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I am submitting here with a thesis written by Gary Taylor Fielden entitled “Survey of Crohn’s diseased patients’ sera utilizing the flow cytometry method.” I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major Wildlife and Fisheries Sciences, and a minor in Statistics.

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SURVEY OF CROHN’S DISEASED PATIENTS’ SERA UTILIZING THE FLOW CYTOMETRY METHOD

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Gary Taylor Fielden
August 2007
DEDICATION

This thesis is dedicated to all those who have inspired me in a variety of ways throughout my life. My sister, Laura, who taught me that there is always another story that could be told, another way to look at things, and a third variable that needs accounting for. My father, Gary Fielden, for fostering a healthy appreciation for the natural world we swirl around in. I’d also like to thank my mother, Vicki Glardon, who showed me that no matter how hard life can be, a positive attitude and hard work will carry you through. Also, everyone involved with our research for your continued patience and guidance over the last two years.
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NOMENCLATURE

ºC: Degrees Celsius
µL: Microliter
cfu: Colony-forming Unit
kbp: Kilobase pair
kDa: KiloDalton
ml: Milliliter
RPM: Revolutions per minute
M: Molar (moles/liter)
mM: Millimolar (milimoles/liter)
V: Voltage

ABBREVIATIONS

2’ Ab: Secondary antibody
AGID: Agar gel immunodiffusion
ASCA: anti-\textit{S. cerevisiae} antibodies
CCFA: Crohn’s and Colitis Foundation of America
CD: Crohn’s Disease
CF: Complement fixation
CWD: Cell wall deficient
ELISA: Enzyme-linked immunosorbant assay
FAE: Follicle Associated Epitheleum.
FBS: Fetal Bovine Serum
FC: Flow cytometry
FCM: Flow cytometry method
FITC: Fluorescine isothiocyanate.
GALT: Gut-associated Lymphoid Tissue
GI: Gastrointestinal
HC: Healthy Controls
IBD: Inflammatory Bowel Disease
IFN-γ: Interferon gamma
IgA: Immunoglobulin A
IgG: Immunoglobulin G
p-ANCA: Atypical perinuclear anti-neutrophil cytoplasmic autoantibodies
JD(+/−): Johne’s Disease positive/negative
LAM: Lipoarabinomannan
MAA: \textit{Mycobacterium avium} subsp. \textit{avium}
MAP: \textit{Mycobacterium avium} subsp. \textit{paratuberculosis}
MHC: Major histocompatibility complex
OD: Optical density
PP: Peyer’s Patch
SEM: Scanning electron microscope
TCR: T cell-receptor
UC: Ulcerative Colitis
Abstract

Crohn’s disease is a chronic inflammatory bowel disease of unknown origin that continues to affect millions of people worldwide. *Mycobacterium avium* subsp. *paratuberculosis* (MAP) has been a suspected etiological agent of Crohn’s disease for nearly two decades. Recently, our laboratory was able to achieve a test sensitivity of 95.2% (n=21) by using a flow cytometry method (FCM) to detect anti-MAP antibodies in Johne’s diseased cattle. Here we investigate whether FCM can differentiate Crohn’s patients’ antibody titers from healthy human control antibody levels. As a second research goal, we investigated other suspected bacteria and serum-differentiating yeast using the FCM. In brief, bacteria/yeast were incubated with serum samples and washed to remove nonspecific antibodies. Antibodies bound to the surface of bacteria/yeast were then labeled with fluorescently-tagged secondary antibody and this binding evaluated by flow cytometry. Serum samples from 37 Crohn’s patients and 37 healthy human controls were tested for antibody (IgG and IgA) binding to bacteria/yeast. When targeting antibodies directed towards MAP, no significant difference was observed between the two populations (ANOVA, alpha=0.05). FCM was also unable to detect an antibody response for either group when directed against a cell-wall-deficient form of MAP, which has been reported to be near infected tissues of Crohn’s diseased patients. However, a differential antibody response to two yeasts that included *S. cerevisiae* and *C. albicans* was detected when looking for both IgG and IgA. This differentiation using yeast agrees with previous studies that have shown Crohn’s diseased subjects to have significantly higher antibody titers to this yeast than do healthy controls. This suggests that the flow cytometry method could be useful in the future for distinguishing Crohn’s subjects from healthy controls.
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CHAPTER I: SCOPE AND OBJECTIVES OF STUDY

Since its’ first description in the early 20th century Crohn’s disease (CD) has continued to confuse investigators and doctors alike, as the exact cause for CD has yet to be fully described. Since 1984, when Mycobacterium avium subsp. paratuberculosis (MAP) was first cultured from the inflamed intestinal tissue of three Crohn’s diseased patients [1], the possibility of there being a bacterial etiology for Crohn’s disease has been under intense scrutiny. This question remains unresolved; however the plethora of research on the topic includes many findings that indicate MAP as an agent of CD. It is of great importance to note that MAP is shed in the milk of dairy cows with Johne's disease (JD) and can survive the pasteurization process. Thus, there is an immediate need to study the involvement of this potentially zoonotic bacterium in the pathogenesis of Crohn’s disease. Yet, a consistently sensitive test for MAP is lacking even for JD-infected animals.

In 2005, our lab was able to differentiate populations of JD-infected and JD-negative dairy cattle using a novel detection method that utilized a Flow cytometer. We obtained a sensitivity of 95%, which was considerably higher than commercially available diagnostic ELISAs that typically having sensitivities in the 15-30% range [2]. The method differs from these ELISAs in that it uses whole-cell MAP and therefore is able to detect antibodies directed against an array of MAP surface antigens. Our primary objective in this study was to investigate the prevalence MAP-directed antibodies in Crohn’s diseased subjects and healthy controls. We would utilize a similar technique to that used in the JD studies. Since MAP is not the only bacterial suspect to cause CD, we also investigated other bacteria as a secondary objective. The method is universally designed to work for any bacteria and could be performed on any of these suspects. Our third objective was to investigate antibodies directed to certain yeast species which have been known to improve the diagnostic prognosis of CD from other similar inflammatory bowel diseases. If our method could increase the sensitivity of this test, those with unknown IBD could be more accurately diagnosed and begin receiving the appropriate treatments.
CHAPTER II: BACKGROUND

General Information on Crohn’s Disease

An Introduction to Crohn’s Disease

Crohn’s disease (CD) is a chronic debilitating inflammatory bowel disease that affects millions of people each year. The disease is named for Dr. Burrill B. Crohn, one of three authors to first describe the disease in 1932 [3]. In America alone, the disease is believed to affect about a million individuals according to The Crohn’s and Colitis Foundation of America (CCFA) [4]. Interestingly, developed countries seemed to be more prone to high CD prevalences within their populations, but Western Europe and the United States seem to be particularly vulnerable [5].

Crohn’s Disease is a member of a broad group of inflammatory bowel diseases (IBDs) of unknown origin occurring in the gastrointestinal (GI) region. The similarities between CD and a second IBD called ulcerative colitis (UC) are striking and thus a differential diagnosis can often be difficult. CD inflamed tissue regions can occur at nearly any region of the GI tract, but nearly 70% of those infected with CD will have granulomatous legions of inflamed tissue in the small intestine or the junction region of the cecum [6]. Diseased tissue is often described as cobblestoned with patches of healthy intestinal lumen often interrupting diseased portions [7]. Patients stricken with CD often experience bouts of stomach pain and have their lives severely affected by the unpredictability of their digestive system, a continuous malaise, and the coexisting malnutrition which often occurs. Severe weight loss and diarrhea are common signs that CD may be setting in [7].

In about 50% of CD patients, inflammations can become severe enough to bring about a blockage (stenosis) that requires surgery [6]. The Crohn’s and Colitis Foundation of America estimates that, overtime, nearly two-thirds of CD patients will require a surgery to relieve a disease symptom. One common surgery is the removal of blockages of the GI tract caused by extremely inflamed tissue [8]. Another is the removal of fistulas, which are regions of the intestine that have become connected with other, unintended organs and are leaking GI constituents to these organs [8]. An average of 25% of CD patients are expected to have such fistulas [8]. The bladder, the vagina, as well as other portions of the GI tract are commonly connected by such fistulas. Internal bleeding within the intestines is a third disease severity which often calls for surgery. About 5% of CD patients develop other more obscure symptoms like eye, skin, or joint inflammations due to CD onset [7].

Disease onset generally occurs in those 16-25 years of age but has also been seen to occur both in early childhood and late adulthood [9]. There is no standard method for diagnosing CD and a plethora of tools are generally used. The average wait time for specific diagnosis after presenting symptoms was recently reported to be 2 years [6]. Diagnostic approaches include X-ray
There is no cure for CD, only pharmaceuticals to relieve symptoms and severity of disease. Of the many treatments available general immunosuppressants are used for severe cases of CD. This is because immunosuppressants in the form of steroids tend to weaken the immune system's ability to fight other pathogens which is a dangerous venture. Anti-inflammatories are very popular treatment choices for CD patients who have more moderate symptoms. Antibiotics are also commonly administered to aid the downgraded immune system in fighting infections.

### The Three Main Crohn’s Disease Etiological Theories

The name “Crohn’s disease” is often described as a misnomer as it may be better characterized as a syndrome, rather than a specific disease. Disease often entails a single causative agent. In CD, this etiological agent has yet to be fully proven [7]. Since its description in the 1930’s, a rigorous search for the root cause of CD has raged on. To this end, three main theories have emerged. The two noninfectious theories include an autoimmune theory and the genetic predisposition theory.

Since many other chronic inflammatory responses are considered to be autoimmune diseases (rheumatoid arthritis, diabetes mellitus type 1, etc) it is not hard to see why some have favored an autoimmune theory for CD. This focuses on the overactive immune responses to what many consider to be self-antigens. It is supported by evidence of manifestations of disease that are outside of the GI tract, such as the rheumatoid arthritis and other inflammations that can also occur with CD. It is further supported by the extreme effectiveness of anti-inflammatory agents which are effective on immune responses. CD subjects have much higher titers of IL-12, IFN-γ, and TNF-α which are all hallmark cytokines of cell-mediated immune responses (Th1 responses) [5] Thus, many have proposed that CD too may be of similar autoimmune etiology. That is, our own immune cells may be triggered to attack self-cells due to an over expression of certain inflammatory response cytokines.

The genetic predisposition theory seems to be gaining more ground as of recently. Roughly 20% of CD patients have relatives that also carry the symptoms of CD [7]. Twin studies show that at least 50% have of identical twins are simultaneously affected with CD [6]. In addition, particular ethnic groups (like the Jewish, Caucasian, and African-American communities) seem to be at a higher risk which also seems to indicate a common genetic link to acquisition [7]. Lastly, certain mutations in particular chromosomes seem to indicate higher risk for disease. Approximately 10-30% of CD patients have a mutated NOD2/CARD15 gene on chromosome 16 [7, 9]. This gene is thought to be expressed as a surface protein on human macrophages and monocytes as well as intestinal epithelial cells [9]. In particular, the protein may interact with bacteria and the antigens they express on their outer walls [7]. The protein is thought to control strong pro-inflammatory responses via the cell-mediated pathway, and
hence mutations may delay appropriate bacterial elimination responses. One study by Hisamatsu et al. showed intestinal epithelial cells that expressed a mutant NOD2/CARD15 gene to be ineffective at removing a well known foodborne pathogen, Salmonella typhimurium [10]. It is important to note that the NOD2/CARD15 data thus far elucidated are far from conclusive and up to 5% of the general population may have the mutation; most of which will not develop CD [7].

