease present abnormality in their immune system, but it is not known yet whether these abnormalities are causes or results of the disease.

CD and UC are known as important causes of gastrointestinal disease worldwide but occurring more frequently in the United States, United Kingdom and Scandinavia (19), it is known that Crohn's disease affects 13 out of every 100,000 of the population in the UK and other countries in Europe (59), over 500,000 carries the disease in the United States, the incidence of Crohn's disease is increasing worldwide (25). CD is not a sex or race related, frequently affects people between the ages of 16 and 25, but it can also occur in early childhood or later in life. CD can affect any part of the gastrointestinal tract inflamed segments are discontinuous in form of patches separated by normal bowel. Inflammation is transmural resulting in fistula formation and the most common location is the ileum and small bowel (32). CD prevails in two distinct forms in which it is analogous to two other mycobacterial diseases, leprosy and tuberculosis: a perforating form and a non-perforating form (27, 33, 43). Leprosy and tuberculosis both manifest in two distinct clinical forms: a contained form and an aggressive form (28, 61, 64). UC, in contrast to CD, is a condition in which the inflammatory response is restricted to the colon. Inflammation is limited to the mucosa and is continuous with ulceration and edema. The etiology of any of these two disorders remains unknown, however granuloma and pathologic manifestations in CD resemble aspects found in mycobacteria-caused diseases such as tuberculosis, leprosy and paratuberculosis (16, 53, 8). MAP has been reported as a possible causative agent for CD, this possibility is increasingly gaining momentum due to the recent findings, MAP was isolated from blood, tissue, milk of CD patients (7, 46, 47). Early studies had shown that MAP is resistant to killing by macrophages and/or Polymorphonuclear cells (44)

In 2001, three research groups had reported that three different mutations in NOD2/CARD15 gene are associated exclusively with CD (31; 35, 49). The first susceptibility gene for Crohn's disease was then identified: NOD2 (nucleotide-binding oligomerization domain) better known as CARD15 (Caspase activation recruitment domain) gene annotated as NOD2/CARD15 is expressed as cytoplasmic protein in monocytes, and it is comprised of N-terminus caspase-activation recruitment domains (CARD), a central nucleotide-binding domain, and a C-terminus leucine-rich repeat (LRR) domain. When the LRR domain senses muramyl dipeptide (MDP), the minimal motif of bacterial cell wall peptidoglycan, CARD15 will activate NF κ B. It is also known that NOD2/CARD15 is regulated by inflammatory cytokines, such as TNF α and IFN- γ . Mutations in NOD2/CARD15 were associated with CD, suggesting a role for intracellular pathogen-host interactions in the etiology of the disease.

Medical application of Microarray A study done by Whitney et al. provides a strong support for the feasibility of RNA Microarray analysis of gene expression patterns in peripheral blood as a basis for detection and diagnosis of disease in humans. This study showed that the degree of variation in gene expression in the blood of healthy individuals is smaller than the one seen in the individuals who had cancer or infection (62). An RNA Microarray analysis of the expression profile of genes from CD and UC patients using the buffy coats from peripheral blood had shown that 5630 genes (17% of total number of genes) were differentially expressed in both IBD disorders 200 of which were genes involved in immune response. Interestingly enough, the study showed that IFNGR1 (interferon-gamma receptor) gene, which encodes the ligand-binding chain of the IFN-

gamma receptor, is Downregulated exclusively in CD patients. In this study, we evaluated the expression of IFNGR1 gene using quantitative real time RT-PCR in an attempt to confirm the Microarray findings. Numerous studies had shown that deficiencies in IFNGR1 gene are associated with familial disseminated atypical mycobacterial infection and disseminated BCG infection. Individuals carrying these deficiencies have an immunologic defect predisposing them to infection with mycobacteria (38, 39, 40). If confirmed our finding will add a strong argument to the role of MAP in CD etiology.

In this study, we intend to investigate blood samples from patients with CD, UC, NIBD and healthy controls for the presence of viable MAP using real time RT-PCR described in Chapter 1. The same clinical samples will be subjected to *IFNGR1* gene analysis using qRT-PCR investigating possible downregulation as shown previously in our lab using microarray chip analysis. Ultimately the study will provide for the first time whether there is a link between the IFNGR gene downregulation and susceptibility to MAP infection in patients with active Crohn's disease.

Materials and methods

Patient Selection and Specimen Source

A total of 18 subjects consisting of 6 CD, 2 UC and 10 NIBD were included in this study. Informed consent was obtained from the subjects in accordance with Institutional Review Board regulations at the University of Florida, Gainesville VAMC, and the University of Central Florida. The diagnosis of CD or UC was established on standard clinical, endoscopic, histologic, and radiographic criteria (3). One whole blood sample drawn into a K₂-EDTA sterile Vacutainer[®] tube was collected from each subject. All samples were coded and a self-reported health status and medication use of each subject was recorded using a standardized questionnaire in order to minimize variables such as age, gender, and medication interference.

Peripheral Blood Withdrawal and Processing

The samples were immediately processed in a class II biosafety cabinet. For buffy coat extraction, the tubes were centrifuged at 3000 rpm for 10 min. The buffy coat layer from each tube was transferred into an RNase-free tube containing 750 ul of RNA later solution (Ambion, CA) and kept at 4°C until processing.

RNA extraction:

Total RNA was isolated from Buffy Coats using modified protocol from RNAqueous-4PCR Kit (Ambion, CA). whole blood was centrifuged at 3000 rpm for 10 min, buffy coat layer was then transferred into a 2ml RNase free tube containing 750 ul of RNA later then processed immediately by centrifugation at 13000rpm for 1 minute , cell pellet was treated with lysing solution and Nucleic acid was separated using phenol chlorophorm the aqueous phase was then transferred to new RNase free 2ml tube RNA was washed, eluted then treated with DNase I for 30 min at 37°C. The RNA purity and integrity was assessed by Agarose gel and stored at -80°C to be reverse-transcribed.

cDNA Analysis:

The RNA isolated from buffy coats was subjected to reverse transcription using the super script III kit (Envitrogen, CA) as described in Chapter 1. The cDNA was then used for evaluation by real time RT-PCR.

Quantitative Real time RT-PCR:

A real time RT-PCR was optimized and normalized to evaluate quantitatively the gene IFNGR1 using syber green and MyIQ system (BioRad, CA), 12.5ul of SYBR Green mix were added to MgCl₂ (5mM), Betaine (0.5mM), primers (6 mM), 2.5ul of cDNA,

and sterile distilled water to a final volume of 25ul into sterile PCR tube and were incubated at 95°C for 5 min as hot start step followed by 40 cycles : 20 sec denaturation step at 95°C then 30 sec annealing step at 60°C were the Fluorescence was captured for analysis. Primers were designed with Primer 3 software and blasted against the gene bank data base for specificity; we used Primers MI1/MI2 that amplifies 200bP from the 5' end of the gene. The IFNGR1 gene is GC rich,

MI1: 5'CCCCTTGTCATGCAGGGTGTGA 3'

MI2: 5' CAGGGACCTGTGGCATGATCTGG 3'

To avoid secondary structures, Betaine was added as described in Chapter 1. B-actine was used in order to normalize the reaction as reference gene and the results were analyzed.

IFN gamma ELISA Test:

Interferon gamma was evaluated in sera and plasma from patients with CD, UC and from NIBD individuals using an interferon gamma based ELISA kit (R&D Systems, Minneapolis, MN). Assay Diluent was added to each well in the ELISA plate, followed by Standards, samples, and control as described by the manufacturer. Microtiter plates were incubated for 2 hours at room temperature, followed by washes as described in the kit. IFN- γ Conjugate was added to each well. The plates were then incubated for 2 hours at room temperature, washed again and then incubated in the dark for 30 min. in the

presence of substrate solution. The optical density of each well was determined using a microplate reader set to 450 nm and 570 nm. Reading at 570nm was then subtracted from reading at 450nm in order to correct optical imperfections in the plate.

Results

To evaluate the expression of *IFNGR1* gene, RNA samples isolated from buffy coats of 6 CD patients and 2 UC subjects were analyzed against RNA from buffy coats of 10 NIBD individuals (4 of them were healthy individuals). A quantitative real time RT-PCR was developed and optimized for this purpose. The results were compared to microarray outcomes from previous study done on the same samples. cDNA of *IFNGR1* gene was sequenced for mutation analysis.

Quantitative real time RT-PCR:

Isolated RNA was evaluated using quantitative real time RT-PCR assay developed and optimized for this purpose. The assay was normalized using β -actine gene as reference gene. Primers used were specific for *IFNGR1* gene and amplify a 200 base pair from the 5' end of the gene and specificity of sequence amplified was assisted using melt curve analysis. As shown in Table 8 a and b, all 10 (100%) NIBD subjects including 4 healthy controls expressed normal and expected level of *IFNGR1* gene. Normal gene expression was also observed in both (100%) patients with UC. However, out of the 6

CD patients, 5 (83%) showed defect in the *IFNGR1* gene expression suggesting a possible downregulation. Only one CD (17%) patient showed normal *IFNGR1* gene expression. Our findings also confirmed the microarray analysis data which showed that the *IFNGR1* gene is exclusively downregulated in CD patients. This could give a strong insight to the role of Map in CD since *IFNGR1* defect was associated with Mycobacterial infection (12, 36, 37, 38). Interestingly, evaluation of buffy coat from the patient who expressed *IFNGR1* gene normally, with culture and nested PCR as well as with real time RT-PCR confirmed presence of MAP in blood samples of this patient (table9). An *IFNGR1* gene mutation analysis for samples from this patient is undergoing for further investigating the gene and its role in MAP infection.

Evaluation of levels of IFN- γ in Clinical Sera:

Along with Downregulation analysis, sera from CD and UC patients as well as from NIBD individuals were analyzed to evaluate the level of IFN- γ in blood. Table 10 illustrates data from an ELISA analysis and as expected shows clear increase in concentration of IFN- γ in sera from the majority of CD patients (5/6; 83%), 1/2 (50%) of UC patients and 0/5 of healthy individuals.

One UC and one CD patients presented a normal level of IFN- γ this could be explained by the antimycobacterial treatment they followed. Interestingly, the patients with higher concentration of IFN- γ had downregulated *IFNGR1* gene.

Discussion

IFNGR1 gene encodes the ligand binding chain alpha of the IFN- γ receptor. As shown in this study, using real time RT-PCR, IFNGR1 was downregulated exclusively in patients with CD but not in UC patients. This data confirm what we had found earlier by microarray analysis (figure 15). And correlate with theory that associate MAP with CD (7, 33, 28, 46). Downregulation of IFNGR1 gene may cause a failure of IFN- γ to up-regulate production of TNF- α by macrophages hence dysregulation of antigen processing and presentation (48) even if IFN- γ concentration in plasma from patient with CD and UC is relatively higher as shown by data from ELISA Table 10

In 1996, Newport et al revealed in a case study that deficiency in IFNGR1 expression due to mutation lead to a defect in immune response in patients carrying this mutation predisposing them to mycobacterial infection. Our finding, correlate with Newport study and suggest strongly a host susceptibility condition for establishing a mycobacterial infection. It also support why UC patients having MAP exposure don't develop the same clinical manifestations as CD patients or don't respond to anti-mycobacterial treatments. Preliminary data from the ELISA measurement of interferon gamma level in clinical sera were not surprising. It is well known that INF- γ level is elevated in inflammatory bowel disease patients. The significance of this observation is

important in order to associate that with downregulation of the gene encoding for the receptor of INF- γ . Although, this study is still in the early stages, it clearly indicates the need for a large scale investigation of the susceptibility of the INFGR1 gene in CD patients. We believe we may just have discovered the genetic susceptibility factor in hosts with Crohn's disease.

CHAPTER FOUR: GENERAL DISCUSSION

Association of MAP with CD has been subject of vigorous debate among scientists, Evidence supporting a link between Map and Crohn's disease such as higher detection rates of Map by PCR and culture in clinical samples from Crohn's patients compared with controls; detection of anti-MAP IgG antibodies in sera from CD patients and anti-Map antibiotic therapy resulting in remission, or significant improvements, in many cases. The general opinion among the scientific community is that there isn't enough evidence to prove or deny the role of MAP in CD etiology; nevertheless, the hypothesis is still valid. The etiology of CD had been controversial; many agents were reported to have a role in this etiology; thus, Crohn's disease is now believed to have a multifactorial etiology with involvement of genetic predisposition (NOD2 mutations), environmental factors (infectious agent, diet or smoking), and abnormal inflammatory response (25).

Isolation and culture of MAP from clinical samples from CD patients have been tried in the past. The outcome depended significantly on the technology used and the specimen selection and processing. We previously reported the isolation of MAP from full-thickness tissue samples obtained from CD patients, from Breast milk and from blood samples from CD patients following short term incubation in mycobacterial growth indicator tube (MGIT) culture media (46,47). In all reports, MAP presence in the culture

was evaluated using IS900-based PCR.

As reported by Chiodini, MAP in CD clinical samples is in Spheroplastic form (cell-wall deficient form) which is different from those in animals with JD this contribute greatly to the challenges faced when culturing MAP from Clinical samples from CD patients (7); using technology designed for isolation of bacillary form of MAP as in JD sources may not be applicable to that in human with CD. That alone will play a major factor in the outcome of the study. There is a possibility that exposure to MAP could occur through environmental fecal contamination or from the milk or blood from JD-infected animals which can explain isolation of MAP from NIBD individuals (20, 23, 24, 25).

Development of Real time RT-PCR that detect IS900 MAP specific sequence will eventually aid other conventional techniques such as culture media, despite the need for long term incubation, in rapid detection of viable MAP in clinical samples. Most importantly, the application of this technology will significantly aid the elucidation of MAP role in Crohn's disease pathogenesis. Regardless of the outcome, this test may aid in resolving the MAP debate in Crohn's disease etiology.

The preliminary data of this study along with microarray data suggest that downregulation in INFGR1 gene and the detection of viable MAP together in CD patients provide yet the strongest evidence toward the linkage between MAP and CD etiology. It also supports why UC patients having MAP exposure don't develop the same clinical manifestations as CD patients or don't respond to anti-mycobacterial treatments.

APPENDIX A: FIGURES

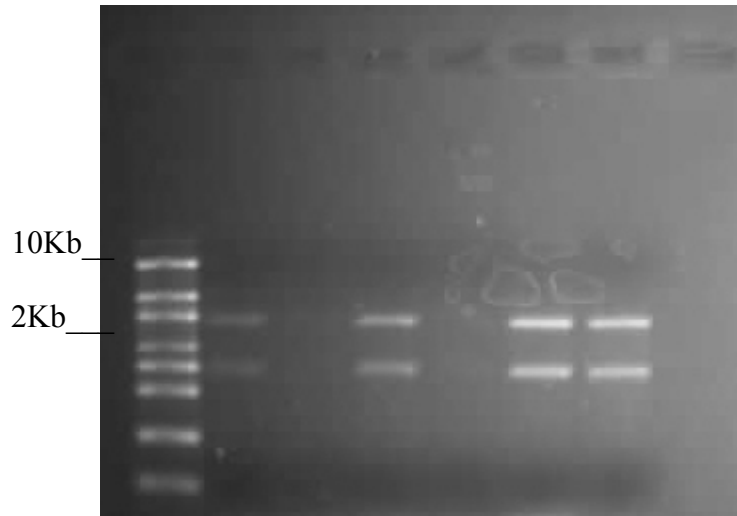


Figure1: comparison of RNA yield with Qiagen protocol, Modified Qiagen protocol and combination of Mechanical and chemical extraction.

RNA samples were mixed with sample loading buffer and analyzed on 1% agarose gel electrophoresis. M: marker, lane 1 corresponds to RNA extraction from manufacturer protocol, Lane 2 corresponds to RNA extraction from modified manufacturer method, and lane3 & 4 correspond to RNA extraction using combination of physical disruption and chemical extraction.



Figure 2: Evaluation of RNA extracts for the presence of DNA using DNase treatment using agarose gel electrophoresis.

RNA extracts isolated using our modified procedure was treated with DNase for 30 min at 37° C.

Band 1 corresponds to 23S ribosomal RNA; Band 2 corresponds to 16S ribosomal RNA

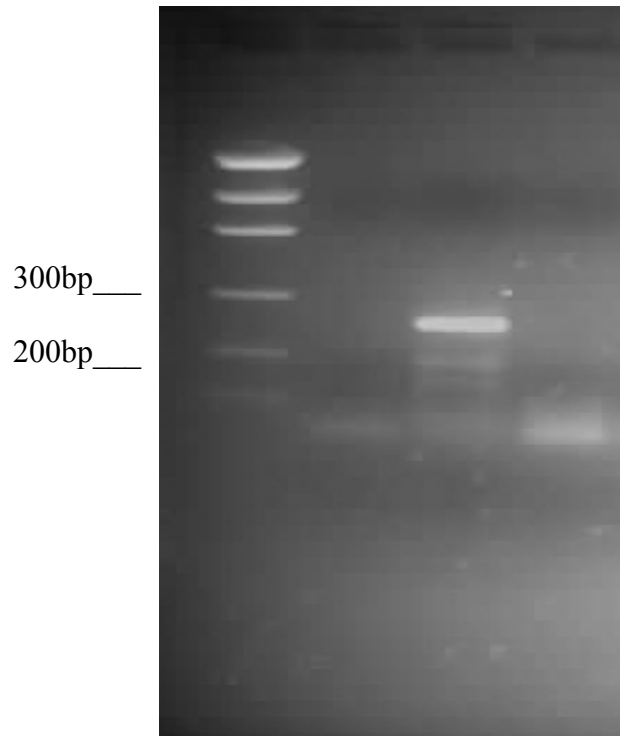


Figure3: Evaluation of RNA extracts for the presence of DNA in the presence and absence of DNase using PCR analysis.

M: corresponds to marker. Lane 1 corresponds to negative controls; Lane 2 corresponds to PCR product using RNA without DNase treatment, Lane 3 corresponds to PCR using RNA with DNase treatment as template.

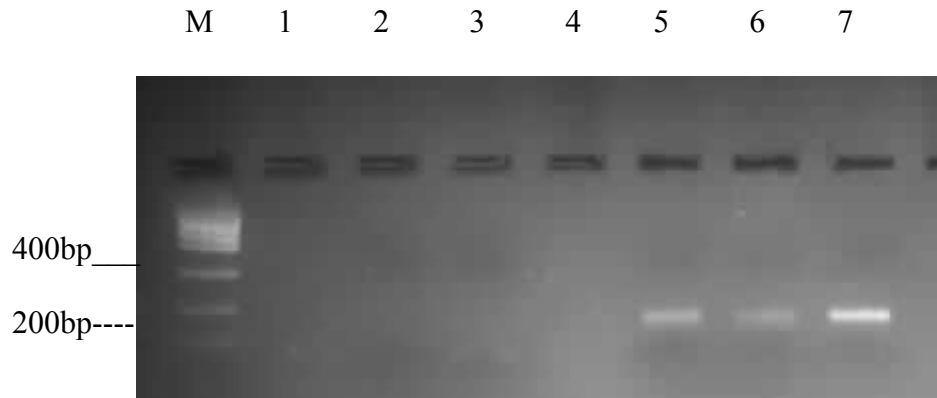


Figure 4: Evaluation of the RNA extracts for the presence of MAP DNA in the presence and absence of cDNA synthesis step.

M corresponds to Marker, Lane 5&6 corresponds to RNA extracts treated with DNase and then subjected to cDNA synthesis before RT-PCR was employed. Lane 3 same as 3 except the cDNA synthesis step was eliminated. Lanes 1 and 2 corresponds to negative control where templates were not included in the PCR amplification. Lane 7: corresponds to positive control where DNA template was used.