The last of the theories is that of an infectious etiology. This is the idea that a foreign biological entity is responsible for causing the chronic inflammatory disease. Within the granulomas of the CD-infected tissues, numerous lymphocytes and macrophages conglomerate. Such granulomas are witnessed in other infectious disease and hence, there is a strong suspicion that CD may involve a similar pathway. There are a number of suspect microbial agents that are associated with this theory [5].

**Non-MAP Microbial Suspects Postulated to Cause Crohn’s Disease**

An array of non-MAP bacterial suspects has been postulated to cause CD. A number of different methods have been employed to try and prove a bacterial association with CD. Since a number have been postulated, this review highlights only the most convincing cases of bacterial suspects for CD. These prominent suspects are listed in Figure 1 along with their reported prevalence upon publication.

A study conducted by Liu et al. attempted to characterize the typical flora found in CD patients [11]. The study examined tissue samples from CD patients. Primary antibodies against a select group of bacteria were used to detect bacteria presence. Positive control tissues for each probed bacteria/virus were used to ensure the antibodies were specifically binding to the microorganisms. The groups of tissues were diverse with some coming from Europe and others from North America. Immunolabeling for 12 of the 16 CD tissue samples (75%) were positive for Listeria monocytogenes, a bacteria responsible for a large percentage of food poisoning cases each year. In contrast, none of the tissues from healthy individuals and those from patients with other cases of colitis
<table>
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<th>Prevalence</th>
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<td><em>E. coli</em> (EIEC)</td>
<td>3/64 or 4.5% (Weber et al.)</td>
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<td></td>
<td>12/21 or 57% (Liu et al.)</td>
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<tr>
<td></td>
<td>11/16 or 69% (Cartun et al.)</td>
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<tr>
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<tr>
<td><em>Listeria monocytogenes</em></td>
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<tr>
<td><em>Campylobacter jejuni</em></td>
<td>1/64 or 1.5% (Weber et al. 1992)</td>
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<tr>
<td><em>Streptococcus gallolyticus</em></td>
<td>7/16 (44%) (Liu et al.) from <em>Streptococcus</em> group F</td>
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<td><em>Enterococcus faecalis</em> (Previously <em>Streptococcus faecalis</em>)</td>
<td>7/16 (44%) (Liu et al.) from <em>Streptococcus</em> group F</td>
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**Figure 1:** Microbes postulated to be associated with Crohn’s Disease
showed antibody binding to *Listeria monocytogenes*. Most of the reactivity was observed within macrophages, indicating host cell invasion by the bacteria. Yet, *L. monocytogenes* was also shown to coexist with two specific bacteria in 10 of the 12 tissues it was observed in. It is, therefore, possible that *L. monocytogenes* was a secondary invader. Further, another study conducted by Blaser et al. was unable to show increased antibody reactivity to the bacteria using the serum of 40 CD subjects and 40 healthy subjects [12].

The same study conducted by Liu et al. also showed a marked distinction in *E. coli* reaction with an overall positive reaction of 57% [11]. For *E. coli* 12 of the 21 CD tissues examined were positive for immunoreaction to *E. coli* antigens. Cartun et al. also found evidence of *E. coli* in 69% of formalin-fixed CD tissue [13]. These findings agreed with Tabaqchali et. al who found a higher antibody titer to *E. coli* O antigens in CD subjects in comparison to healthy controls [14]. Importantly, UC subjects also showed high antibody levels to *E. coli* antigens and thus, a secondary infection due to the chronic inflammation was not ruled out by these authors.

Further, Liu et al. found a CD seroreactivity of 44% to the F group antigens of the genus Streptococcus [11]. This finding was similar to Cartun et al. who found that 63% of their CD serum reacted strongly to the bacterial antigens [13]. Yet, 25% of UC subjects in the Liu study also showed a positive immunoreactivity to these antigens indicating a lack in specificity for CD within the IBD group [11].

In 1992, Weber et al. surveyed IBD patients’ stool samples and biopsies; including both CD and UC subjects [15]. Out of the 49 CD patients, 5 or 10.2% were positive for *Clostridium difficile*. This was the most prevalent of the several microbes the investigators probed which also included MAP. Yet, Keighly et al. provided evidence that *C. difficile* may not be involved when they noted a spontaneous disappearance of the bacteria in CD patients’ stool when the study continued to follow a group which had previously tested positive for the bacteria [16]. Weber et al. also cultured a peculiar microbe well known for its role in food poisoning in *Salmonella typhimurium* from the stool of a UC patient [15].

*Yersinia enterocolitica* had been postulated as a possible suspect for UC but not CD for years. Yet a seven year study conducted by Saebo et al. involving 1041 subjects are suggestive of some involvement of these bacteria for CD [17]. Of the 1041 subjects that were tested, only 41 of them tested positive for the bacteria via an agglutination test. Of these, 3 of the 41 turned out to be CD positive or 7.3%. While the prevalence rates are not as high as others that have been reported for CD, the unexpected finding that the bacteria was found in both UC and CD patients but not controls calls for further testing.

*Campylobacter jejuni* also has raised suspicions lately, after being cultured by Weber et al. from the stool of a CD patient [15]. The common disease it is known to cause in humans, *Campylobacter jejuni enterocolitis*, was also found to share common host autoantibodies to UC and CD in a study conducted by Berberian et al. [18]. This autoantibody directed against host red blood cells was found to be highest in CD serum, but the *Campylobacter jejuni* chronically
infected subjects also were found to produce the autoantibody in comparable amounts.  

*Yersinia pseudotuberculosis* and *Yersinia enterocolitica* can cause an acute terminal ileitis that can be a preceding symptom to Crohn’s disease [19]. Others have shown elevated blood titers of the *Yersinia* genus in CD patients. Homewood et al. discuss the evidence for this and conclude that the *Yersinia* correlation is likely due to secondary infections of the bacteria primed by Crohn’s like conditions.

**Yeasts and Their Importance in Diagnosing Crohn’s Disease**

A eukaryotic microbe that we are all familiar with in our daily lives has also been making an impact on the diagnosis of inflammatory bowel diseases. Yeast, both baker’s and brewer’s, is the common name for the eukaryote *Saccharomyces cerevisiae*. For years food antigens were thought to play an important role in worsening IBD symptoms. Main et al. reported the first known antibody response to a food antibody that was specific for CD but not UC [20].

The authors probed a serum population of CD and HC for an antibody response to baker’s yeast. A subsequent ELISA-like test utilized an isolated yeast antigen to elicit a response. Serum populations included healthy controls, UC patients, and CD patients. The results revealed an astoundingly strong response by CD sera with virtually no response at all by the remaining sera populations. The marked differences are clearly visible in Figure 2. Levels of antibody (IgG and IgA) binding to *S. cerevisiae* in serum samples of CD patients were much higher than that in control and UC groups (Figure 1).

The differentiation of the CD group from other groups was statistically more significant when IgA against *S. cerevisiae* was detected. Since it is often difficult to distinguish between UC and CD, this test may offer an easy, virtually pain-free method of determining which IBD an individual may have incurred. Since the release of that study, a plethora of other papers differentiating sera populations based upon yeast antigens (usually called anti-*S. cerevisiae* antibodies or ASCAs) has peppered the scientific literature [21]. Most of these consist of an ELISA format, with around 60% of the CD subjects showing an elevated antibody response to yeast. Since only 10% of UC subjects show a similar high response, CD-UC differentiation is possible. Further, a second differentiation is possible because only UC patients have been shown to produce autoantibodies against self-neutrophils (called atypical perinuclear anti-neutrophil cytoplasmic autoantibodies or p-ANCA). The percentages detected using the p-ANCA assay are a virtual reciprocal of those mentioned with the yeast ELISA. Thus, blood may be drawn and both assays can help give the gastroenterologist a good indicator of which disease may be present. It is important to note, however, that there may be a difference in antibody production to yeast depending on one’s country of origin. In one study conducted by Preda et al. only a low prevalence of ASCA (12.5%, n=40) existed within the CD group of a serum population.
Another yeast, *Candida albicans*, has also been surveyed for its use as a disease marker for CD. *C. albicans* has common surface antigens (notably a surface polysaccharide called mannan). Thus in 2006, Standaert-Vitse et al. were able to show that *C. albicans* can act as an immunogen to the ASCA tests [23]. This is because it is able to mimic *S. cerevisiae* with its mannan-protein production. A second study conducted by Savage et al. showed a marked increase in antibody production to *C. albicans* in CD sera as compared with healthy controls [24]. Yet, it must be noted that an older study conducted by McKenzie et al. found no significant difference when they tested CD subjects and healthy controls for antibodies to *C. albicans* [25].

**Figure 2:** Differentiation of serum populations based on IgG and IgA antibody reactions to yeast (from Main et al. [20])
An Overview of the Case for MAP as an Etiological Agent of Crohn’s Disease

Presence of MAP or Antibodies Against MAP in CD patients

Of all the microbial suspects touched on above, none has garnered as much attention as *Mycobacterium avium* subsp. *paratuberculosis*. The linkage between MAP and Crohn’s disease is one that has been long suspected, but received enormous attention after Chiodini et al. isolated an uncharacterized mycobacteria from the diseased intestinal tissue of three Crohn’s patients [1]. DNA probes later revealed these mycobacteria to be MAP [26]. Interestingly, many of the MAP particles which have been identified thriving in the intestinal regions of infected humans have taken on a cell-wall deficient form (spheroplast) [27]. This meant that the classical acid-fast staining used to identify mycobacteria is not able to differentiate these microbes [28]. However, IS900 DNA probes are still able to detect the bacteria and one in situ hybridization study by Sechi et al. revealed that up to 70% of the Crohn’s patients tested positive for the MAP insertion sequence [29]. A follow-up study by the same group revealed that another 70% of CD patients were both positive for MAP by IS900 but, in addition, were also positive for the NOD2 mutation mentioned previously [30]. In 2004 Naser et al. identified 46% of Crohn’s patients as having MAP DNA in the buffy coat of their blood while only 10% of the healthy controls were PCR positive [31]. Naser et al was also able to culture MAP from the breast milk of two CD mothers, while none of the healthy controls cultivated bacteria [32].

It must be noted, however, that IS900 PCR methods aiming to detect MAP within Crohn’s patients have not always been as successful. While some studies show IS900 PCR detection methods to detect the bacterium in up to 76% of Crohn’s patients, others have found prevalence rates that hover around 30-40% [33]. In addition, some data revealed no significant difference between healthy controls and Crohn’s patients. Such inconsistencies raise the possibility of cross-reactivity of IS900 PCR primer, with the most likely candidate being the insertion sequence derived from MAA.

Mycobacteria-derived antigen p36 tests have provided further support for the MAP-Crohn’s connection. Naser et al. found that this antigen reacted with 86.5% of Crohn’s diseased patients and 89% of patients with leprosy or tuberculosis [34]. Conversely, only 10% of control subjects who had not received a BCG immunization tested positive. These results broadly suggest mycobacterial involvement in CD acquisition [34].

It should be noted that, despite the similarities of CD and UC in their symptoms, UC-infected patients rarely test positive for MAP, showing that the presence of MAP is specific to CD. A 2001 review by El-Zaatari et al. compiled recent research utilizing various detection methods including: direct culturing from diseased tissue, DNA hybridization, serological methods, and in situ hybridization[34]. All were able to detect MAP in CD patients with MAP
prevalence values ranging from 40% to 90% of CD patients tested. On the contrary, UC patients rarely exhibited MAP presence with prevalence values ranging around 0% with only one DNA-DNA hybridization test result having an outlier 33% prevalence. Non-IBD negative controls also tended towards zero prevalence rate with, again, the exception of the hybridization test which revealed a 17% prevalence of MAP [35]. Still, there exists the possibility that CD-infected tissues somehow provide a more lucrative environment for MAP to establish itself. Therefore, mere presence of MAP within CD patients is not enough to prove MAP causation. This may be supported by the very mixed success rates of using anti-mycobacterial treatments reviewed by Chamberlain et al. [28].