PCR Amp/Cycle Graph for SYBR-490

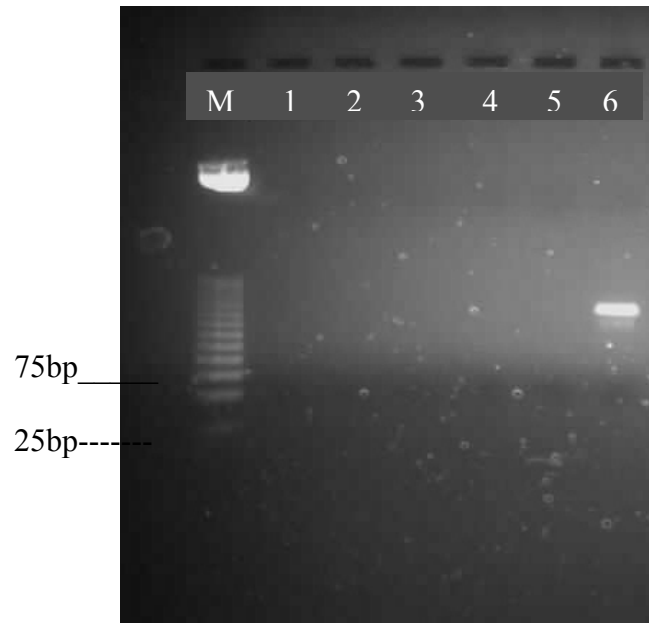
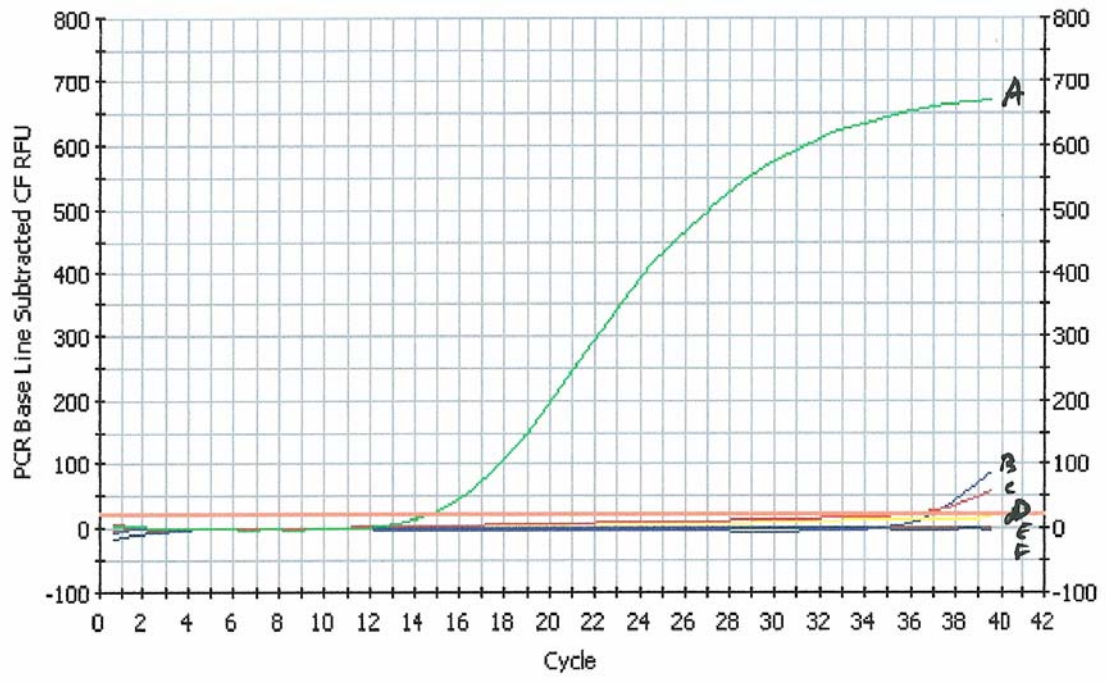
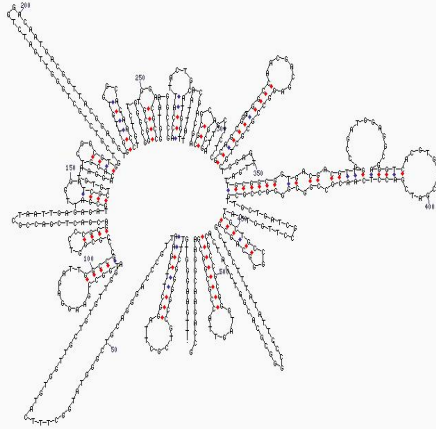


Figure 5 Evaluation of our real-time RT-PCR for specific detection of MAP. RNA templates from 4 different microorganisms were subjected to real-time RT-PCR analysis using our developed protocol. A corresponds to graphical illustration of the real time PCR and B corresponds to agarose gel electrophoresis of PCR products shown in figure 5-I, the green color corresponds to tubes containing MAP cDNA. The blue, red and yellow correspond respectively to MAV, MTB and MSM. IN Figure 5-II, lane 6 corresponds to tubes containing MAP cDNA, lane 1 and 2 correspond to negative controls, lane 3 corresponds to MSM, lane 4 corresponds to MTB and lane 5 correspond to MAV.

A

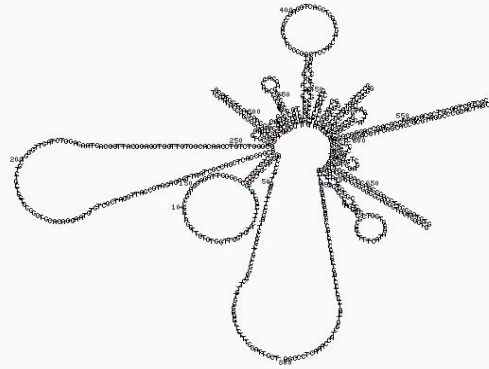
p1422jpa by D. Stewart and M. Zuker
© 2004 Washington University



dG = -28.9 04Jul14-14-21-16

B

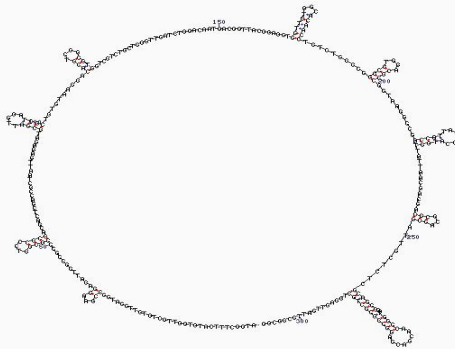
p1422jpa by D. Stewart and M. Zuker
© 2004 Washington University



dG = -10.8 IS900

C

p1422jpa by D. Stewart and M. Zuker
© 2006 Washington University

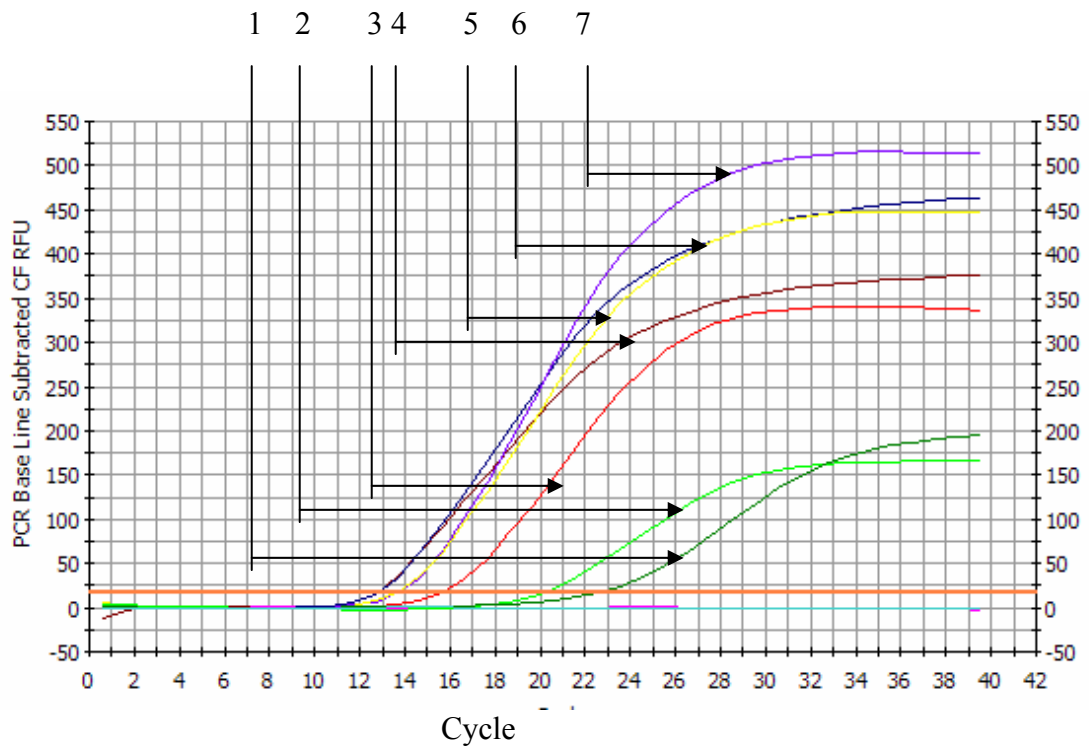


dG = -10.9 IS900

Figure 6: Effect of annealing Temperature on secondary structure formation in IS900 sequence.

Alignment of IS 900 sequence: A: at 55°C, B: at 60°C, C: at 69°C

D)



II) M 1 2 3 4 5 6 7 8 M

150 bp _____

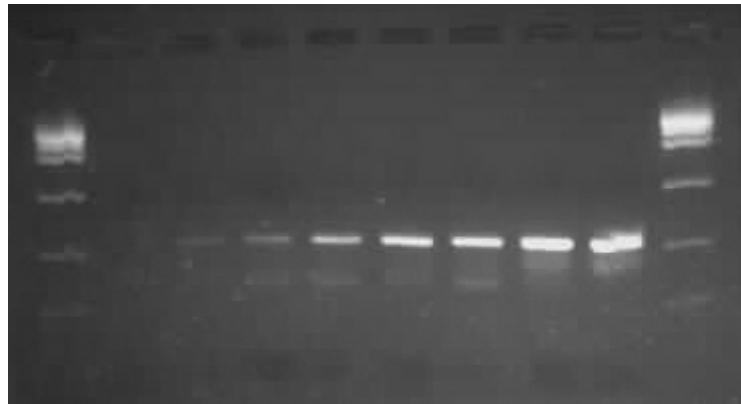
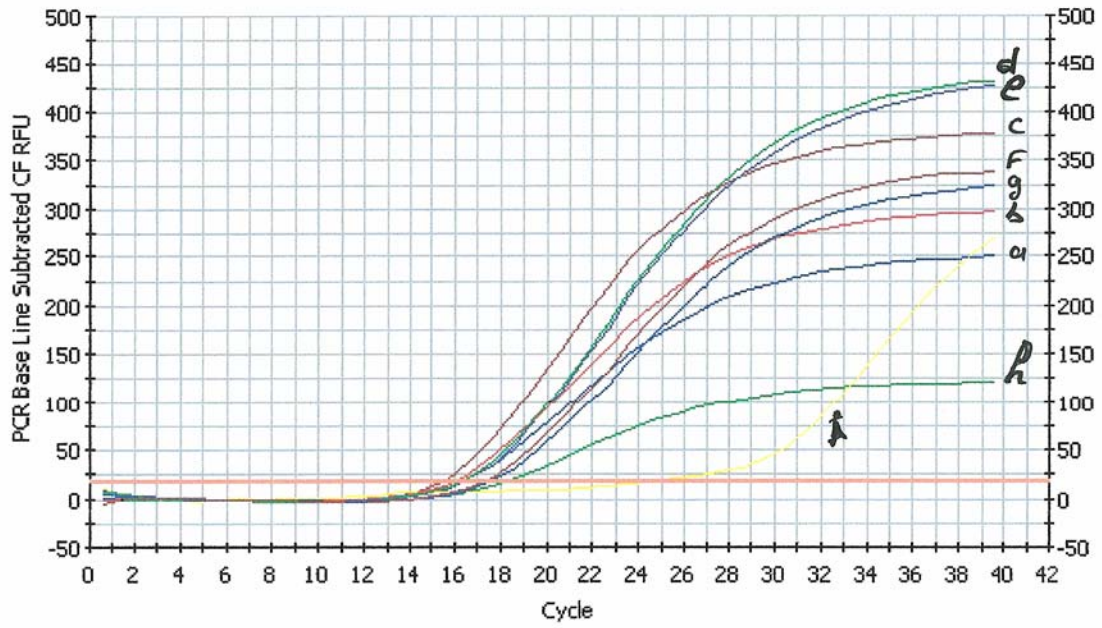


Figure 7: Optimization of the concentration of Oligonucleotide primers used in this study. A serial dilution of F/R1 primers ranged from (0 to 33 pmol/ul; lanes 2-8) were evaluated by real time RT-PCR. Curve7 correspond to a primer concentration of 6pmole/ul M: corresponds to Marker, Lane 1 corresponds to negative control.

I)

PCR Amp/Cycle Graph for SYBR-490



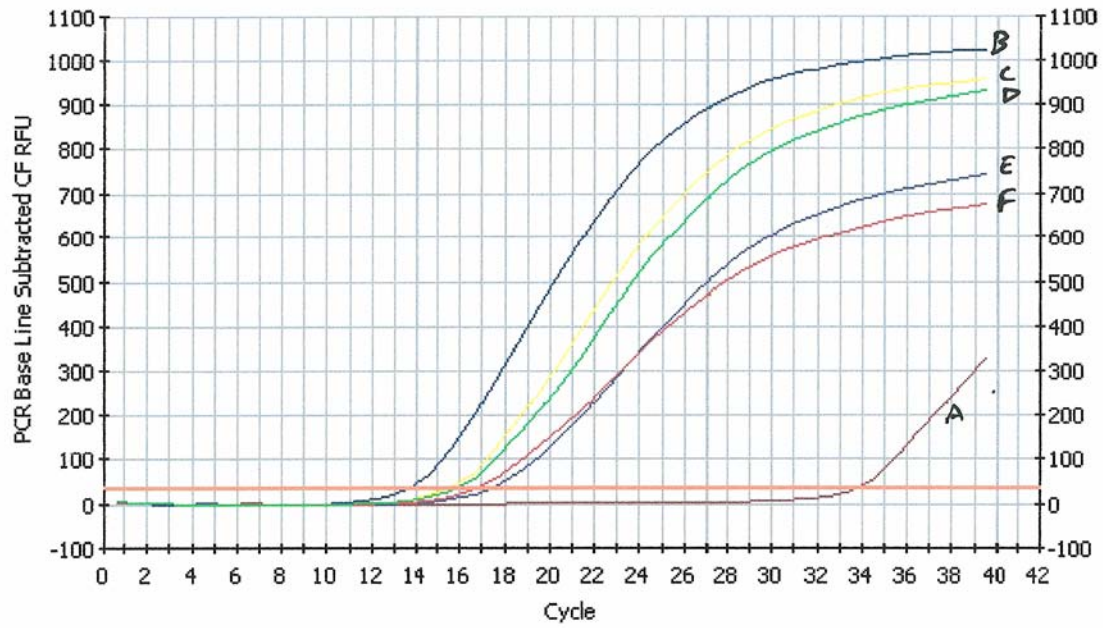
II) M 1 2 3 4 5 6 7 8 9



Figure 8: evaluation of the annealing temperature used in this study:

figure 8-I corresponds to Real time RT-PCR Graph, where curves a to h : corresponds to reaction with annealing temperature that varies from 54°C to 64°C, curve I corresponds to negative control. In figure 8-II, M corresponds to marker, lane 1 corresponds to negative control, and lane 2 to 9 correspond to reactions with annealing temperature from 54°C to 64°C

PCR Amp/Cycle Graph for SYBR-490



b)

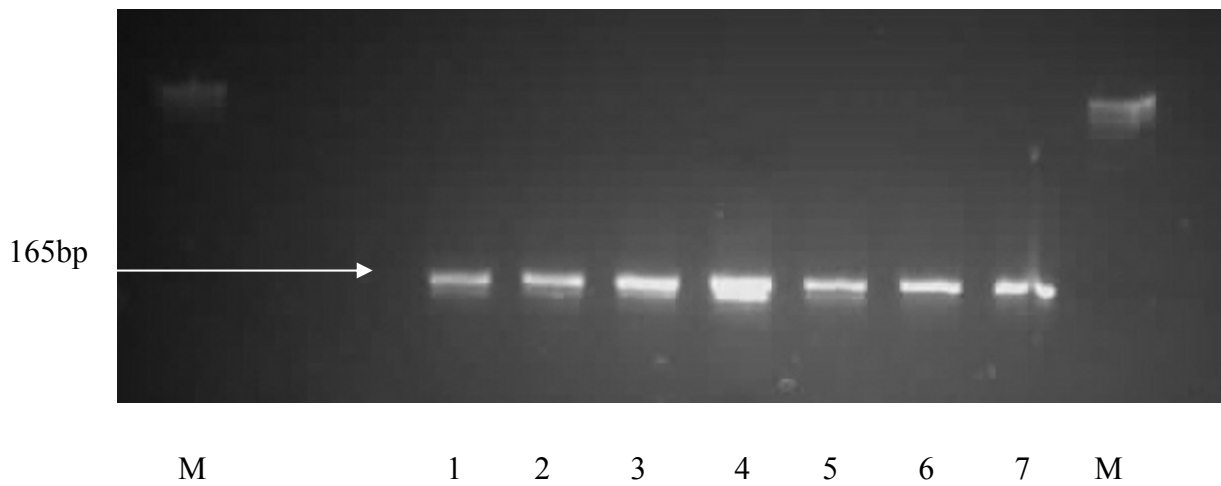
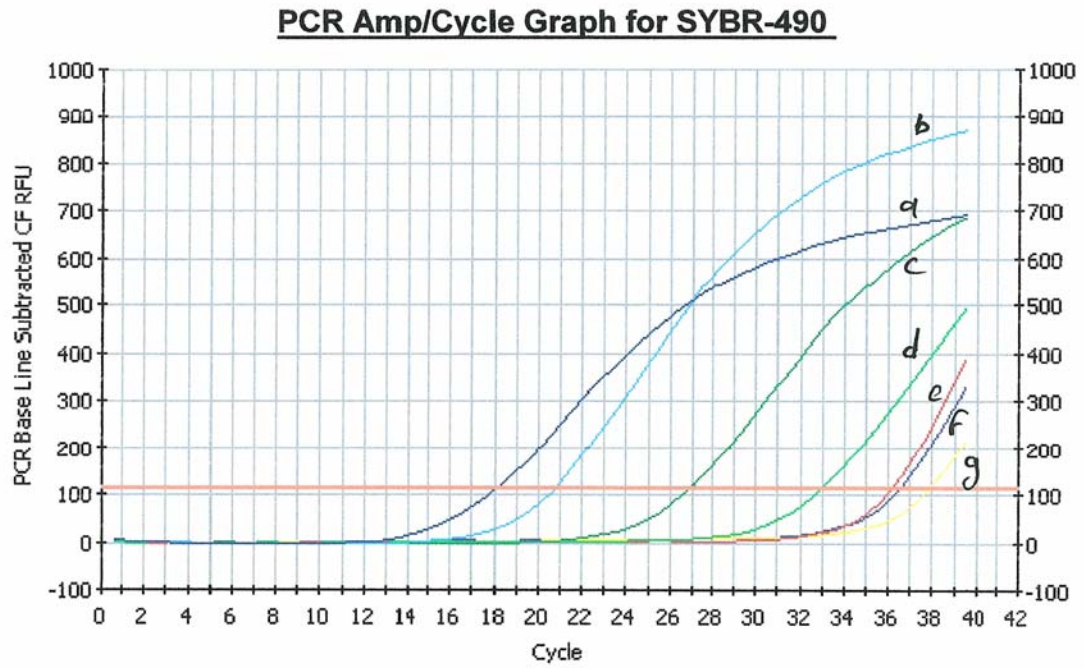


Figure 9: Optimization of the concentration of MgCl₂ used in this study.

A serial concentrations of MgCl₂ from (0 to 30 mM; lanes 1-7) were evaluated by real time RT-PCR. M: corresponds to Marker.

D)



II)

M 1 2 3 4 5 6 7 8

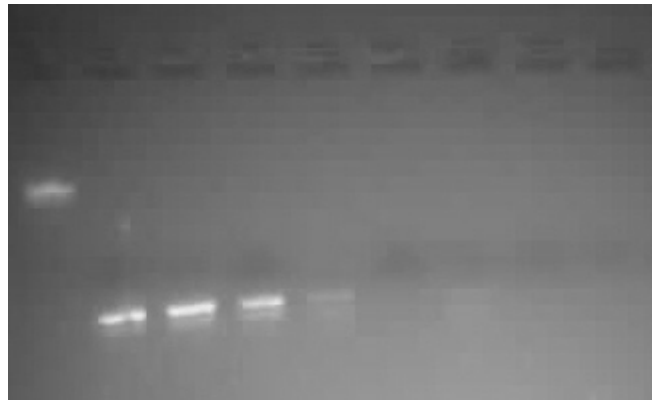


Figure 10: Evaluation of our real-time RT-PCR for sensitive detection of MAP RNA.

Dilution series from 1:10 to 1:1000000 of RNA templates with a starting concentration of 33ng/ul were subjected to real-time PCR analysis using our developed protocol: **Figure10-I)** corresponds to graphical illustration of the real time PCR. Letters from a to g corresponds respectively to tubes containing MAP RNA in respectively 1 to 1:1000000 dilutions. **Figure10-II)** corresponds to agarose gel electrophoresis of PCR products shown in figure 10-I, lanes 1 to 7 corresponds to tubes containing MAP RNA , lane 8 correspond to negative control, lane 1 to 7 correspond to PCR products from RNA dilutions with 1 been RNA with no dilution and 7 been RNA with the higher dilution. M corresponds to marker.