Symptomatic Similarities Between CD and JD

MAP is well-known as the causative agent of an intestinal chronic disease in ruminant animals, called Johne’s disease (JD), and symptomatic similarities between CD and JD have supported the hypothesis of an etiological role of MAP in CD. Both diseases can result in a constant diarrhea and, in severe cases, can lead to lethal blood loss or anemia. There are important similarities when one observes the inflamed tissue taken from diseased ruminants and humans as well. Granulomas are the main common feature to both tissue types. CD- and JD-infected tissues have goblet cells that are responsible for proper mucous secretion along infected areas [7]. Transmural inflammation, while rare in JD, is also a common feature of CD tissue samples [5]. In a 2004 review, Chacon et al. highlights the similarities in diseased tissues of UC, CD, JD seen in Figure 3 [5]. However, there are some differences in symptoms of CD and JD as well. JD-infected tissues usually inflame the lamina propria while this is rarely seen in CD patients [5]. Another important distinction is the lack of fistulas, fissures, and pseudopolyps in JD-diseased tissue – these features are common in CD tissues.

Since MAP has been studied intensively as a candidate causative agent of CD, details of MAP biology and MAP infection in animals, JD, are described below. A complete understanding of the biology of the disease – and how it may make it through the food chain to our refrigerators – helps one to understand the significance of this bacteria as a zoonotical suspect.

MAP General Characteristics

MAP belongs to the genus *Mycobacterium*, which encompasses nearly 100 species [5]. MAP bacilli resemble 1-2 micrometer long, plump rods when viewed microscopically [36]. Taxonomically, the *Mycobacterium* genus is most closely related to the genera *Corynebacterium, Nocardia, Rhodococcus*, and *Streptomyces*. Microbiologists often characterize bacteria by the guanine-cytosine makeup within the microbe’s DNA; often called he G-C percentage.
Figure 3:  Histology comparison between normal, UC, CD, and JD tissues (from Chacon et al [5])

MAP DNA has a characteristic G-C content of approximately 69% [37].

A defining trait of all Mycobacteria is their propensity for acid-fast staining. Members of the Mycobacterium genus contain high concentrations of mycolic acid in their cell wall. This component binds to the basic fuchsin dye used in the first step of the Ziehl-Neelsen Acid-fast staining procedure. It does so when the carboxylic acid portion of the mycolic acid forms an ionic bond to one of the amine groups of the fuchsin [38]. This cell wall component is impermeable to the acid-alcohols used for decoloration. The mycolic acid is also covalently bound to the peptidoglycan of the cell wall. The cell wall of mycobacteria is covered further by capsular structure (also called outer layer) composed primarily of carbohydrates and proteins. Further, the bacteria tend to produce a large amount of extracellular matrix which can be seen in a recent scanning electron microscopy image (Figure 4) taken by our laboratory in April of 2007. Extracellular pili resembling those in the figure have recently been described by Alteri et al. in MAP’s close relative M. tuberculosis [39]. Further, the investigators revealed that MAP was found to carry the same gene responsible for pili in M. tuberculosis.
Amongst the Mycobacterium, there are two large groups; fast-growing and slow-growing. Those in the latter group comprise most of the known pathogenic strains of the *Mycobacterium* genus, including MAP, along with its infamous pathogenic relatives *M. tuberculosis* and *M. lepra* [40]. These can be easily distinguished from fast-growers because slow growers only contain one set of rRNA genes, whereas fast growers contain two sets [41]. Susceptible hosts vary considerably: MAA causes tuberculosis in birds, and opportunistic infections in immunocompromised humans. Yet, MAA does not cause disease in cattle or other ruminants [42].

MAP is one of three subspecies belonging to the mycobacterium species *M. avium* [41] – the other two being *Mycobacterium avium* subsp. *avium* (MAA) and *Mycobacterium avium* subsp. *silvaticum*. Of the three, MAP is the slowest growing with a generation time that can exceed 20 hours [5]. A recent genomic analysis between *M. avium* subsp. *avium* and MAP revealed greater than 97%
genomic similarity between the large genomic sequences chosen for the comparison [43]. Analysis of the dnaJ gene, a highly conserved heat shock protein used for phylogenetic analysis, revealed a 99% homology between the same two subspecies [44]. While the genetic and physiological make-up of these two seem to be related, the diseases and

Recently, Li et al. used shotgun sequencing to determine that the entire genome of a laboratory strain of MAP, K10 was about 4.83 kbp long [37]. M. avium subsp. avium is substantially larger, with a genomic size of 5.48 kbp [5]. The homology is such that the two subspecies probably differ by fewer than 75 genes. Lastly, many other mycobacterial species, including MAP’s closest relative MAA, have plasmid DNA in their genomic arsenal. No such native plasmids have been found in MAP [45].

Two other key features distinguish MAP from related bacteria. These are mycobactin J-dependence and the presence of a specific genetic insertion sequences [45]. Unlike all other known mycobacterial species MAP is a mycobactin auxotroph. Mycobactins (lipid soluble iron chelators), along with the exochelins (water soluble iron chelators) are responsible for iron scavenging to ensure survival in iron-depleted environments. Mycobactins contain a phenyloxazolidine ring while exochelins do not [46]. In MAP and other mycobacterial species, both mycobactins and exochelins are necessary. Since MAP lacks mycobactin J, it must utilize foreign, extracellular mycobactin J in iron-starved conditions. This is why the bacterium was so difficult to culture. Twort was able to get around this because the M. phlei was providing the mycobactin J for MAP.

A distinguishing feature of MAP is the presence of the insertion sequence IS900 [45]. This is a small, highly motile bit of genomic material which contains transposition-related genes. Specifically, IS900 is a member of the IS110 family of insertion sequences [45]. It therefore lacks both the terminal inverted repeats and direct repeats expected in most transposable DNA. Typically, 17-20 copies of these insertional sequences are found in MAP. Other mycobacteria have similar, but distinct, insertion sequences. As expected, MAA has the insertional sequence most closely resembling IS900. Its version, IS1626, shares 82% homology with IS900 and is also in the IS110 family. Yet we expect to find IS1626 in copies of three when probing MAA cells [45].

Even within the MAP subspecies, significant variation is evident. This seems to be related to where the MAP subspecies is isolated from. Two major subspecies strain include the cattle (c strain) and the sheep (s strain) strains. In reference to the only fully sequenced C strain (K10), a DNA microarray hybridization revealed significant deletions an S strain. These deletions totaled approximately 30,000 base pairs which are about 0.6% of the genomic size of the reference C strain. However, this test is only unidirectional and a second hybridization with a reference S strain must be conducted to ensure the deletions exist [47]. Lastly, it is important to note that there are subtle key differences within the IS900 sequence of MAP which allow us to determine our strain type [48]. We include further discussion of IS900 in the diagnostic methods section.
Role of MAP in Animal Disease

Johne’s Disease

Johne’s Disease (JD), often referred to as ‘paratuberculosis’, caused by MAP, is a chronic, often fatal enteric disease which mostly infects ruminant animals. In particular, it affects cattle and sheep -- the important economic cornerstones of many agricultural economies. It is prevalent on every continent except Antarctica; in the United States prevalences reach as high as 85% on some farms [49]. Various characteristics of this microbe have made the eradication of Johne’s disease a very difficult prospect. The economic implications of Johne’s disease can be quite severe and thus, a great deal of research has gone into JD. For example, it is now known that non-ruminants also harbor the acid-fast bacterium: in 1977, Matthews and Sargent isolated MAP from a brown hare [50]. Such a vast host range represents an extremely tough obstacle for designing effective management techniques.

In general, animals are most susceptible to infection during the first few months of life [51]. While initial exposure occurs early, classical clinical conditions may take years to develop. Transmission is believed to be primarily linked to oral ingestion of MAP-laden material. Experimental infection models with adults have proven that disease resistance is acquired with age [8]. JD can be spread via direct and indirect contacts mechanisms. The most common means for direct transmission is through an infected mother’s milk or colostrum (first milk after calf delivery) [51].

Indirect transmission occurs most commonly via MAP-contaminated food or water sources. Teats contaminated by fecal material can also cause oral-fecal transmission [51]. The minimal infective dose of MAP appears to be $10^3$ bacilli [52]. Infected animals with clinical symptoms shed $10^6$ to $10^8$ MAP per gram of fecal material [52]. Thus, just a small amount of fecal contamination will go a long way in spreading disease. To make matters worse, the onset of fecal shedding of MAP by infected cattle is nearly impossible to predict.

Once infection is initiated, JD is mostly undetectable for the first couple years. Thus, the disease spends much of its time in a subclinical phase [51]. Only 5-10% of the subclinically infected animals will progress to more overt symptoms [51]. A late subclinical phase is marked by heavy bacterial shedding with no overt symptoms prevalent [53]. Clinically infected animals shed high numbers of bacteria in their feces, rapidly lose weight, and exhibit low milk production. Symptomatic animals will eventually die due to malnutrition.

MAP’s ubiquitous distribution in the environment makes it impractical to eliminate the bacteria from a suspected farm. Once infected, chemotherapeutical agents may be employed which will may reduce the severity of disease and in turn increase survival time. However, no chemotherapeutic agent has been found that will completely relieve all symptoms and guarantee relapse prevention. Vaccines
represent a second form of protection. Yet, there have been only a few successful demonstrations of both live and killed bacteria vaccinations [54].

Survival of MAP in environment and milk

As mentioned earlier, MAP bacilli are surrounded by multiple layers, including cell membrane, cell wall, capsular structure and extracellular matrix, and are known to be extremely resistant to various chemical and physical treatments. Consequently, MAP can survive in environments (e.g. soil and water) and food matrices (e.g. milk) for a relatively long term with maintaining their infectivity. Since the majority of MAP transmission occurs through the oral route, contaminated materials are likely to cause the spread of MAP infections in animals and possibly in humans. If MAP is a causative agent or deteriorating factor of CD, survival of MAP in drinking water and milk will be a food safety concern especially in countries where JD prevalence is known to be high.

MAP’s Physical Resilience In Vitro

MAP is an extremely durable organism. In the lab, it is able to resist high temperatures better than any of its closely related family members including *M. avium* subsp. *avium*, *M. phlei*, *M. scrofulaceum*, and *M. xenopi*. Normal doses of UV light are adequately capable of killing the organism, yet the organism is slightly more capable of surviving sunlight than *M. bovis*. Slight differences in overall pH in the 5-8 range have no effect on MAP’s survival. Like MAA, chlorine has been showed to have a less pronounced effect on MAP survivability when compared to most other bacteria.

MAP’s Environmental Resilience

With such an enduring nature under laboratory conditions it is hardly surprising that this mycobacterium has also proven to be hearty in various environmental sampling experiments. The bacterium remains extremely viable under various environmental conditions. For instance it has been cultured after 163 days in river water, 270 days in pond water, and 11 months in cattle feces [55]. Low temperatures can also be tolerated, as it has been cultured @ 14 degrees Celsius for at least a year [55].

MAP’s Survival of Pasteurization Process

If MAP does cause a zoonotic disease in humans, its survival within commercially available dairy products would represent a very serious risk. The mycobacterium has been isolated from both pasteurized and unpasteurized milk of infected dairy cattle. Pasteurization is a process which was designed to eliminate possible bacteria (namely *Mycobacterium bovis* the etiological agent of tuberculosis) or other biologically active entities that can be found in collected
milk. The mycobacterium is found in milk by either direct secretion from the udder or a contamination of the udder by MAP-laden fecal matter. While milk shedding is less prevalent than fecal shedding, it has been shown that about 30% of heavily infected animals will shed the bacteria in their milk [55]. While most studies have found low MAP counts from milk, shortcomings in current methodology prevent us from knowing the true viable MAP count in milk. The processes used to rid the milk of faster growing organisms, are also harmful for MAP. Secondly, fecal-contaminated milk enumeration methods have yet to be undertaken [36].

Different methods exist for milk pasteurization. Mainly these consist of holder pasteurization and high temperature, short time pasteurization. Holder pasteurization entails heating the milk to 63 degrees Celsius for an extended 30-minute period. High temperature, short time pasteurization involves a higher temperature of 72 degrees Celsius for just 15 seconds. Several studies have been conducted using experimentally inoculated milk samples which were subsequently pasteurized using both methods. Both methods were proven unable to completely rid the milk of viable MAP. In addition, minimal starting concentrations of MAP in the milk are important if we are to seriously consider the possibility of MAP transmission through commercially available milk. It has been shown that if milk is present at levels as low as 100 cuff/ml (colony-forming units per milliliter) some MAP survival is plausible under high-temperature, short-time pasteurization [36]. Thus, cows with at least this concentration of viable MAP in their milk could be considered a public health risk; at least until the Johne’s disease/Crohn’s disease connection has been convincingly invalidated [55].

MAP Detection/Diagnosis

Preamble

Most of the information garnered thus far on MAP detection has been acquired using animals affected with JD. Since this is the only disease MAP has been proven to cause, this is hardly surprising. Thus, this section focuses on MAP detection techniques that have been developed for JD, but which might also allow us to look for MAP in CD patients to help evaluate the putative connection between the two.

Detecting MAP in an Infected Host

The jury is still out as to which diagnostic method should be utilized in accurately assessing one’s MAP infection status. Since the disease has only been proven to cause Johne’s disease, most studies concentrating on finding direct or indirect evidence of disease have concentrated on its known animal hosts. Disagreement between labs using the same methods are common in the literature. There are many reasons why this may be true, including strain differences, the
slow-growth of MAP, and the heartiness of the bacteria to name a few. Two important facets of any diagnostic method are its intrinsic sensitivity and specificity values. These statistical terms are straightforward in their meanings. Sensitivity refers to a test's ability to detect all true positives. Its value is only lowered by false-negatives. Specificity refers to a test's ability to accurately assess the true negatives for a disease. High combined specificities and sensitivities indicate that the test is both able to detect disease and differentiate the healthy from the infected. Early detection of Johne's via serological or non-serological methods remains a difficult task. Thus while high specificities may be possible due to the low prevalence of false-positives, true negatives may be much less prominent. This is because an infected animal may take months to years to progress to a disease state where detection is even possible due to the pathology of MAP. The following discussion represents a synthesis of the newest research conducted and tries to gather some of the general sensitivity and specificity trends of each diagnostic method.

**Fecal Culturing**

Though there may be some who disagree, most researchers familiar with the diagnostic aspects of Johne’s disease would consider the fecal culture test the gold standard by which all other tests are usually compared against. Though it has taken many years since fecal culturing began, today we are able to detect the presence of the bacteria both earlier and at lower initial prevalence. However, many questions still exist as to whether or not fecal culturing should be assumed to be the gold standard for infection status. The bacteria can be extremely difficult to free from the fecal matter, and for this reason many low-shedding hosts may be misdiagnosed as being fecal culture negative. In addition, since the mycobacteria are often consumed via contaminated feed or milk, there is a possibility that some fecal positive cultures are the result of these bacteria simply being processed and excreted with the ingested material.

Thus, there is the risk that the bacteria could be detected without ever establishing an infection. Cultures testing positive in this manner are said to be ‘pass-through’ positives [56]. Other problems with fecal culturing are the considerable efforts which must be expended to determine a given samples disease status. The filtration and decontamination stages can be completed in 3 days by the Whitlock method [57], however even with sophisticated growth detectors, some cultures may take up to 60 days until microbial growth becomes evident [57]. With such prolonged incubation times, laboratory costs can be very high.

Despite these drawbacks, fecal culturing still represents one of the only direct methods to detect MAP. Consequently, many still rely upon fecal culturing to obtain a cow or herd’s disease status when looking for Johne’s diseased animals. While fecal culturing has become the standard for detecting MAP within the Johne’s disease animal hosts, the method has yet to be found useful in any Crohn’s disease studies. However, as previously mentioned, there have been
several accounts of culturing MAP from various tissues and non-fecal fluids of CD subjects [1, 31].

**Serological Methods: An Introduction**

Blood can be physically separated into different components of which the main constituents are plasma (the liquid portion) and red blood cells (the solid portion). These can be obtained by simple centrifugation. Plasma has many proteins associated with clotting called clotting factors which can be removed immobilized with special coagulants. Once these clotting factors have been removed serum is what remains. The sera contains antibodies and complement proteins. In addition, other proteins exist within the serum that act as chemical signalers (cytokines) for different components of the innate and adaptive immune responses.

Thus, it is clear that serum can provide us with a wealth of information about a particular organism’s immune responses. Because we cannot readily detect the actual invasive organisms within the serum of an animal, serological testing remains an indirect, but very effective detection method. Many different serological testing techniques have been developed to detect different aspects of the host organisms’ immune system.

**Humoral Immune Responses**

The humoral immune response is directed by a strong T\textsubscript{H}2 response. That is, type 2 helper T-cells activated by the antigens direct B lymphocytes to mature into antibody secreting plasma B cells. The antigen-specific antibodies may then bind to the surface antigen of free bacteria and mark the bacteria for eventual destruction via one of a number of available pathways including opsonization and complement killing. Antibodies to a common antigen may exist in a variety of isotypes. A variety of isotypes exist to allow the antibodies to capture antigen in multiple ways. These may be produced by the same T\textsubscript{H}2-activated B lymphocyte [38]. IgG is structurally very simple and is generally the most prevalent antibody circulating in tissue and blood of animals [58].

For years, the humoral immune response was thought to take a significant amount of time to develop in a typical paratuberculosis infection [59]. Thus it was considered by many to be an immune response dominating the later, clinical stages of MAP infection. One major reason for this is that the enzyme-linked immunosorbant assays (ELISAs) often used to detect antibodies to MAP antigens are mostly seropositive after the animal has been shown to be shedding bacteria via fecal culturing. Yet, recent work by Koets et al. challenges many of these assumptions [60]. An advanced knowledge of the immunology concerning MAP infection will likely improve humoral immunity detection methods. We will now discuss some of the common methods used to detect MAP-targeting antibodies.
AGID and CF Tests

There are agar gel immunodiffusion (AGID) and complement fixation (CF) tests for the presence of antibody to MAP antigens. Developed prior to the more sophisticated ELISA, these two assays offer an easy, quick visual affirmation of antibody presence. Both tests are fairly specific, but lack the sensitivity achieved by most ELISAs in cattle, goats, and sheep [61-63].

AGID tests are extremely simple as they require only MAP antigen, the serum to be tested, and a known positive control. Test results are available in two days [64]. The three constituents are placed equidistant from each other and injected into an agar gel. If the antibodies to the antigen are present in the serum, the two will bind to form a precipitant. A precipitant describes an insoluble complex formed from a soluble antigen and antibody [38]. This line should be compared to precipitant line from the positive control. The Johne’s Information Center recommends that this test only be conducted on animals which have been clinically diagnosed due to their malnourished appearance or constant diarrhea [64]. There are no records of AGID testing with CD subjects.

The CF test utilizes an indirect indicator system to portray antibody presence. The presence of antigen binding antibody is indicated by adding complement proteins. Complement proteins exist as a part of our immune response to trigger a cascading effect of activating proteins which lead to effective clearing of pathogenic cells by creating holes in their cell membranes [65]. Complement proteins can be activated by a variety of different pathways.

The classical pathway exists as an antibody-activated pathway [38]. The test works in the following manner; if the antibody is present within the serum, it will bind both the antigen and activate or “fix” the complement protein. A second indicatory antibody is added which leads to sheep red blood cell lysis upon complement binding. If all of the complement is already bound to the antibodies against MAP antigen, there will be a drop off in complement available to bind the anti-sheep RBC antibody. Therefore, when we add sheep red blood cells hemolysis will occur to a lesser degree and the lawn of red blood cells will remain intact. This would appear in the test well as a solid red lawn.

However, if there is no antibody binding to MAP antigen, the inactivated complement proteins will be more freely available to bind to the anti-sheep RBC antibodies. This will activate the classical complement system to eventually lead to hemolysis. This would appear as a white or pink lawn in the test. There have been no similar CF tests done on CD patients.

ELISA

The enzyme-linked immunosorbant assay or ELISA has become the preferred method of antibody detection since its development by Yokomizo et al. in 1983 [66]. The ELISA is the preferred immunoglobulin detection method because it requires the least amount of antibody required to give a positive reaction [38]. It is also relatively cheap, and easier to standardize making
duplication easier [64]. Lastly, it is important to note that specificity of the ELISA significantly improved when *M. phlei*-absorbed ELISAs were introduced to reduce non-specific antibody binding and hence, false positives [67].

The ELISA for JD works by having some MAP antigens attached the wells of a ELISA plate [38]. Serum at a specific dilution is then added to the wells and allowed proper incubation time for the reaction between antigen and antibody to occur. The wells are then washed to remove any materials that did not bind to the antigen. A secondary enzyme-conjugated antibody specific for the animal and isotype of your targeted antibody is then pipetted into the wells. After a second incubation the substrate for the enzyme is added to the wells. If there is any antibody present for the targeted antigen, the secondary antibody with its conjugated enzyme will also be present and reacts with the substrate producing a coloration of the solution in the well. The coloration of each individual well is directly proportional to the amount of antibody present. These are read on an ELISA plate reader which quantifies the optical density or OD [38].

Like IFN-γ, a number of different antigens have been used with ELISAs. This includes LAM proteins, heat-shock proteins, whole-cell MAP, ethanol extracted antigen, antigen 85, antigen 35, and antigens removed by whole-cell sonication techniques [60, 68-71]. In addition, different methods for antibody detection have been tried. This has been mostly dominated by the extremely popular ELISA. Thus far, very few ELISAs have been able to separate the populations with much effectiveness (See table 1-1). Yet, there is hope with the ethanol-ELISA, the formaldehyde-treated ELISA, and the p35 ELISA having sensitivities in the 85-100% range [69-71]. It must be noted, however, that whilst the p35 ELISA has shown a high sensitivity, it has a lower specificity (86.4%) than either of the other two aforementioned ELISAs which approach 100% [69-71].

ELISAs have been the most popular method for testing CD patients for *MAP*-directed antibody production. Kreuzpaintner et al. used their own ELISA that utilized 45/48 doublet antigens shared between *M. tuberculosis* and MAP [72]. Bernstein et al. used a commercial ELISA to test seroreactivity between CD and HC groups [33].

**Using Flow Cytometry to Detect Bacteria**

**An Introduction to Flow Cytometry**

Over the years, flow cytometry has proved to be an extremely useful tool in the medical and scientific community. It has served as an extremely potent investigative tool for researchers wishing to address a wide array of biological questions. These may include the monitoring of foreign or self-antigens, antibodies, chemokines, and key immunological markers. Its applicability stems from the extreme sensitivity and specificity one is able to achieve with flow cytometry.
By its name the machine may seem deceptively simple. Cytometry refers to the equipment for the characterization and measurement of cells and cellular constituents. The ‘flow’ portion refers to how this cytometry is accomplished; by flowing a liquid stream containing the target of interest through an array of detectors. This flow procedure allows for the subjects of interest to be lined up in the stream. Once these targets are lined up, they pass through a laser beam and florescence emitted from or light scattered by the targets be captured by a detector. The flow rate through the stream is controlled and constant, thus only the particles size affects how long the laser is kept from hitting its detector. Intensity of the fluorescent and light is converted into voltage, amplified and digitally recorded.