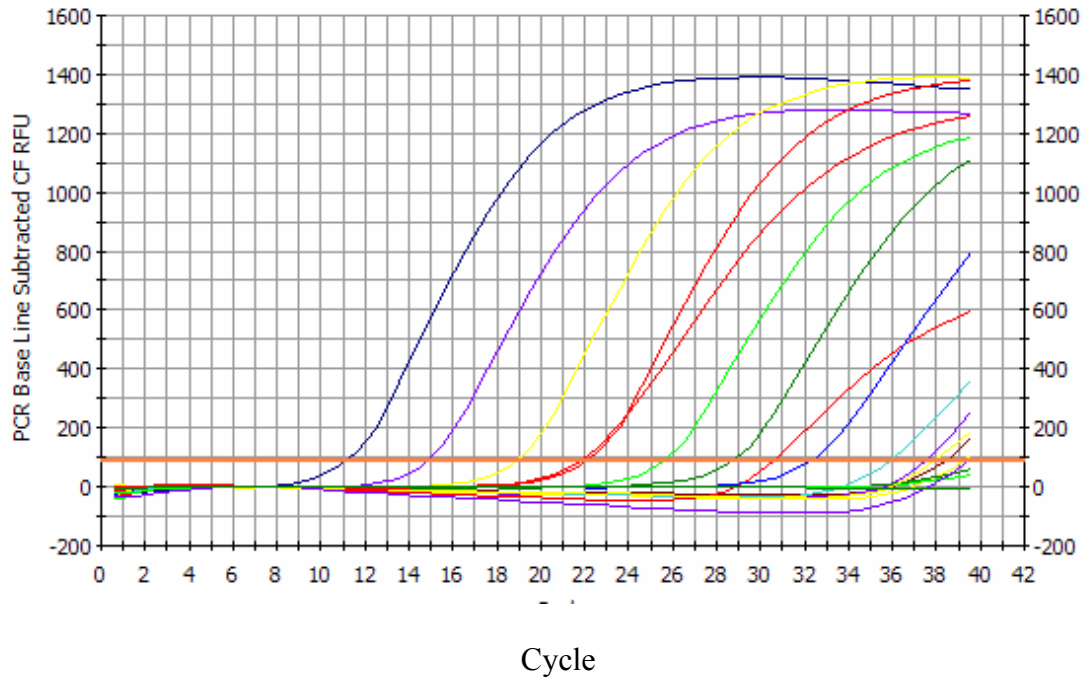


Figure11: Representative figure for evaluation of samples from patients using real time RT-PCR

Each sample is represented by different color according to its position on the plate.

Standards were used in this assay as well as controls.

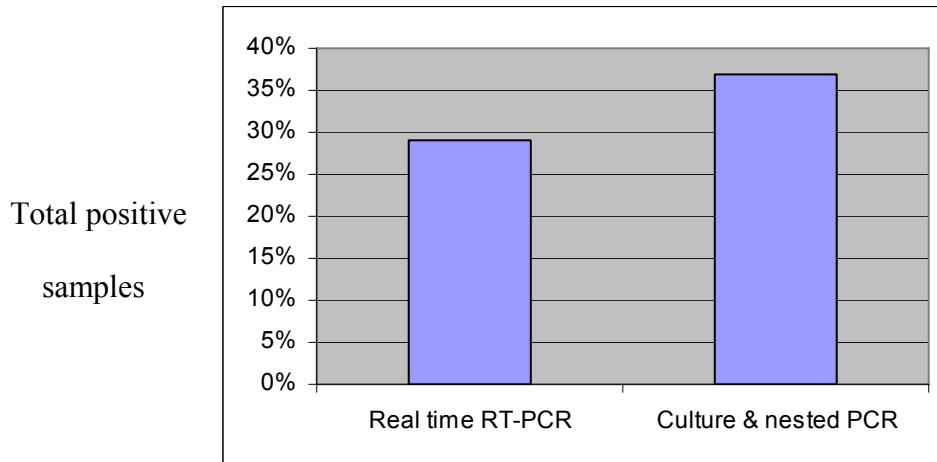
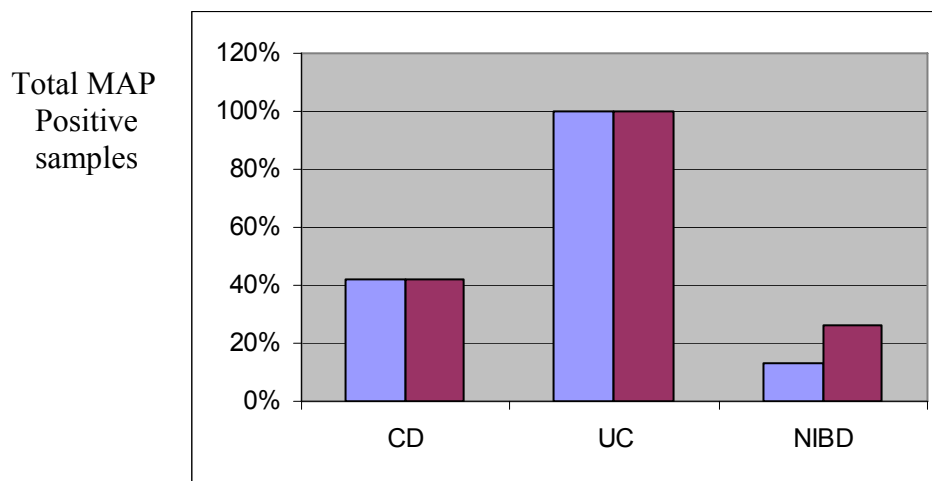


Figure 12: Detection of MAP in human samples by Real time RT-PCR (12hours) and culture confirmed by nested PCR (12weeks).



: Real time RT-PCR
 : Culture and nested PCR

Figure13: Evaluation of MAP positive samples from CD, UC and NIBD individuals using real time RT-PCR compared to culture confirmed by nested PCR

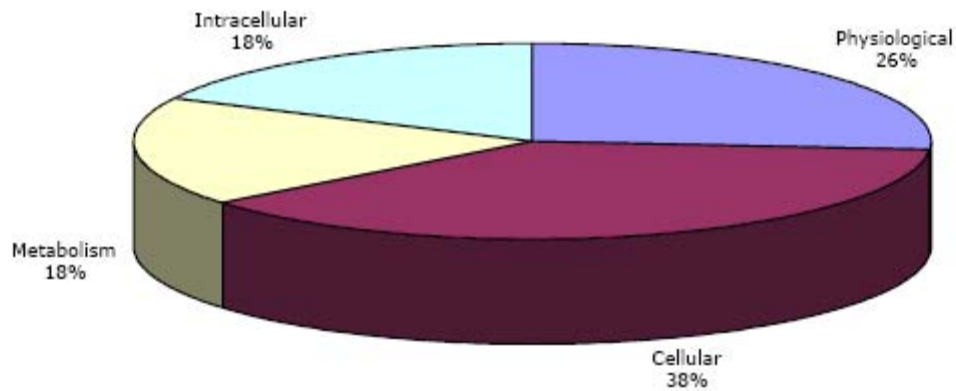


Figure 14: Biological themes predicted by MeV with EASE microarray analysis software

We found that 17% (5630 genes) of the total number of genes was differentially expressed in both IBD disorders compared to the reference pooled sample. These genes were distributed mainly within four biological themes classified as cellular processes (38%), physiological processes (26%), intracellular genes (18%), and genes involved in metabolism (18%). (This figure was obtained from Doctor Romero PhD thesis)

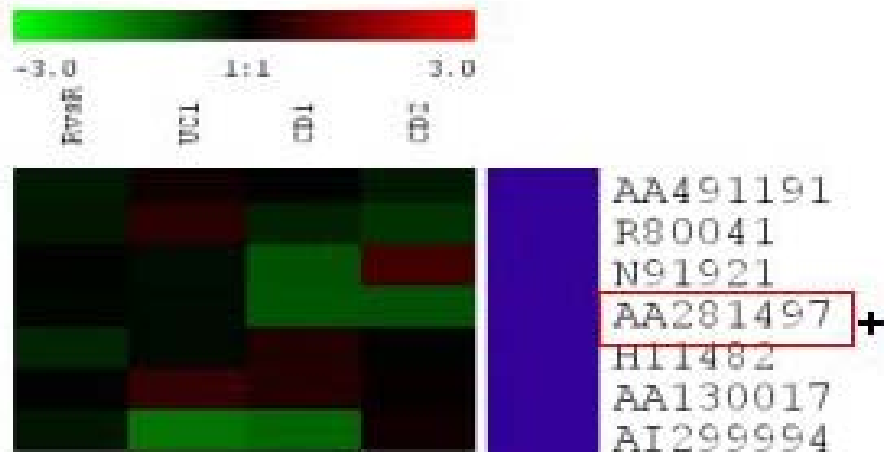
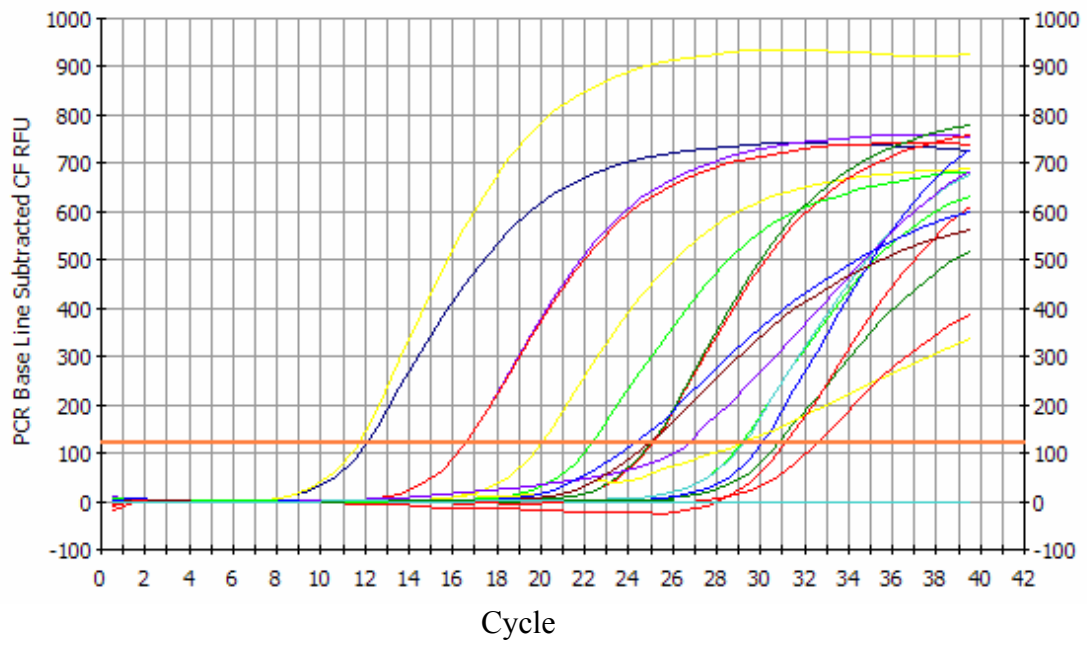


Figure 15: variation in some of the immune response gene expression patterns including IFNGR1 in human white blood cells.

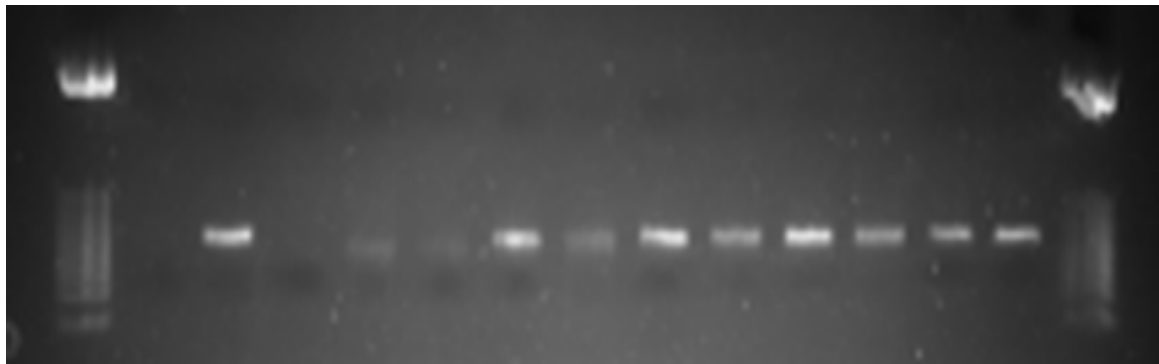
The differentially expressed genes IFNGR1 is boxed in red and indicated by a plus sign next to the red box. Each element is red, green, black, or gray. Black elements have a log ratio (Cy5/Cy3) of 0, while green elements have a log ratio of less than 0 and red elements have a log ratio greater than 0. The further the ratio from 0, the brighter the element is. Gray elements have invalid values and are not used in any analysis.

(This figure was obtained from Doctor Romero PhD thesis)

I)



II)



M N P N 1 2 3 4 5 6 7 8 9 10 M

III)

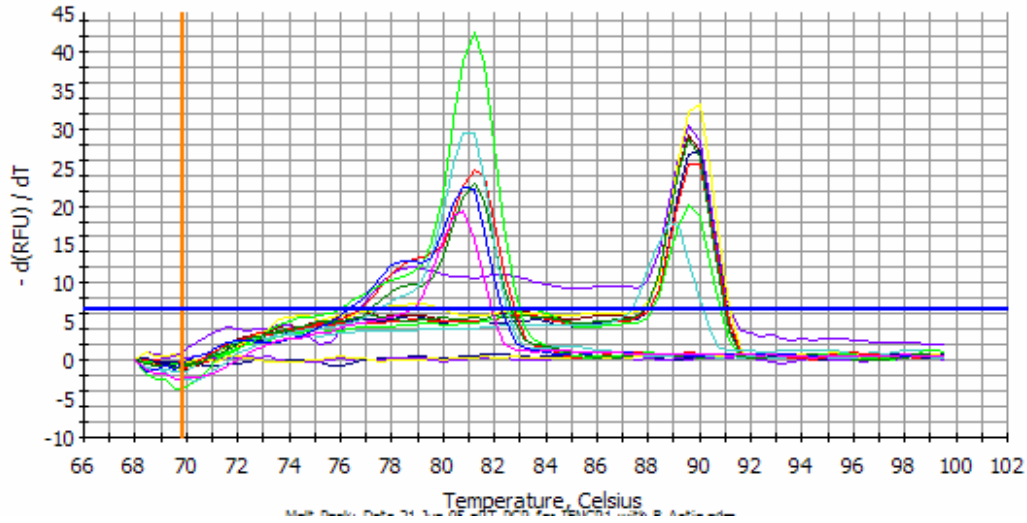


Figure 16: Representative figure for IFNGR1 gene expression analysis using real time RT_PCR.

I) correspond to real time RT-PCR graph, II) correspond to representative gel picture and III) correspond to melt curve which show 2 peaks, one for IFNGR1 around 81°C and another one around 90°C for beta-Actine.

N: correspond to negative controls, P corresponds to positive control, and M corresponds to marker, in the graph each sample is represented by distinguished color

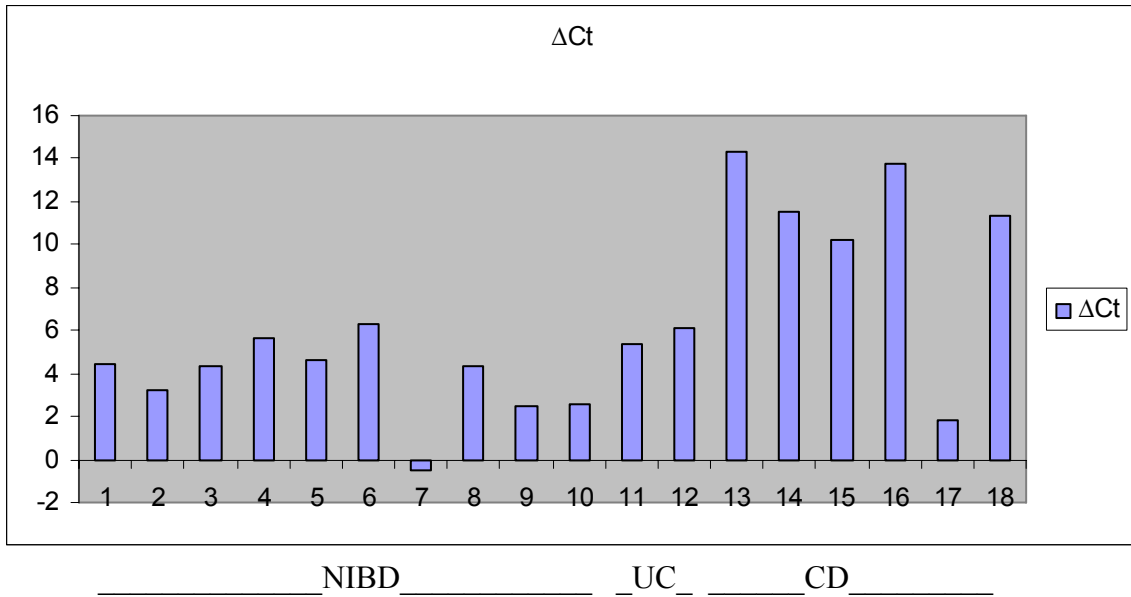


Figure17: graph illustrating quantitative real time RT-PCR data

The higher the ΔCt the lower is the expression.

Figures 1 to 10 correspond to NIBD samples, figures 11 & 12 correspond to UC samples and figures 13 to 18 correspond to CD samples

APPENDIX B: TABLES

Table1: List of microorganisms used in the study.

<i>Microorganism</i>	<i>Strain</i>
Mycobacterium avium <i>subspecies</i> paratuberculosis	ATCC Ben
Mycobacterium avium <i>subspecies</i> avium	Clinical isolate strain LM1
Mycobacterium tuberculosis	ATCC 25177
Mycobacterium smegmatis	MC ² 155

Table2. Oligonucleotide primers used in this study.

<i>Nucleotide sequence</i>	<i>Primer's name</i>	<i>Reference</i>
5' GTT-CGG-GGC-CGT-CGC-TTA-GG-3'	P90	(31, 33, 35)
5' GAG-GTC-GAT-CGC-CCA-CGT-GA-3'	P91	
5' ATGTGGTTGCTGTGTTGGATGG 3'	AV1	
5'CCGCCGCAATCAACTCCAG 3'	AV2	
5' CACGTCGGCGTGGTCGTCT 3'	F1	In this study
5' CGTCACCGCCGCAATCAACT 3'	R1	In this study

Table 3: Optimized protocols for PCR analysis used in this study:

Conditions	conventional Nested PCR	Real time RT-PCR
Thermal cycler	iCycler	My IQ system
Reaction duration	2X 3 h	1:00 h
Reaction volume	50 ul	25 ul
Analysis	gel electrophoresis	real time fluorescence
Amount analyzed	5 ul (10%)	25 ul (100%)
Target/size	IS900 /(400 bp)	IS900 /(165 bp)
Primers/concentration.	P90, 91/(12 pmole/ul each) AV1/AV2 (10pmole/ul)	F1/R1/(6 pmole/ul each)
MgCl2 conc.	4 ul (5mM)	1ul (5mM)
PCR buffer	5ul [200mM Tris-HCl (pH8.4), 500mM KCl]	12.5ul (Buffer, dNTP & enzyme all mixed in one tube)
dNTP concentration	1ul (10mM)	
DNA polymerase	HiFi Taq polymerase (5U/ul)	
PCR parameters	hot start: 5 min/95 oC 36 cycles: 45 s/95 oC 45 s/62 oC 120 s/72 oC	hot start: 3 min/95oC 40 cycles: 10 s/95 oC 30 s/60 oC

Table 4A: Analysis of culture samples For MAP by real time RT-PCR versus culture and nested PCR.

Sample Code	Diagnosis	Real time RT-PCR	Culture& Nested PCR
B2	CD	Positive	Positive
B3	CD	Positive	Positive
R14A	CD	Positive	Positive
R14B	CD	Positive	Positive
B20	CD	Positive	Positive
R33B	CD	Negative	Negative
UCF6	CD	Positive	Positive
UCF7	CD	Positive	Positive
UCF8	CD	Positive	Positive
B34	CD	Positive	Positive
B50	CD	Positive	Positive
B55	CD	Negative	Positive
R60B	CD	Negative	Negative
Total	13	10/13(77%)	11/13(85%)
B39	UC	Negative	Negative
Total	1	0/1	0/1
R6	NIBD	Negative	Negative
B30	NIBD	Negative	Negative
R36	NIBD	Positive	Positive
Total	3	1/3 (33%)	1/3 (33%)

Table 4B: Over all summaries of MAP detection in culture samples by direct analysis using real time RT-PCR versus culture and nested PCR.

Patients Diagnosis	MAP positive by real time RT-PCR	MAP positive by culture & nested PCR
CD	10/13(77%)	11/13(84%)
UC	0/1(0%)	0/1(0%)
NIBD	1/3(33%)	1/3(33%)
Total	11/17(65%)	12/17(70%)

Table5A: Analysis of peripheral blood buffy coat samples for Map detection by real time RT-PCR versus culture and nested PCR.