Thus flow cytometry is both quantitative (frequency) and qualitative (size). This beam breakage between a source and a detector is called “forward scatter”. In addition, secondary detectors will be set up to observe where the beams which encounter the particle are deflected. This deflection is called “side scatter” and is observed by side scatter detectors (usually oriented about 90 degrees to the laser). While forward scatter mostly measures cell surface area or general size, side scatter can give good approximations of how a cell is deflecting light. This deflection pattern is a qualitative property that details cell topography and can even give hints as to intra-cellular structure. A cell with a rough surface is not going to deflect light in as ordered of a manner as a nearly perfectly smooth sphere bead. It is important to note that both side-scatter and forward scatter can be utilized to estimate particle size [73].

In both cases, computers allow these measurements to be recorded and as many as $3 \times 10^5$ measurements can be made in a single minute [73]. In addition to size and frequency recordings, most flow cytometers will have the ability to excite a fluorescent marker that may be attached to the target of interest. Secondary detectors will detect this florescence as it occurs and store it alongside the particle size information. In this manner, the flow can be extremely specific. Fluorescent monoclonal antibodies can be made which only bind to very specific epitopes. If they are not bound, no florescence can occur and although a false positive may have a similar sized particle, it is nearly impossible that it will also contain the same epitope. Another crucial aspect of modern day flow cytometry is the ability to make several simultaneous readings upon a single cell. For instance, if one wants to measure the expression of antigens A, B, C, D within a given population of cells, it would be possible to measure these for both the individual cells and within the population as a whole, as well. A diagram of a typical flow cytometer is outlined in Figure 5.
Figure 5: General schematic of flow cytometer courtesy of University of Arizona Department of Microbiology http://microvet.arizona.edu/courses/mic419/ToolBox/f2fasc.gif
A Brief History of FCM Development Towards Studying Microbes:

Though it has its conceptual origins under Moldovan as early as the 1934, it was not until the 1950’s -- with the advent of a detector developed by Coulter that could differentiate cell volumes [73] - that flow cytometry (FC) began to make significant progress. These early flow cytometers were equipped with “Coulter Counters” that used the differences in electrical signal absorption from their media to determine approximate volumes [74]. Optical means of cell volume differentiation later replaced the Coulter Counters due to their superiority in many different aspects of cell volume differentiation. Cell sorters were a second important milestone in the evolution of the flow cytometer. This feat was achieved by Fulwyler who was able to successfully sort human and mouse red blood cells based on differences in their total cell volume [75]. This apparatus would allow the investigator to single out only the cells of interest to be studied and eliminated an enormous amount of possible background noise.

Improvements in optical technologies and fluorescent markers allowed researchers to begin investigating the microbial world using flow cytometry around the 1970’s [74]. In an important early experiment, Hutter and Eipel were able to observe previously undetectable microbial entities. Various measurement parameters including, protein content, DNA content, and chlorophyll content were measured from a diverse group of microorganisms that included some yeasts (S. cerevisiae), a prokaryote (E. coli), and some filamentous fungi [76]. This work was conducted using a flow cytometer developed primarily for working with larger, mammalian cells. A necessary final step had to be taken in order for flow cytometry to be of use for microbiologists: bacteria and similarly small particles needed to be studied using instruments that had a high signal to noise ratio. This meant eliminating background noise. In 1979, Steen and Lindmo addressed this issue by redesigning the chamber through which the fluid and laser interact [77]. They aimed for the fluid to flow over a glass cover slip in an ordered manner which created a laminar flow [77]. This cut down on background noise and allowed for a high enough sensitivity to detect smaller prokaryotes. There was a catch, however, because these flow cytometers utilized arc lamps instead of lasers. With arc lamp flow cytometers, multi-parameter flow cytometry of more than two fluorescent parameters is not possible [74]. Cell sorting is also not an option with arc lamp models. With time, further improvements in laser and computer technology allowed the laser-based flow cytometers to become sensitive enough to detect the smallest bacteria. Even smaller biological materials, such as DNA, can now be observed. Armed with these more precise instruments, research on bacteria using FCM has become much more prevalent as evidenced in Figure 6 by Davey et al. in their review of flow cytometry [74].
Figure 6: Graph displaying increasing flow cytometry papers concerning microbes during the period from 1980-1996. Davey et al. did a database search on two criteria: for any paper concerning flow cytometry (open circles) and for any papers containing both the words ‘flow cytometry’ and ‘bacteria’ (solid circles). The percentage of flow cytometry papers dealing with bacteria were then mapped as a percentage of the total papers dealing with flow cytometry in general (open triangles) [74].
Fluorescent Staining in Flow Cytometry

When an atom absorbs light energy, its electrons enter an excited state in which they may jump to a higher energy shell level. When they return from this level to the normal, ground state a photon with a characteristic energy can be emitted. This everyday phenomenon is termed “fluorescence” and it can be a valuable tool when optimized in flow cytometry. Since fluorescence is always of lower energy than the light energy that initiated the phenomenon, the emitted fluorescent light may be detected and quantified [74]. Different fluorescent dyes have characteristic extinction coefficients which dictate at what wavelengths of light a particular dye will absorb the maximum amount of light. In addition, different fluorescent dyes emit at different wavelengths. This makes possible multiparameter techniques not only using flow cytometry but also simple fluorescent dye microscopy.

Fluorescent staining in flow cytometry allows us some very distinct experimental advantages [73]. First, a fluorescent emission is directly proportional to the fluorescently-tagged biological materials. Secondly, very low concentrations of any fluorescent dye may be detected, due to the possibility of multiple fluorescent emissions per passage through the beam. This provides a general amplification of the signal and leads to greater overall sensitivity. Lastly, non-fluorescent compounds can be altered by enzymes to allow for fluorescence. This allows us to saturate a sample and only detect those which have the appropriate enzymes.

Ideal dyes for flow cytometry have extinction coefficients which are highest at the corresponding wavelength energies of the lasers of the flow cytometer being used. Other crucial properties include how many times a molecule can be excited before it breaks down, what is the maximum energy a dye can withstand before it breaks down, the duration of fluorescence, and how sensitive it may be to its chemical environment [74].

Fluorescin isothiocyanate (FITC) is one of the most widely used fluorescent dyes in all of flow cytometry. FITC has its maximum absorption wavelength at 490 nm. This is ideal because most flow cytometers use a argon laser which has a blackbody radiation curve peaking at 488nm [74]. FITC emits a photon with a characteristic peak at 520 nm and these photons are generally detected at sensors adjacent to the side-scattering detectors. FITC also can exert multiple signals per molecule because it exerts its photon over a very short period of time of 4 nanoseconds [78]. Since the typical sample passing through a flow chamber contacts the laser for 10 to 100 microseconds, the detector will observe more than one signal from a tagged item and thus will be more sensitive to even trace amounts of FITC-tagged material. Lastly, FITC also has a high quantum yield at biological pHs of 8 making detection optimal for typical biological assays [74].

FITC can either be used in two important, but distinct manners. First, researchers may use FITC to bind to all available proteins in a given sample [79]. This allows access to a number of data collecting possibilities including the ability
to distinguish cell cycle status, differentiate different cell types based on overall protein expression, or any other investigation when the amount of protein per constituent may be of interest. Secondly, more specific assays may be done by conjugating FITC with antibodies to bind only one particular antigen type [73]. Once these antibodies bind their target, the attached FITC will fluoresce when the complex passes through the argon laser. Similarly, FITC secondary antibodies which bind to species-specific antibodies are commonly used.

**Fluorescent-based FCM Method by Dietrich et al.**

Humoral responses to bacteria have been measured in a number of different ways, as discussed previously. However, in 1991 Dietrich et al. outlined a method for detecting host immune responses to whole-cell bacteria using flow cytometry [80]. In this original study, the authors used a secondary antibody conjugated with FITC which targeted the F’ab regions of the bovine IgG antibody. The study focused on a group of 18 cattle which were challenged with live *B. abortus*. They sampled blood from the cattle at prechallenge, 1, 2, and 4 weeks post-inoculation. In the initial step of the assay, they incubated bovine serum with the *B. abortus* which was fresh from a culture. After the incubation, nonspecific antibody binding the bacteria was prohibited by washing twice with PBS. A second incubation with the FITC conjugated-anti bovine secondary antibody was performed. Washes were performed after an incubation period and the bovine antibody binding to *B. abortus* was assessed using flow cytometry. The results of the study proved very promising: antibody titer increased from a near zero level as expected as the weeks progressed and the cattle’s immune systems began producing immunoglobulins to the challenge bacteria. The study ended with exciting results as the authors proclaimed,

“In summary, we have developed a fluorescence based flow cytometric assay to measure antibody binding to intact bacteria using blood samples as the source of antibodies. The assay was as reproducible as the standard EIA (ELISA-immunoassay), it was easier to run and it produced data in a format that was more applicable to our research needs. We are using this assay to measure specific antibodies in bovine plasma samples to *B. abortus* but the assay could be suited to the measurement of antibody to binding any bacteria.”

**Developing a FCM Method for MAP antibodies**

In 2005 our lab developed a similar flow method to detect antibody binding to MAP in sera of cattle on JD+/- dairy herds [2]. Like the Johne’s ELISA, the Flow Cytometry Method (FCM) also uses a secondary antibody as an indicator of the presence of antibodies against MAP. In principle, the idea is to incubate serum with whole-cell MAP to allow antibody to bind to the surface of bacilli. Washes remove unbound materials and the bound host antibodies are then tagged using FITC-labeled secondary antibodies that are specific for bovine antibody. The general idea is captured in Figure 7. Here we have a complex
Figure 7: Schematic of the antibody binding complex that is detected by FCM
composed of antigen, bound primary antibody from the serum, and bound labeled secondary antibody that is labeled with FITC for detection.

The complex is then run through the flow cytometer to detect antibody binding by immunofluorescence. When tested with other related mycobacterium, this study proved the assay to be specific for MAP antibodies. The lab used 21 serum samples from cows which had produced 2 or more positive fecal samples as the positive controls. Serum samples from 30 cows which had been testing serum negative by ELISA for 20 years were used as the negative control and gave both the diagnostic specificity and cut-off value. The mean fluorescence of this group was determined and the cut-off was set three standard deviations above mean negative fluorescence value. Using this method, the assays’ sensitivity was estimated to be 95.2% with a specificity of 96.7%. This differentiation is shown in the S/P dot plot depicted in **Figure 8**.

**Figure 8**: Differentiation of JD negative (1) and JD positive (2) cattle sera, based on FCM-derived S/P values (from Eda et al [2])
CHAPTER III: Materials and Methods

Serum Populations

Serum samples from a total of 74 individuals were tested using the FCM; 37 were from known CD patients (the CD group - all volunteers) and 37 from healthy controls (the HC group). All 37 CD patients came from our collaboration with M.D. of Gastroenterology, Dr. Anderson. Dr. Anderson is assistant professor and Chief of the Gastroenterology Division at UT Medical Center. In April 2005 his patients were sent a letter asking them to participate in our study. The packages included a cover letter (Figure 9) and an agreement to the terms of the study (Figure 10). The patients were directed to come to UT Medical center and have blood collected at their convenience. Patients diagnosed CD by Dr. Anderson or any previous M.D. were considered to be truly positive for the assay purposes. Any donated blood was collected by trained phlebotomist personnel and placed in BD Vacutainer © (BD bioscience, Franklin, New Jersey) serum collecting tubes. The tubes were usually picked up and transported back to our lab for proper storage at -20°C. The samples were documented with names, ages, and gender.

Healthy controls (HC) were obtained from three independent companies. Bioreclamation Inc provided us with 20 samples which were donated by those claiming to be CD-negative by questionnaire (Bioreclamation Inc. New York, NY). Since CD-positive blood is more valuable and therefore the donor can receive more compensation, the questionnaire is rarely falsified when it comes to disease status. Seventeen other CD-negative samples came from Profile Diagnostics Inc. and were guaranteed to be CD-negative by questionnaire, as well (Profile Diagnostics Inc. Sherman Oaks, CA).

Once in the lab, aliquots of sera were made by first spinning down the BD vacutainer tubes and collecting the remaining supernatant. Aliquots were subdivided into 2.5 ml master tubes, and smaller 600 µL tubes. This was done to assure we had appropriate back-ups in case that a serum is contaminated with other sample or that antibodies in serum are inactivated by multiple freeze-thaw processes. The larger volume, stocking tubes were kept in freezer at -80°C. The smaller tubes to be used directly in the experiments were kept at -20°C for easier access and shorter thawing periods. All CD-negative and CD-positive sera were relabeled with a numbering system for ease of experimentation and labeling purposes.
Dear Crohn's Patient,

I would like to invite you to participate in a study that is being conducted by Dr. CA Speer’s Laboratory at the University of Tennessee, Agriculture Campus. Your participation requires a one time blood draw of 10-20 mls (a little more than a tablespoon) and a disclosure of your age and gender. The blood will be drawn at UT medical center’s phlebotomy laboratory and the procedure should take less than 15 minutes. There will be no cost to you or your insurance company for the blood draw.

As you are aware Crohn’s disease (CD) is currently diagnosed by a combination of tests, including blood/stool test, X-ray examination, colonoscopy and biopsy. Since some of these tests are difficult, invasive and hard to interpret, there is a need for a simple and less-invasive test for the disease. It has been shown by some investigators that most CD patients have serum antibodies against a bacterium, called Mycobacterium paratuberculosis, which may be involved in CD. Dr. Speer’s Lab has developed a method to detect such antibodies with high sensitivity and specificity, and the method requires only a small amount of blood from patients with CD. Further efforts on the development of a diagnostic test for CD based on the Speer Lab method would benefit CD patients by providing more accurate diagnosis and by reducing risk, cost, and time required for diagnosis.

Mycobacterium paratuberculosis (MP) causes a disease similar to Crohn’s disease in cattle and other animals called Johne’s disease. MP involvement in CD has also been indicated in a number of scientific studies but to date no conclusive evidence has been found. However, recent studies showed that antibiotic treatments that are effective against MP resulted in long-term remission of CD, supporting the hypothesis that CD might be caused by mycobacteria. The test for MP developed in Dr. Speer’s Lab is far more sensitive and specific for MP bacteria than anything that has been provided previously. If these studies help prove that MP is involved in CD it may lead to new therapies for Crohn’s disease. If you have questions about the Informed Consent Form or if you would like scientific literature or more information on MP please contact Cathy Scott at 865-974-0465 or mcscott@utk.edu.

If you are willing to participate in the study please fill out, sign and take the enclosed Informed Consent and Dynacare forms to the University of Tennessee, Patient Service Center, POB (Physician Office Building Ste 155) and ask to have your blood drawn for the Speer Lab Study. A copy of your Informed Consent Form will be mailed to you at the address you provide on the Dynacare form.

Sincerely,
Dr. Mark Anderson M.D.

Figure 9: Cover letter for Crohn’s volunteers
Detection of *Mycobacteria paratuberculosis* Antibodies in Crohn's Patient Serum.

Dr. C.A. Speer Ph.D.
Dr. Shigetoshi Eda Ph.D.
Forestry, Wildlife and Fisheries
University of Tennessee
274 Ellington Plant Science
Knoxville, Tn 37996

Dr. Mark Anderson M.D.
1928 Alcoa Highway
Medical Office Building B, Suite 100
Knoxville, TN 37920

The study in which I am being asked to participate by your Gastroenterologist Dr. Mark Anderson has the goal of producing a quick and accurate test for Crohn’s Disease (CD). It has been shown by some investigators that most CD patients have serum antibodies against a bacterium, called *Mycobacterium paratuberculosis*, which may be involved in CD. We have developed a method to detect such antibodies with high sensitivity and specificity, and the method requires only a one time blood draw of 5 to 10 mls from patients with CD. UT medical center’s phlebotomy lab [University of Tennessee, Patient Service Center, POB (Physician Office Building Ste 155)] will draw a little more than a tablespoon of your blood, the procedure should take less than 15 minutes, there is a possibility some redness or swelling could occur. If redness and swelling do occur they will not last long. This is a preliminary study involving 20 to 40 subjects; if the initial findings are promising more Crohn’s patients will be enrolled. Further efforts on the development of a diagnostic test for CD based on our method would benefit CD patients by possibly providing more accurate diagnosis and by reducing risk, cost, and time required for diagnosis.

I have read the description of the study and I have freely volunteered to participate. I understand that I do not have to take part in this study and that my refusal to participate will involve no penalty or loss of rights to which I am entitled and it will not adversely affect my subsequent medical care. I have had an opportunity to ask questions of the doctor and have received acceptable answers. I understand that I will receive the same treatment for my condition weather I participate in the study or chose not to participate. No treatment decision will be made from the results of the tests.

There is no additional medical cost to me or my insurance involved for the test, all expenses will be paid for by Dr. Speer’s lab through The University of Tennessee, Institute of Agriculture. My Blood will be drawn by Dynacare a UT contracted phlebotomy lab, Dr. Speer will not be involved in any medical procedure.

**Figure 10:** Volunteer consent form
Figure 10 continued

I understand that I am not waving any legal rights or releasing the hospital or its agents from liability for negligence. I understand that in the event of physical injury resulting from the research procedure, The University of Tennessee does not have funds budgeted for compensation either for lost wages or for medical treatment. There is no additional medical cost to me or my insurance involved for the test, all expenses will be paid for by Dr. Speer’s lab at the University of Tennessee, Institute of Agriculture.

PATIENT CONFIDENTIALITY
Although study results may be published, your confidentiality will be maintained. Your name or information identifying you will not be released without written permission unless required by law. Under federal privacy regulations, you have the right to determine who has access to your personal health information (called “protected health information” or PHI). PHI collected in this study may include your medical history, the results of physical exams, lab tests, x-ray exams, and other diagnostic and treatment procedures, as well as basic demographic information. By signing this consent form, you are authorizing the researchers at the University of Tennessee Medical Center to have access to your PHI collected in this study and to receive your PHI from, Dr. Mark Anderson M.D., and the University of Tennessee Medical Center where you have received health care. In addition, your PHI may be shared with other persons involved in the conduct or oversight of this research, including the (FDA) Food and Drug Administration, the University of Tennessee Medical Center, and the University of Tennessee Graduate School of Medicine Institutional Review Board. Your PHI will not be used or disclosed to any other person or entity, except as required by law, or for authorized oversight of this research study by other regulatory agencies, or for other research for which the use and disclosure of your PHI has been approved by the IRB. Your PHI will be used indefinitely. You may cancel this authorization in writing at any time by contacting the Principal Investigator listed on the first page of the consent form. If you cancel the authorization, continued use of your PHI is permitted if it was obtained before the cancellation and its use is necessary in completing the research. However, PHI collected after your cancellation may not be used in the study. If you refuse to provide this authorization, you will not be able to participate in the research study. If you cancel the authorization, then you will be withdrawn from the study. Finally, the federal regulations allow you to obtain access to your PHI collected or used in this study. However, in order to complete the research, your access to this PHI may be temporarily suspended while the research is in progress. When the study is completed, your right of access to this information will be reinstated.
Figure 10 continued

If you have any questions regarding this study, you may contact the Co-Principal Investigators, Dr. C.A. Speer at 865-974-0467 or Dr. Shige Eda as well as the Study Coordinator Cathy Scott at 865-974-0465. If you have any questions regarding your health or risks associated with participating in the study you may contact your physician Dr. Mark Anderson at 865-544-6570. If at any time you have any further questions about your rights as a participant in this research protocol, you may contact the University of Tennessee Graduate School of Medicine Institutional Review Board (a group of people who review the research to protect your rights) at 865-544-9781. Thank you for your consideration.

You will receive a copy of this consent form for your records.

Signature of Subject ___________________________ Date __________

Signature of Principal Investigator ___________________________ Date __________

Subject’s Age- ___________________________ Date __________

Subject’s Gender- M or F

Dynacare- Draw two red top no additive vacutaner tubes of blood attach this form and please call 974-0465 or 974-0479 immediately for sample pick up. Thanks.
**Bacterial Cultures**

**Whole-cell MAP**

To culture MAP we used a Middlebrook broth that has become standard for many mycobacteria since its discovery by Middlebrook et al. during the 1950’s [81, 82]. Specifically, Linda strain MAP (ATCC© 43015) was cultured in Middlebrook 7H9 medium (Becton Dickinson, Cockeysville, Maryland) with 10% oleic acid, albumin, dextrose, and NaCl (OADC). This particular strain was thought to be ideal for such a study as it had been originally isolated from the intestinal mucosa of a 15 year-old Crohn’s patient named Linda [1]. Interestingly, the strain was reactive to antibodies in sera obtained from JD-positive cattle in the FCM assay as well [2]. Since mycobactin J is critical for the growth of MAP, 2 μg/ml of the iron chelator was added to the growth media [45]. Media were prepared aseptically, with autoclaving for the M7H9/mycobactin J solution and vacuum filtration for the OADC due to denaturing concerns under high temperature conditions. In our previous work, we found that MAP bacilli were not antigenic until they had been growing in media for at least 3 months. This may be due to the slow-growing nature of MAP. Visible films of bacteria along the bottommost region of the flask were a good visual indicator for proper growth state. Periodic culture antigenicity assays were conducted to identify those cultures which exhibited the strongest antibody reactivity to pooled samples of JD-positive serum. Those with strong fluorescence using a JD-positive, and weak to negligible fluorescence using the JD-negative pooled sera were chosen as positive and negative controls for testing serum samples corrected from CD patients.

**MAP Spheroplasts**

Spheroplasts or cell wall deficient (CWD) forms of MAP have been isolated from CD patients, so it was important to examine if CWD forms of MAP can be used to detect anti-MAP antibodies in CD patients [27]. In 2003, Hines et al. successfully transformed a strain of MAP with a full cell wall to a CWD form of MAP [83]. We, therefore, prepared CWD form of MAP (Linda strain) according to their protocol with minor modifications. First we spun down 10 ml of Linda strain MAP in a viable culture. The pelleted bacteria was then resuspended in 20 ml of M7H( w/ OADC and glycerol) with additional 1% glycine added. The solution was incubated for 3 days at 37ºC. A saline wash followed with a subsequent treatment in a CWD solution that includes 1.2% glycine, 30μL/ml lysozyme, 60% M7H9 w OADC, 40% 0.5M sucrose and 20mM MgCl₂. The bacteria were placed in the chemical bath for an eight day incubation period. Post-incubation the CWD forms of MAP had to be examined quickly as gross reversions to normal MAP can occur in as little as 8 days when treated bacteria are placed back into nutrient rich environments like M7H9 broth [83].
Yeasts

Several different strains of dried *Saccharomyces cerevisiae* were obtained from a local brewer’s store and placed in nutrient broth for overnight culture. Additionally an official strain of *S. cerevisiae* purchased from the ATCC© (47058) was cultured in a same manner. Since yeasts are facultative anaerobes, both aerobic and anaerobic culture conditions were applied and tested. A *Candida albicans* culture was obtained from Dr. Pamela Small, a Professor in Microbiology at the University of Tennessee, Knoxville.