Sample code	Diagnosis	Real time RT-PCR^c	Culture & Nested PCR^d
BS77	CD	Negative	Negative
BS78	CD	Negative	Negative
BS82	CD	Positive	Positive
BS88	CD	Positive	Positive
BS92	CD	Positive	Positive
BS96	CD	Negative	Negative
BS99	CD	Negative	Negative
Total	7	3/7(42%)	3/7(42%)
BS94	UC	Positive	Positive
BS95	UC	Positive	Positive
Total	2	2/2(100%)	2/2(100%)
BS72	NIBD	Positive	Positive
Bs73	NIBD	Negative	Negative
BS74	NIBD	Positive	Negative
BS75	NIBD	Negative	Negative
BS76	NIBD	Negative	Negative
BS79	NIBD	Negative	Negative
BS83	NIBD	Negative	Positive
BS84	NIBD	Negative	Positive
BS85	NIBD	Negative	Negative
BS86	NIBD	Negative	Positive
BS87	NIBD	Negative	Negative
Total	11	2/11(18%)	4/11(36%)
NS	Healthy	Negative	Negative
MC	Healthy	Negative	Negative
Md	Healthy	Negative	Negative
Bn	Healthy	Negative	Negative
Total	4	0/4 (0%)	0/4 (0%)

CD: corresponds to patients with Crohn's disease

UC: corresponds to patients with ulcerative colitis

NIBD: corresponds to individuals with non-inflammatory bowel diseases.

RT-PCR was performed using R1/F1 oligonucleotide primers

^dbuffy coats were inoculated into MGIT (MYCOBACTERIA GROWTH INDICATOR TUBE) culture media, incubated for a minimum of 12 weeks and then confirmed for MAP using nested PCR based on the p90/91 oligonucleotide primers.

Table 5B: Over all summaries of MAP detection in peripheral blood buffy coat samples by direct analysis using real time RT-PCR versus culture and nested PCR.

Patients diagnosis	MAP Positive by Real time RT-PCR	MAP Positive by Culture & Nested PCR	Real time RT-PCR versus culture & Nested PCR
IBD CD	3/7(42%)	3/7(42%)	100%
UC	2/2(100%)	2/2(100%)	100%
Total	5/9(55%)	5/9(55%)	
NIBD	2/11(13%)	4/11(26%)	50%
Healthy	0/4(0%)	0/4(0%)	100%
Total	2/15(13%)	4/15(26%)	

Table 6: Analysis of fresh tissue samples for MAP detection using real time RT-PCR versus culture and nested PCR.

Sample Code	Diagnosis	Real time RT-PCR	Culture & Nested PCR
R61A	CD, inflamed tissue	positive	Pending
R61B	CD, non-inflamed	negative	Pending
R62A	CD, inflamed	Negative	Pending
R62B	CD, non-inflamed	negative	Pending

Table7: Genes with Differences in the Expression Levels in each subtype of IBD Disorder. (This table was used from doctor Romero PhD thesis).

Gene Bank	Description	Expression in CD patients	Expression in UC patients
AA083407	Tripartite motif-containing 22	No change	Downregulated
AA476221	Unnamed protein product	Downregulated	No change
AA495985	Small inducible cytokine A18 precursor (CCL18)	Downregulated	Upregulated
AI123732	EBV-induced G protein-coupled receptor 2 (EBI2)	Downregulated	No change
AA281497	Interferon-gamma receptor alpha 1	Downregulated	No change
R47893	Small inducible cytokine A3 precursor (CCL3)	Downregulated	Upregulated
AA454646	Lymphotoxin-beta receptor	No change	Upregulated
AI298976	Lymphotactin precursor (XCL1)	Downregulated	No change

Table 8 a: Analysis of Buffy coats from peripheral blood of patients to evaluate IFNGR1 gene expression using quantitative real time RT-PCR

Sample code	Diagnosis	microarray	RT-PCR	Gene expression
		Gene expression	Δ Ct	
BS 25	Healthy	Normal	4.42	Normal
BS 26	Healthy	Normal	3.23	Normal
BS 27	Healthy	Normal	4.38	Normal
BS 28	Healthy	Normal	5.62	Normal
BS54	NIBD	No data	4.61	Normal
BS83	NIBD	No data	6.28	Normal
BS84	NIBD	No data	-0.53	Normal
BS85	NIBD	No data	4.33	Normal
BS86	NIBD	No data	2.46	Normal
BS87	NIBD	No data	2.61	Normal
BS 33	UC	No change	5.33	No change
BS47	UC	No change	6.09	No change
BS 35	CD	Down-regulated	14.31	Down-regulated
BS 37	CD	Down-regulated	11.56	Down-regulated
BS 42	CD	Down-regulated	10.19	Down-regulated
BS 43	CD	Down-regulated	13.78	Down-regulated
BS61	CD	No change	1.87	No change
BS88	CD	No change	11.31	downregulated

Table 8b: overall Summary of IFNGR1 gene expression in CD patients and Non CD individuals.

Patients Diagnosis	down-regulated gene
NIBD	0/10(0%)
UC	0/2 (0%)
CD	5/6(83%)

Table9: Evaluation of IFNGR1 gene expression in with regard to MAP diagnosis in samples from CD, UC and NIBD subjects.

Sample's code	Diagnosis	Q RT-PCR Analysis	MAP Diagnosis
BS25	NIBD	normal	negative
BS54	NIBD	normal	negative
BS85	NIBD	normal	negative
BS87	NIBD	normal	negative
BS33	UC	normal	positive
BS47	UC	normal	negative
BS35	CD	downregulated	negative
BS37	CD	downregulated	positive
BS42	CD	downregulated	positive
BS43	CD	downregulated	positive
BS88	CD	normal	positive

Table 10: Evaluation of level of IFN- γ in sera from peripheral blood of CD, UC and NIBD subjects using ELISA:

Sample	Diagnosis	mean OD reading	IFN- γ concentration	Cut off value
NS	Healthy	0.09	12.5	Normal
MC	Healthy	0.077	6	Normal
Md	Healthy	0.083	9	Normal
Bn	Healthy	0.075	5	Normal
BS84	Healthy	0.067	1	Normal
BS33	UC	0.111	23	Elevated
BS47	UC	0.083	9	Normal
R27	CD	0.087	11	Normal
R28	CD	0.785	360	Elevated
BS35	CD	0.108	21.5	Elevated
BS43	CD	0.11	22.5	Elevated
BS44	CD	0.259	97	Elevated

LIST OF REFERENCES

1. Autschbach F, Eisold S, Hinz U, Zinser S, Linnebacher M, Giese T, Loffler T, Buchler MW, Schmidt J. High prevalence of *Mycobacterium avium* subspecies paratuberculosis IS900 DNA in gut tissues from individuals with Crohn's disease. *Gut*. 2005 Jul, 54(7):944-9.
2. Bernstein, C.N., Blanchard, J.F., Rawsthorne, P. and Collins, M.T. Population-based case control study of seroprevalence of *Mycobacterium paratuberculosis* in patients with Crohn's disease and ulcerative colitis. *J Clin Microbiol*. 2004, 42, 1129–1135.
3. Bull, T.J., McMinn, E.J., Sidi-Boumedine, K., Skull, A., Durkin, D., Neild P., Rhodes, G., Pickup, R. et al., Detection and verification of *Mycobacterium avium* subsp. paratuberculosis in fresh ileocolonic mucosal biopsy specimens from individuals with and without Crohn's disease. *J Clin Microbiol*. 2003, 41, 2915–2923.
4. Bustin, S.A., Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J. Mol. Endocrinol*. 2000, 25, 169-193
5. Bustin S.A., Quantification of mRNA using real time reverse transcription PCR (RT-PCR): Trends and problems. *J. Mol. Endocrinol*. 2002, 29, 23-39
6. Chiodini, R. J. and H. J. van Kruiningen. Characterization of *Mycobacterium paratuberculosis* of bovine, caprine, and ovine origin by gas-liquid

- chromatographic analysis of fatty acids in whole-cell extracts. *Am. J. Vet. Res.* 1985, 46:1980–1989
7. Chiodini RJ, Van Kruiningen HJ, Thayer WR, et al.; Spheroplastic phase of mycobacteria isolated from patients with Crohn's disease. *J Clin Microbiol*; 1986; 27: 357-63.
 8. Chiodini, R.J., Crohn's disease and the mycobacterioses: a review and comparison of two disease entities. *Clin Microbiol Rev.* 1989, 2, 90–117.
 9. Chiodini, R.J. and Hermon-Taylor, J.; The thermal resistance of *Mycobacterium paratuberculosis* in raw milk under conditions simulating pasteurization. *J Vet Diagn Invest.* 1993, 5, 629–631.
 10. Collins, M.T., Update on paratuberculosis: 3. Control and zoonotic potential. *Ir Vet J.* 2004,57, 49–52.
 11. Collins, M.T., Lisby, G., Moser, C., Chicks, D., Christensen, S., Reichelderfer, M., Hoiby, N., Harms, B.A. et al., Results of multiple tests for *Mycobacterium avium* subspecies *paratuberculosis* in patients with inflammatory bowel disease and in controls. *J Clin Microbiol.* 2000, 38, 4373–4381.
 12. Cooper AM, Dalton DK, Stewart TA, et al. Disseminated Tuberculosis in Interferon-gamma Gene-disrupted Mice. *J Exp Med* 1993; 178: 2243-47.
 13. Council for Agricultural Science and Technology, Johne's disease in Cattle. Issue Paper No. 17 (May 2001). Ames, IA: Council for Agricultural Science and Technology.
 14. Corinne Le Dantec et al., Chlorine Disinfection of Atypical Mycobacteria Isolated

- from a Water Distribution System, Applied and environmental microbiology, 2002, 68.3.1025–1032
15. Crohn, B., Ginzberg, L. and Oppenheimer, G. Regional ileitis, a pathological and clinical entity. J Am Med Assoc. 1932, 99, 1323–1329.
 16. Dalziel, T.K. (1913) chronic interstitial enteritis. BMJ ii, 1068–1070.
 17. Danbing et al, Developpement of conventional and Real-Time PCR assays for the rapid detection of group B streptococci, Clinical Chemistry, 2000, 46:3 324-331
 18. European Commission ; Possible links between Crohn's disease and paratuberculosis – report of the Scientific Committee on Animal Health and Animal Welfare. Available at: http://www.europa.eu.int/comm/food/fs/sc/scah/out38_en.pdf ,2000
 19. Everhart JE. Digestive diseases in the United States: Epidemiology and impact; *NIH Publication* 1994; 94: 1447.
 20. Gao, A., Mutharia, L., Chen, S., Rahn, K. and Odumeru, J., Effect of pasteurization on survival of Mycobacterium paratuberculosis in milk. J Dairy Sci 200285, 3198–3205.
 21. Kathy Granger et al, 2004, Recovery of Mycobacterium avium subspecies paratuberculosis from the natural host for the extraction and analysis in vivo-derived RNA, Journal of Microbiological Methods, 2004, 57, 241– 249
 22. Grant, I.R., Ball, H.J. and Rowe, M.T., Effect of high temperature, short time