Other Bacteria

*Escherichia coli* was purchased from ATCC© (23258) and cultured overnight in nutrient broth. This strain was isolated from the urine of a human patient. *Streptococcus faecalis* (ATCC© 19433) was cultured anaerobically overnight in brain heart infusion broth. *Streptococcus gallolyticus* (ATCC© 9809) was cultured in trypticase soy broth with defibrinated sheep blood under aerobic conditions. A human isolate of *Salmonella typhimurium* (ATCC© 1311) was cultured aerobically in nutrient broth. *Yersinia enterocolitica* (ATCC© 23715) was grown aerobically in tryptose broth. *Clostridium difficile* (ATCC© 43600) isolated from the feces of a patient with colitis was cultured in reinforced Clostridial broth in anaerobic conditions. *Campylobacter jejuni* subsp. *jejuni* (ATCC© 33291) isolated from human feces was cultured in brucella broth with 0.16% agar in aerobic conditions. A human isolate of *Listeria monocytogenes* (ATCC© 19115) was cultured in brain heart infusion broth in aerobic conditions. A human isolate of *Yersinia pseudotuberculosis* (ATCC© 29910) was cultured in nutrient broth in aerobic conditions. All cultures were checked for their opaqueness (indicating growth) and examined under light microscopy to affirm bacterial presence post-inoculation.

The FCM Procedure

Microorganisms harvested from liquid cultures were centrifuged to pellet the organisms. The pellet was resuspended in Buffer A (PBS, 10% SuperBlock, 0.05% Tween) at a 1:1 ratio of the initial culture volume extracted. Using a Nunc® 96-well microarray plate which had a conical wells for centrifugation steps (Rochester, NY), 2µL of room temperature serum were introduced into the each well. Pooled positive sera from JD+ farms and pooled negative sera from JD- farms were used as positive and negative controls, respectively. Fetal Bovine Serum (FBS) was also used as a negative control. Two wells were left completely devoid of any serum to examine the level of non-specific (background) binding of secondary antibody to the bacteria. All sera and controls were tested in duplicate to ensure quality control. Next, 100µL of suspension was inoculated into each well of a 96-well plate for a final serum-to-bacteria ratio of 1:50. The solution was resuspended for proper mixing with the individual sera. The plate was then
covered and placed upon a plate rocker for 1 hour to incubate. After the incubation, each well was washed with Buffer A to remove any non-specific antibody binding to the whole-cell bacteria. The washing steps consisted of three principle parts. First, the plates were centrifuged at 3500 rpm and 20°C for 10 minutes. This was done to move the heavier and stickier materials (the bacteria and bound antibodies to the bottom of the well where they became pellet). Next, we removed the supernatant using a suction apparatus. The remaining pellet was resuspended in Buffer A. This procedure was repeated a second time to ensure the proper removal of any unwanted, non-specific antibodies that may have been left in the wells. A final centrifugation and suction step was completed to prepare the samples for FITC tagging.

Following the washes, a secondary antibody (2’ Ab) was added to tag the remaining bound antibody-bacterial complexes. Since it has been shown that yeast can be a marker of disease when detecting both IgG and IgA, both antibodies were probed for in many of the assays [20]. The FITC-labeled 2’ Ab (Jackson Immunology, West Grove, PA) was diluted to 1:25 using Buffer A. The 96-well plates were covered with aluminum foil to prevent premature photoexcitation of FITC during the second incubation period. Secondary antibody reactions were carried out at room temperature for 1 hour on a rocker. The complex was then diluted in 1 ml of PBS and run on the flow cytometer (LSR II, BD bioscience, San Diego, CA). Detector voltages were set at 691 V for the Forward Scatter detector, 307 V for the Side Scatter, and 550 V for the FITC fluorescence detector. The side scatter threshold was set at 200 V one that has been shown to preferentially select bacterium from surrounding materials. Measurements were conducted over a cell count of 10,000 units. FITC fluorescent intensity was recorded as a measure of antibody presence. This could be visualized by the histogram recorded by the FCM software. A statistical analysis of the histogram was also included. An example of one flow cytometry histogram and complimentary analysis recorded during an assay designed to detect antibodies to whole-cell yeast is shown in Figure 11.

Statistical Analysis

The CD and HC serological groups were compared by ANOVA using a completely randomized design (SAS 2001). If the serological groups were found to be significantly different with an $\alpha<0.05$ then sensitivity and specificities were calculated using fixed cut-off values. Cut-off values were estimated using the HC serum values: the cut-off was defined as the mean of this HC population plus two standard deviations. Points lying above the cut-off values within the CD-free serum group were considered false positives. Specificity was calculated as ($n_{false\ positive}/n_{all\ negatives}$) x 100%. Sera from the CD group that fell below the cut-off were considered false negatives. Sensitivity was calculated as ($n_{tested\ positive}/n_{all\ positives}$) x 100%. Occasionally, gender was analyzed in a similar manner to that of disease-state to determine whether there were any differences in reactivity based upon sex.
Figure 11: Example of the histogram and other statistical data recorded by the FCM equipment
CHAPTER IV: Assay Results

MAP Results

Whole-cell MAP

Antibody (IgG and IgA) binding to MAP was observed in CD and HC groups, whereas MAP (Linda Strain) treated only with 2’ Ab for IgG and IgA (Figure 12 and 13) showed no preferential binding for either serum population (both p>0.05). This was true for the Linda strain, and also for several other MAP strains that we evaluated (data not shown). Further, absorption with another related bacteria, M. phlei (which is commonly used to reduce antibody cross-reactivity to other mycobacterium antigens in ELISA tests for CD), failed to improve the differentiation between the sera groups (data not shown) [84].

![Human Sera IgG binding to MAP](image)

**Figure 12:** IgG FITC probe with MAP-Human Ab Complex
Analysis of MAP using an IgA human antibody probe. There were no significant differences between the two sera populations (p>0.05).

**Figure 13:** IgA FITC probe with MAP-Human Ab Complex
Spheroplasts

The CWD form of MAP prepared in this study seemed to resemble the spheroplasts’ rounder and slightly larger morphology described by Hines et al. when observed under a light microscope [83]. Yet in our pilot experiments, the spheroplasts failed to bind any antibody in either serum population.

Other Bacteria

Non-MAP Suspects

*C. difficile, E. coli, C. jejuni,* and *S. faecalis* all reacted strongly with antibodies in the sera of both the HC and CD groups. Of these, *C. difficile* resulted in the greatest level of antibody binding -- as indicated by the fluorescent intensities that reached as high as 40,000 (unitless). However, while it appears that these four bacteria showed some antibody binding, a differentiation of the two sera groups was not possible using FCM (p>0.05). Our pilot studies with *E. coli* and *S. faecalis* led us to evaluate the two bacteria in separate all-sera studies. The results are shown in Figures 14 & 15.

Many of the non-MAP bacteria that we surveyed produced a very low level of antibody binding. Non-MAP bacteria with little to no antibody response included: *L. monocytogenes, Y. entercolitica,* and *S. enterica.* In these cases, the bacteria in question failed to achieve fluorescence appreciably larger than that of the 2’ antibody-only control.

Yeasts

*S. cerevisiae* differentiated the CD and HC sera groups with both IgG and IgA (Figures 16 & 17; both p<0.05). Overall reactivity seemed to be higher in those within the IgG assay, with FITC values with a mean FITC value of 25,449 within the CD group. The HC group in the IgG assay had a mean FITC fluorescent value of 15,123. FCM analysis using IgA was the more successful of the two assays.
Analysis of *E. coli* using an IgG human antibody probe. There were no significant differences between the two sera populations (p>0.05).

**Figure 14:** IgG FITC probe with *E. coli*-Human Ab complex
Analysis of *E. faecalis* using a IgG human antibody probe. There were no significant differences between the two sera populations (p>0.05).

**Figure 15:** IgG FITC probe with *E. faecalis*-Human Ab complex
Figure 16: IgG FITC probe with *S. cerevisiae*-Human Ab complex
Human Sera IgA binding to *S. cerevisiae*

![Graph showing IgA binding levels for Crohn's Disease and Healthy Controls](image)

Analysis of Baker’s yeast using a IgA human antibody probe. CD and HC groups significantly different (p<0.05). CD mean = 4,325. HC mean = 2,076. At a CD cut-off of 3,558 sensitivity was 13/37 x 100% = 35.1%. Specificity was much higher than with IgG at (1-(2/37)x100%) = 95.0%

**Figure 17:** IgA FITC probe with *S. cerevisiae*-Human Ab complex
sensitivity of 35.1%. This assay’s specificity was accurate to 95% (35/37). While the IgG assay had a better specificity of 97.3% (36/37 HC controls were true negatives), the tests’ sensitivity of 29.7% was slightly lower than that of its counterpart. Further testing using 11 different strains of *S. cerevisiae* under both aerobic and anaerobic conditions were unable to improve upon diagnostic sensitivity (data not shown).

*C. albicans* w/ IgA (Figure 18) also differentiated the CD and HC sera groups (p<0.05). There was significantly high reactivity within the CD group with FITC values as high as 100,000. The FITC mean amongst the CD group was 30,939. The HC group had a mean FITC value of 16,878. Sensitivities for the *C. albicans* FCM test was 24.1% (9/37 CD sera tested positive using our cut-off value). This was the lowest of the FCM methods which were able to differentiate serum populations. Specificity for this assay was 91.9% as 3 false negatives were detected. In addition, there were no differences between gender as a subpopulation of the disease groups (p>0.05).
Analysis of C. albicans using a IgA human antibody probe. CD and HC groups determined to be significantly different (p<.05). CD mean determined to be 30,939. HC group mean found to be 16,878. CD cut-off value determined to be at 49,486. Using this value sensitivity determined to be 9/37 x 100% or 24.3%. Specificity was much lower than with IgG and determined to be (1-(3/37)x100%) or 91.9%.

**Figure 18:** IgA FITC probe with C. albicans-Human Ab complex
CHAPTER V: Discussion

Summary of Results

The results of this study are summarized in Figure 19. While MAP were unable to differentiate the CD and HC groups under various experimental conditions, there were some encouraging aspects from our FCM studies using CD and HC sera. Our study confirmed previous data obtained by many researchers which showed a higher overall antibody reactivity to S. cerevisiae for CD subjects when compared to healthy individuals. While our sensitivities were not as high as some that have been reported, they approached those reported by Bossuyt in his review of antibody markers of CD [21]. Similarly, C. albicans was able to differentiate the sera populations supporting the common antigen association discussed by Standaert-Vitse et al. [23].

Many bacteria reacted strongly, but did not elicit sufficient differentiation to justify a full-length test. Lastly, many of the bacteria failed to elicit any antibody binding whatsoever.

Discussion of MAP Non-differentiation

While there was certainly reactivity to MAP in our FCM, our MAP assays did not allow for differentiation of the two sera groups. Few serological tests probing for MAP antibodies have been applied to CD populations, but one large population study by Bernstein et al. [34] revealed similar results our own. Their study used 283 CD sera, 144 UC sera, and 402 HC sera to look for antibodies to MAP using a commercially available ELISA (IDEXX, Westbrook, ME) which had been manufactured for use in dairy cattle tests, but had been validated for human use as well. Its validation in humans may come into question, though, as the positive control came from the pooled sera of a man who had accidentally inoculated himself with a killed MAP vaccine and had incurred a granulatous legum at the injury site. Negative controls of well-studied American Cross subjects who had no history of HC made up their control negative population to allow them to obtain S/P ratios. A final strength of their study was that it used CD and HC volunteers from Manitoba, Candida; a region with a very high CD rate that coincides with high rate of MAP prevalence within the dairy cattle of the agricultural town [33].

While there was a high rate of sera in Bernstein’s study which were considered positive, these sera were spread across all sera groups with an approximate overall seropositivity of 35%. The authors found no true statistical difference between the two populations. To be exact the CD sera were 37.8% positive while the HC group was 33.6% positive. Further, gender was unable to predict disease, along with seven other variables which included predispositions.
### Reactive/Differentiating

- *S. cerevisiae* w/ IgG probe
- *S. cerevisiae* w/ IgA probe
- *C. albicans* w/ IgA probe

### Reactive/Non-differentiating

- *M. avium subsp. paratuberculosis* w/ IgG
- *M. avium subsp. paratuberculosis* w/ IgA
- *E. coli* w/ IgG
- *C. jejuni* w/ IgG
- *S. faecalis* w/ IgG
- *C. difficile* w/ IgG

### Non-reactive

- *L. monocytogenes* w/ IgG probe
- *Y. enterocolitica* w/ IgG probe
- *S. gallolyticus* w/ IgG (did not grow)
- *S. enterica subsp. enterica* serovar Typhimurium w/ IgG
- *M. avium subsp. paratuberculosis* (spheroplast) w/ IgG

**Figure 19:** Summary of results
to MAP exposure that the authors surveyed as MAP antibody-positive indicators. Unlike Bernstein et al., we lacked a true positive control to set cut-off values for the MAP FCM. Thus we cannot properly estimate the sensitivity and specificity of our test. Yet, like the authors, we were also unable to detect an antibody difference between the sera populations. Either we do not have the correct antigen on our bacteria to elicit a differentiating response, or else MAP is sufficiently common bacteria for both HC and CD subjects to have built-up some antibody defense for it. In addition, the preabsorption experiments with *M. phlei* were conducted to reduce potential cross-reactivity to other mycobacteria, but perhaps another future experiment could be designed using preabsorption with other mycobacterium. Two promising candidates would be *M. kansassi* and *M. tuberculosis*. Thayer et al found many MAP seropositive CD subjects to also be seropositive for these two mycobacteria [85]. Surprisingly, they found that 100% of CD subjects that were seropositive to MAP were also seropositive to *M. tuberculosis*. In addition, 43% of CD subjects seropositive to MAP, were also seropositive for *M. kansassi.*

The genetic susceptibility of certain patients to CD suggests that MAP may need a G.I. system malfunction in order to invade and cause disease [5]. Presently, we lack the proper genetic information on our subjects to explore this issue further. Other important information we lack is the disease history of the CD subjects and their past treatment. These data might help to explain if there has been a sufficient period of time for the subjects to develop a strong immune response, or, in some cases, if disease has been ongoing for too long, so that response has declined. This latter possibility is highlighted in a study conducted by Olsen et al. in 2001 that compared the antibody titers to a MAP derived protein in subjects with CD and UC [86]. While the CD subjects seemed to have an overall higher antibody response to the MAP proteins, a peculiar thing had occurred. It seemed that the longer the period had been since each CD subject had been diagnosed with disease, the lower the antibody responses tended to be. This negative correlation was very strong: \( r_s = -0.85 \) [86].

A second patient history consideration we must take into account is what type of treatment has been administered. For example it would have been useful to know whether or not immunosuppressant therapy and possibly even antimycobacterial medication had been administered to the CD patients. Immunosuppressant would lower the entire immune system, including humoral immunity. Although this treatment is usually reserved for subjects with severe CD who don’t respond to other treatments [4], we cannot rule out the possibility that our subjects were on immunosuppressants.

Our findings provide no affirmation of MAP’s possible role in CD via serological means, it would be premature to conclude that the mycobacterium is not involved in any manner. Serological tests merely detect antibodies and thus are considered an ‘indirect’ means of determining causation. Their presence, even in high titers, does not directly imply active, ongoing infection. Our bodies possess memory B-cells which continually produce antibodies to specific antigens long after the body has cleared those antigens.
As previously discussed, much of the literature describing actual MAP within CD tissue has involved a CWD form [1]. Thus using assays designed to detect antibodies against surface antigens of an intact bacteria (with an outer cell wall) may be unlikely to produce a successful differentiation. Further, while we did attempt to test for reactions to spheroplastic forms of MAP, it cannot be assumed that this spheroplastic form is identical to that observed in vivo. As Beran et al. note in their review of spheroplastic mycobacteria, no study has been produced which reveals the in vivo behavior of these chemically-formed CWD MAP [87]. Thus, further research is needed to better verify the in vivo behavior exhibited chemically-induced spheroplastic so as to determine whether or not treated bacteria are sufficient for this sort of assay. Secondly, spheroplastic bacteria may lack the necessary antigens to induce an immune response in the first place. To date, no study has directly investigated the antibody-inducing ability of spheroplastic MAP. However, an extremely indirect evaluation of the spheroplastic antibody induction ability was conducted in a very recent study conducted by Hines et al. [88]. In this study the authors attempted to look for antibodies to chemically-induced spheroplastic MAP vaccines in goat kids. The MAP bacteria were treated in a similar manner to our experiments to induce the spheroplastic form. The bacteria were then killed and injected into the animals to evaluate vaccine effectiveness. The researchers utilized a commercial Parachek ELISA kit (Prionics, Zurich, Netherlands) to evaluate antibody responsiveness pre and post-challenge with live whole-cell pelleted MAP. It is important to note that this ELISA uses antigens removed by a 30 minute sonication. This step is likely to remove all antigens, even the most basal ones which a CWD MAP might possess. In their study, none of the CWD vaccines were able to induce an appreciable humoral immune response.

The water is further clouded by numerous reports of serological data that conflict with MAP DNA probe data. This suggests that there is a disconnect between MAP presence within an individual, and the antibodies to that bacterium that have been stockpiled by that person’s humoral immune response. This can be evidenced by a study conducted by Collins et al. in which used 2 separate diagnostic tests, that of PCR and that of the IDEXX© ELISA [89]. A subset of their studies included a group of 181 individuals which consisted of HC, CD, and UC subjects. Of these 36 were positive for MAP IS900 PCR. Another 11 were positive for MAP antibodies by ELISA. However, in not a single instance was a subject positive for both tests.

Lastly, the possibility exists that there is a strain of MAP which is more infectious than others. If this was the case, it may elicit an entirely different antibody response than those for the non-infectious MAP. Thus, while we may be detecting an antibody response in both HC and CD subjects, antibodies to the more infectious strain may have differentiated the populations. Different strains of MAP may express unique surface antigens which have a complimentary set of antibodies predominantly present in those with CD. This has been shown to be the case with other bacteria such as Helicobacter pylori which had differential antibody responses depending on strain type [90].
Antibody Reactions in Non-MAP Bacteria

Of the non-MAP bacteria surveyed, 4 out of 9 had an appreciable antibody response across all sera groups. One of the 4 non-reactive bacteria, *S. gallolyticus*, simply failed to grow. The remaining three that failed to react may have behaved this way because these are bacteria that are less common and thus haven’t elicited a corresponding humoral immune response for those individuals tested. For example, *L. monocytogenes* -- a gram-positive bacteria that can survive refrigeration temperatures -- is more known for its role in a small percentage of food poisoning cases than as a commensal human gut microbe [38]. Another, *S. enterica* subsp. *typhimurium*, is also being monitored carefully by the CDC, even though it is considered to be part of the normal natural flora of the human gut. Of the remaining 5 that did react, none were able to differentiate the sera populations (p>0.05). High environmental exposure rate to these microbes may have resulted in a generalized, high antibody titer that is common to both the HC and CD groups.

Differentiating Populations Using Whole-cell Yeast FCM

Both of the yeast species that we used to investigate for unique antibody responses were able to differentiate the CD and HC populations. Antibodies to *S. cerevisiae* were much more acute in their differentiating achieving overall assay sensitivities of 29.7% and 35.1% for IgG and IgA responses, respectively. While one could look at the IgG responses and presume the two-fold higher FITC levels to be indicative of different antibody levels to MAP according to immunoglobulin type, this is not a safe assumption.

The finding that the yeasts were able to differentiate the two sera groups is important because it proves that FCM can be a valuable means of detecting differential antibody responses. It affirms studies by our lab, and by others, that the flow cytometer is sensitive enough to detect minute microbe-antibody complexes [2, 80]. In 2006, Reese et al. conducted a rigorous metaanalysis on articles concerning serological markers within CD that included sifting through hundreds of articles to obtain the highest quality studies on ASCA and pANCA markers as predictors of disease [91]. Sixty studies were choosen for a detailed statistical analysis. None of the articles dealt with differentiating CD from HC populations, as the test is more commonly used to test CD from UC subjects.

One subgroup of articles compared IBD versus non-IBD groups using ASCA IgA ELISAs, with an overall sensitivity of 31.4% obtained from a test population of 1,651 subjects. This was very similar to the 35.1% sensitivity we achieved when differentiating CD from HC groups using 74 subjects. A review article detailing ASCA sensitivity and specificity information show our data to be consistent with many of the lower-end sensitivity estimates (ranging from 40-70%). [21]. If sensitivity for ASCAs could be improved for the FCM,
technological advances in microfluidic flow cytometers would allow for quicker, in-house tests to be conducted.

Antibodies to *C. albicans* were less apt to separate the sera populations. These were the lowest assay sensitivities achieved by detecting only 9 of the 37 or 24.3% of the CD sera as positive. There are four common cell wall macromolecules shared between *S. cerevisiae* and *C. albicans* [92]. These consist of chitin, mannoproteins, \( \beta(1,6) \) glucan, and \( \beta(1,3) \) glucan. Since these cell wall constituents are expressed on the outer surface of both yeasts (albeit in different concentrations), antibodies directed towards the entities are likely to cause a cross-reactivity event. That is, antibodies directed towards the cell wall of *S. cerevisiae* are also binding to that of *C. albicans*. As stated previously, this has been proven as *C. albicans* has been shown to cause false positive results in the ASCA test [23].

**Final Thoughts**

The experiments reported here represent a first attempt to utilize FCM to improve CD diagnostics. Our previous success with the method in an animal model made this attempt to apply FCM to human sera the logical next step. Given our present lack of success, it is clear that the problem is not a simple one, but it certainly seems worth continuing to pursue. Meanwhile, CD continues in its enigmatic ways among the human population. There are those who feel that the disease is strictly a bacterial issue and others who feel another mechanism may be at the center of the cause – given the present state of knowledge, we suggest the answer probably lies somewhere in between.
LIST OF REFERENCES


VITA

Taylor Fielden was born in Maryville, TN. He spent most of his life in Tennessee enjoying the wildlife of the mountains and country. His time at Maryville High School saw Taylor pursuing both his scientific curiosities and his interest in writing within a variety of disciplines. Taylor attended Oglethorpe University in the Fall of 2000 on a scholarship and began studies for a pre-medical path. Taylor transferred to the University of Tennessee in the Spring of 2002 and received his Bachelor’s of Science in Biological Sciences with a concentration in Microbiology. Throughout his studies, Taylor has maintained a healthy fascination with the natural world from the microscopic to the macroscopic.