- (HTST) pasteurization on milk containing low numbers of *Mycobacterium paratuberculosis*. *Lett Appl Microbiol*. 1998, 26, 166–170.
23. IRENE R GRANT, *Mycobacterium avium* ssp. *Paratuberculosis*: its incidence, heat resistance and detection in milk and dairy products, *International Journal of Dairy Technology*, 2001, Vol 54
24. Grant, I.R., Ball, H.J. and Rowe, M.T., Incidence of *Mycobacterium paratuberculosis* in bulked raw and commercially pasteurized cows_ milk from approved dairy processing establishments in the United Kingdom. *Appl Environ Microbiol*. 2002a, 68, 2428– 2435.
25. Grant I. R., Zoonotic potential of *Mycobacterium avium* ssp. *paratuberculosis*: the current position, *Journal of Applied Microbiology*, 2005, 98, 1282–1293
26. Green, E.P., Tizard, M.L.V., Moss, M.T., Thompson, J., Winterbourne, J.J., McFadden, J.J. and Hermon-Taylor, J., Sequence and characteristics of IS900, an insertion element identified in a human Crohn's disease isolate of *Mycobacterium paratuberculosis*. *Nucleic Acids Res*. 1989, 17, 9063–9072.
27. Greenstein RJ, Greenstein AJ. Is There Clinical, Epidemiological, and Molecular Evidence for Two Forms of Crohn's Disease? *Mol. Med. Today*. 1995; 1(7): 343-348.
28. Greenstein AJ, Lachman P, Sachar DB, et al. Perforating and Non-perforating Indications for Repeated Operations in Crohn's Disease: Evidence for Two Clinical Forms. *Gut*. 1988; 29: 588-592.
29. Greenstein, R.J., Is Crohn's disease caused by a mycobacterium? Comparisons

- with leprosy, tuberculosis, and Johne's disease. *Lancet Infect Dis.* 2003, 3, 507–514.
30. Greig, A., Stevenson, K., Perez, V., Hughes, V., Pavlik, I., Hines, M.E., McKendrick, I. and Sharp, J.M. Epidemiological study of paratuberculosis in wild rabbits in Scotland. *J Clin Microbiol.* 1999, 37, 1746–1751.
31. Hampe, J., Cuthbert, A., Croucher, P.J.P., Mirza, M.M., Mascheretti, S., Fisher, S., Frenzel, H., King, K. et al., Association between insertion mutation in NOD2 gene and Crohn's disease in German and British populations. *Lancet* 2001, 357, 1925–1928.
32. Hendrickson BA, Gokhale R, Cho JH. Clinical Aspects and Pathophysiology of Inflammatory Bowel Disease. *Clin Microbiol Rev* 2002; 15(1): 79-94.
33. Hermon-Taylor J, Barnes N, Clarke C, et al., *Mycobacterium paratuberculosis* Cervical Lymphadenitis Followed Five Years Later by Terminal Ileitis Similar to Crohn's Disease. *Br. Med. J.*, 1998, 316:449-453.
34. Hermon-Taylor, J. and Bull, T., Crohn's disease caused by *Mycobacterium avium* subspecies paratuberculosis: a public health tragedy whose resolution is long overdue. *J Med Microbiol.* 2002, 51, 3–6.
35. Hugot, J.P., Chamaillard, M., Zouali, H., Lesage, S., Cezard, J.P., Belaiche, J., Almer, S., Tysk, C. et al., Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature*, 2001, 411, 599–603.
36. Jouanguy E, Altare F, Lamhamedi S, et al., Interferon-gamma receptor

Deficiency in an Infant with Fatal Bacille Calmette-Guerin Infection. *N Engl J Med.* 1996, 335: 1956-61.

37. Jouanguy E, Lamhamedi S, Lammas D, et al., A human IFNGR1 small deletion hotspot associated with dominant susceptibility to mycobacterial infection. *Nat Genet*, 1999, 21: 370-78.
38. Jouanguy E, Lamhamedi-Cherradi S, Altare F, et al., Partial interferon - γ - receptor 1 deficiency in a child with tuberculoid bacillus Calmetter-Guerin infection and a sibling with clinical tuberculosis. *J Clin Invest.* 1997, 100: 2658-64.
39. Kim DS, Jeon YG, Shim TS, et al., The value of interleukin-12 as an activity marker of pulmonary sarcoidosis. *Vasc Diffuse Lung Dis.* 2000; 17: 271-6.
40. Kobayashi K, Blaser MJ, Brown WR., Immunohistochemical examination for mycobacteria in intestinal tissues from patients with Crohn's disease. *Gastroenterol.* 1989, 96: 1009-15.
41. Janet E. Harris, Anna M. Lamberding, Crohn's disease and Mycobacterium avium sbsp. Paratuberculosis: current issues, *Journal of food protection.* 2001, 64, 12, 2103-2110
42. J.A.Mangan, K.M. Sole, D.A. Mitchison and P.D. Butcher An effective method of RNA extraction from bacteria refractory to disruption including mycobacteria. *Nucleic acids research.* 1997, vol 25 No 3 675-676

43. Mishina D, Katsel P, Brown ST, et al; On the etiology of Crohn's disease. *Proc Natl Acad Sci U S A*. 1996, 93: 9816–20.
44. Mohagheghpour N, van Vollenhoven A, Goodman J, et al., Interaction of *Mycobacterium avium* with human monocyte-derived dendritic cells. *Infect Immun*. 2000, 68: 5824-9.
45. Naser, S.A., Hulten, K., Shafran, I., Graham, D.Y. and El-Zaatari, F.A.K. Specific seroreactivity of Crohn's disease patients against p35 and p36 antigens of *Mycobacterium avium* subsp. *paratuberculosis*. *Vet Microbiol*. 2000a, 77, 497–504
46. Naser, S.A., Schwartz, D. and Shafran, I. Isolation of *Mycobacterium avium* subsp. *paratuberculosis* from breast milk of Crohn's disease patients. *Am J Gastroenterol*. 2000b, 95, 1094–1095.
47. Naser SA, Ghobrial G, Romero C, et al. Culture of *Mycobacterium avium* subspecies *paratuberculosis* from the blood of patients with Crohn's disease. *Lancet*. 2004; 364: 1039-44.
48. Newport MJ, Huxley CM, Huston S, et al. A mutation in the interferon- γ -receptor gene and susceptibility to mycobacterial infection. *N Engl J Med*. 1996; 335: 1941-9.
49. Ogura, Y., Bonen, D.K., Inohara, N., Nicolae, D.L., Chen, F.F., Ramos, R., Britton, H., Moran, T. et al. A frame shift mutation in Nod2 associated with susceptibility to Crohn's disease. *Nature*. 2001, 411, 603–606.
50. I.R. Peters et all, Real time RT-PCR: considerations for efficient and sensitive

- assay design, journal of immunological methods. 2004, 286, 203-217
51. Romero, C., Hamdi, A., Valentine, J.F. and Naser, S.A. Evaluation of surgical tissue from patients with Crohn's disease for the presence of *Mycobacterium avium* subsp. *paratuberculosis* DNA by in situ hybridization and nested polymerase chain reaction. *Inflamm Bowel Dis*. 2005, 11, 116–125.
52. Sartor RB, Does *Mycobacterium avium* subspecies *paratuberculosis* cause Crohn's disease? *Gut*. 2005 1;54(7):896-8.
53. Saxegaard F. Isolation of *Mycobacterium paratuberculosis* from intestinal mucosa and mesenteric lymph nodes of goats by use of selective Dubos medium. *J Clin Microbiol* 1985; 22: 312–3.
54. Schwartz, D., Shafran, I., Romero, C., Piromalli, C., Biggerstaff, J., Naser, N., Chamberlin, W. and Naser, S.A. Use of shortterm culture for identification of *Mycobacterium avium* subsp. *paratuberculosis* in tissue from Crohn's disease patients. *Clin Microbiol Infect*. 2000, 6, 303–307.
55. Sechi, L.A., Manuela, M., Francesco, T., Amelia, L., Antonello, S., Giovanni, F. and Stefania, Z. Identification of *Mycobacterium avium* subsp. *paratuberculosis* in biopsy specimens from patients with Crohn's disease identified by in situ hybridization. *J Clin Microbiol*. 2001,39, 4514–4517.
56. Sechi, L.A., Mura, M., Tanda, E., Lissia, A., Fadda, G. and Zanetti, S. *Mycobacterium avium* subsp. *paratuberculosis* in tissue samples of Crohn's disease patients. *Microbiologica*. 2004, 27, 75–77.

57. Selby, Mycobacterium avium subspecies paratuberculosis bacteraemia in patients with inflammatory bowel disease, *Lancet*. 2004,18; 364(9439):1013-4.
58. Shafran, I., Piromalli, C., Decker, J.W., Sandoval, J., Naser, S.A. and El-Zaatari, F.A.K. Seroreactivities against *Saccharomyces cerevisiae* and *Mycobacterium avium* subsp. paratuberculosis p35 and p36 antigens in Crohn's disease patients. *Dig Dis Sci* 47. 2002a, 2079–2081.
59. Shivananda, S., Lennard-Jones, J., Logan, R., Fear, N., Price, A., Carpenter, L., Van Blankenstein, M. and the EC-IBD Study Group Incidence of inflammatory bowel disease across Europe: is there a difference between north and south? Results of the European Collaborative Study on Inflammatory Bowel Disease (EC-IBD). *Gut* 39. 1996, 690–697.
60. Targan S. et al, Inflammatory bowel disease from Bench to bed side. 2nd edition
61. Vidal Pessolani MC, Marques MA, Reddy VM, et al. Systemic dissemination in tuberculosis and leprosy: do mycobacterial adhesins play a role? *Microbes Infect.* 2003; 5(7): 677-84.
62. Whitney A, Diehn M, Popper S, et al. Individuality and variation in gene expression patterns in human blood. *Proc Natl Acad Sci*. 2003; 100:1896-1901.
63. Whitt S., et al, Bacterial pathogenesis, 2nd edition
64. Yamamora M, Uyemura K, Deans RJ, et al., Defining Protective Responses to Pathogens: Cytokine Profiles in Leprosy Lesions. *Science*. 1991; 254: 277-279.